Antigenic relationships between strains of A. naeslundii Genotypes 1 and 11, A. viscosus and the animal species A. howellii, A. denticolens and A. slackii

by

Edward E. Putnins

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Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science

Department of Medical Microbiology
University of Manitoba
Winnipeg, Manitoba

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<ins>For my parents, brothers, sister and sister-in-law</ins>.

They taught me the importance of having dreams and the need to work for those dreams.

<ins>For Shelley</ins>.

Without her never ending support and encouragement the past three years would have been difficult.

<ins>For Jedi</ins>.

Last but not least my dog, who's wagging tail at the end of a long day helped keep problems in perspective.

TABLE OF CONTENTS

	PAGE
ABSTRACT	i
CHAPTER 1	1
LITERATURE REVIEW	1
1) Oral Cavity	1
I) Introduction	1
II) Periodontium	1
a) Health	1
b) Disease	3
III) Teeth	4
a) Health	4
b) Disease	4
IV) Factors Influencing Oral Homeostasis	5
a) Saliva	5
i) Lubrication	5
ii) Protective Properties	6
b) Gingival Crevicular Fluid	6
c) Nutrition	8
d) Atmosphere	9
V) Conclusion	9
2) Resident Oral Flora	11
I) Introduction	11
II) Resident Oral Flora	11
a) Oral Mucosa and Tongue	12
i) Oral Mucosa	12
ii) Tongue	12
b) Supragingival Dental Plaque (SGDP)	13
i) Acquired Pellicle	13
ii) Structure and Chemical Composition of SGDP	14
iii) Histology of SGDP	15
iv) Microbiology of SGDP	17
-Adherence	17
-Supragingival Dental Plaque (Cultural)	18
-Sulcular Dental Plaque (Cultural)	20
III) Conclusion	20
3) Oral Microflora Associated With Disease	22
I) Oral Microbial Ecology	22
II) Non-specific Plaque Hypothesis	23
III) Specific Plaque Hypothesis	23
a) Opportunistic Oral Pathogens	23
b) Exogenous Oral Pathogens	24

PAGE

4)A. viscosus and A. naeslundii-Pathogenicity, Physiology and Taxonomy 27
I) Introduction 27
II) Pathogenicity 27
a) Extraoral Infections 27
b) Intraoral Infections 28
i) Animal Studies 28
ii) Human Studies 29
-Enamel Caries 29
-Rootcaries 29
-Gingivitis/Periodontitis 30
III) Physiological Characteristics 31
a) Morphology 31
b) Growth in Culture 31
c) Atmospheric Requirements 32
d) Physiology and Metabolism 32
IV) Taxonomy of Actinomyces 32
V) Taxonomy of A. viscosus and A. naeslundii 33
a) Historical 33
b) Physiology-Based Taxonomy 34
c) Significant Changes in The Taxonomy of A. viscosus And A. naeslundii 35
VI) Taxonomy of Animal Actinomyces Species 39

5)Antigenic Structure of A. viscosus and A. naeslundii and the Humoral Response 40
I) Introduction 40
II) Antigenic structure 40
a) Carbohydrate Antigens 41
b) Protein Antigens 41
III) Humoral Immune Response to A. viscosus and A. naeslundii 43
a) Serum Immunoglobulins 43
b) Mucosal Immunoglobulins 44
c) Gingival Crevicular Fluid Immunoglobulins 45

CHAPTER 2 46

RATIONALE AND APPROACH FOR THE PRESENT STUDY 46

I) Rationale 46
II) Approach 47
a) Agglutination 47
b) Polyacrylamide Gel Electrophoresis/Immunoblotting 48
III) Clinical Applications in Species Identification 48

	PAGE
CHAPTER 3	50
MATERIALS AND METHODS	50
I) Species Selection and Growth	50
II) Physiological Characterization	50
a) Atmospheric Requirements	50
b) Catalase	50
c) Carbohydrate Fermentation	52
d) Starch Hydrolysis	52
e) Aesculin Hydrolysis	52
f) Urea Hydrolysis	52
g) Nitrate/Nitrite Reduction	53
III) Sample Preparation	53
a) Bulk Culture	53
b) Whole Cells For Agglutination	53
c) Pronase Treatment of Whole Cells	54
d) Whole Cell Glass Bead Extracts	54
e) Whole Cell Sonicates	54
f) Cell Walls	54
g) Pronase Treatment of Cell Walls	55
h) Extraction of Carbohydrates From Cell Walls	55
IV) Transmission Electron Microscopy	56
V) Immunological Techniques	57
a) Antiserum Preparation	57
b) Agglutination	58
i) Whole Cells	58
ii) Cell Walls	60
c) Double Diffusion	60
d) Rocket Immunoelectrophoresis	60
e) Polyacrylamide Gel Electrophoresis and Immunoblotting	61
i) Sample Preparation	61
ii) Electrophoresis	62
iii) Immunoblotting	62
iv) Control of Immunoblotting Reagents	63
VI) Immunoblot Analysis	63
a) Scanning Densitometer	63
b) Calculation of the Molecular Weights of Antigens Present on Immunoblots	64
VII) Absorption of Antigens Common to the Genus	65
VIII) Preparation of Cluster Specific Antisera	65
a) Cell Wall Absorptions	65
b) Whole Cell and Sonicated Cell Absorptions	66
CHAPTER 4	67
RESULTS	67
I) Physiological Characteristics	67
a) Fermentation	67
b) Hydrolysis	67

PAGE

c) Growth 67
d) Catalase 68
e) Nitrate/Nitrite Reduction 68
II) Agglutination Reactions 71
a) Whole Cells 71
i) Non-pronase-treated Whole Cells 71
ii) Pronase-treated Whole Cells 72
b) Pronase-treated Cell Walls 75
III) Carbohydrate Cross-reactivity 77
a) Double Diffusion 77
b) Rocket Immunoelectrophoresis 77
IV) Antigenic Similarity (Sj) Determined For Cell Wall Extracts 77
a) Morphology of Cell Wall Preparations 77
i) Whole Cells 78
ii) Non-pronase-treated Cell Walls 78
iii) Pronase-treated Cell Walls 78
b) Whole Cell Glass Bead Extracts and Cell Wall Extracts 82
c) Cell Wall Extract Antigenic Similarity (Sj) Using Cell Walls Produced in Different Batches 85
d) Similarity (Sj) of Cell Wall Extracts Reacted With Homologous Antisera Produced in Different Rabbits 86
V) Antigenic Similarity (Sj) Between the Fillery Clusters 1-7 and A. israelii Cell Wall Extracts Assessed by Immunoblotting 89
a) Intercluster Antigenic Similarity (Sj) 89
b) Intracluster Antigenic Similarity (Sj) 96
c) Antigenic Similarity of Cell Wall Extracts From The Fillery Clusters and Animal Actinomyces Species 99
d) Antigenic Similarity (Sj) of Cell Wall Extracts Prepared to Fillery Clusters And A. naeslundii-serotype II & III and Actinomyces-serotype NV Species 101
VI) Antigenic Cross-reactivity Due to Antigens Common to Actinomyces 104
VII) Intercluster Cell Wall Absorptions 109
a) Whole Cell Agglutination Using Cluster Specific Antisera 109
VIII) Intracluster Specificity of Intercluster Specific Antisera 113
IX) Immunological Relationship of A. naeslundii-Serotypes II & III and Actinomyces-serotype NV to the Clusters 117
X) Specificity of Cluster Antisera Using Glass Bead Extracts and Whole Cell Sonicate 117

CHAPTER 5 119

DISCUSSION 119

I) Introduction 119
II) Sample Preparation 119
III) Antigenic Relationship of A. viscosus and A. naeslundii 121
a) Cross-reactivity Assessed by Agglutination and Rocket Immunoelectrophoresis 121
b) Antigenic Cross-reactivity Assessed by Immunoblotting 121
i) Intercluster Cross-reactivity 122
ii) Intracluster Cross-reactivity 125

PAGE
iii) Animal Strain Cross-reactivity 126
IV) Conclusions 127
a) Antigenic Cross-reactivity Between Clusters 127
b) Use of Serovar Specific Antisera as a Screening Test 128
c) Strain Selection For Studies That Examine the Response of the Humoral Immune System to A. viscosus and A. naeslundii. 128

REFERENCES 132

APPENDIX 1-Media and Agar Formulas 146
Appendix 1A-B.M. Medium 147
Appendix 1A-B.B.L. Actinomyces Broth 147
Appendix 1B-Innoculum Broth 148
Appendix 1B-Streptococcus Sugar Agar 148
Appendix 1C-Starch Agar 149
Appendix 1C-Aesculin Agar 149
Appendix 1C Urea Agar 149
Appendix 1D Nitrate Broth 150
Appendix 1D Nitrite Broth 150
Appendix 1E Actinomyces Defined Medium (ADM) 151

APPENDIX 2 152

Percent Antigenic Similarity (Sj) of Cell Wall Extracts For the Fillery Clusters 1-7 and A. israelii Using Antisera Produced to Each of the 7 Fillery Clusters. 153

APPENDIX 3 156

Percent Antigenic Similarity (Sj) of Cell Wall Extracts for Three Fillery Clusters and Three Animal Actinomyces Species Using Antisera Produced to these Six Strains 157

APPENDIX 4 159

Molecular Weights of Antigens that Remain for Each Cluster After Reaction of Cell Wall Extracts With Cluster Antisera That Was Absorbed With A. israelii (NGB17) Cell Walls. 160

FIGURES

	PAGE
Figure 1.1. Diagram of Periodontal Health and Periodontitis.	2
Figure 1.2. Actinomyces Similarity Matrix.	36
Figure 1.3. Antigenic Structure of A. viscosus and A. naeslundii Cell Wall.	40
Figures 4.1a-f. Transmission Electron Microscopic Picture of ATCC 12104 Whole Cells, Cell Walls and Pronase Treated Cell Walls.	80
Figure 4.1g. Whole cells of WVU 627 (cluster 1) showing fimbriae.	81
Figures 4.2a-b. Whole Cell Glass Bead and Cell Wall Extracts Run on a Polyacrylamide Gel Stained With Coomassie Blue.	84
Figure 4.3. Immunoblots of Cell Wall Extracts Reacted With Antiserum Produced in Different Rabbits. Both Rabbits Immunized With The Same Strain.	87
Figure 4.4. Examples of Scanning Densitometer Results.	91
Figures 4.5a-b. Immunoblots of Cell Wall Extracts Prepared to a Representative From Each of The Fillery Clusters and A. israelii.	93
Figure 4.6. Immunoblots Showing Intracluster Similarities of Cell Wall Extracts From Clusters 1 and 5.	97
Figure 4.7. Immunoblot of Cell Wall Extracts From the Fillery Clusters and Three Animal Actinomyces Species.	100
Figure 4.8. Immunoblot of Cell Wall Extracts From the Fillery Clusters and A. naeslundii-serotype II & III and Actinomyces-serotype NV.	102
Figure 4.9. Immunoblot of Cell Wall Extracts From The Fillery Clusters and A. israelii Reacted With Nonabsorbed Cluster 4 Antiserum and Antiserum Absorbed With A. israelii Cell Walls.	107
Figure 4.10. Immunoblot of Cell Wall Extracts From The Fillery Clusters and A. israelii Reacted With Nonabsorbed Cluster 7 Antiserum and Antiserum Absorbed With A. israelii Cell Walls.	108
Figures 4.11a-d. Four Immunoblots of Cell Wall Extracts Reacted With One of Four Cluster Specific Antisera Showing Intercluster Specificity.	112
Figures 4.12a-b. Two Immunoblots of Cell wall Extracts Reacted With Cluster Specific Antiserum Showing Intracluster Specificity.	116

TABLES

	PAGE
Table 1.1. Bacterial Genera Found in the Human Mouth.	11
Table 1.2. Histological Studies of Early Plaque Formation.	16
Table 1.3. Bacteria Detected at Various Stages of Plaque Development on Cleaned Surfaces in the Mouth.	19
Table 1.4. Species Within the Genus Actinomyces.	33
Table 1.5. Taxonomic Changes in the Classification of A. viscosus and A. naeslundii.	38
Table 3.1. Origin and Designation of the Microorganisms used in this Study.	51
Table 3.2. Actinomyces Strains Used for Rabbit Immunization.	59
Table 4.1. Physiological Characteristics of Actinomyces.	69-70
Table 4.2. Non-pronase and Pronase-treated Whole Cell Agglutinations.	73
Table 4.2b. Additional Non-pronase-treated Whole Cell A. slackii Agglutinations.	74
Table 4.3. Pronase-treated Cell Wall Agglutinations.	76
Table 4.4. Percent Antigenic Similarity (Sj) of Different Batches of Cell Walls When Reacted With Their Homologous Antiserum.	85
Table 4.5. Percent Antigenic Similarity (Sj) of Cell Wall Extracts Reacted With Homologous Antisera Produced in Different Rabbits.	88
Table 4.6. Mean and Range of Antigenic Similarities (Sj) of Cell Wall Extracts From Fillery Clusters 1-7 and A. israelii Using Antisera Against Fillery Clusters 1-7.	94
Table 4.7. Mean and Range of Antigenic Similarities (Sj) Between Cell Wall Extracts of Each of the Fillery Clusters and A. israelii.	95
Table 4.8. Percent Intracluster Antigenic Similarity (Sj) Using Cell Wall Extracts Reacted With Antisera Homologous to that Cluster	98
Table 4.9. Percent Antigenic Similarity (Sj) of Cell Wall Extracts From Fillery Cluster Representatives and Animal Actinomyces Species.	99
Table 4.10. Percent Antigenic Similarity of Cell Wall Extracts of Fillery Cluster Representatives and A. naeslundii-serotype II &III and Actinomyces-serotype NV	103

Table 4.11. The Number of Antigens Detectable in Cell Wall Extracts for Each Fillery Cluster and A. israelii Using Cluster Antisera that was Unabsorbed and Absorbed With A. israelii (NGB17) Cell Walls. 105

Table 4.12. Absorptions With Cell Wall Extracts and Subsequent Specificity to the Clusters (C) and A. israelii Cell Wall Extracts. 110

Table 4.13. Intracluster Specificity to Cluster Absorbed Antisera. 114

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ABSTRACT

Actinomyces have a limited habitat in the human and animal oral cavity and tonsillar region. The close physiological relationship between A. viscosus and A. naeslundii makes identification and grouping of these species difficult. Strains belonging to A. viscosus and A. naeslundii have been grouped into 7 Fillery clusters using numerical taxonomy applied to the results of 65 physiological tests. Immunological techniques like whole cell agglutination and immunofluoresence also show high degrees of antigenic cross reactivity between and within these species.

The aim of this study was to quantitate the degree of immunological cross-reactivity and identify if antigenically distinct serovars exist within A. viscosus and A. naeslundii. Once immunologically distinct strains or species can be identified, antiserum specific to these groups can be used to screen fresh isolates from areas of health and disease. In addition, immunological responses to these distinct strains can be measured in health and disease.

A) Agglutination of pronase and non-pronase-treated whole cells and pronase-treated cell walls with antisera was measured to provide an overview of the extent of cross reactivity. Antisera was produced by immunizing rabbits with a strain from each of the 7 Fillery clusters and three animal species A. slackii, A. howellii and A. denticolens. In general, a high degree of cross reactivity existed between each of the 7 Fillery clusters and A. slackii. It appeared that cluster 5 (typical A. naeslundii), A. denticolens, and A. howellii were quite distinct as they primarily agglutinated with their homologous antiserum. Treating the cells with pronase had little effect on the agglutination pattern suggesting that the antigens responsible for agglutination may be carbohydrate in nature. A study was undertaken to examine the reaction of carbohydrate antigens with antibody using Ouchterlony double diffusion and rocket immunoelectrophoresis. Extraction of carbohydrates from pronase-treated cell walls

was accomplished with acid. The results confirmed that cross-reactivity does exist within the carbohydrate component of the cell walls. The inability to develop good carbohydrate precipitating antisera in this component of the study made it impossible to draw any firm conclusions on carbohydrate cross reactivity.

B) The cross-reactivity that exists within the protein component of the cells was examined with polyacrylamide gel electrophoresis and immunoblotting. Whole cell glass bead extracts and cell wall extracts were made and both showed good protein extraction on a polyacrylamide gel stained with Coomassie blue. Cell wall extracts was chosen for two reasons. Firstly, the cell surface antigens are those most readily presented to the hosts immune system. It would be expected that antibody to these antigens would be a major component in antisera. Secondly, whole cell glass bead extracts contain many cytoplasmic proteins that would likely be conserved between the two species as A. viscosus and A. naeslundii are physiologically very similar. Since this study was attempting to identify immunologically distinct antigens it would help to remove proteins that are alike and not complicate the immunoblot profiles.

The cross-reactivity shown on immunolbots was quantitated using the Jaccard coefficient. The degree of cross reactivity within the clusters was significantly higher than the cross reactivity between the clusters. In addition, relatively high cross reactivity between the clusters and A. israelii was identified suggesting that some of the cross reactivity was due to antigens common to Actinomyces. Cross-reactivity between the Fillery clusters and A. naeslundii-serotypes II & III and Actinomyces-serotype NV and the three animal species A. slackii, A. howellii and A. denticolens was shown to be high when cell wall extracts were used.

Absorptions of each of the 7 cluster antisera with A. israelii cell walls removed antigens common to Actinomyces. The immunoblot profile changed significantly. The seven different absorbed antisera to each of the clusters reacted weakly with cell wall

extracts from clusters 5 and 7. Therefore, the cross-reactivity between clusters 5 and 7 and the rest of the clusters was due to antigens common to Actinomyces. Cross-reactivity between clusters 1,2,3,4 and 6 was still observed with each of the 7 absorbed cluster antisera. Intercluster absorptions were then performed to identify if clusters 1,2,3,4 and 6 were immunologically distinct. Three serovars were identified. A polyclonal antisera specific to each of the clusters 2 and 3 was produced. Clusters 1,4 and 6 formed one serovar to which a specific polyclonal antiserum was produced.

To clarify the specificity of the polyclonal antisera, a study was undertaken to see if several strains from the same cluster belonged to the same serotype. Strains from a given cluster usually reacted most strongly with the antiserum that was specific to the clusters.

These results support a recent paper that has reclassified A. viscosus and A. naeslundii. Animal A. viscosus (cluster 7) would be retained as A. viscosus and A. naeslundii (cluster 5) would be placed into A. naeslundii-genospecies I (Johnson et al. 1990). The present study identified these two groups as been immunologically distinct. The cross reactivity of these two species with other A. viscosus and A. naeslundii was due to antigens common to Actinomyces. Johnson et al. (1990) placed strains which would fall into Fillery clusters 1,2,3,4 and 6 into A. naeslundii-genospecies II. This study showed that three serovars exist within this group.

C) Three strains identified as A. naeslundii-serotypes II & III and Actinomyces-serotype NV were compared. The results showed that A. naeslundii-serotype II and Actinomyces-serotype NV reacted most strongly with the antiserum specific to clusters 1,4 and 6. A. naeslundii-serotype III reacted most strongly with the antiserum specific to cluster 3.

D) Any future clinical trial in humans examining the humoral response to A. viscosus and A. naeslundii would involve choosing four strains as antigens. That is, one

strain from each of clusters 2, 3 and 5 and one representative of the group consisting of clusters 1, 4 and 6. To remove any cross reacting antigens, it would be necessary to pre-absorb the patients antiserum with the appropriate strain. The final titres of absorbed antisera measured for each of the four strains selected would be a reflection of a challenge from that strain and not a result of extensive cross reactivity between several strains.

CHAPTER 1

LITERATURE REVIEW

1) Oral Cavity

I) Introduction

The oral cavity includes both hard and soft tissue surfaces which provide various habitats for microorganisms. A broad division of habitats can be based on cell shedding mucosal surfaces and the nonshedding enamel of the tooth, both of which are bathed by saliva. The junction between these areas occurs at the gingival sulcus and the unique anatomical arrangement in this junctional area and its close association with dental plaque provides the environment for the development of periodontal disease. The brief review presented in this chapter will serve to introduce a non-dental reader to some of the significant aspects of the mouth and their relationship to oral disease.

II) Periodontium

a) Health

In health, teeth remain stable in the gingiva because of their secure connective tissue attachment to alveolar bone. This connective tissue, the periodontal ligament, forms a link between alveolar bone and a calcified matrix on the root of the tooth called cementum (Figure 1.1). At the junction of the cementum and enamel lies a collar of epithelial tissue that is attached to the tooth.

Teeth are the only structures that perforate epithelium anywhere in the body. It is this unique perforation of epithelium that requires a special junctional tissue. In health, the epithelial attachment around each tooth is called the dentogingival junction. This junction causes a slight invagination (0-3mm) in the tissue around the tooth which is called the gingival sulcus.

If local irritation of the gingival tissue occurs certain histologic changes arise in the tissues mediating attachment (Page & Schroeder, 1976). In brief, this entails acute inflammation and subsequent loss of connective tissue (gingivitis). If the irritation

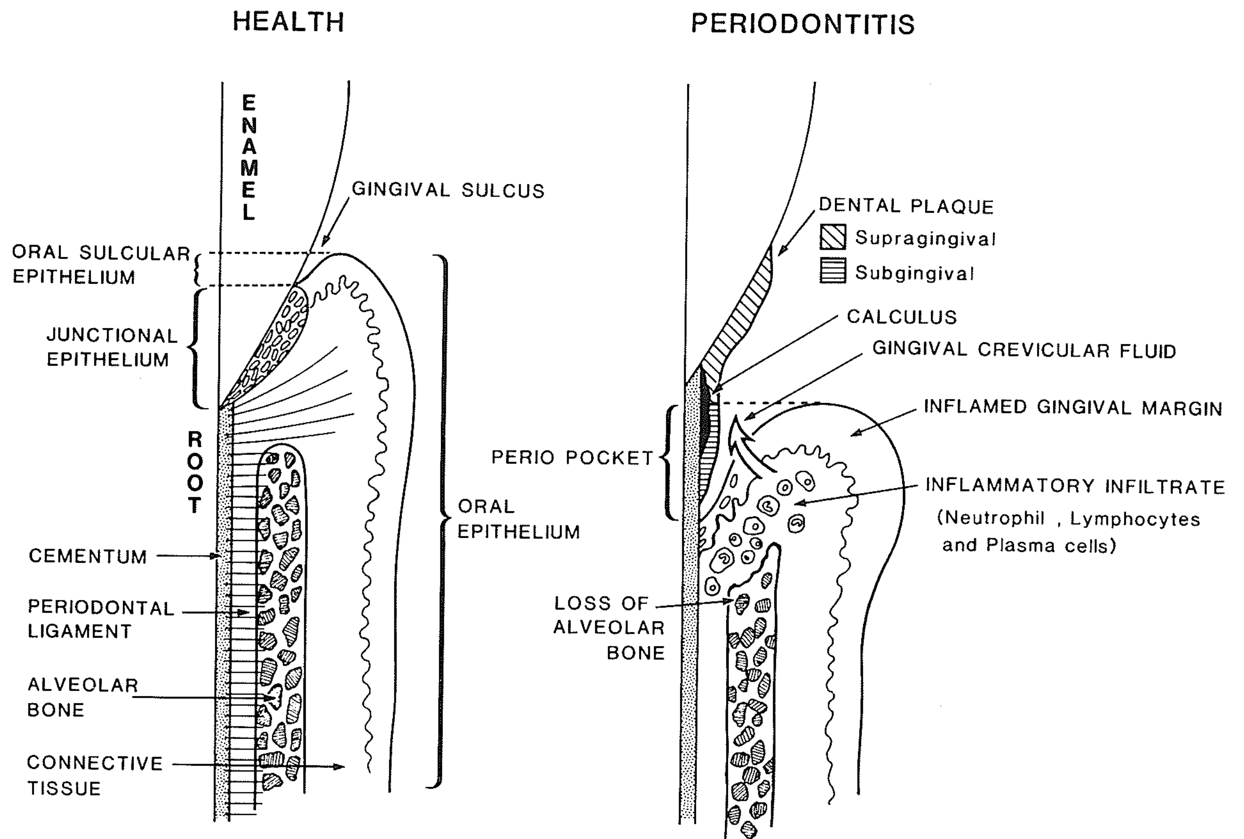


Figure 1.1. Diagram of periodontal health and periodontitis.

persists then chronic inflammatory cells are generated and there is slow resorption of the alveolar bone with loss of connective tissue attachment between the tooth and the gingiva (periodontitis).

The ability to maintain the gingival sulcus and periodontium intact depends on a balance between the irritation that arises from dental plaque, the response of the host's immune system and environmental factors that control plaque. One of the difficulties in explaining the aetiology of periodontal disease is the problem of deciding when this balance has shifted in favor of disease or when the disease is inactive.

b) Disease

Diagnosis of periodontal disease is based on subjective methods a describing disease that has occurred. For example, radiographic bone loss, pocket depth (loss of gingival attachment), and tooth mobility have all been used to describe periodontitis. Currently, periodontal researchers are trying to identify active disease using objective markers.

For many years, it was assumed that periodontal disease was chronic and that all teeth in a given individual would lose the support of alveolar bone at the same time (continous disease hypothesis). However, Goodson et al. (1982) showed that loss of attachment followed a cyclic pattern of loss of supporting connective tissue, repair of lost support, and also periods of disease inactivity. When sophisticated statistical methods were applied to analyze the occurrence of periodontal disease, the continous disease hypothesis was again brought into doubt (Haffajee et al. 1983). Socransky et al. (1984) showed that bursts of disease activity occurred randomly at periodontal sites throughout the mouth. A given site could show a burst of disease over a period of a few days to months and, subsequently, a period of remission. This pattern was called the random burst model. This model takes into account that periodontal disease occurs in an episodic fashion in different areas of the mouth and at different times. The concept of periodic disease activity has not been accepted by all. Ralls & Cohen (1986) suggested

that bursts of change can arise due to measurement error and the model should be subjected to further, more rigorous testing. In a review of disease activity, Rezende et al. (1986) pointed out that it would be desirable to detect periods of disease activity at individual sites. Research to define objective markers of active disease has focused on microbiology of dental plaque and the composition of the gingival crevicular fluid.

III) Teeth

a) Health

The integrity of enamel in health, is controlled in part by saliva. Salivary flow physically removes bacteria but it also has a buffering capacity to neutralize the effects of acids on tooth enamel. Enamel does not have an active repair process if it is decalcified, but saliva is able to promote re-mineralization of early enamel demineralization. Saliva is maintained supersaturated with calcium phosphate (Gron & Hay, 1976) by acidic proline rich proteins, statherin, and cysteine-containing phosphoproteins that bind calcium (Hay et al. 1982 and Mandel, 1989). This allows saliva to recalcify early subsurface demineralization without spontaneous precipitation of minerals onto the teeth (Hay, 1984).

b) Disease

The development of caries is dependent on the interrelationship between three factors. These are: availability of substrates, host factors (mainly teeth and saliva) and associated microflora. The availability, duration, and frequency of a substrate (carbohydrate) will ultimately affect the caries process. A characteristic of a cariogenic microflora is its ability to use this substrate and form acid which causes enamel demineralization (Gibbons, 1964). Caries occurs when the rate of demineralization exceeds the ability of saliva to remineralize teeth. All areas of a tooth are not equally susceptible to the development of a carious lesion. Though any area of a tooth can develop caries, it is the pit and fissure, approximal (Boyar & Bowden, 1985), and exposed root surfaces (van Houte et al. 1990) that are most commonly affected.

The importance of saliva and its effect on microbial populations in dental plaque is exemplified in patients that suffer from radiation induced xerostomia when aciduric organisms predominate. With a decrease in salivary flow, there are pronounced microbial population shifts in the mouth. Cariogenic organisms gain prominence at the expense of organisms associated with health. There are pronounced increases in Streptococcus mutans, Lactobacillus and Candida with decreases in Streptococcus sanguis, Neisseria and Fusobacterium (Brown et al. 1975). There is a marked increase in caries in these patients associated with this increase in opportunistic organisms.

IV) Factors Influencing Oral Homeostasis

a) Saliva

Saliva serves many important functions including lubrication, defence of the oral cavity against invading microorganisms and maintenance of enamel integrity (Shannon et al. 1974; Mandel & Wotman, 1976; Mandel, 1987 and Mandel, 1989).

i) Lubrication

The lubricating properties of saliva have been attributed to mucin glycoproteins. These help coat food for easier swallowing but also maintain mucous membrane integrity. The mucins are able to concentrate on mucous membranes where they form a barrier against desication and environmental insult (Tabak et al. 1982). Saliva coats all areas of the mouth to a thickness of 0.07-0.10 mm (Collins & Dawes, 1987) and this thin layer of saliva moves at different rates in different areas of the mouth (Dawes et al. 1989). Slow movement of saliva prolongs the clearance from dental plaque of metabolic products like acid. A computer model suggested that a low salivary film velocity resulted in areas of low pH for long periods of time which would promote enamel demineralization (Dawes, 1989).

ii) Protective Properties

The major protective function is to maintain an ecological balance by lavage, reducing the adherence of bacteria by both immunological and nonimmunological means, and direct antibacterial activity.

Although saliva is low in total protein compared to serum (Mandel & Wotman, 1976), more than 40 proteins have been identified in salivary secretions (Mandel, 1989). Proteins in saliva are produced by the acinar cells and stromal (ductal) cells. Another contributor to total salivary protein is leakage of serum proteins into saliva.

The first group of proteins that are produced by the acinar cells is the proline-rich proteins. This family is important as they serve as receptors for bacteria during early microbial colonization of a cleaned tooth surface (Gibbons, 1989). These proteins account for 60%-70% of the total protein in submandibular and parotid saliva (Mandel, 1989). Also produced by the acinar cells are the antibacterial agents lyzozyme, lactoferrin and lactoperoxidase along with the important secretory component that makes secretory IgA (sIgA) more resistant to proteases (Lindh, 1975).

The second group of proteins are produced by ductal cells and include the major mucosal immunoglobulin, sIgA (Mestecky and McGhee, 1987). One function of sIgA is to inhibit attachment for certain microorganisms to oral surfaces. Williams and Gibbons (1972) showed that if Streptococcus salivarius was coated with sIgA, it was unable to attach to buccal epithelial cells, although uncoated cells did attach.

The third group of proteins found in saliva is derived from leakage of serum via gingival crevicular fluid. The major contributors to saliva from serum are albumin and IgG. Immunoglobulins, like IgG, can also find their way into saliva via leakage into the gingival crevicular fluid.

b) Gingival Crevicular Fluid (GCF)

The attachment of the gingiva to the teeth (dentoginigval junction) is anatomically complex (Figure 1.1). The dentogingival junction can be divided to two.

parts. The first is the sulcular epithelium and the second the junctional epithelium (Listgarten 1972). The junctional epithelium forms a collar around the tooth and consists of non-keratinized, flattened squamous cells with a large amount of intercellular space. The junctional epithelium consists of 18% intercellular space, while the sulcular epithelium is 12% intercellular space (Schroeder & Munzel-Pedrozzoli, 1970). It is this large amount of intercellular space that allows for leakage of a serum like fluid called the gingival crevicular fluid. The composition and function of gingival crevicular fluid has been extensively reviewed by Cimasoni (1974 & 1983) and Golub & Kleinberg (1976).

The rate of flow of GCF through the junctional epithelium increases with increasing gingival inflammation (Brill, 1959 and Loe & Holm-Pedersen, 1965). The GCF is considered a serum transudate since Tollefsen and Saltvedt (1980) examined GCF and serum from the same patient by crossed immunoelectrophoresis and showed that the protein composition of the GCF mirrored that of plasma. Schroeder (1977) showed that molecules in GCF of molecular weight less than 700,000 daltons were able to pass into and out of the connective tissue at the junction between the tooth and the gingiva.

