

5-2012

Identifying *Aggregatibacter actinomycetemcomitans* periodontal antigens by immunoscreening.

Gerald Bernard Pevow
University of Louisville

Follow this and additional works at: <https://ir.library.louisville.edu/etd>

Recommended Citation

Pevow, Gerald Bernard, "Identifying *Aggregatibacter actinomycetemcomitans* periodontal antigens by immunoscreening." (2012).
Electronic Theses and Dissertations. Paper 1124.
<https://doi.org/10.18297/etd/1124>

This Master's Thesis is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact thinkir@louisville.edu.

**IDENTIFYING AGGREGATIBACTER ACTINOMYCETEMCOMITANS
PERIODONTAL ANTIGENS BY IMMUNOSCREENING**

By

Gerald Bernard Pevow

A Thesis
Submitted to the Faculty of the
University of Louisville School of Dentistry
In Partial Fulfillment of the Requirements
For the Degree of

Master of Science

Department of Oral Biology
University of Louisville
Louisville, KY

May 2012

Copyright 2012 by
GERALD BERNARD PEVOW

All rights reserved

**IDENTIFYING *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*
PERIODONTAL ANTIGENS BY IMMUNOSCREENING**

By

Gerald Bernard Pevow

A Thesis Approved on

April 17, 2012

By the following Thesis Committee:

Douglas Darling, Ph.D

Margaret Hill, D.M.D.

Robert Staat, Ph.D.

DEDICATION

This thesis is dedicated to my wife Mrs. Robin Roberts Pevow

who has always shown strength, loyalty, and devotion,

to

my parents Dr. Frederick Merrill Pevow

and

Mrs. Francine Jacobs Pevow,

who have instilled within me their tremendous drive and quest for knowledge,

and to

my children Jonathan Zachary Pevow and Garrett Benjamin Pevow

who have displayed tremendous understanding and patience in my scholastic endeavors.

ACKNOWLEDGMENTS

I wish to thank my mentor and guide, Dr. Douglas Darling, for his efforts in training and support, excellence in research, and unending patience. I would like to thank the committee members, Dr. Margaret Hill and Dr. Robert Staat for their assistance and guidance. I also would like to thank my wife Robin and boys Jonathan and Garrett for their encouragement and tolerance of my time commitments.

ABSTRACT

IDENTIFYING *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* PERIODONTAL ANTIGENS BY IMMUNOSCREENING

Gerald B. Pevow

May 4, 2012

Periodontitis is a chronic, destructive inflammatory disease of the supporting tissues of the teeth with a high prevalence among adults. While the complete pathogenesis of periodontitis remains unclear, it is initiated and sustained by dental plaque containing pathogens such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Bacteroides forsythus*, and *Prevotella intermedia*.

Our hypothesis is that multiple antigens are recognized by human antibodies, and antigens can be identified by use of a genomic expression library. We developed an unbiased global approach to define *A. actinomycetemcomitans* protein antigens that elicit humoral immune responses and developed a screening method to identify periodontal antigens. We identified 19 unique antigens, 17 of which have not been studied in *A. actinomycetemcomitans*.

Future studies should 1) use additional human sera to identify additional antigens, 2) elucidate the role of identified antigens, and 3) determine how *A. actinomycetemcomitans* antigen-antibody profiles relate to disease progression.

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	v
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
CHAPTER 1	
INTRODUCTION.....	1
<i>Periodontitis Definitions and Etiology</i>	1
<i>A. actinomycetemcomitans Definitions and Role in Periodontal Disease</i>	2
<i>Identifying A. actinomycetemcomitans Periodontal Antigens by Immunoscreening</i>	3
CHAPTER 2	
METHODS AND MATERIALS.....	8
<i>Bacteriophage Lambda Genomic DNA Expression Libraries</i>	8
<i>Partial Digestion of Genomic DNA</i>	12
<i>Library Creation Using Lambda Bacteriophage Vector</i>	12
<i>Library Characterization</i>	16
<i>Immunoscreening Genomic DNA Lambda Phage Expression Libraries</i>	21
<i>Immunoscreening A. actinomycetemcomitans Genomic DNA Libraries</i>	23
<i>Identifying and Characterizing A. actinomycetemcomitans Antigens</i>	25
<i>Sequence Identification</i>	26
CHAPTER 3	
RESULTS.....	26
<i>Genomic DNA Lambda Expression Library Creation</i>	26
<i>Partial Digestion of A. actinomycetemcomitans Genomic DNA</i>	26
<i>Ligation and Packaging of Genomic DNA λ Expression Library</i>	29
<i>Characterization of the A. actinomycetemcomitans Expression Libraries</i>	29
<i>Immunoscreening Genomic DNA Lambda Phage Expression Libraries</i>	32
<i>Western Blot Analysis of Individual Serum Antibody Responses to Bacteria</i>	32
<i>Immunoscreening A. actinomycetemcomitans Genomic DNA Libraries</i>	34
<i>Identifying Positive A. actinomycetemcomitans Gene Sequences</i>	37
CHAPTER 4	
DISCUSSION	41
REFERENCES.....	46
CURRICULUM VITA.....	52

LIST OF TABLES

1. <i>Antigenic Genes Identified Through Whole Genome Expression Libraries</i>	38
2. <i>A. actinomycetemcomitans Antigen Table</i>	39

INTRODUCTION

Periodontitis is a chronic, destructive, multifactorial inflammatory disease of the supporting tissues of the teeth with a high prevalence among the adult population. Whereas the complete pathogenesis and mechanism of periodontal disease progression remains unclear, it is initiated and sustained by dental plaque containing bacterial pathogens. Some of the most commonly investigated periodontal pathogens include *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Bacteroides forsythus*, and *Prevotella intermedia* (Lovegrove, 2004).

Plaque-induced periodontal diseases are mixed infections associated with relatively specific groups of oral bacteria (Armitage, 2009, Lovegrove, 2004, Socransky, 2002). Patient susceptibility and disease progression is highly variable, depending on both environmental factors and the host responses to periodontal pathogens (Zambon, 1996; Kornman, 1997; Van Dyke, 2005; Darveau, 2009). Risk factors for periodontal disease include but are not limited to smoking, age, diabetes mellitus, stress (depression, anxiety, job or financial strain), gender, medication, socio-economic status and HIV infection (Ng, 2006; Armitage, 2003, Corbet 2011).

To arrive at a periodontal diagnosis, clinicians observe the presence or absence of signs of inflammation such as 1) bleeding on probing; 2) probing depths; 3) extent and pattern of loss of clinical attachment and bone; 4) patient's medical and dental histories;

and 5) presence or absence of additional clinical indicators such as pain, ulceration, and the amount of plaque and calculus. Inflammation without observable connective tissue attachment loss is termed gingivitis while inflammation with attachment loss is termed periodontitis (Armitage, 2010).

Periodontitis is a common dental disorder. However, estimates of the exact prevalence of periodontitis in the U.S. and worldwide vary due to inconsistent disease definition and study methodology (Costa, 2009). If periodontitis is defined as the identification of at least one site with Clinical Attachment Loss¹ (CAL) of ≥ 2 mm, around 80% of all adults are affected, and around 90% of those aged 55 to 64 (Centers for Disease Control, 1997). However, if the case definition is at least one site with CAL of ≥ 4 mm, the prevalence in those aged 55 to 64 drops to around 50%, and with a case definition of CAL ≥ 6 mm, prevalence is less than 20% (Burt, 2005; Baelum, 2003). Despite efforts by the Center for Disease Control and Prevention and the American Academy of Periodontology to develop consensus, the discussion of definitions of periodontal diseases and periodontitis continues (Corbet, 2011; Meisel, 2009). Nevertheless, by any definition, a large portion of the U.S. population suffers from periodontitis.

