

Construction and Characterization of a Single-Chain Variable Fragment
Antibody Library against *Fusobacterium nucleatum*

by

Farhan M. Khan

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DALHOUSIE UNIVERSITY

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

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Dated: July 26, 2012

External Examiner:

Co-Supervisors:

Readers:

Departmental Representative: _____

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AUTHOR: Farhan M. Khan

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To my parents, thank you for your continued and everlasting encouragement and support.

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ABSTRACT

The accumulation of oral bacteria forming dental plaque, or biofilm, promotes the development of dental caries and periodontal diseases. These biofilms are formed through a sequential process of bacterial accretion. Early colonizing bacteria are mainly commensal streptococci and *Actinomyces* species. Pathogenic late colonizers consist largely of Gram-negative anaerobes. A key bridge organism capable of coaggregating with both the early and late colonizers is *Fusobacterium nucleatum*. Despite being the focus of research for over 20 years, very few *F. nucleatum* adhesins are known due to the lack of an effective tool to identify these adhesins. We hypothesize that a single-chain variable fragment (scFv) antibody library will enable the identification of *F. nucleatum* adhesins and help to elucidate the mechanism of coaggregation between *F. nucleatum* and other oral bacteria. Therefore, a scFv M13 phage display library, consisting of 4×10^8 clones, was created using RNA from the spleen of a mouse immunized with *F. nucleatum*. The library was enriched by biopanning 6 times against *F. nucleatum* whole cells. Individual clones of the enriched library were analyzed by ELISA to identify scFvs specific for *F. nucleatum*. All 292 individual clones tested in ELISA reacted strongly to *F. nucleatum*. Sixty two of the 292 clones inhibited *F. nucleatum* interaction with *Streptococcus sanguinis* in coaggregation assays. The 62 scFvs were further grouped into 5 categories based on their reactivity with the *F. nucleatum* outer membrane proteins in western immunoblotting. Analysis of 11 representative clones from the 5 categories revealed differences in coaggregation inhibition with *S. sanguinis*, recognition of outer membrane proteins and BstOI restriction pattern. DNA sequencing of the 11 representative clones revealed 6 unique scFvs and of them, 3 strongly inhibited interaction with 5 *Streptococcus* species. Mass spectrometry identified the protein recognized by the function-blocking scFvs as RadD, a previously identified *F. nucleatum* adhesin involved in coaggregation with *S. sanguinis*. The construction, characterization and use of this anti-*F. nucleatum* scFv library to identify an adhesin involved in coaggregation, demonstrates the utility of this library and its potential for future use in the discovery of adhesins and cognate receptors.

LIST OF ABBREVIATIONS USED

ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BSA	bovine serum albumin
cDNA	complementary DNA
CDR	complementary determining region of antibody
CFU	colony-forming units
C _H	constant region of heavy chain of antibody
C _L	constant region of light chain of antibody
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ET SSB	extreme thermostable single-strand DNA binding protein
Fab	fragment antigen-binding
Fc	fragment crystallisable
FR	framework region of antibody
Fv	fragment variable
HMW	high molecular weight
IgG	immunoglobulin G
LB	Luria-Bertani
LPS	lipopolysaccharide
OD	optical density
OM	outer membrane
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-20
PCR	polymerase chain reaction
PFU	plaque-forming units
SB	super broth
scFv	single-chain variable fragment
SD	standard deviation

SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	tris-buffered saline
V _H	variable region of heavy chain of antibody
V _L	variable region of light chain of antibody

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CHAPTER 1 INTRODUCTION

1.1 THE FORMATION OF DENTAL PLAQUE

A microorganism first entering the oral cavity is like a small boat in a stormy sea, buffeted in the waves and currents of saliva that continually flow through the mouth and across the oral surfaces. In this dynamic environment of the oral cavity, bacterial adherence mechanisms are crucial. In their absence, unanchored bacteria become part of the salivary flow and are swallowed. Consequently, oral bacteria have evolved specific mechanisms to allow colonization of the oral cavity. Three principle surfaces exist for oral bacterial colonization: teeth, epithelial mucosa, and the nascent surface created by new bacterial cells as they form a multiple-species community known as biofilm.

Biofilm, or dental plaque as it is known in the oral cavity, tends to form on the hard surface of teeth, as opposed to the oral mucosa, which desquamate the surface layer of epithelia and with it any attached microbiota. In this regard, a tooth in the mouth is similar to a rock in a stream; both sites favour bacterial colonization and the accretion of biofilm. A prerequisite for bacterial colonization to any surface is the ability of the bacteria to adhere to that surface. Bacteria can adhere to the host through a variety of mechanisms (Scannapieco, 1994). One general mechanism involves nonspecific forces such as, ionic, hydrophobic, hydrogen bonding, and van der Waals forces, between the microbe and host surface. Another mechanism involves specific interactions between bacterial surface proteins, designated adhesins, and receptors on host components or bacterial binding partners (Beachey, 1981). Receptors can be another protein or glycoprotein or glycolipid. Specific interactions, similar to antibody-antigen or enzyme-substrate interactions, depend on recognition of molecular shapes between proteins. These interactions account in part for selective colonization in the oral cavity.

The formation of dental plaque occurs through a sequential process (Kolenbrander *et al.*, 1993). Immediately following cleaning of the teeth, a thin layer of salivary components, called the acquired pellicle, covers the tooth surface. The pellicle consists of numerous

components, including mucins, glycoproteins, proline-rich proteins, histidine-rich proteins, enzymes like alpha-amylase, phosphate-containing proteins like statherin, and sialic acid. The earliest colonizers are overwhelmingly streptococci, which constitute up to 85% of the cultivable cells found in the first 4 h after teeth cleaning (Nyvad and Killian, 1987). Streptococci bind to components of the acquired pellicle, including acidic proline-rich proteins (Gibbons *et al.*, 1991; Hsu *et al.*, 1994), alpha-amylase (Scannapieco *et al.*, 1989; Scannapieco *et al.*, 1995), and sialic acid (Duan *et al.*, 1994). As well, streptococci bind to other streptococci (Kolenbrander *et al.*, 1990), which provides them an additional advantage in colonizing, since this allows them to bind to the nascent monolayer of already bound streptococci. Within 12 h the population grows to include the early colonizers: actinomyces, capnocytophagae, haemophili, prevotellae, propionibacteria, and veillonellae (Nyvad and Killian, 1987). These early colonizers adhere to the acquired pellicle (Gibbons *et al.*, 1988) and to the streptococci (Kolenbrander, 1989).

The primary colonizers, streptococci and actinomyces, are facultative anaerobes and grow quickly in the first 4 h of plaque development (Weiger *et al.*, 1995). Consequently, Weiger *et al.* (1995) propose that streptococci and actinomyces prepare the environment for late colonizers that have more fastidious growth requirements. Late colonizers are mainly Gram-negative anaerobes and include: *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola*, *Selenomonas flueggei*, and *Aggregatibacter actinomycetemcomitans*. As bacteria accrete they metabolize and change the environment, allowing cells that could not survive the original aerobic environment to now be able to colonize the anaerobic plaque (Kolenbrander, 2000). However, these cells still require a place to attach. This is where the intermediate colonizer fusobacteria play a key role. As proposed by Kolenbrander and London (1993) fusobacteria act as bridges to anchor the pathogenic late colonizers to the commensal early colonizers (Figure 1.1).

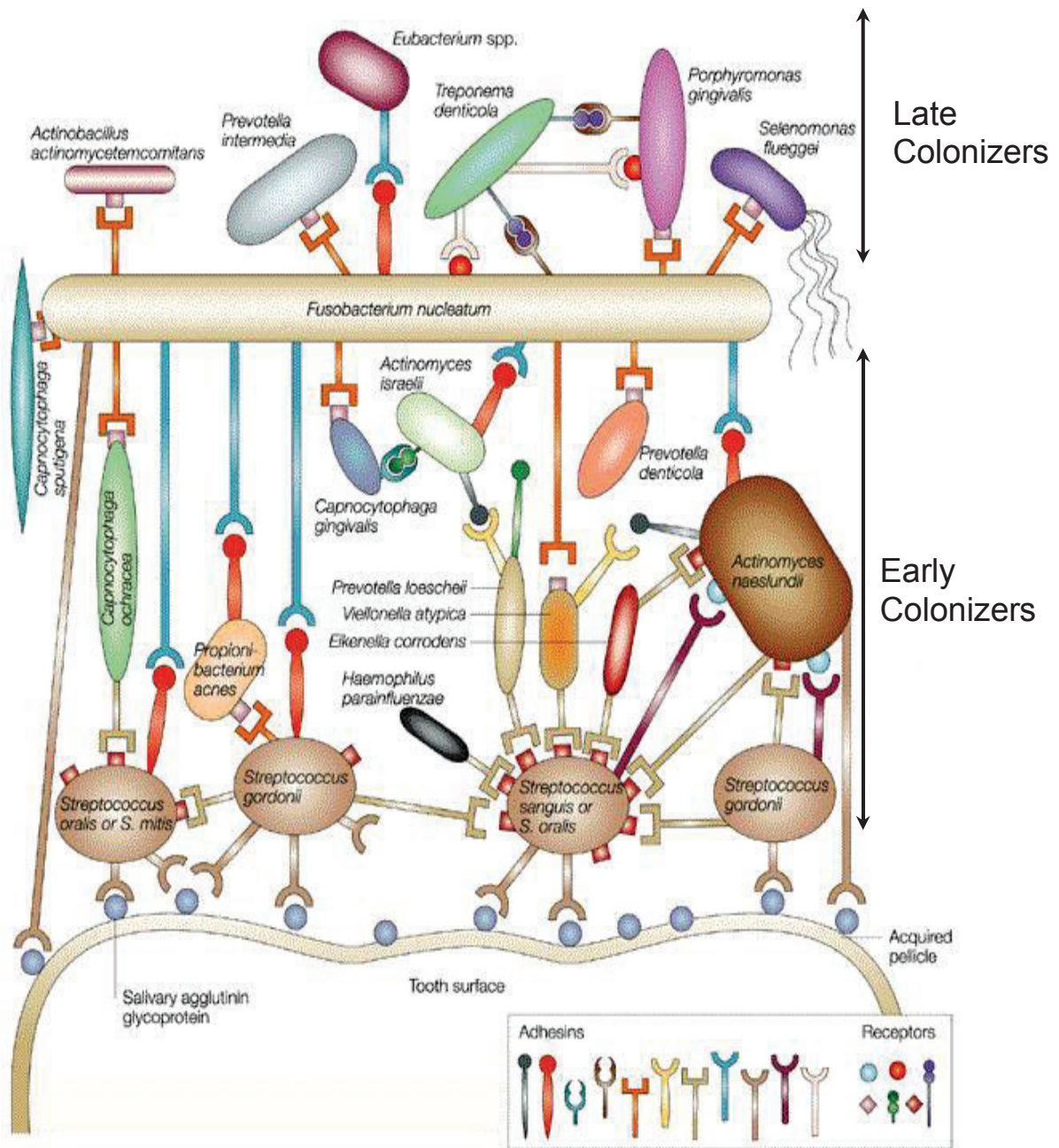


Figure 1.1. *F. nucleatum*: The microbial bridge. The tooth surface is covered by an acquired pellicle comprised of lipids, glycoproteins and mucins. The pellicle is recognized by primary colonizing bacteria, mostly streptococci, which express receptors for specific salivary components. *F. nucleatum* coaggregates with early colonizers and provides a nascent surface for late colonizers, generally Gram-negative anaerobes, to colonize sequentially on and eventually form dental plaque. Reproduced with permission from Kolenbrander *et al.* (2002).

1.1.1 The Unique Role of Fusobacteria

Fusobacteria are able to recognize and adhere to different bacteria. When these interactions occur among suspended cells, the process is called coaggregation (Whittaker *et al.*, 1996). When it occurs between suspended and already attached cells in biofilms, it is called coadhesion (Bos *et al.*, 1995). In principle, the mechanisms of coaggregation and coadhesion are identical. The two are interconnected in that suspended coaggregates, as well as single cells, may accrete onto the biofilm.

As a group, fusobacteria coaggregate with some strains of all 17 genera of oral bacteria that have been tested, but each strain of *Fusobacterium nucleatum* coaggregates only with a certain set of partners (Kolenbrander *et al.*, 1989). *F. nucleatum* is able to take part in intrageneric coaggregation, which is coaggregation within the bacteria's genera (Kolenbrander *et al.*, 1995). As well, *F. nucleatum* can take part in intergeneric coaggregation with early colonizing bacteria (Kolenbrander and London, 1992) and is the preferential coaggregation partner of late colonizers (Kolenbrander *et al.*, 1989).

F. nucleatum is one of the most commonly isolated species in both healthy plaque, which is composed of mostly commensal early colonizers, and diseased plaque, which is over-represented with pathogenic late colonizers (Tanner and Bouldin, 1989; Papapanou *et al.*, 1993). Thus, due to the ability of *F. nucleatum* to participate in a broad range of coaggregations and since it is very frequently isolated in established dental plaque, in both healthy and diseased sites, it is proposed that fusobacteria acts as a bridge between early and late colonizing bacteria (Kolenbrander and London, 1993).

1.2 GENERAL CHARACTERISTICS OF *F. NUCLEATUM*

F. nucleatum is a species of the genus *Fusobacterium*, which belongs to the family *Bacteroidaceae*. The name *Fusobacterium* has its origin in *fusus*, a spindle, and *bacterion*, a small rod. Thus, the bacterium is shaped like a long spindle-shaped rod or fusiform, which presumably helps it form multiple simultaneous coaggregation

interactions. The term *nucleatum* originates from the nucleated appearance frequently seen in light and electron microscope preparations due to the presence of intracellular granules (Bolstad *et al.*, 1996). *F. nucleatum* is nonsporeforming, nonmotile, and Gram-negative (Robrish *et al.*, 1987). Most cells are 5 to 10 µm long and have rather sharply pointed ends. The bacterium is anaerobic but is aerotolerant, growing in the presence of up to 6% oxygen (Hofstad, 1981). This helps it act as an intermediate colonizer allowing it to bind to and survive in the accreting biofilm before even more fastidious bacteria can.

Organisms within the species *F. nucleatum* are quite heterogeneous. Therefore, on the basis of electrophoretic patterns of whole-cell proteins and DNA homology, Dzink *et al.* (1990) proposed dividing *F. nucleatum* into three different subspecies: *nucleatum*, *polymorphum*, and *vincentii*. However, Gharbia and Shah (1990) on the basis of DNA-DNA hybridization patterns and electrophoretic patterns of the enzymes glutamine dehydrogenase and 2-oxoglutarate reductase, have divided *Fusobacterium* species into four subspecies: *nucleatum*, *polymorphum*, *fusifforme*, and *animalis*. Strain American Type Culture Collection (ATCC) 25586, which is used in this study, is classified as *F. nucleatum* subspecies *nucleatum*.

1.3 F. NUCLEATUM AND PERIODONTAL DISEASES

Periodontal diseases and dental caries are two of the most chronic and epidemic human diseases (Callister *et al.*, 2008). In Canada, 21% of adults have, or have had, a moderate or a severe periodontal problem and 96% of adults have had a history of dental caries (Health Canada, 2010). These oral diseases are the unfortunate consequence of dental plaque (Newbrun, 1979). When the cariogenic bacterium *Streptococcus mutans* becomes abundant in dental plaque, dental caries occur. Similarly, periodontal diseases occur when pathogenic Gram-negative anaerobes become prevalent in dental plaque along the gingiva producing periodontal pockets. Periodontal diseases consist of a group of inflammatory conditions broadly classified as gingivitis and periodontitis. *F. nucleatum* promotes the development and stability of dental plaque through its function as a microbial bridge between commensal early colonizers and pathogenic late colonizers

(Kolenbrander and London, 1993). However, the pathogenic potential of *F. nucleatum* is not limited to its ability to form aggregates with suspected pathogens. In addition, *F. nucleatum* has the ability to produce toxic metabolites. These cytotoxins produced by *F. nucleatum* inhibit proliferation of human gingival fibroblasts, may have the ability to penetrate the gingival epithelium, and are present in elevated levels in plaque associated with periodontitis (Bartold *et al.*, 1991). Furthermore, *F. nucleatum* is associated with periodontal disease due to its abundance in periodontal lesions and its synergism with other bacteria in mixed infections.

Moore and Moore (1994) examined over 51,000 isolates from the human gingival crevice and found that *F. nucleatum* is one of the most commonly occurring species. During the progression from healthy plaque to diseased plaque, the number of *F. nucleatum* increases in proportion (Ritz, 1967; Socransky *et al.*, 1988). *F. nucleatum* appears to be closely correlated with gingivitis as described by studies on the bacteriology of experimental gingivitis in children (4 to 6 years) and young adults (22 to 31 years) (Moore *et al.* 1982; Moore *et al.* 1984). The most common subspecies found in the gingival crevice is *F. nucleatum* subspecies *vincentii*, with *F. nucleatum* subspecies *nucleatum* and *F. nucleatum* subspecies *polymorphum* following in a ratio of 7:3:2 (Moore and Moore, 1994). In addition, oral *F. nucleatum* can act as the origin of disease for other human infections. Of the periodontal species that are statistically associated with periodontal disease, *F. nucleatum* is the most common in clinical infections of other body sites (Moore and Moore, 1994). It has been isolated from infections such as tropical skin ulcers, peritonsillar abscesses, pyomyositis and septic arthritis, bacteremia and liver abscesses, intrauterine infections, bacterial vaginosis, urinary tract infections, pericarditis and endocarditis, and lung and pleuropulmonary infections (Bolstad *et al.*, 1996). *F. nucleatum* is also associated with pre-term births (Tateishi *et al.*, 2012) and has been linked to colorectal carcinoma (Castellarin *et al.*, 2012).

F. nucleatum plays a synergistic role in periodontal disease. In experimentally induced infections in mice, a mixed culture of *F. nucleatum* with either *Porphyromonas gingivalis* or *Prevotella intermedia* was significantly more pathogenic than *F. nucleatum* in pure

culture and more pathogenic than *P. gingivalis* or *P. intermedia* alone (Baumgartner *et al.*, 1992). *F. nucleatum* positively correlates with the formation of periodontal lesions when combined with *Campylobacter rectus*, *Prevotella intermedia*, and *Peptostreptococcus micros* (Dzink *et al.*, 1985; Dzink *et al.*, 1988). In a study of Sudanese adults with untreated periodontitis, *Prevotella intermedia* was never found unless *F. nucleatum* was also present, underscoring the key role played by *F. nucleatum* in periodontal disease (Ali *et al.*, 1994). Feuille *et al.* (1994) demonstrated that when *F. nucleatum* is combined with *P. gingivalis* there is a synergistic effect on tissue destruction. Alone, *F. nucleatum* and *P. gingivalis* do not create as large periodontal lesions as when they infect together.

Collectively, the pathogenic potential of *F. nucleatum* is significant. Whether it is due to the ability of *F. nucleatum* to act as a microbial bridge linking pathogenic bacteria to commensal biofilm, or the production of cytotoxins, or its abundance in diseased plaque, or its synergistic role in periodontal disease, *F. nucleatum* has gained the interest of many researchers.

1.4 F. NUCLEATUM COAGGREGATION PROPERTIES

F. nucleatum coaggregation is grouped into lactose-, heat- and EDTA-sensitive and non-sensitive interactions (Kolenbrander *et al.*, 1989). It is thought that early colonizing Gram-positive and late colonizing Gram-negative bacteria have different properties of coaggregation (Figure 1.2). Kolenbrander *et al.* (1989) revealed that heating *F. nucleatum* to 85 °C for 30 min prevented coaggregation to Gram-positives. However, the addition of 60 mM lactose or 1 mM EDTA (ethylenediaminetetraacetic acid) had no effect. Since heat treatment prevented coaggregation, it is thought that one or more proteins are involved as coaggregation mediators with Gram-positive bacteria. In the case of Gram-negative bacteria, lactose, EDTA, and heating *F. nucleatum* all inhibited coaggregation. Since all treatments cause inhibition of coaggregation, it is suggested that coaggregation of *F. nucleatum* with Gram-negative partners are mediated by lectin-carbohydrate interactions. The partner bearing the adhesin (lectin) is inactivated from participating in

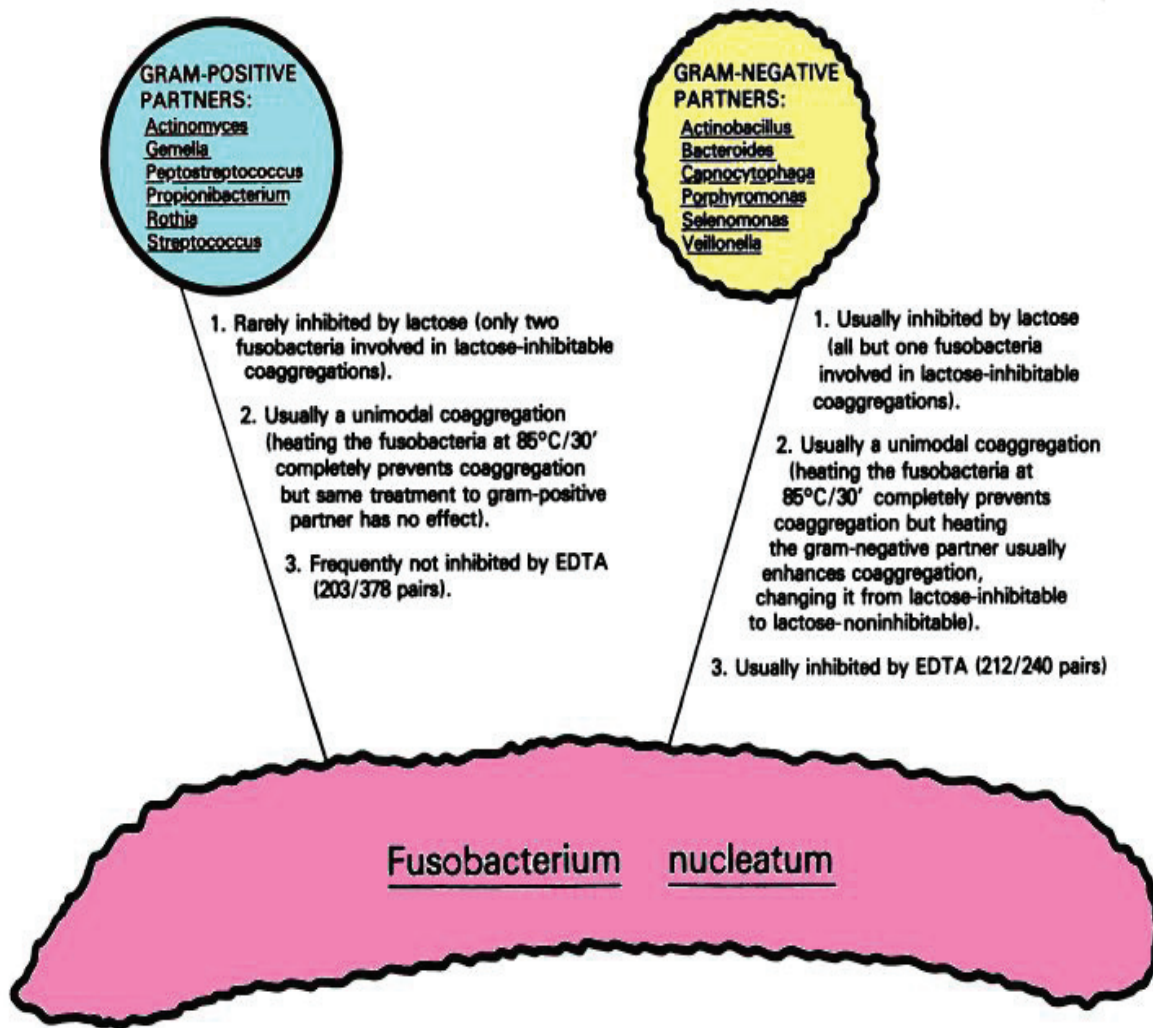


Figure 1.2. Properties of *F. nucleatum* coaggregation between Gram-positive and Gram-negative partners differ. Most fusobacteria coaggregate with some Gram-positive and some Gram-negative cell types, but adherence mechanisms appear to be quite different. Coaggregation phenotypes can be described as lactose-, heat-, or EDTA-sensitive or non-sensitive. Reproduced with permission from Kolenbrander *et al.* (1989).

coaggregation when it is heated, but the partner bearing the complementary receptor polysaccharide is unaffected when it is heated (Kolenbrander *et al.*, 2006). Thus, it appears that the adherence mechanism between *F. nucleatum* and early colonizing Gram-positive and late colonizing Gram-negative bacteria have some underlying differences.

1.5 *F. NUCLEATUM* COAGGREGATION ADHESINS

Despite being the focus of study for over twenty years, very little is known of the *F. nucleatum* adhesins involved in coaggregation. Currently, only five adhesins involved in biofilm formation have been described in varying amounts of detail.

1.5.1 FomA

The most researched outer membrane protein of *F. nucleatum* is by far the major outer membrane porin FomA. Many outer membrane proteins of approximately 40 kDa have been the focus of interest and are presumably FomA (Bolstad *et al.*, 1996). Kaufman and DiRienzo (1989) isolated a 39.5 kDa protein that is involved in fusobacterial corncob intergeneric coaggregation. Corncobs are distinct morphological units, in which several streptococci surround a filamentous microorganism, producing an arrangement similar in appearance to a corncob. Kaufman and DiRienzo (1989) show that *F. nucleatum* ATCC 10953 coaggregation with *S. sanguinis* CC5A is mediated by this 39.5 kDa protein, presumably FomA. Separately, Kinder and Holt (1993) isolated a 42 kDa protein from *F. nucleatum* T18 involved in coaggregation with *P. gingivalis* T22. The coaggregation is mediated by a protein adhesin, presumably FomA, on *F. nucleatum* and a carbohydrate receptor on *P. gingivalis*. The proteinaceous nature of the adhesin was demonstrated using proteolytic pre-treatment of the *F. nucleatum* outer membrane fraction, which resulted in loss of coaggregation inhibition compared to the untreated fraction. If both the 42 kDa and 39.5 kDa proteins are the same 40 kDa porin that has previously been described (Bakken *et al.*, 1989), then these results imply a dual role played by porins.