Gingival crevicular fluid flow has been viewed as both protective and detrimental to the host tissues (Loe, 1968). The protective role includes the positive flow out of the gingival sulcus which washes out noxious substances produced in dental plaque. One of the major cellular elements present in the GCF are leukocytes which contribute to the phagocytosis of microrganisms, as well as liberating various antibacterial agents like lactoferrin and lysozyme (Fine & Mandel, 1986). In addition to these non-specific agents, Holmberg and Killander (1971) showed that GCF contained IgG, IgM and IgA, but found no secretory piece, possibly reflecting the serum transudate nature of GCF. Loe (1968) proposed potential detrimental effects of GCF. Serum proteins and ions present in the GCF would provide an excellent medium for bacterial growth. This in turn may help the proliferation of certain bacterial species in the gingival crevice.

Renewed interest in GCF has resulted from attempts to find a component that is associated with active periodontal disease. A review by Curtis et al. (1989) stated that GCF analysis was a promising area for research because collection was straight forward and non-invasive. Although no clear evidence has yet been found for a marker of active disease, people have looked at the levels of proteins like the subclasses of IgG (Reinhardt et al. 1989) and fibronectin (Lopatin et al. 1989). Also levels of enzymes like aspartate aminotransferase, beta-glucuronidase, lactate dehydrogenase, and aryl sulfatase have been investigated to see if they are associated with disease (Harper et al. 1989; Lamster et al. 1989 and Persson et al. 1990).

c) Nutrition

Consumption of food is not needed for plaque formation. Bacterial communities develop even when animals and human are fed by intubation (Bibby, 1976). Bibby felt that the natural environment of the mouth was more important for the development of a given bacterial community than transient food. The host's contribution of nutrients would consist of saliva (DeJong & Van der Hoeven, 1987) and the gingival crevicular fluid.

For example, saliva is known to contain cortisol at levels found in plasma (Walker et al. 1978). Investigations have shown that estradiol and progesterone can substitute for vitamin K, an essential growth factor for Bacteroides intermedius (Prevotella intermedia)(Kornman & Loesche, 1979). It was postulated that elevated levels of progesterone and estradiol found during pregnancy were associated with increased levels of Prevotella intermedia (Kornman & Loesche, 1980), although this was not corroborated by Jonsson et al. (1988).

Nutritional requirements may also affect the virulence of certain oral organisms. Gibbons and Macdonald (1960) showed that hemin is a requirement for growth of Bacteroides gingivalis (Porphyromonas gingivalis). Similarly, McKee et al. (1986) showed that if a strain of Porphyromonas gingivalis (W50) cells were grown in excess

hemin the strain became highly virulent and caused 100% mortality in mice, whereas cells grown under hemin restriction were less virulent and only caused a 20%-50% mortality in mice. Therefore, the presence of hemin and the ability to compete successfully for this substrate may affect the virulence of Porphyromonas gingivalis. Mayrand and McBride (1980) showed that infectivity of Porphyromonas gingivalis was dependant on the presence of a second organism. The second organism had to be one that produced succinate as this could replace the hemin requirement, consequently, organisms which produced succinate would stimulate growth of Porphyromonas gingivalis. The local atmospheric conditions of a given oral area may also explain why certain microrganisms are present in a given site.

d) Atmosphere

In general the mouth provides an aerobic environment, although areas like the maxillary and mandibular buccal folds have a much lower oxygen concentration than the tongue (Eskow & Loesche, 1971). In general, anaerobic bacteria are not found in large numbers on the tongue although Milnes (1987) was able to isolate anaerobic organisms like Prevotella melaninogenica and Fusobacterium from the tongue surface. The surface topography of the tongue with its crypts and papillae may provide some areas with a low redox potential that would be suitable for these anaerobes. Other areas of low oxygen tension also occur in dental plaque and periodontal pockets.

Increase in plaque thickness (Ritz, 1967), or increase in pocket depth due to loss of attachment (Kenny & Ash, 1969), also tend to reduce oxygen tension. The change in a bacterial population (aerobic to anaerobic) seen in these areas may in part be explained by the reduced environment.

V) Conclusion

The oral cavity provides a series of habitats like teeth, tongue and periodontal pockets that are colonized by different oral microorganisms. This, in part, is dependent on the substrata that is present. One interesting area is the gingival sulcus where soft

tissue is traversed by hard tissue (teeth). This unique area requires a special junctional attachment that is susceptible to inflammatory degenerative changes (periodontal disease). Gingival crevicular fluid and saliva contain various antibacterial substances but also contain factors like hormones that may be exploited by the oral microrganisms. Nutrients and local atmospheric conditions may also affect which types of oral bacteria develop in a given habitat.

2) Resident Oral Flora

I) Introduction

All individuals carry a microbial flora resident in the oral cavity. This resident flora is generally stable but does show some variation from individual to individual. The flora also varies within different areas (habitats) of the oral cavity. The following is a brief overview of the oral microflora in health and the structural composition of dental plaque. Dental plaque on tooth surfaces and in the gingival sulcus represents bacterial communities composed of several populations. Growth as a biofilm on teeth ensures that organisms do not get removed easily.

II) Resident Oral Flora

The mouth is sterile at birth (Carlsson et al. 1970). The establishment of the early flora is, in part, initiated by contact between the neonate and the mother. Bacteria that colonize the oral cavity have developed special characteristics which enable them to survive against the cleaning and antibacterial effects of saliva, immunological mechanisms and competition from other members of the developing flora (Bowden, Ellwood, and Hamilton, 1979). Table 1.1 provides a list of bacterial genera that have been islolated from the oral cavity.

Table 1.1-Bacterial Genera Found In the Human Mouth

Streptococcus	*Lactobacillus*	*Neisseria*
Haemophilus	*Arachnia*	*Bifidobacterium*
Actinomyces	*Eubacterium*	*Propionibacterium*
Rothia	*Bacterionema*	*Veillonella*
Bacteroides	*Fusobacterium*	*Campylobacter*
Peptococcus	*Peptostreptococcus*	*Selenomonas*
Eikenella	*Leptotrichia*	*Micrococcus*
Spirochaeta		

Bowden, Ellwood and Hamilton-1979

The isolation of a given bacterial species from the mouth depends in part on the area that one examines. The diversity in the environment within the oral cavity is reflected in the complexity of the flora (Bowden, Ellwood and Hamilton, 1979). For

example, the oral mucosa and tongue provides a surface with a high rate of cellular turnover, while teeth provide a stable nonshedding surface for colonization and the development of bacterial communities (dental plaque). In order to simplify this overview of the resident oral microflora, the oral cavity can be divided into two general areas; firstly, the tongue and the oral mucosa, and, secondly on the tooth surface there exists supragingival and sulcular plaque.

a) Oral mucosa and Tongue

i) Oral Mucosa

Few studies have been made of the flora associated with the palatal and vestibular mucosa of the mouth. Continous cell desquamation prevents the accumulation of large masses of bacteria. The number of microorganisms found on the mucosa is a reflection of their ability to attach to the mucosa, their rate of growth and also their numbers in the saliva that washes the mucosal surface (van Houte, 1982). For example, Streptococcus mitior, Streptococcus sanguis and Streptococcus salivarius have been frequently isolated in high numbers on the buccal mucosa (Gibbons, 1984), but Veillonella and Neisseria are found on the buccal mucosa in low numbers (Liljemark & Gibbons, 1971).

ii) Tongue

The tongue, with its large surface area due to the crypts and papillae, is an effective reservoir for oral bacteria. The most common isolate from the tongue is Streptococcus salivarius (Milnes, 1987). Other prominent Streptococcal species associated with the tongue include Streptococcus mutans, Streptococcus mitior and Streptococcus milleri in decreasing frequency of isolation. Other frequently isolated genera include, Neisseria, Veillonella and Actinomyces. Of the Actinomyces associated with the tongue, Actinomyces naeslundii was present in higher numbers than Actinomyces viscosus (Milnes, 1987).

Since the mouth of the neonate is edentulous, the tongue represents the largest colonizable area for bacteria. Streptococcus salivarius is one of the first organisms to

colonize the mouth (Carlsson et al. 1970) and remains a major component of the flora associated with the tongue in later life. The tongue provides an excellent example of how a community initially develops, based on the type of colonizable habitat available. When teeth erupt they provide a stable, non-shedding enamel surface that is colonized by bacteria which develop into a complex bacterial community (dental plaque).

b) Supragingival Dental Plaque (SGDP)

The microbial composition of SGDP has been one of the most extensively studied aspects of the microbiology of the oral cavity. Dawes et al. (1963) defined dental plaque as the soft, concentrated mass consisting mainly of a large variety of bacteria, together with a certain amount of cellular debris, which develops within a short time of refraining from toothbrushing. Growth of dental plaque follows a series of steps. The initial step being deposition of the acquired pellicle to which various bacteria can attach.

i) Acquired pellicle

The acquired pellicle has an acellular structure that is essentially bacteria free and is deposited after the eruption of a tooth (Dawes et al. 1963). The amount of the pellicle that can be removed from a tooth surface increases for the first 1.5 hours after cleaning of the teeth (Sonju & Rolla, 1973). It is thought that cleaning of the teeth exposes phosphate groups which interact with calcium ions in saliva to form bridges with salivary components.

Pellicle will form on oral hard tissues (i.e., teeth and dental restorations) as well as oral soft tissues (keratinized and non-keratinized oral epithelium). The composition of the pellicle has been reported by Meckel, 1965; Armstrong, 1967; Leach et al. 1967 and Rykke et al. 1990). Leach et al. (1967) found that saliva, acquired pellicle, and the aqueous extract of the plaque matrix all had an amino acid composition characteristic of glycoproteins, but only the acquired pellicle was composed of seemingly intact and specific salivary glycoproteins. Further investigation showed that approximately 45% of the pellicle consisted of amino acids and a high concentration of

carbohydrates including significant quantities of hexosamines. Rykke et al. (1990) showed the amino acid profiles of the acquired pellicle were consistent both between and within individuals over a 2 year period. The fact that the acquired pellicle does not vary from tooth to tooth indicates that the pellicle is formed by a mechanism of selective adsorption of salivary proteins to the enamel surface. By use of indirect immunofluorescent antibody staining other constituents have been demonstrated. These included IgA and lysozyme which were common components, and amylase and IgG which occur less frequently. Albumin and fibrinogen are rarely detected (Orstavik & Kraus, 1973). Formation of the acquired pellicle serves as a base upon which dental plaque will be deposited.

ii) Structure and Chemical Composition of Supragingival Dental Plaque

Dental plaque is composed of members of the resident flora within an acellular matrix. Formation of dental plaque starts with isolated bacteria growing in microcolonies on the teeth and, with time, these microbial colonies coalesce. As the microcolonies increase in size the intermicrobial matrix that helps to bind the organisms together also increases. This mass of bacteria and matrix forms on the tooth surface a bacterial community described as dental plaque.

Dental plaque is composed of approximately 80% water and 20% solids (Bowen, 1976). The solids of dental plaque consist of inorganic and organic components. Inorganic components include calcium and phosphorus and the organic component is composed extensively of protein and carbohydrates derived from saliva and bacteria.

Plaque is able to concentrate calcium, inorganic phosphate, sodium and potassium relative to saliva. It is possible those cations that are in excess of salivary levels are bound non-specifically to plaque proteins. However, potassium may be primarily intracellular in bacteria (Dawes & Jenkins, 1962). The calcium content of plaque obtained from different areas of the mouth varies, for example, plaque from the

mandibular teeth can contain significantly more calcium than plaque from other areas. The high calcium binding capacity of plaque is probably important in movement of calcium in and out of the enamel and in the initiation of calculus formation (Mandel, 1966).

Protein comprises 35-47% of the bulk of the solid phase of plaque (Bowen, 1976). The amino acid composition of the plaque appears to be little influenced by diet. Eastoe and Bowen (1971) showed in primates that the amino acid composition of plaque did not vary greatly between animals fed a normal diet and those fed their entire diet by gastric intubation. They concluded that proteins in dental plaque, whether synthesized by microorganisms or not, were derived from saliva and not newly ingested food. The type of carbohydrate utilized by the host determines the type of extracellular polysaccharides formed by oral bacteria (Guggenheim & Schroeder, 1967). The bulk of polysaccharide in plaque is extracellular matrix and contributes 10-20% to the plaque volume (Bowen, 1976). If a person consumes a diet rich in sucrose, glucan is by far the major polysaccharide in plaque. The level of fructan in plaque depends on the period of time since the last intake of sucrose. It has been suggested that such carbohydrate polymers may be responsible for the change from reversible to a irreversible adherence by bacteria to the teeth. These extracellular polysaccharides stabilize the developing community of bacteria so it does not get washed away by saliva or the cleansing action of mastication.

iii) Histology of SGDP

Formation of plaque has been studied by various histological techniques. Sample preparation in terms of preparing calcified materials without damaging the loosely adhering material is difficult. Some authors have used different materials like millipore filters (Eastcott & Stallard, 1973) and plastic films (Brecx et al. 1981) as a subtrata for plaque formation. Brecx et al. (1981) felt that the use of a plastic film may prevent the loss of loosely adhering material during histological preparation. More

recent papers have tried mimicking the oral cavity more closely by using enamel slivers embedded in acrylic holding devices (Nyvad & Fejerskov, 1987 a, b).

Table 1.2 Histological Studies of Early Plaque Formation

	Histology			Time	Technique
Author	Light Mic.	Scan EM.	Trans Em	frame-hrs	used
Saxton-73		*		24	Impression of plaque
Eastcott & Stallard -73		*		1-48	Millipore filter
Listgarten-76	*		*	Normal gingiva	Extracted teeth
Brecx et al.-81			*	4-8	Plastic film
Nyvad et al.-87a		*		4-48	Enamel sliver
Nyvad et al.-87b	*		*	4-48	Enamel sliver

There is general agreement from the studies listed in Table 1.2 on the sequence of events in plaque development. After the surface of the tooth is cleaned, there is deposition of an acellular layer on the tooth which is consistent with the acquired pellicle. The publication by Nyvad and Fejerskov (1987a) provides a nice summary. Initially a few coccoid or coccobacillary organisms can be seen in microcolonies adhering to the surface in pits, grooves, or enamel irregularities. After 8 hours there is an increase in the number of rod shaped bacteria on the surface. Bacteria proliferate out as a monolayer with eventual fusion of neighboring microcolonies. Central parts of the microcolonies eventually show a multilayered structure with the bacteria being embedded in considerable amounts of intercellular material. By 48 hours, the surface is covered by a morphologically heterogenous biofilm consisting of coccoid, filamentous and rod shaped bacteria.

iv) Microbiology of SGDP

-Adherence

In its mature form, dental plaque is mainly a product of microbial growth. The formation of plaque follows a dynamic process that can be divided into two catagories: first, the adherence of bacterial aggregates, and secondly, growth of organisms (Saxton, 1973). Initial adherence occurs between specific bacteria and the acquired pellicle. This process is not random but depends on specific interactions between bacterial adhesins and receptor molecules of the acquired pellicle (Gibbons, 1989).

The ability of microorganisms to attach to teeth can be viewed in at least three ways. First, bacteria may have developed certain ligands that allow for their attachment to various host substrata. A. viscosus cells possess two types of protein fimbriae (Cisar et al. 1984) which facilitate adhesion. Type 1 fimbriae mediate binding of A. viscosus cells to salivary pellicle on apatitic surfaces (Clark et al. 1986) while type 2 fimbriae have a galactosyl-binding lectin which mediates attachment of A. viscosus to mammalian cells and bacteria (Ellen et al. 1980).

Many bacteria bind to saccharides, but some can bind to protein receptors. Studies of components of saliva that adsorb onto hydroxyapatite and promote the attachment of plaque bacteria have revealed that the family of acidic proline-rich proteins (PRPs) promote attachment of strains of A. viscosus, Porphyromonas gingivalis and Streptococcus mutans (Gibbons, 1989). A second fraction of saliva that promotes attachment contains the protein statherin and both fractions (PRPs and statherin) are phosphoproteins. It was an unexpected finding that A. viscosus adhered avidly to PRPs that were bound to apatitic surfaces but not to PRPs free in solution. It was postulated that PRPs undergo conformational changes when they adsorb onto apatitic surfaces exposing a receptor for A. viscosus 'cryptic region' (Gibbons, 1989). Adhesins of A. viscosus recognize these exposed cryptic regions of PRPs once they have undergone this conformational change. The hidden receptors for bacterial adhesins were named

cryptitopes (Gibbons, 1989) and this concept would provide an explanation of how A. viscosus, which is an early colonizer of dental plaque (Nyvad & Kilian, 1987c), can preferentially bind to teeth even though PRPs are present in saliva.

A second mode of attachment occurs by the organism exploiting host mechanisms such as secretory IgA. Liljemark et al. (1979) showed that IgA was required for the adsorption of S. sanguis to saliva treated hydroxyapatite. The removal of the IgA from the coating saliva lead to a decrease in adherence. Although IgA is part of the host defence mechanism, it is evident that it can also be exploited by certain bacteria to facilitate their establishment in the oral cavity.

A third mode of attachment is by coaggregation between bacteria. This was initially shown by the histological study of Listgarten (1976) when cocci were often found to coat the surface of filamentous organisms. Kollenbrander and Williams (1981) showed that strains of A. viscosus and A. naeslundii could aggregate with whole cells of S. sanguis. Cisar et al. (1989) identified a role for type 2 fimbriae of Actinomyces species in their coaggregation with S. sanguis and their attachment to epithelial cells and polymorphonuclear leukocytes.

Although it is likely that other mechanisms of bacterial adhesion operate in the mouth, there is good evidence that the examples shown above contribute to colonization of the oral cavity and to the development of dental plaque.

-Supragingival Dental Plaque (Cultural)

As shown by the histological studies, formation of the dental plaque matrix is initiated by the attachment of certain Gram-positive members of the indigenous oral flora with subsequent changes over time. There have been several cultural studies which describe these changes. Nyvad and Kilian (1987c) studying the bacteriology of early plaque formation, found 77% of the bacteria present in the monolayer were Streptococcus sanguis, Streptococcus mitis and Streptococcus oralis and another 7% were Gram-positive rods. Thirty nine percent of the rods were identified as

Actinomyces, while 29% were assigned to Arachnia, Corynebacterium, Rothia and Propionibacterium. One third of the Gram-positive rods could not be identified either due to insufficient growth or incomplete results. Of the identified Actinomyces, A. naeslundii was the most frequently isolated followed by A. viscosus, but this varied among the plaque samples from different patients.

As plaque matures, the number and type of bacterial species changes. Table 1.3 taken from Bowden, Ellwood and Hamilton, (1979) is a summary that shows the microbiological changes that occur in plaque over a period of 14 days

Table 1.3 Bacteria Detected at Various Stages of Plaque Development on Cleaned Surfaces in the Mouth.

Time after cleaning	Species present
0-15 min	*S. sanguis, S. salivarius, A. viscosus, "corynebacterium"*
1-18 hrs	*S. sanguis, S. mitis, S. epidermidis, A. viscosus, Peptococcus sp.*
24-48 hrs	*S. sanguis, S. salivarius, A. viscosus, R. dentocariosa, L. casei, Veillonella sp., Fusobacterium sp., Neisseria sp.*
3-5 days	*S. sanguis, S salivarius, A. viscosus, A. naeslundii, A. odontolyticus, R. dentocariosa, L. buccalis, E. saburreum, A. israelii, B. melaninogenicus, Neisseria sp., Veillonella sp., Lactobacillus sp.*
6-14 days	At this time, the plaque reaches its most complex community

Bowden, Ellwood and Hamilton-1979

As shown by the above table, some anaerobic species like Veillonella and Fusobacterium are not detected for at least 24 hours. Ritz (1967) showed that growth of these anaerobic organisms was dependant on prior growth of aerobic and facultative organisms. These first colonizers used the available oxygen and therefore suitable anaerobic conditions were created.

Bowden et al. (1975) sampled approximal dental plaque from the distal surface of the maxillary first premolar of ten 13-14 year old children over a period of 12 weeks. The mean percentages of the predominant genera were Actinomyces 34.92, Streptococcus 22.9, Veillonella 13.07 and "bacteroides" 7.97. Species percentages

were Streptococcus mutans 0.51, A. israelii 16.5 and A. viscosus together with A. naeslundii 19.05.

The above data shows that Actinomyces and Streptococcus form a significant percentage of supragingival plaque. This was also true for plaque allowed to develop for 2 days (Nyvad and Kilian, 1987c).

-Sulcular Dental Plaque (Cultural)

A shallow sulcus exists around each tooth. In health, this sulcus can vary in depth from 0 to 3mm. The flora associated with this area in health has been poorly studied, while the most information on the microbial flora of the sulcus is that which is associated with disease. If one compares the results of Slots (1977) for the microflora of a healthy gingival sulcus to the control (health) group results of Newman and Socransky (1977), and the time zero results of the longitudinal gingivitis study of Syed and Loesche (1978) there are many similarities. Newman and Socransky (1977) found the flora to be made up largely of Gram-positive organisms and Slots (1977) found that the flora of healthy sites included 44.6% and 40.4% Gram-positive rods and cocci, respectively. In general, healthy gingival sulcus sites had a low proportion of Gram-negative organisms. Syed and Loesche (1978) found that as sulcular plaque matured Streptococcus species started to decline and there was an increase in Actinomyces species.

III) Conclusion

Normal healthy individuals all carry a resident oral flora which will vary in the different ecosystems in the mouth. In disease, local changes in the flora occur, and in specific instances e.g., xerostomia and chemotherapy, the composition of the flora of the ecosystem may be changed.

Plaque results from a balance between the formation and removal of microorganisms on the tooth surface. Although certain bacterial genera like Actinomyces and Streptococcus are major components of the supragingival flora there

are many other representatives that appear as plaque matures. If a "normal" balance is maintained between formation of dental plaque and its removal then the oral tissues remain in a state of health.

3) Oral Microflora Associated With Disease

I) Oral Microbial Ecology

The oral cavity supports communities of commensal bacteria in local habitats and the composition of these communities can vary significantly in different habitats. The habitats include nonshedding tooth surfaces, shedding mucosal surfaces and finally habitats formed at the junction of the teeth and gingiva. The development of a given community is enhanced by the presence of a relatively complex nutrient supply normally present (e.g., GCF), as well as an intermittent supply of rich and varied nutrients (e.g., carbohydrate) from the host diet. Each different organism (species, strain) within dental plaque is termed a population, a variety of populations forming a community (dental plaque). Each organism in the community occupies or has a niche which implies a functional and not a specific physical area. Thus, carbohydrate fermentation would be one parameter of the niche of Streptococcus. Elimination of the niche from a habitat will result in the elimination of the population 'occupying' that niche. Similar bacteria will compete for the same niche and the most successful will be able to increase the size of its population. The type of habitat and competition for a niche will affect which bacteria achieve prominence in a given area (Bowden et al. 1977) Finally, the apparent composition of plaque revealed by cultural analysis is affected by the area to be sampled, the method of sampling and dispersion techniques, isolation and culture procedures, and the identification techniques (Hardie & Bowden, 1976). These problems in conjunction with the inability to easily identify active disease sites in caries, periodontal diseases and gingivitis have frustrated oral microbiological researchers.

Studies that have attempted to relate successful competition (prominence) of a microorganism to the cause of the disease have produced two general philosophies. These are the non-specific plaque hypothesis and the specific plaque hypothesis.

II) Non-specific Plaque Hypothesis

Loesche (1976) explained that the uniqueness of dental infections relates to: a) lack of tissue invasion by bacteria, (b) their non-specific nature, (c) their chronic nature which makes documentation difficult, and (d) their common occurence in the adult population. The apparent non-specific nature of the microflora associated with the disease (i.e., no single pathogen) led people to believe that oral disease was due to the accumulation of the entire plaque flora rather than one or a few members of the community. This dilemma is typified by the studies on the association of bacteria to periodontal disease.

In one extensive study of patients with moderate (chronic) periodontitis 171 taxa representing 1900 bacterial isolates were identified (Moore et al. 1983). In this paper and a previous paper by Moore et al. (1982a), which reviewed the bacteriology of severe periodontitis, no one bacterial species was identified as the causative agent of this disease. The non-specific plaque hypothesis may, therefore, be valid in certain cases, but there is also evidence that the specific plaque hypothesis may also be valid in a given situation.

III) Specific Plaque Hypothesis

This hypothesis proposes that only certain pathogenic organism(s) are associated with the different oral diseases (Loesche, 1976). These pathogenic bacteria are often prominent at a diseased site being at an advantage over other members of the community. If a specific organism causes a given disease then it can either be an opportunistic member of the resident oral flora, or an exogenous oral pathogen which is transient in the oral cavity (Genco et al. 1988).

a) Opportunistic Oral Pathogens

This situation can be illustrated with studies that examine the change in the oral microflora with developing gingivitis. Actinomyces are members of the resident flora that appear as early colonizers of the tooth surface. As gingivitis develops, it is

associated with increases in Actinomyces in dental plaque, but the species involved vary from site to site (Loesche & Syed, 1978; Syed & Loesche, 1978 and Moore et al. 1982b). Gusberti et al. (1988) reduced bleeding gingivitis with 0.12% chlorhexidine and 1% hydrogen peroxide and associated with the decrease in gingivitis was a marked reduction in A. viscosus and to a lesser degree a reduction in A. naeslundii. These data suggest that a decrease in gingivitis promoted by chlorhexidine is associated with a decrease in A. viscosus and A. naeslundii. The effect produced by chlorhexidine may be due to its ability to inhibit the colonization of the tooth surface by Actinomyces and, thereby, prevent maturation of dental plaque (Gusberti et al. 1988). Although gingivitis appears to be related to overgrowth by members of the resident flora, periodontitis may be due to exogenous infecting organisms.

b) Exogenous Oral Pathogens

Evidence for this aspect of the specific plaque hypothesis may be found with localized juvenile periodontitis. This disease causes severe loss of alveolar bone associated with the incisor and molar teeth in teenage patients. There is a strong association between Actinobacillus actinomycetemcomitans (Aa) and areas of bone loss. Zambon et al. (1983) found that 97% of localized juvenile periodontitis (LJP) patients harbored Aa in their subgingival plaque compared to levels that were 4-5 times lower in healthy subjects. A role for Aa as an exogenous oral pathogen is a difficult to establish and opinions vary. A review by Zambon (1985) suggests that the primary ecological habitat for Aa is the oral cavity and that it is present in up to 36% of the normal population. In contrast, Genco et al. (1988) suggest that Aa is an exogenous oral pathogen and sites a paper by Zambon et al. (1986) in which subgingival plaque samples in 283 patients were examined for the presence of Aa using indirect immunofluorescence. Sixty-four percent of the patients did not show detectable levels of Aa and none of these subjects had levels greater than 5% of the total subgingival flora. The fact that Aa is present either in low levels, or not at all, suggests that this

microorganism may not be a member of the indigenous oral flora and is an exogenous oral pathogen associated with localized juvenile periodontitis (Genco et al. 1988). Further evidence that Aa may be an exogenous oral pathogen comes from studies of families with multiple cases of LJP. All members of a family harbored Aa of the same biotype and serotype (Zambon et al. 1983) and, in one case, Aa from a child was of the same clonal type as the family dog (Preus & Olsen, 1988).

It would be ideal to fulfill Koch's postulates to confirm that Aa was the causative organism of LJP . The organism should be isolated from a lesion and, when reinfected into a model system, it should cause the disease. When studies were undertaken to see if an infected periodontal site with Aa could infect a healthy periodontal site in the same patient during probing, Aa was only cultured from the transfer site for a period of 2-3 weeks (Christersson et al. 1985). In this study, the infecting dose may not have been high enough, but one could also question if it is the microorganism alone, or if the environment (substrata and associated microorganisms) must be such that allows Aa to increase. That is, a healthy pocket would not have a niche available for the organism. The question whether an oral disease is the result of a given pathogenic microorganism, or is the result of environmental pressures that occur prior to the disease which allow a specific microorganism(s) to become predominant is difficult to answer.

Carlsson (1988) addressed the above concern about periodontal pathogens and their association with disease. Although he referred to periodontal pathogens and their relation to periodontal disease, the concept is applicable to many oral diseases. Bowden et al. (1977); Carlsson (1988) and Newman (1990) feel that oral pathogens may not be virulent in isolation. The organism is always a member of a complex bacterial community and will not fulfill Koch's postulates in pure culture. Carlsson suggested abandoning the concept of a periodontal pathogen and proposed the term pathogenic microbiota. With this term, one accepts that periodontal diseases (and other oral diseases) have a polymicrobial etiology. An oral disease, therefore, occurs due to shifts

in the proportion of species within plaque (Newman, 1990). Commensal plaque species occur in health and still may cause disease if the organism is pathogenic and the appropriate microbial interactions from other organisms are present. In addition, environmental pressures like gas changes, (aerobic-anaerobic) presence of nutrients that arise from increasing inflammation (e.g.,hemin) can provide new and/or varied ecological niches. The changed niches may provide a selective advantage to specific microorganisms allowing them to become prominent. This prominence of a given microorganism should not be interpreted as evidence that this is the sole causative agent of an oral disease.

4) A. viscosus and A. naeslundii-Pathogenicity, Physiology and Taxonomy

I) Introduction

This chapter reviews the association between A. viscosus and A. naeslundii and disease. The relationship between these organisms and disease demonstrated by various workers are at times contradictory and this may be explained in part by the very close physiological characteristics that these species share, making differentiation difficult. This inability to adequately differentiate the species makes taxonomic classification confusing and one that is still evolving.

II) Pathogenicity

Actinomyces have a limited habitat that consists of the human or animal oral cavity and tonsils, where they are members of the resident flora (Bowden & Hardie, 1973 and Slack & Gerencer, 1975). Actinomyces species have been isolated from the gut but they do not constitute the indigenous flora of these areas (Gossling & Slack, 1974).

a) Extraoral Infections

Actinomyces are not considered virulent pathogens and most infections associated with this genera are mixed (Bowden, 1991). Actinomyces infections called actinomycosis are usually classified as cervicofacial, thoracic, or abdominal with the highest prevalence seen in the cervicofacial region (Jordan, 1982). A study of 3000 cases of human actinomycosis noted that bacteria commensal to the oral cavity were most often associated with the infection (Pulverer & Schaal, 1984). Most infections of the cervicofacial tissues arise from initially localized oral infections that have spread. Also, spreading to the lung from the oral cavity can occur by aspiration, hematogenously or along facial planes of the neck while abdominal actinomycosis is usually preceded by some disease or trauma (Bowden & Hardie, 1973; Jordan, 1982 and Bowden, 1984).