Periodontitis is caused by inflammation and infection of the tissues that support the teeth and is characterized by the loss of attachment between the teeth and the surrounding tissues. It is classified as chronic or aggressive periodontitis based on the differing rates of loss of attachment of the teeth and the patient's age and medical status (Armitage, 1999; Armitage, 2010; Schaudinn, 2009). Chronic periodontitis, the most

¹ Clinical attachment loss (CAL) is the predominant clinical manifestation and determinant of periodontitis. It is calculated as the clinician's probing depth measurement plus his/her gingival margin level measurement.

common type of periodontitis, is typically a slowly progressing disease that emerges clinically in adulthood and continues throughout life. By contrast, aggressive periodontitis is less common than chronic periodontitis and principally affects young people (Armitage, 2003; Armitage, 2010).

Further, chronic and aggressive periodontitis is characterized by extent (localized or generalized) and severity (slight, moderate, and severe). By definition, localized periodontitis affects less than 30% of sites while generalized periodontitis affects 30% or more sites (Savage, 2009). Also by definition, severity is measured by clinical attachment loss (CAL) as follows: Slight = 1-2mm, Moderate = 3-4 mm, and Severe \geq 5 mm (Armitage, 2003; Armitage, 2010).

The disorder is initiated by dental plaque, a microbial biofilm. Like other biofilm infections, periodontitis is refractory to antibiotic agents and host defenses because the causative microbes live in complex communities that persist despite challenges. Oral health or disease depends on the interface between the host and the microbial community as a whole. Further, both the pathogenicity of specific microbes and the community within which they reside are important to study (Jenkinson, 2005; Kuramitsu, 2007; Schaudinn, 2009).

Bacteria play an essential role in the etiology of periodontal disease. Over 700 bacterial species have been identified in the oral cavity and nearly 300 species have been cultured and found to contribute to the biofilm of the periodontal pocket (Dewhirst, 2010; Jenkinson, 2005; Loesche, 2001; Nibali, 2009; Paster, 2001; Pocolos, 2005). However, the direct involvement of each individual microbe in pathogenesis remains unclear. Some of the most commonly investigated periodontal pathogens include *Porphyromonas*

gingivalis, *Aggregatibacter actinomycetemcomitans*, *Bacteroides forsythus*, and *Prevotella intermedia* based on earlier work associating bacterial species with severity of disease (Haffajee, 1994).

Aggregatibacter actinomycetemcomitans, formerly *Actinobacillus actinomycetemcomitans*, is a gram-negative, facultative anaerobic coccobacillus bacterium that colonizes the oral cavities of humans (Norskov-Lauitsen, 2006; Henderson 2011). Several studies have shown that *A. actinomycetemcomitans* is significantly associated with both aggressive periodontitis and chronic periodontitis (Ebersole, 1995a; Zambon, 1996; Loesche, 2001; Cortelli, 2005; Rylev, 2008). The association is clearly greatest with aggressive periodontitis. However, strong associations have been reported for *A. actinomycetemcomitans* with chronic periodontitis patients, 74% (Choi, 2000); 50% (Kuboniwa, 2004); >75% (Rodrigues, 2004); 30% (Slots, 1990); 60% (Sixou, 1991); 75% (Savitt, 1991); 28% (Sandholm, 1987); 55% (Flemmig, 1996).

Additional evidence linking *A. actinomycetemcomitans* with periodontitis is that there is a clear immune response of the host to *A. actinomycetemcomitans* infection (Kinane, 1999; Apatzidou, 2004). Further, certain anti-*A. actinomycetemcomitans* serum antibodies appear to protect patients from disease (Flemmig, 1996; Lamster, 1998). Such data have led reviewers to generally conclude that *A. actinomycetemcomitans* is likely a periodontal pathogen in humans (Zambon, 1996; Kinane, 2000b; Kinane, 2001a; Loesche, 2001; Henderson, 2002; Mombelli, 2002; Henderson, 2003; Lovegrove, 2004). This conclusion is supported by experimental evidence in animal models including miniature pigs, dogs, rats, and mice which demonstrates that *A. actinomycetemcomitans* does cause periodontal disease (Freire, 2011; Fine, 2009; Oz, 2011).

The onset and severity of periodontal disease is largely controlled by the effectiveness of the immune response of the patient to the oral pathogens. As contributors to the immune response, several lines of evidence indicate that peripheral serum antibodies are relevant to disease progression: Immunization against *A. actinomycetemcomitans* bacteria can attenuate infection-induced periodontitis-like disease, and total serum antibody levels correlate with decreased severity of disease (Casarin, 2010; Rams, 2006).

However, high serum antibody titers do not always prevent disease. Thus, analysis of specific host antibodies to specific bacterial antigens is likely to be more informative than gross analysis of total serum immunoglobulin levels. This is the premise of the present work and from which we developed the project hypothesis: Antibodies to certain *A. actinomycetemcomitans* bacterial proteins are very highly associated with the severity of, and response to, treatment of periodontal disease.

Several such studies of specific host antibodies have investigated one or very few antibody/antigen relationships at a time. These studies indicate the clinical significance of serum antibodies. However, it remains unclear whether some groups of antibodies elicit better protection than others.

The purpose of this study was to identify immunoreactive *A. actinomycetemcomitans* proteins by use of an unbiased genomic DNA lambda phage expression library. The method allows for a global screening approach to identify all protein epitopes encoded on the bacterial genome that may elicit a human antibody response. Bacterial protein antigens that elicit a humoral response can then be further

studied and associations drawn between the immune response and clinical indices. The four main goals of this research project are to:

1. create bacteriophage lambda genomic expression libraries expressing peptides encoded on the *A. actinomycetemcomitans* genome,
2. develop the immunoscreening method as a broad approach to find novel bacterial antigens,
3. optimize the immunoscreening method for this project and immunoscreen whole cell lysates from *A. actinomycetemcomitans* JP2, and
4. identify and characterize *A. actinomycetemcomitans* antigens obtained through the optimized immunoscreening method.

While the research used an unbiased global approach to define novel bacterial protein antigens that elicit humoral immune responses, there are other methods to identify novel protein antigens, most notably IVIAT, *In Vivo* Induced Antigen Technology (Handfield, 2000; Cao 2004). IVIAT also uses bacterial genomic DNA expression libraries, supporting the utility of such an approach. However, the goal of the IVIAT approach is the opposite of the goal of this research. IVIAT seeks to identify the bacteria's response to living inside the host, whereas we seek to identify a major component of the host's response to the bacteria. Further, the IVIAT technology requires pooled sera and is not easily compatible with creation of microarrays.

A future goal for this project is to identify a discrete set of the most informative antigen proteins for use as prognostic diagnostic indicators. Such indicators may be of interest to determine whether the antibody profile of patients with gingivitis will develop

periodontitis in the future. This is based on the idea that certain antigen-specific antibodies confer protection from disease whereas other antigen-specific antibodies do this poorly. A microarray would be a simple approach to identify whether a given patient has serum antibodies that correlate with a protective response. Arranged in a microarray, the antigens have the potential for broad use as a prognostic diagnostic tool in the setting of a clinical lab.

Further, such antigenic proteins may be useful as a vaccine to confer resistance against periodontal disease. If certain *A. actinomycetemcomitans* antigenic proteins cause an immune response in humans that protects against periodontitis, those proteins could then be used as a vaccine candidate to prevent disease.