1.5.2 300-350 kDa Galactose-binding Adhesin

Murray *et al.* (1988) described a lectin-carbohydrate interaction with a 300 to 350 kDa galactose-binding adhesin of *F. nucleatum* FN-2. Binding by this adhesin is not only inhibited by galactose, but also to a greater extent by lactose, p-aminophenyl galactosides, and asialoglycopeptides. This finding suggests this lectin adhesin is capable of recognizing carbohydrates other than the simple monosaccharide galactose. The authors proposed that this adhesin is involved in adherence with components of the acquired pellicle. Galactosyl residues are often terminal sugars on salivary glycoconjugates and could be functional receptor sites for bacterial galactose-binding lectins. Alternatively, the adhesin may mediate coaggregation with bacterial partners that have not yet been described.

1.5.3 30 kDa Galactose-binding Adhesin

Using the strain *F. nucleatum* PK1594, Shanitzki *et al.* (1997) discovered a 30 kDa galactose-sensitive adhesin. A monoclonal antibody was used to inhibit galactose-sensitive interactions and determine the identity of the adhesin. The 30 kDa outer membrane polypeptide mediates coaggregation with *P. gingivalis* PK1594 and *A. actinomycetemcomitans* JP2. As well, a putative role in hemagglutination was proposed, since *F. nucleatum* interactions with erythrocytes are blocked by the monoclonal antibody. Hemagglutination may represent a more general phenomenon in which *F. nucleatum* helps anchor other poorly adhering pathogenic bacteria and itself to the epithelium and macromolecules. Thus, the findings suggest that this 30 kDa protein is involved in coaggregation with the late colonizers and possibly the acquired pellicle.

1.5.4 N-acetylneuraminic Acid Specific *F. nucleatum* Adhesin

Shanitzki *et al.* (1998) used the same method they previously employed to identify the 30 kDa galactose-binding adhesin (Shanitzki *et al.*, 1997) to find this N-acetylneuraminic

acid specific adhesin. Using monoclonal antibody to inhibit coaggregation, Shaniztki *et al.* (1998) determined that *F. nucleatum* PK1597 coaggregation with *Actinomyces israelii* PK16, *Streptococcus oralis* H1, *S. oralis* 522, *Capnocytophaga ochracea* ATCC 33596, *Prevotella denticola* PK1277 and *Prevotella intermedia* PK1511 are mediated by the same protein. Although, the size and cellular location of this adhesin is not known, it is known that the interaction is both lactose- and galactose-insensitive. This N-acetylneuraminic acid-sensitive adhesin is also proposed to play a role in mediating interactions with host tissues and macromolecules, including glycoproteins of the acquired pellicle. Therefore, this newly described adhesin may have a dual role, similar to other identified adhesins, and bind both bacterial partner strains and the acquired pellicle.

1.5.5 RadD

The most recently discovered adhesin is Fn1526 (RadD). Kaplan *et al.* (2009) demonstrated that this 350 kDa outer membrane autotransporter protein is necessary for the formation of biofilms between *S. sanguinis* ATCC 10556 and *F. nucleatum* ATCC 23726. This arginine-inhibitable adhesin is also involved in coaggregation with the Gram-positive early colonizers, *A. naeshundii* ATCC 12104, *S. oralis* ATCC 10557, and *S. gordonii* ATCC 10558. As well, gene inactivation of Fn1526 reduced agglutination of Jurkat cells (human T lymphocyte) by *F. nucleatum*. Thus, Fn1526 may allow *F. nucleatum* to adhere to not only early oral colonizers, but also human lymphocytes.

1.6 COGNATE RECEPTORS ON PARTNER BACTERIA

The identity of receptors on partner bacteria that participate in coaggregation with *F. nucleatum* is even more limited. To date, only three receptors have been described.

1.6.1 Polar Fimbriae of *S. sanguinis* Mediate Corncob Formation

Pioneering a transposon mutagenesis method to insertionally inactivate the genes associated with the synthesis or assembly of the polar fimbriae of *S. sanguinis* CC5A, Correia *et al.* (1995) investigated the molecular basis for corncob formation with *F. nucleatum* ATCC 10953. A partial reduction in binding was demonstrated, implying the existence of multiple binding components. At least 4 types of mutants of *S. sanguinis* showed reduced binding to *F. nucleatum*, indicating that a number of specific loci in *S. sanguinis* are involved in adherence to *F. nucleatum*. Therefore, it appears that polar fimbriae play a role in mediating *S. sanguinis* coaggregation with *F. nucleatum*.

1.6.2 O-Polysaccharide of *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans serotype b and f strains, but not serotype a, c, d, or e strains, coaggregate with *F. nucleatum* (Rupani *et al.*, 2008). It is thought that this specificity is due to the galactose-containing O-polysaccharide region of the lipopolysaccharide (LPS) in serotypes b and f. Rosen *et al.* (2003) showed that coaggregation between *A. actinomycetemcomitans* serotype b strains Y4, JP2 and *F. nucleatum* PK1594 is mediated by serotype b LPS. Coaggregation was inhibited by N-acetylgalactosamine, galactose, and by purified serotype b LPS. The *F. nucleatum* adhesin that binds to *A. actinomycetemcomitans* serotype b O-polysaccharide is a 30-kDa galactose-binding adhesin (Shaniztki *et al.*, 1997). Rupani *et al.* (2008) demonstrated that *A. actinomycetemcomitans* strain CU1060N (serotype f) coaggregation with *F. nucleatum* PK1594 is also mediated by the O-polysaccharide region of LPS. *A. actinomycetemcomitans* serotype f O-polysaccharide mutants failed to coaggregate with *F. nucleatum*. Thus, *A. actinomycetemcomitans* coaggregation with *F. nucleatum* is mediated by the O-polysaccharide of *A. actinomycetemcomitans* serotypes b and f.

1.6.3 *Treponema denticola* Major Outer Sheath Protein

The major outer sheath protein of *T. denticola* ATCC 35405 acts as an adhesin for coaggregation with *F. nucleatum* PK1594 (Rosen *et al.*, 2008). Purified major outer sheath protein inhibited coaggregation in a concentration-dependent manner. Periodic acid-Schiff staining of the major outer sheath protein revealed that it is a glycoprotein. Deglycosylation of this glycoprotein was found to inhibit its binding to *F. nucleatum*. Interestingly, deglycosylation did not effect coaggregation to *P. gingivalis* (PK1924, ATCC 33277, and W381) cells. Therefore, the major outer sheath protein of *T. denticola* is thought to act as an adhesin during coaggregation with *P. gingivalis* and *F. nucleatum* through its protein and carbohydrate moieties, respectively.

1.7 WHY ARE SO FEW *F. NUCLEATUM* ADHESINS AND COGNATE RECEPTORS KNOWN?

Despite over two decades of research, the mechanisms of coaggregation between *F. nucleatum* and partner bacteria remain to be fully understood. We believe this is due to a lack of an effective tool to identify *F. nucleatum* adhesins. Currently, adhesins are usually discovered by identifying a sugar or amino acid that can inhibit coaggregation and using that inhibitor to isolate the adhesin. However, this method is limited by whether an inhibitor for the novel adhesin can be found.

A library of single-chain variable fragment antibodies raised against *F. nucleatum* provides a powerful and convenient technology to identify novel adhesins and even their cognate receptors.

1.8 SINGLE-CHAIN VARIABLE FRAGMENT (SCFV) ANTIBODIES

As part of our normal immune defence system, our bodies create antibodies against foreign pathogens. Each antibody can recognize a specific antigen unique to its target since the antibody possesses an antigen-binding site, called paratope (a structure analogous to a lock) located at the upper tip of the “Y” shaped antibody molecule. This

paratope is specific for one particular epitope (analogous to a key), displayed on the particular antigen, allowing these two structures to specifically bind together like a lock-and-key (Janeway *et al.*, 2001). Thus, this natural immune response can be harnessed to create the powerful biological tool known as single-chain variable fragment (scFv) antibodies.

The variable fragment (Fv) is the smallest unit of an immunoglobulin molecule that is still able to functionally bind its antigen. A recombinant antibody in scFv format consists of variable regions of light (V_L) and heavy (V_H) chains joined together by a glycine/serine linker sequence (Figure 1.3). This flexible peptide linker allows the V_L and V_H regions to fold into the proper antigen-binding conformation. The small, compact size of the scFv particle (approximately 27.5 kDa compared to 150 kDa for immunoglobulin G molecule) allows it to be easily expressed in functional form in *Escherichia coli* (Barbas *et al.*, 2001). Moreover, tags or peptides attached to the scFvs could allow the purification of these antibody fragments. The addition of a hexa-His sequence and a haemagglutinin tag provides mechanisms for purification and detection, respectively.

scFvs are a robust and potent technology that has been applied for several diverse purposes. Expressed as either soluble proteins or surface expressed in both eukaryotic and prokaryotic cells, scFvs have been used as a treatment for infectious diseases (Chen *et al.*, 2004; Lamarre *et al.*, 1997; Mabry *et al.*, 2005; Matthews *et al.*, 1995), targeting cancer cells (Liu *et al.*, 2004; Deckert *et al.*, 2003), and modulating immune response to antigen cargo (Knight *et al.*, 2008).

1.9 CONSTRUCTION OF scFV ANTIBODY LIBRARY

scFv antibodies have been constructed primarily from hybridoma (Galeffi *et al.*, 2006; Huston *et al.*, 1988; Chaudhary *et al.*, 1990; Deng *et al.*, 2003), spleen cells from immunized mice (Clackson *et al.*, 1991; Chaudhary *et al.*, 1989; Finlay *et al.*, 2006), and from human B lymphocytes (Marks *et al.*, 1991; Shadidi and Sioud, 2001; Zhang *et al.*, 2006). To obtain them, total RNA is first isolated from the spleen, lymph cells, bone marrow, or hybridoma followed by reverse transcription of the mRNA into

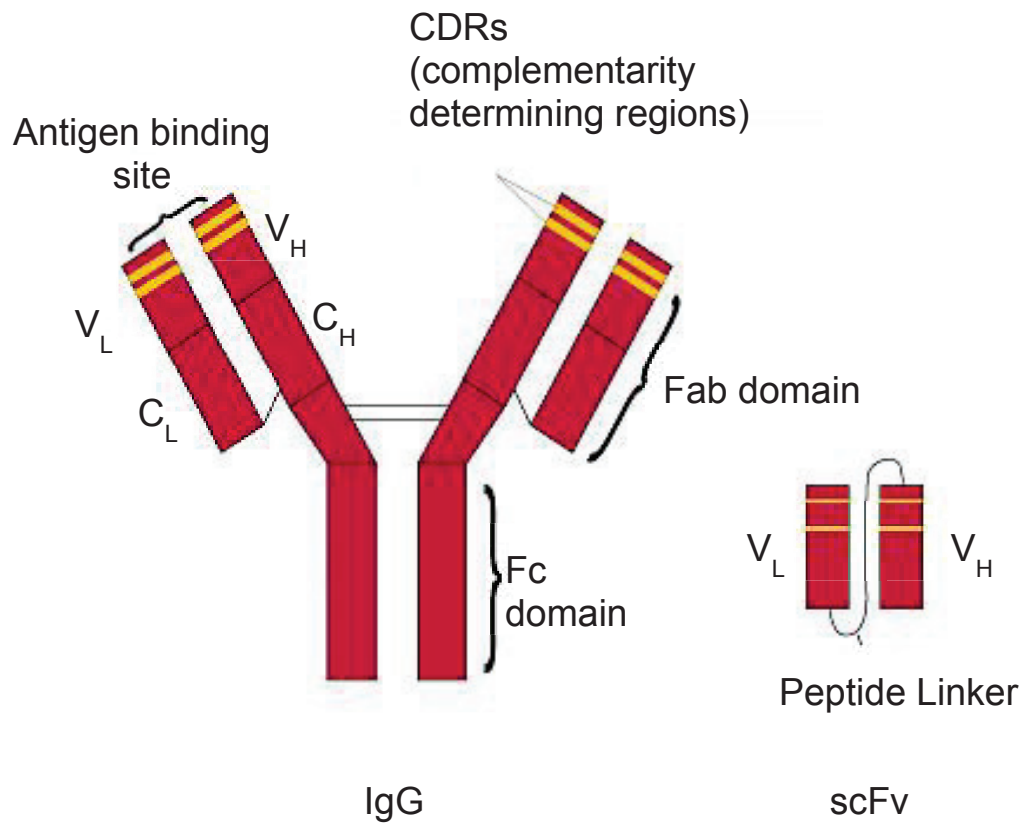


Figure 1.3. Single-chain variable fragment antibody. Antibody model showing subunit composition and domain distribution along the polypeptide chains of an immunoglobulin G (IgG) antibody. To the right is the much more compact single-chain fragment variable (scFv) antibody generated by recombinant antibody technology. V_L and V_H: variable regions of light and heavy chains; C_L and C_H: constant regions of light and heavy chains; Fab domain: fragment antigen-binding; Fc domain: fragment crystallisable. Reproduced with permission from Roth (2001).

complementary DNA (cDNA) to serve as a template for amplification of antibody genes by the polymerase chain reaction (PCR). Using this method, large libraries with high diversity of antibody V_H and V_L genes can be created (Marks *et al.*, 1991). McCafferty *et al.* (1990) pioneered a successful approach to recombinant antibody production in which phage recombinants that display the scFv antibody at their tips were utilized together with a technique of affinity selection, called biopanning. This work by McCafferty and coworkers unlocked the potential for using *in vitro* selection of scFvs from large, diverse libraries.

The order of the domains in scFv construction can be either V_H -linker- V_L or V_L -linker- V_H , both orientations have been applied successfully (Dai *et al.*, 2003; Hu *et al.*, 2005; Sheikholvaezin *et al.*, 2006). PCR assembly of the scFv was first described by Horton *et al.* (1989) and has become the standard. This method permits the scFv gene to be ligated into a phagemid vector for expression in both *E. coli* and phage (Hogrefe *et al.*, 1993). Extensive knowledge of the genetics and biochemistry of *E. coli* makes this organism a preferred expression host (Baneyx, 1999). Furthermore, *E. coli* affords a good folding environment and economical expression of scFv antibodies. Moreover, *E. coli* can be infected by the M13 phage, which is commonly used in scFv phage display libraries.

1.9.1 Phage Display of scFv Antibody Library

Smith (1985) established the method of fusing foreign DNA to the gene encoding the pIII coat protein of the M13 bacteriophage (Figure 1.4). The scFv-pIII fusion protein was expressed on the virion surface without disturbing the infectivity of the phage. Following this discovery, McCafferty *et al.* (1990) successfully demonstrated that a scFv antibody can be displayed on the phage surface as a functional protein which retains an active antigen-binding domain. Thus, these two important milestones have allowed the scFv to be displayed on the surface of M13 phage. This advancement paved the way for the easy and rapid selection of scFvs that recognize a desirable antigen through the enrichment process known as biopanning.

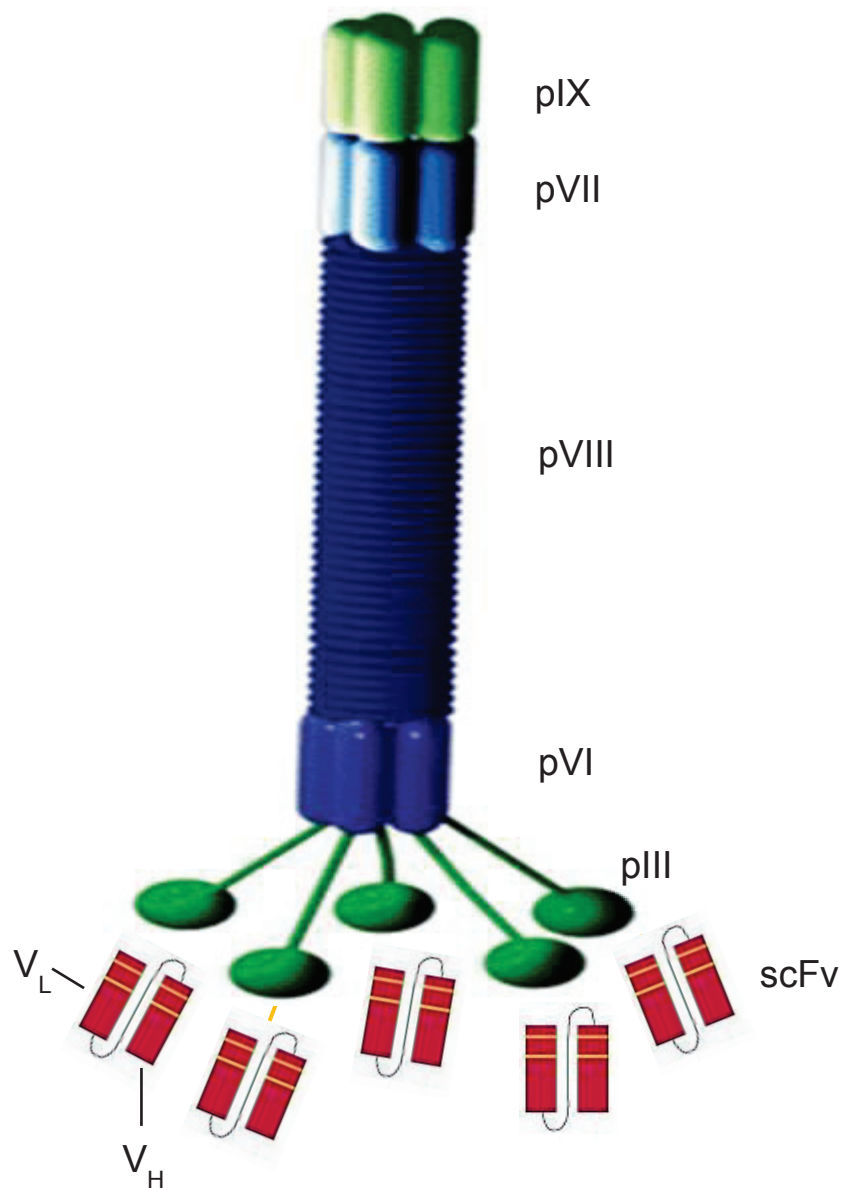


Figure 1.4. M13 phage displaying scFv antibody on its surface. The scFv antibody is surface displayed on the M13 phage linked to the pIII minor coat protein. The gene for the scFv-pIII fusion protein is on a phagemid carried by the phage. pIII, pVI, pVIII, pVII and pIX are phage proteins. Adapted from Marco Antonio Arap (2005) and used under the Creative Commons Attribution-Non-Commercial 3.0 Unported License (<http://creativecommons.org/licenses/by-nc/3.0/>).

The scFv gene can be fused to either the pVIII gene (Kang *et al.*, 1991) or the pIII gene (McCafferty *et al.*, 1990) for expression on the surface of the M13 phage. About 2700 copies of the major coat protein pVIII surround the M13 phage. The pVIII coat protein can be fused together with the scFv antibody resulting in multivalent display of the scFv. The minor coat protein pIII has three to five copies located on the tip of the phage and is responsible for attachment of the phage to its bacterial host during infection. Display on pIII allows monovalent display, making it desirable in affinity selection. Phage display is derived from the use of the phagemid vector, which contains the fusion of the scFv gene and the gene for the coat protein.

1.9.2 Biopanning: An Enrichment Process

Phage display was initially invented to allow affinity selection of scFv phages that can bind to a target antigen of interest with high affinity from a huge number of nonspecific phage clones. This is achieved by multiple rounds of phage binding to the antigen, washing to remove the unbound phage, followed by elution in acidic conditions and retrieval of specifically bound phages. After each round, the neutralized eluted phages are amplified by infection of *E. coli* for the subsequent selection step. Soluble antibodies can be produced in the final round using the bacteria (Barbas *et al.*, 2001).

A number of selection strategies exist to obtain specific antibodies (Hoogenboom *et al.*, 1998; Hoogenboom *et al.*, 2000). Biopanning has been carried out by incubating phage on antigen immobilized onto a solid support, such as immunotubes (Knappik *et al.*, 2000), microtiter plate wells, or columns (McWhirter *et al.*, 2006). As well, the phage library can be incubated using biotinylated antigen in solution, followed by capture of the antigen-phage complex on streptavidin surface (Griffiths *et al.*, 1994).

1.10 INNOVATIVE APPROACH OF STUDY

The affinity selected scFv phages provide an excellent tool for the identification of novel coaggregation adhesins of *F. nucleatum* and even their cognate receptors. The approach is briefly described below.

Once specific scFv phages are obtained via biopanning, these scFv phages can be used to inhibit coaggregation between *F. nucleatum* and partner bacteria. Function-blocking scFvs can then be examined in western immunoblotting to determine what *F. nucleatum* outer membrane protein they recognize, allowing identification of the adhesin. The scFv-adhesin can then be used as bait to capture the cognate receptor on the partner bacteria. In this way, the scFv library provides great potential for furthering the study of *F. nucleatum* coaggregation and elucidating the mechanisms of bacterial adherence that have remained undiscovered for far too long.

1.11 IMPORTANCE OF STUDY

Adherence by bacteria is the essential first step in the colonization and establishment of oral infection. It is a highly specific process, allowing a bacterium to localize in a favourable ecological niche. Thus, adherence itself is an important virulence factor. Therefore, understanding the basis of this initial, but essential interaction, should lead to the development of strategies to prevent adherence and thereby prevent infection. Consequently, *F. nucleatum* which has the ability to coaggregate with all key oral bacteria (Kolenbrander and London, 1993) is and should continue to be the focus of intensive research, for it provides the best opportunity for the development of effective strategies to prevent periodontal diseases and dental caries.

Currently, however, the molecular basis for *F. nucleatum* coaggregation is poorly understood. Thus, my dissertation aims to address this deficiency in knowledge of *F. nucleatum* adhesins and by doing so contribute to an improved understanding of *F. nucleatum* coaggregation.

1.11.1 Objectives and Conclusions of Dissertation

Dental caries and periodontal diseases are caused by the accumulation of dental plaque (Newbrun, 1979). *Fusobacterium nucleatum* plays a central role in bridging pathogenic

late colonizers to commensal early colonizers, promoting the development of dental plaque (Kolenbrander and London, 1993). Despite over two decades of research, very few *F. nucleatum* adhesins involved in coaggregation are known. Thus, this study focuses on ameliorating this paucity of knowledge, through the construction of a single-chain variable fragment antibody library against *F. nucleatum* ATCC 25586 and the use of the library to identify adhesins.

A scFv library of 4×10^8 clones was constructed and enriched by biopanned against *F. nucleatum* whole cells six times. Use of individual scFv clones to inhibit coaggregation between *F. nucleatum* and the early oral colonizer *Streptococcus sanguinis* V920 and use of the same coaggregation-blocking scFvs in inhibition assays with five other early colonizers allowed the identification of common coaggregation-blocking scFvs. Mass spectrometry analysis of the *F. nucleatum* outer membrane protein band being recognized by the coaggregation-blocking scFvs revealed that the *F. nucleatum* adhesin involved in coaggregation is RadD. Previous identification by Kaplan *et al.* (2009) of RadD as a *F. nucleatum* adhesin necessary for interaction with *S. sanguinis* validates our approach, the construction of a scFv library against *F. nucleatum* and its use in adhesin identification. Screening of our scFv library in coaggregation assays between *F. nucleatum* and *S. sanguinis*, an already characterized interaction, resulted in the identification of the previously known adhesin RadD. This finding suggests that our library is capable of identifying adhesins and has the potential to be used to identify novel adhesins and cognate receptors when applied to uncharacterized coaggregation interactions.

CHAPTER 2 MATERIALS AND METHODS

2.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

All bacterial strains used in this study were from Dr. Song Lee's (Dalhousie University) personal collection. Bacterial strains were maintained as frozen cultures at -80 °C in 25% glycerol with the appropriate broth medium as described below.

2.1.1 *Fusobacterium nucleatum*

F. nucleatum subspecies *nucleatum* American Type Culture Collection (ATCC) 25586 was grown at 37 °C under anaerobic conditions (BD GasPack Anaerobic System; BBL Microbiology Systems, Cockeysville, Md.). *F. nucleatum* from frozen culture was inoculated onto blood agar (5% sheep blood) and allowed to grow for approximately 3 days. Then, a single colony of *F. nucleatum* was streaked onto Brain Heart Infusion (BHI; BD BBL Microbiology Systems) 1.5% (wt/vol) agar plates and was incubated for 2 days. When grown in liquid culture, a single colony was inoculated in a starter culture of BHI and incubated overnight. The starter culture was used to inoculate a larger volume of BHI (pre-reduced) and incubated overnight.

2.1.2 Binding Partners

Streptococcus sanguinis V920, *S. gordonii* DL1, *S. sobrinus* B13, *S. mutans* UA159, *S. downei* ATCC 33748, and *Actinomyces naeslundii* WVU 627 were grown overnight at 37 °C in 5% CO₂ on BHI 1.5% (wt/vol) agar plates or BHI broth.

2.1.3 *Escherichia coli*

E. coli was grown aerobically with shaking at 200 rpm at 37 °C in super broth (SB) medium (1% MOPS [morpholinepropanesulfonic acid], 3% tryptone, and 2% yeast extract [wt/vol]) or Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl). If required, antibiotics were added to the medium at 100 µg/mL ampicillin, 10 µg/mL tetracycline, 70 µg/mL kanamycin, or 34 µg/mL chloramphenicol. All antibiotics were purchased from Sigma-Aldrich Chemical Co. (Oakville, Ontario, Canada). For the cultivation of *E. coli* on plates, Luria-Bertani agar (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar [wt/vol]) was used.