A. israelii is generally considered the major etiological agent in human actinomycosis (Pulver & Schaal, 1984). Studies in animals have found that A. naeslundii and A. viscosus injected intraperitoneally cause abscesses and death in mice (Coleman & Georg, 1969 and Behbehani et al. 1979), however, A. viscosus and A. naeslundii were considerably less pathogenic than A. israelii in this animal model. A. naeslundii can be pathogenic in man and has been isolated from human infections (Coleman et al. 1969). Although most actinomycotic infections are mixed, one case of empyema of the gall bladder was reported as a monoinfection with A. naeslundii (Coleman et al. 1969).

b) Intraoral Infections

A. viscosus and A. naeslundii are associated with early plaque formation (Nyvad & Kilian, 1987c) and remain a dominant component of established dental plaque, accounting for 20% of the total cultivable flora (Bowden et al. 1975). It is difficult to determine if A. viscosus and A. naeslundii are associated with oral disease because of their isolation frequency in health and the difficulty in differentiating these two species physiologically and serologically.

i) Animal studies

Both A. viscosus and A. naeslundii have been shown to cause fissure caries in gnotobiotic rats. A human strain of A. viscosus innoculated into gnotobiotic rats fed a diet high in sucrose developed fissure caries (Llory et al. 1971). However, other studies have not corroborated this and suggest that the cariogenicity of A. viscosus is low (van der Hoeven et al. 1972). This inconsistency may in part be explained by the observation that the former study used a human strain and the latter used an animal strain (Ny1) of A. viscosus. Subsequent gnotobiotic studies of rats mono-infected with strains isolated from humans and identified as A. naeslundii and A. israelii confirmed that both species caused caries (Guillo et al. 1973).

Other studies using gnotobiotic rats have also shown an association between A. viscosus and A. naeslundii and root caries and periodontal disease (Socransky et al. 1970; Jordan & Hammond, 1972 and Jordan et al. 1972). One interesting aspect of the Jordan & Hammond (1972) study was that roughly one third of the Actinomyces isolated from human teeth with root caries could not be identified to the species level. This inability to consistently identify strains of A. viscosus or A. naeslundii makes it difficult to make firm conclusions on the role that these species play in disease.

ii) Human studies

Human studies show the same varied results in the association of A. viscosus and A. naeslundii with disease.

-Enamel Caries

In a detailed study of the development of caries in children there was no difference in Actinomyces in carious and non-carious sites (Hardie et al. 1977). Subsequent studies of nursing caries identified strains to the species level and found that A. naeslundii decreased and A. viscosus increased (Milnes & Bowden, 1985). In the latter study species were separated as caries developed using whole cell agglutination with sera prepared to single representatives of A. viscosus and A. naeslundii. This technique would not allow identification of atypical strains of these species.

-Rootcaries

Early studies have shown that Actinomyces were present within carious lesions of the tooth root with A. viscosus the species most frequently isolated (Sumney & Jordan, 1974). However, recent studies that compared the flora of non-carious to carious root surface sites did not find a positive correlation with Actinomyces species and root caries (Keltjens et al. 1987; Emilson et al. 1988 and van Houte et al. 1990). The first two papers grouped A. viscosus and A. naeslundii together which makes it difficult to assess the significance of each species in the root caries process.

Actinomyces form a significant proportion of the flora from root caries sites. Careful examination of the isolates within Actinomyces would be a good starting point in deciding their relative importance in disease as little is known of the 'intermediate' strains that can be included into both A. viscosus and A. naeslundii (Bowden, 1990). One recent paper examined the isolates in detail and showed that typical A. naeslundii (serovar 1) was not isolated from root surface lesions whereas A. viscosus serovar 2 was positively associated with some root lesions (Bowden et al. 1990). These papers support the observation of Milnes & Bowden (1985) of dominance by A. viscosus in the lesions of nursing caries.

-Gingivitis/Periodontitis

Gingivitis may be considered an intermediate stage between health and periodontal disease. Composition of the subgingival flora in gingivitis is significantly different from the flora associated with health or periodontitis (Moore et al. 1987). Detailed studies of the Actinomyces species associated with experimentally induced gingivitis (Moore et al. 1982b) and naturally occuring gingivitis (Moore et al. 1987) found increases in A. naeslundii serotype 3 as gingivitis developed. In a study of experimentally induced gingivitis, it was shown that rinsing with 0.12% chlorhexidine was associated with a reduction in the severity of gingivitis and associated with this reduction was a 99.9% and a 91-96% decrease in the numbers of A. viscosus and A. naeslundii respectively (Gusberti et al. 1988). Unfortunately, identification of Actinomyces species was based on selective media and it is known that the use of selective media may be inappropriate for differentiation of Actinomyces as atypical strains may not grow on such media (Bowden, 1990).

In contrast to the data for gingivitis there is a decease in Actinomyces as periodontitis develops. Here, research indicates that A. viscosus and A. naeslundii have been shown to decrease as the community becomes dominated by Gram negative bacteria more commonly associated with periodontitis. (Moore et al. 1982a; 1983 and 1987).

The flora associated with gingivitis is consistent with a flora in transition from health to disease (Moore et al. 1987).

III) Physiological Characteristics

a) Morphology

Actinomyces are irregular, nonsporing Gram positive rods (Schaal, 1986). The microscopic appearance varies from branching filaments (e.g.,A. israelii) to short pleomorphic rods (e.g.,A. viscosus and A. naeslundii). When examined by transmission electron microscopy the appearance of Actinomyces is typical of Gram-positive cells (Duda & Slack, 1972 and Slack & Gerencer, 1975). The cytoplasmic membrane is tightly bound to the cell wall and is not readily discernible. Within the cytoplasm, the nuclear region is spherical and centrally located (Slack & Gernecer, 1975). The cell wall varies from 30-64 nm depending on the species examined and appears to be composed of two layers. The outer fuzzy layer of the wall appears to be polysaccharide by its staining characteristics, whereas, the inner layer of the cell wall is thin and more darkly staining. A. naeslundii and A. viscosus show fibril-like structures extending from the cell wall. These fibrils may serve as a mechanism for attachment to teeth, tissues or other organisms (Girard & Jacius, 1974).

b) Growth in Culture

Actinomyces have complex nutritional requirements. Various species of Actinomyces grow well in the fully defined media of Kiel and Porteus (1962) or its modification (Bowden et al. 1976). The modified medium is suitable for physiological and antigenic studies as it can be standardized and all components are dialysable.

The growth of Actinomyces in broth reflects that seen on solid media. Species that grow as smooth regular colonies will grow as smooth suspensions. Strains of A. viscosus and A. naeslundii will produce a smooth ropy sediment presumably because of production of extracellular polymers. In contrast, A. israelii serotype 1, which

produces rough colonies on agar, grows as granular deposits in broth. Most Actinomyces species grow well on a quality nutrient agar such as Blood Agar Base No. 2 CM271 (Oxoid, England) or Brain Heart Infusion Agar (Difco Laboratories, Michigan) supplemented with 5% V/V defibrinated horse or sheep blood (Bowden & Hardie, 1973 and Bowden, 1991). The colonial morphology depends to some extent on the culture medium (Bowden & Goodfellow, 1990).

c) Atmospheric Requirements

Actinomyces are described as facultative anaerobes and most species are preferentially anaerobic although some grow well aerobically. Strains of A. bovis and A. meyeri require strict anaerobic conditions, while A. israelii will occasionally grow aerobically (Bowden, 1991). Incubation in an anaerobic environment with added carbon dioxide is important as Actinomyces can fix carbon dioxide (Schaal, 1984). In broth culture, the addition of sodium carbonate (1-2% of a 1% W/V of sodium carbonate) will provide sufficient carbon dioxide for growth (Bowden, 1991).

d) Physiology and Metabolism

In general, Actinomyces show little capacity to degrade proteins although some species will hydrolyse starch, Tween 40 or 60 and aesculin and they show a wide range of fermentative capacity including mono, di, trisaccharides and sugar alcohols (Schaal, 1986 and Bowden, 1991). They produce formic, acetic, lactic and succinic acids if grown anaerobically with carbon dioxide although varying the atmosphere will alter the end products (Buchanan & Pine, 1965 and 1967).

IV) Taxonomy of Actinomyces

The name Actinomyces was first used by Harz (1879) to describe an organism isolated from cattle. The organism appeared as masses that resembled fungal mycelia therefore the name Actinomyces='ray fungus' was selected (Bowden & Goodfellow, 1990). The confusion that existed in deciding whether the organisms were bacteria or fungi was resolved once chemotaxonomic methods were introduced. Cummins & Harris

(1958) and Cummins (1962) showed that Actinomyces was a distinct genus of bacteria and separate species could be identified. At present there are at least 13 species included into Actinomyces (Table 1.4).

Table 1.4 Species Within the Genus Actinomyces

Species	Habitat	Serotype
A. israelii	Human Oral Cavity	1
A. gerencseriae* Formerly-A. israelii-2	Human Oral Cavity	?
A. bovis	Animals	1,2
A. georgiae*	Human Periodontal flora	?
A. naeslundii*	Human and animal oral cavity	genospec. 1 genospec. 2
A. viscosus*	Animal oral cavity	1
A. odontolyticus	Human and animal oral cavity	1,2
A. denticolens	Oral cavity (cattle)	?
A. howellii	Oral cavity (cattle)	?
A. slackii	Oral cavity (cattle)	?
A. hordeovulneris	Oral cavity (dogs)	?
A. meyeri	Human oral cavity	?
A. pyogenes	Mucosal surfaces,animal and human	?

Bowden and Goodfellow 1990 & *Johnson et al. 1990

V) Taxonomy of A. viscosus and A. naeslundii

a) Historical

A naeslundii named after Carl Naeslund (Naeslund, 1925), was an actinomycete-like organism isolated from the human mouth. A. naeslundii was separated from A. israelii by Thompson & Lovestedt (1951). A closely related species, A. viscosus was first isolated from plaque in hamsters. This catalase-positive species was named Odontomyces viscosus (Howell, 1963; Howell & Jordan, 1963 and Howell et al. 1965) to differentiate it from Actinomyces, which were described as catalase negative (Howell et al. 1965). The description of Actinomyces was better defined on chemotaxonomic grounds and was changed to include catalase positive and negative

organisms. Hence, Odontomyces viscosus was reclassified as Actinomyces viscosus (Georg et al. 1969).

Gerencer & Slack (1969) felt that since A. viscosus differed from A. naeslundii in cell wall carbohydrate composition, grew better aerobically, and differed antigenically they should be maintained as two separate species. However, a number of workers noted the close physiological relationship between A. viscosus and A. naeslundii (Holmberg & Hallander, 1973 and Holmberg & Nord, 1975). It was also proposed that A. viscosus and A. naeslundii should be combined into a single species in which A. viscosus would be designated a catalase-positive variety or subspecies of A. naeslundii (Gerencer & Slack, 1976).

b) Physiology-Based Taxonomy

Various physiological tests such as acid-end product analysis, cell wall composition, the catalase test, sugar fermentation, hydrolysis of starch, aesculin, urea, and reduction of nitrate have been used to differentiate Actinomyces species (Schaal, 1986 and Bowden, 1991). To date, there are no phenotypic tests available to clearly separate the species A. viscosus and A. naeslundii (Johnson et al. 1990). For example, although many isolates have 6-deoxytalose in the cell wall some lack this carbohydrate. It is possible that strains lacking 6-deoxytalose that are currently classified as A. viscosus or A. naeslundii may represent different species (Bowden, 1991). Catalase is another of the tests that have been used to differentiate between these two species (Gerencer & Slack, 1976). A numerical taxonomic study placed a catalase negative human strain (W 752) (designated as A. naeslundii) with typical A. viscosus (catalase positive)(Fillery et al. 1978) suggesting that the catalase test was not a reliable test to differentiate these species. Johnson et al. (1990) also found strains of each species that differed in their catalase reactions. The physiological tests that appear to be useful for differentiating these species include nitrate reduction, aesculin hydrolysis, production

of acid from certain sugars and production of ammonia from urea (Schofield & Schaal, 1981; Schaal, 1986 and Bowden & Goodfellow, 1990).

c) Significant Changes in the Taxonomy of A. viscosus and A. naeslundii

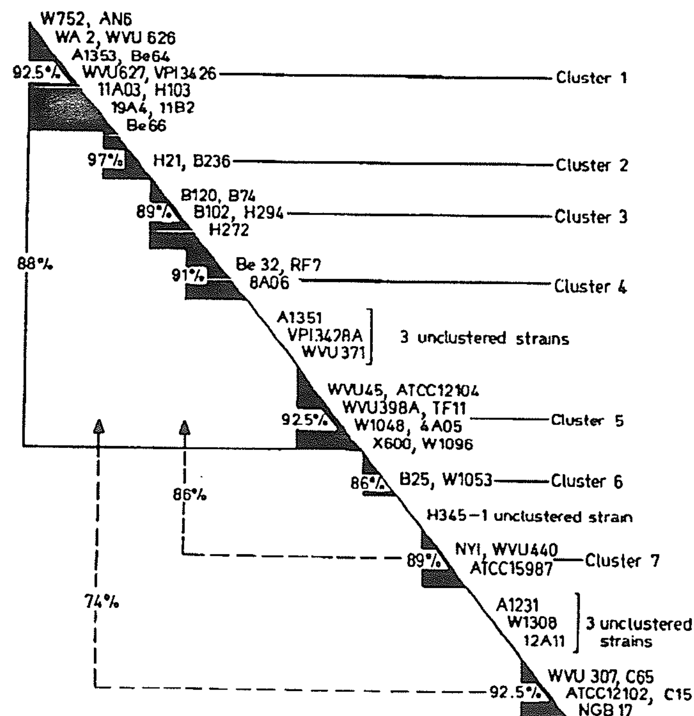
A number of significant changes have occured within the taxonomy of A. viscosus/naeslundii over the last 20 years. The following classifications are presented in chronological order and include studies of the serology, DNA homology and large numbers of physiological tests. To aid the reader please refer to Table 1.5 for the significant relationships of one species to the other.

Gerencer & Slack (1969), using fluorescence antibody techniques and selective absorption, were able to group A. viscosus into serotypes 1 (animal) and 2 (human) and this division remained relatively unchanged (Schaal, 1986 and Bowden & Goodfellow, 1990) until recently (Johnson et al. 1990). Using flourescent antibodies Slack & Gerencer (1970) showed cross-reactivity existed between A. naeslundii and A. viscosus. Only one serotype of A. naeslundii was reported at that time, however, in 1976 A. naeslundii was divided into four serotypes (Gerencer & Slack, 1976). In the latter study using fluorescent antibodies, they showed that A. viscosus serotypes 1 and 2 cross-reacted with A. naeslundii serotype 1, 2 and 3.

Fillery et al. (1978) examined the similarity between 43 strains of A. viscosus and A. naeslundii with the results of 65 physiological tests and numerical taxonomy. The species were grouped into 7 clusters (Figure 1.2) with cluster 1 representing typical human A. viscosus (serotype 2), cluster 7 representing animal A. viscosus (serotype 1) and clusters 2,4,6 representing atypical A. viscosus. Cluster 5 included typical A. naeslundii (serotype 1) and cluster 3 atypical A. naeslundii. There was a similarity of 88% between human strains of A. naeslundii and A. viscosus. The similarity dropped to 86% when animal A. viscosus was included. One additional study using numerical taxonomy showed that isolates of A. viscosus and A. naeslundii clustered together with a

similarity of 84.5% and within this cluster there were 3 subclusters (Schofield & Schaal, 1981). These data suggest that the division between strains of human A. viscosus and A. naeslundii are serotypes, and not true individual species (Fillery et al. 1978).

Figure 1.2 Actinomyces Similarity Matrix
Fillery et al. 1978



The validity of the Fillery cluster divisions was examined by DNA hybridization (Coykendall & Munzenmaier, 1979) and the data from this study support the separation of clusters 1,3,5 and 7. The strain representative of cluster 6 (W 1053) hybridized best (70%) with those of cluster 1, however, this was lower than the intracluster 1 hybridization of 85-100% suggesting that although cluster 6 is related to cluster 1 it may still warrant being placed in a different cluster. Coykendall & Munzenmaier (1979) did not examine strains representative of clusters 2 and 4.

Firtel and Fillery (1988) used monoclonal antibodies (MCA) to analyze further the validity of the clusters proposed in 1978. Using a total of 18 MCA, they concluded that cluster 3 and 5 should be considered distinct but clusters 1,2,4 and 6 were closely related, however, clusters 1,2,4 and 6 had a unique determinant not shared by cluster 3 and 5.

McCormick et al. (1985) also studied the relatedness of various Actinomyces species including A. viscosus serotype 1,2 and A. naeslundii 1,2,3. Urea extracts of whole cells analyzed with polyacrylamide gel electrophoresis yielded a complicated array of protein bands. The protein band patterns were compared with numeric taxonomic studies and showed that the serotypes of A. viscosus were more closely related (85%) than the serotypes of A. naeslundii (82-83%). McCormick et al. (1985) suggested that additional species existed among strains now classified as A. naeslundii.

Johnson et al. (1990) most recently examined the classification of A. viscosus and A. naeslundii using DNA homology. They proposed that A. viscosus (Fillery cluster 7) should be limited to animal strains. A. naeslundii (Fillery cluster 5) would be designated as A naeslundii-genotype 1 whereas human A. viscosus (Fillery clusters 1,2,4,6 or serotype 2), A. naeslundii (cluster 3 or serotype 3) and Actinomyces NV strains would be grouped together as A. naeslundii-genospecies II. Since no physiological test differentiated the two genospecies, they would retain this designation until some phenotypic difference can be found (Wayne et al. 1987).

Table 1.5 Taxonomic Changes in the Classification of A. viscosus and A. naeslundii

1969 Slack and Gerencer	**1978 Fillery et al**	**1990 Johnson et al**
Technique: Fluorescent Antibodies	Technique: Numerical taxonomy	Technique: DNA hybridization
A. viscosus-Serotype 1 (animal)	Cluster 7-Animal *A. viscosus*	Animal *A. viscosus*
A. viscosus-Serotype 2 (human)	Cluster 1-Typical *A. viscosus* Cluster 2-Atypical *A. viscosus* Cluster 4-Atypical *A. viscosus* Cluster 6-Atypical *A. viscosus*	/ /*A. naeslundii*-genospecies II / /
1976 Slack and Gerencer		
Technique: Fluorescent Antibodies *A. naeslundii*		
Serotype 1	Cluster 5-Typical *A. naeslundii*	*A. naeslundii*-genospecies I
Serotype 2		/*A. naeslundii*-genospecies II
Serotype 3	Cluster 3-Atypical *A. naeslundii*	/*A. naeslundii*-genospecies II
Serotype 4 (WVA 963)		Genospecies ----*Actinomyces* serotype WVA 963

Genospecies 2 also contains *Actinomyces* serotype NV

VI) Taxonomy of Animal Actinomyces Species

A. viscosus (animal cluster 7 or serotype 1) has been included in the previous section for the ease of discussion. To date these animal strains represent *A. viscosus* (Johnson *et al*. 1990). Recently three new animal species of *Actinomyces* have been isolated from dental plaque in cattle. *A. denticolens* and *A. howellii* were originally classified as *A. naeslundii* (Dent & Williams, 1984a, b) and *A. slackii* was originally classified as *A. viscosus* (Dent & Williams, 1986). All three species show DNA homology to one another of about 30% but share the common characteristic of absence of 6-deoxytalose and presence of rhamnose in their cell walls (Dent & Williams, 1986). The lack of 6-deoxytalose is one physiological characteristic that separates these three species from most isolates of *A. viscosus* and *A. naeslundii*. At present little information is available on their antigenic structure of these new animal isolates and their cross-reactivity with *A. viscosus* and *A. naeslundii*. Serological cross-reactions between *A. naeslundii* and *A. denticolens* appear to be minimal even though they share many physiological characteristics (Schaal, 1986).

5) Antigenic Structure of A. viscosus and A. naeslundii and the Humoral Response

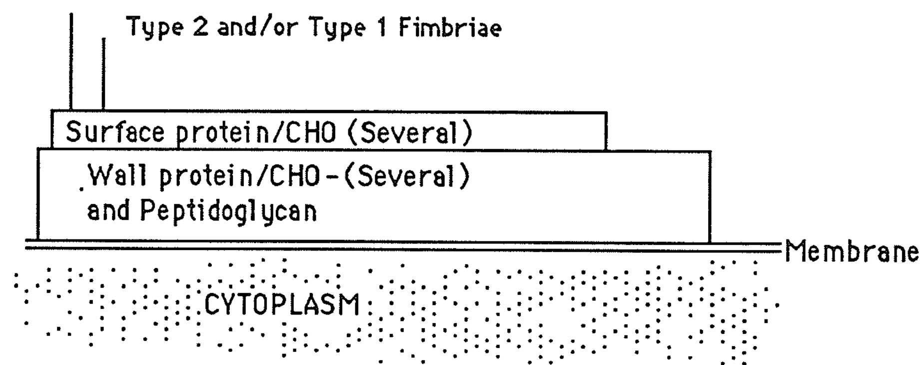
I) Introduction

Serology has been a useful technique in the classification of Actinomyces (Slack & Gerenser, 1970; Gerenser & Slack, 1969; 1976 and Schaal & Gatzer, 1985). These divisions have been based on fluorescent antibody reactions but with little understanding of the location and makeup of the antigens. Antigenic variation within species should be recognized and antigens that are selective for a given species or strain should be identified. Once individual strains or species can be identified immunologically, antibody responses in a patient specific to these strains can be measured (Bowden & Fillery, 1978).

II) Antigenic Structure

Figure 1.3 shows a simplified diagram of the antigenic structure of the cell walls of A. viscosus and A. naeslundii. A broad serological division of Actinomyces can be based on carbohydrate polymers and proteins or polypeptides (Bowden & Goodfellow, 1990).

Figure 1.3. Antigenic Structure of A. viscosus and A. naeslundii Cell Wall



Antigenic Structure

1) Carbohydrate Polymers
2) Proteins-Surface/Cell Wall
-Fimbriae

a) Carbohydrate Antigens

Carbohydrates constitute 5-24% by weight of the wall and can be extracted by 5% trichloracetic acid, formamide, or sodium hydroxide (Bowden et al. 1976; Bowden & Fillery, 1978 and Schleifer & Seidl, 1985). It is likely that wall carbohydrate may provide a useful group of antigens to define species and serotypes (Bowden & Goodfellow, 1990). A. israelii has a least two carbohydrate antigenic determinants, one common to all A. israelii strains and one common to A. israelii serotype 2 (Bowden & Fillery, 1978).

Study of A. viscosus and A. naeslundii carbohydrate antigens is limited (Bowden et al. 1976 and Fillery et al. 1978). The former study showed by precipitin tests that cross-reactivity exists between A. viscosus (serotype 2) antiserum and A. naeslundii carbohydrate. The latter study showed that cell wall carbohydrate from human A. viscosus (serotype 2) gave two precipitin lines when examined by rocket line electrophoresis, both of which cross-reacted with A. naeslundii, suggesting that there are specific wall carbohydrate antigens which relate to some of the serotypes (Fillery et al. 1978).

b) Protein Antigens

Pronase sensitive charged antigens extracted from various species of Actinomyces exhibit extensive cross-reactivity. A. viscosus serotype 1 antiserum cross-reacts with protein antigens extracted from A. viscosus serotype 2 and cross-reactivity also exists between A. israelii serotype 1 antiserum and A. israelii serotype 2, A. naeslundii and A. viscosus serotype 1 and 2 protein antigens. A. israelii serotype 2 antiserum also shows cross-reactivity with protein antigens extracted from A. israelii serotype 1 and A. naeslundii (Bowden et al. 1976).

Extracts of various Actinomyces whole cells with urea have yielded a variety of proteins but little is known of their origin in the cell (McCormick et al. 1985). It would be useful if protein antigens that are specific to each of the clusters could be

identified as this would facilitate serological identification and detection of specific organisms in health and disease

The one group of proteins associated with Actinomyces that have been extensively studied is the surface fimbriae (Masuda et al. 1983; Ellen & Grove, 1985 and Ellen et al. 1989). Masuda et al. (1983) examined the fimbriae from a representative for each of clusters 1 through 6 as described by Fillery et al. (1978). Chemical analysis showed that the major component of all fimbriae was protein and each cluster representative had similar proportions of basic, acidic, polar uncharged and nonpolar amino acids. The use of SDS-polyacrylamide gel electrophoresis to study fimbrial proteins has been unsuccessful as the proteins from the fimbriae failed to migrate or penetrated only slightly into polyacrylamide gel (Masuda et al. 1983).

Cross-reactions between fimbriae demonstrated by anti-fimbriae antibodies is common. Absorption of anti-fimbriae antibodies with cross-reactive strains produced cluster specific antibodies to cluster 5 when tested by whole cell agglutination. However, this absorbed cluster 5 anti-fimbriae antisera recognized both clusters 3 and 5 when assessed by indirect immunofluoresence. Cluster 1 fimbriae antisera also cross-reacted with all other A. viscosus clusters suggesting that fimbriae antigens specific to each of the clusters may be difficult to identify (Masuda et al. 1983).

Indirect immunofluoresence with antifimbrial antisera was used to screen 213 A. viscosus and A. naeslundii isolates. The antisera were very successful in separating the two taxonomic groups-cluster 3 and 5 but could not separate A. viscosus clusters 1,2,4 and 6, supporting the close association of these latter four clusters (Ellen & Grove, 1985).

Monoclonal antibodies raised against strains of A. viscosus and A. naeslundii showed a high specificity to each of A. viscosus, atypical and typical A. naeslundii (Firtel & Fillery, 1988). The results closely matched those using fibril antisera of Ellen & Grove (1985) suggesting that the epitopes recognized by the monoclonals may lie on the

fibrils. Cisar et al. (1984) showed that typical A. viscosus possessed type 1 and type 2 fimbriae and typical A. naeslundii possessed only type 2 fimbriae. Type 1 fimbriae were associated with adherence to saliva-treated hydroxyapatite and type 2 fimbriae with attachment to epithelial surfaces. Ellen et al. (1989) used monoclonals in immunogold electron microscopy and found that anti-type 2 fimbriae antibodies localized on long A. viscosus fimbriae and anti-type 1 fimbriae was associated with short appendages close to the cell body, while typical A. naeslundii (cluster 3) labelled in a pattern similar to that of A. viscosus. Typical A. naeslundii (cluster 5) did not react with the anti-fimbriae type 2 antibody even though typical A. naeslundii strains are known to carry type 2 fibrils. The nonreactivity with A. viscosus anti-type 2 fibrils suggests some dissimilarity between the type 2 antigenic determinant of A. viscosus and A. naeslundii.

III) Humoral Immune Response to A. viscosus and A. naeslundii

The data available on the humoral antibody response to A. viscosus and A. naeslundii in tissues is limited. Studies have shown that these Actinomyces species can penetrate into diseased gingival tissues where they are complexed with specific antibodies (Pekovic' & Fillery, 1984). The fact that bacteria reside within the tissue and are complexed with antibodies suggests that they are recognized by the immune system. A. viscosus was found more commonly complexed with specific antibodies than was A. naeslundii. The specificity of the antibodies is difficult to assess because studies involving animal A. viscosus in gnotobiotic mice have shown that extracellular heteroglycan and sonicated cell supernatant exhibit B-cell mitogenic properties and operate as polyclonal B cell activators (Burckhardt et al. 1977 and Engel et al. 1977).

a) Serum Immunoglobulins

A. naeslundii is an organism that is found commonly in the mouth at a young age (Ellen, 1976). A study that followed the changes in the antibody titre to A. naeslundii in 35 insulin dependant diabetic subjects, ages 7-1, found the antibody titre increased a

statistically significant amount with increasing age (Morinushi et al. 1989). Studies have shown that as inflammation increases (gingivitis-periodontitis), there is also a statistically significant increase in the mean serum antibody titre to A. naeslundii-ATCC 12104 (Nisengard & Beutner, 1970 and Gilmour & Nisengard, 1974). Both studies found no statistically significant increase in the titre to A. israelii. Gilmour and Nisengard (1974) absorbed the human sera with A. israelii to remove cross-reactions and this had little effect on the titre to A. naeslundii. They concluded the cross-reactivity between these A. israelii and A. naeslundii was small. An increase in the antibody titre to A. naeslundii with increasing inflammation was not shown in the study of Williams et al. (1976), although they did shown that Actinomyces was the predominant genera in the subgingival plaque of their patients with periodontal disease.

A recent study, using enzyme-linked immunosorbent assay (ELISA) examined the association between IgG serum antibody titre to A. viscosus (ATCC 15987) and A. israelii and various forms of periodontal diseases (Murayama et al. 1988). The A. israelii antibody titre was found to be statistically elevated in disease but the titre to A. viscosus was not. The lack of response to A. viscosus can, in part, be explained by the fact that A. viscosus (ATCC 15987) was used as the antigen. This strain is the type strain of A. viscosus but it is an animal strain first isolated from hamsters. The use of a strain of human origin may have given a more accurate representation of the human response.

b) Mucosal Immunoglobulins

Oral mucosal immune responses can be generated by immunizing directly into the salivary glands (Crawford et al. 1978) or via Peyers patches of the gut associated lymphoid tissues with subsequent homing of precursor cells to salivary glands (Challacombe, 1987) Sprague-Dawley gnotobiotic rats were immunized with strains of A. naeslundii and A. viscosus in the region of each major salivary gland and then mono-infected with the same strain as used for the immunization. Rats mono-infected with A.

naeslundii showed higher levels of salivary antibodies than serum antibodies and a definite trend to decreased alveolar bone loss. Rats immunized with A. viscosus exhibited increased vertical bone loss associated with a delayed hypersensitivity response (Crawford et al. 1978). This suggested that both species stimulate the immune system, but in a different manner. It should be noted that salivary antibodies can be contaminated during gingival inflammation with immunoglobulins that are released with gingival crevicular fluid. The immunoglobulins released with gingival crevicular fluid reflect the serum and not the mucosal immune system (Holmberg & Killander, 1971).

c) Gingival Crevicular Fluid Immunoglobulins

Tynelius-Bratthall and Ellen (1985) explored the impact of various strains of A. viscosus and A. naeslundii on the mucosal immune system by determining the antibody titre in gingival crevicular fluid, unstimulated and stimulated saliva, in patients that were undertaking treatment to resolve gingivitis. Salivary antibodies to typical (ATCC 12104) and atypical A. naeslundii (B 74) were barely detectable in pre- and post-treatment samples. Crevicular fluid IgG, and salivary IgA and IgG to A. viscosus (WVU 627) showed no statistical difference before and after treatment, but did show a tendency to an increase in the titre in post-treatment samples. It was postulated that this increase in titre may account for some of the beneficial effects of periodontal therapy by scaling. Scaling may stimulate immunocompetent cells by innoculating bacterial antigens into the gingival tissues.

CHAPTER 2

RATIONALE AND APPROACH FOR THE PRESENT STUDY

I) Rationale

Actinomyces have a limited habitat in the human and animal oral cavity and tonsilar region where they are members of the resident flora (Bowden & Hardie, 1973 and Slack & Gerenser, 1975). A. viscosus and A. naeslundii can be pathogens, although they appear to be of low virulence. A. naeslundii-serotype 3 is associated with the development of gingivitis (Moore et al. 1987) and A. viscosus-serovar 2 is associated with the development of root caries (Bowden et al. 1990). Although Actinomyces naeslundii can cause periodontal disease in experimental animals, (Socransky et al. 1970) human studies suggest that both A. naeslundii and A. viscosus decrease in frequency in periodontal disease sites (Moore et al. 1982a; 1983 and 1987). The close physiological relationship between A. viscosus and A. naeslundii makes identification and grouping of these species difficult and this, in turn, obscures the association of the typical and atypical strains of these two species with disease. (Holmberg & Hollander, 1973; Holmberg & Nord, 1975; Gerencer & Slack, 1976; Fillery et al. 1978; Schofield & Schaal, 1981; Schaal, 1986; Johnson et al. 1990 and Bowden, 1991).