METHODS AND MATERIALS

Bacteriophage Lambda Genomic DNA Expression Libraries

The goal of this research is to identify bacterial proteins which are antigenic in patients. This is done by using human sera to screen an unbiased bacterial genomic DNA expression library. Libraries are collections of cloned DNA fragments. Ideally, they contain a complete representation of genomic or cDNA sequences. They are commonly “packaged” in bacteriophage vectors. Different library types have different utilities and starting material. The most common types are cDNA libraries or genomic libraries.

cDNA libraries contain only complementary DNA molecules synthesized from mRNA in a cell. Because cDNA libraries are copied directly from mRNA molecules they will only contain DNA representing mature mRNAs, i.e. intron sequences are not present. cDNA libraries have been essential for isolation of novel mRNAs. However, they typically over-represent abundant RNAs and may lack rare mRNAs. Also, cDNA libraries lack information about enhancers, introns, and other regulatory elements found in a genomic DNA library.

Genomic libraries contain random DNA fragments representing the entire genome of an organism. Genomic libraries are useless for expression of eukaryotic genes since these genes contain introns. However, bacterial genes lack introns. Use of bacterial genomic DNA for an expression library provides a better representation of bacterial

peptides than a bacterial cDNA library constructed from mRNAs since different mRNAs are likely expressed during *in vitro* culture than *in vivo*. Hence, this approach is not biased by the initial abundance of mRNAs. This research project takes advantage of the extremely high gene density of the bacterial genome, which lacks introns, and the project uses genomic libraries since bacterial genes expressed selectively in the oral cavity may be severely under-represented in a cDNA library.

The first step in genomic library preparation is the isolation of large fragments of chromosomal DNA (Figure 1). Extraction conditions should break open the cells to release the DNA but should minimize the shearing of DNA into small pieces. Also endogenous nuclease activity should be minimized in order to keep the DNA intact.

Once large fragments of DNA are isolated, they need to be cut into the right size to be inserted into a vector. Not only do the fragments need to be sized correctly, but they also need to cut the DNA randomly so that the library will contain a collection of overlapping fragments. Such a collection ensures that all regions of the chromosome are equally represented. The best way to accomplish this is to partially digest the DNA with restriction endonucleases. Restriction digests not only cut DNA into the appropriate sizes, but also generate fragments with sticky ends that allow for easy insertion into the vector of choice. A restriction digest will leave some DNA fragments that are too large and some that are too small to use in a library. Thus, upon completion of a restriction digest, the appropriate sized fragments are fractionated by gel electrophoresis, cut from the gel and then purified to isolate the fragments from the gel. Once the fragments of DNA are isolated, they need to be inserted into a vector to generate the genomic library. The most commonly used vectors for genomic libraries are lambda derivatives that have large

Figure 1. Genomic DNA Library Construction

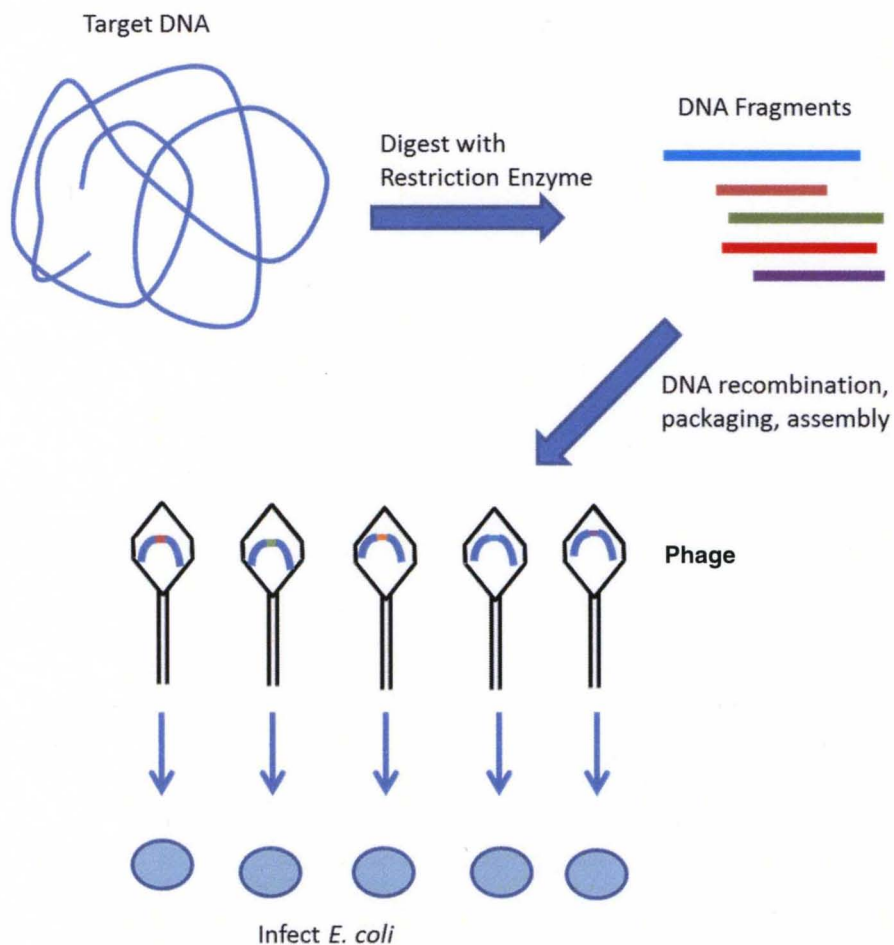


Figure 1. Genomic DNA Library Construction. Target DNA is partially digested with a restriction endonuclease resulting in overlapping DNA fragments of varying lengths. DNA fragments are packaged and assembled in phage with each phage containing a different DNA fragment. *E. coli* is spread on an agar plate and infected *E. coli* create a plaque on the bacterial lawn. One phage infects one bacterium so each plaque represents a unique DNA sequence.

fragments that can be removed and substituted for equally large pieces of genomic DNA. Excess lambda arms are added to restriction endonuclease-digested genomic DNA. The sticky ends of the DNA fragments hybridize with the lambda arms and are subsequently ligated together with DNA ligase. Finally, the DNA is packaged into lambda capsids, resulting in lambda phages with random DNA inserts. The product is a library of many thousands of recombinant lambda phage each containing a unique fragment of chromosomal DNA.

Preliminary work used genomic DNA from *A. actinomycetemcomitans* strain HK1651 (American Type Culture Collection) and a modified λ TriplEx2 vector (Clontech) that allows use of the EcoR1 site for making the library. HK1651 is serotype b, based on its immune responses to surface antigens. Serotype b is the most prevalent *A. actinomycetemcomitans* serotype in both aggressive and chronic periodontitis (Yang, 2004).

λ TriplEx2 was initially used because it expresses in all three reading frames, decreasing the number of screens necessary to immunoscreen the entire *A. actinomycetemcomitans* genome. However, it did not appear to have the expression levels necessary to provide a strong positive signal and clearly differentiate positive from negative plaques. As such, all later work (and all results) used Lambda ZAP II Expression Library and Gigapack Gold III packaging kit purchased from Stratagene (USA). Further, *A. actinomycetemcomitans* strain JP2 cultures were received from the laboratory of Dr. Don Demuth (University of Louisville) and used instead of HK 1651 genomic DNA. JP2, like HK 1651, is a well characterized serotype b strain.

Partial Digestion of Genomic DNA

To give overlapping fragments, *A. actinomyctemcomitans* genomic DNA (5 µg) was partially digested with 5 Units of TSP5091 restriction endonuclease (New England Biolabs) for approximately 60 min. Tsp5091 is not sensitive to methylation and cuts at the 4-base recognition sequence AATT leaving a 4 base 5'-overhang identical to the EcoR1 overhang on the vector. Since Tsp5091 cuts at a 4-nucleotide target, it is predicted to cut random sequence DNA with a frequency of once every 256 bases.