2.1.4 Harvesting Bacteria

Bacteria were grown to late-exponential or early-stationary phase, harvested and washed once in phosphate-buffered saline (PBS) containing 0.02% NaN₃ (8.7 mM Na₂HPO₄, 1.5 mM NaH₂PO₄•H₂O, 145 mM NaCl, 5 mM MgCl₂•6H₂O, 0.02% [wt/vol] NaN₃) by centrifugation (10,000 × g, 5 min, 4 °C). Cells were stored in a final volume of 1 mL at 4 °C until used. The optical density (OD_{600 nm}) was measured by a spectrophotometer (Shimadzu, UV-1700 PharmaSpec) and the bacterial concentration was estimated by the conversion factor 1 × 10⁸ colony-forming units (CFU) equal 0.2 OD₆₀₀.

2.2 IMMUNIZATION AND TOTAL RNA ISOLATION

To obtain the RNA coding for the anti-*F. nucleatum* antibodies and the mouse polyclonal serum against *F. nucleatum*, a BALB/c mouse was immunized via intraperitoneal injection of *F. nucleatum* subspecies *nucleatum* ATCC 25586 whole cells (10⁹ CFU in PBS) on days 1, 14, and 21. The animal was bled for serum collection and euthanized on day 28.

Following euthanasia, the whole spleen was removed, immediately minced, and placed into 1 mL of TRIzol (Invitrogen Life Technologies, Burlington, Ontario, Canada). The sample was incubated for 5 min at room temperature and centrifuged at $2,500 \times g$ for 10 min. The supernatant fluid was vortex mixed with 0.1 mL of 1-bromo-3-chloro-propane (Sigma-Aldrich) for 15 s and incubated at room temperature for 15 min. The mixture was then centrifuged at $17,500 \times g$ for 15 min, and the upper aqueous layer was saved. Total RNA was precipitated from the aqueous layer by the addition of 0.5 mL of isopropanol and incubation at room temperature for 15 min. The precipitated RNA was recovered by centrifugation at $17,500 \times g$ for 15 min, washed with 75% ethanol, and redissolved in RNase-free water (Diethylpyrocarbonate-treated water). The amount of RNA was determined by absorbance measurements at 260 nm.

2.2.1 ELISA of Anti-*F. nucleatum* Polyclonal Serum

The mouse polyclonal serum against *F. nucleatum* that was used to create the scFv library was tested by ELISA. A polystyrene plate coated with 1×10^7 *F. nucleatum* cells/well was blocked for 1 h with 1% gelatin in phosphate-buffered saline with tween-20 (PBST; 8.7 mM Na_2HPO_4 , 1.5 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 145 mM NaCl, 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 3.07 mM NaN_3 , and 0.1% [vol/vol] Tween 20) at room temperature. Following blocking, the pre-immune and immune sera were applied in triplicate at the following dilutions: 1:25 600, 1:51 200, 1:102 400, 1:204 800, and 1:409 600. The plate was incubated overnight at 4 °C. Following incubation, the plate was washed with PBST, and the reactivity of the serum was detected after overnight incubation with goat anti-mouse immunoglobulin G–alkaline phosphatase conjugate (1:8,000; Sigma-Aldrich).

2.3 PHAGE DISPLAY LIBRARY CONSTRUCTION

The anti-*Fusobacterium nucleatum* phage display library was constructed using the method previously employed by Hussein *et al.* (2007) and described in Barbas *et al.* (2001). The steps in library construction are summarized in Figure 2.1.

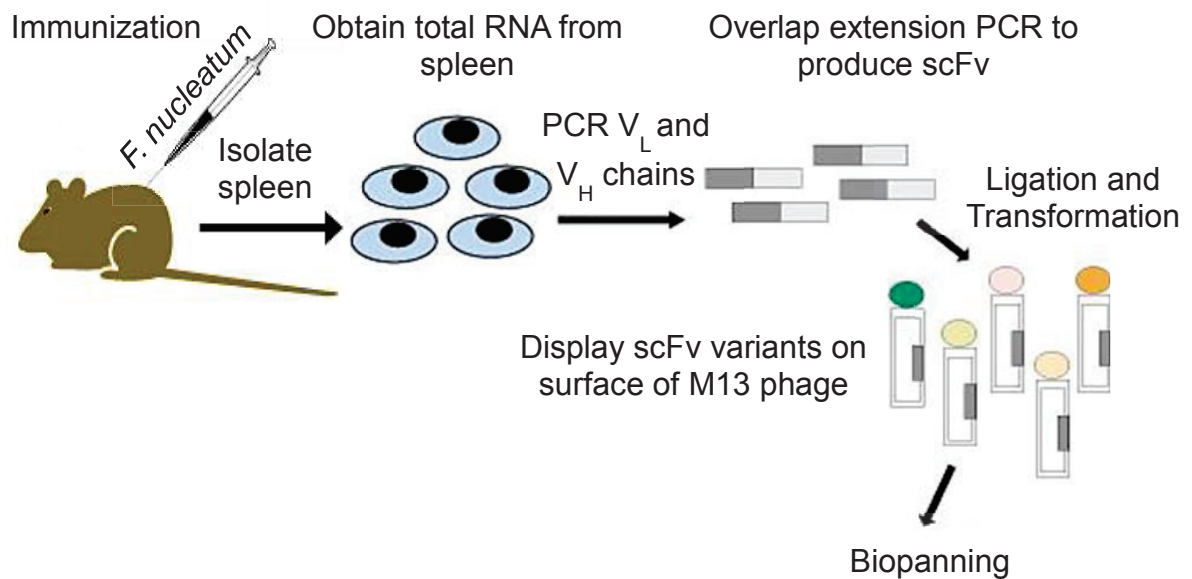


Figure 2.1. Construction of scFv phage display library. Briefly, an anti-*F. nucleatum* phage display library was constructed by immunization of a mouse with *F. nucleatum* whole cells. The total RNA was obtained from the spleen of the immunized mouse and used to amplify the variable light (V_L) chains and the variable heavy (V_H) chains of the antibodies. Overlap extension PCR was conducted to obtain the scFv. Through genetic engineering the scFv was displayed on the surface of the M13 phage fused to the pIII coat protein. Following construction of the phage display library, the library was enriched by biopanning. Adapted from Roth (2001) and used with permission.

2.3.1 First-strand cDNA Synthesis from Total RNA

Complementary DNA (cDNA) was produced from 2 μg total RNA using oligo(dT) as the primer and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The 2 μg total RNA, 1 μL oligo(dT)₁₂₋₁₈ (500 $\mu\text{g}/\text{mL}$), and 1 μL dNTP mix (10 mM each) were combined and adjusted to a final volume of 13 μL in RNase-free water. The mixture was heated at 65 $^{\circ}\text{C}$ for 5 min and then quickly chilled on ice. Four microliters of 5X First-Strand Buffer and 2 μL of 0.1 M dithiothreitol were added and then incubated at 42 $^{\circ}\text{C}$ for 2 min. One microliter of 200 Units/ μL SuperScript II was added and incubated at 42 $^{\circ}\text{C}$ for 50 min, and then at 70 $^{\circ}\text{C}$ for 15 min to inactivate the reaction. Finally, 1 μL of 2 Units/ μL RNaseH (Invitrogen) was added and incubated at 37 $^{\circ}\text{C}$ for 20 min.

2.3.2 First Round of PCR

Polymerase chain reaction (PCR) was conducted to produce the DNA that encodes the variable light (V_L) chains (V_{κ} and V_{λ}) and the variable heavy (V_H) chain from the cDNA using a procedure adapted from Lim *et al.* (2010). To construct a large single-chain library, 9 reactions with V_{κ} primers (SL239-SL258), one reaction with V_{λ} primers (SL259 and SL260) and 12 reactions with V_H primers (SL261-SL282) were performed (Table 2.1). Each reaction consisted of 1 μL cDNA template, 60 pmole of the appropriate forward and reverse primers, 1X Phusion High Fidelity Reaction Buffer (New England BioLabs), 0.2 mM dNTP mix and 2 Units of Phusion DNA Polymerase (New England BioLabs) in a final reaction volume of 100 μL made with sterile distilled water. For amplification of V_{κ} and V_H , 400 ng extreme thermostable single-stranded DNA binding protein (New England BioLabs) was added to the reaction mixture. Hot start PCR was conducted by heating the reaction components to 98 $^{\circ}\text{C}$ before adding the polymerase. The thermocycling conditions were: initial denaturation at 98 $^{\circ}\text{C}$ for 30 s, followed by 30 cycles of: denaturing at 98 $^{\circ}\text{C}$ for 10 s, annealing at 56 $^{\circ}\text{C}$ for 30 s, and extension at 72 $^{\circ}\text{C}$ for 30 s, and then final extension at 72 $^{\circ}\text{C}$ for 10 min.

Table 2.1. Primers used in this study. All primers were synthesized by Alpha DNA (Montréal, Québec). All primers were described by Barbas *et al.* (2001), except for SL283 and SL284 which were described by Hussein *et al.* (2007). Underlined sequences are the SfiI restriction sites. Degenerate nucleotide symbols are defined below.^a

Primer	Nucleotide sequence (5'- 3')	Direction
SL239	<u>GGGCCCAGGCGGCCGAGCTCGAYATCCAGCTG</u> CCTCAGCC	Forward
SL240	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGTTCTC</u> WCCAGTC	Forward
SL241	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGTGMTM</u> ACTCAGTC	Forward
SL242	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGTGYTR</u> ACACAGTC	Forward
SL243	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGTRATG</u> ACMCAGTC	Forward
SL244	<u>GGGCCCAGGCGGCCGAGCTCGAYATTMAGATR</u> AMCCAGTC	Forward
SL245	<u>GGGCCCAGGCGGCCGAGCTCGAYATTCAGATG</u> AYDCAGTC	Forward
SL246	<u>GGGCCCAGGCGGCCGAGCTCGAYATYCAGATG</u> ACACAGAC	Forward
SL247	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGTTCTCA</u> WCCAGTC	Forward
SL248	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGWGCTS</u> ACCCAATC	Forward
SL249	<u>GGGCCCAGGCGGCCGAGCTCGAYATTSTRATGA</u> TGACCCARTC	Forward
SL250	<u>GGGCCCAGGCGGCCGAGCTCGAYRTTKTGATG</u> ACCCARAC	Forward

Primer	Nucleotide sequence (5' - 3')	Direction
SL251	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGTGACB</u> CAGKC	Forward
SL252	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGTGATA</u> ACYCAGGA	Forward
SL253	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGTGATG</u> ACCCAGWT	Forward
SL254	<u>GGGCCCAGGCGGCCGAGCTCGAYATTGTGATG</u> ACACAACC	Forward
SL255	<u>GGGCCCAGGCGGCCGAGCTCGAYATTTTGCTG</u> ACTCAGTC	Forward
SL256	GGAAGATCTAGAGGAACCACCCCAACCACCGC CCGAGCCACCGCCACCAGAGGATTTKATTTTC CAGYTTGGTCCC	Reverse
SL257	GGAAGATCTAGAGGAACCACCCCAACCACCGC CCGAGCCACCGCCACCAGAGGATTTTATTTCC AACTTTGTCCC	Reverse
SL258	GGAAGATCTAGAGGAACCACCCCAACCACCGC CCGAGCCACCGCCACCAGAGGATTTTCAGCTC CAGCTTGGTCCC	Reverse
SL259	<u>GGGCCCAGGCGGCCGAGCTCGATGCTGTTGTG</u> ACTCAGGAATC	Forward
SL260	GGAAGATCTAGAGGAACCACCCCAACCACCGC CCGAGCCACCGCCACCAGAGGAGCCTAGGAC AGTCAGTTTGG	Reverse
SL261	GGTGGTTCCTCTAGATCTTCCCTCGAGGTRMAG CTTCAGGAGTC	Forward
SL262	GGTGGTTCCTCTAGATCTTCCCTCGAGGTBCAG CTBCAGCAGTC	Forward
SL263	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGCAG CTGAAGSASTC	Forward

Primer	Nucleotide sequence (5' - 3')	Direction
SL264	GGTGGTTCCTCTAGATCTTCCCTCGAGGTCCAR CTGCAACARTC	Forward
SL265	GGTGGTTCCTCTAGATCTTCCCTCGAGGTYCAG CTBCAGCARTC	Forward
SL266	GGTGGTTCCTCTAGATCTTCCCTCGAGGTYCAR CTGCAGCAGTC	Forward
SL267	GGTGGTTCCTCTAGATCTTCCCTGGAGGTCCAC GTGAAGCAGTC	Forward
SL268	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGAAS STGGTGGAAATC	Forward
SL269	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGAWG YTGGTGGAGTC	Forward
SL270	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGCAG SKGGTGGAGTC	Forward
SL271	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGCAM CTGGTGGAGTC	Forward
SL272	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGAAG CTGATGGARTC	Forward
SL273	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGCAR CTTGTTGAGTC	Forward
SL274	GGTGGTTCCTCTAGATCTTCCCTCGAGGTRAAG CTTCTCGAGTC	Forward
SL275	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGAAR STTGAGGAGTC	Forward
SL276	GGTGGTTCCTCTAGATCTCTCGAGGTTACTCTR AAAGWGTSTG	Forward

Primer	Nucleotide sequence (5'-3')	Direction
SL277	GGTGGTTCCTCTAGATCTTCCCTCGAGGTCCAA CTVCAGCARCC	Forward
SL278	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGAAC TTGGAAGTGTC	Forward
SL279	GGTGGTTCCTCTAGATCTTCCCTCGAGGTGAAG GTCATCGAGTC	Forward
SL280	CCTGGCCGGCCTGGCCACTAGTGACACATGGG GSTGTYGTTTTGGC	Reverse
SL281	CCTGGCCGGCCTGGCCACTAGTGACAGATGGG GCTGTTGTTGT	Reverse
SL282	CCTGGCCGGCCTGGCCACTAGTGACATTTGGGA AGGACTGACTCTC	Reverse
SL283	GAGGCGGGGCCCAGGCGGCCGAGCTC	Forward
SL284	GAGCCTGGCCGGCCTGGCCACTAGTG	Reverse

^a Nucleotide notation was used according to International Union of Pure and Applied Chemistry standards as follows:

- W:** A or T (weak bond)
- S:** C or G (strong bond)
- M:** A or C (amino)
- K:** G or T (keto)
- R:** A or G (purine)
- Y:** C or T (pyrimidine)
- B:** C, G, or T
- D:** A, G, or T
- H:** A, C, or T
- V:** A, C, or G
- N:** A, C, G, or T

2.3.3 Isolation and Purification of V_L and V_H Fragments

After PCR, the DNA encoding the V_L and V_H fragments were combined into two separate pools and ethanol precipitated. Two-volumes of 95% ethanol (vol/vol) containing 2.5% (wt/vol) potassium acetate was added to the pooled samples and allowed to precipitate at -80 °C for 30 min. The DNA was pelleted by centrifugation at 14,000 × g, 20 min, 4 °C and washed with 1 mL of 70% (vol/vol) ethanol. The precipitated DNA encoding the V_L and V_H fragments were analyzed by gel electrophoresis on a 1.5% agarose gel and the 0.4 kb bands were excised.

The DNA encoding the V_L and V_H fragments were recovered from the agarose gel pieces by electro-elution in dialysis bags (prepared by boiling 10 min in 2% [wt/vol] sodium bicarbonate and 1 mM ethylenediaminetetraacetic acid [EDTA]) containing 1 mL TE (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). The DNA was eluted at 100 V for 1 h, reversing current for 20 s to detach DNA from sides of the dialysis bag. The DNA was purified using the Elutip-D column (Whatman Schleicher & Schuell, Keene, NH). The column was prepared by passing 1 mL high salt solution (1 M NaCl, 20 mM Tris-HCl [pH 8], 1 mM EDTA), followed by 3 mL low salt solution (20 mM NaCl, 20 mM Tris-HCl [pH 8], 1 mM EDTA) through the column. The content of the dialysis bag was passed through the column twice and a 0.5 mL low salt solution wash of the dialysis bag was passed through once. The column was washed with 3 mL low salt solution and the DNA eluted with 0.5 mL high salt solution and precipitated using ethanol. The quantity of the purified DNA encoding the V_L and V_H fragments were estimated by band intensity compared to a DNA standard (New England BioLabs 1-kb DNA Ladder) using ImageJ (National Institutes of Health, Bethesda, MD).

2.3.4 Overlap Extension PCR

The single-chain variable fragment (scFv) antibody was produced from the DNA encoding the V_L and V_H chains using overlap extension PCR. To obtain enough product, 20 overlap extension PCR reactions were conducted. Each reaction consisted of 100 ng

V_L DNA and 100 ng V_H DNA, 60 pmole of the forward and reverse overlap extension primers (SL283 and SL284; Table 2.1), 1X Phusion High Fidelity Reaction Buffer (New England BioLabs), 0.2 mM dNTP mix and 2 Units of Phusion DNA Polymerase (New England BioLabs) in a final reaction volume of 100 µL made with sterile distilled water. Hot start PCR was conducted by heating the reaction components to 98 °C before adding the polymerase. The thermocycling conditions were: initial denaturation at 98 °C for 30 s, followed by 30 cycles of: denaturing at 98 °C for 10 s, annealing at 56 °C for 30 s, and extension at 72 °C for 45 s, and then final extension at 72 °C for 10 min.

2.3.5 pComb3X Phagemid DNA Amplification and Isolation

To obtain a large quantity of the pComb3X phagemid, 100 mL LB broth containing 50 µg/mL ampicillin was inoculated with 4 mL overnight culture of *E. coli* XL1-Blue carrying pComb3X and grown to OD₆₀₀ of 0.4 at 37 °C with shaking at 200 rpm. Chloramphenicol, to a final concentration of 170 µg/mL, was added and the culture was further incubated overnight.

To isolate the phagemid, the culture was centrifuged at 10,000 × g, 10 min, 4 °C and resuspended in 4 mL Tris-Sucrose buffer (50 mM Tris and 25% Sucrose). Five milligrams of lysozyme and 0.5 mL EDTA, pH 8 were added to the mixture. The mixture was vortex mixed and then incubated on ice for 15 min. Three milliliters of Triton lysis buffer (1% Triton X-100, 50 mM Tris [pH 8] and 50 mM EDTA [pH 8]) was added, followed by gentle mixing and 15 min incubation on ice. The mixture was centrifuged 35,000 × g for 45 min at 4 °C and the supernatant was extracted with phenol/chloroform once and chloroform twice. Phagemid DNA was ethanol precipitated and resuspended in 0.5 mL TE buffer and treated with 100 µg/mL RNase at 37 °C for 1 h. Following incubation, 100 µg/mL proteinase K was added and the sample was incubated for another hour. The phagemid DNA was then extracted once with phenol/chloroform and twice with chloroform, ethanol precipitated and redissolved in 0.5 mL TE.

2.3.6 Library Ligation and Transformation

The chloroform extracted overlap extension PCR product and purified phagemid were digested with SfiI (New England BioLabs) at 50 °C overnight. The scFv fragments (0.8 kb) and phagemid vector (3.4 kb) were eluted from agarose gels and purified by the Elutip-D column (as described in section 2.3.3). Three library ligations using 1.4 µg purified SfiI-cut pComb3X phagemid and 0.7 µg purified SfiI-cut scFv PCR product per ligation were performed. An illustration of the ligated scFv + pComb3X vector is shown in Figure 2.2. *E. coli* XL1-Blue were transformed via electroporation (actual volts 2.52 kV; capacitance 25.0 µF; time constant 6.0 ms) with the ligated product according to Barbas *et al.* (2001). Following electroporation, the cultures were titered to determine the library size. Two microliters of each culture was diluted in 200 µL of SB medium and 100 µL and 10 µL of this 1:100 dilution was inoculated on to LB + ampicillin (100 µg/mL) and tetracycline (10 µg/mL) plates. The plates were incubated overnight at 37 °C. The total number of transformants was calculated by counting the number of colonies, multiplying by the culture volume, and dividing by the plating volume.

Recombinant phages were prepared from the library with the helper phage VCSM13. The transformed *E. coli* were grown in 15 mL SB medium containing ampicillin (20 µg/mL) and tetracycline (10 µg/mL) for 1 h at 37 °C, shaking at 200 rpm. The concentration of ampicillin was increased to 50 µg/mL and the culture was incubated for an additional 1 h. The culture was infected with approximately 2×10^{13} plaque-forming units [PFU] VCSM13 helper phage. The culture volume was brought up to 200 mL with SB medium and incubated for another 2 h. Kanamycin (70 µg/mL) was added to the culture and incubated overnight. Kanamycin was used to select for *E. coli* infected with the VCSM13 helper phage, since only the helper phage contains a kanamycin resistance gene. The next day, the bacteria were removed by centrifugation (3000 × g, 15 min, 4 °C) and the phage containing supernatant was precipitated with polyethylene glycol-NaCl (4% [wt/vol])

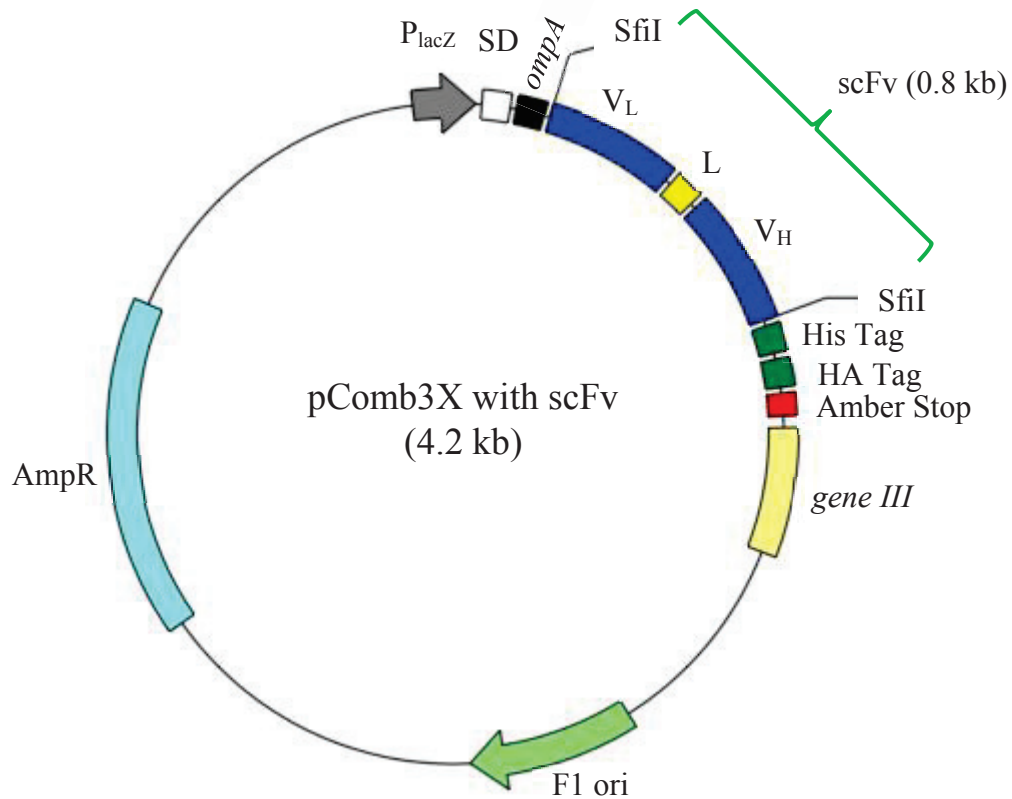


Figure 2.2. Phagemid map of scFv in pComb3X. The scFv was cloned into pComb3X via two non-identical SfiI sites. P_{lacZ}: inducible promoter; SD: Shine-Delgarno sequence; *ompA*: signal sequence; V_L and V_H: light and heavy variable chains; L: linker sequence; His and HA tag: hexa-histidine and decapeptide hemagglutinin tags; Amber stop: amber stop codon; *gene III*: phage minor coat protein; F1 ori: phage origin of replication; Amp^R: ampicillin resistance marker.

polyethylene glycol-8000 and 3% [wt/vol] NaCl) on ice for 30 min. The phage library was collected by centrifugation (15,000 × g, 15 min, 4 °C) and stored in 2 mL Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 8.0], 150 mM NaCl) containing 1% (wt/vol) bovine serum albumin (BSA) at 4 °C.

2.4 BIOPANNING

To enrich for phages that surface displayed anti-*F. nucleatum* scFv, the phage library was biopanned against *F. nucleatum* whole cells. A schematic of the biopanning process is shown in Figure 2.3. Two wells of a 96-well polystyrene microtiter plate (Corning CoStar 9017) were coated with 1×10^9 *F. nucleatum* cells in TBS containing 0.125% glutaraldehyde for 30 min at room temperature. The wells were blocked with 3% BSA in TBS for 1 h at 37 °C, and 100 µL of the phages were added to each well. The plate was incubated at 37 °C for 2 h and washed five times with TBS containing 0.5% Tween 20. The adherent phages were eluted with 100 µL of 0.1 M glycine-HCl (pH 2.2). The eluate was neutralized immediately with 3 µL of 2 M Tris base. The eluted phages were grown in *E. coli* XL1- Blue with VCSM13, and the phage preparation was obtained by polyethylene glycol precipitation (method as in section 2.3.6). The phages were panned a total of six times as described above. The final phage preparation was used for individual clone screening.