Immunological techniques, like whole-cell agglutination and immunofluoresence, also show high degrees of antigenic cross-reactivity between and within these species (Slack & Gerencer, 1970; Gerencer & Slack, 1976; Bowden & Fillery, 1978 and Fillery et al. 1978). Cross-reactivity within the carbohydrate component of the cell walls (Bowden et al. 1976 and Bowden & Fillery, 1978) and cross-reactivity that occurs between the protein component of cell surface fimbriae has been examined (Masuda et al. 1983; Ellen & Grove, 1985 and Ellen et al. 1989), but no final decision is available on the location and nature of any antigens unique to numerical taxonomic clusters of these species.

A. viscosus and A. naeslundii have been grouped by numerical taxonomy using 65 physiological tests and seven clusters have been identified (Fillery et al. 1978). The current study examined the antigenic similarities between and within these taxonomic clusters. If antigenic differences between the clusters are identifiable then cluster-specific antisera would be useful as a screening technique in identifying unknown species and also providing useful information for planning of clinical trials that examine the relationships between organisms isolated during health and disease and humoral immunity. Specifically, clarification of the degree of cross-reactivity, the presence of specific antigens and the position of such strains as those designated as A. naeslundii-serotypes I,II,III,IV and Actinomyces serotype NV would provide useful information and would aid in identifying relationships between Actinomyces and disease. This study was intended to provide some insight into these questions.

II) Approach

Strains from the 7 clusters of A. viscosus and A. naeslundii as designated by Fillery et al. (1978), 3 recent isolates of Actinomyces-A. naeslundii serotype II and III and Actinomyces serotype NV (Johnson et al. 1990), and three animal species A. howellii, A. slackii, and A. denticolens, were included in this study. Cross-reactivity due to antigens common to the genus were identified using A. israelii-serotype 1 (NGB 17) as the representative strain for Actinomyces.

a) Agglutination

The degree, location, and nature of the surface antigens responsible for the agglutination of whole cells were examined initially to give a broad overview of the extent of the cross-reactivity. Agglutination of the various clusters of A. viscosus, A. naeslundii and other Actinomyces species was included. Pronase and non-pronase-treated whole cells and pronase-treated cell walls were used to identify the location and nature of the agglutinating antigens. Pronase treatment of the whole cells and cell walls removed the protein component allowing analysis of the association of carbohydrates

with agglutination (Bowden, 1976). Carbohydrate extracted from pronase-treated cell walls provided antigens useful in determining the role of carbohydrates in cross-reactivity. Reaction of carbohydrate antigens with antibodies was examined by Ouchterlony double diffusion (Roitt, 1988) and rocket immunoelectrophoresis (Weeke, 1973)

b) Polyacrylamide Gel Electrophoresis/Immunoblotting

While agglutination of whole cells provided data on the cross-reactivity between different organisms, the test gave no indication of the number of antigens or polypeptides involved in the reaction and specific antigens could not be visualized. Polyacrylamide gel electrophoresis and immunoblotting of cell extracts provided profiles of protein antigens in the extracts and also allowed numerical values of similarity to be calculated. Testing several types of extracts containing cell surface and cytoplasmic antigens would show the location of both common and specific protein antigens.

Extracts of whole cells made by shaking with glass beads, whole cell sonicates, and cell walls of the above strains and species were examined to determine their antigenic similarity. The protein components of these extracts were detected by polyacrylamide gel electrophoresis and their antigens by immunoblotting.

III) Clinical Applications in Species Identification

To date physiological tests have had limited success in separating isolates of *A. viscosus* and *A. naeslundii* (Johnson *et al*. 1990). The grouping of strains based on antigenic profiles, as determined from this study will provide the foundation for the selection of isolates as antigens in studies of the immune response to *A. viscosus* and *A. naeslundii* in humans. Use of physiological tests together with immunoblotting with the cluster specific antisera will be of value in screening unknown strains.

Determining the immunological similarity of each cluster would define organisms that are immunologically distinct. This would help in planning future clinical studies directed at examining the immunological association of *A. viscosus* and *A.*

naeslundii with disease. A battery of strains identified as distinct in this study would be used as antigens for quantitation of humoral antibody. The high degree of immunological cross-reactivity that is known to exist between typical and atypical strains of A. viscosus and A. naeslundii would be controlled by selective absorption of patients antisera with the defined strains. The final titre measured for antibody to a given cluster, species or strain would then be a reflection of the challenge to the host by that serotype and not a value resulting from extensive cross-reactivity between several strains.

CHAPTER 3

MATERIALS AND METHODS

I) Species Selection and Growth

A total of 22 strains of Actinomyces were examined. One strain of A. israelii; 16 strains of A. viscosus and A. naeslundii; four animal species A. howellii, A. slackii, A. denticolens, and A. viscosus and one strain designated Actinomyces-serotype NV. The origin of these species are presented in Table 3.1. Freeze-dried strains of Actinomyces were resuspended in B. M. Medium (Appendix 1A) and then plated on blood agar plates (Oxoid CM 271 Blood Agar Base No. 2) supplemented with haemin, Vitamin K_1 and 5% defibrinated sheep blood (Atlas Lab., Winnipeg) (Bowden & Hardie, 1973 and Bowden, 1991) and maintained in an aerobic atmosphere containing 10% CO_2. After 48 hours, single colonies were subcultured to ensure a pure culture. Subsequently, the test strains were maintained on blood agar plates in a candle jar at 37°C and were subcultured every 7 days for the remainder of the study.

II) Physiological Characterization

a) Atmospheric Requirements

Aerobic (O_2), anaerobic (N-80%, H_2-10% and CO_2-10%), and candle jar growth (CO_2) were assessed on blood agar plates that were incubated at 37°C for 5 days.

b) Catalase

Bacteria grown anaerobically on blood agar plates were placed in aerobic conditions for 1 hour and then a sample from each plate was placed on a glass slide to which was added 1 drop of 30% hydrogen peroxide (Mallinckrodt, Quebec)(Cowan & Steel, 1974). A positive result was indicated by bubbling within 15 seconds.

Table 3.1 Origin and Designation of the Microorganisms Used in this Study

Human Strains	Designation	Cluster[a]	Source
NGB 17	*A. israelii*-Serotype 1		G. Bowden
WVU 627 WVU 626 WA 2 W 752	Typical *A. viscosus*	1	E. Fillery
B 236	Atypical *A. viscosus*	2	E. Fillery
B 120 B 102 B 74	Atypical *A. naeslundii*	3	E. Fillery
BE 32	Atypical *A. viscosus*	4	E. Fillery
ATCC 12104 WVU 398A TF 11 4 AO5	Typical *A. naeslundii*	5	E. Fillery
W 1053	Atypical *A. viscosus*	6	E. Fillery
Add. Strains			
ATCC 49339	*A. naeslundii*-Serotype II		W. Moore
ATCC 49340	*A. naeslundii*-Serotype III		W. Moore
VPI N11A-6	*Actinomyces*-Serotype NV		W. Moore
Animal Strains			
ATCC 15987	Animal *A. viscosus*	7	E. Fillery
NCTC 11636	*A. howellii*		A. Williams
NCTC 11928	*A. slackii*		A. Williams
NCTC 11490	*A. denticolens*		A. Williams

a) Cluster designation of Fillery et al. 1978.

c) Carbohydrate Fermentation

The medium used to assess fermentation consisted of Streptococcus sugar agar (Appendix 1B). Each sugar was dissolved in distilled water at a concentration that would give a final carbohydrate concentration of 1% when added to the basal agar medium. The sugars were filter sterilized and then added to the sterilized basal media just prior to pouring. The carbohydrates tested were amygdalin, arabinose, cellobiose, glycerol, glucose, glycogen, inulin, lactose, mannitol, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, and trehalose.

The test strain was first innoculated into innoculum broth (Appendix 1B) and incubated anaerobically for 24 hours. Subsequently, the cells were mixed and then streaked onto the various sugar plates with a sterile pasteur pipette and incubated anaerobically. The plates were examined on days 2 and 5 with a color change from purple to yellow indicating the production of acid. The innoculum broth was plated onto blood agar plates and incubated anaerobically for 5 days to rule out any obvious contamination.

d) Starch Hydrolysis

Strains were tested for starch hydrolysis on starch agar plates (Appendix 1C). Iodine was added to the the plates at 5 days and a positive result was indicated by clearing of the blue stained agar around the colonies within 10 minutes.

e) Aesculin Hydrolysis

Strains were tested for hydrolysis of aesculin by plating onto aesculin agar plates (Appendix 1C). The results were assessed after 2 and 5 days of incubation. Blackening around the colonies was considered a positive result.

f) Urea Hydrolysis

Urea hydrolysis was tested on urea agar plates (Appendix 1C). After 2 and 5 days a positive result was recorded when the agar around the colonies turned from orange to pink.

g) Nitrate/Nitrite Reduction

For nitrate reduction, nitrate broth (Appendix 1D) was innoculated and incubated anaerobically for 2 days. At this time, gas in the Durham tubes was noted and 1 ml of each reagent was added to the tubes with a red color indicating nitrite. Negative results were confirmed by the addition of zinc metal dust (Fisher Scientific Company, Alberta), which produced a red color in the presence of nitrate. Nitrite reduction was tested in nitrite broth innoculated and tested after 7 days of incubation. Addition of the reagents with the production of a red color showed that nitrite was still present and was not reduced and the absence of colour indicating that the organism reduced nitrite.

III) Sample Preparation

a) Bulk Culture

Cells for rabbit immunization and for antigenic studies were grown in 10 litre bottles containing the semi-defined complex media (ADM) (Appendix 1E) suggested by Bowden (1976). Prior to innoculation into the batch culture bottles, the strain was first innoculated into BBL medium (Appendix 1A) and maintained anaerobically for 48 hours. The culture was mixed and 2-5ml of the suspension was innoculated into the culture bottles. The ADM media was supplemented after innoculation with filter-sterilized sodium carbonate (1-2% of a 1% W/V) and then maintained aerobically at 37°C for 5 days. Cells were harvested at day 5 by centrifugation at 16,300 G (Sorvall GSA, Dupont, Connecticut) for 10 minutes and stored in EDTA buffer pH 7.4 until needed. All cultures were Gram-stained and plated on blood agar plates that were incubated aerobically and anaerobically to confirm purity before progressing to the next step.

b) Whole Cells For Agglutination

Cells grown on blood agar plates for 4 days were collected into 5 ml sterile saline and centrifuged in a Dynac Centrifuge (Clay Adams, New Jersey). The cells were washed three times and resuspended in 5 ml of sterile saline.

c) Pronase Treatment of Whole Cells

Pronase treatment of whole cells was accomplished by removing cells from one blood agar plate and adding them to 1.0 ml of 0.1 M Tris HCl buffer at pH 8.0 containing 0.5 mgm/ml of pronase E (Merck, Germany). The cell suspensions were incubated at 37°C for 2 hours, centrifuged, and washed three times with sterile saline. Pronase E activity was tested by the addition of the enzyme to 10 mg of Azocoll (Sigma) dissolved in 1 ml of the buffer. The control was completely denatured in 5 minutes.

d) Whole Cell Glass Bead Extracts

Cells grown in one litre of *Actinomyces* defined medium (ADM) were collected by centrifugation at 16,300 G (Sorval-GSA) and then packed into a 5 ml bijou bottle with 40/60 mesh glass beads (Chromatographic Specialties, Brockville, Ontario) at a volume ratio 3:1 (cell:beads). Cell were disrupted in a Mickle Tissue Disintegrator (Mickle Engineering Company, Gomshall, Surrey) for approximately 2 hours with breakage assessed by negative Gram staining. The broken cells and glass beads were centrifuged in a Dynac Centrifuge (Clay Adams, New Jersey) and the clear supernatant removed and frozen (-70°C) until needed. An extract from a representative of each of the Fillery clusters (Fillery *et al*. 1978) was prepared in this manner.

e) Whole Cell Sonicates

Representative strains from clusters 1,3 and 5 were selected, innoculated onto 4 blood agar plates and incubated for 4 days. The cells were collected into 1 ml of sterile saline and sonicated for 5 minutes (Kontes Micro-Ultrasonic Cell Disrupter, Vineland, New Jersey) on ice. The suspension was centrifuged at 100,000 G (Beckman LM80, Rotor-50Ti, Ontario) for 30 minutes at 10°C.

f) Cell Walls

Approximately 3.5 gm wet weight of cells was loaded into each of two glass tubes belonging to a Mickle Tissue Disintegrator (Mickle Engineering Company, Gomshall, Surrey). The tubes were filled about one quarter of the way with 40/60 mesh glass

beads (Chromatographic, Specialties) and the cells were added. The tubes were then filled about three quarters with EDTA buffer (pH 7.4) and a few drops of octanol were added to minimize the frothing, which slows the disruption of the cells. When the tubes were filled correctly there was a 0.5 inch air space present below the bung. The machine was run at 4°C and checked every thirty minutes by Gram staining to determine degree of disruption. When all the cells were stained Gram negative, the tubes were removed and the suspension allowed to settle (Bowden, 1976). The supernatant was collected and the glass beads were washed with distilled water three times to collect cell fragments, with the washing pooled with the original supernatant. The suspension of cell fragments was then centrifuged at 1,000 G for 10 minutes (Sorvall-SS34, Dupont, Connecticut) to remove any residual glass beads and whole cells. The supernatant was collected and centrifuged again at 17,000 G for 15 minutes (Sorvall-SS34, Dupont, Connecticut). The resulting pellet was washed three times by resuspending in 50 ml of distilled water and then centrifuged as before. After the final wash the cell walls were suspended in 15 ml of distilled water, frozen, and freeze dried (Edwards High Vacum-Model EF03, Sussex, England).

g) Pronase Treatment of Cell Walls

Pronase treatment of cell walls was accomplished by suspending 300 mg of freeze dried cell walls in 50 ml of 0.1 M Tris HCl buffer at pH 8.0 containing 0.5 mg/ml of pronase E (Merck, Germany) and a few drops of chloroform to inhibit bacterial growth. This suspension was incubated at 37°C for 16 hours (Bowden, 1976) and then centrifuged at 17,000 G (Sorvall-SS34, Dupont, Connecticut) for 15 minutes. The pellet was washed three times and freeze dried as described for cell walls

h) Extraction of Carbohydrates From Cell Walls

Approximately 300 mg of freeze dried pronase treated cell walls were suspended in 25 ml of 5% W/V of trichloracetic acid and maintained at 37°C for 16 hours with stirring (Bowden, 1976 and Bowden et al. 1976). The suspension was centrifuged at

17,000 G (Sorvall-SS34, Dupont, Connecticut) for 15 minutes and the supernatant poured off and kept. The pellet was resuspended in 25 ml of distilled water and centrifuged for 15 minutes. The distilled water wash was added to the original supernatant.

Five volumes of acetone containing a few crystals of sodium acetate were added to the carbohydrate-containing supernatant and the carbohydrate allowed to precipitate at 4°C for 24 hours. The carbohydrate precipitated on the bottom of the flask and the acetone supernatant was carefully poured off leaving the precipitated carbohydrate. Residual acetone was evaporated by blowing with air and then 5 ml of distilled water was added to dissolve the carbohydrate. The acetone supernatant that contained the smaller molecular weight carbohydrates that did not settle out was centrifuged at 17,000 G (Sorvall-SS34, Dupont, Connecticut). The supernatant was poured off, the acetone was allowed to evaporate, and 1 ml of distilled water was added to each centrifuge tube to redissolve any carbohydrate. This solution of carbohydrate was added to the rest and then dialysed against distilled water in a 1000mw cut off dialysis tubing (Spectrum-Type 6, Los Angeles) for 24 hours. After dialysis the sample was frozen and freeze dried.

IV) Transmission Electron Microscopy

Sample fixation was accomplished by suspending 100 mg of whole cells or cell walls in a solution of 1.0 ml of 0.1M Tris buffer (pH 7.1) containing 40 μl of freshly purified 50% glutaraldehyde and incubated at 4°C for one hour. Subsequently, the suspension was washed twice in a buffer of 0.1 M sodium cacodylate and 0.01 M calcium chloride (pH 7.4), fixed with 1% osmium tetroxide and rewashed in the above buffer (Hayat, 1981). The whole cells or cell walls were suspended in 3% agarose and chilled to 4°C (Yuan & Gulyas, 1981). The blocks were diced and fixed en bloc with saturated aqueous uranyl acetate. Dehydration of the blocks was accomplished in a series of washes using increasing concentrations of acetone (10%-100%) and then passed through a

transition solution of increasing concentrations of propylene oxide (25%-100%). The blocks were infiltrated and then polymerized with fresh JEMBed 812 (J.B. EM Services, Quebec) (Luft, 1961). The tissue blocks were sectioned on a Microstar Diamond Knife (Microengineering Inc., Huntsville, Texas) using an LKB Ultratome III (LKB Produkter-AB, Bromma, Sweden). Sections of 500 nm were collected on 400 hexagonal mesh copper grids. These sections were stained for 10 minutes in a saturated solution of uranyl acetate in 50% ethanol (Stempek & Ward, 1964) and poststained for five minutes with lead citrate (Venables & Coggeshall, 1965) with procedure modifications of Heinrich (1985).

The sections were viewed on a Phillips Electron Microscope-model 201 (Toronto, Canada) with an accelerator voltage of 60 keV, a gun bias setting of 5 and a 20μ French Gold Foil Objective Aperture. Images were recorded on Kodak Fine Grain Release Positive Film-#5302 (Toronto, Ontario).

V) Immunological Techniques

a) Antiserum Preparation

For rabbit immunization, one strain was chosen from each Fillery cluster (Fillery et al. 1978) and the animal species A. slackii, A. howellii and A. denticolens (Table 3.2). Each strain was grown in semi-defined media (Bowden, 1976)and the cells were aseptically into a sterile 15 ml centrifuge tube (Corning, New York) and sedimented in a Dynac Centrifuge (Clay Adams, New Jersey). Generally approximately 1 gm wet weight of cells was collected. The cells were washed three times by resuspending in 10 ml of sterile saline and centrifuging as above. Purity checks of the antigen suspensions were made by innoculating blood agar plates and incubating them aerobically and anaerobically for 2 days. Washed cells were resuspended into 5 ml of sterile saline and stored frozen.

New Zealand white male rabbits (3-5 kgs) were used for preparation of the antisera. Each rabbit was test bled prior to immunization and the preimmune sera was

tested against whole cells using Ouchterlony double diffusion to determine whether natural antibodies to the test strain were present (Roitt, 1988). Three millilitres of the cell suspension were emulsified with 3 ml of Freunds complete adjuvant (Difco, Detroit, Michigan) and 0.2 ml of the emulsion was injected subcutaneously into each of 4 sites on the back of the rabbit and 0.2 ml was injected intramuscularly into the hind flank (Bowden et al. 1976). After two weeks, the rabbits were test bled from the ear vein and double diffusion was used against homologous sonicated whole cells to assess the production of antibodies.

The animals were then boosted by injection of 0.2 ml of the frozen whole cell suspension into the ear vein. This booster dose was repeated and then the animals were rested for a week and then sacrificed. The blood was allowed to coagulate for 16 hours at 4°C in sterile bottles, the serum was removed and clarified by centrifugation (Dynac Centrifuge Clay Adams, New Jersey) and stored frozen until needed (Bowden, 1976).

b) Agglutination

Pronase-treated cell walls and non-pronase and pronase-treated whole cells were used as antigen in agglutination tests.

i) Whole Cells

The whole cells from 1 blood agar plate were suspended in 0.5 ml of sterile saline with mild sonication (Kontes Micro-Ultrasonic Cell Disrupter) and then one drop of this suspension was added with one drop of antisera (1:20 in sterile saline). Agglutination was assessed by examining the suspension using a small hand lens (X10).

Table 3.2 *Actinomyces* Strains Used for Rabbit Immunization

Cluster*	Species	Immun. Strain	# Of Rabbits Immunized.
1	*Typical *A. viscosus*	WVU 627	3
2	*Atypical *A. viscosus*	B 236	2
3	*Atypical *A. naeslundii*	B 102	2
4	*Atypical *A. viscosus*	BE 32	2
5	*Typical *A. naeslundii*	ATCC 12104	2
6	*Atypical *A. viscosus*	W 1053	2
7	*Animal *A. viscosus*	ATCC 15987	2
-	*A. howellii*	NCTC 11636	2
-	*A. slackii*	NCTC 11928	2
-	*A. denticolens*	NCTC 11490	2

*Fillery et al. 1978

ii) Cell Walls

Ten milligrams of pronase-treated cell walls were suspended in 40 ml of sterile saline. The titre of the antiserum was determined with dilutions of sera ranging from 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320 and 0.2 ml of the diluted sera was mixed an equal volume of the antigen and maintained at 45°C for 2 hours and then for 16 hours at room temperature. Agglutination was checked by examination with a hand lens (X10).

c) Double Diffusion

One tablet of I.D Agar (Oxoid, England) was dissolved in 12.5 ml of 0.1 M Barbitone Acetate Buffer (Oxoid, England) and diluted to 50 ml with distilled water (pH 8.6). The agar was brought to a boil and then cooled to 55-60°C in a water bath. Approximately 12 ml of agar was poured onto a 8 cm X 8 cm glass plate and then allowed to cool. Wells (4 mm diameter) were cut in the agar 5 mm from each other with each well capable of holding approximately 30 μl of antisera or carbohydrate antigen (1mg/ml). The plates were maintained at 4°C for 48 hours, washed for 24 hours in 0.85% sodium chloride, fixed, stained with Coomassie blue (BioRad, Ontario), and then destained as described for polyacrylamide gel electrophoresis. Precipitin bands were stained blue on a clear background.

d) Rocket Immunoelectrophoresis

This technique was based on those of Weeke (1973). Approximately 15 ml of a 1% Agarose (BioRad, Ontario) with 0.025 M barbitone buffer (Oxoid, England) was boiled, cooled to 55-60°C and then poured onto a 9.4 cm X 8.3 cm glass plate and allowed to set. Six wells were cut 2.0 cm from the end of the glass plate and the gel cut to leave a 2.5 cm section of agarose containing the 6 wells. The exposed area of the plate was then covered with 12 ml of 1% agarose with 0.025 M barbitone containing 1/20 of test antibody. Each well was loaded with 20 μl of carbohydrate at a concentration of 1 mg/ml. Electrophoresis was carried out for 16 hours at 2 volts/cm at 10°C. After electrophoresis the agarose was washed for 24 hours in 0.85% sodium chloride then the

gel was dried, fixed, stained with Coomassie blue and destained as described for polyacrylamide gel electrophoresis.

e) Polyacrylamide Gel Electrophoresis and Immunoblotting

i) Sample Preparation

The following procedure was followed for preparation of cell wall extracts. Approximately 20 mg of non-pronase-treated cell walls were suspended in 100 μl of X3 sample buffer (glycerol 15 ml, mercaptoethanol 7.5 ml, SDS(Bio-Rad, Ontario) 3.45 g, and Tris (Bio-Rad, Ontario) 0.38 g) without the tracking dye and 200 μl of EDTA buffer pH 7.4. The preparation was sonicated for 1 minute (Kontes Micro-Ultrasonic Cell Disrupter) and then boiled for 20 minutes. The protein level in this extract was determined using the Bradford BioRad Protein Assay (BioRad, Ontario) with the protein standard containing sample buffer equivalent to that in the extract in order to control for the effect of the sample buffer. The cell wall extract was diluted with one-third sample buffer containing tracking dye (3.0 ml of a 0.1% bromophenol blue) and two-thirds EDTA buffer (pH 7.4), so that a small volume (< 5μl) could be loaded to the gels. For each cell wall extract, 1.5 μg of protein was loaded per lane if the gel was to be stained, while 0.5 μg of protein was loaded for Western blotting.

The whole cell glass bead extracts and whole cell sonicates were prepared in a slightly different manner. Protein was calculated on the original sample before the EDTA buffer and the X3 sample buffer with tracking dye was added. After determining the protein concentration, 500 μl of sample was added to 500 μl of X3 sample buffer with the tracking dye, and 500 μl of EDTA buffer at pH 7.4. The same amount of protein was added to the gel for staining and blotting as used for the cell wall preparations.

All preparations were stored frozen and reused but new low molecular weight standards (Pharmacia LKB, New Jersey) were prepared fresh each day.

ii) Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis was carried out in a Mini-Protean™ II Dual Slab Cell system (Bio-Rad, Ontario). A 15 lane gel (1.5 mm) was prepared with a resolving gel of 12% and a stacking gel of 3% and a low molecular weight standard was included (Pharmacia LKB, New Jersey) within each gel. Electrophoresis was carried out at 200 V constant current for approximately 1 hour in running buffer (Glycine 71.5 g, Tris 16.25 g, SDS 5 g, distilled water to 5000 ml with no adjustment of the pH).

The gels to be stained were fixed in 45% methanol and 10% acetic acid for one hour, stained in 0.25% Coomassie blue, 45% methanol, and 10% acetic acid for 1 hour and then destained until clear in 30% methanol and 10% acetic acid. Once the gel was destained a black and white photograph was taken.

iii) Immunoblotting

The method used was based on those reviewed by Towbin and Gordon (1985). Approximately 0.5 µg of protein was loaded per lane and after the electrophoresis, the gel was washed in blotting buffer (Tris 15.15 g, glycine, 72.05 g, methanol 1000 ml, and distilled water 3900 ml with an adjusted pH to 8.3). Prior to assembly of the gel/membrane immunoblotting apparatus, 0.5% pyronin was used to mark the LMW standard lane. The blotting membrane was Immobilon-P Transfer Membrane (Millipore, Ontario) and the proteins were transferred in blotting buffer using a Transfor system (Hoeffer, Technical Marketing Inc., Ontario). The transfer was accomplished in 16 hours at 10°C using 0.5 Amp.

After the transfer was completed the lane with the standard marked by the pyronin was cut out and this was stained with Coomassie blue. The remainder of the membrane for blotting was washed three times in Tris-saline (sodium chloride 17 g, Tris 2.42 g, Tween 20 (Fisher Scientific Company, New Jersey) 4 grams, distilled water 2000 ml adjusted pH of 8.2). The antisera used as first antibody in immunoblotting the

membranes are listed on Table 3.2. The optimum dilution that gave maximum resolution of bands was 1:1000 for unabsorbed sera and 1:500 for absorbed sera that was diluted during absorption. The membrane was reacted with the first antiserum diluted in 15 ml of Tris-saline with 0.8% globulin-free bovine serum albumin (Sigma, St. Louis) in a plastic bag and rotated constantly for 1 hour at 37°C. The membrane was then washed in Tris-saline for a total of 30 minutes with three changes. The second antibody used was peroxidase conjugated swine immunoglobulin to rabbit immunoglobulins (Dako Corporation, California) at a dilution of 1:2000 in Tris-saline containing 0.8% globulin free bovine serum albumin. The membrane and antibody were again rotated for 1 hour at 37°C and the membrane was washed for 30 minutes in Tris-saline with three changes. The washed membranes were then reacted with 50 mgm of $3,3^1$-diaminobenzidine (Sigma, St Louis), and 100 ul of 30% hydrogen peroxide (Mallinckrodt, Quebec) in 0.1 M phosphate buffered saline (Oxoid, England) (pH 7.3). The immunoblots were allowed to develop for about 3 minutes, washed in 2000 ml of distilled water for 1 hour and then dried on filter paper.

iv) Control of Immunoblotting Reagents

To show if the swine second antibody contained natural antibodies to Actinomyces, an immunoblot with the various Actinomyces cell wall extracts was reacted with the second antibody only and then developed. The results showed no natural antibodies to the test strains. In addition, controls to check for the presence of immunoglobulin Fc receptors within the protein extracts were carried out by reacting with peroxidase-conjugated Fc fragments from rabbit IgG (Jackson ImmunoResearch Laboratories, PA). No Fc receptors were detected.

VI) Immunoblot Analysis

a) Scanning Densitometer

The immunoblots were scanned by reflectance with a video densitometer coupled to a Perkin Elmer integrator (Bio-Rad, Ontario) (Bowden & Nolette, 1990). Strains were

compared to one another within the same gel. The strain that was homologous to the first antibody was run in two lanes that were spaced about 5 lanes apart. The gel was aligned on the densitometer platform so that the retention times listed for the two identical lanes were as close as possible.

Comparisons of cross-reactivity were based on the relative mobility of the bands with the similarity between the profiles on a immunoblot expressed as the Jaccard Coefficient (Sj) (Sokal & Sneath, 1963). The retention times of each band within a gel were entered into an IBM XT computer which placed a zero into the position where there was no match. Differences in band position due to differences in the gel polymerization or differences in peak size were corrected by the sequential addition of 0.01-0.05 to each peak before comparison. The highest Sj value calculated was selected as the similarity between the profiles. The value of similarity for the two identical control lanes on each gel was taken as 100%. To assess the reproducibility of the technique two different batches of the same strain were prepared, run on the same gel and then their Sj value calculated. In addition, the antigenic variability that occurs between different rabbits immunized with the same strains was compared. The degree of intercluster cross-reactivity between two strains was presented as the mean of the Sj values given for each of the seven different cluster antisera. The comparison of Fillery cluster 1 and 5 to the four animal strains was recorded as two values, determined from reaction of each with the homologous antisera against the two strains compared.

b) Calculation of the Molecular Weights of Antigens Present on Immunoblots

The immunoblots were read in the densitometer as described previously, the information was transfered to the IBM XT personal computer and stored on a floppy disc. The relative mobility of the major bands and the standard from the same immunoblot were calculated with the help of a 1-D Analyst Data Analysis Software program (Version 2.01, BioRad, Ontario).

The molecular weights of the major bands were calculated with the help of two software computer programs run on a Macintosh SE computer. The first program was Cricket (Cricket Software Inc, Malvern, PA) which provided the formula for the slope of the line when the log of the molecular weight was plotted against the relative mobility. The slope calculated was then entered into the spreadsheet program Excel (Microsoft Excel 2.2, Redmond, WA). The relative mobility for each of the bands was recorded and the program calculated the Log molecular weight based on a set formula. Calculating the antilog (10^x) gave the molecular weight for each band on the immunoblot.

VII) Absorption of Antigens Common to the Genus

Approximately 20 mg of A. israelii cell walls were suspended in 1.5 ml of antisera in an eppendorf tube and then placed on a rotator (Allied Fisher Scientific, Alberta) for 2 hours at 37°C. The cell walls were pelleted in a Micro-Centrifuge (Allied Fisher Scientific, Alberta) and the antisera was reabsorbed an additional two times. Both the unabsorbed and absorbed antisera were reacted with the immunoblot membrane using 1:1000 first antibody and 1:2000 second antibody. To confirm that bands detected by the antisera after absorption ran to the same level as those of the homologous strains, unabsorbed antisera and absorbed antisera were run on the same gel. Two gels were needed to compare all the representatives from the 7 clusters. The molecular weights for the antigens that were detected using the absorbed serum were calculated as previously described .

VIII) Preparation of Cluster Specific Antisera

a) Cell Wall Absorptions

Absorption of antisera that were raised against the clusters with cell walls from the other clusters was undertaken using the same technique as that for the absorption of antigens that were common to Actinomyces.

b) Whole Cells and Sonicated Cells Absorptions

The labour intensive method of preparing cell walls led us to try an alternate method of preparing cluster specific antiserum. Whole cell glass bead extracts and whole cell sonicates were prepared and transfered to immunoblot membranes. These immunoblots were reacted with antisera that was absorbed four times with whole washed cells that were grown in ADM medium. Subsequently, further absorptions with sonicated cells were undertaken. One ml of sterile saline was mixed with 0.75 g wet weight of ADM grown whole cells and the suspension sonicated for one minute (Kontes) on ice. To this was added 1 ml of the serum that had been previously absorbed with whole cells. This was placed on a rotator (Fisher) for 5 hours at 37°C and then centrifuged at 100,000 G (Beckman-Type 50Ti, Ontario) for 30 minutes at 10°C. Immunoblots were reacted with a dilution of 1:500 of this absorbed antisera.