Digestion was assessed by running an aliquot of digested DNA on 1% agarose gel to verify generation of random length DNA fragments. Fragments from 400-1500 bp in size were excised from the gel. The DNA was purified from the gel plugs using Qiagen gel purification columns (Qiagen, USA).

Library Creation Using Lambda Bacteriophage Vector

Lambda Zap II arms (Stratagene, cat#236612), commercially EcoRI digested and Calf Intestinal Alkaline Phosphatase treated, were added to the partially digested *A. actinomyctemcomitans* DNA fragments inserts at a ratio of 1:1 (500 ng arms to 500 ng of DNA) to 3:1. T4 DNA Ligase (New England Biolabs) was used, in a reaction volume of 6 µL, for an 18 hour incubation at 16°C to ligate lambda arms with the insert to form a recombinant lambda phage-genomic DNA strand (Figure 2). The recombinant arms were then packaged (ie. addition of phage protein coat) using a lambda packaging reaction, Gigapack III Gold Packaging Reaction (Stratagene, cat#200206) for 2 hours at room temperature. This phage packaging kit is capable of producing 1×10^8 PFU/µg DNA. Recombination efficiency was determined by measuring the loss of β-galactosidase activity in recombinant phages. The host bacteria, XL1-Blue MRF', was infected with the

packaging reaction and plated in the presence of IPTG and X-gal. Since phage that are packaged *in vitro* are not very stable, half the library was immediately amplified and stored at -80°C.

Figure 2. Lambda ZapII Cloning Procedure

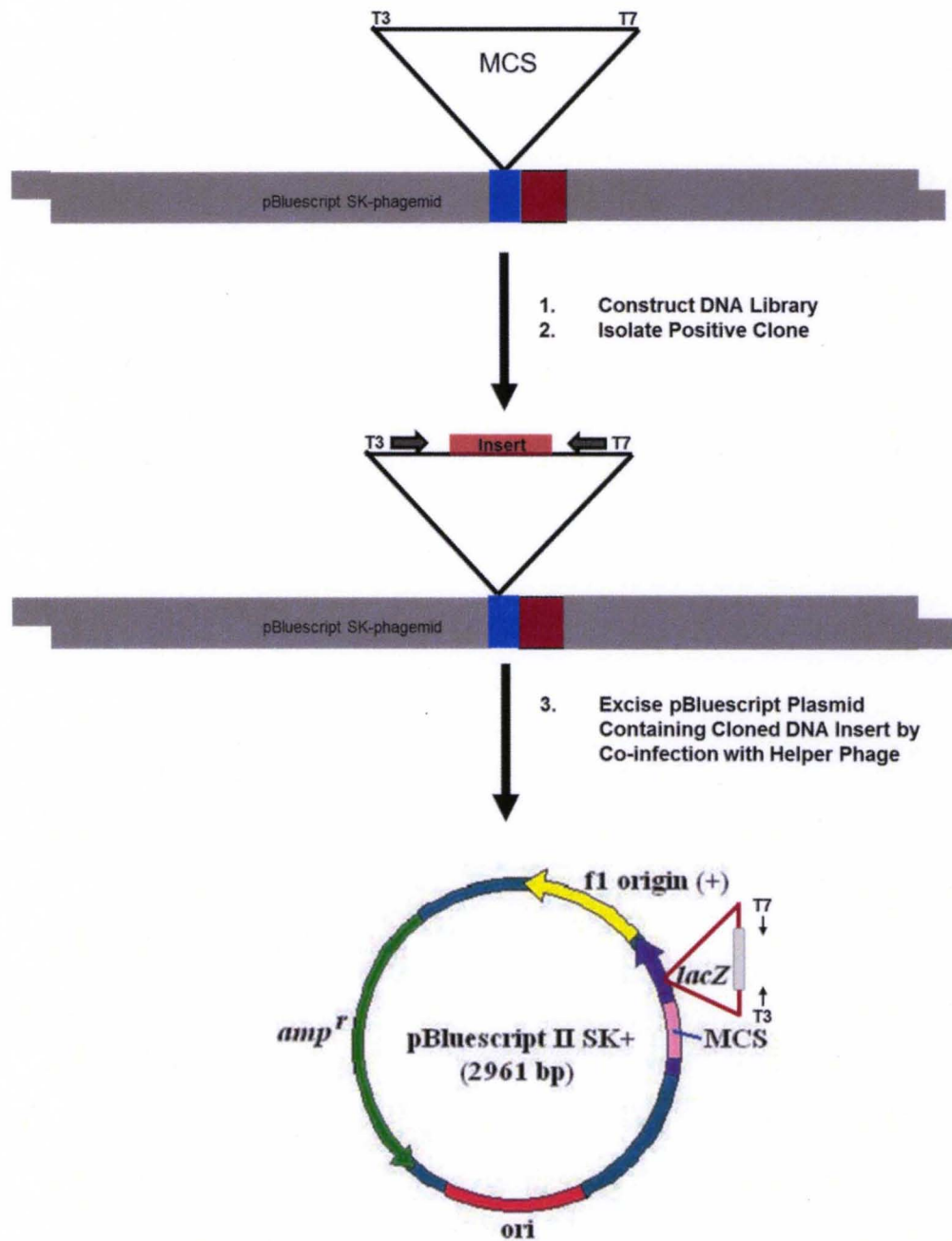


Figure 2. Lambda ZapII Cloning Procedure. Lambda ZAP vectors simplify the construction of high-titer cDNA libraries and the characterization of inserted DNA. They allow easy excision and recircularization of cloned insert DNA from lambda phage and eliminate the need for subcloning procedures.

Library Characterization

Libraries were characterized in order to assure a high representation of the bacterial genome. Genomic DNA insert sizes were determined by restriction digests of cloned inserts. The Lambda ZAP vector allows *in vivo* excision and recircularization of cloned inserts to form a plasmid (phagemid) containing the cloned insert. Signals for packaging the phagemid are contained within the f1 terminator origin DNA sequence and permit the circularized DNA to be packaged and secreted from the *E. coli*. Once secreted, the *E. coli* cells used for excision of the cloned DNA can be removed from the supernatant by heat treatment. Once the packaged pBluescript DNA is mixed with fresh *E. coli* cells and re-plated, DNA from minipreps of these colonies can be used for analysis of insert DNA.

Details of this method are as follows: Plaques are cored from the agar plate and transferred to a sterile microcentrifuge tube containing 500 μ l of SM buffer and 20 μ l of chloroform. The microcentrifuge tube is vortexed to release the phage particles into the SM buffer and incubated overnight at 4°C. Separately, 50 ml of XL1-Blue MRF' cells in LB broth with supplements is cultured overnight at 30°C. The next day the cells are centrifuged at 1000 \times g and resuspended in MgSO₄. 200 μ l of cells at an OD600 of 1.0 are combined with 1 μ l of the R408 helper phage and the phage from the plaque and incubated at 37°C for 15 minutes to allow the phage to attach to the cells. 3 ml of LB broth with supplements is added and incubated at 37°C overnight. Next, the tube is incubated at 65–70°C for 20 minutes to lyse the lambda phage particles and the cells and centrifuged at 1000 \times g for 15 minutes to pellet the cell debris. The supernatant containing the excised pBluescript phagemid packaged as filamentous phage particles is

decanted into a sterile 14 ml round-bottom tube. 200 µl of freshly grown XL1-Blue MRF' cells are placed in two microcentrifuge tubes and 100 µl of the phage supernatant is added to one of the tubes and 10 µl to the other microcentrifuge tube. The tubes are incubated at 37°C for 15 minutes and plated on LB–ampicillin agar plates and incubate overnight at 37°C. Colonies contain the pBluescript double-stranded phagemid with the cloned DNA insert.