2.5 ANALYSIS OF PHAGE POOLS BY ELISA

The reactivity of the 6 enriched libraries to *F. nucleatum* whole cells was compared by enzyme-linked immunosorbent assay (ELISA). A 96-well polystyrene plate was coated with 1×10^8 *F. nucleatum* cells/well in duplicate for each library tested. The plates were then blocked for 1 h at 37 °C with 5% skimmed milk in PBS. Following blocking, 1/6 dilution of the phage libraries in skimmed milk were added (17 µL phage + 83 µL 5% skimmed milk). Mouse polyclonal serum against *F. nucleatum* whole cells (1:7,000), which was used to make library and 100 µL of parent phage that do not display a scFv

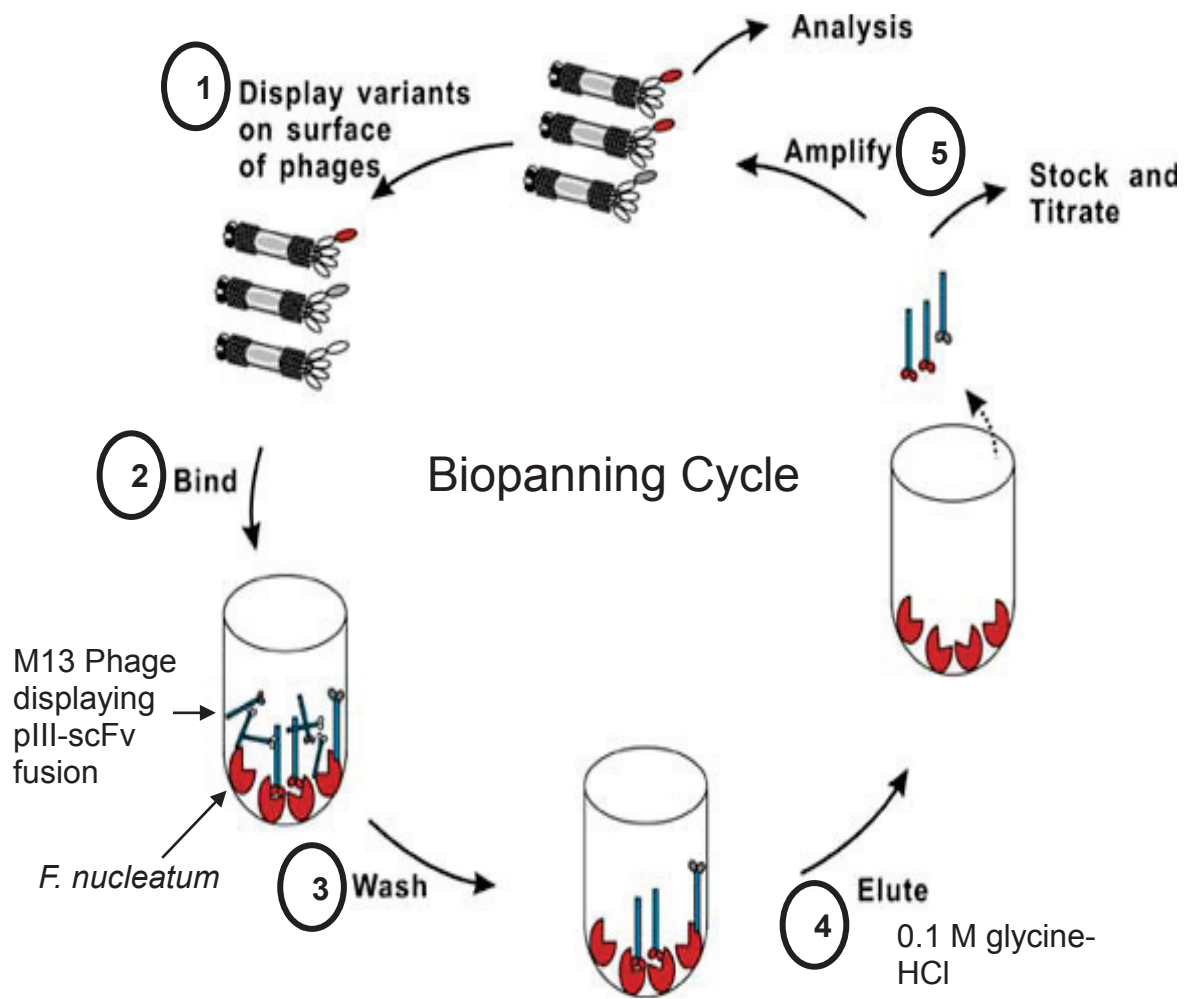


Figure 2.3. Biopanning: An enrichment process. Since the phage display library expresses all antibodies produced by the mouse and was created by random pairing of V_L and V_H chains, the library must be biopanned to enrich for clones that are reactive to *F. nucleatum*. The scFv phage display library (1) was added to fixed *F. nucleatum* whole cells in a well of a microtiter plate (2). The unbound phages were washed off with 0.5% Tween 20 in TBS (3) and the bound phages were eluted using the acidic solution 0.1 M glycine-HCl (pH 2.2) (4). The eluted phages were amplified by infecting *E. coli* and used again in another round of biopanning (5). The scFv phage display library was biopanned a total of 6 times. Adapted from Creative Biolabs (<http://www.creative-biolabs.com/phagedisplay1.htm>) and used with permission.

(ca. 2×10^{10} PFU) were included as controls. The plates were incubated overnight at 4 °C. Following incubation, the plates were washed, and bound phages were detected with anti-M13 monoclonal antibody (1:7,000; GE Healthcare Bio-Sciences) followed by the goat anti-mouse immunoglobulin G–alkaline phosphatase conjugate (1:8,000; Sigma-Aldrich).

2.6 INDIVIDUAL PHAGE CLONE PREPARATION

For the initial screening of 292 clones in ELISAs, phage particles were prepared as follows: SB medium (3 mL) was inoculated with a single colony and grown for 4 h at 37 °C with shaking at 200 rpm. Thirty microliters of VCSM13 helper phage (10^{12} PFU/mL) was added. The culture was incubated for another 2 h, at which time kanamycin was added (70 µg/mL final concentration) and left to shake overnight. Kanamycin was used to select for *E. coli* infected with the VCSM13 helper phage, since only the helper phage contains a kanamycin resistance gene. The following day, the culture was split in half (1.5 mL each half) and centrifuged at $2,800 \times g$ for 15 min at 4 °C. The supernatant (2×1.2 mL) was saved, and 300 µL of 5X polyethylene glycol-NaCl (20% polyethylene glycol-8000 and 15% NaCl [wt/vol]) was added. The mixture was incubated on ice for 1 h to precipitate the phage particles. To collect the precipitate, the solution was centrifuged at $10,000 \times g$ for 5 min at 4 °C. The two phage pellets were resuspended in a total of 100 µL of 1% (wt/vol) BSA in TBS and used without determining the titer. For large-scale phage preparations to be used as the primary antibody in western immunoblots, 100 mL of culture was used, and the phages were resuspended in 2 mL of 1% BSA in TBS. Phage titer was determined by plating serially diluted phage and *E. coli* XL1-Blue cells onto agar plates (Barbas *et al.*, 2001).

2.7 ELISA OF INDIVIDUAL PHAGE CLONES

For the initial screening of 292 phage clones, a single well was used for each clone. The 96-well polystyrene plates were coated with 1×10^9 *F. nucleatum* cells/well. The plates

were then blocked for 2 h with 5% skimmed milk in PBS. Following blocking, 70 μL of phages and 30 μL of PBS were added. In the ELISA, a 1:7,000 dilution of mouse polyclonal serum against *F. nucleatum* whole cells, which was used to make the library, was included as a positive control and 100 μL of parent phage that does not display scFv (ca. 2×10^{10} PFU) was included as a negative control. The plates were incubated overnight at 4 °C. Following incubation, the plates were washed, and bound phages were detected with anti-M13 monoclonal antibody (1:7,000; GE Healthcare Bio-Sciences) followed by the goat anti-mouse immunoglobulin G–alkaline phosphatase conjugate (1:8,000; Sigma-Aldrich).

2.8 VISUAL COAGGREGATION INHIBITION ASSAYS

To further screen the 292 individual phage clones, coaggregation inhibition assays were performed. The individual phage clones were screened for their ability to inhibit coaggregation between *F. nucleatum* and the partner bacterium *S. sanguinis* V920 using a procedure modified from Cisar *et al.* (1979). Assays were conducted in 1 cm diameter test tubes. *F. nucleatum* (1×10^8 CFU) was allowed to incubate with 55 μL untitrated phage at room temperature for 1 h with gentle rocking, before addition of 2×10^8 CFU *S. sanguinis* and coaggregation buffer (1 mM Tris [pH 8], 0.1 mM CaCl_2 , 0.1 mM MgCl_2 , 0.02% NaN_3 , and 0.15 M NaCl) to 500 μL . The cell and phage suspension was vortex mixed for 5 s, allowed to stand at room temperature for 10 min and scored for coaggregation inhibition. The following controls were included: *F. nucleatum* and *S. sanguinis* with TBS added instead of phage; addition of control parent phage (5×10^{11} PFU) that does not display a scFv; and addition of the mouse anti-*F. nucleatum* serum (1:250 dilution).

In the initial screen, coaggregation inhibition was scored as either presence or absence of inhibition. Presence or absence of inhibition was determined visually by comparison to the no inhibition of coaggregation controls (*F. nucleatum* plus *S. sanguinis* control and the addition of parent phage control) and the complete inhibition of coaggregation control (addition of mouse anti-*F. nucleatum* serum).

When analyzing the representative scFv phages, the degree of coaggregation was scored using a scale modified from Cisar *et al.* (1979). Scores ranged from 0 to 4+ and were assigned by the following criteria: 0, no visible coaggregation in the cell suspension; 1+, small coaggregates in suspension; 2+, definite coaggregates easily seen but suspension remained turbid and coaggregates settled gradually; 3+, large coaggregates which settled rapidly leaving some turbidity in the supernatant fluid; 4+, clear supernatant fluid and large coaggregates which settled immediately.

2.9 PREPARATION OF *F. NUCLEATUM* OUTER MEMBRANE

The procedure for obtaining the *F. nucleatum* outer membrane was adapted from Reddy *et al.* (2007). *F. nucleatum* was grown anaerobically in 1 L of Brain Heart Infusion broth to mid-exponential phase of growth ($OD_{600} = 0.6$). The cells were harvested by centrifugation ($10,000 \times g$, 15 min, 4 °C) and washed once in 100 mL of cold 30 mM Tris-HCl, pH 7.5. The pellet was resuspended in 10 mL of the same buffer and the cells were lysed using sonication with a Vibra-Cell sonicator (Sonics and Materials Inc., Danbury, CT). Twenty sonication events at amplitude of 60 were conducted by pulsing for 20 s at a time and allowing the suspension to cool on ice for 20 s between intervals. Intact cells were removed by centrifugation ($10,000 \times g$, 10 min, 4 °C). The crude envelope was acquired by ultracentrifugation ($150,000 \times g$, 1 h, 4 °C; Optima L-100 XP Beckman Coulter) and washed once with 10 mM HEPES buffer, pH 7.4 containing 10 mM $MgCl_2$. The cytoplasmic membrane was dissolved by incubation in 2% Triton X-100 in HEPES- $MgCl_2$ buffer (pH 7.4) for 1 h at room temperature. The outer membrane remained intact under this treatment and was collected by ultracentrifugation ($100,000 \times g$, 40 min, 4 °C) and washed once. The resulting outer membrane was resuspended in 30 mM Tris-HCl buffer, pH 7.5.

2.10 FRACTIONATION OF *F. NUCLEATUM* OUTER MEMBRANE

The outer membrane proteins were obtained by subjecting the outer membrane to a Triton X-114 extraction procedure modified from Sela *et al.* (1997). The outer membrane was resuspended in 4% (vol/vol) Triton X-114 in PBS and 1 mM phenylmethylsulfonyl fluoride, which is a protease inhibitor, and gently rotated for 2 h at 4 °C. To obtain the remaining outer membrane after Triton X-114 extraction, the sample was centrifuged at $20,000 \times g$ for 30 min at 4 °C. The supernatant was then warmed to 37 °C for 5 min to allow the detergent to undergo phase separation and centrifuged ($15,000 \times g$, 5 min, 25 °C). The top aqueous layer was saved. The bottom detergent layer was washed by mixing with 1 mL of ice-cold PBS, warmed to 37 °C for 5 min, and centrifuged ($15,000 \times g$, 5 min, 25 °C). The washing process was repeated two additional times. The four aqueous layers were pooled and the proteins were recovered by precipitation with 8 volumes of cold acetone and incubated overnight at 4 °C. The precipitated proteins were collected by centrifugation ($3,500 \times g$, 15 min, 4 °C), washed once with cold acetone, allowed to air-dry and then dissolved in 30 mM Tris-HCl buffer, pH 7.5, and designated as the aqueous fraction. Proteins in the final detergent layer were precipitated similarly, except that centrifugation was at $14,000 \times g$ for 5 min at 4 °C. The pellet was dissolved in 30 mM Tris-HCl buffer, pH 7.5 and designated as the Triton X-114 fraction.

2.11 SDS-PAGE

The protein profiles of the whole isolated outer membrane, the remaining outer membrane after Triton X-114 extraction, Triton X-114 fraction and aqueous fraction were examined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the buffer system of Laemmli (1970). The samples were prepared by adding 2 μL of the remaining outer membrane to 6 μL of distilled water and 2 μL of 5X sample buffer (50% [vol/vol] β -mercaptoethanol, 30% [vol/vol] 1 M Tris-HCl [pH 6.8], 10% [wt/vol] sodium dodecyl sulphate, 50% [vol/vol] glycerol, and 3 mM bromophenol blue). The whole outer membrane (5 μL), Triton X-114 fraction (5 μL), and aqueous fraction (5 μL) were added separately to 3 μL of distilled water and 2 μL of 5X sample

buffer. The samples were boiled for 5 min followed by centrifugation ($10,000 \times g$, 5 min, 4 °C). The supernatants containing the denatured proteins were loaded onto a 10% polyacrylamide gel. A broad range prestained protein marker (New England Biolabs Ltd; Pickering, ON) was used as the molecular weight ladder. SDS-PAGE was conducted at 200 V in a mini Trans-Blot cell (BioRad; Mississauga, ON) powered by a Power Pac 300 (BioRad). The gel was stained with Coomassie Brilliant Blue R-250 and subsequently destained with 5% (vol/vol) acetic acid.

2.12 PHAGE IMMUNOBLOTTING

Western immunoblotting using the scFv expressing phage as the primary antibody was conducted similarly to Ding *et al.* (2005). The *F. nucleatum* outer membrane protein sample was first separated by 10% SDS-PAGE. Then the gel was placed on a nitrocellulose membrane (BioRad) sandwiched between two pieces of filter paper using the method previously described by Towbin *et al.* (1979). The proteins were transferred at 200 mA for 60 min, with a minimum voltage of 60 V while submerged in transfer buffer (0.025 M Tris base, 20% methanol, 0.192 M glycine). Subsequently, the nitrocellulose membrane was blocked with 5% skimmed milk in PBST. After incubation for 1 h at room temperature with gentle rocking, the membrane was incubated overnight with either 55 μ L untitrated phage in skimmed milk for the initial screen of the 62 coaggregation inhibiting scFv phage or 1×10^{12} PFU scFv phage for later blots at 4 °C overnight. The membrane was washed 3 times with PBST and then incubated with the anti-M13 monoclonal antibody (1:7,000; GE Healthcare Bio-Sciences) for 1 h at room temperature in skimmed milk. Following 3 washes, the membrane was incubated with the goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate (1:8,000; Sigma-Aldrich) in skimmed milk. Following 1 h incubation at room temperature, the membrane was washed another 3 times with PBST. To develop the blot, the membrane was incubated in 10 mL of alkaline phosphatase buffer (0.01 mM $MgCl_2 \cdot 6H_2O$, 0.1 M NaCl, and 0.2 M Tris-HCl [pH 9.8]) with 33 μ L of 5-bromo-4-chloro-3-indoyle phosphate (BCIP; 50% BCIP [wt/vol] in distilled water) and 66 μ L of nitroblue tetrazolium (NBT;

50% NBT [wt/vol], 70% dimethyl-formamide). The blot was covered with aluminum foil, to protect it from light, and gently rocked until colour developed to desired intensity.

2.13 PCR AND RESTRICTION ANALYSIS OF CLONES

The scFv DNA from the 11 representative recombinant scFv phages were amplified by PCR using the primer pair SL283/SL284 and Taq DNA polymerase. Primer sequences are listed in Table 2.1. The PCR products were digested with BstOI (Promega, Madison, WI) for 3 h at 60 °C and analyzed on 2% agarose gels.

2.14 DNA SEQUENCING

The scFv genes (in the pComb3X phagemid) were sequenced from both ends using universal scFv forward and reverse primers SL283 and SL284, respectively (Barbas *et al.*, 2001; Table 2.1). Commercial Sanger Sequencing was conducted by McGill University and Génome Québec Innovation Centre (Montréal, Québec). The deduced amino acid sequences were aligned using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>).

2.15 QUANTITATIVE COAGGREGATION INHIBITION ASSAY

To identify a common inhibitory scFv among early colonizing oral bacteria, the 6 unique scFvs were tested for their ability to prevent coaggregation between *F. nucleatum* and the binding partners *S. sanguinis* V920, *S. gordonii* DL1, *S. sobrinus* B13, *S. mutans* UA159, *S. downei* ATCC 33748 and *A. naeslundii* WVU 627. Phage (1×10^{12} PFU [for *S. sanguinis* only] or 5×10^{12} PFU) were incubated with 1×10^8 CFU *F. nucleatum* at room temperature for 1 h with gentle rocking. Following incubation, 2×10^8 CFU partner bacteria and coaggregation buffer to 500 μ L was added. The cell and phage suspension was vortex mixed for 5 s, allowed to stand at room temperature for 10 min. From the uppermost liquid in the coaggregation test tube 150 μ L was carefully removed and the

OD₆₀₀ was measured. A no coaggregation unincubated control was included, where the *F. nucleatum* and binding partner were mixed and immediately 150 µL was removed without allowing time to coaggregate. The unincubated control displays the total cell density. The coaggregation control consisted of *F. nucleatum* and partner bacteria with TBS buffer added instead of phages. The phage control used the parent phage that does not display a scFv. The positive control for inhibition was mouse anti-*F. nucleatum* serum (1:250 dilution). The experiment was conducted in triplicate.

2.16 MASS SPECTROMETRY FROM NITROCELLULOSE MEMBRANE

To identify the protein being recognized by the scFv phage, the band from the nitrocellulose membrane detected by the scFv phage was analyzed by mass spectrometry using a method adapted from Luque-Garcia *et al.* (2008). The remaining *F. nucleatum* outer membrane after Triton X-114 extraction fraction was separated by SDS-PAGE on 7.5% polyacrylamide gel and transferred to nitrocellulose membrane. Phage immunoblotting was conducted using scFv phage 198 on one-half lane. The other half-lane was left undisturbed in transfer buffer. After completing western immunoblotting the high molecular weight band was lined up to the undeveloped half-lane and the undeveloped nitrocellulose membrane was excised at the location of the band.

2.16.1 Tandem Mass Spectrometry Analysis

Mass spectrometry analysis was conducted by Dr. Alejandro Cohen (Proteomic Core Facility, Atlantic Research Centre, Dalhousie University) using the procedure previously described by Lee *et al.* (2009). The nitrocellulose membrane was dissolved in 90 µl acetone/4 mm² nitrocellulose and then digested with trypsin. Following digestion, the peptides were resuspended in 30 µL of a 2% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid solution, analyzed by LC-MS/MS on a nano-LC-Packings HPLC system which is coupled online to AB-Sciex Q-TRAP 2000 tandem mass spectrometer. Chromatographic separation of the peptides was performed on a Phenomenex Onyx Monolythic C-18 capillary column using a linear water-acetonitrile gradient (0.1% [vol/vol] formic acid)

from 2 to 30% acetonitrile over a 40 min period. Mass spectrometry data were acquired using an Information Dependant Acquisition mode and analyzed using Analyst 1.5 software. Database identification was done using the National Center for Biotechnology Information nonredundant protein database with the Mascot algorithm through a MS/MS ion search.

CHAPTER 3 RESULTS

3.1 CONSTRUCTION OF ANTI-*F. NUCLEATUM* SCFV LIBRARY

A BALB/c mouse was immunized with 10^9 CFU of *F. nucleatum* subspecies *nucleatum* American Type Culture Collection (ATCC) 25586. Following 3 immunizations, serum was collected, the whole spleen was harvested, and total RNA was isolated. A total of 1397 μg of RNA was obtained. From the total RNA, complementary DNA (cDNA) was reverse transcribed and used as a template for polymerase chain reaction (PCR) to amplify the DNA encoding the variable light and heavy chains (V_L and V_H) of all antibodies produced by the immunized mouse.

3.1.1 Mouse Serum Reacts Strongly to *F. nucleatum*

The mouse polyclonal serum against *F. nucleatum*, which was used to make the scFv library, was tested by ELISA for its reactivity to *F. nucleatum* whole cells. When compared to the pre-immune serum, the anti-*F. nucleatum* serum showed a strong reaction to *F. nucleatum*, suggesting the mouse was successfully immunized against *F. nucleatum* (Figure 3.1).

3.1.2 Successful Amplification of V_L and V_H Chains

Initially, difficulties with amplifying the DNA encoding the V_L (V_κ and V_λ) and V_H chains were encountered. The method described by Barbas *et al.* (2001) using 20 μg RNA for cDNA synthesis and *Taq* DNA polymerase for amplification of the DNA encoding the V_L and V_H chains failed to produce the desired product. Ultimately, it was found that using 2 μg total RNA to reverse transcribe the cDNA and using Phusion DNA polymerase in hot start PCR produced the highest quantity of the 0.4 kb V_L and V_H chains DNA (Figure 3.2). Additionally, for the amplification of the DNA encoding the V_κ light chain and the V_H chain, the extreme thermostable single-strand DNA binding protein (ET SSB) was added to increase the amount of product. The DNA encoding the V_λ light chain did not require the ET SSB for amplification.

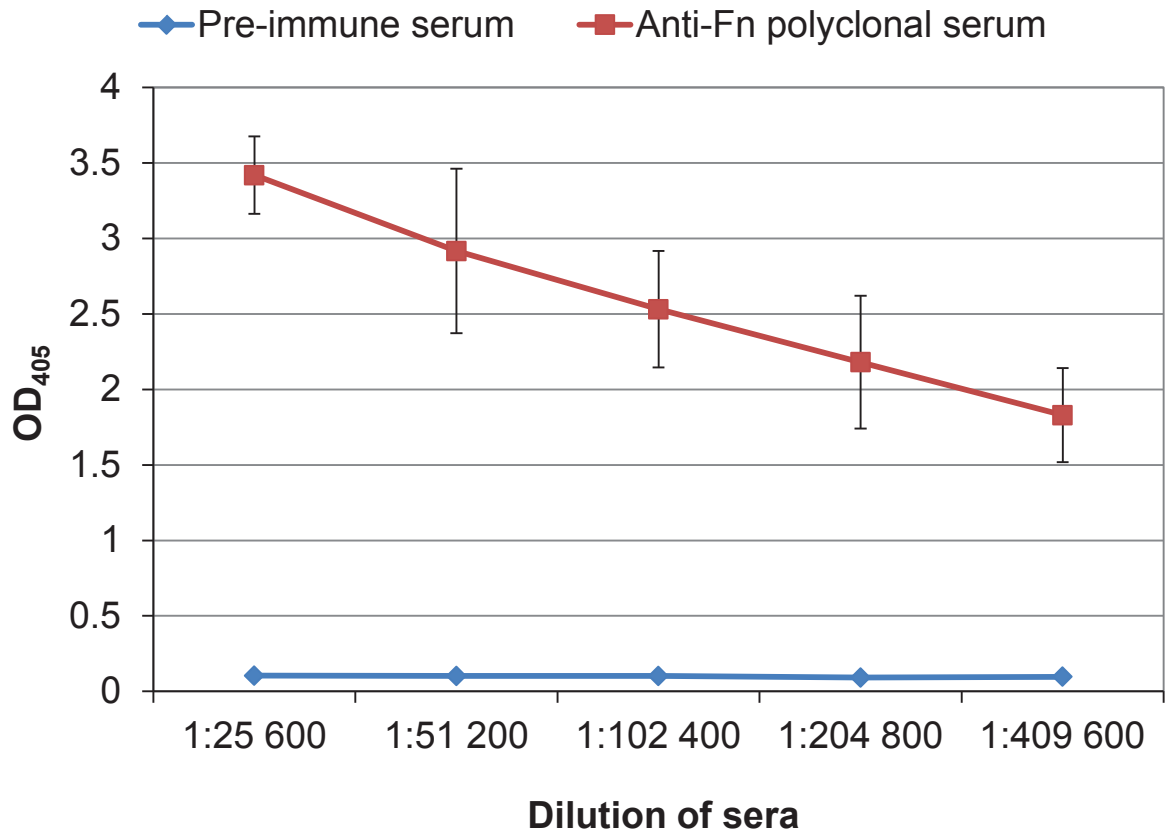


Figure 3.1. Anti-*F. nucleatum* serum shows strong reaction in ELISA. Compared to the pre-immune serum, the anti-*F. nucleatum* serum is highly reactive to *F. nucleatum*. Results are means (\pm SD) of triplicate measurements.