CHAPTER 4

RESULTS

I) Physiological Characteristics

a) Fermentation

Table 4.1 shows the results of carbohydrate fermentation tests on the strains included in the study. Results assessed after 2 days were often negative but by day 5 acid production became evident. The carbohydrates amygdalin, cellobiose, mannitol and sorbitol were poorly fermented. Mannitol was only fermented by NGB 17 (A. israelii), while amygdalin was only fermented by NGB 17 (A. israelii) and WA 2 (cluster 1). Cellobiose was fermented by NGB 17 (A. israelii), ATCC 15987 (cluster 7) and VPI NIIA (Actinomyces-serotype NV). Glycerol, glucose, melibiose, raffinose and rhamnose were fermented by almost all the strains tested except NGB 17 (A. israelii) and WVU 398A (cluster 5). In contrast, melibiose and raffinose were not fermented by 3 strains and 1 strain, respectively, on day 2, but were fermented by all the strains on day 5. Sorbitol was fermentated on day 2 by B 120 and B 102, and by NGB 17 and W 752 by day 5. No fermentation test was specific for any of the clusters.

b) Hydrolysis

Strains NGB 17 (A. israelii) and ATCC 12104 (cluster 5) hydrolysed aesculin while starch hydrolysis was negative for all strains. Five strains (WVU 626, WA 2, ATCC 12104, TF 11 and 4AO5) hydrolysed urea, however, urea hydrolysis was not typical of a given cluster.

c) Growth

Almost all strains were able to grow under aerobic, anaerobic and carbon dioxide supplemented (candle jar) conditions. A. israelii grew aerobically, but only weakly, and two strains from cluster 3 (B 102 and B 120) did not grow under aerobic conditions.

d) Catalase

All strains designated as _A. viscosus_ (clusters 1,2,4,6 and 7), except for W 752, tested as catalase positive, and most strains designated as _A. naeslundii_ were catalase negative, except for B 74 (cluster 3) and VPI NIIA (_Actinomyces_-Serotype NV).

e) Nitrate/Nitrite Reduction

All strains tested were able to reduce nitrate to nitrite after 2 days, but two strains ATCC 15987 (cluster 7) and ATCC 49340 (_A. naeslundii_-serotype III), were only weakly positive for reduction of nitrate to nitrite at day 2. When retested at day 5 these two strains were also positive. No strains tested were able to reduce nitrite.

Table 4.1 Physiological Characteristics of Actinomyces

	A. is.	Cl 1				Cl 2	Cl 3		
	NGB 17	WVU 627	WVU 626	WA 2	W 752	B 236	B 120	B 102	B 74
FERMENT.									
amygdalin	-/+	-/-	-/-	-/+	-/-	-/-	-/-	-/-	-/-
arabinose	-/-	+/+	-/-	+/+	+/+	+/+	-/-	-/-	+/+
cellobiose	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
glycerol	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
glucose	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
glycogen	-/-	+/+	-/+	+/+	-/+	+/+	-/+	-/+	+/+
inulin	-/-	-/-	+/+	+/+	-/+	+/+	-/+	-/-	+/+
lactose	-/+	+/+	+/+	+/+	-/-	-/-	+/+	+/+	+/+
mannitol	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
melibiose	-/+	+/+	+/+	+/+	+/+	-/+	+/+	+/+	+/+
raffinose	-/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
rhamnose	-/-	+/+	+/+	+/+	-/+	+/+	+/+	+/+	+/+
ribose	-/+	-/+	-/+	-/+	-/+	+/+	+/+	+/+	+/+
salicin	-/+	-/-	-/-	+/+	-/-	-/-	+/+	+/+	-/-
sorbitol	-/+	-/-	-/-	-/-	-/+	-/-	+/+	+/+	-/-
trehalose	-/-	-/-	-/-	+/	-/+	+/+	+/+	+/+	+/+
HYDROLYSIS									
aesculin	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
starch	-	-	-	-	-	-	-	-	-
urea	-/-	-/-	-/+	-/+	-/-	-/-	-/-	-/-	-/-
GROWTH									
O_2	w	+	+	+	+	+	-	-	+
AnO_2	+	+	+	+	+	+	+	+	+
CO_2	+	+	+	+	+	+	+	+	+
CATALASE	-	+	+	+	-	+	-	-	+
NO_3 BROTH									
NO_3-no change	-	-	-	-	-	-	-	-	-
NO_3->NO_2	+	+	+	+	+	+	+	+	+
NO_2->reduced	-	-	-	-	-	-	-	-	-
N_2 gas	-	-	-	-	-	-	-	-	-
NO_2 BROTH									
NO_2->reduced	-	-	-	-	-	-	-	-	-

continued...

Table 4.1 ...continued

	CL 4	Cl 5				Cl 6	Cl 7	naII	naIII	NV
	BE 32	ATCC 12104	WVU 398A	TF 11	4AO5	W 1053	ATCC 15987	ATCC 49339	ATCC 49340	VPI NIIA
FERMENT.										
amygdalin	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
arabinose	- / -	- / -	- / -	+/+	- / +	- / +	- / -	+/+	+/+	+/+
cellobiose	- / -	- / -	- / -	- / -	- / -	- / -	+/+	- / -	- / -	+/+
glycerol	+/+	+/+	- / -	+/+	+/+	+/+	+/+	+/+	+/+	+/+
glucose	+/+	+/+	+/+	- / -	+/+	+/+	+/+	+/+	+/+	+/+
glycogen	- / +	- / -	- / -	- / +	- / +	- / +	+/+	- / +	+/+	+/+
inulin	- / +	- / +	- / +	- / +	- / +	+/+	+/+	+/+	- / +	+/+
lactose	+/+	- / -	+/+	+/+	+/+	- / -	+/+	- / -	+/+	+/+
mannitol	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
melibiose	+/+	+/+	+/+	+/+	+/+	- / +	+/+	+/+	+/+	+/+
raffinose	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
rhamnose	+/+	+/+	- / +	- / -	- / -	+/+	+/+	+/+	+/+	+/+
ribose	+/+	- / +	- / +	- / -	- / +	- / +	- / -	+/+	+/+	+/+
salicin	- / -	+/+	+/+	+/+	+/+	+/+	+/+	- / -	- / -	+/+
sorbitol	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
trehalose	- / -	+/+	+/+	- / +	- / -	+/+	+/+	+/+	+/+	- / +
HYDROLYSIS										
aesculin	- / -	+/+	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
starch	-	-	-	-	-	-	-	-	-	-
urea	- / -	- / +	- / -	- / +	- / +	- / -	- / -	- / -	- / -	- / -
GROWTH										
O_2	+	+	+	+	+	+	+	+	+	+
AnO_2	+	+	+	+	+	+	+	+	+	+
CO_2	+	+	+	+	+	+	+	+	+	+
CATALASE	+	-	-	-	-	+	+	-	-	+
NO_3 BROTH										
NO_3-no change	-	-	-	-	-	-	-	-	-	-
NO_3-->NO_2	+	+	+	+	+	+	w	+	w	+
NO_2->reduced	-	-	-	-	-	-	-	-	-	-
N_2 gas	-	-	-	-	-	-	-	-	-	-
NO_2 BROTH										
NO_2->reduced	-	-	-	-	-	-	-	-	-	-

II) Agglutination Reactions

a) Whole Cells

Whole cells from each of the 7 clusters, A. naeslundii-serotypes II & III, Actinomyces-serotype NV and the animal strains A. denticolens (NCTC 11490), A. slackii (NCTC 11928) and A. howellii (NCTC 11636) were assessed for their ability to agglutinate with sera produced against seven clusters and the three animal strains (Table 4.2).

i) Non-pronase-treated Whole Cells

Whole cells from clusters 1-4 and 6 agglutinated with each of the antisera to clusters 1-4 and 6. Whole cells from clusters 1,2 and 4 also agglutinated weakly with cluster 7 antiserum while clusters 3 and 6 whole cells were unable to agglutinate with cluster 7 antiserum. Whole cells from cluster 5 were apparently quite distinct in that they only would agglutinate with their homologous antiserum and cluster 7 antiserum. Whole cells from cluster 7 were also unique in that they agglutinated with antisera raised to clusters 1-3 and 7. The results based on whole cell agglutinations suggest that cluster 5 (ATCC 12104) is immunologically quite distinct from the rest of the 6 clusters. Cluster 7 (ATCC 15987) is slightly different from clusters 1-4 and 6, but a high degree of cross-reactivity exists between clusters 1-4 and 6.

A. naeslundii-serotype II and Actinomyces-serotype NV whole cells showed an agglutination pattern identical to that of whole cells from clusters 3 and 6. Since strain A. naeslundii-serotype III agglutinated weakly with cluster 7 antiserum, it was more similar to clusters 1, 2 and 4.

The antisera produced by immunizing with the animal strains were quite specific for two of the species. A. denticolens antiserum only agglutinated with their homologous cells and A. howellii agglutinated with their homologous cells and non-pronase-treated B 102 whole cells. A. slackii sera was unusual as this sera agglutinated whole cells from clusters 1-4, 6,7, A. naeslundii-serotype III and Actinomyces-serotype NV. When

whole cells of other strains of *A. slackii* (received from Dr. A. Williams) were tested for agglutination by the *A. slackii* antiserum the results were unexpected (Table 4.2b). The strain used in this study to immunize (NCTC 11928) represents the parent of the type strain for *A. slackii*. Strain 4036 of *A. slackii* agglutinated with *A. denticolens* antiserum.

ii) Pronase-treated Whole Cells

Pronase treatment of whole cells had little effect on the agglutination patterns. Pronase-treated cluster 5 whole cells only agglutinated with their homologous antiserum. The cross-reactivity between cluster 5 whole cells and cluster 1 and 7 antisera was eliminated. In addition, pronase treatment of cluster 3 whole cells removed the cross-reactivity to *A. howellii* antiserum.

The antigens responsible for agglutination are unlikely to be cell surface proteins because the agglutination patterns remain after treatment with pronase.

Table 4.2 Non-pronase and Pronase-treated Whole Cell Agglutinations

	cluster 1	cluster 2	cluster 3	cluster 4	cluster 5	cluster 6	cluster 7	A. dent	A. slackii	A. howellii
Strain Number	WVU 627 Serum[a)]	B 236 Serum	B 102 Serum	BE 32 Serum	ATCC 12104 Serum	W 1053 Serum	ATCC 15987 Serum	NCTC 11490 Serum	NCTC 11928 Serum	NCTC 11636 Serum
WVU 627 cluster 1	+/+[b)]	+/+	+/+	+/+	- / -	+/+	w / w[c)]	- / -	+/+	- / -
B 236 cluster 2	+/+	+/+	+/+	+/+	- / -	+/+	w / w	- / -	+/+	- / -
B 102 cluster 3	+/+	+/+	+/+	+/+	- / -	+/+	- / -	- / -	+/+	+/-
BE 32 cluster 4	+/+	+/+	+/+	+/+	- / -	+/+	w / w	- / -	w/+	- / -
ATCC12104 cluster 5	w / -	- / -	- / -	- / -	+/+	- / -	+/-	- / -	- / -	- / -
W 1053 cluster 6	+/+	+/+	+/+	+/+	- / -	+/+	- / -	- / -	+/-	- / -
ATCC15987 cluster 7	+/+	+/+	+/+	- / -	- / -	- / -	+/+	- / -	+/+	- / -
ATCC49339 A. naes II	+/+	+/+	+/+	+/+	- / -	+/+	- / -	- / -	- / -	- / -
ATCC49340 A. naes-III	+/+	+/+	+/+	+/+	- / -	+/+	w / w	- / -	+/++	- / -
VPI NIIA Actino-NV	+/+	+/+	+/+	+/+	- / -	+/+	- / -	- / -	+/+	- / -
NCTC11490 A. dent	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+/+	- / -	- / -
NCTC11928 A. slackii	+/+	+/+	+/+	+/+	- / -	+/+	w / w	- / -	+/+	- / -
NCTC11636 A. howellii	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+/+

a) Antisera diluted 1:20.

b) +/=Nonpronase treated whole cells /+=Pronase treated whole cells

c) w=weak reaction.

Table 4.2b

Additional Non-pronase-treated Whole Cell A. slackii Agglutinations

A. slackii Strains From A. Williams	A. Slackii Serum	A. Dent Serum
4563 (NCTC 11928)*	+	
4503	-	
4444	-	
4476	-	
4528	-	
4036		+

*-Immunizing Strain

b) Pronase-treated Cell Walls

Pronase-treated cell walls for a strain in each of the clusters, A. naeslundii II, III, and the three animal species A. denticolens, A. slackii and A. howellii were assessed for their ability to agglutinate with antisera raised to each of the clusters and the three animal species A. denticolens, A. slackii and A. howellii (Table 4.3). In general a high degree of cross-reactivity existed. The highest dilutions showing agglutinations were usually those between the homologous cell walls and antiserum, except for B 102 (cluster 3) and BE 32 (cluster 4). Cross-reactivity was high between clusters 1-6, A. naeslundii II, III and A. slackii using antisera to the same groups. Cross-reactivity was minimal between the pronase-treated cell wall extracts of animal strains ATCC 15987 (cluster 7), A. denticolens and A. howellii and the 10 antisera tested.

In order to determine whether the antigens in the pronase-treated cell walls, were protein an SDS extract of the pronase-treated cell walls was subjected to polyacrylamide gel electrophoresis and stained with Coomassie blue. No proteins stained on the gel.

Table 4.3 Pronase-treated Cell Wall Agglutinations

	cluster 1	cluster 2	cluster 3	cluster 4	cluster 5	cluster 6	cluster 7	A. dent	A. slackii	A. howellii
Strain Number	WVU 627 Serum	B 236 Serum	B 102 Serum	BE 32 Serum	ATCC 12104 Serum	W 1053 Serum	ATCC 15987 Serum	NCTC 11490 Serum	NCTC 11928 Serum	NCTC 11636 Serum
WVU 627 cluster 1	80[a)]	20	10	40	0	10	0	10	20	10
B 236 cluster 2	10w[b)]	80	10	0	10	0	0	0	0	0
B 102 cluster 3	0	10	0	20	0	20	0	0	20	0
BE 32 cluster 4	40	20	20	20	20	40	0	0	80	0
ATCC12104 cluster 5	0	20	0	20	80	10	0	0	20	0
W 1053 cluster 6	40	10	20	40	40	320	0	20	160	0
ATCC15987 cluster 7	0	10	0	10	0	0	20	0	0	0
ATCC49339 A. naes II	10	20	0	0	40	160	0	0	80	0
ATCC49340 A. naes III	0	10	10	0	0	10	10	0	0	20
VPI NIIA Actino.NV	NT[c)]	NT	NT	NT	NT	NT	NT	NT	NT	NT
NCTC11490 A. dent	0	0	10w	0	0	0	0	80	10	0
NCTC11928 A. slackii	10	0	0	20	20	20	0	0	160	0
NCTC11636 A. howellii	0	0	0	0	0	0	0	0	20	80

a) The highest dilution of antiserum giving a positive result.
b) w=weak reaction.
c) NT=Not Tested

III) Carbohydrate Cross-reactivity

a) Double Diffusion

Carbohydrate acid extracts were made from the pronase-treated cell walls of strains representing each of the seven clusters and the three animal strains *A. denticolens*, *A. slackii*, and *A. howellii*. No protein was present in the carbohydrate extracts tested as assessed using the BioRad Protein Assay (BioRad, Ontario). Carbohydrates were used in the double diffusion study at concentrations of 1 mg/ml. When the extracts were assessed for the ability to precipitate with each of the 21 antisera only a few gave weakly positive reactions (data not shown) and, consequently, the results were inconclusive.

b) Rocket Immunoelectrophoresis

To further demonstrate the cross-reactivity that may exist between the carbohydrate component of the cell walls, we tested the carbohydrate extracts using rocket immunoelectrophoresis with agarose containing 1/20 antiserum. The sera that appeared to be able to precipitate carbohydrate in the double diffusion study was selected as the test antisera. The results were weak so it was difficult to draw any conclusion about the nature of the carbohydrate cross-reactivity. However, antiserum produced by immunizing with ATCC 12104 (cluster 5) produced two precipitin lines with ATCC 12104 carbohydrate. One cross-reacting band also appeared with wall carbohydrate from B 236 (cluster 2), *A. denticolens* and *A. howellii*. Due to the weak reactivity of the carbohydrate extracts with antisera, it is not possible to draw any firm conclusions on the cross-reactivity detected.

IV) Antigenic Similarity (Sj) Determined for Cell Wall Extracts

a) Morphology of Cell Wall Preparations

In order to gain some insight into the origin of the cell surface antigens whole cells, cell walls, and pronase-treated cell walls were examined by transmission electron

microscopy. Figures 4.1a-f shows an example of the results with whole cells and non-pronase and pronase-treated cell walls of ATCC 12104 (cluster 5) and Figure 4.1g shows an example of whole cells of WVU 627 (cluster 1).

i) Whole Cells

The appearance was consistent with a Gram positive rod (Figures 4.1a & b). Cells were pleomorphic rods that formed chains and branched occasionally. The cell wall appeared as an outer lighter staining fuzzy layer associated with an inner darker staining fine band. Adjacent to the cell wall the cell membrane was visible. Long fimbriae extending from the cell wall were clearly seen on WVU 627 (Figure 4.1g) but similar structures were not visible on ATCC 12104 (Figures 4.1 a & b).

ii) Non-pronase-treated Cell Walls

The cell wall preparation showed cell walls with little extracellular material (Figure 4.1c). No plasma membrane was apparent and the cell walls appeared occasionally to be coiled inside out and packed together (Figure 4.1d). This packing was most likely a result of the freeze drying process.

iii) Pronase-treated Cell Walls

Pronase treatment had little effect on the transmission electron microscopic picture (Figures 4.1e & f). The outer layer of the cell wall appeared to be less fuzzy and, in general, the cells were less coiled and not packed together as tightly as the non-pronase-treated cell walls.

Figure 4.1a-f. Transmission electron microscopic picture of ATCC 12104 (cluster 5) whole cells (a & b), cell walls (c & d) and pronase-treated cell walls (e & f).

CW-cell wall
OCW-outer cell wall
ICW-inner cell wall
PM-plasma membrane

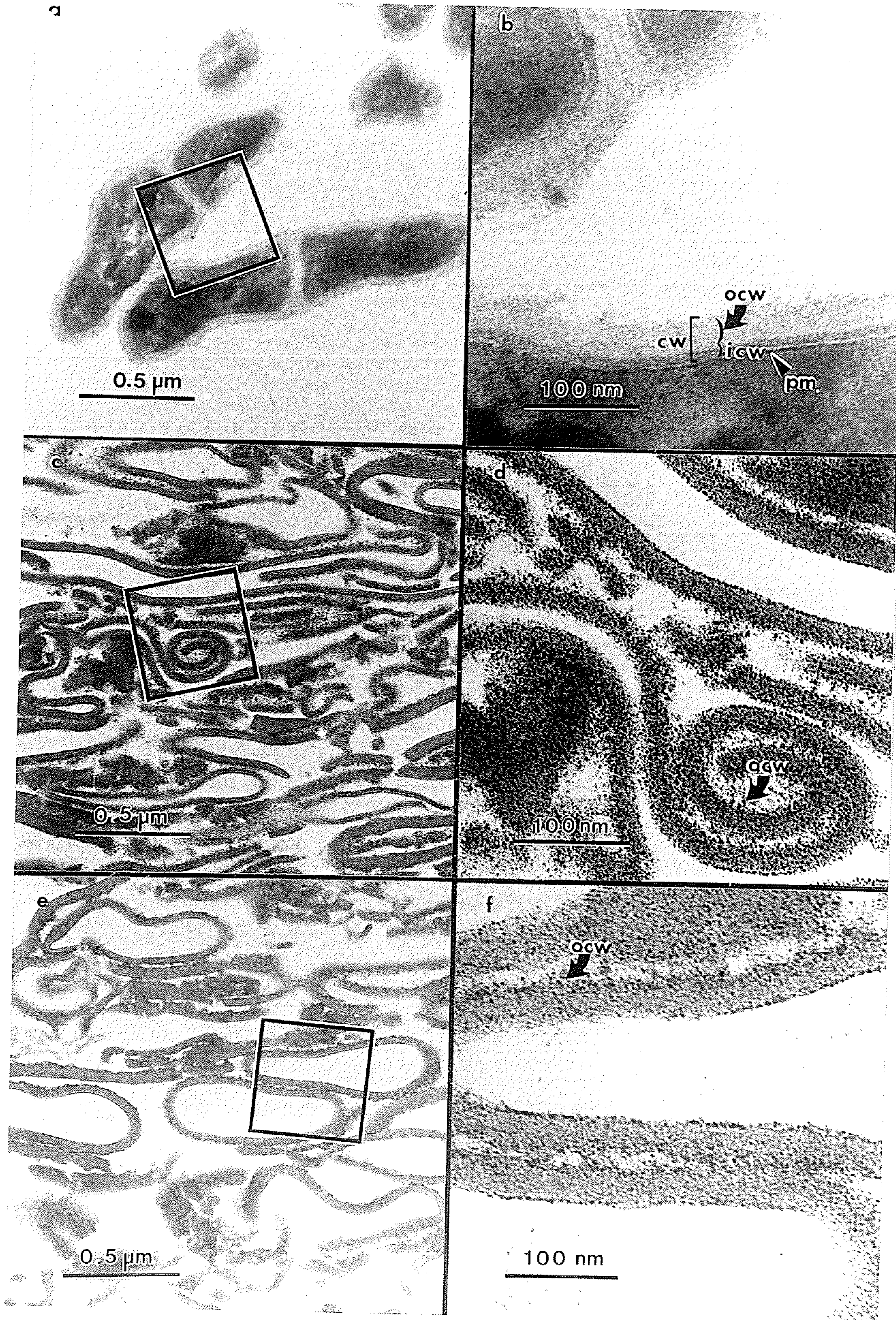


Figure 4 .1a-f. Legend on previous page.

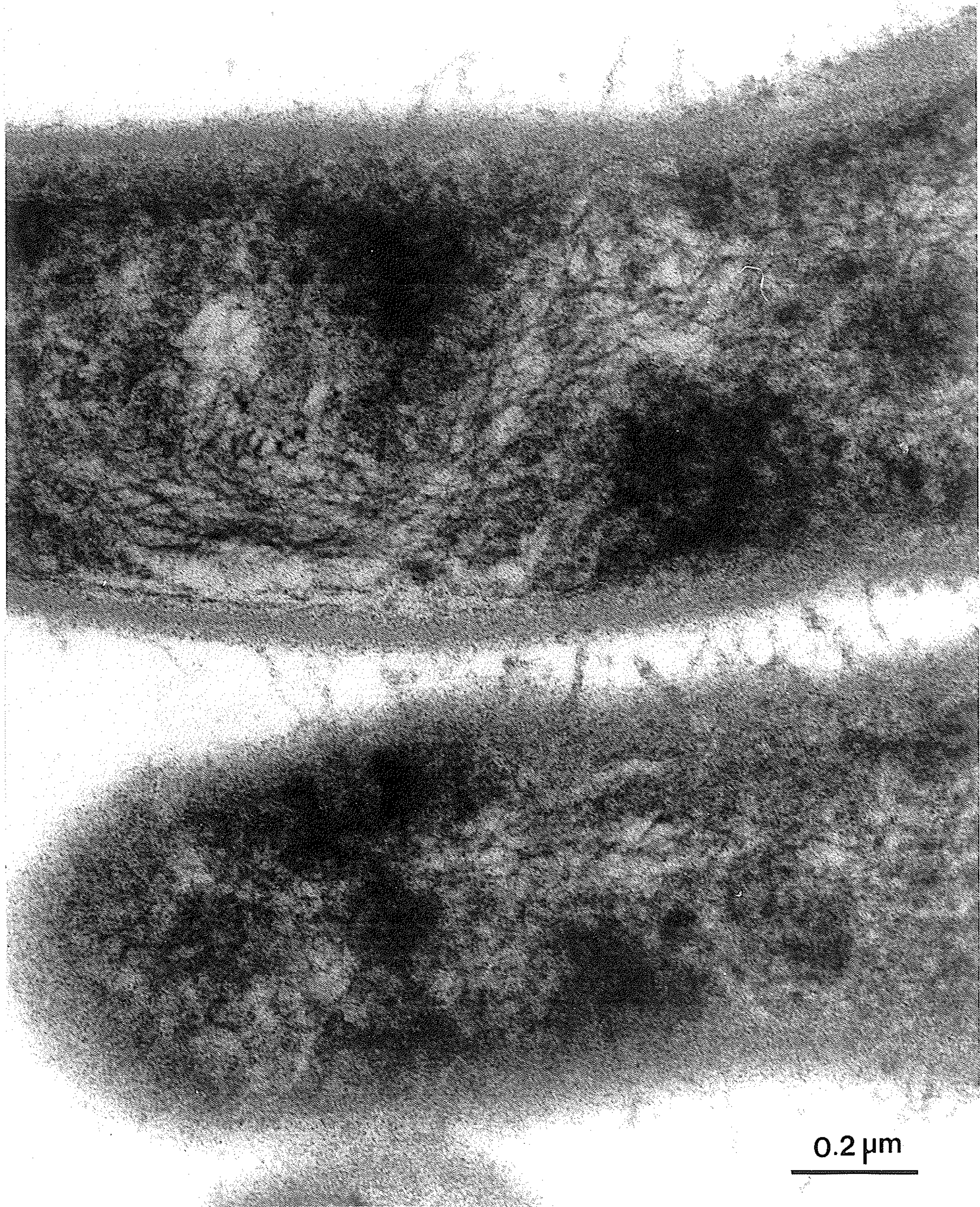


Figure 4.1g. Whole cells of WVU 627 (cluster 1) showing fimbriae.

b) Whole Cell Glass Bead Extracts and Cell Wall Extracts

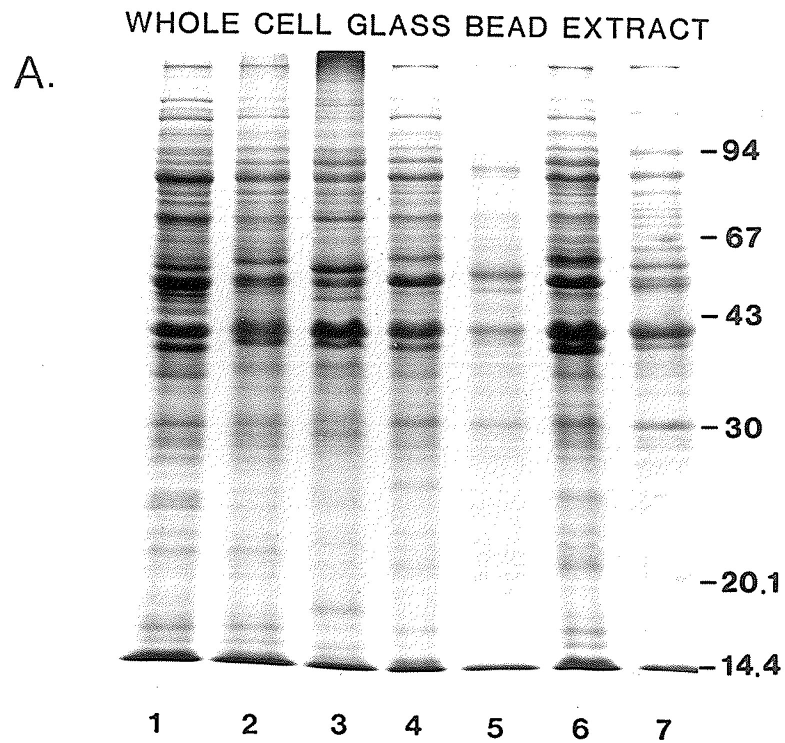
Since this study is trying to identify immunologically distinct antigens, it would be best to remove proteins that are similar in antigenic structure as these would complicate the immunoblot profiles. The cell wall extracts were chosen for additional study for two reasons. Firstly, cell surface antigens are most likely to be presented to the immune system and secondly, whole cell glass bead extracts contain many cytoplasmic proteins that could be common to A. viscosus and A. naeslundii as they are physiologically similar (Fillery et al. 1978). Figure 4.2 shows two Coomassie blue stained polyacrylamide gels. Figure 4.2a is of whole cell glass bead extracts and Figure 4.2b of cell wall extracts. Both techniques gave good extraction of protein.

Figure 4.2a Whole cell glass bead extracts run on a polyacrylamide gel stained with Coomassie blue.

Lane 1 WVU 627 (cluster 1)
Lane 2 B 236 (cluster 2)
Lane 3 B 102 (cluster 3)
Lane 4 BE 32 (cluster 4)
Lane 5 ATCC 12104 (cluster 5)
Lane 6 W 1053 (cluster 6)
Lane 7 ATCC 15987 (cluster 7)

Figure 4.2b. Cell wall extracts run on a polyacrylamide gel stained with Coomassie blue.

Lane 1 WVU 627 (cluster 1)
Lane 2 B 236 (cluster 2)
Lane 3 B 102 (cluster 3)
Lane 4 BE 32 (cluster 4)
Lane 5 ATCC 12104 (cluster 5)
Lane 6 W 1053 (cluster 6)
Lane 7 ATCC 15987 (cluster 7)
Lane 8 NGB 17 (*A. israelii*)



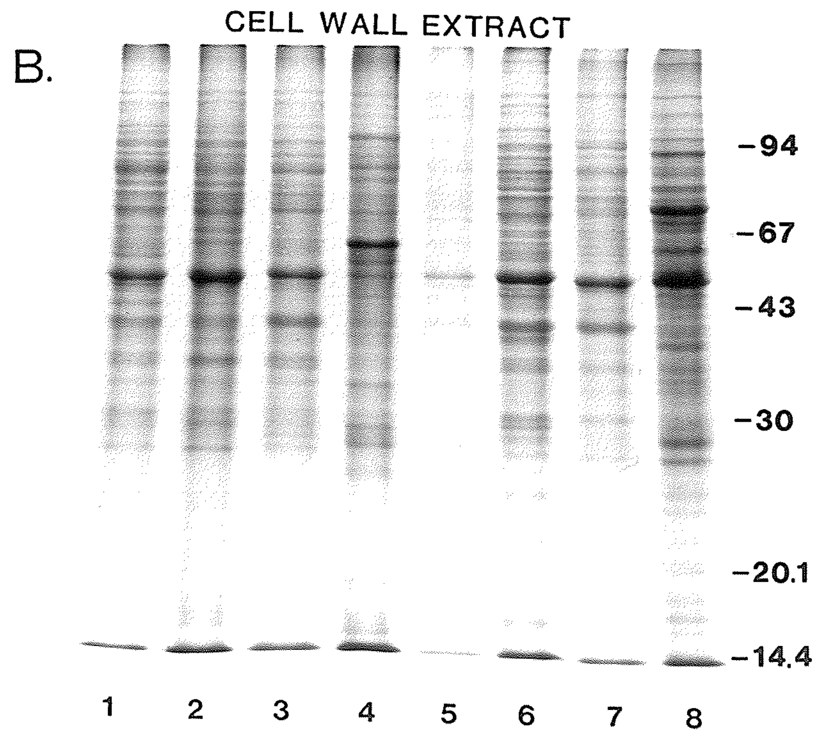


Figure 4.2a & b. Legends on previous page.

c) Cell Wall Extract Antigenic Similarity (Sj) Using Cell Walls Produced in Different Batches

Table 4.4 shows the similarity between different batches of cell walls from strains that were grown separately in batch culture. Each strain from the two different batches was run on the same gel/immunoblot and reacted with its homologous antiserum. The percent antigenic similarity (Sj) ranged from 85%-92% for each of the three strains tested.