After isolating the excised plasmid DNA by miniprep (Qiagen), insert sizes and numbers were determined by restriction analysis. Acceptable libraries had an average insert size greater than 750 bp (3 times the average frequency of Tsp5091 sites), ensuring overlapping fragments. The complexity of the library was calculated as the number of plaques with inserts per µg of insert DNA (Ausubel, 2000). Phage infected bacteria were plated at a density of about 4000 plaques per plate. Screening 50,436 plaques was calculated to provide a 95% probability that every section of the genome is represented in an expression clone, based on the size of the bacterial genome (2.1 Mb *A. actinomycetemcomitans*), the average size of the inserts, and the fraction of clones in the correct orientation and reading frame (Sambrook, 1989). The probability of having any given genomic DNA sequence in the *A. actin* library can be calculated from the equation

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where P is the desired probability, f is the fractional proportion of the genome in a single recombination, and N is the necessary number of recombinants. To achieve a 95%

probability of having a given DNA sequence represented in a library of 750 bp fragments of the 2.1 Mb *A. actinomycetemcomitans* genome $N = \ln 0.05 / \ln(1 - 750 / 2.1 \text{ Mb}) = 8,406$ plaques. N is then multiplied by 3 to correct for reading frame and by 2 for orientation. Thus, $8,406 \text{ plaques} \times 6 = 50,436$ plaques are necessary to screen in order to have a 95% probability of having any given DNA sequence in the library.

To characterize later libraries, PCR was conducted directly on phage particles to determine the frequency and size of inserts. Clear plaques lacking β -gal activity were picked and amplified using Amplifier forward and Amplifier reverse primers that span the EcoRI insert site and yield a band of 160 bp when no insert is present. PCR products were run on a 1.0% agarose gel, for size determination. Gel slices were purified using the Qiagen PCR clean-up kit (Qiagen, US) and the PCR products were collected for further analysis.

Figure 3. PCR Analysis of Inserts

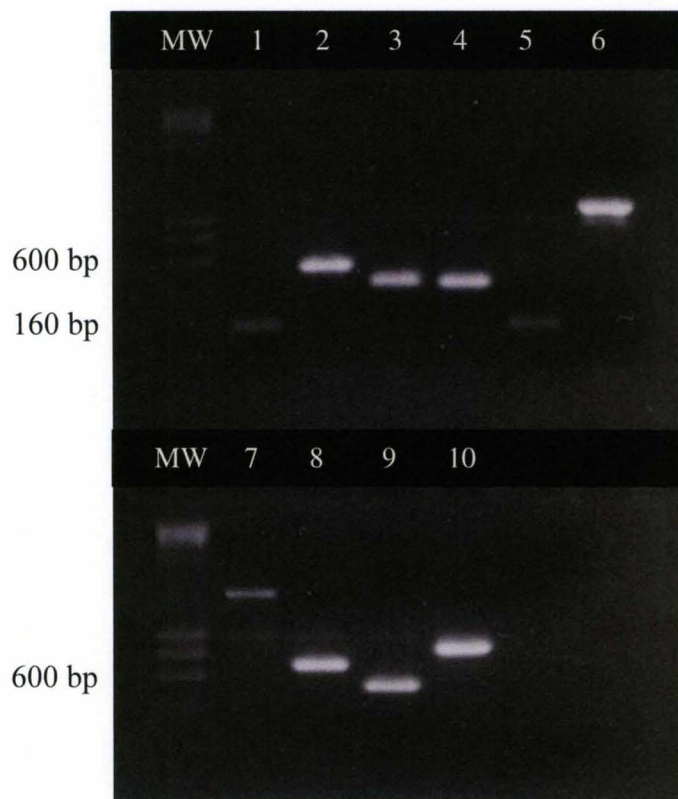


Figure 3. PCR Analysis of Inserts. PCR Analysis of Inserts. PCR was conducted on inserts using M13 primers for Lambda Zap II. Bands cut from digest were between 400 bp and 1500 bp. No inserts would give a fragment of approximately 160 bp. 2 out of the 10 fragments had no inserts (Lanes 1, 5). 80% of fragments contained inserts of the anticipated size range based on the partial digest of *A. actinomycetemcomitans* genomic DNA.

Immunoscreening Genomic DNA Lambda Phage Expression Libraries

Analyzing Serum Pools by Western Blotting

Eleven human serum samples (Innovative Research, Southfield, MI) were pre-screened to identify highly immunogenic serum by western blotting of bacterial proteins. A western blot is an analytical technique used to detect specific proteins in a given sample. It uses gel electrophoresis to separate proteins that are then transferred to a membrane. Membranes are subsequently probed using antibodies specific to the target protein.

For the western blot analysis of individual serum antibody responses to oral bacteria, liquid cultures of *A. actinomycetemcomitans*, *P. gingivalis* and *E. coli* strain XL1- Blue were centrifuged to pellet bacteria. The resulting pellets were washed and suspended in 100 μ L of LB. The suspensions were then boiled for 15 min at 98°C to lyse the bacteria. Lysates were quantified using a Nanodrop (Nanodrop Technologies, USA) by absorbance at 280 nm wavelength. The bacterial proteins were separated on a 20% polyacrylamide gel (NuSEP, USA) by gel electrophoresis and transferred to a PVDF membrane. After blocking with a 5% milk-TBST blocking solution (25 mM Tris, 140 mM NaCl, 3 mM KCl, 1.0% Tween-20, pH 8.0), the membranes were cut into strips with each of the three bacterial lysates on each strip. Strips were then probed overnight at 4°C with a 1:500 dilution of human serum, purchased commercially from Innovative Research, Inc. (Southfield, MI) and blocking buffer. Strips were subsequently washed liberally with TBST and incubated for 1 hour at room temperature with goat anti-human total Ig horseradish peroxidase (HRP) conjugated secondary antibody (Southern Biotech, USA) at a dilution of 1:5000 in blocking buffer. Bound antibodies were visualized by the

addition of DAB/CN (Pierce, USA). Chloronaphthol (CN) and diaminobenzidine (DAB) are chromogenic peroxidase substrate compounds combined in a single, stable, 10X stock solution. The CN/DAB solution produces an intense dark black precipitate at sites of bound HRP-conjugated antibodies on probed blots. This identified sera exhibiting a high titer of anti- *A. actinomycetemcomitans* antibodies. Three sera were combined for screening the genomic expression library.

Immunoscreening *A. actinomycetemcomitans* Genomic DNA Libraries

Once a library has been constructed, a robust assay is needed to detect and isolate the few clones containing antigens out of the millions of clones in the library.

Identification of a novel antigen involves the following steps: titering the library to determine the PFU/ml (plaque forming units per ml of library stock), primary plating of the library, preparing filters of phage plaques, screening of filters with human serum, secondary screening, tertiary screening, preparation of DNA, and subcloning plus sequencing of *A. actinomycetemcomitans* inserts.