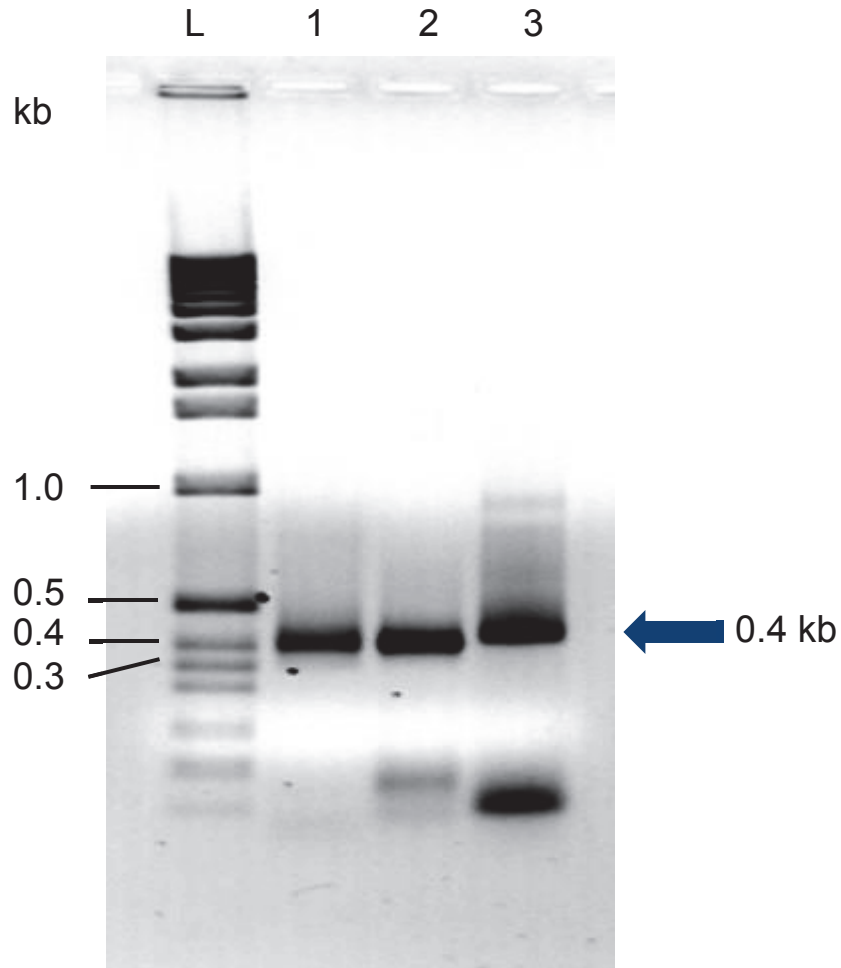


Figure 3.2. PCR-dependent amplification of the expected 0.4 kb variable light and heavy chain DNA. The expected 0.4 kb bands for the DNA encoding the variable light chains, V_{κ} (lane 1) and V_{λ} (lane 2), and the variable heavy chain (lane 3) were observed on a 1.5% agarose gel. L, 1-kb DNA ladder (Invitrogen). Hot start PCR with Phusion DNA polymerase was used. The extreme thermostable single-strand DNA binding protein was added to the PCR reactions for V_{κ} and V_H .

3.1.3 Production of the scFv

The DNA encoding the single-chain variable fragment antibodies (scFvs) were produced by overlap extension PCR that combines the V_L and V_H chains. As with the amplification of the V_L and V_H chains, *Taq* DNA polymerase failed to produce the desired scFv product. Optimization of the PCR reaction conditions resulted in using Phusion DNA polymerase and hot start PCR to amplify the 0.8 kb scFv (Figure 3.3). Some larger size non-specific product was formed and some 0.4 kb individual V_L and V_H chain DNA was also obtained.

3.1.4 Phage Display and Production of Large scFv Library

The 0.8 kb scFv DNA was gel purified and ligated into the *Sfi*I sites of the phagemid pComb3X. Electrocompetent *E. coli* XL1-Blue cells were transformed with the ligated DNA to produce the library. Titering of the transformed *E. coli* revealed that a library containing 4×10^8 clones was constructed. The library was expressed on the surface of recombinant phages using the helper phage VCSM13.

3.2 ENRICHED LIBRARY 6 IS HIGHLY REACTIVE TO *F. NUCLEATUM*

The scFv phage display library was enriched for clones reactive to *F. nucleatum* by biopanning against 1×10^9 CFU *F. nucleatum* whole cells. After 4 rounds of biopanning, the library did not show a great enrichment for anti-*F. nucleatum* clones as was expected from previous experience with the anti-*Bordetella pertussis* scFv phage library (Hussein *et al.*, 2007). However, after 2 additional biopanings the library became highly enriched for anti-*F. nucleatum* clones. The ability of the scFv phage pools to bind to *F. nucleatum* (10^8 cells/well) was measured by enzyme-linked immunosorbent assay (ELISA). Bound phages were detected by the anti-M13 monoclonal antibody (1:7,000, GE Healthcare Biosciences) followed by the goat anti-mouse IgG alkaline phosphatase conjugates (1:8,000, Sigma-Aldrich). Enriched library 6 showed strong reactivity to *F. nucleatum* compared to the control parent phage, which does not display a scFv, and the

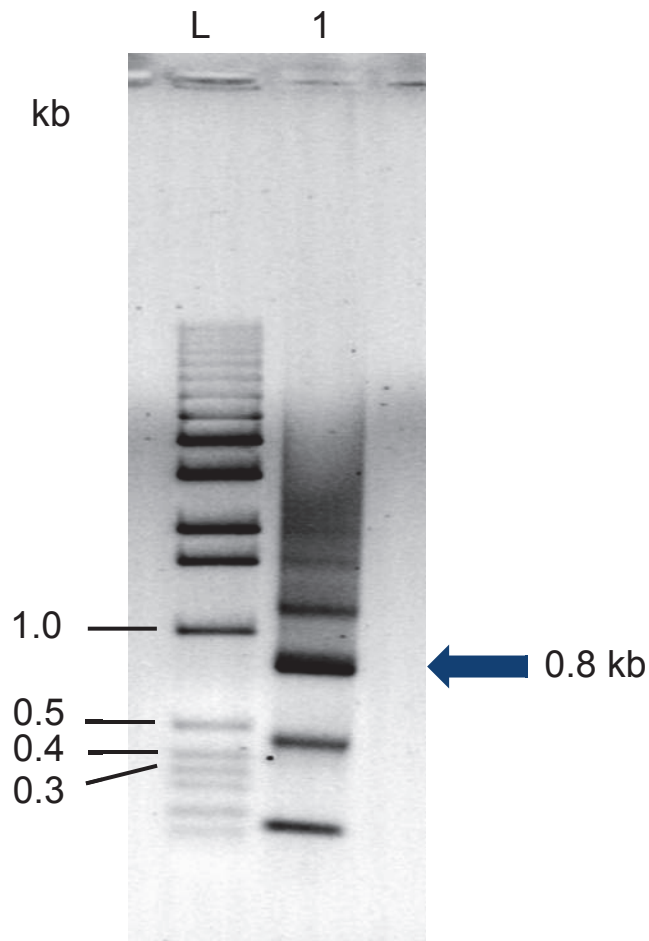


Figure 3.3. Overlap extension PCR produces the expected 0.8 kb scFv DNA. The expected 0.8 kb band for the DNA encoding the scFv produced by joining the DNA for the variable light and heavy chains was observed on a 1.5% agarose gel (lane 1). Some larger size non-specific product was formed and some 0.4 kb individual V_L and V_H chain DNA was also obtained. L, 1-kb DNA ladder (Invitrogen). Hot start PCR using Phusion DNA polymerase was performed.

other enriched and unenriched libraries (Figure 3.4). The anti-*F. nucleatum* mouse polyclonal serum was used as a positive control for *F. nucleatum* recognition and reacted strongly as expected.

3.3 SCREENING 292 INDIVIDUAL scFv PHAGES

From enriched library six, 292 individual scFv phages were grown and amplified separately. First, the scFv phages were screened by ELISA to determine their *F. nucleatum* binding ability. Second, after reamplification of the same 292 scFv phages they were tested for their ability to inhibit coaggregation between *F. nucleatum* and the early oral colonizer *Streptococcus sanguinis*.

3.3.1 Screening Individual scFv Phages by ELISA

A single well coated with 1×10^9 *F. nucleatum* cells/well was used for the initial screening of the untitered 292 scFv phages. Bound phages were detected with anti-M13 monoclonal antibody followed by the goat anti-mouse immunoglobulin G–alkaline phosphatase conjugate. The initial screen demonstrated that the majority of the 292 scFv phages reacted strongly to *F. nucleatum* in comparison to the control parent phage, which does not display a scFv (Table 3.1). The positive control, mouse polyclonal serum against *F. nucleatum* whole cells, reacted strongly to *F. nucleatum* in the ELISAs. This further confirmed that biopanning was successful in enriching the library for scFv phage clones that recognize *F. nucleatum*.

3.3.2 Screening Individual scFv Phages by Coaggregation Inhibition Assay

The 292 untitered scFv phages were tested for their function-blocking ability to prevent *F. nucleatum* from binding to the early oral colonizer *S. sanguinis*. In order to determine the *F. nucleatum* adhesin involved in *F. nucleatum* coaggregation with *S. sanguinis*, the

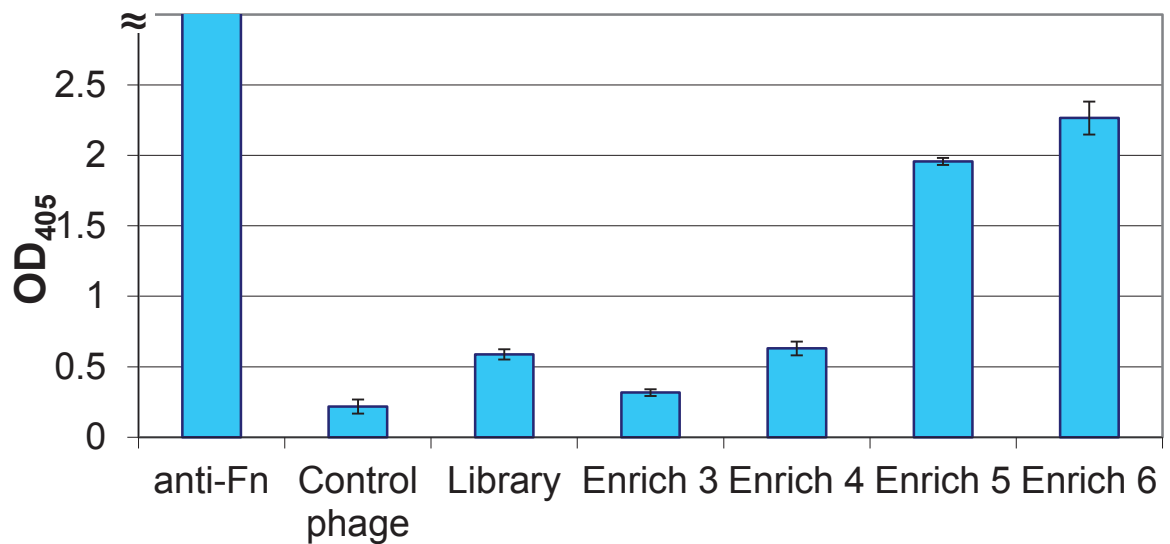


Figure 3.4. Enriched library 6 is highly reactive to *F. nucleatum*. ELISA results comparing the binding of the scFv phage display library before and after biopanning. Microtiter plates were coated with 1×10^8 *F. nucleatum* cells and reacted with aliquots of the phage library. Bound phages were detected by the anti-M13 monoclonal antibody (1:7,000, GE Healthcare Biosciences) followed by the goat anti-mouse IgG alkaline phosphatase conjugates (1:8,000, Sigma-Aldrich). anti-Fn, mouse antiserum against *F. nucleatum* whole cells. Control phage, phage that does not display a scFv. Results are means (\pm SD) of duplicate.

Table 3.1. Summary of ELISA and coaggregation inhibition data for individual clones. The 292 individual clones from the enriched library were screened by ELISA for their ability to recognize *F. nucleatum*. The 292 clones were also tested for their ability to inhibit coaggregation between *F. nucleatum* and *S. sanguinis*. The majority of the 292 clones reacted strongly to *F. nucleatum* and 62 clones inhibited coaggregation. Inhibited coaggregation (+). Did not inhibit coaggregation (-).

scFv	OD ₄₀₅	Inhibits Coaggregation	scFv	OD ₄₀₅	Inhibits Coaggregation
1	1.173	+	37	1.715	-
2	1.02	-	38	2.062	-
3	1.651	+	39	2.06	-
4	1.539	-	40	1.929	+
5	1.304	-	41	2.122	+
6	1.461	+	42	2.007	+
7	1.44	+	43	1.945	-
8	1.338	-	44	2.037	-
9	1.471	-	45	2.123	-
10	0.626	-	46	1.999	+
11	0.477	+	47	1.953	+
12	1.608	-	48	1.776	-
13	1.816	-	49	1.865	-
14	2.044	-	50	2.34	-
15	1.886	-	51	1.855	-
16	1.751	-	52	1.393	-
17	1.287	-	53	2.248	+
18	1.605	-	54	1.817	-
19	1.882	-	55	1.965	+
20	1.908	-	56	2.031	-
21	1.924	+	57	2.17	-
22	1.639	-	58	1.949	-
23	1.876	-	59	1.973	-
24	1.693	-	60	1.775	-
25	1.838	-	61	1.617	-
26	2.056	+	62	2.091	+
27	2.084	-	63	1.889	-
28	1.964	-	64	1.949	-
29	1.822	-	65	1.771	-
30	1.838	-	66	1.989	+
31	1.945	+	67	1.954	-
32	1.963	-	68	1.155	-
33	2.049	-	69	2.282	-
34	1.987	-	70	1.887	-
35	2.043	-	71	2.169	+
36	1.168	-	72	1.858	-

scFv	OD₄₀₅	Inhibits Coaggregation	scFv	OD₄₀₅	Inhibits Coaggregation
73	1.204	–	109	1.211	–
74	1.372	–	110	1.72	–
75	2.153	–	111	1.807	–
76	1.755	+	112	1.387	–
77	1.904	–	113	1.776	–
78	1.517	+	114	1.84	–
79	1.966	–	115	1.61	–
80	1.885	+	116	1.738	–
81	1.882	–	117	1.76	–
82	1.816	–	118	1.701	–
83	2.036	+	119	2.074	–
84	1.715	+	120	1.888	+
85	1.587	–	121	1.78	+
86	2.016	–	122	1.889	+
87	1.741	–	123	1.201	–
88	1.959	–	124	1.64	–
89	1.972	–	125	2.09	–
90	1.971	+	126	1.265	–
91	2.044	–	127	2.029	–
92	1.502	–	128	2.054	–
93	2.036	–	129	2.209	–
94	1.993	–	130	1.95	–
95	1.494	–	131	1.949	–
96	1.961	–	132	1.995	–
97	0.847	–	133	2.079	–
98	1.686	+	134	1.574	–
99	1.602	+	135	2.179	–
100	1.819	–	136	2.077	+
101	0.941	–	137	2.347	–
102	1.835	+	138	2.239	–
103	1.768	+	139	2.14	–
104	1.464	–	140	1.065	–
105	1.296	–	141	2.116	–
106	1.013	–	142	2.041	–
107	1.634	+	143	2.087	–
108	1.672	–	144	1.916	–

scFv	OD ₄₀₅	Inhibits Coaggregation	scFv	OD ₄₀₅	Inhibits Coaggregation
145	2.084	-	181	1.041	-
146	2.045	+	182	1.699	+
147	2.234	-	183	1.16	-
148	1.904	-	184	0.593	-
149	2.145	-	185	0.475	-
150	2.215	-	186	1.168	-
151	2.288	-	187	1.178	-
152	2.258	+	188	0.727	-
153	2.008	-	189	1.632	-
154	2.237	-	190	1.924	-
155	1.835	-	191	1.998	-
156	1.802	-	192	1.748	-
157	2.03	-	193	1.64	-
158	2.128	-	194	2.025	-
159	2.256	+	195	1.682	-
160	2.178	-	196	2.095	+
161	1.981	-	197	2.337	+
162	2.235	+	198	1.884	+
163	1.986	+	199	1.673	-
164	1.586	-	200	2.44	-
165	2.061	-	201	1.985	-
166	1.886	-	202	1.826	+
167	2.042	+	203	1.869	-
168	1.973	-	204	1.847	+
169	2.301	-	205	2.319	-
170	2.033	-	206	2.043	-
171	2.065	+	207	1.953	+
172	1.995	-	208	1.659	+
173	1.602	-	209	2.046	-
174	1.615	-	210	1.825	-
175	1.515	-	211	2.024	-
176	1.47	+	212	1.336	+
177	1.569	-	213	2.435	+
178	1.571	-	214	1.685	-
179	1.931	+	215	1.888	-
180	1.492	-	216	1.986	-

scFv	OD ₄₀₅	Inhibits Coaggregation	scFv	OD ₄₀₅	Inhibits Coaggregation
217	1.423	–	256	1.945	–
218	1.998	–	257	1.94	–
219	1.98	–	258	2.288	–
220	1.81	–	259	1.926	–
221	1.556	–	260	2.159	–
222	2.161	–	261	2.085	+
223	1.429	–	262	2.374	–
224	1.831	–	263	2.166	–
225	2.168	–	264	2.215	–
226	2.08	+	265	1.912	–
227	2.313	+	266	2.023	–
228	1.901	–	267	2.072	–
229	2.126	–	268	1.966	–
230	1.913	–	269	1.893	–
231	0.382	–	270	1.552	–
232	0.148	–	271	1.392	–
233	0.159	–	272	2.02	–
234	0.473	–	273	1.59	–
235	0.3	–	274	0.255	–
236	0.806	–	275	0.587	–
237	1.659	–	276	0.36	–
238	0.846	–	277	0.395	–
239	0.445	–	278	0.376	–
240	0.51	+	279	0.14	–
241	0.164	+	280	0.251	–
242	0.108	–	281	0.312	–
243	1.832	–	282	0.256	+
244	2.101	–	283	0.818	+
245	2.263	–	284	0.267	+
246	1.886	+	285	0.301	–
247	2.145	–	286	0.76	+
248	2.216	–	287	0.485	–
249	1.43	–	288	0.294	–
250	2.182	–	289	0.433	–
251	1.767	–	290	1.327	–
252	2.346	–	291	0.594	–
253	1.623	–	292	1.683	–
254	1.721	–	Anti-Fn serum	>3	+
255	1.886	–	Parent Phage	0.209	–

scFv library was screened for scFv phages that prevented coaggregation, presumably because the scFv recognizes the *F. nucleatum* adhesin and blocks *S. sanguinis* adherence.

The 292 scFv phages (55 μ L untitered) were individually allowed to incubate with 1×10^8 CFU *F. nucleatum* with gently rocking, to allow ample contact. The binding partner *S. sanguinis* (2×10^8 CFU) was added, the suspension was mixed using a vortex mixer for 5 s and then any coaggregates of bacteria were allowed to settle to the bottom of the tube for 10 min. Following the incubation, the turbidity of the reaction tube was compared to the controls and assessed visually for the presence or absence of inhibition of coaggregation (Figure 3.5). The buffer (no phage) negative control, showed no inhibition of coaggregation (Figure 3.5 B); there was a clear supernatant fluid, with all bacteria clumped together at the bottom. The addition of the parent phage that does not express a scFv also did not prevent coaggregation (Figure 3.5 C). The addition of mouse antiserum against *F. nucleatum* whole cells acted as a positive control and inhibited coaggregation strongly (Figure 3.5 D); there was a cloudy suspension, with no clumping at the bottom of the tube. In preliminary tests, autoaggregation was not observed with *F. nucleatum* or *S. sanguinis* during the 10 min incubation. From the 292 scFv phages tested, 62 scFvs produced reaction tubes that showed obvious inhibition of coaggregation (Table 3.1). scFv 1, a representative function-blocking scFv is shown in Figure 3.5 A. The results show that even though many scFv phages reacted strongly in the ELISA recognizing *F. nucleatum*, only 62 scFv phages inhibited *F. nucleatum* coaggregation with *S. sanguinis*. This suggests that many scFvs recognize *F. nucleatum* proteins that are not involved in coaggregation with *S. sanguinis*, but could be involved in coaggregation with other binding partners.

After identifying 62 function-blocking scFvs that likely bind to a *F. nucleatum* adhesin, we isolated the outer membrane of *F. nucleatum* in order to determine the binding specificity of the scFvs.

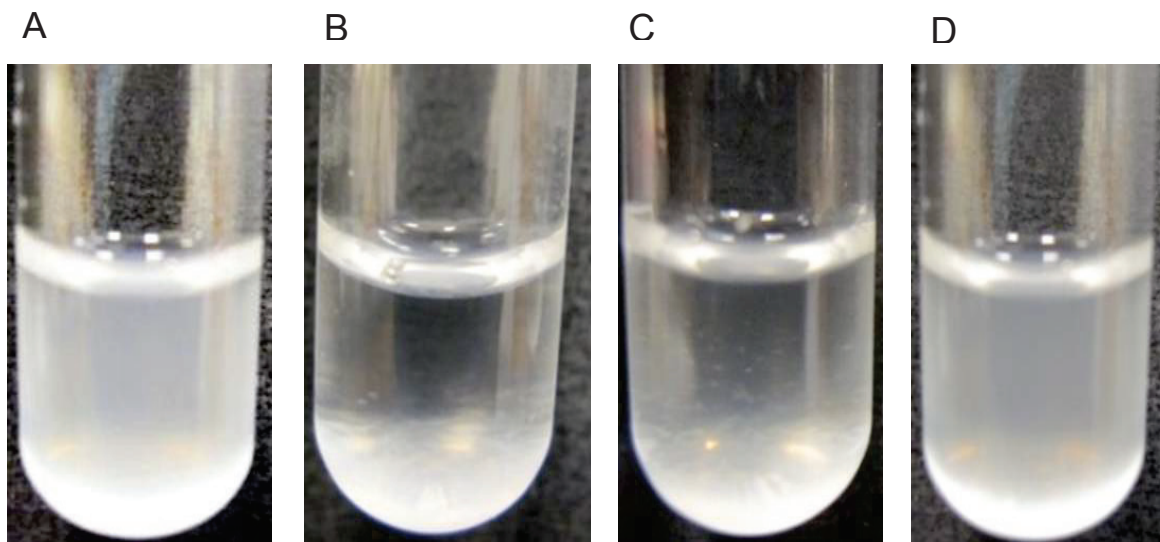


Figure 3.5. Representative coaggregation inhibition result by scFv 1 against *F. nucleatum*. The assays were conducted to screen 292 individual scFv phages for function-blocking scFvs. (A) Inhibition of coaggregation between 1×10^8 CFU *F. nucleatum* and 2×10^8 CFU *S. sanguinis* by the function-blocking scFv 1. (B) Coaggregation between *F. nucleatum* and *S. sanguinis* alone. (C) Coaggregation between *F. nucleatum* and *S. sanguinis* in the presence of 5×10^{11} PFU parent phage that do not display a scFv. (D) Coaggregation between *F. nucleatum* and *S. sanguinis* inhibited by 1:250 diluted mouse antiserum against *F. nucleatum* whole cells. Of the 292 individual scFv phages tested, 62 strongly inhibited coaggregation.

3.4 ISOLATION OF *F. NUCLEATUM* OUTER MEMBRANE

F. nucleatum was grown to mid-exponential phase. The bacteria were sonicated and centrifuged to obtain the broken cells in the supernatant. The supernatant was ultracentrifuged to pellet the crude envelope, which was resuspended in 2% (vol/vol) Triton X-100 containing Mg^{2+} to solubilize the cytoplasmic membrane. After ultracentrifugation, the outer membrane was acquired. The outer membrane was fractionated via Triton X-114 extraction into 3 fractions: Triton X-114 fraction, aqueous fraction, and remaining outer membrane after Triton X-114 extraction. The Triton X-114 fraction and aqueous fraction were precipitated with acetone and resuspended in buffer.

The three fractions generated, as well as the whole isolated outer membrane, were separated on a 10% polyacrylamide gel by SDS-PAGE (Figure 3.6). The whole outer membrane and remaining outer membrane showed similar band profiles (Figure 3.6, lanes 1 and 2). The Triton X-114 fraction had the least number of protein bands, but was still representative of the whole outer membrane sample (Figure 3.6, lane 3). The aqueous fraction had a number of distinct bands below 30 kDa (Figure 3.6, lane 4).

To confirm that the isolation of the *F. nucleatum* outer membrane procedure was successful, the intensely stained bands A and B from the Triton X-114 fraction (Figure 3.6, lane 3) were analyzed using tandem mass spectrometry (Elden Rowland, Proteomic Core Facility, Atlantic Research Centre, Dalhousie University). It was found that band A is the 302 kDa outer membrane autotransporter protein Fn1893 (Figure 3.7). The Mascot search result for Fn1893 had a score of 572 and sequence coverage of 11%. Band B was classified as the protein Fn1859 with a Mascot score of 154 and sequence coverage of 14% (Figure 3.8). This 40 kDa protein is the major outer membrane porin FomA (Jensen *et al.*, 2006) and likely is the same protein studied by Kaufman and DiRenzo (1989) involved in *Streptococcus sanguinis* CC5A binding.