Table 4.4 Percent Antigenic Similarity (Sj) of Different Batches of Cell Walls When Reacted With Their Homologous Antiserum

Cluster & Strain	Jaccard (Sj) Similarity
Cluster 1 WVU 627	85
Cluster 3 B 102	92
A. slackii (animal) NCTC 11928	89

d) Similarity (Sj) of Cell Wall Extracts Reacted With Homologous Antisera Produced in Different Rabbits

Cell wall extracts were made from strains in cluster 1 (WVU 627) and cluster 5 (ATCC 12104). In one case, the extracts of the strains WVU 627 and ATCC 12104 were run in three pairs on the same immunoblot and in the second case two pairs were run on the same immunoblot. Each pair on the first immunoblot was reacted with each of the three WVU 627 antisera produced in different rabbits and each pair on the second immunoblot was reacted with ATCC 12104 antisera produced in two different rabbits (Figure 4.3). The antigenic similarity (Sj) of cell wall extracts reacted with antiserum from different rabbits immunized with the homologous strain is shown in Table 4.5. The differences noted appear only to be one of degree of reactivity. One major antigen of WVU 627 was only detected by one rabbit antiserum against ATCC 12104 (Figure 4.3). As there is no corresponding antigen in ATCC 12104, the band visible in the WVU 627 cell wall extract must be a immunologically similar to an antigen in ATCC 12104 of different molecular weight.

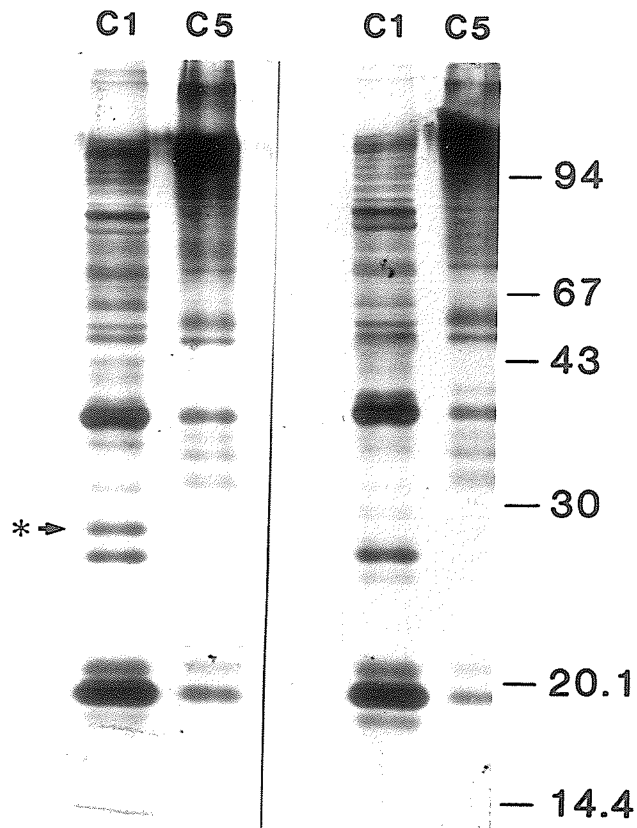


Figure 4.3. Two immunoblots of cell wall extracts of WVU 627 (C1) and ATCC 12104 (C5). Each immunoblot was reacted with antiserum produced in one of two different rabbits. Both rabbits were immunized with the same strain-ATCC 12104 (cluster 5).

*-a cross-reacting antigen recognized in cluster 1 cell wall extracts by antibodies in one rabbit antiserum but not the other.

Table 4.5

Percent Antigenic Similarity (Sj) of Cell Wall Extracts Reacted With Homologous Antisera Produced in Different Rabbits (R)

A) Cluster 1-WVU 627 Cell Wall Extract Reacted With Homologous Rabbit Antisera (R101-103)

	R101	R102	R103
R101	-	74	67
R102	-	-	77
R103	-	-	-

Average similarity for 3 rabbits =73%.

Cluster 5-ATCC 12014.Cell Wall Extract Reacted With Homologous Rabbit Antisera (R104-R105).

	R104	R105
R104	-	71
R105	-	-

V) Antigenic Similarity (Sj) Between the Fillery Clusters 1-7 and A. israelii Cell Wall Extracts Assessed by Immunoblotting

This study was undertaken to assess whether the Fillery clusters, based on physiological differences, would also exhibit cluster related immunological differences. Intercluster and intracluster antigenic similarity was calculated together with the similarity of the clusters to three additional A. naeslundii species and three recently described animal species.

a) Intercluster Antigenic Similarity (Sj)

Cell wall extracts were prepared from one representative of each of the seven Fillery clusters and A. israelii, the latter organism being used to determine the cross-reactivity due to antigens common to Actinomyces. Cross-reactivity was analyzed by immunoblotting with each of the seven antisera raised against the Fillery clusters. Figure 4.4 shows an example of the bands identified in densitometer scans and Figure 4.5 shows two immunoblots of cell wall extracts that were developed with two different cluster antisera. The complete data comparing the antigenic similarity of each cluster extract to one another using the seven different cluster antisera is shown in Appendix 2. In general, a high degree of cross-reactivity existed with the percent antigenic similarity ranging from 79.2% for WVU 627 (cluster 1) to B 236 when developed with B 236 (cluster 2) antiserum to 21.1% for B 236 (cluster 2) to ATCC 12104 (cluster 5) when developed with W 1053 (cluster 6) antiserum. Cross-reactivity between the clusters and A. israelii was also extensive, ranging from 15.4%-61.1% suggesting that antigens common to Actinomyces were present in the extracts.

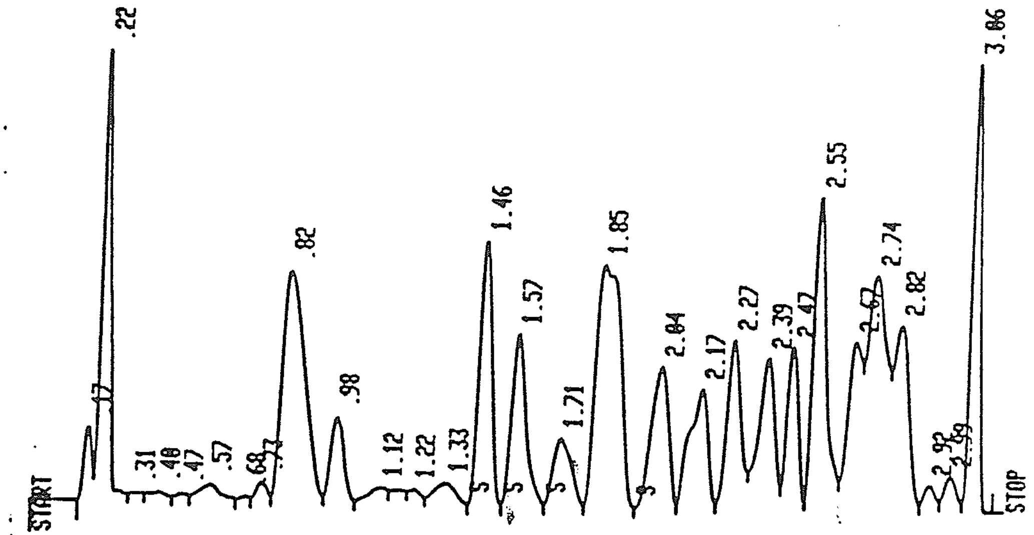
The data shown in Appendix 2 has been simplified in Table 4.6 by representing the mean and standard error of antigenic similarity between each of the seven clusters and A. israelii. The highest antigenic similarity (71%) occurs between WVU 627 (cluster 1) and B 236 (cluster 2), a value calculated as a mean of the similarities given by reaction of extracts with the seven different cluster antisera. The lowest

similarity within the seven Fillery clusters was 37% between B 102 (cluster 3) and ATCC 12104 (cluster 5). None of the 7 clusters appeared antigenically unique. Antigenic similarities between each cluster and A. israelii ranged from 26%-43%.

Table 4.7 further simplifies the data and shows the mean similarity between each cluster, and the other clusters and A. israelii using the seven different cluster antisera. The mean antigenic similarity between the seven clusters ranged from 41-55%. The three lowest antigenic similarities were for clusters 5 (ATCC 12104)-41%, 3 (B 102)-45% and 7 (ATCC 15987)-46% suggesting that these clusters differ from the others (clusters 1,2, 4 and 6). A. israelii (NGB 17) showed an antigenic similarity of 35% to the clusters of A. viscosus and A. naeslundii.

Figure 4.4. Examples of Scanning Densitometer Results

Scan 1
WVU627
(cluster 1)



Scan 2
ATCC12104
(Cluster 5)

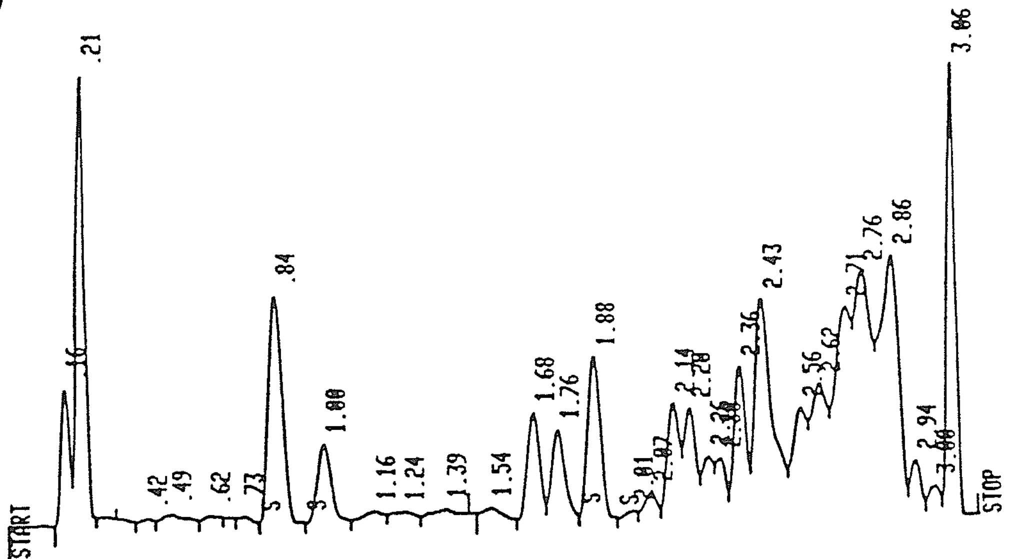
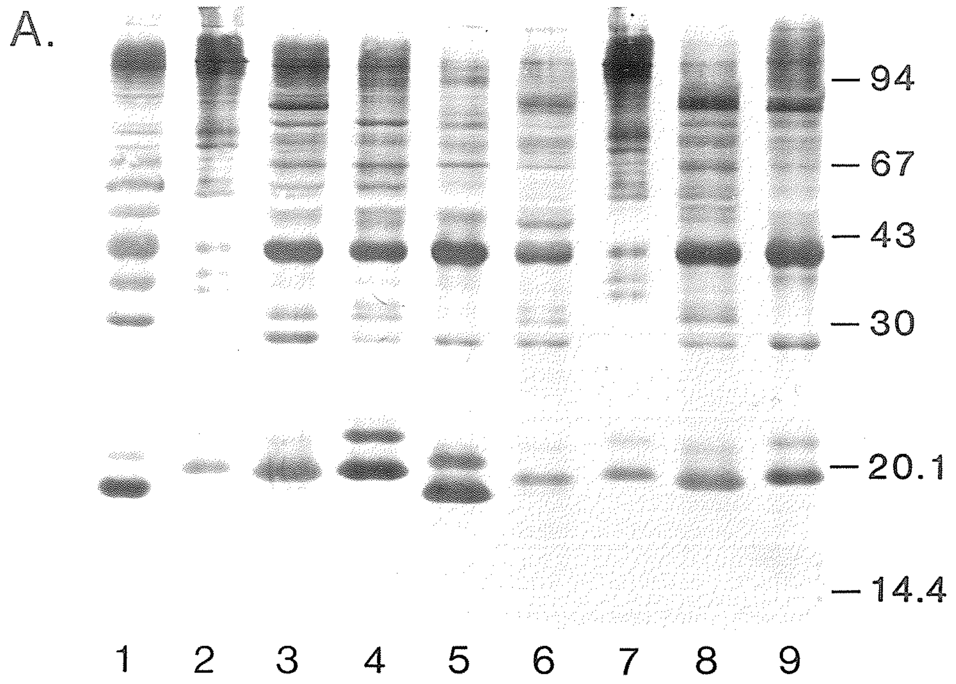


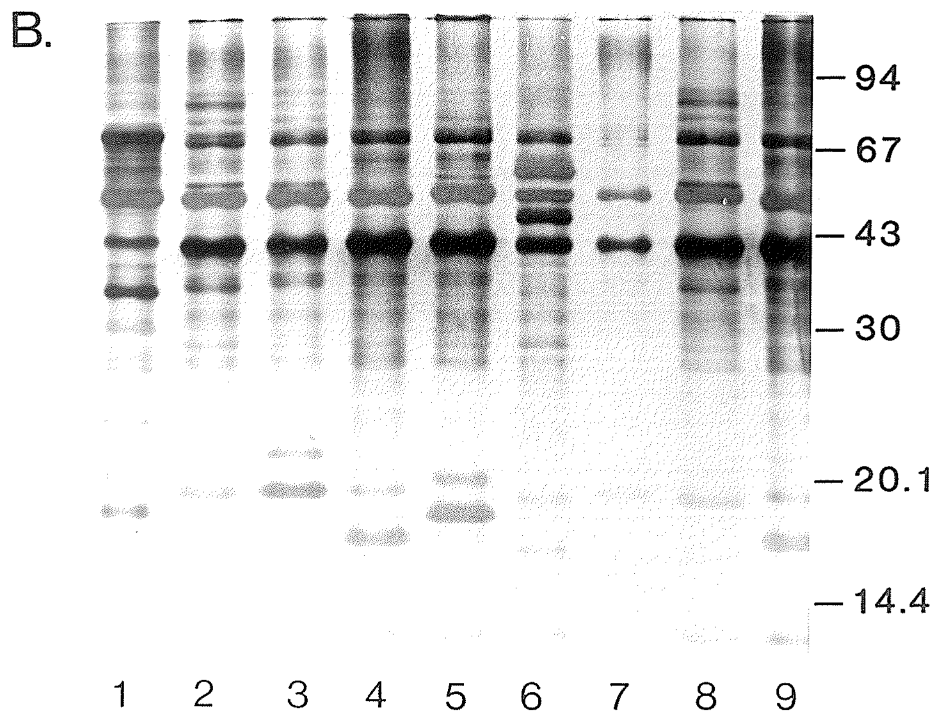
Figure 4.5a. Immunoblot of cell wall extracts prepared to a representative from each of the seven clusters and A. israelii. Immunoblot reacted with antiserum prepared by immunizing with ATCC 12104 (cluster 5).

Lane 1 NGB 17 (A. israelii)
Lanes 2 & 7 ATCC 12104 (cluster 5)
Lane 3 WVU 627 (cluster 1)
Lane 4 B 236 (cluster 2)
Lane 5 B 102 (cluster 3)
Lane 6 BE 32 (cluster 4)
Lane 8 W 1053 (cluster 6)
Lane 9 ATCC 15987 (cluster 7)

Figure 4.5b. Immunoblot of cell wall extracts prepared to a representative from each of the seven clusters and A. israelii. Immunoblot reacted with antiserum prepared by immunizing with ATCC 15987 (cluster 7).

Lane 1 NGB 17 (A. israelii)
Lane 2 WVU 627 (cluster 2)
Lane 3 B 236 (cluster 2)
Lanes 4 & 9 ATCC 15987 (cluster 7)
Lane 5 B 102 (cluster 3)
Lane 6 BE 32 (cluster 4)
Lane 7 ATCC 12104 (cluster 5)
Lane 8 W 1053 (cluster 6)





Figures 4.5a & b. Legend on the previous page.

Table 4.6

Mean and Range of Antigenic Similarity (Si) Determined by Immunoblotting of Cell Wall Extracts Between Extracts of Fillery Clusters and *A. israelii* Using Antisera Against Fillery Clusters 1-7

	cluster 1	cluster 2	cluster 3	cluster 4	cluster 5	cluster 6	cluster 7	A. israelii
	WVU 627	B 236	B 102	BE 32	ATCC 12104	W 1053	ATCC 15987	NGB 17
WVU 627		71±3* 58-79†	46±5 24-59	55±3 42-65	41±3 30-47	62±4 46-76	54±6 29-75	39±4 26-52
B 236			47±3 33-64	56±4 42-71	48±7 21-80	62±7 43-90	53±4 35-67	37±3 26-53
B 102				45±3 33-52	37±4 25-53	51±3 42-61	46±4 30-57	43±4 25-55
BE 32					45±4 31-64	53±1 46-59	43±5 26-69	35±4 24-50
ATCC 12104						48±3 39-65	40±4 21-52	26±4 15-46
W 1053							55±4 36-72	34±5 17-61
ATCC 15987								34±4 22-53

* Mean of 7 cluster antisera ±SE
† Minimum - maximum values

Table 4.7

Mean and Range of Antigenic Similarity (Sj) Between Cell Wall Extracts of Each of the Fillery Clusters and *A. israelii*

Strain/Cluster Cell Wall Extracts	Extracts Tested For Similarity	Mean Antigenic Similarity (Sj) ± SE Min-Max
WVU 627 cluster 1	Cluster 2-7 and *A. israelii*	53±4 39-71
B 236 cluster 2	Cluster 1, 3-7 and *A. israelii*	55±5 37-71
B 102 cluster 3	Cluster 1-2,4-7 and *A. israelii*	45±2 37-51
BE 32 cluster 4	Cluster 1-3,5-7 and *A. israelii*	48±3 35-56
ATCC 12104 cluster 5	Cluster 1-4,6-7 and *A. israelii*	41±3 26-48
W 1053 cluster 6	Cluster 1-5,7 and *A. israelii*	53±4 34-70
ATCC 15987 cluster 7	Cluster 1-6 and *A. israelii*	46±3 34-55
NGB 17 (A. *israelii*)	Cluster 1-7	35±2 26-43

b) Intracluster Antigenic Similarity (Sj)

Four strains from each of clusters 1 and 5, and three strains from cluster 3 were chosen. Cell wall extracts to each strain in the cluster were prepared and then run as two sets on an immunoblot. Each set was reacted with the two different rabbit antisera representing that cluster. Figure 4.6 shows an example of two different immunoblots of cell wall extracts of four strains from cluster 1 reacted with cluster 1 antiserum and four strains from cluster 5 reacted with cluster 5 antiserum.

Intracluster antigenic similarities were high (Table 4.8). Cluster 1 had an average intracluster antigenic similarity of 78% or 84% (1 for each rabbit representing that cluster) and cluster 5 showed an intracluster antigenic similarity of 72% or 80%. The values for cluster 3 were very interesting in that strain B 74 showed a relatively low antigenic similarity to the two other cluster 3 representatives (B 102 and B 120). The average antigenic similarity between B 74 and the two other strains of cluster 3 was only 36% or 45%. Since both rabbit antisera showed a similar result, it would be unlikely to be a false value due to a technical error. The similarity between B 120 and B 102 was 77% or 84% depending on the antiserum used. These numbers are similar to the intracluster values of clusters 1 and 5. Therefore, these data show an intracluster antigenic similarity for cluster 1, 3 (excluding B 74) and 5 of around 72%-84%. The low value for B 74 suggests this strain possibly should not be in cluster 3.

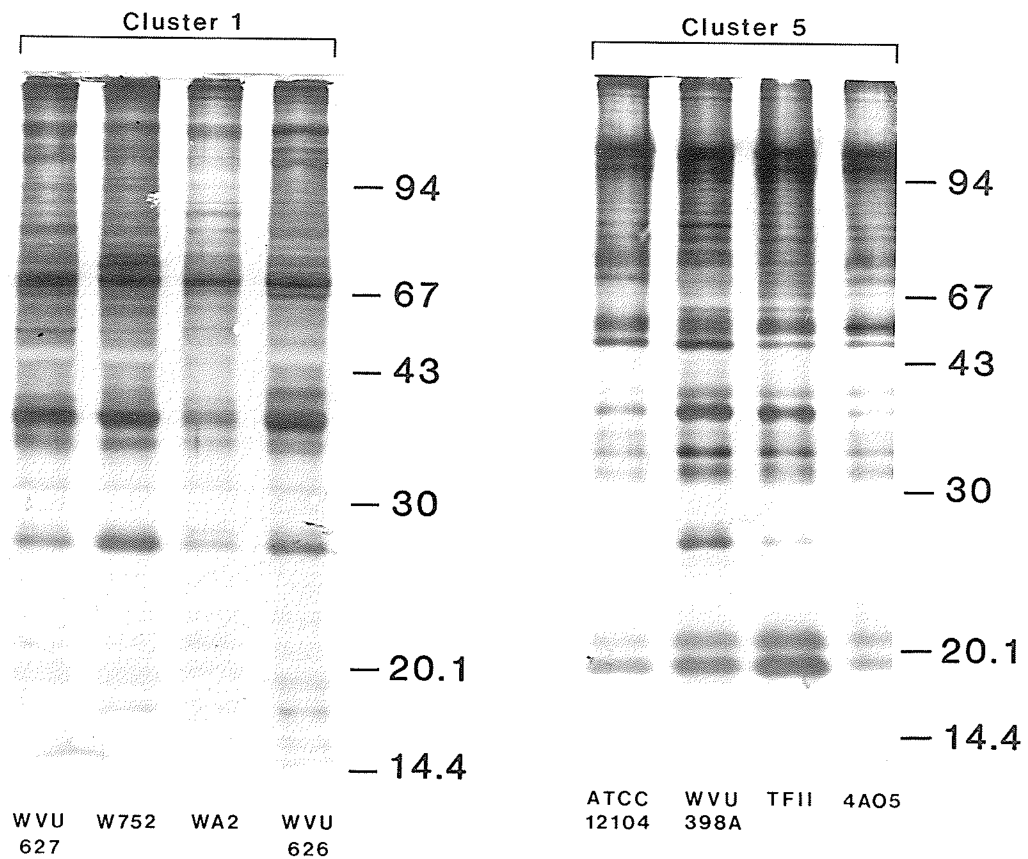


Figure 4.6. Immunoblots of cell wall extracts from clusters 1 and 5.

Cluster 1 representative strains WVU 627, W 752, WA 2 and WVU 626. This immunoblot was reacted with antiserum produced by immunizing with WVU 627.

Cluster 5 representative strains ATCC 12104, WVU 398A, TF 11 and 4AO5. This immunoblot was reacted with antiserum produced by immunizing with ATCC 12104.

Table 4.8

Percent Intracluster Antigenic Similarity (Si) Using Cell Wall Extracts Reacted With Antisera Homologous to that Cluster

A) Intracluster 1 Antigenic Similarity.(Si)

	WVU 627	W 752	WA 2	WVU 626
WVU 627	-	69,80*	71,88	82,82
W 752	-	-	81,83	85,85
WA 2	-	-	-	82,85
WVU 626	-	-	-	-

Average within cluster=78-84%.

B)Percent Intracluster 3 Antigenic Similarity (Si)

	B 102	B 120	B 74
B 102	-	77,84	39,42
B 120	-	-	33,47
B 74	-	-	-

Average within the cluster=Value would be falsely low due to the low similarity of B 120 and B 102 to B 74.

C)Percent Intracluster 5 Antigenic Similarity (Si)

	ATCC 12104	WVU 398A	TF 11	4AO5
ATCC 12104	-	65,77	67,70	70,81
WVU 398A	-	-	65,89	74,83
TF 11	-	-	-	83,89
4AO5	-	-	-	-

Average within cluster=72-80%.

*-One value for each of 2 rabbits immunized with the homologous cluster strains

c) Antigenic Similarity of Cell Wall Extracts From the Fillery Clusters And Animal Actinomyces Species

Appendix 3 contains all the values of the antigenic similarities between the cell wall extracts of cluster 1 (WVU 627), cluster 5 (ATCC 12104), cluster 7 (ATCC 15987), A. howellii (NCTC 11636), A. slackii (NCTC 11928) and A. denticolens (NCTC 11490) using antisera prepared to each of the 6 strains listed.

For ease of presentation, Table 4.9 summarizes Appendix 3 and Figure 4.7 shows an example of one of the immunoblots. The values presented within each cell of Table 4.9 represent the antigenic similarity values using the two antisera homologous to the two cell wall extracts being compared. Generally, there exists a fairly high degree of cross-reactivity between the three Fillery cluster representatives and the three animal strains, but, no one species appeared to be antigenically distinct.

Table 4.9

Percent Antigenic Similarity (Si) of Cell Wall Extracts From Fillery Cluster Representatives and Animal Actinomyces Species

	Cluster 1 WVU 627	Cluster 5 ATCC 12104	Cluster 7 ATCC 15987	A. howellii NCTC 11636	A. slackii NCTC 11928	A. dent NCTC 11490
WVU 627	-	67,56	64,79	62,52	74,67	59,72
ATCC12104	-	-	65,45	61,50	77,61	56,50
ATCC15987	-	-	-	74,60	69,68	54,71
A. howellii	-	-	-	-	58,77	63,48
A. slackii	-	-	-	-	-	58,61
A. dent	-	-	-	-	-	-

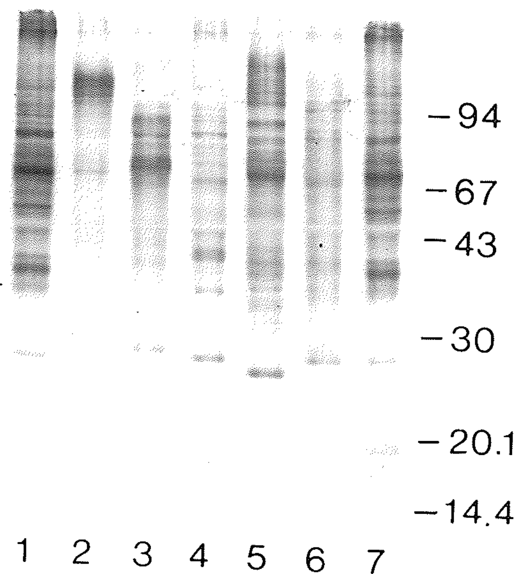


Figure 4.7. Immunoblot of cell wall extracts from cluster 1,5 and 7 and three animal *Actinomyces* species. Immunoblot reacted with antiserum produced by immunizing with WVU 627 (cluster 1).

Lanes 1 & 7 WVU 627 (cluster 1)
Lane 2 ATCC 12104 (cluster 5)
Lane 3 NCTC 11490 (*A. denticolens*)
Lane 4 NCTC 11928 (*A. slackii*)
Lane 5 NCTC 11636 (*A. howellii*)
Lane 6 ATCC 15987 (cluster 7)

d) Antigenic Similarity (Sj) of Cell wall Extracts Prepared to Fillery Clusters and A. naeslundii-serotype II & III and Actinomyces-serotype NV Species

Cell wall extracts were prepared to three Fillery cluster representatives (cluster 1-WVU 627, cluster 3-B 102 and cluster 5-ATCC 12104). These were compared to cell wall extracts prepared to A. naeslundii-serotypes II (ATCC 49339) and III (ATCC 49340) and Actinomyces-serotype NV (VPI NIIA). Figure 4.8 shows an immunoblot developed with these extracts using antisera to one of the three Fillery clusters. The values presented on Table 4.10 shows that a high degree of cross-reactivity exists between all strains tested. Strain Actinomyces-serotype NV was most similar to WVU 627 with an antigenic similarity of 85% and A. naeslundii-serotype II was most similar to WVU 627 with an antigenic similarity of 77%. Strain A. naeslundii-serotype III was most similar to B 102 with an antigenic similarity of 76%. All three of these similarities were based on data from immunoblots developed with cluster 1 antisera.

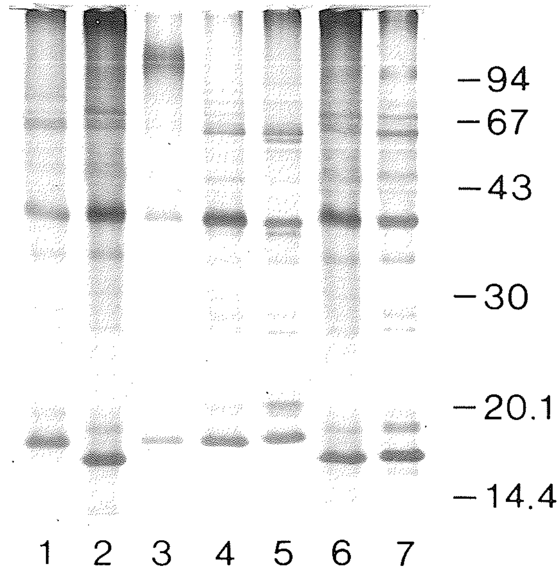


Figure 4.8. Immunoblot of cell wall extracts from clusters 1,3 and 5 and A. naeslundii-serotypes II & III and Actinomyces-serotype NV. Immunoblot reacted with antiserum produced by immunizing with B 102 (cluster 3).

Lane 1 WVU 627 (cluster 1)
Lane 3 ATCC 12104 (cluster 5)
Lane 5-ATCC 49339 (A. naeslundii-II)
Lane 7 ATCC 49340 (A. naeslundii-III)

Lanes 2 & 6 B 102 (cluster 3)
Lane 4 VPINIIA (Actinomyces-NV)

Table 4.10

Percent Antigenic Similarity (Si) of Cell Wall Extracts of Fillery Cluster Representatives and A. naeslundii-serotypes II & III and Actinomyces-serotype NV

	Cluster 1	Cluster 3	Cluster 5	Actino NV	A. naes II	A. naes III
	WVU 627	B 102	ATCC 12104	VPI NIIA	ATCC 49339	ATCC 49340
WVU 627						
*S1	-	72	64	85	77	73
S3	-	58	38	46	39	35
S5	-	52	48	67	65	42
B 102						
S1	-	-	46	59	63	76
S3	-	-	31	52	40	62
S5	-	-	32	50	50	36
ATCC 12104						
S1	-	-	-	44	48	48
S3	-	-	-	47	33	28
S5	-	-	-	38	50	46
VPINIIA						
S1	-	-	-	-	78	66
S3	-	-	-	-	73	73
S5	-	-	-	-	70	58
ATCC 49339						
S1	-	-	-	-	-	64
S3	-	-	-	-	-	62
S5	-	-	-	-	-	64

*S=Antiserum produced to that cluster indicated.

VI) Antigenic Cross-reactivity Due to Antigens Common to Actinomyces

Each of the seven cluster antisera were absorbed with A. israelii cell walls. These and the original antisera were reacted with immunoblots of cell wall extracts of the 7 clusters and A. israelii (NGB 17). The number of the antigenic bands visible before and after absorption with NGB 17 cell walls are listed in Table 4.11.