Figure 4. Immunoscreening Technique

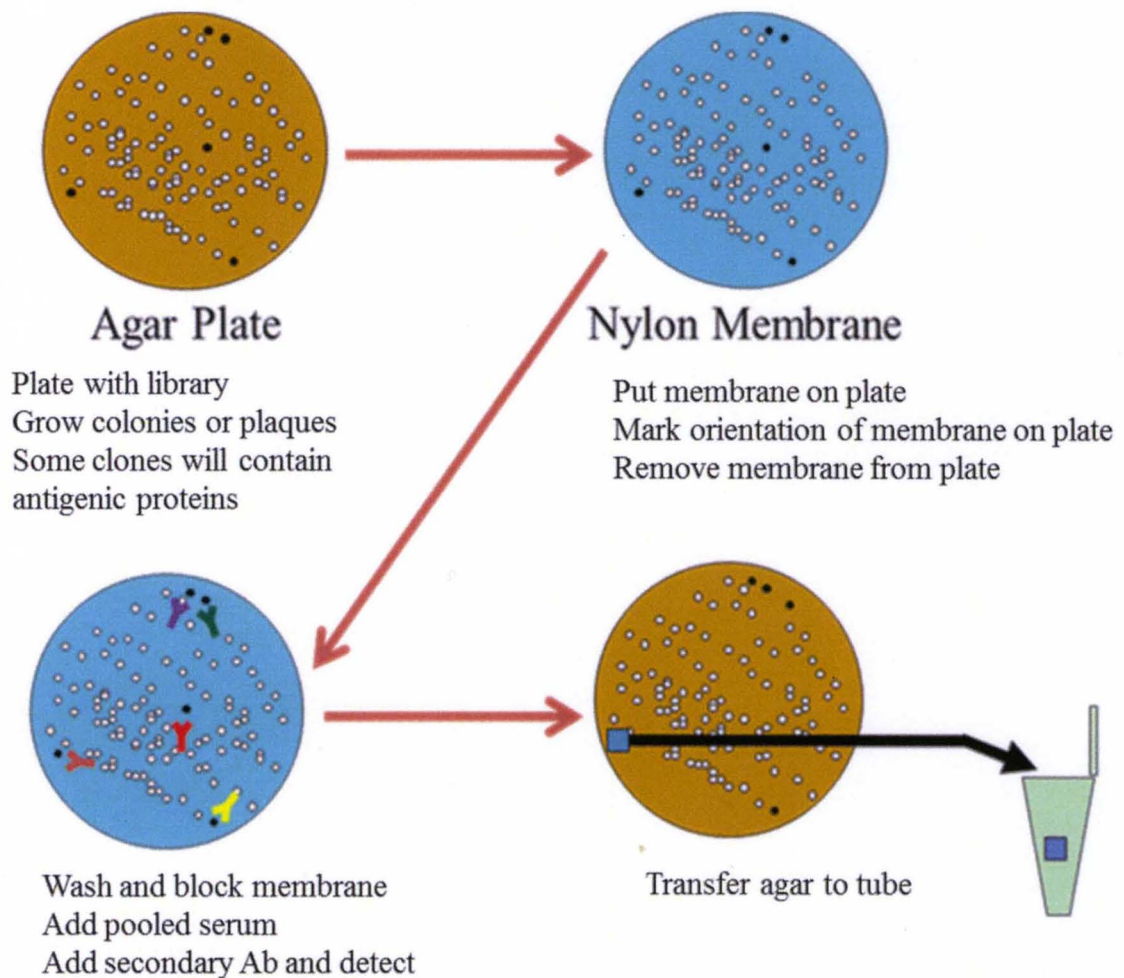


Figure 4. Immunoscreening Technique. *A. actinomycetemcomitans* genomic DNA library is incubated with the growth culture of excess *E. coli* bacteria and plated to yield approximately 2000-4000 plaques. The plaques appear on a lawn of bacteria whereby each plaque represents a unique genomic DNA sequence of *A. actinomycetemcomitans*. A nitrocellulose filter is placed onto the agar plate and by capillary action the plaques are replicated on the nitrocellulose. *A. actinomycetemcomitans* antigens of interest are then detected using an immune detection protocol.

Once titered, an appropriate amount of the bacteriophage lambda library is incubated with the growth culture of excess *E. coli* bacteria. The bacteriophage infect the *E. coli* and the cells are subsequently spread on agar plates. Plaques which contain the debris from the lysed bacteria including the highly expressed *A. actinomycetemcomitans* peptide (antigen), appear on the lawn of bacteria whereby one phage creates one plaque, and each plaque represents a unique genomic DNA sequence of *A. actinomycetemcomitans*. For immunoscreening, a library of 2000 to 4000 plaque-forming units (PFU) was plated on each petri dish. For each screening, 10-15 petri dishes were grown to give approximately 24,000 plaques total.

As the plaques grow, the lambda bacteriophage lyse the adjacent infected cells. Each plaque expresses a high concentration of the peptide cloned into that phage. A nitrocellulose filter is placed onto the agar plate and by capillary action the plaques are replicated on the nitrocellulose.

An immune detection protocol is then used to detect the antigens of interest. Antigens in different plaques immobilized on nitrocellulose membranes are detected with human antibodies in a three-step process. Nitrocellulose membranes are placed into individual petri dishes and diluted serum from three pooled patient samples is added to each dish. Antibodies in the human serum are termed primary antibodies, and will bind to those antigens on the membranes that the antibodies recognize. Since serum presumably contains many different antibodies, it is likely that on an individual plate containing approximately 2000 unique clones of *A. actinomycetemcomitans* antigens, that several different antibodies will recognize different clones. This means that there will likely be one or two antibody/antigen reactions on each membrane.

In a second step, a secondary antibody-horseradish peroxidase (HRP) conjugate, which recognizes general features of all human antibodies, is added to find locations where the primary antibody is bound. In the third step, HRP, the enzyme conjugated to the secondary antibody, catalyzes a colorimetric reaction when the appropriate substrate is added, resulting in the deposition of colored product on the membrane at the individual immunoreactive plaque. This color provides a visual indication of potential primary antibody recognition. Positive, reactive plaques are identified, picked from the agar plates, and preserved.

Because of the density of the plates, the wet surface of the plates, and the difficulty in picking individual plaques, preserved plaques may contain some contaminating phage. To decrease the likelihood of contamination and to confirm that a positive plaque was picked, a secondary and tertiary screening is performed. In particular, the tertiary screening is conducted at a low enough plaque density to ensure that the final preserved plaque isolate is homogenous in nature.

Once screenings have been completed, each preserved, lambda phage containing tertiary plaque is converted into a plasmid. By subcloning the *A. actinomyctemcomitans* insert from the lambda phage containing plaque into a plasmid vector, the positive *A. actinomyctemcomitans* gene sequence is preserved and is more easily manipulated for future studies.

Details of the above screening process are as follows: Phage infected bacteria were plated at a density of 2000 to 4000 plaques per plate. IPTG soaked nitrocellulose membranes were placed on each plate and the plates were incubated for 14 hours at 37°C. Membranes were removed and washed in TBST-5% milk for 2 hours.

Membranes were probed overnight with pooled human sera diluted 1:500 in 5% milk in TBST. Goat anti-human total Ig human horse radish peroxidase (HRP) conjugated secondary antibody (Southern Biotech, USA) were used to identify human serum Ig bound plaques. Secondary antibodies were incubated with the membranes for 1 hour. To visualize reactive plaques, chloronaphthol (CN) or DAB/CN (Pierce, USA) was added to form a colored precipitate reaction. Plaques corresponding to the colored reactive spots on the membranes were picked using disposable glass Pasteur pipettes. Recombinant clones were purified through repeated plating, serum screenings, and picking of reactive plaques.

Identifying and Characterizing *A. actinomycetemcomitans* Antigens

Each plasmid generated from identified immuno-positive tertiary plaques was sequenced to identify the proteins expressed. Purified phage clones were amplified and the pBluescript plasmid with the insert was excised following manufacturer's protocols. Plasmid clones were sequenced at the University of Louisville Nucleic Acids Core facility. pBluescript forward and reverse sequencing primers were provided to the Core facility for sequencing and to confirm correct insert orientation.