The identification of *F. nucleatum* outer membrane proteins in the preparation suggests that the *F. nucleatum* outer membrane was successfully isolated. Since it is hypothesized that the outer membrane contains adhesins or proteins that mediate coaggregation, the

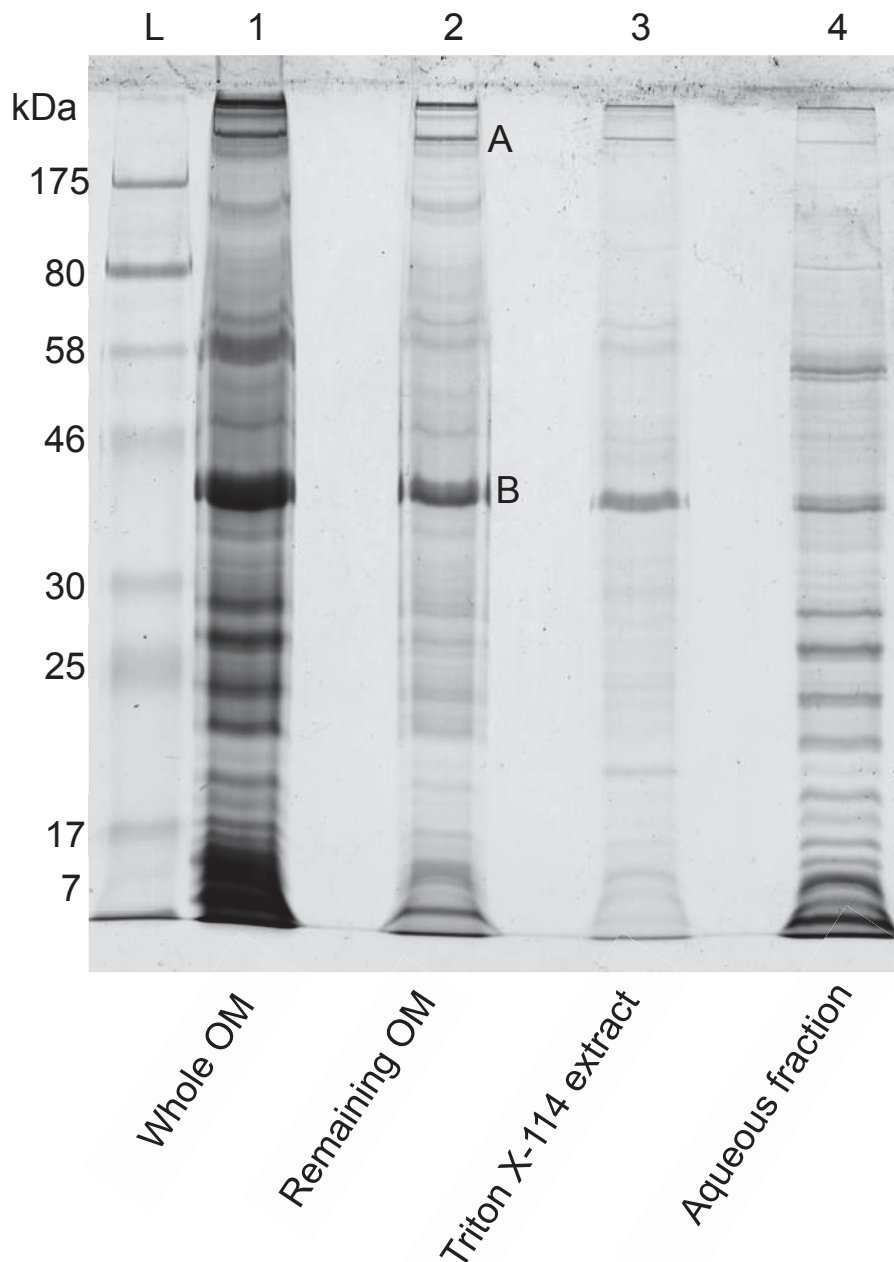


Figure 3.6. SDS-PAGE gel showing isolation of *F. nucleatum* outer membrane protein fractions. *F. nucleatum* whole outer membrane (OM), 5 μ l (lane 1). Remaining *F. nucleatum* outer membrane after Triton X-114 extraction, 2 μ l (lane 2). Triton X-114 fraction of outer membrane, 5 μ l (lane 3). Aqueous fraction of outer membrane, 5 μ l (lane 4). L, broad range prestained protein marker (New England BioLabs). The protein bands A and B were identified by tandem mass spectrometry as the outer membrane proteins Fn1893 and Fn1859, respectively. Fn1893 is a member of the autotransporter family of outer membrane proteins and Fn1859 is the major outer membrane porin FomA. The gel is stained with Coomassie Brilliant Blue R-250.

Fn1893

1 MDFTASNVTI NENISVTGSK AGKVIIAQAN YNGATNSSTI KVTNKGKIDY
51 SGNKSTALAT DFGQVTNETS GTIRMSGDNS IGLYGAANSI VTNKGTIEMG
101 **KAGVGIWGAN NLSNKYANRN INIVNSGTIR** GISGKEGVFG IYAKNSHAGA
151 TSNISHSGNI DLSQAKKSTG IFMTKGTLS SGNISVNEGS VGVNAEDSTV
201 NVNGGTHTIG ANSIGFNLKG NSSLLANSGN ISITGKGSVA YLFEGVNLTS
251 GTNFKDNLTL TATNGYTYIN LTNSTLNYKN QKTINNDETI FVNSKNSTVN
301 LLEGNDISST KNKVVG VYSE GGVS NAGKM TLMGDGSSAL YSKGAATVNG
351 APGKITIGAN GSGIYV NAG STGSNYGEIT IGAGSVGMRA ENGIKKNST
401 GKISSTA EKA TGMSQSGNEN LENEGTITLT GNQSVGMHSE GVTAAGHQMI
451 NKGIVTVGHS ATATSPSIGM YAANTDKTTI VNNGKVIAGN KSTGIYGGNI
501 TLNNSSETS GNGGIGVYSK GGTVDIKENA KISVGD TLDG KQEGVGVYLA
551 GNNQTLNSDT DNLTIGKGSF GYVMTGQGNT VRTGKAGTTR MINLTHSSIF
601 MYSADR**TGTA VNYNNLRSTG DLNYGIYASG RVDNYGTIDF** SQGIGNIGAY
651 SYTKGAT TTP NSIRNYGTIN VSKSDLQ TNP DDRKYGIGMA AGYSEESPAG
701 SGRKVTRGIG SIENHGLIRV TTPDSIGMYA TGKGSRIYNG PTGRIELSGR
751 KRNIGIFAEN GAEVVNEG TI TTVGSGNVGQ IGIGITSGAT LINRGNIHVN
801 AAR**GYGLFVA GGIVK**NYGNI TVAGGAQKTK EVSASDTSKA LGDEGLDRVG
851 IKSPAGASKG TITSNGKVKK PTIVQAIPNR KPSEIPKSSI GMYLDTSGIN
901 YTKPINNVGA LAGLKQGD LI VGTEAADYTN SKYIQLGQDI IKPYNEMIRK
951 SGIEKWSIYS ASLTWMA SIT QLPDY TIRNA YLVK**IPYTVF AGDKNTTRDT**
1001 **YNFTDGLEQR YGVEGLNSRE** KELFKLKI GNNERILLQQ AFDEMMGHQY
1051 ANVQQRIYAT GQILDKEFDY LRNEWKT VSK DSNKIKIFGI KGEYKTD TAG
1101 VIDYKNEAYG MAYVHENE DI KLGK**GIGWYT GIVDNTEFKFK** DIGKSKEEQI
1151 QAKVGLL KSI PFDDNNSL NW TISGDIFVGY NKMHRK**YLVV NEIFNAKSKY**
1201 **YTYGIGIKNK** ISKDFRLED FSLVPYGSLN LEYGRV NKIK EKVGEIRLEV
1251 KENYYVSNP EIGAELTYKH LLASRK TFRM **GLGIAYENEL GKVANGKNKA**
1301 **RVAYTNADWF NIRGEKEDRK** GNIK**FDLNIG LDNQRVGVTA** NAGYDTKGHN
1351 VRGGLGLRVI F

Figure 3.7. Mass spectrometry identified the 302 kDa protein as Fn1893. The matched peptides generated from trypsin digestion and identified by tandem mass spectrometry are in red boldface. The Mascot search result for Fn1893 has a score of 572 and the protein sequence coverage is 11%. Fn1893 is an outer membrane autotransporter protein.

Fn1859

1 MKKLALVLGS LLVVGSVASA KEVMPAPTPA **PKK**VVEYVEK** **PVIVYR****DREV
51 APAWRPNGSV DVQYRWYGNV ENRTPKKEDP ASPWLGDNVN AGRLQTLTKV
101 NFTEKQMLEI RTRNYHTLMN PKDSQAADDQ VRVRHFYKFG KLGSSKIDVT
151 SRLEYKKNNG DAGRK**QAEAS** **VLDFADYIY** **SNNFFK**ADKF GFRLGYQHKW
201 AGHNSGVVQ PFNKGTDNY FINFESEYTL PWGFS AELNA YNYYNVHNKK
251 FATYKGNKK **SQFYGEIEAY** **LYQHTPLYT** NNVELSDFE GGYDPYTWHQ
301 YKVVSADSN KYEVYMLPTL QVSYKPTDFV KLYAAAGAEY RNWAVTAESK
351 AKNWRWQPTA WAGMKVTF

Figure 3.8. Mass spectrometry identified the 40 kDa protein as Fn1859. The matched peptides generated from trypsin digestion and identified by tandem mass spectrometry are in red boldface. The Mascot search result for Fn1859 has a score of 154 and the protein sequence coverage is 14%. Fn1859 is the major outer membrane porin FomA.

next avenue of investigation was to probe the outer membrane proteins with the 62 coaggregation-blocking scFv phages to determine their binding specificity.

3.5 COAGGREGATION-BLOCKING scFVS CATEGORIZED BY REACTIVITY TO *F. NUCLEATUM* OUTER MEMBRANE PROTEINS

The whole *F. nucleatum* outer membrane protein sample was probed with the untitered 62 scFv phages in western immunoblots. Bound scFv phages were detected by the anti-M13 monoclonal antibody (1:7,000; GE Healthcare Biosciences) followed by the goat anti-mouse IgG alkaline phosphatase conjugates (1:8,000; Sigma-Aldrich). On the western blots, a high molecular weight (HMW) doublet band and a low molecular weight band (7 kDa) were observed. The relative intensity of the each of the bands was used to categorize the 62 scFv phages into 5 groups (Table 3.2 and Figure 3.9). From the table, 11 representative scFv phages were chosen for further study (boldface scFv numbers in Table 3.2).

3.6 CHARACTERIZING THE REPRESENTATIVE scFVS

The 11 representative scFv phages were titered and further analyzed in coaggregation inhibition assays between *F. nucleatum* and *S. sanguinis*, immunoblotting against the *F. nucleatum* outer membrane proteins, BstOI restriction digestion of the scFv DNA and by DNA sequencing the scFvs.

3.6.1 scFvs Have Different Coaggregation-Blocking Abilities

To develop a better understanding of the ability of each of the scFv phages to inhibit *F. nucleatum* coaggregation with the early oral colonizer *S. sanguinis*, standardized amounts of the scFv phages were used. Standard amounts of 1×10^{11} PFU, 5×10^{11} PFU and 1×10^{12} PFU scFv phages were allowed to incubate with 1×10^8 CFU *F. nucleatum* for 1 h

Table 3.2. Categorization of the 62 scFv phages that inhibit coaggregation between *F. nucleatum* and *S. sanguinus*. The scFvs were grouped based on reactivity to the *F. nucleatum* outer membrane by western immunoblotting. The numbers displayed are the scFv number given to the individual clones. HMW, high molecular weight protein band. Boldface, representative scFvs selected for further studies. For reference, an example immunoblot is shown for scFv phage 42 in Figure 3.9.

7 kDa only		Faint HMW, strong 7 kDa		Strong HMW, faint 7 kDa	Equal HMW, 7 kDa		HMW only	No HMW or 7 kDa
7	163	90	213	26	6	80	1	21
11	179	162	226	31	40	103	3	98
71	182	167	227	47	41	107	66	122
76	207	171	240	83	42	120	78	212
84	208	196	241		46	121	136	
99	283	197	246		53	176	152	
102	284	202			55	261	198	
146	286	204			62	282		
159								

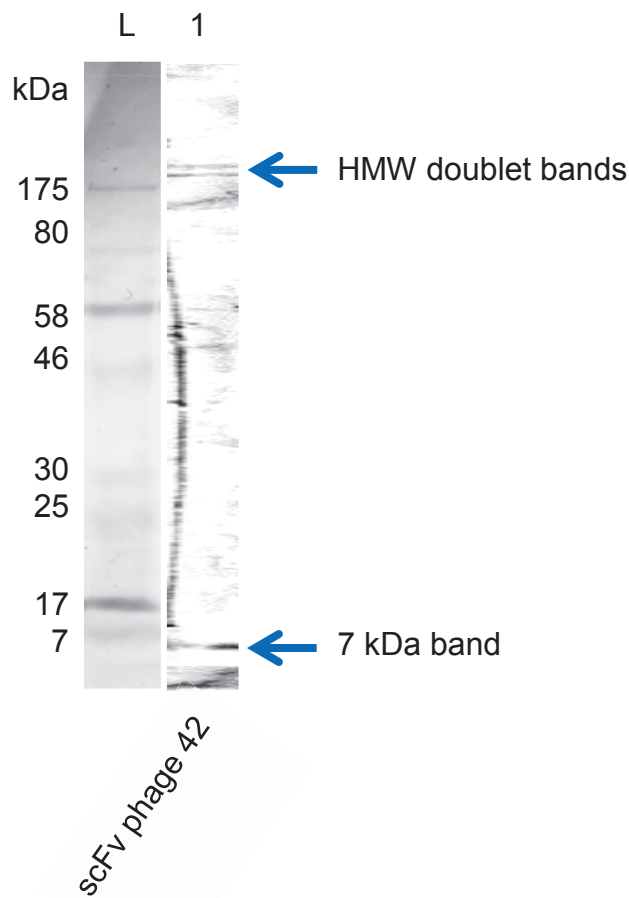


Figure 3.9. Representative immunoblot for scFv phage 42. In Table 3.2, the scFv phages were categorized based on their relative recognition of the HMW doublet and 7 kDa bands from the whole *F. nucleatum* outer membrane protein sample. As shown in this example blot, scFv phage 42 recognizes both the HMW doublet and 7 kDa bands equally. The HMW doublet bands were observed above the 175 kDa mark. The 7 kDa band migrated close to the 7 kDa mark. L, broad range prestained protein marker (New England BioLabs).

with gentle rocking. Following incubation the binding partner *S. sanguinis* (2×10^8 CFU) was added, the suspension was mixed using a vortex mixer for 5 s, and after waiting 10 min for any coaggregates of bacteria to settle, the coaggregation was scored. The controls included the addition of buffer instead of phage (negative control), addition of 5×10^{11} PFU parent phage (phage control), and the addition of 1:250 dilution of mouse antiserum against *F. nucleatum* whole cells (positive control).

The coaggregation was scored on a scale of 0 to 4+, where 0 means no coaggregation (cloudy suspension, with no clumping at bottom) and 4+ means maximum coaggregation (clear supernatant fluid, with all bacteria clumped together at bottom). See Figure 3.10 for visual representation of coaggregation scores.

The negative control showed excellent coaggregation and was given a score of 4+. The parent phage did not produce any noticeable inhibition and was also scored 4+. The antiserum against *F. nucleatum* (1:250 dilution) eliminated coaggregation and was scored 0. The scFv phages produced a dose-dependent effect (Table 3.3). scFv phages 1, 21, 83, 102, 167, 176, 182 and 226 eliminated coaggregation at 1×10^{12} PFU. Thus, the scFvs differ in their ability to inhibit coaggregation.

3.6.2 scFvs Recognize HMW Doublet Band to Different Intensities

The whole *F. nucleatum* outer membrane protein sample was probed with a standard amount of 1×10^{12} PFU of the 11 representative scFv phages and bound scFv phages were detected by the anti-M13 monoclonal antibody followed by the goat anti-mouse IgG alkaline phosphatase conjugates.

All 11 scFv phages recognized the high molecular weight (HMW) doublet band (Figure 3.11). scFv 26, 83, 102, 167, 176, 182, and 198 recognize the lower band in the doublet most strongly (Figure 3.11, band B). scFv 1, 21, and 42 recognize both bands in the doublet equally (Figure 3.11, bands A, B). While, scFv 226 recognizes the top band in the doublet most strongly (Figure 3.11, band A). scFv 21 and 226 also recognize mid-

Table 3.3. Coaggregation scores^a for assays with titered scFv phages. The ability of the 11 representative scFv phages to inhibit *F. nucleatum* coaggregation with the early oral colonizer *S. sanguinis* was examined. The amount of inhibition was compared to the buffer added negative control^b, phage control^c and anti-*F. nucleatum* serum positive control^d.

scFv Phage (PFU)	scFv Number										
	1	21	26	42	83	102	167	176	182	198	226
1×10^{11}	4+	4+	3+	3+	4+	4+	4+	4+	2+	4+	2+
5×10^{11}	1+	1+	2+	2+	1+	1+	1+	2+	0	2+	0
1×10^{12}	0	0	1+	1+	0	0	0	0	0	1+	0

^a Coaggregation scores: Ranked on a scale from 0 for no coaggregation to 4+ for maximum coaggregation. See Figure 3.10 for visual representation.

^b Negative control: addition of buffer instead of phage had no effect on coaggregation and was scored 4+.

^c Phage control: the addition of 5×10^{11} PFU parent phage, which does not express a scFv, had no effect on coaggregation and was scored 4+.

^d Positive control: the addition of 1:250 dilution of mouse antiserum against *F. nucleatum* whole cells eliminated coaggregation and was scored 0.

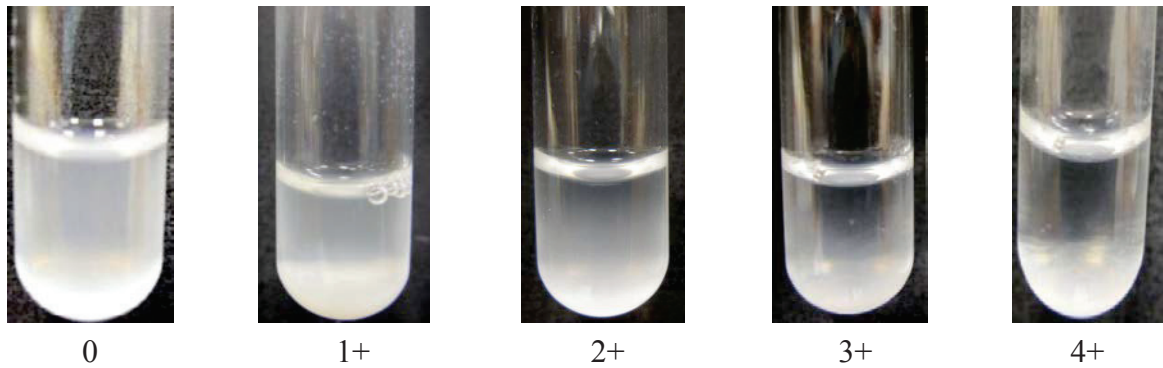


Figure 3.10. Visual representation of coaggregation scores. The extent of coaggregation was scored using a scale modified from Cisar *et al.* (1979). Scores ranged from 0 to 4+ and were assigned by the following criteria: 0, no visible coaggregation in the cell suspension; 1+, small coaggregates in suspension; 2+, definite coaggregates easily seen but suspension remained turbid and coaggregates settled gradually; 3+, large coaggregates which settled rapidly leaving some turbidity in the supernatant fluid; 4+, clear supernatant fluid and large coaggregates which settled immediately.

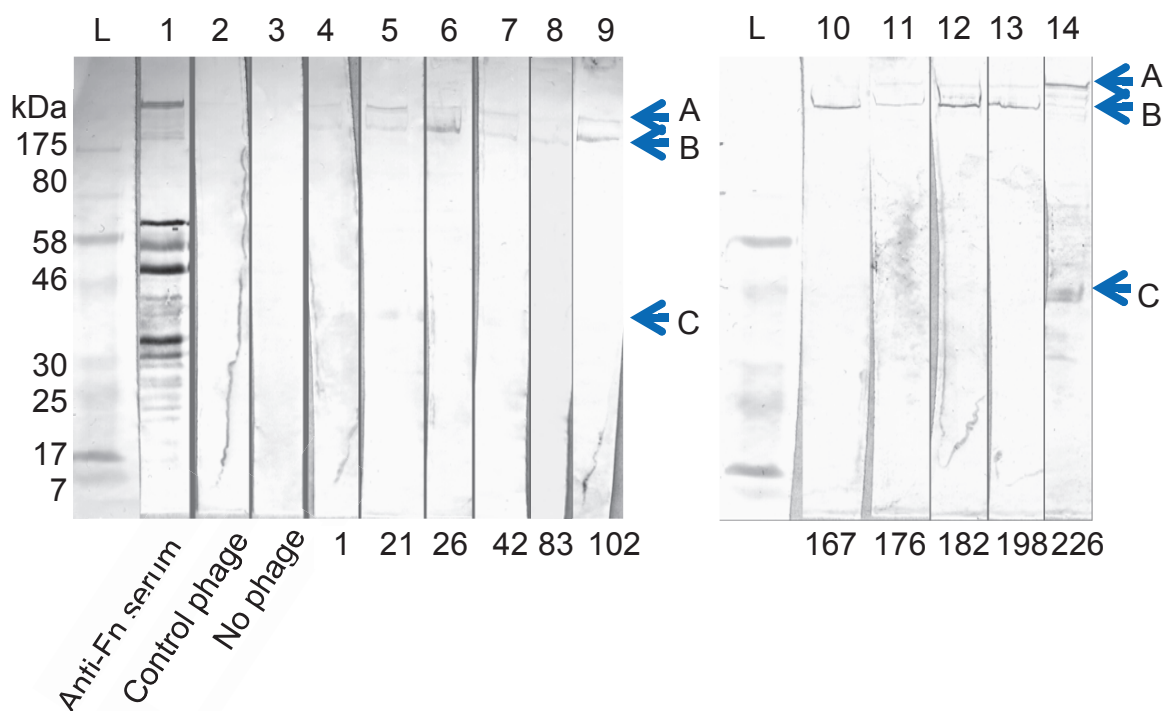


Figure 3.11. Western blot reveals representative scFv phages recognize HMW protein. *F. nucleatum* outer membrane proteins was probed with a standard amount of 1×10^{12} PFU scFv phages and reactive bands were detected by the anti-M13 monoclonal antibody followed by the goat anti-mouse IgG alkaline phosphatase conjugates. All scFvs displayed on phage recognized the HMW doublet (bands A, B). scFv phage 21 and 226 also recognize mid-weight bands around 40 kDa (band C). Anti-Fn serum, mouse antiserum against *F. nucleatum* whole cells (lane 1). Control phage, parent phage that does not display a scFv (lane 2). No phage, buffer added instead of phage (lane 3). The numbers shown at bottom are the scFv number given to the individual clones (lanes 4-14). L, broad range prestained protein marker (New England BioLabs).

molecular weight bands (Figure 3.11 band C). Taken together these results suggest that there are differences between the epitopes recognized by some of the scFvs.

3.6.3 BstOI Digestion Reveals 3 Distinct Restriction Fragment Patterns

The scFv DNA of the 11 representative scFv phages were amplified through PCR using the primer pair SL283/SL284 (Table 2.1). SL283 and SL284 are the universal forward and reverse primers for scFv amplification, respectively. To further characterize the scFvs and quickly identify scFvs with different DNA sequences, the scFv DNA was digested with BstOI restriction enzyme. This provides a quick method to analyze diversity among scFv phages since BstOI sites are known to occur within the complementary determining regions of antibodies (Barbas *et al.*, 2001).

BstOI restriction analysis revealed 3 different fragment patterns (Figure 3.12). scFv 1, 21 and 176 have the same pattern. scFv 26, 42, 83, 102, 167, 182, and 198 have their own pattern. scFv 226 has a distinct pattern. Thus, it can be inferred that there are a minimum of 3 different scFvs. These different patterns are the result of the scFvs having different DNA sequences and therefore different restriction patterns were observed.

To confirm these differences between scFvs, the scFv DNA was sequenced.

3.6.4 DNA Sequencing Reveals 6 Unique scFvs

DNA sequencing was performed on the scFv gene in the isolated pComb3X phagemids. The universal forward and reverse primers for scFv amplification, SL283 and SL284 respectively, were used to sequence the scFvs from both directions.

The deduced amino acid sequences derived from the DNA sequencing results were aligned and identical scFvs were identified. Three groups of identical scFvs were determined: scFv 1, 21 and 176; scFv 26 and 182; and scFv 83, 102 and 167. scFv 42,

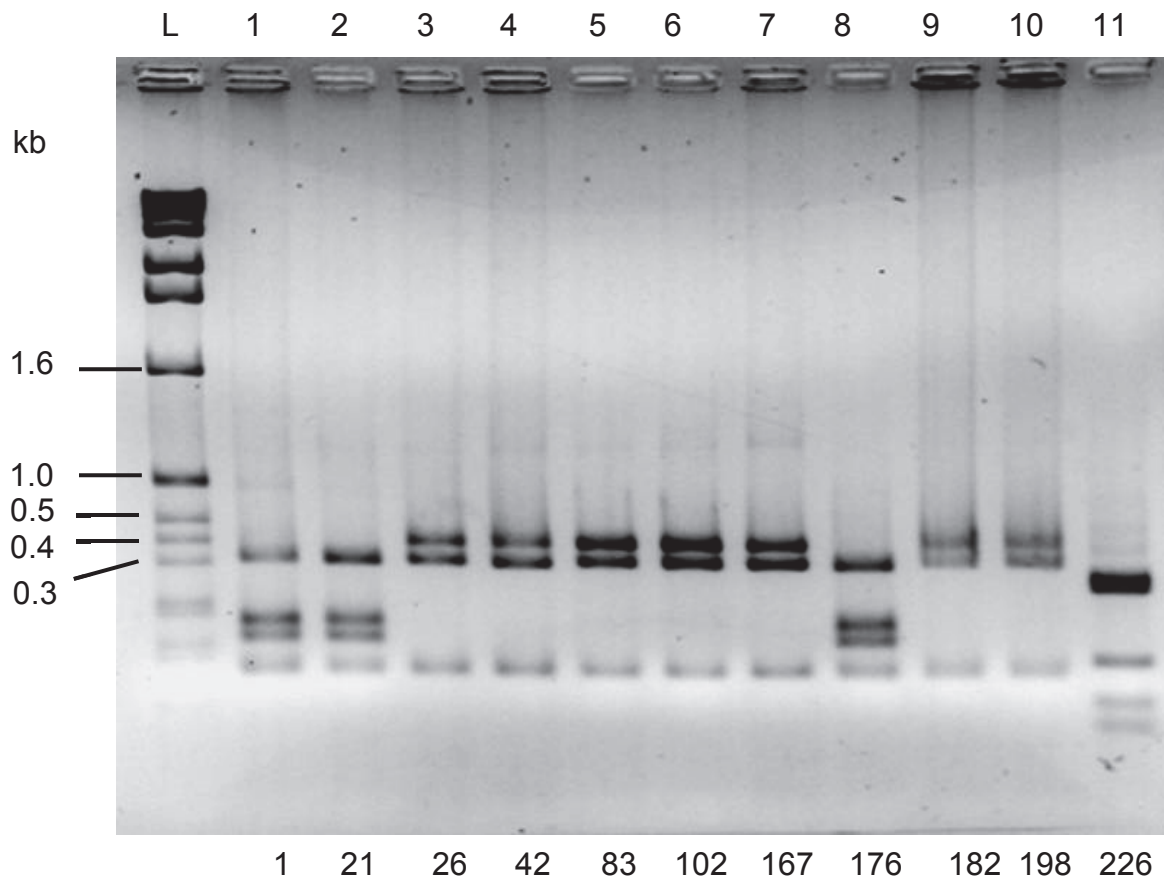


Figure 3.12. BstOI digestion reveals 3 distinct restriction fragment patterns.