Absorption of the cluster antisera with NGB 17 cell walls removed most of the cross-reactivity with NGB 17 cell wall extracts. Table 4.11 shows that in 6 of 7 immunoblots developed with absorbed antisera, there were not more than two antigens visible on the immunoblot of the NGB 17 cell wall extract. The antigen which was developed most frequently by absorbed antisera had a molecular weight of approximately 30kDa. Reabsorption of the antisera to each of the clusters was not effective in removing these one or two remaining bands. Further examination of these antigens was undertaken. The possibility existed that antibodies present in the peroxidase labelled second antibody developed these antigens. An immunoblot developed with the peroxidase labelled antibody did not show the 30 kDa antigen. Also peroxidase labelled IgG Fc fragment was reacted with one immunoblot to determine if any Fc receptors were present in the cell wall extracts. None were detected.

The absorption of each cluster antiserum with A. israelii (NGB 17) cell walls produced antisera that still identified many of the antigens in the homologous cell wall extracts. Cluster 7 antiserum showed less reactivity after absorption compared to its homologous antiserum because many of the antigens on the immunoblot had a density just below the threshold of the Bio Rad scanning program.

Regardless of which of the 7 cluster antisera was absorbed with A. israelii cell walls, two of the clusters appear to be distinct. Cluster 5 (ATCC 12104) and cluster 7 (ATCC 15987) cell wall extracts showed a high degree of cross-reactivity to the other clusters regardless of which of the unabsorbed cluster antisera was used. In contrast,

after absorption of the cluster antisera with A. israelii (NGB 17) cell walls, they exhibited only minor cross-reactivity with cell wall extracts of clusters 5 and 7. Figure 4.9 shows an example of an immunoblot reacted with cluster 4 antiserum unabsorbed and absorbed with A. israelii (NGB 17) cell walls. Cross-reactivity between the antisera and extracts of clusters 5 and 7 disappeared using the absorbed antiserum.

It is also interesting to note one additional point. Table 4.6 shows that the antigenic similarities of cluster 5 (ATCC 12104) and cluster 7 (ATCC 15987) to A. israelii (NGB 17) are 26% and 34%, respectively, using any of the 7 cluster antisera. However, absorption of each cluster antisera with A. israelii (NGB 17) cell walls removed close to 100% of the cross-reactivity to clusters 5 and 7. A number of the antigens detected on the immunoblots may be immunologically similar, but migrate to different levels due to differences in their molecular weights.

One interesting antigen in each of the 7 clusters was developed using ATCC 15987 (cluster 7) antisera absorbed with A. israelii. This apparent highly immunogenic antigen with a molecular weight of 46 kDa appears to be a major antigen present in all of the strains from the 7 clusters but not A. israelii (Figure 4.10).

Appendix 4 lists the molecular weights of the antigens detected in each cell wall extract when reacted with cluster antisera absorbed with A. israelii cell walls. The antigens are aligned to match bands of similar molecular weights. A high number of antigens were still detectable for cluster 1,2,3,4 and 6 and no one antigen could identify a cluster. To further reduce the number of cross-reacting antigens between clusters 1,2,3,4 and 6 additional intercluster absorptions were made.

Table 4.11

The Number of Antigens Detectable In Cell Wall Extracts For Each Fillery Cluster And A. israelii Using Cluster Antisera That Was Not Absorbed and Absorbed With A. israelii (NGB 17) Cell Walls

	Cell Wall Extracts							
	WVU 627 Cluster 1	B 236 C2	B 102 C3	BE 32 C4	ATCC12104 C5	W 1053 C6	ATCC15987 C7	NGB 17 *A. israelii*
Cluster 1 antisera	17[a)] 14[b)]	21 14	15 6	18 11	13 1	18 11	17 0	20 1
Cluster 2 antisera	15 15	23 22	6 6	13 13	4 3	14 14	3 2	6 2
Cluster 3 antisera	9 6	17 14	18 15	6 3	4 1	14 8	3 1	13 1
Cluster 4 antisera	14 8	19 15	6 1	15 10	4 0	16 14	9 0	12 1
Cluster 5 antisera	12 10	14 11	14 6	10 9	12 12	12 11	9 6	9 6
Cluster 6 antisera	9 5	17 10	7 4	8 3	3 2	14 8	3 2	10 1
Cluster 7 antisera	10 1	13 2	13 1	9 1	3 1	12 1	14 2	13 0

a) Number of antigens detected using nonabsorbed antisera on top and absorbed antisera below.

b)= The molecular weights (kDa) of antigens that were detected using absorbed antisera are presented in Appendix 4.

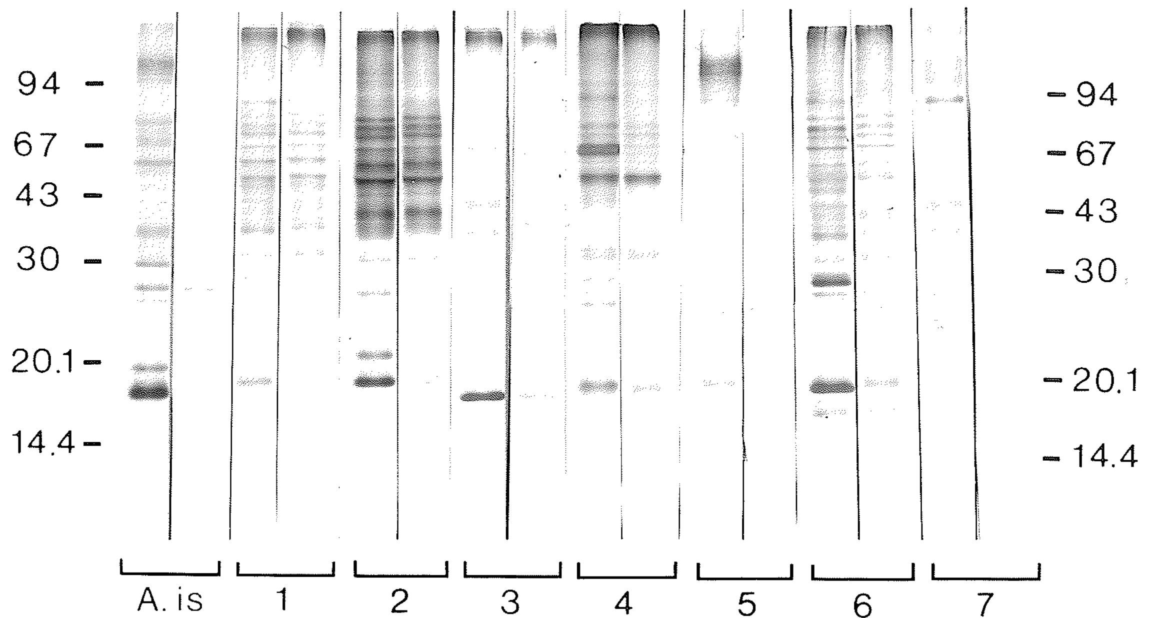


Figure 4.9. Immunoblot of cell wall extracts of each of the seven clusters and A. israelii. Immunoblots are presented in pairs. The left lane of the pair was reacted with BE 32 (cluster 4) antiserum and the right lane of the pair was reacted with BE 32 antiserum absorbed with A. israelii (NGB 17) cell walls.

Lane A. is. NGB 17 (A. israelii)
Lane 1 WVU 627 (cluster 1)
Lane 2 B 236 (cluster 2)
Lane 3 B 102 (cluster 3)
Lane 4 BE 32 (cluster 4)
Lane 5 ATCC 12104 (cluster 5)
Lane 6 W 1053 (cluster 6)
Lane 7 ATCC 15987 (cluster 7)

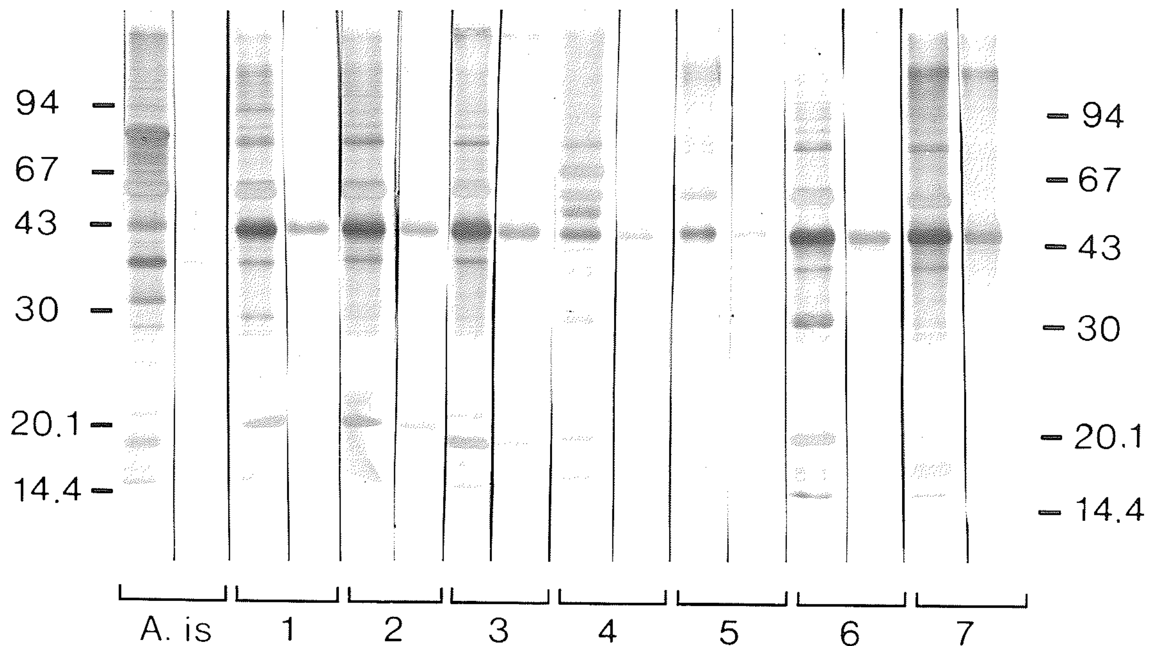


Figure 4.10. Immunoblot of cell wall extracts of each of the seven clusters and A. israelii. Immunoblots are presented in pairs. The left lane of the pair was reacted with ATCC 15987 (cluster 7) antiserum and the right lane of the pair was reacted with ATCC 15987 antiserum absorbed with A. israelii (NGB 17) cell walls.

Lane A. is. NGB 17 (A. israelii)
Lane 1 WVU 627 (cluster 1)
Lane 2 B 236 (cluster 2)
Lane 3 B 102 (cluster 3)
Lane 4 BE 32 (cluster 4)
Lane 5 ATCC 12104 (cluster 5)
Lane 6 W 1053 (cluster 6)
Lane 7 ATCC 15987 (cluster 7)

VII) Intercluster Cell Wall Absorptions

Further intercluster absorptions were preformed to identify any immunologically distinct clusters. Each cluster antiserum was absorbed with cell walls from the other clusters. These antisera were then reacted with immunoblots containing cell wall extracts to clusters 1-7 and A. israelii (NGB 17).

Intercluster absorptions automatically removed the cross-reactivity based on Actinomyces eliminating the need to preabsorb the cluster antisera with A. israelii. Most of the cluster antisera were absorbed with cluster 2 (B 236) and cluster 1 (WVU 627) cell walls as this was most effective in removing cross-reactivity. Table 4.12 summarizes the various intercluster absorptions that were performed. Three immunologically distinct groups were detected within clusters 1,2,3,4 and 6. Cluster 1 antiserum absorbed with cluster 2 (B 236) cell walls produced an antiserum specific to clusters 1,4 and 6. These three clusters could not be further separated immunologically using this technique. Antiserum specific to cluster 2 was produced by absorption with cluster 1 (WVU 627) cell wall extracts and an antiserum specific to cluster 3 was produced by absorption with cluster 2 (B 236) cell walls. Antiserum specific to cluster 5 was produced by absorption with cluster 1 cell wall extracts. Figures 4.11 shows 4 immunoblots containing cluster and A. israelii cell wall extracts developed with the 4 cluster specific antisera.

a) Whole Cell Agglutination Using Cluster Specific Antisera

The agglutination patterns between the above cluster specific antisera with whole cells was examined. The agglutinations occurred slower and were finer than for unabsorbed antisera. The antisera were still specific to their given cluster suggesting that common carbohydrate antigens were removed with the proteins during the absorption process.

Table 4.12

Absorptions With Cell Wall Extracts and Subsequent Specificity to the Clusters (C) and A. israelii Cell Wall Extracts

Sera Absorptions	C1	C2	C3	C4	C5	C6	C7	A. israe.	Cluster Specificity
C1 sera abs with C2	+			+		+			C1,4,6
C1 sera abs with C3	+	+		+		+			
C1 sera abs with C6	+	+							
C4 sera abs with C1		+		+		w			
C4 sera abs with C2				+					
C6 sera abs with C1				w					
C6 sera abs with C2				w					
C2 sera abs with C1		+							C2
C2 sera abs with C3	+	+		+		+			
C2 sera abs with C6	+	+							
C3 sera abs with C2			+						C3
C3 sera abs with C1		+	+						
C5 sera abs with C1					+	w	w		C5
C5 sera abs with C2	w	w	w	w	+	w	w		

w=weak

Strains and cell walls used for immunizations and absorptions:

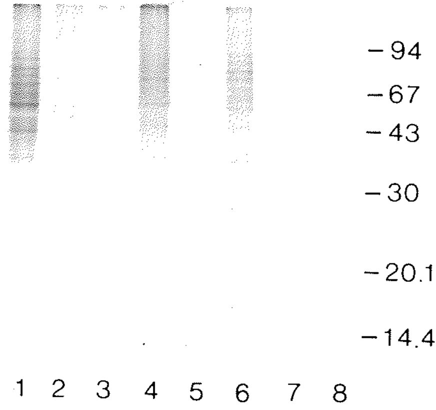
Cluster (C) 1=WVU 627
Cluster 2=B 236
Cluster 3=B 102
Cluster 4=BE 32
Cluster 5=ATCC 12104
Cluster 6=W 1053
Cluster 7=ATCC 15987

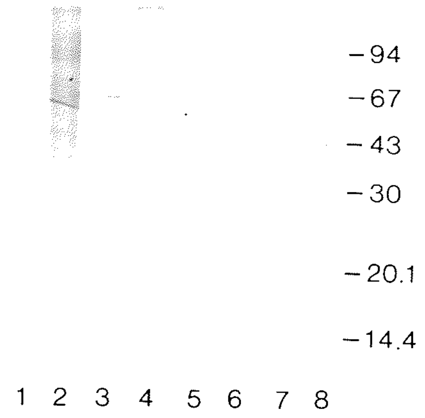
Figures 4.11a-d. Four immunoblots of cell wall extracts representing each of the clusters and A. israelii.

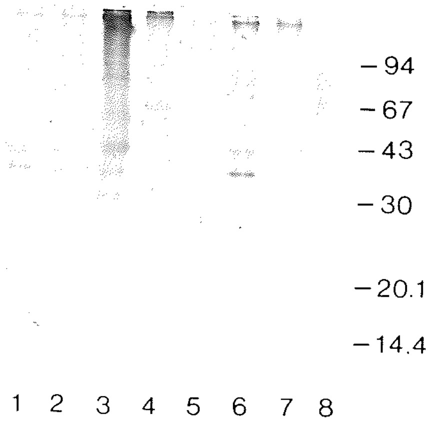
Lane 1WVU 627 (cluster 1)	Lane 2 B 236 (cluster 2)
Lane 3 B 102 (cluster 3)	Lane 4 BE 32 (cluster 4)
Lane 5 ATCC 12104 (cluster 5)	Lane 6 W 1053 (cluster 6)
Lane 7 ATCC 15987 (cluster 7)	Lane 8 NGB 17 (A. israelii)

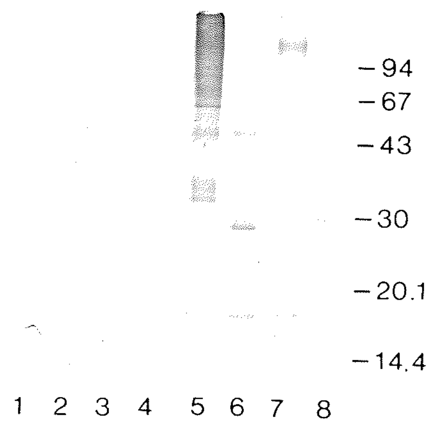
Each immunoblot reacted with one of four cluster specific antiserum:

Figure 4.11a. Immunoblot reacted with antiserum specific to clusters 1,4 and 6.
Figure 4.11b. Immunoblot reacted with antiserum specific to cluster 2.
Figure 4.11c. Immunoblot reacted with antiserum specific to cluster 3.
Figure 4.11d. Immunoblot reacted with antiserum specific to cluster 5.









Figures 4.11a-d. Legend on the previous page.

VIII) Intracluster Specificity of Intercluster Specific Antisera

Once antisera specific to certain clusters were identified then an additional study was undertaken to determine if the absorbed antisera were specific to that cluster or only to the strain representing that cluster. Cell wall extracts from four strains representing each of clusters 1 and 5, 3 strains from cluster 3 and 1 strain from cluster 2 were prepared. Immunoblots with the cell wall extracts were reacted with the four cluster specific antisera prepared in the previous section. Table 4.13 shows the results of the intracluster cross-reactivity and Figure 4.12 shows two of the immunoblots of cell wall extracts of strains from clusters 1, 3 and 5 reacted with the cluster specific antiserum. Generally, the antisera prepared to each cluster were quite specific to that cluster. Antiserum specific to clusters 1,4 and 6 cross-reacted with strain B 74 (cluster 3). The antiserum specific to cluster 3 cross-reacted weakly with the extracts of strain W 752 (cluster 1) and the antiserum specific to cluster 5 cross-reacted weakly with the extract of strain WA 2 (cluster 1).

Table 4.13

Intracluster Specificity to Cluster Absorbed Antisera

	Cluster 1				Cluster 2	Cluster 3			Cluster 5			
Cluster Specific Antisera	WVU 627	W752	WA 2	WVU 626	B 236	B 102	B 120	B 74	ATCC 12104	WVU 398A	TF 11	4AO5
Clusters 1,4 and 6	+	+	+	+	-	-	-	w	-	-	-	-
Cluster 2	-	-	-	-	+	-	-	-	-	-	-	-
Cluster 3	-	w	-	-	-	+	+	+	-	-	-	-
Cluster 5	-	-	w	-	-	-	-	-	+	+	+	+

w=weak

Figures 4.12 a & b. Two immunoblots of cell wall extracts from clusters 1,3 and 5.

Cluster 1

Lane 1 WVU 627	Lane 2 W 752
Lane 3 WA 2	Lane 4 WVU 626

Cluster 3

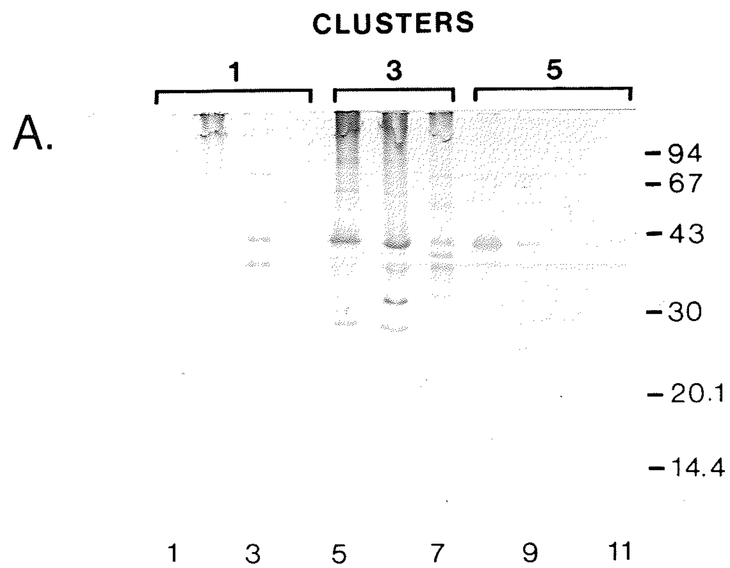
Lane 5 B 102	Lane 6 B 120
Lane 7 B 74	

Cluster 5

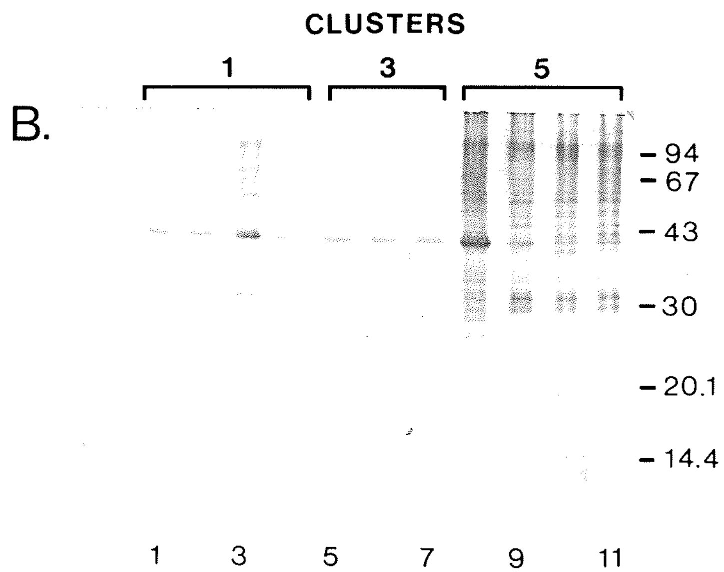
Lane 8 ATCC 12104	Lane 9 WVU 398A
Lane 10 TF 11	Lane 11 4AO5

Figure 4.12a. Immunoblot reacted with antiserum specific to cluster 3.
Figure 4.12b. Immunoblot reacted with antiserum specific to cluster 5.

A.



B.



Figures 4.12a & b. Legend on the previous page.

IX) Immunological Relationship of A. naeslundii-serotypes II & III and Actinomyces-serotype NV to the Clusters

Cell wall extracts, prepared from A. naeslundii-serotype II (ATCC 49339), A. naeslundii-serotype III (ATCC 49340) and Actinomyces-serotype NV (VPINIIA), were blotted and reacted with the four cluster specific antisera prepared in the previous section (data not shown). Actinomyces-serotype NV and A. naeslundii-serotype II reacted most strongly with the antiserum specific to clusters 1,4 and 6. A. naeslundii-serotype III reacted most strongly with cluster 3 specific antiserum. All 3 strains exhibited weak reactions with the cluster 2 and 5 specific antisera.

X) Specificity of Cluster Antisera Using Glass Bead Extracts and Whole Cell Sonicates

Reaction of the four cluster specific antisera with immunoblots of whole cell glass bead extracts showed a high degree of cross-reactivity between the seven clusters. Therefore, cluster specific antisera produced by absorption with cell walls was only specific if cell wall extracts were used on the immunoblots. Cross-reacting antibodies must still exist in the antisera after cell wall absorptions and these will detect antigens in glass bead and sonicated extracts. An attempt was made to remove cross-reacting antibodies by absorption with whole cells. This was totally ineffective in removing the cross-reactivity between any of the 7 clusters. This antisera was reabsorbed with a suspension of sonicated cells and then reacted with immunoblots containing glass bead and sonicate extracts. The resulting immunoblots were mostly clear with few antigens remaining in immunoblots of any of the 7 cluster extracts. It appears that the antigens detected in wall extracts by the cluster antisera absorbed with cell walls must account for a significant amount of the protein in these extracts. However, these same proteins must only account for a very small fraction of the protein present in glass bead and sonicate extracts. It is likely therefore, that these antigens are present in too low a concentration to be detected by immunoblotting whole cell extracts. The labour intensive

method of cell wall preparation seems necessary to detect the five immunologically distinct groups within the 7 clusters.

CHAPTER 5

DISCUSSION

I) Introduction

The close physiological and immunological relationships of A. viscosus and A. naeslundii makes identifying individual species difficult (Gerencer & Slack, 1969; Slack & Gerencer, 1970; Gerencer & Slack, 1976; Bowden et al. 1976; Bowden & Fillery, 1978; Fillery et al. 1978; Schofield & Schaal, 1981; Schaal, 1986; Firtel & Fillery, 1988 and Bowden & Goodfellow, 1990). Antigenically unique serotypes of A. viscosus and A. naeslundii have been identified but there is little understanding of the nature of the cross-reacting antigens. The aim of this study was to locate and quantitate this immunological cross-reactivity, and to decide if antigenic differences were sufficient to propose that distinct serovars exist within A. viscosus and A. naeslundii. In addition, the cross-reactivity of these two species with other Actinomyces species was examined.

II) Sample Preparation

The Coomassie stained gels of whole cell glass bead extract and cell wall extracts confirmed that proteins were present in both preparations. Cell wall extracts were chosen for the study because the antigenic cross-reactivity of whole cell glass bead extracts would likely be high because of conserved cytoplasmic proteins (Parent et al. 1986) resulting from the physiological relationship between A. viscosus and A. naeslundii (Fillery et al. 1978). The antigenic cross-reactivity of cell wall proteins would be a measure of the cross-reactivity between A. viscosus and A. naeslundii based on those antigens presented most readily to the immune system. The identification of antigenically distinct strains using cell wall extracts would help in planning the selection of antigens to use in future clinical trials of the immune response to Actinomyces.

The origin of proteins present in the cell wall extracts is difficult to determine. The proteins detected on the immunoblots are not likely to be of fimbrial origin. The work of Masuda et al. (1983) showed that fimbrial proteins would not penetrate into gels during sodium dodecyl sulfate-polyacrylamide gel electrophoresis even after boiling, reduction, or alkylation. This was further substantiated with one additional experiment using 2 monoclonal antibodies provided by Firtel and Fillery (Toronto). The monoclonal antibody 2c4 was raised against WVU 627 (cluster 1) and the monoclonal antibody 3g5 was raised against TF 11 (cluster 5). These two antibodies were specific to typical A. viscosus (cluster 1) and typical A. naeslundii (cluster 5), respectively, (Firtel & Fillery, 1988) and matched closely the fibril antisera profiles of Ellen & Grove (1985) suggesting that the epitopes of these monoclonals may lie on the fimbriae. Reaction of the monoclonals recognized few antigen bands when reacted with our cell wall extracts to clusters 1-7. Monoclonal antibody 2C4 recognized one cell wall antigen in cluster 1, three in cluster 2, three in cluster 4, and one in cluster 6, while monoclonal antibody 3G5 recognized one antigen in each of clusters 4 and 5.

The transmission electron microscopic pictures are similar to those published by Duda & Slack (1972), Girard & Jacius (1974), and Slack & Gerencer (1975). In some of the whole cell pictures, long fimbriae were visible (Figure 4.1g), but cell wall preparations showed no obvious fimbriae. The cell walls were composed of a thick lighter staining outer component and a thin darker staining inner component but no attendant cell membrane. This was similar to structures demonstrated in previous studies (Girard & Jacius, 1974). Therefore, it appears that the cell wall extracts contained primarily proteins from the cell walls and protein concentrations from the plasma membrane and fimbriae were probably low.

III) Antigenic Relationship of A. viscosus and A. naeslundii

a) Cross-reactivity Assessed by Agglutination and Rocket Immunoelectrophoresis

Whole cell agglutination of strains from the 7 clusters of A. viscosus and A. naeslundii showed extensive cross-reactivity. Only typical A. naeslundii (cluster 5) and the animal species A. denticolens and A. howellii appeared to be unique. Pronase treatment had little effect on the agglutination pattern as most of the cross-reactions remained. Carbohydrates could be the antigen responsible for this agglutination as cross-reactivity of carbohydrates present on Actinomyces does occur. Bowden et al. (1976) showed that trypsin- and pronase-treated cell walls of A. naeslundii-serotype 1 agglutinated with A. viscosus-serotype 2 antiserum.

The use of double diffusion and rocket immunoelectrophoresis in this study was not successful in defining the extent of the cell wall carbohydrate cross-reactivity, due to our inability to develop good carbohydrate precipitating antisera. A previous study showed that only 1 of 30 rabbits immunized with whole cells produced an antisera that was able to precipitate carbohydrate (Bowden et al. 1976).

b) Antigenic Cross-reactivity Assessed by Immunoblotting

Immunoblotting of cell wall extracts proved very effective for studying antigenic cross-reactivity. These data have shown extensive cross-reactivity between the 7 clusters representing A. viscosus and A. naeslundii, and also extensive cross-reactivity between these species and A. israelii, A. naeslundii-serotype II and III, Actinomyces-serotype NV, and the 3 animal species, A. slackii, A. howellii, and A. denticolens. For ease of discussion, the intercluster, intracluster and animal strain cross-reactions will be discussed separately.

i) Intercluster Cross-reactivity

The high degree of cross-reactivity shown in this study was not unexpected, as previous studies showed that cross-reactivity between A. viscosus and A. naeslundii exists (Gerencer & Slack, 1969; Slack & Gerencer, 1970; Gerencer & Slack, 1976; Bowden et al. 1976 and Fillery et al. 978). A. viscosus was divided into 2 serotypes based on selective absorption of fluorescent-labelled antibodies (Gerencer & Slack, 1969). Based on numerical taxonomy A. viscosus-serotype 1 (animal strains) were placed into cluster 7 and A. viscosus-serotype 2 (human strains) were placed into cluster 1 (typical A. viscosus) and clusters 2,4 and 6 (atypical A. viscosus). Although clusters 1 and 7 were distinct they still showed a 86% similarity (Fillery et al. 1978). The data from this study (Table 4.7) shows that cluster 7 does exhibit a high degree of antigenic cross-reactivity to the other 6 clusters (Sj=46%). Although fluorescent labelled antibodies were able to identify these strains within clusters 1 and 7 as distinct, they have not identified the antigens involved, their site of origin on the cell or the degree of cross-reactivity.

Cross-reactivity exists between the cell wall associated carbohydrates of cluster 7 and clusters 1,2,3,4 and 6 based on wall agglutinations. However, the cross-reactivity of protein antigens associated with the cell walls of cluster 7 and the other 6 clusters was the result of antigens common to Actinomyces. Table 4.11 showed that absorption of each of the 7 cluster antisera with A. israelii (NGB 17) cell walls removed almost all of the cross-reactivity between clusters 1-6 and animal A. viscosus (cluster 7). One cross-reacting antigen between animal A. viscosus and the remaining 6 clusters was identified using cluster 7 antiserum absorbed with A. israelii cell walls (Figure 4.10). This antigen was a protein with a molecular weight of 46 kDa and it was present in all cell wall extracts prepared to each of the 7 clusters. This antigen should be examined in further detail as it may represent a protein common to all A. viscosus and A. naeslundii. A fluorescent-labelled polyclonal antiserum specific to this antigen may be

an effective means of screening Actinomyces isolates for those included in A. viscosus and A. naeslundii.

The stimulation of antibodies to this 'common' antigen by immunization with animal A. viscosus (cluster 7) and not strains from the other clusters is interesting. It suggests that this antigen is immunogenic when it is associated with cells of A. viscosus (cluster 7) but less so with other strains. This provides some evidence for structural differences in the location of this antigen on different Actinomyces.

Previous studies suggest that cluster 5 (A. naeslundii-serotype 1) is antigenically unique. Early studies showed that cross-reactivity exists between A. naeslundii-serotypes 1,2,3 and A. viscosus-serotypes 1 and 2 (Gerencer & Slack, 1976). A more recent study created specific anti-fimbrial antibodies to cluster 5 as assessed by whole cell agglutination (Masudo et al. 1983). Using this serum in indirect immunofluoresence to screen oral A. viscosus and A. naeslundii, proved very effective for identifying strains included in cluster 5 (Ellen & Grove, 1985). In addition, a monoclonal antibody (3g5) labelled 100% of typical A. naeslundii (cluster 5) strains supporting the separation of cluster 5 as antigenically distinct (Firtel & Fillery, 1988). Therefore, studies to date have suggested that A. naeslundii (cluster 5) is physiologically distinct and also that its antigen profile supports it being maintained as a separate entity.