Sequence Identification

A. actinomycetemcomitans clone sequences were compared to the published genome using the **Basic Local Alignment Search Tool (BLAST)** on the JCVI website (cmr.jcvi.org), on the ORALGEN website (www.oralgen.lanl.gov) or the NIH website (blast.ncbi.nlm.nih.gov). The sequences of the insert DNA and 7 codons upstream of the

EcoRI insert site were compared to the databases to identify *A. actinomycetemcomitans* genes encoded within the insert. DNA inserts disrupt the coding sequence for β -galactosidase since they are inserted within that sequence. Peptides are expressed as a fusion protein with the first 7 amino acids of β -galactosidase. This ensures the presence of an initiator methionine. However, it also means that only one reading frame can be expressed. The BLASTx option was utilized to ensure that the insert was in the correct reading frame. Since a group of three nucleotides code for a single peptide, a totally different polypeptide can be transcribed depending on whether reading starts with the first, second, or third nucleotide. BLASTx (Basic Local Alignment Search Tool) is used to compare a newly determined DNA sequence against already existing sequences in the NCBI non redundant protein database.

RESULTS

Genomic DNA Lambda Expression Library Creation

The steps in the creation of the *A. actinomycetemcomitans* Genomic DNA Lambda Expression Library are as follows:

1. Partial Digestion of *A. actinomycetemcomitans* genomic DNA
2. Ligation and packaging of the Genomic DNA Lambda Expression Library
3. Characterization of the *A. actinomycetemcomitans* Expression Libraries

Partial Digestion of *A. actinomycetemcomitans* Genomic DNA

A. actinomycetemcomitans genomic DNA (0.4 µg) was partially digested with varied amounts of TSP5091 restriction endonuclease to identify the optimal ratio of enzyme to DNA. Digestion was assessed by running an aliquot of digested DNA on 1% agarose gel to verify generation of random length DNA fragments. Based on the results in Figure 5, the ideal ratio of TSP5091 to *A. actinomycetemcomitans* DNA was 3.3 Units/ug DNA. Subsequently, a preparative digestion was done using 16.5 U TSP5091 and 5 µg genomic DNA. The entire reaction was fractionated on an agarose gel. Fragments from 400-4000 bp in size were excised from the gel. The gel plugs were purified using Qiagen gel purification columns (Qiagen, USA). The pooled DNA was used for library construction.

Figure 5. Analytical and Preparative Partial Digests of *A.a.* Genomic DNA

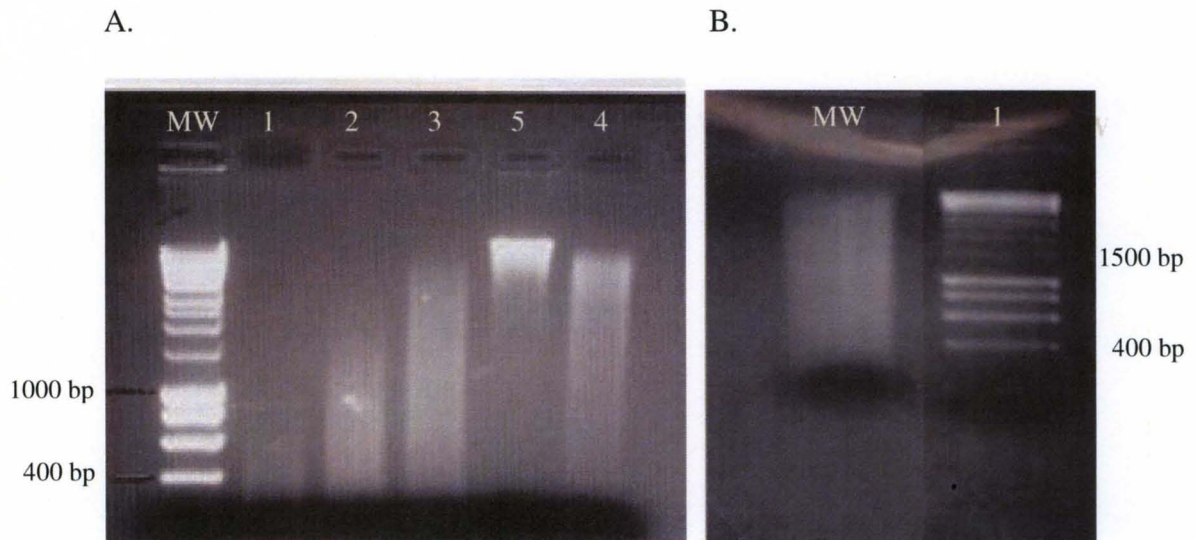


Figure 5. Analytical and Preparative Partial Digests of *A. actinomycetemcomitans* Genomic DNA. A. For analysis, *A. actinomycetemcomitans* genomic DNA (0.4 μ g) was partially digested with TSP5091 restriction endonuclease. Tube 1 contains 4 Units of TSP5091. Tubes 2-5 were serial dilutions as follows: 1.33 U, .44 U, .15 U, .07 U). Conditions were optimized by running the partial digestions on 1% agarose gels. Based on the results, the ideal ratio of TSP5091 to *A. actinomycetemcomitans* genomic DNA was 3.3 U/ μ g DNA (Lane 2). B. For library construction, 5 μ g of *A. actinomycetemcomitans* genomic DNA was partially digested with 16.5 Units of TSP5091 restriction endonuclease. Fragments from 400-1500 bp in size were excised from the gel, and purified using Qiagen gel purification columns (Qiagen, USA).

Ligation and Packaging of Genomic DNA λ Expression Library

Commercially available Lambda Zap II arms (Stratagene) were added to the partially digested *A.actinomyctemcomitans* DNA fragment with T4 DNA Ligase (New England Biolabs) and incubated overnight to ligate lambda arms to the insert. The overhang left by the TSP5091 digestion matches and ligates with the EcoR1 overhangs on the ends of the lambda arms. The resultant recombinant lambda phage-genomic DNA strands were then packaged by the addition of phage coat proteins using a lambda packaging reaction, Gigapack III Gold Packaging Reaction (Stratagene).

Characterization of the *A. actinomyctemcomitans* Expression Libraries

Recombination efficiency was determined by measuring the loss of β -galactosidase activity in recombinant phages. Lambda arms which ligate to each other without a DNA insert encode the beta-galactosidase enzyme across the ligation site. The presence of an insert disrupts the galactosidase sequence. The host bacteria, XL1-Blue MRF', was infected with the packaging reaction and plated in the presence of IPTG and β -galactosidase. Lambda Zap II:*A. actinomyctemcomitans* expression libraries were found to have relatively high titers of approximately 1×10^7 PFU/ug DNA. Less than 10% of the plaques showed beta-galactosidase activity, demonstrating a recombination efficiency of >90%.

We next investigated whether the insert sizes in the phage library matched the expected size range. PCR amplification of random recombinant clones was done using M13forward and M13reverse primers and published PCR protocols. Results in Figure 6 show bands were diverse sizes between 200-1400 bp consistent with random fragments.

Further, to confirm diverse portions of the *A. actinomycetemcomitans* genome were cloned, Ten inserts were sequenced and aligned to the *A. actinomycetemcomitans* genome. Inserts appeared to be randomly selected from different areas of the bacterial chromosome. Since phage that are packaged *in vitro* are not very stable, half the library was immediately amplified and stored.

Figure 6. Size Determination of Expression Library Inserts



Figure 6. Size Determination of Expression Library Inserts. Phage particles from “white” (β -gal activity negative) plaques were directly PCR amplified using Amplifier Forward and Reverse primers that encompass the EcoR1 insert site. Negative inserts yield a 160 bp band. Bands range in size from 200-1400 bp.