Differences in digestion patterns of the 11 scFvs suggest there are at least 3 unique scFvs. scFv 1, 21 and 176 have a similar pattern. scFv 26, 42, 83, 102, 167, 182, and 198 have their own pattern. scFv 226 has a distinct pattern. The numbers shown at the bottom are the scFv number given to the individual clones. L, 1-kb DNA ladder (Invitrogen).

198, and 226 were each unique. The alignments of the deduced amino acid sequences for the 6 unique scFv sequences are shown in Figure 3.13. The first 2 lines of the sequence is the light chain followed immediately by the glycine/serine long linker sequence. At the other end of the long linker is the heavy chain. The framework (FR) regions were all nearly identical between different scFvs. Conserved cysteine residues, in both the light and heavy chains, were found. These residues are responsible for disulfide bond formation, which permits proper folding of the scFv. Conserved glycine markers were also observed in both chains. Variability was seen in the complementary determining regions (CDRs) between the different scFvs. The CDRs are responsible for the antibody-antigen complex formation.

3.7 IDENTIFYING COMMON INHIBITORY SCFVS AMONG EARLY ORAL COLONIZERS

To determine the utility of the 6 unique scFvs in inhibiting *F. nucleatum* coaggregation with various early oral colonizing bacteria, spectrophotometric quantitative coaggregation inhibition assays were performed. The binding partners *S. sanguinis* V920, *S. gordonii* DL1, *S. sobrinus* B13, *S. mutans* UA159, *S. downei* ATCC 33748 and *Actinomyces naeslundii* WVU 627 were tested.