Immunoblotting of cell wall extracts showed extensive cross-reactivity between A. naeslundii-serotype 1 (cluster 5) and the rest of the 6 clusters and A. israelii. The mean antigenic similarity of 41% was the lowest between the 6 clusters suggesting that cluster 5 strains were distinct. The whole cell agglutination data presented here confirms that cluster 5 has unique antigens, as it would only agglutinate with its homologous antiserum. Immunoblotting of cell wall extracts showed that typical A. naeslundii (cluster 5) resembles animal A. viscosus (cluster 7) in that the cross-reactivity was due to antigens common to Actinomyces. Absorption of each cluster

antiserum with A. israelii cell walls removed most of the cross-reactivity to cluster 5 cell wall extracts. As long as cell wall extracts were used in the immunoblots a polyclonal antiserum specific to cluster 5 was produced. This result along with the whole cell agglutination data suggests that typical A. naeslundii (cluster 5) is sufficiently antigenically different from the other clusters to be maintained as a separate cluster.

Atypical A. naeslundii (cluster 3) has been identified as being immunologically distinct by using indirect immunofluorescent labelled anti-fimbrial antisera (Ellen & Grove, 1985) and a monoclonal antibody (1d10), which identified three quarters of the strains grouped in cluster 3 (Firtel & Fillery, 1988). The fact that all of the strains were not identified by the monoclonal may suggest that other strains possibly grouped with cluster 3 do not belong in that cluster. Table 4.7 showed that cluster 3 had the second lowest antigenic similarity to the rest of the clusters (Sj=45%). Reaction of immunoblots with antisera absorbed with A. israelii cell walls removed some of the cross-reactivity between cluster 3 and the rest of the clusters. It appears that there are cross-reacting antigens common to A. viscosus and A. naeslundii in the cell wall extracts of cluster 3. Absorption of cluster antisera with cell walls from other clusters produced a polyclonal antiserum specific to atypical A. naeslundii (cluster 3). It appears that cluster 3 represents a group of organisms that occupy an intermediate antigenic similarity between clusters 5,7 and clusters 1,2,4,6. Cluster 3 shows a fair degree of cross-reactivity due to antigens common to Actinomyces (e.g., clusters 5 and 7), but also shows cross-reactivity due to antigens common to A. viscosus and A. naeslundii (like clusters 1,2,4 and 6). The extent of cross-reactivity due to antigens common to A. viscosus and A. naeslundii between cluster 3 and clusters 1,2,4 and 6 group appears to be less than the cross-reactivity within clusters 1,2,4 and 6. That is, cluster 1,2,4 and 6 appear antigenically very similar and share many antigens common to A. viscosus and A. naeslundii.

Cross-reactivity between clusters 1,2,4 and 6 is extensive with many common antigens, however, immunologically distinct groups have not been identified. These clusters have been shown to be very similar using numerical taxonomy (Fillery et al. 1978), indirect immunofluoresence of absorbed antifibril antisera (Ellen & Grove, 1985), and monoclonal antibodies (Firtel & Fillery, 1988). Fillery et al. (1978) designated cluster 1 as typical A. viscosus and clusters 2,4 and 6 as atypical A. viscosus. This study corroborates the high degree of cross-reactivity between these 4 clusters. Absorbing cluster antisera with A. israelii removed some of the cross-reactivity to each cluster, but many cross-reacting antigenic bands were identified suggesting that these four clusters exhibit a high degree of cross-reactivity due to antigens that are common to A. viscosus and A. naeslundii. Intercluster absorptions were able to identify cluster 2 as being antigenically different, but clusters 1,4 and 6 effectively formed one antigenic group. The separation of cluster 2 on the basis of antigenic structure has not been reported before.

ii) Intracluster Cross-reactivity

Three clusters were examined for the degree of antigenic cross-reactivity within the clusters. Cluster division based on physiological testing showed higher intracluster similarities (Fillery et al. 1978). All three clusters (1,3 and 5) showed a much higher intracluster antigenic similarity (Table 4.8) than intercluster antigenic similarity (Table 4.7) suggesting that immunological differences mirror the physiological differences. Clusters 1 and 5 had intracluster similarities of >70%. Cluster 3 was unusual in that strain B 74 showed a much lower similarity to the other two cluster 3 strains (B 102 and B 120). Since antisera from both rabbits identified this difference it is unlikely to be a technical error. Other studies examining the similarity of strains in cluster 3 showed a numerical taxonomic intracluster similarity of 89% (Fillery et al. 1978) and data available on DNA homology showed that B 74 and B

120 were 89% homologous (Coykendall & Munzenmaier, 1979). Both of these studies suggest that B 74 belongs to cluster 3.

Based on our physiological tests, strain B 74 differs from B 120 and B 102 in cluster 3. Strains B 102 and B 120 did not grow aerobically, were catalase negative and fermented sorbitol. Since physiological tests have failed to clearly differentiate species to date one should not depend too heavily on individual tests (Holmberg & Hallander, 1973; Holmberg & Nord, 1975; Fillery et al. 1978; Schaal, 1986; Bowden & Goodfellow, 1990 and Johnson et al. 1990). The low antigenic similarity of B 74 to other cluster 3 strains, the fact that B 74 reacts more weakly with cluster 3 specific antiserum and cross-reacts with antiserum specific to clusters 1,4 and 6, suggests B 74 may not belong in cluster 3.

iii) Animal Strain Cross-reactivity

Three animal species were examined for their antigenic similarity to clusters 1, 5 and 7. The two strains A. howellii and A. denticolens appear to be quite distinct as whole cell agglutinations showed these two species would only agglutinate with their homologous antiserum. A. slackii 4563 (NCTC 11928) which represents the parent of the type strain agglutinates with the other clusters and also showed the highest antigenic similarity to the clusters of the three animal strains suggesting A. slackii (NCTC 11928) was antigenically different from other animal species (Table 4.9). Other strains identified as A. slackii do not agglutinate with A. slackii antiserum (Table 4.2b) suggesting that a parent strain may be mislabelled or that different serovars may exist within A. slackii. This should be confirmed by ordering the type strain from the National Collection of Type Cultures and examining its agglutination with the A. slackii antiserum. In addition, producing antiserum to other A. slackii strains and examining the degree of cross-reactivity that exists may help decide if serovars exist.

All three animal species appear to be unique as they show a DNA homology of only 30% to one another (Dent and Williams, 1986). Little antigenic cross-reactivity

between A. denticolens and A. naeslundii has been identified (Schaal, 1986). The fact that cross-reactivity exists between the 3 animal species and 3 of the clusters when cell wall extracts are used may be related to antigens that are common Actinomyces. Table 4.9 shows that cluster 7, which represents animal A. viscosus, shows almost the same degree of cross-reactivity to cluster 1 and 5 as the other animal species. The cross-reactivity of cluster 7, detected by using cell wall extracts, was due to antigens common to Actinomyces. Further studies on the taxonomy of animal Actinomyces species may show that the cross-reactivity between A. slackii, A. howellii and A. denticolens, and strains in the clusters, is due to antigens common to Actinomyces as was shown for animal A. viscosus (cluster 7).

IV) Conclusions

a) Antigenic Cross-reactivity Between Clusters

The degree of antigenic cross-reactivity detected in this study between the 7 clusters representing A. viscosus and A. naeslundii appears to be due to antigens common to Actinomyces and antigens common to A. viscosus and A. naeslundii. The results support a recent publication that reclassified the closely related organisms. A. viscosus and A. naeslundii (Johnson et al. 1990). Based on DNA homology Johnson et al. (1990) proposed that animal A. viscosus (cluster 7) should be retained as A. viscosus and typical A. naeslundii (cluster 5) would be classified as A. naeslundii-genospecies I. Cross-reactivity between cluster 5 and 7 wall extracts and the other cluster extracts appears to be due to antigens that are common to Actinomyces. Organisms that would fall into the remaining clusters (1,2,3,4 and 6) were reclassified by Johnson et al. (1990) into A. naeslundii-genospecies II. A major component of the cross-reactivity detected in these clusters was due to antigens common to A. viscosus and A. naeslundii. Polyclonal antisera produced by intercluster absorptions with cell walls suggests that 3 serovars

may exist within A. naeslundii-genospecies II. These serovars consist of:

a) Strains from cluster 1,4 and 6.
b) Strains from cluster 2.
c) Strains from cluster 3.

b) Use of Serovar Specific Antisera as a Screening Test

Three strains representative of different serovars that are included into A. naeslundii-genospecies II were examined for their antigenic relationship to A. viscosus and A. naeslundii. Cell wall extracts were prepared to A. naeslundii-serotypes II and III and Actinomyces-serotype NV. The degree of antigenic similarity to cluster representatives was calculated and then cell wall extracts on immunoblots were reacted with the four cluster specific polyclonal antisera. The Jaccard antigenic similarities suggested that Actinomyces-serotype NV and A. naeslundii-serotype II were most similar to cluster 1 and A. naeslundii-serotype III was most similar to cluster 3. This supports the placing of these three strains into A. naeslundii-genospecies II by Johnson et al. (1990). Actinomyces-serotype NV and A. naeslundii-serotype II reacted most strongly with the antiserum that was specific to clusters 1,4 and 6, and A. naeslundii-serotype III reacted most strongly with cluster 3 specific antiserum. These three strains do appear to be a little different from the cluster strains in that there is also some cross-reactivity detected with the cluster 2 and 5 specific antiserum. Preparation of cell wall extracts from fresh isolates of these serovars and reaction to each of the 4 polyclonal specific antisera may be helpful in identifying the position which these strains should occupy in relation to A. naeslundii-genospecies I or II.

c) Strain Selection for Studies that Examine the Response of the Humoral Immune System to A. viscosus and A. naeslundii

A previous study used the type strain for A. viscosus as the test antigen to determine the humoral immune response in humans to A. viscosus (Murayama et al.

1988). This may have been a poor choice because the type strain was isolated from animals. The decision of which strain(s) to select when studying the impact A. viscosus and A. naeslundii has on the immune system must take into account the high degree of antigenic cross-reactivity that exists between these two species. The clusters will be reviewed and the most appropriate strains for use as antigens in human clinical trials in order to represent antigenically distinct groups will be proposed.

Clusters 1,4 and 6 appear to form one serovar. Two strains from cluster 1 (W752 and WA 2) cross-react with cluster 3 and 5 specific antisera and should not be used as the test antigens. Strain WVU 626 reacts only with cluster 1,4 and 6 antiserum but a previous study by Firtel and Fillery (1988), using cluster specific monoclonal antibodies, suggested that WVU 626 should be reclassified to cluster 3. Our data does not support this, but it would be best to avoid this strain. Selection of the strain WVU 627 as the representative for serovar 1,4 and 6 appears to be the best choice.

The immunoblotting study used one strain as the cluster 2 representative. If one examines the similarity matrix proposed by Fillery et al. (1978) strain H21 also belongs to cluster 2 but the authors in their discussion stated that H21 could have been placed in cluster 1. Therefore, selection of B 236 as the cluster 2 representative appears to be most appropriate.

A suitable representative from cluster 3 is more difficult to identify. Previous studies have placed B 74 in cluster 3, but the results of this study shows that this strain shows antigenic cross-reactivity with other serovars (i.e., clusters 1,4 and 6 group). This precludes using B 74 as the test antigen. Selecting either B 120 or B 102 would appear to be more appropriate.

Cluster 5 is distinct from the other clusters based on whole cell agglutination and intercluster absorption of cluster antiserum with cell walls. Since all 4 strains from cluster 5 reacted strongly with the cluster 5 specific antiserum, it would be appropriate to select any one of the four strains tested.

Due to the high degree of cross-reactivity between all the strains, it would be necessary to pre-absorb the patient's antiserum with cell walls of various clusters prior to determination of the antibody titre to a given serovar. In this way, cross-reactivity between common proteins and carbohydrates would be removed. Cross-reactions due to the common carbohydrates were removed with this technique as whole cell agglutination with the cluster specific antisera were specific to their respective clusters. During the absorption process cross-reacting carbohydrates were removed with the cross-reacting proteins.

Preabsorption of the patients antiserum would give an antibody titre that reflects the immune response to that serovar and not to cross-reacting antigens. That is:

A) Cluster 1,4 and 6.
WVU 627 antigen reacted with patient's antiserum absorbed with cluster 2 cell walls.

B) Cluster 2.
B 236 antigen reacted with patient's antiserum absorbed with cluster 1 cell walls.

C) Cluster 3.
B 120 or B 102 antigen reacted with patient's antiserum absorbed with cluster 2 cell walls.

D) Cluster 5.
ATCC 12104 antigen reacted with patient's antiserum absorbed with cluster 1 cell walls.

The reaction of absorbed antisera was not cluster specific when reacted with whole cell sonicates and glass bead extracts representatives of the different clusters. Antisera specific to the different clusters could not be produced using extracts of whole cell sonicates and glass bead extracts. The more labour intensive method of cell wall extract preparation appears to be necessary to identify the 3 serovars-cluster 1,4 and 6, cluster 2 and cluster 3. Cluster 5 can be identified by whole cell agglutination but a cluster specific antiserum was also created.

In a clinical study, cell walls and not whole cells of these strains would be used as the antigen on the ELISA plates. The cluster specific antisera was only specific if cell walls were used. Antibodies to whole cell glass bead extract proteins are still present in the cluster specific antisera and would cross-react with whole cell antigens.

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Appendix 1

Media and Agar Formulas Used for this Study.

Appendix 1A

B.M Medium

Tryptone	1%
Tryptose	1%
Yeast Extract	0.5%
Glucose	0.5%
Sodium Chloride	0.5%
L-Cysteine HCl	0.075%
Haemin Stock Solution*	10 ml/l
Menadione (vitamin K_1)**	20 drops/l

Dissolve ingredients in distilled water and adjust pH to 7.4.
Autoclave at 121°C for 30 minutes.

*Haemin Stock Solution
50 mg in 1 ml-1N NaOH and make up to 100 ml of distilled water.
Autoclave at 121°C for 15 minutes

**Menadione Stock Solution
0.15ml of menadione in 30 ml of 95% ethanol

B.B.L. Actinomyces Broth

Potassium Phosphate (monobasic)	0.6 %
Potassium Phosphate (dibasic)	0.9%
Ammonium Sulphate	0.1%
Magnesium Sulphate	0.02%
Calcium Chloride	0.001%
Nutrient Broth No. 2	0.1%
Glucose	0.5%
L-Cysteine HCl	0.05%
Tryptone (Difco)	0.4%
Yeast Extract	0.4%

Dissolve in distilled water. Adjust pH to 7.0.
Sterilize at 15 lbs/10 minutes.

Appendix 1B

Innoculum Broth

Tryptose	2%
Yeast Extract	0.5%
Sodium Chloride	0.5%
Sodium Phosphate Dibasic	0.1%
Bromocresol Purple	0.001%
Glucose	0.1%

Dissolve ingredients in distilled water and adjust pH to 7.6.
Dispense in 5 ml bijou bottles.
Autoclave 121°C for 30 minutes.

Streptococcus Sugar Agar

Tryptose	2%
Yeast Extract	0.5%
Sodium Chloride	0.5%
Sodium Phosphate Dibasic	0.1%
Bacto-Difco Agar	1.5%
Bromocresol Purple	0.002%

Dissolve ingredients and adjust pH to 7.2.
Autoclave 121°C for 30 minutes.

Prepare 1% sugar in distilled water.
Filter sterilize and add to basic agar medium and then pour plates.

Appendix 1C

Starch Agar (Cowan & Steel 1974)

Flask 1
Starch 20%

Flask 2
Nutrient Agar 1.3%

Dissolve starch to a smooth cream
Autoclave Nutrient Agar at 115°C for 30 minutes.
Mix flask 1 with flask 2

Aesculin Agar (Cowan & Steel 1974)

Tryptone (Oxoid)	0.5%
Tryptose	0.5%
Aesculin	0.1%
Dextrose	0.1%
Ferric Ammonium Citrate	0.05%
Bacto-Difco Agar	1.5%

Dissolve in distilled water and adjust pH to 7.2.
Autoclave at 121°C for 30 minutes.

Urea Agar (Cowan & Steel 1974)

Flask 1
Blood Agar Base No. 2 40 gm
Distilled water 750 ml
Autoclave at 121°C for 30 minutes.

Flask 2
Urea Broth (Difco) 38.7 gm
Distilled water 250 ml
Filter sterilize

Combine when agar cooled to 55°C

Appendix 1D

Nitrate Broth (Cowan & Steel 1974)

Potassium Nitrate	1 gm
Nutrient Broth	1000 ml

Distribute into Bijou bottles with inverted Durham tubes.
Autoclave at 115°C for 20 minutes.

Nitrite Broth (Cowan & Steel 1974)

Sodium Nitrite	0.01 gm
Nutrient Broth	1000ml

Distribute in Bijou bottles.
Autoclave at 115°C for 20 minutes.

Test Reagents

Solution A

Sulphanilic Acid	0.8%
5N Acetic Acid	
Dissolve by gentle heating.	

Solution B

Dimethyl-a-napthylamine	0.6%
5N Acetic Acid	
Dissolve by gentle heating.	

Appendix 1E

Actinomyces Defined Medium (ADM)-Bowden (1976)

Potassium Phosphate Monobasic	0.6%
Potassium Phosphate Dibasic	0.9%
Calcium Chloride	0.002%
Magnesium Sulphate	0.02%
Sodium Acetate	0.03%
Glucose	0.5%
L-Cysteine HCL	0.02%
Glutathione	0.005%
L- Asparagine	0.01%
L-Tryptophane	0.004%
Bacto-tryptone	0.2%
L-Glutamic Acid	0.05%
Solution A*	10ml/l
Solution B**	1ml/l
Solution C***	1ml/l

*Solution A

p-Amino Benzoic Acid	200mg
Thiamine (Aneurine)	200mg
Riboflavin	200mg
Nicotinic Acid	200mg
Pyridoxal HCl	200mg
Inositol	200mg
Ca Pantothenate	200mg

Dissolve in 1 litre of distilled water and adjust pH to 7.0.

**Solution B

Dl Thioctic Acid	10mg
Biotin	10mg
Haemin	10mg
Folic Acid	20mg

Dissolve Haemin in 1 drop of distilled water and 1 drop of ammonium hydroxide.
Dissolve above in 100 ml of distilled water

***Solution C

Ferrous Sulphate	400mg
Manganous Sulphate	15mg
Sodium Molybdate	15mg

Dissolve ingredients in 100ml of distilled water.

Appendix 2

Percent Antigenic Similarity (Sj) of Cell Wall Extracts for the Fillery Clusters 1-7 and A. israelii Using Antisera produced to Each of the 7 Fillery Clusters

Appendix 2

	WVU627	B236	B102	BE32	ATCC12104	W1053	T6	NGB17
WVU627								
S1*	--	72.2	52.4	60.1	30	63.2	63.2	52.4
S2	--	79.2	51.7	41.9	43.3	76.0	51.9	35.5
S3	--	70.0	59.1	52.4	40.0	45.5	52.6	47.6
S4	--	75.0	23.5	56.3	46.2	53.8	29.4	29.2
S5	--	76.2	56.5	53.8	46.2	63.6	42.9	35.7
S6	--	57.9	52.4	64.7	31.6	68.4	63.2	47.6
S7	--	68.4	26.9	54.5	47.4	60.0	75.0	25.9
B236								
S1	--	--	45.0	70.6	43.8	75.0	64.7	52.6
S2	--	--	64.0	57.7	60.0	57.7	58.3	30.0
S3	--	--	48.0	47.8	30.0	47.8	34.8	43.5
S4	--	--	33.3	60.0	80.0	90.0	50.0	31.3
S5	--	--	45.8	50.0	48.0	75.0	44.4	37.0
S6	--	--	42.9	62.5	21.1	42.9	52.6	38.1
S7	--	--	50.0	41.7	55.6	45.5	66.7	25.9
B102								
S1	--	--	---	42.9	33.3	61.1	52.6	42.9
S2	--	--	--	50.0	51.9	50.0	30.0	37.9
S3	--	--	--	52.2	29.2	45.8	42.9	54.5
S4	--	--	--	33.3	28.6	46.2	50.0	50.0
S5	--	--	--	52.0	38.5	41.7	35.7	44.0
S6	--	--	--	47.4	25.0	60.0	55.0	47.6
S7	--	--	--	40.0	52.6	50.0	56.5	25.0

*S=Antiserum produced to the cluster indicated.

	WVU627	B236	B102	BE32	ATCC12104	W1053	T6	NGB17
BE32								
S1	--	--	--	--	33.3	52.6	45.0	50.0
S2	--	--	--	--	64.0	55.6	39.3	33.3
S3	--	--	--	--	47.4	52.4	26.1	29.2
S4	--	--	--	--	46.7	53.3	47.1	25.0
S5	--	--	--	--	42.9	46.2	44.8	42.9
S6	--	--	--	--	31.3	58.8	68.8	42.1
S7	--	--	--	--	50.0	54.5	32.1	24.1
ATCC12104								
S1	--	--	--	--	--	43.8	21.1	33.3
S2	--	--	--	--	--	46.4	46.2	30.0
S3	--	--	--	--	--	47.4	47.1	17.4
S4	--	--	--	--	--	54.5	35.7	18.8
S5	--	--	--	--	--	38.5	52.0	46.2
S6	--	--	--	--	--	38.9	41.2	20.0
S7	--	--	--	--	--	64.7	34.8	15.4
W1053								
S1	--	--	--	--	--	--	64.7	61.1
S2	--	--	--	--	--	--	50.0	29.0
S3	--	--	--	--	--	--	52.6	29.2
S4	--	--	--	--	--	--	53.8	25.0
S5	--	--	--	--	--	--	35.7	38.5
S6	--	--	--	--	--	--	72.2	40.9
S7	--	--	--	--	--	--	59.1	17.2

	WVU627	B236	B102	BE32	ATCC12104	W1053	T6	NGB17
T6								
S1	--	--	--	--	--	--	--	52.6
S2	--	--	--	--	--	--	--	23.3
S3	--	--	--	--	--	--	--	21.7
S4	--	--	--	--	--	--	--	29.4
S5	--	--	--	--	--	--	--	33.3
S6	--	--	--	--	--	--	--	42.9
S7	--	--	--	--	--	---	--	32.0

Appendix 3

Percent Antigenic Similarity (Sj) of Cell Wall Extracts for Three Fillery Clusters and Three Animal *Actinomyces* Species Using Antisera Produced to these Six Strains

Appendix 3

	Cluster 1	Cluster 5	Cluster 7	A. howellii	A. slackii	A. dent
	WVU 627	ATCC12104	ATCC15987	NCTC11636	NCTC 11928	NCTC11490
WVU 627						
S1*	-	66.7	64.3	62.1	74.1	58.6
S5	-	55.9	72.7	70.0	55.6	64.5
S7	-	38.9	78.9	40.0	76.5	56.3
Sh	-	54.5	52.0	52.0	50.0	54.2
Ss	-	64.5	76.7	74.2	66.7	39.5
Sd	-	61.9	50.0	59.1	66.7	72.0
ATCC12104						
S1	-	-	48.3	51.7	57.1	50.0
S5	-	-	64.7	61.3	77.4	56.3
S7	-	-	45.0	35.3	47.1	25.0
Sh	-	-	50.0	50.0	41.7	40.0
Ss	-	-	71.4	58.1	61.3	65.5
Sd	-	-	52.4	55.0	50.0	50.0
ATCC15987						
S1	-	-	-	55.2	60.7	43.8
S5	-	-	-	74.2	68.6	54.3
S7	-	-	-	39.1	70.0	38.1
Sh	-	-	-	60.0	72.7	56.0
Ss	-	-	-	82.1	67.7	61.3
Sd	-	-	-	57.1	46.2	70.8
A. howellii						
S1	-	-	-	-	53.3	46.9
S5	-	-	-	-	65.6	50.0
S7	-	-	-	-	40.0	43.8
Sh	-	-	-	-	58.3	62.5
Ss	-	-	-	-	76.7	64.5
Sd	-	-	-	-	37	48.1

*S=Antiserum produced to that cluster or animal species

continued...

Appendix 3-continued

	cluster 1	cluster 5	cluster 7	A. howellii	A. slackii	A. dent
A. slackii						
S1	-	-	-	-	-	56.7
S5	-	-	-	-	-	60.6
S7	-	-	-	-	-	47.1
Sh	-	-	-	-	-	60.9
Ss	-	-	-	-	-	57.6
Sd	-	-	-	-	-	60.7
A. dent.						
S1	-	-	-	-	-	-
S5	-	-	-	-	-	-
S7	-	-	-	-	-	-
Sh	-	-	-	-	-	-
Ss	-	-	-	-	-	-
Sd	-	-	-	-	-	-

Appendix 4

Molecular Weights of Antigens that Remain for Each Cluster After Reaction of Cell Wall Extracts With Cluster Antisera That Was Absorbed With A. israelii (NGB 17) Cell Walls

Appendix 4

Molecular Weights of Antigens Detected For WVU 627 Cell Wall Extracts Reacted With The 7 Different Cluster Antisera After Absorption With A. israelii (NGB 17) Cell Walls

WVU 627 SERA	B 236 SERA	B 102 SERA	BE 32 SERA	ATCC 12104 SERA	W 1053 SERA	ATCC 15987 SERA
					119.3	
107.9 a)	110.9	108.4	112.0			
93.8	94.4			92.3		
87.4	88.1			88.3		
81.5	80.4	83.5				
77.8				77.2		
72.5	73.3	72.5		72.2		
69.2			69.1		69.4	
64.6	66.8				64.7	
61.6	62.4			63.1		
56.1	56.9		59.8			
	55.6		54.3			
48.8	50.7	48.4	47.0			
44.4	45.2			46.1	46.5	45.9
42.4	42.2	43.0				
40.5	40.3		41.6	40.3		
	35.1		36.0	34.5		
				21.1		
	18.4	19.7	18.8	18.8	18.1	

a) Molecular weight in kDa.

Molecular Weights of Antigens Detected For B 236 Cell Wall Extracts Reacted With The 7 Different Cluster Antisera After Absorption With A. israelii (NGB 17) Cell Walls.

WVU 627 SERA	B 236 SERA	B 102 SERA	BE 32 SERA	ATCC 12104 SERA	W 1053 SERA	ATCC 15987 SERA
					119.3	
110.4 a)	113.5		114.8			
	103.5	105.9				
	96.6		96.9			
91.6	90.2			92.3		
					87.8	
83.4	84.1	83.5				
79.6	78.5	76.0	79.9	77.2		
72.5	73.3	72.5	74.3	72.2		
	70.0	69.1	70.8	69.0	71.1	
			69.1			
67.6	66.8	64.4			64.7	
61.6	62.4	60.0	62.8	63.1		
			59.8			
56.1	56.9		54.3		57.5	
51.1	51.9	53.3			53.6	
48.8	48.4	47.3	45.8	46.1	48.7	45.9
44.4	44.2		43.7		45.4	
	41.2	42.0	41.6			
40.5	40.3			40.3		
	35.1	34.8	36.0	35.2		
29.2	29.2	30.9	30.4	29.5		
	24.8	22.2		22.0		
	21.6				20.9	
	18.4	19.2	19.2	19.3	18.1	18.2
16.7	16.8					

a) Molecular weight in kDa.

Molecular Weights of Antigens Detected For B 102 Cell Wall Extracts Reacted With The 7 Different Cluster Antisera After Absorption With A. israelii (NGB 17) Cell Walls

WVU 627 SERA	B 236 SERA	B 102 SERA	BE 32 SERA	ATCC 12104 SERA	W 1053 SERA	ATCC 15987 SERA
	113.5	116.4	114.8		119.3	
110.4 a)		111.0				
		105.9				
100.6						
96.0						
	94.4	91.9				
83.4		81.6				
		76.0		77.2		
		72.5				
	62.4	64.4		64.5		
		58.6				
		48.4		46.1	47.6	45.9
40.5	39.4	41.0		40.3		
		34.8				
27.2		30.9				
	19.3	20.2		20.1	19.0	
	17.2	17.9		17.6	16.9	

a) Molecular weight in kDa.

Molecular Weights of Antigens Detected For BE 32 Cell Wall Extracts Reacted With The 7 Different Cluster Antisera After Absorption With A. israelii (NGB 17) Cell Walls

WVU 627 SERA	B 236 SERA	B 102 SERA	BE 32 SERA	ATCC 12104 SERA	W 1053 SERA	ATCC 15987 SERA
					122.0	
110.4 a)	113.5	116.4	114.8	110.9		
102.9		105.9				
	96.6					
81.5	86.1			89.3		
	78.5			79.2		
	71.6		70.8	70.2		
63.1	66.8		64.3			
	62.4		61.3			
57.4	58.2					
52.3	53.1		54.3			
43.4	47.3			46.6	48.1	46.9
34.4	35.9		36.9	36.6		
27.9			28.3	31.7		
	20.7			21.5		
18.7	18.4	19.2	18.8	19.1	18.1	
16.7			16.6			
			15.8			

a) Molecular weight in kDa.

Molecular Weights of Antigens Detected For ATCC 12104 Cell Wall Extracts Reacted With The 7 Different Cluster Antisera After Absorption With A. israelii (NGB 17) Cell Walls

WVU 627 SERA	B 236 SERA	B 102 SERA	BE 32 SERA	ATCC 12104 SERA	W 1053 SERA	ATCC 15987 SERA
				113.0		
				103.3		
97.1[a)]				96.6		
				82.6		
				70.6		
				66.0		
				57.7		
				46.1	48.1	46.9
				41.2		
	36.3			37.7		
	21.8			21.5		
	19.4	19.5		18.8	18.5	

a) Molecular weight in kDa.

Molecular Weights of Antigens Detected For W 1053 Cell Wall Extracts Reacted With The 7 Different Cluster Antisera After Absorption With A. israelii (NGB 17) Cell Walls

WVU 627 SERA	B 236 SERA	B 102 SERA	BE 32 SERA	ATCC 12104 SERA	W 1053 SERA	ATCC 15987 SERA
					119.2	
111.8 a)	113.7	112.2	109.4			
	106.0	104.8				
99.4	98.8		96.9	98.3		
88.4	88.0				89.5	
82.4		83.5	81.8	79.2		
73.3	76.5	71.2	72.5	70.2	70.5	
68.3			67.5			
	65.0		64.3		65.6	
62.2			61.3	63.7		
58.0	60.6	58.1			59.7	
			55.6			
	52.7		51.7			
		47.3		46.6	47.0	
44.8	45.8					45.7
40.8	41.8	41.3	42.6	40.3		
			38.7			
	36.3		34.3	35.7	35.3	
31.5	30.8		29.0	31.7		
	21.3			21.5		
	18.9	19.1	17.9	19.1	18.1	
				16.5		

a) Molecular weight in kDa.

Molecular Weights of Antigens Detected For ATCC 15987 Cell Wall Extracts Reacted With The 7 Different Cluster Antisera After Absorption With *A. israelii* (NGB 17) Cell Walls

WVU 627 SERA	B 236 SERA	B 102 SERA	BE 32 SERA	ATCC 12104 SERA	W 1053 SERA	ATCC 15987 SERA
				98.3 a)		97.3
				91.5		
				79.2		
				70.2		
	48.0			46.6	48.1	46.9
	18.9	19.5		19.6	18.5	

a) Molecular weight in kDa.