F. nucleatum (1×10^8 CFU) was reacted with scFv phage (1×10^{12} [for *S. sanguinis* only] or 5×10^{12} PFU) for 1 h with gentle rocking. Following incubation, 2×10^8 CFU partner bacteria was added, the suspension was mixed using a vortex mixer for 5 s, and after waiting 10 min for any coaggregates of bacteria to settle, 150 μ L was carefully removed from the uppermost liquid in the tube and the OD₆₀₀ was measured. The unincubated control displays the total cell density. The coaggregation control consisted of *F. nucleatum* and partner bacteria with TBS buffer added instead of phage. In the phage control, parent phage that does not display a scFv was added. The positive control for inhibition included mouse anti-*F. nucleatum* serum (1:250 dilution). In this assay, inhibition is indicated by optical density (OD) values higher than that of the coaggregation controls shown by the dashed line (Figure 3.14). Any scFv phage that

```

←-----FR1-----→      CDR1      ←----FR2----
scFv 1  FLGGGPGAELDIVLTQSHKFMSTSVGDRVSITCKASQDVS---TAVAWYQQKPGQS PKLL
scFv 42  FEGGGPAAELDIQMIQSHKFMSTSVGDRVSITCKASQDVG---TAVAWYQQKPGQS PKLL
scFv 167 FEGGGPAAELDIVMTQSQKFMSTSVGDRVSITCKASQDVG---TAVAWYQQKPGQS PKLL
scFv 198 FWGGGPGAELDIVMTQSHKFMSTSVGDRVSITCKASQDVG---TAVAWYQQKPGQS PKLL
scFv 26  CLGGGAAAELDIVMTQSHKFMSTSVGDRVSITCKASQDVG---TAVAWYQQKPGQS PKLL
scFv 226 VWGGGPAAELDAVVTQES-ALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHFLTGL

→      CDR2 ←-----FR3-----→      CDR3 ←----FR4--
scFv 1  IYWASTRHTGVPDRFTGSGSGTDFTLTI SNVQSEDLADYFCQQYSSYP LTFG-AGTKLEI
scFv 42  IYWASTRHTGVPDRFTGSGSGTDFTLTI SNVQSEDLADYFCQQYSSYP LTFG-GGTKLEI
scFv 167 IYWASTRHTGVPDRFTGSGSGTDFTLTI SNVQSEDLAEYFCQQYSSYSLYTFGGGTKLEI
scFv 198 IYWASTRHTGVPDRFTGSGSGTDFTLTI SNVQSEDLAEYFCQQYSSYSLYTFGGGTKLEI
scFv 26  IYWASTRHTGVPDRFTGSGSGTDFTLTI SNVQSEDLAEYFCQQYSSYSLYTFGGGTKLEI
scFv 226 IGGTNNRAPGVPARFSGSLIGDKAALTI TGAQTEDEAIYFCALWYSNHWVFGGGTKLTVL

→      Linker      ←-----FR1-----→ CDR1←----
scFv 1  KSSGGGGSGGGGGGS SRS SLEVQLQQSGPELVKPGASVKI SCKASGYTFTDYNMHWVKQS
scFv 42  KSSGGGGSGGGGGGS SRS SLEVKLMEGPELVKPGASVKI SCKASGYTFTDYNMHWVKQS
scFv 167 KSSGGGGSGGGGGGS SRS SLEVQLQQSGPELVKPGASVKI SCKATGYTFTDYNIHWVKQS
scFv 198 KSSGGGGSGGGGGGS SRS SLEVQLQQSGPELVKPGASVKI SCKASGYTFTDYNMHWVKQS
scFv 26  KSSGGGGSGGGGGGS SRS SLEVKLVEGPELVKPGASVKI SCKASGYTFTDYNMHWVKQS
scFv 226 GS SGGGGSGGGGGGS SRS SLEVQLQQSGTVLAR PGASVKLSCKASGYSFTNYWMNWVKQR

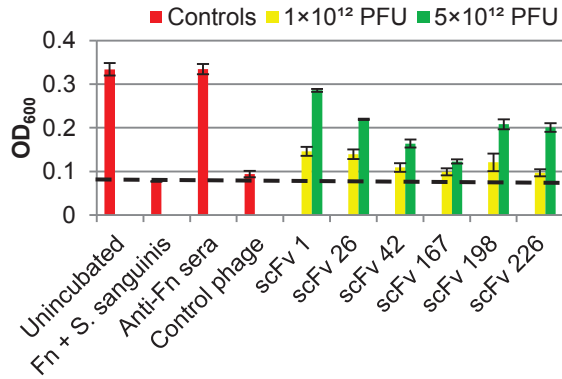
--FR2--→      CDR2      ←-----FR3-----→
scFv 1  HGKSLEWIGHIY PYNGGTDYNQKFKKATLTVDNS SRTAYMELRSLTSEDSAVYYCTR--
scFv 42  HGKSLEWIGHIY PYNGGTDYNQKFKSKATLTVDNS SSTAYMELRSLTSEDSAVYYCAR--
scFv 167 HGKSLEWIGYIY PYNGGTVYNQKFRSKATLTVDNS SSTAYMELRSLTSEDSAVYYCAR--
scFv 198 HGKSLEWIGYIY PYNGGTVYNQKFRSKATLTVDNS SSTAYMELRSLTSEDSAVYYCAR--
scFv 26  HGKGLEWIGYIY PHNGGTEYNQKFKSKATLTVDNS SNTAYMELRSLTSEDSAVYYCAR--
scFv 226 PGQGLEWIGMIHPFDSE SRLNQNFKDKATLTVDKS SSTAYMQLSSPTSEDSAVYYCARGG

      CDR3 ←----FR4----→
scFv 1  -ETMITPFAYWGQGLVTVSAAKTT PPSVT SGQAGKGSK
scFv 42  -ETMITPFAYWGQGLVTVSAAKTT PPSVT SGQAAPGSK
scFv 167 -ETMITPFAYWGQGLVTVSAAKTT APSVT SGQAAPGSK
scFv 198 -ETMITPFAYWGQGLSRLAQNAFPAPP P-----
scFv 26  -ETFYRPF SYWGQGLVTVSAAKTT APSVT SGQAPKGSK
scFv 226 YFGNYKDFAYWGQGLVTVSAAKTTAQTVTMAR PQGLKK

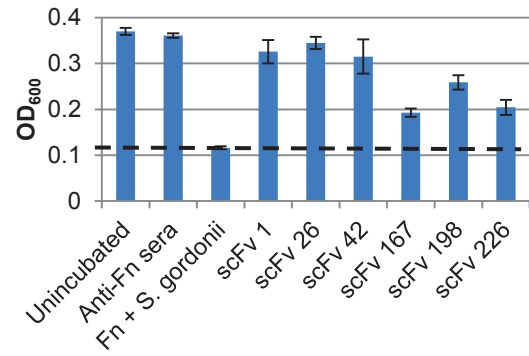
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Figure 3.13. DNA sequencing reveals 6 unique scFv sequences. The aligned deduced amino acid sequence of the 6 anti-*F. nucleatum* scFvs are shown. Sequences shown are V_L followed by V_H. FR, framework. CDR, complementary determining region. Underlined residues in the FR correspond to either conserved cysteine residues responsible for disulfide bond formation or conserved glycine markers found in immunoglobulin chains. Boldface indicates unique residues in the scFvs.

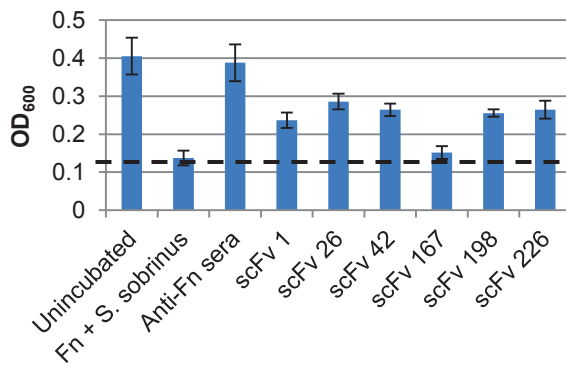
A. *S. sanguinis*



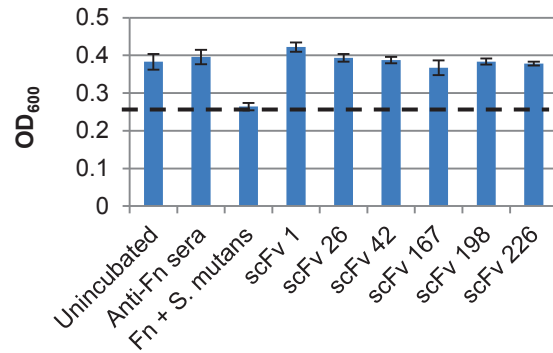
B. *S. gordonii*



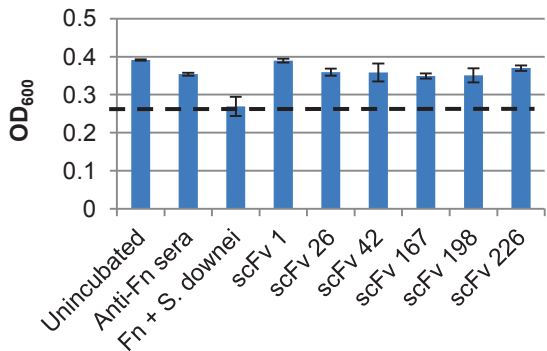
C. *S. sobrinus*



D. *S. mutans*



E. *S. downei*



F. *A. naeslundii*

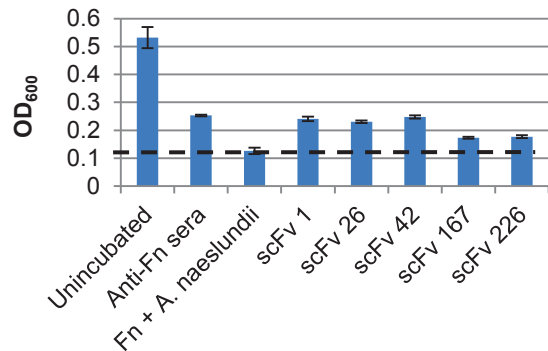


Figure 3.14. The unique scFVs inhibit multiple early oral colonizers. Inhibition of coaggregation between *F. nucleatum* (Fn) and various early oral colonizers was measured by the spectrophotometric coaggregation inhibition assay (mean of triplicate \pm SD). In this assay, inhibition is indicated by OD values higher than that of the coaggregation controls (e.g., *F. nucleatum* + *S. sanguinis*) shown by the dashed line. Unincubated, bacteria were mixed and OD read immediately to give the total cell density. Fn + partner bacteria, is the coaggregation controls, inhibition is shown above dashed line. Anti-Fn sera, mouse antiserum against *F. nucleatum* whole cells is the positive control for inhibition. Inhibition of coaggregation between 1×10^8 CFU *F. nucleatum* and 2×10^8 CFU partner bacteria was measured.

produced an OD value above the OD value of the coaggregation control is considered to have inhibited coaggregation.

Coaggregation with *S. sanguinis* was inhibited by all the 6 unique scFvs tested (Figure 3.14 A). Inhibition was dose-dependent. scFv phage at 5×10^{12} PFU produced more inhibition than at 1×10^{12} PFU for all scFv phages.

All scFv phages tested inhibited coaggregation with *S. gordonii* (Figure 3.14 B). scFv 1, 26, and 42 inhibited coaggregation the best.

S. sobrinus coaggregation with *F. nucleatum* was inhibited by 5 out of the 6 scFvs tested (Figure 3.14 C). scFv 1, 26, 42, 198 and 226 inhibited coaggregation, but not scFv 167.

F. nucleatum coaggregation with *S. mutants* and *S. downei* was inhibited by all 6 of the unique scFvs tested (Figure 3.14 D, E). However, compared to the other streptococci tested, *S. mutants* and *S. downei* did not coaggregate well with *F. nucleatum* as seen in the coaggregation controls.

Coaggregation with *A. naeslundii* was inhibited by all scFvs tested (Figure 3.14 F). scFv 1, 26, and 42 inhibited coaggregation the most.

Thus, all scFv inhibit coaggregation to different degrees among the early oral colonizers tested. Among the streptococci tested, scFv 1, 26 and 198 produced the greatest inhibition of coaggregation.

3.8 MASS SPECTROMETRY IDENTIFIES ADHESIN AS RADD

To identify the protein that scFv phages bind on *F. nucleatum*, the band from the nitrocellulose membrane detected by the scFv phages was analyzed by mass spectrometry. The remaining *F. nucleatum* outer membrane after Triton X-114 extraction fraction was separated by SDS-PAGE on a 7.5% polyacrylamide gel and transferred to

nitrocellulose membrane. Following transfer, the sample lane was cut in half. scFv phage 198 was used as the primary antibody for immunoblotting on one-half lane. The other half-lane was left undisturbed in transfer buffer. After completing immunoblotting the high molecular weight band was lined up to the undeveloped half-lane and the undeveloped nitrocellulose membrane was excised at the location of the band. The band that was used for mass spectrometry identification is band B in Figure 3.11. Band B was recognized by all scFv tested (Figure 3.11).

Mass spectrometry analysis produced six results for identification:

- Outer membrane protein of *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 23726
- Outer membrane protein of *Fusobacterium* sp. 4_1_13,
- Hypothetical protein Fn1526 of *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586 (the subspecies used in this study)
- Outer membrane protein of *Fusobacterium* sp. 7_1
- Hypothetical protein HMPREF9942_01844 of *Fusobacterium nucleatum* subsp. *animalis* OT 420, and
- Outer membrane protein of *Fusobacterium nucleatum* subsp. *vincentii* ATCC 49256

When the amino acid sequences of the results were aligned using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2>), it was found that all sequences had considerable homology. The other five protein sequences were the same as Fn1526 in the C-terminal region. However, they also have longer N-terminal regions, which extended beyond the start codon annotated for Fn1526. Upon investigation on the Oralgen Database (<http://www.oralgen.lanl.gov>) it was found that immediately upstream of Fn1526 is an intergenic region, IGR26. When IGR26 was aligned with the six identified protein sequences, IGR26 aligned with the N-terminal region missing from Fn1526. Thus, we hypothesize that Fn1526 was incorrectly annotated and is actually a larger protein that includes IGR26. Therefore, the adhesin being recognized by the scFvs is Fn1526 (Figure 3.15).

Fn1526

1 MKDYNKVESC LKSFLLKNNKR LSYSMALLIT FLINGGFSYA DEAVQVPLRT EIKTRIEKEQ ENISQMLKEA DESMKDIELK IKKLTQRGEF WVKPLEKSYQ
101 GFIFANWGNV SKNKNKTESN FNGPEYSASY GRNMGYQOFS NGKYVGEYGI VKNPLEFVDK IDFGANITPK AVIEKTIVEK TVIKKIDITAP SVTPPTVEVG
201 EITLTAPEEV AIGEMTPPNE PNPSTVTPST VPALOGITVA AVSEVNVTPS IPEVAAAPTI NARGVQPPAT PAGETPRLLT PPEAPEDIVV TPPDEVOANL
301 TGSANPTTH YYWWDGNGGA ISQITLKAGT FNIKPGNIIN VNGYDAQAYP GTNPOQTRPN GDHTVNOQTF HTLLNVVPYSE YGSGVVINND RONSTLINLE
401 TEGIVAGNLG NRETDGYITS TKKTTLKGYO IYSDIK**QDD TELLEFINKGT** VNLNAKNTTY LFTTSHDYGN LR**TNYIDNEG TISAK**GENSI IIKHSPDTSQ
501 AKAWIYSNNG TMKAEGKCSI VYGAAYRNLA HGRAAFINDG TIEVTGESAI GVILPKDSVN SKLANGHLVW LKKSILLKKG KSMGFVAQNT NISNDKNLVK
601 FNIDA EKAIG ILQDVEGAGT AKTAGKIAIE GNSSGSMGIY AKQGTLELIA PHANSGETES SIELKAGSNN IGIYAKGAST VNFNGITKIT GGEGQ**KIALA**
701 **TEGSTINLK** AVTAGDKATS NFVKDAVPLY STGSNSKINV ENPDSLKFYL SGNSTAAYAK DKGLINMNRN SLPTTEPTIYI KGTNNKGIGL FAKDGGVINA
801 KHNYIKVENG SVGLSSIGEN GSDKSNIDFS GSKLEYTGNG YAVYSDGTGT VNLSDAELNL YGSSTAFDVD FNATTLPTIL NANTRIHANS DDVIAFNLKN
901 ASGLTTVGGI ETSIKSKIET KLGSGSGLN NLFTGSTSTK YKVAVDGGE ITVGNLDKSG KKGDTDQDKI DGYQYFNREL AQRLKATADG STIKAVLTTE
1001 DATERFNGQV VGFEMNSSKN ANGVDDTAIN FKNGAKIAD RTDAGTGAIG AFINYGEVNI DATSKIEVEK ENNVVNQOAV GYAVVNGSKV DNKGTIDVGG
1101 DOSVGILGMA YRKGSSNNPI VNEFGGK**SGE GTVNITNEKD** IKMSGKDAIG IYAMNNKAGS SSTEHIKNT GNIIEIGDSVE KTAUVGIYAKG VDVKPESGKI
1201 KIGKAVGIY AEDSOVGEAN KDLGTVDVFDG DDGCVGIYLYG SGSNLLGNKV TLTSQSKDSKN KVGILADRGT SSIKTEVAV GAINNVIAYY SKGNHEFNVO
1301 SNVTLNENSI GISGEDDLLY GDGTNTYTMK LGKGSTGLFG TKKIGLKDKT NIELNGENSV GAYASGADGK ITSEKIKFL KEKSIGLYGA NGATVKDKTT
1401 MDFSDANAKN NMGAYLAGAK WERDSVLTF SAEHEKNIYL FAQGSGEGAT DKGSTVTLKN TFKVSPDGT TKNKTIGMY LDTAVKKGST YVDNTIDMSN
1501 NNAKVEVEKS GIGIYTKNTT GSGKNNIINT LKVSDDGQGT VGVFTDGNLK LSGNGGLIEA KNSGIGLYGN KGKISIENTH KVEVTSAGTG IYLTKGSHLD
1601 GSKLELENKT AGTSAAGIYY EGTNNEVDHN TDIEVTAGEN LLAIYANGLK LNNKEILIK KGKNNVAAYI TGNSTFKNKG KIQLGHPQTQ DFESGIGIYV
1701 VDGEAINESG KTIIDIYDFEN TASGGSLSVG MLAKAGAGKT AKVTNKGITIN VNGEVIGMVV EDTSEGLNDT GAIEIVAKDKE DINAKAIGAY VKGANAKFEN
1801 KGKISAENIA LALQGTGANK ILSGTLNLT KTGAAGVYAK DSVVDFNIAP TVAGADKTVV LYASGTTKIK SQITSASGKA HIGVYAEEDA EFLSGSKVTV
1901 GNGDNDYGI GYVTKSGYNK TVNTDIQLGG EKTIGFYLGA TGGTGSTVTH TGTIDVSGSI GTYIPEHSKF IAQNTTFNVG DNGTAVYKLG GEVDLGTGT
2001 ANINFNGTNG RAIYQDGGTI TTGTGLHIQG SGSFLTLKNA NSSINSLVEV GASGIGINGI YDMAGKDYTL TLESPNGHIK LGGNKATGIA AVAKSTVGNP
2101 KVVVINKGTI ETTSGEKTG IYKGANIEN ATGAKINIGA KGVGIYTTND NSLENTLLN AGEINLTGDE ATGLVAVKAK TSQDFIVGKI SGTDKDLVGA
2201 YFKDSQAVTK VKDFNISLGT NAKGLVFNGG KDFITSSST NKVTIGATTG NSRGIGIAAL GVNGNISKTD VVVGKSLGL YAKDKLTFD LASGKLESSD
2301 ANRSSILAYA DGNTSEVALN GGGTLK**VGAN GIALGTRKGGK** VSANATTVE VDGVKGLGAY VENGGSIDNN FDIKVKSAEG IGMYAKGGAL TSVAKVSEIK
2401 GNKSIGYVFE NITSAITMPN SVQLTDTNAT GQGVVAQGT GNGLTVAGVS VVSGSNTGVY SSTGKAVINN GLTLVGDSTG KSSIGIYSKG GAVTSTGSAT
2501 IGKNSIAIYG KDTAATLNGN LTIGEKIGL YVDNTATSKG DTAVNGNITV GANGAIGIQT TNSKVNLTGD LSVASGDSKG IFSMGAGNIE TTGNITVGSN
2601 SVGIYKNGSG EVKTAAGKTL TVADSAYGIF SKGAKLINNM NVTVGVDIAG AYVDGNDLTS TGTVTVDKG VGLLVKGTGK TLTSTGNITV GSNSVGLYA
2701 GDNANIAQSG NITVANNGI GYVSKGNV STIGAITVGK DSIYVYKDGK GTMNIASSP IQTMTIAEK YGLYKGNR ADSIINSNMN MTLGKEAVGI
2801 YAKNTTVNHV GDITVGETNI GSSGFTTSPD NKNSIGIFGD NSNINFKGNM LVDKPLSVGI YGSNGGSITV KSGSTITVKN GATGIMTGSK VESITLESKS
2901 TLNVGKVDI SVYTNATKSN VSFIAAAYSG AINNQGTINV KNGATGIYLA GTASLVNQTG ITVDAISKQI GRPDT**KASAE LGGIKV**TDKG EVTINNKVIN
3001 GGTVNIKGD LNMAGMGLDVS TGKTIVDARS ISGVAEVLN FSKGNSEQKV TIKDVFRGTG VGFSGDVKS KSVSWIAKIS KEPGSSTTK DITMVR**IPYN**
3101 **SLISGER**YKN LAKGLEDIRS KIGKSSSPI FKSLDNISSH RDFARAIANI **RGDIYSNVQE** RMKTVENAFD KSYNELLSSY NKTRNVNFKG IYYTGEHKD
3201 **STLGVSGYK**Y KSTGVLYLND REAFTYGGKY GWSAGIVGSN FEFNGDTNKG SKERVVSGKL GLHYQAPLNK EDDNAKLKWL TRGEVTVNNH RTNRYSQVGK
3301 DTYQNKASFY STELSWKNII SYDYDINTNW MVKPYTGIDM SYGHIFNIKE KNEGLPLEVK GKDYFVITPN VGVETKYVLP LGATHQVFAK ADTEFSYDVA
3401 KLYHGVNQAQ MKNASSGYD LSKPERRAR VAVGAELGLE KENAYGITFR AEYQYKKS**Q LNYGVRL**NYK F

Figure 3.15. Mass spectrometry identified the 360 kDa protein as RadD. The matched peptides generated from trypsin digestion and identified by tandem mass spectrometry are in red boldface. Intergenic region 26 (IGR26) is shown as underlined. The protein sequence coverage is 3%. RadD (Fn1526) is a *F. nucleatum* outer membrane autotransporter protein that is a known adhesin for *S. sanguinis* (Kaplan *et al.*, 2009).

Fn1526, when including IGR26, is a 360 kDa protein. It is a member of the autotransporter family of outer membrane proteins. Furthermore, Kaplan *et al.* (2009) identified Fn1526 as the adhesin RadD and described the involvement of RadD in *F. nucleatum* coaggregation with *Streptococcus sanguinis* ATCC 10556. Hence, we have identified RadD (Fn1526), a known *F. nucleatum* adhesin for *S. sanguinis*, as the protein being recognized by the scFvs.

CHAPTER 4 DISCUSSION

4.1 RATIONALE AND OBJECTIVES OF STUDY

As a group, *Fusobacterium nucleatum* has the ability to coaggregate with all oral bacteria examined (Kolenbrander *et al.*, 1989). Consequently, the mechanism of *F. nucleatum* coaggregation, which promotes the progression to pathogenic dental plaque, has been the focus of research for over twenty years. However, to date only five *F. nucleatum* adhesins involved in adherence to the acquired pellicle or in coaggregation are known.

This deficiency in knowledge about *F. nucleatum* adhesins is likely due to the lack of an effective tool to identify adhesins of *F. nucleatum*. We thought that an anti-*F. nucleatum* single-chain variable fragment (scFv) antibody library would provide a powerful tool for adhesin identification. Thus, the objective of this study is to construct, characterize and use an anti-*F. nucleatum* scFv library to identify *F. nucleatum* adhesins involved in coaggregation.

An anti-*F. nucleatum* ATCC 25586 scFv library consisting of 4×10^8 clones was constructed. A library size in the range of 10^7 to 10^8 is considered a comprehensive library that captures all possible antibody variants (Barbas *et al.*, 2001). Therefore, our library has great complexity, making it a valuable tool for studying *F. nucleatum*.

The library was enriched by biopanning against *F. nucleatum* whole cells 6 times and 292 individual scFv clones from the enriched library were tested for their ability to recognize *F. nucleatum* in ELISA. Most scFv phages were highly reactive to *F. nucleatum* in ELISA, which suggests that biopanning successfully enriched the library for clones that recognize *F. nucleatum*.

The scFv phages were further screened by coaggregation assay to determine which scFvs prevent *F. nucleatum* from binding to *S. sanguinis*, an early oral colonizer. This provided a direct method to identify adhesins involved in coaggregation. Of the 292 tested, 62 scFv

phages prevented coaggregation between *F. nucleatum* and *S. sanguinis*. Even though many of the 292 scFv phages reacted strongly in ELISA recognizing *F. nucleatum*, only 62 scFv phages inhibited *F. nucleatum* coaggregation with *S. sanguinis*. This suggests that several scFvs recognize *F. nucleatum* proteins that are not involved in coaggregation with *S. sanguinis*, but could be involved in coaggregation with other binding partners. Therefore, this library has the potential to be used to identify adhesins involved in other *F. nucleatum* coaggregation interactions.

Common function-blocking scFvs were identified by using the coaggregation-blocking scFvs in inhibition assays with the early colonizers *S. gordonii* DL1, *S. sobrinus* B13, *S. mutans* UA159, *S. downei* ATCC 33748 and *Actinomyces naeslundii* WVU 627. Since a number of unique scFv phages were able to inhibit coaggregation with multiple early oral colonizers individually, a common adhesin may exist among the early colonizers.

Mass spectrometry analysis of the *F. nucleatum* outer membrane protein band being recognized by the coaggregation-blocking scFvs revealed that the *F. nucleatum* adhesin involved in coaggregation is RadD. Previous identification by Kaplan *et al.* (2009) of RadD as a *F. nucleatum* adhesin necessary for interaction with *S. sanguinis* validates our approach, the construction of a scFv library against *F. nucleatum* and its use in adhesin identification. Screening of our scFv library in coaggregation assays between *F. nucleatum* and *S. sanguinis*, an already characterized interaction, resulted in the identification of the previously known adhesin RadD. This finding suggests that our library is capable of identifying adhesins and has the potential to be used to identify novel adhesins when applied to uncharacterized coaggregation interactions.

4.2 BIAS IN SCREENING LIBRARY BY COAGGREGATION INHIBITION ASSAY

The successful identification of a coaggregation adhesin using the scFv library demonstrates the utility of this library. However, screening the library by visual coaggregation assays could have biased the results toward identifying only the major adhesin involved in coaggregation. The 292 individual clones were screened by adding

untitered scFv phage to *F. nucleatum*, followed by the addition of *S. sanguinis* and incubation for 10 min to allow coaggregation. Following incubation, the turbidity of the reaction tube was compared to the controls (parent phage, no inhibition, and complete inhibition with anti-*F. nucleatum* serum) and assessed visually for the presence or absence of inhibition of coaggregation. When *F. nucleatum* and *S. sanguinis* are allowed to coaggregate without inhibition the two bacteria clump together and fall to the bottom of the tube during incubation, leaving a clear supernatant fluid on top. When inhibited, the two bacteria are prevented from clumping together and stay suspended in the fluid making the tube appears very turbid. In this way, visual screening favours the selection of scFv phages that only produce a dramatic inhibitory effect, in other words a very turbid tube. Of the 292 scFv phages tested, only 62 scFv phages produced a dramatically more cloudy tube than the no inhibition control. Therefore, it is reasonable to assume that only those scFv phages that recognized the major adhesin involved in coaggregation were identified by this method. If a scFv phage recognized a minor adhesin it would not have been selected in this screen because the effect on inhibition would not be noticeable, since the major coaggregation adhesin would still be available for interaction with *S. sanguinis*.

The visual coaggregation assay used was a quick and convenient method to screen hundreds of scFv phages for their ability to prevent *F. nucleatum* coaggregation with *S. sanguinis*. Untitered phages were used in this experiment because of the unreasonable amount of time it would have taken to titer all 292 phages and since the library, after enrichment six times, will have many identical scFvs. The same scFv was screened more than once in the assay as shown in the DNA sequencing results that identified many identical scFvs (Figure 3.13). A drawback of using untitered phage is that this increases the amount of false-positives and false-negatives for coaggregation inhibition. False-positives are those scFv phages that appear to inhibit coaggregation, but in reality do not. This may have occurred due to the titer of the phage being too high. Conversely, false-negatives are scFv phages that appear to have no effect on coaggregation, but actually do inhibit coaggregation. This occurs when the titer of the phage is too low.

The work of Kaufman and DiRienzo (1989) shows that *F. nucleatum* ATCC 10953 coaggregation with *S. sanguinis* CC5A is mediated by the 39.5 kDa protein FomA. However, the function-blocking scFvs identified by our screen do not recognize this protein. It is possible that this interaction is strain specific; we used *F. nucleatum* ATCC 25586 and *S. sanguinis* V920. Kolenbrander *et al.* (1989) showed that *F. nucleatum* can coaggregate with a wide number of partner bacteria; however, the interactions depended on the strains of *F. nucleatum* and partner bacteria. Alternatively, FomA may be a minor adhesin and could not be detected in this screen because of the limitations of our visual assay.

In order to overcome the limitations of this visual assay and attempt to identify minor adhesins involved in *F. nucleatum* coaggregation with *S. sanguinis*, we could repeat the screen using a strong function-blocking scFv identified in this study, such as scFv 1, and add in other scFvs from the library to determine if inhibition could be improved. To do this a quantitative spectrophotometric coaggregation assay should be used, since this assay provides a more reliable method by which to compare the turbidity of the reaction tubes, and look for increased inhibition. The scFvs that promote greater inhibition could be further characterized as in this study and used in immunoblotting to determine the *F. nucleatum* outer membrane protein they recognize.

4.3 REPRODUCIBILITY OF PHAGE IMMUNBLOTTING

The 62 function-blocking scFv phages that were identified in the coaggregation inhibition assay screen were characterized by what *F. nucleatum* outer membrane protein(s) they recognized in immunoblotting. The scFvs were categorized by their recognition of a high molecular weight (HMW) doublet band and a 7 kDa band (Table 3.2). Untitered scFv phages were used to simplify the procedure. From the 6 categories, 11 representative scFv phages were selected and the immunoblot was repeated with a known titered amount of scFv phages (Figure 3.11). However, this time the HMW doublet band was recognized by all scFvs tested and scFv 21 and 226 also recognized mid-weight bands.

The difference in the bands being recognized could be attributed to the amount of scFv phage used. Since the titered amount of 1×10^{12} PFU was used in the second blot, it is possible that there were not enough scFv phage to recognize the HMW doublet and mid-weight bands in the previous blot. As well, the freshness of the phage preparation has a great impact on the quality of the scFv displayed (Barbas *et al.*, 2001). When the phages are stored, the displayed scFv can become cleaved off the phage by proteases in the environment. Therefore, the scFv phages that were used in the characterization of the 62 scFvs may not have been optimal since they were stored for a longer time than the fresh phage used in the second blot. If the scFvs were missing, this would explain why the HMW doublet and mid-weight bands were not recognized in the initial characterization immunoblot.

The 7 kDa band observed in the initial screen is likely due to over-development of the immunoblot. Since the bands were faint and took a long time to become visible, the blot was allowed to develop for an extended period of time. This overdevelopment likely permitted time for the 7 kDa non-specific band to appear. Since the whole *F. nucleatum* outer membrane protein sample that was run on the immunoblot is very complex (Figure 3.6, lane 1) it is likely that cross-reactive antibodies could have caused the 7 kDa non-specific band to appear.

4.4 CHARACTERIZING THE DIVERSITY OF THE SCFV LIBRARY

Since the 11 representative scFvs were selected from an enriched library following 6 rounds of biopanning, it is likely that many of the scFvs are duplicates. To determine how many of the 11 representative scFvs are actually unique, the scFvs were analyzed by BstOI restriction digestion. BstOI restriction analysis showed 3 distinct restriction patterns. However, DNA sequencing revealed 6 unique scFvs demonstrating the limitation of BstOI restriction analysis. Furthermore, the 6 unique scFvs all appear to recognize the same protein, suggesting the scFvs must differ in the epitope they recognize.

4.4.1 Limitation of BstOI Restriction Analysis

As described by Barbas *et al.* (2001), BstOI restriction analysis offers rapid screening of a large scFv library. However, this method is limited by how spatially close the BstOI sites are on different scFv DNA. In many cases, the BstOI restriction sites are only a few bases up- or down-stream from a site on another scFv, therefore two unique scFvs can appear identical when screened by BstOI restriction analysis.

This limitation of BstOI restriction analysis offers an explanation for why BstOI analysis revealed only 3 distinct restriction patterns among the 11 representative scFvs (Figure 3.12), but DNA sequencing of the same 11 scFvs revealed 6 unique scFvs (Figure 3.13). Since the BstOI restriction sites were very close together on the scFv DNA, the differences in migration of the digested DNA bands were not obvious on the 2% agarose gel. DNA sequencing was able to pick up these minor differences in DNA sequence and provided a more definitive characterization.

4.4.2 Unique scFvs Recognize the Same Antigen Due to Differences in Epitope

The 6 unique scFvs recognize the same HMW protein band on the immunoblot against the *F. nucleatum* outer membrane proteins (Figure 3.11, band B). When band B was analyzed by mass spectrometry it was identified as the *F. nucleatum* outer membrane autotransporter protein RadD (Figure 3.15). Since RadD is a large 360 kDa protein it is reasonable to assume that the scFvs recognize different parts, or epitopes, of the antigen. Epitopes, also called antigenic determinants, are the part of an antigen that is recognized by antibodies. Epitopes can be conformational or linear (Janeway *et al.*, 2001). Conformational epitopes are composed of a discontinuous amino acid sequence from different parts of the polypeptide chain that have been brought together by protein folding in a three-dimensional structure. In contrast, a linear epitope is composed of a single continuous segment of polypeptide chain.

In this way, the unique scFvs likely recognize different epitopes of the antigen RadD. This may explain why more than one band on the immunoblot was recognized by some scFvs (Figure 3.11). It is possible that breakdown products of RadD were formed during the preparation of the outer membrane sample. On the immunoblot, the HMW doublet was recognized by all 6 unique scFvs and mid-weight bands by scFvs 21 and 226. Since the individual scFvs recognize different epitopes, they likely would bind different breakdown products with different affinities. Each breakdown product may differ in the epitopes they have and in the way they display them. Therefore, not all breakdown products will be recognized by the individual scFvs due to the binding specificity of the scFvs. For example, the amino acid sequence of scFv 226 is very different from all other scFvs sequenced (Figure 3.13). This corresponds to a difference in the intensity of the bands scFv 226 recognizes in the immunoblot (Figure 3.11). scFv 226 recognizes the higher HMW band more strongly than the lower band, unlike the other scFvs, and recognizes some mid-weight bands.

Furthermore, the coaggregation inhibition assays using the 6 unique scFvs to prevent *F. nucleatum* coaggregation with a number of early colonizing oral bacteria (Figure 3.14) suggests that the difference in epitope recognition makes a big difference in how well each scFv blocks coaggregation. Where the scFv binds the adhesin RadD seems to influence the scFv's ability to inhibit coaggregation. Among the streptococci tested, scFv 1, 26 and 198 produced the greatest amount of inhibition. It is possible that these scFvs bind RadD more closely to where the cognate receptor on the streptococci adheres, thereby promoting greater inhibition.

4.4.3 The Distinctive Character of scFv 226

Characterization of scFv 226 revealed that it has a distinctive DNA sequence and unique binding properties. The BstOI restriction pattern of scFv 226 did not match the pattern of any other scFv (Figure 3.12). This difference was confirmed with DNA sequencing, which showed that the complementary determining regions of scFv 226 have many unique residues when compared to the other 5 scFvs (Figure 3.13). The differences

observed in the DNA sequence of scFv 226 corresponded to differences seen in immunoblots with the *F. nucleatum* outer membrane protein sample. Band A in Figure 3.11 was recognized more strongly by scFv 226, than by any other scFv. Taken together, these results suggest that scFv 226 might be an entirely different class of scFv and may recognize a different *F. nucleatum* protein. Further investigation is warranted to discover what adhesin scFv 226 recognizes and what similarities exist between this adhesin and RadD.

4.5 COMMON ADHESIN FOR MANY EARLY COLONIZERS

The 6 unique scFvs were screened for their ability to inhibit *F. nucleatum* coaggregation with a number of early oral colonizing bacteria (Figure 3.14). It was found that all scFvs could inhibit coaggregation with the binding partners: *S. gordonii*, *S. sobrinus*, *S. mutans*, *S. downei* and *A. naeslundii*. The 6 unique scFvs all recognize a HMW protein that was identified as RadD on *F. nucleatum* (Figure 3.11, band B). This suggests that RadD may be a common adhesin for early colonizing bacteria.

This finding is consistent with those of Kalpan *et al.* (2009). Gene inactivation of *radD* in the transformable *F. nucleatum* strain ATCC 23726 resulted in the inability of mutant *F. nucleatum* to coaggregate with *S. sanguinis* ATCC 10556, *S. oralis* ATCC 10557, *S. gordonii* ATCC 10558, and *A. naeslundii* ATCC 12104. Thus, our work both supports and extends the findings of previous researchers showing that RadD is involved in coaggregation with many early colonizing bacteria.

4.6 USE OF SCFV PHAGES

In this study, the scFv was displayed on the surface of phage during use in coaggregation assays and immunoblotting. The scFv can also be expressed as a soluble protein independent of the phage (Barbas *et al.*, 2001). However, we encountered problems with inducing expression of the scFv in *E. coli* making this approach not feasible.

The use of scFv phage in immunoblotting is well documented. Ding *et al.* (2005) showed that the expression and purification of the soluble scFv is unnecessary and that the scFv expressing phage can be more conveniently used as the primary antibody. Our results agree with this assertion. Using as a control the parent phage, which does not express a scFv, we showed that the scFv phage binds specifically to the antigen recognized by the scFv (Figure 3.11).

In coaggregation assays, the use of the scFv phage creates the possibility of steric hindrance. The 27.5 kDa scFv is displayed on the surface of a phage, which is 6.5 nm in diameter and 930 nm in length (Barbas *et al.*, 2001). *Fusobacterium* cells are generally 5 to 10 μm long (Bolstad *et al.*, 1996) and *S. sanguinis* are 0.8 to 1.2 μm in diameter (Holt *et al.*, 1994). Since the size of the phage is similar to the smallest *S. sanguinis*, it is possible steric hindrance could have taken place. However, in coaggregation assays, a parent phage control, which does not display a scFv, was included. The parent phage did not cause any inhibition of coaggregation (Figure 3.14). As well, the 6 unique scFv phages all produced different degrees of coaggregation inhibition in the assay. These 6 individual scFvs recognize the RadD adhesin (Figure 3.11). This suggests that the variances observed in inhibition are due to the different epitopes that the scFvs recognize on RadD. To further verify the absence of steric hindrance, another control experiment could be to use a phage displaying a scFv that recognizes *F. nucleatum* but does not inhibit coaggregation. This control would show that even when the phage is bound to the surface of *F. nucleatum* it does not prevent the partner bacteria from binding.

4.7 ALTERNATIVE INTERPRETATION OF MASS SPECTROMETRY RESULT

Mass spectrometry was conducted on the band on the nitrocellulose membrane that was recognized by the scFvs. This procedure allowed for more accurate identification of the protein band, than would have the usual method of identifying the protein on a stained polyacrylamide gel. The developed half-lane of the blot and the undeveloped half-lane from which the band for mass spectrometry analysis was excised have the same protein

profile and migration, which should result in the identification of the correct protein band. All trypsin digested peptides generated matched to RadD.

However, there is still room for interpretation and uncertainty. On the immunoblot, the remaining *F. nucleatum* outer membrane after Triton X-114 extraction sample was run because the concentration of individual proteins is high. However, this sample also has a complex protein profile (Figure 3.6, lane 2). Therefore, the incorrect band could have been identified. It is possible that scFv 198 recognizes a band of lower concentration that migrates very close to RadD, but because RadD is in higher concentration only matched trypsin digested peptides generated from RadD were observed. In mass spectrometry, the more abundant species will suppress the signal from the less abundant ones when the mixture is ionized using electrospray (Hernandez *et al.*, 2006). Thus, if the true adhesin was in low concentration, it would not have been identified.

Hence, the mass spectrometry result should be verified by an independent method. Possible approaches to verifying the identity of the adhesin are explored in the following section.

4.8 FUTURE EXPERIMENTS: HOW TO VERIFY THE IDENTITY OF THE ADHESIN

There are a number of approaches that could be taken to verify that the adhesin identified is truly RadD. An obvious method is the production of a genetic mutant. Another is the creation of recombinant proteins.

4.8.1 Knock-out mutant of RadD

A logical future study would be to construct a RadD knock-out mutant of *F. nucleatum* ATCC 25586, the strain used in the current study, in order to verify the role of RadD as an adhesin. However, transformation with the strain *F. nucleatum* ATCC 25586 has not been described in the literature. The likely reason for this is due to severe restriction modification systems of fusobacteria, consisting of methylated DNA and up to three

different restriction enzymes with four-base recognition sites (Lui *et al.*, 1979). As well, difficulties in cloning fusobacterial DNA exist due to a low G+C content of only 30% (Kolenbrander, 2000). Nevertheless, successful transformations with the strains *F. nucleatum* ATCC 10953 and ATCC 23726 are described in the literature (Kinder Haake *et al.*, 2000; Kinder Haake *et al.*, 2006). Kaplan *et al.* (2009) described a successful gene inactivation of RadD in *F. nucleatum* ATCC 23726. It was found that the lack of RadD significantly decreased *F. nucleatum* coaggregation with the early colonizers *S. sanguinis* ATCC 10556, *S. oralis* ATCC 10557, *S. gordonii* ATCC 10558 and *Actinomyces naeslundii* ATCC 12104. As well, the knock-out mutant *F. nucleatum* did not form structured biofilms when grown in the presence of *S. sanguinis* ATCC 10556. These findings indicate that RadD is necessary for coaggregation between the strains used by Kaplan *et al.* (2009).

To confirm that the function-blocking scFvs identified in the present study recognize the same RadD protein that was identified by Kaplan *et al.* (2009), the immunoblotting experiments should be repeated using the outer membrane protein sample of the wild-type and mutant strains of *F. nucleatum* ATCC 23726. If the scFvs recognize the HMW protein RadD in the wild-type strain, but not in the mutant strain, then that would provide conclusive evidence that the function-blocking scFvs recognize RadD.

4.8.2 Recombinant Proteins of RadD

In this study, RadD was identified as a 360 kDa protein, slightly larger than the 350 kDa protein identified by Kaplan *et al.* (2009). This is because trypsin digested peptides in mass spectrometry analysis matched the sequence of the intergenic region IGR26 upstream of RadD (Gene ID: Fn1526), as well as RadD itself (Figure 3.15). Therefore, we combined the translated products of *radD* and *igr26* since the two genes seem to be expressed as one protein.

The 360 kDa protein RadD would be difficult to express as a single recombinant protein due to its large size. Therefore, it is reasonable to produce it as smaller recombinant

proteins. In the middle of RadD are a number of repeat domains as identified by SMART (<http://smart.embl-heidelberg.de>) and within those repeat domains is a carbohydrate-binding module (Oralgen; <http://www.oralgen.lanl.gov>). In the C-terminal region there is an autotransporter beta-domain (Oralgen). Therefore, a reasonable approach would be to express RadD as 3 distinct recombinant proteins: The N-terminal region containing the IGR26, the middle fragment containing the repeat domains and carbohydrate-binding module, and the C-terminal region containing the autotransporter beta-domain.

In this way, not only will the role of RadD as an adhesin be confirmed, but also the region of RadD that is involved in coaggregation and is recognized by the function-blocking scFvs will be identified. Furthermore, the recombinant protein that is recognized by the scFvs could be used in coaggregation assays to determine if the protein alone can promote or inhibit *S. sanguinis* aggregation. Hence, the multiple uses of these potential recombinant proteins make it a fruitful approach to examine RadD in the future.

As well, once the recombinant protein that is recognized by the scFvs is identified, the recombinant protein can then be used to find the cognate receptor on partner bacteria. The recombinant protein could be used as bait to pull out the cognate receptor and allow its identification. Thus, RadD recombinant proteins provide avenues to investigate not only RadD, but also to determine the cognate receptor of RadD.

4.9 SIGNIFICANCE OF STUDY

The construction of a scFv library against *F. nucleatum* provides a powerful tool for the identification of adhesins involved in coaggregation. Most previous adhesins were identified based on their binding properties. The 300-350 kDa galactose-binding adhesin (Murray *et al.*, 1988), 30 kDa galactose-binding adhesin (Shanitzki *et al.*, 1997), and N-acetylneuraminic acid specific adhesin (Shanitzki *et al.*, 1998) were identified based on the ability of the adhesin to bind sugars. Even RadD, the most recently discovered adhesin, was isolated by using arginine agarose beads (Kaplan *et al.*, 2009). Unlike the others, FomA was identified by fractionation of the *F. nucleatum* outer membrane

(Kaufman and DiRienzo, 1989). These two methods of adhesin identification have obvious limitations. Adhesins that are not strongly inhibited by a sugar or amino acid cannot be identified using this method. As well, adhesins that are not in high abundance in any fraction of the outer membrane cannot be discovered using the fractionation technique. Therefore, the scFv library provides another method independent of previous approaches that can identify adhesins regardless of the adhesin's binding specificity or abundance in the outer membrane.

Furthermore, the scFv library has the potential to identify adhesins on *F. nucleatum* involved in coaggregation with other binding partners and even the cognate receptor on the binding partner. The scFv library can be screened in coaggregation assays to find scFvs that inhibit coaggregation with any binding partner of *F. nucleatum*. Additionally, once the adhesin is identified using the scFv, the cognate receptor can then be isolated using the scFv-adhesin as bait for the cognate receptor. Thus, the scFv library holds great promise as a new approach to discovering the mechanisms of *F. nucleatum* coaggregation.

4.9.1 Monoclonal Antibody Approach

Many of the benefits associated with the construction of a scFv library could have also been achieved using monoclonal antibodies. Monoclonal antibodies are normally produced using hybridoma technology by fusing myeloma cells with B-cells from a mouse that has been immunized with the desired antigen (Köhler and Milstein, 1975). This allows the limitless production of monoclonal antibodies of a defined specificity to practically any antigen. However, monoclonal antibody producing technology is very laborious and time consuming. scFv phage display libraries allow the production of recombinant antibodies in bacteria, making this technique much more user friendly. As well, the scFv antibody fragment retains the intact antigen binding site, but is more compact than the monoclonal antibody produced by hybridomas. These minimized antibodies have several advantages in practical use, including better tumor penetration, more rapid blood clearance, lower retention times in non-target tissue and reduced

immunogenicity (Huston *et al.*, 1996; Marasco *et al.*, 1998; Colcher *et al.*, 1998). The key difference between monoclonal antibodies and scFvs is that monoclonal antibodies have two antigen binding sites, while scFvs have only one. This allows monoclonal antibodies to bind an antigen through avidity interactions. This way, an antibody with low affinity for the antigen will bind the antigen through the synergism of multiple antigen binding sites.

The scFv approach was followed in this study due to the ease of producing the scFvs in bacteria and our lab's previous experience with the creation of an anti-*Bordetella pertussis* scFv library (Hussein *et al.*, 2007).

4.10 CONCLUSION

The scFv library reported here is the first such library ever constructed against *F. nucleatum*. The use of the scFv library to identify function-blocking scFvs that inhibit *F. nucleatum* coaggregation with the early oral colonizers: *S. sanguinis*, *S. gordonii*, *S. sobrinus*, *S. mutans*, *S. downeyi*, and *A. naeslundii* and identification of RadD as the adhesin involved in coaggregation confirms and extends the results of Kaplan *et al.* (2009). The creation of this anti-*F. nucleatum* scFv library may offer a powerful new tool for the discovery of *F. nucleatum* adhesins involved in coaggregation and their cognate receptors. The importance of this study is not limited only to the construction and characterization of the first scFv library against *F. nucleatum* and its application in adhesin identification, but also is in the potential this scFv library has as a possible preventative treatment for dental plaque.

The identification of the function-blocking scFvs 1, 26 and 198, which inhibit *F. nucleatum* coaggregation with a number of early colonizing streptococci, provides a tantalizing new approach to biofilm control and prevention of periodontal disease and dental caries. These scFvs are capable of preventing *F. nucleatum* coaggregation with many early oral colonizers (Table 3.3 and Figure 3.14). By inhibiting *F. nucleatum* adherence to early colonizers, *F. nucleatum* could be prevented from becoming part of

dental plaque. This would exclude the pathogenic late colonizers from the biofilm, since most pathogenic bacteria adhere via the microbial bridge *F. nucleatum*. Hence, periodontal disease and dental caries, both of which are caused by pathogenic late colonizing bacteria, could be prevented by using these scFvs. Although more work in characterizing and optimizing these scFvs is required, it is possible to envision the future use of these scFvs as an additive in mouthwash or expressed in a live oral vaccine vehicle providing long-term, or even life-long, protection from these common oral diseases. Thus, this study has accomplished the first steps towards providing our children a future free from the anxiety of the dentist's scaler and drill.

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