Immunoscreening Genomic DNA Lambda Phage Expression Libraries

Western Blot Analysis of Individual Serum Antibody Responses to Bacteria

Western blot analysis of bacterial lysates was used to determine the relative antibody titer in each human serum sample. This step ensures that the original serum samples were immunoreactive to a broad range of *A. actinomycetemcomitans* antigens and had sufficient antibody titers for immunoscreening. Bacterial lysates for *E.coli*, *P. gingivalis*, or *A. actinomycetemcomitans* were run on SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed overnight with individual serum aliquots (1:500 dilution) followed by goat α -human Ig secondary antibody. CN/DAB substrate was added for visualization of results. Antibody titers in some sera (e.g. serum samples #3 and #4) were found to be too low for use in screening of *A. actinomycetemcomitans* libraries. Instead, high titer sera (e.g. serum samples #1, #2, and #8) were chosen to be used for screening of the expression libraries.

The antigenic bands identified differed between bacterial species. *A. actinomycetemcomitans* often had more antigenic bands and more intense staining than *P. gingivalis*, but not always. Serum from different individuals recognized different antigenic proteins in *A. actinomycetemcomitans* extracts, with different intensities. Thus, the high variability in individual serum antibody response must be taken into consideration when choosing sera for screening of expression libraries. This is unsurprising considering it strongly supports the project's approach which is based on the idea that individuals have different profiles of antibodies.

Figure 7. Western Blot Analysis: Individual Serum Antibody Responses to Bacteria

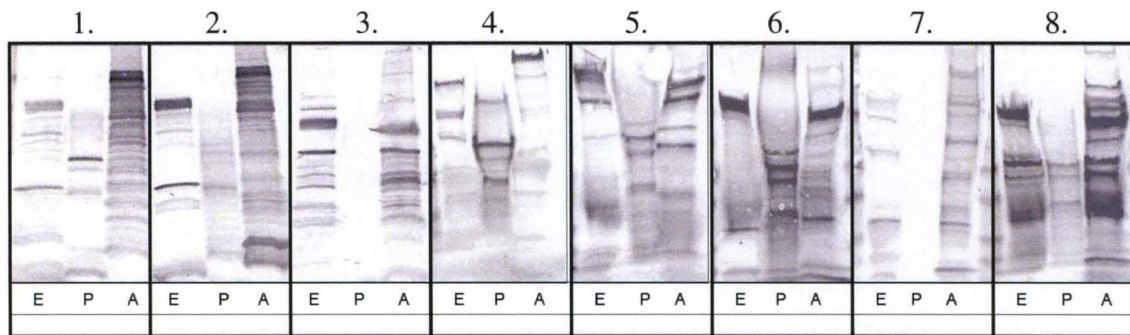


Figure 7. Western blot analysis of individual serum antibody responses to oral bacteria.

1 μ g of total bacterial lysate for *E. coli* (E), *P. gingivalis* (P), or *A. actinomycetemcomitans* (A) was run on a SDS-PAGE gel and transferred to PVDF membrane. Membranes were probed overnight with serum from different individuals using aliquots at 1:500 dilution. Goat α -human total Ig at 1:10k dilution secondary antibody was used and CN/DAB substrate was added for visualization of results. Responses to *A. actinomycetemcomitans* are variable between patients with regard to banding patterns and antibody titers.

Immunoscreening *A. actinomycetemcomitans* Genomic DNA Libraries

Membranes from ZAPII: *A. actinomycetemcomitans* plates were incubated with serum pools, 1:500 in blocking buffer, overnight before incubation for 1 hour with secondary antibody goat α -human total Ig (HRP conjugated) followed by CN:DAB for visualization. In Figure 8A, an individual immunoreactive plaque (box) is seen among approximately 200 plaques in panel field. Plugs from immunoreactive plaques were picked and re-screened for clone isolation. Secondary plating, Figure 8B, increased the percentage of positive clones while tertiary plating (Figure 8C) allowed for isolation of pure clones. As can be seen in Figure 8, each successive plating increased the percentage of positive clones. Each blot also included a positive control spot of crude *A. actinomycetemcomitans* lysate.

About 100,000 plaques have been screened and 72 immunoreactive clones have been isolated. Of the 72 immunoreactive clones picked, 26 made it through primary, secondary, and tertiary screening.

Figure 8. Serum Screening for Immunoreactive Plaques and Clone Isolation

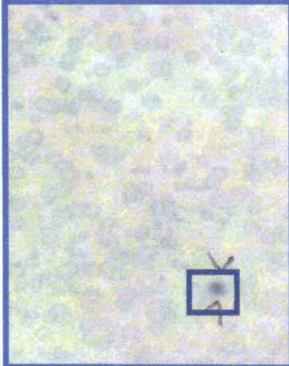
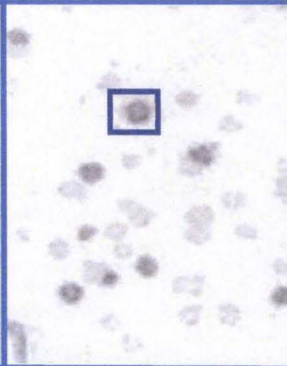

	A.	B.	C.
			
Positive plaques per field	1 of ~200	10 of 25	25 of 25

Figure 8. Serum Screening for Immuno-reactive Plaques and Clone Isolation. Membranes from Lambda ZAPII: A. actinomycetemcomitans plates were incubated with serum pools, 1:500 in blocking buffer (5% milk in TBST), overnight before incubation for 1 hour with HRP conjugated secondary antibody goat α -human total Ig at 1:10,000. In panel A, an individual immunoreactive plaque (box) is seen among ~200 plaques in panel field. Immunoreactive plaques were picked and re-screened for clone isolation. Secondary screening (panel B) of the plaque from panel A exhibits an increased ratio of reactive plaques (dark spots) to non-reactive plaques (light spots). Panel C shows that all the plaques in this tertiary screen are positive.

Identifying Positive *A. actinomycetemcomitans* Gene Sequences

Each plasmid generated from identified immunopositive tertiary plaques was sequenced to identify the proteins expressed. This study used the Nucleic Acids Core at the University of Louisville for sequencing. *A. actinomycetemcomitans* clone sequences were compared to the published genome using BLAST on the JCVI website (cmr.jcvi.org), on the ORALGEN website (www.oralgen.lanl.gov), or on the NIH website (blast.ncbi.nlm.nih.gov). Figure 9 shows a typical BLAST analysis of the DNA sequence of an antigenic peptide found through tertiary screening of clones. Analyses compare the nucleotide sequence of the clone with that found in the database and provide a detailed description of proteins with significant homology.

From the sequences, the insert DNA and 7 codons upstream of the EcoRI insert site were blasted using the TIGR-CMR BLAST database to identify *A. actinomycetemcomitans* genes encoded within the insert. The BLASTx option was utilized to insure that the insert was in the correct reading frame. All sequenced tertiary screened clones were in the correct reading frame. The genes identified are listed in Table 1. Table 2 includes more detailed information about the *A. actinomycetemcomitans* genes. Column 1 of Table 2 gives the Los Alamos National Laboratories gene number. Column 2 gives the gene name. Columns 3, 4, and 5 give the nucleotide identity, gaps in the sequence identification and E value, respectively. Columns 5, 6, and 7 give the protein definition, gene length and the functional class of the protein.

Of these, 7 antigenic peptide sequences appeared to be similar to others we had identified, while the remaining 19 antigenic peptide sequences appeared unique. Of the 19 unique antigens identified, eight appear to be proteins displayed on the bacterial cell

wall, while the remaining eight appear to be protoplasmic proteins. 17 of the 19 unique antigens have not been studied in *A. actinomycetemcomitans* and were instead found through homology to proteins in other species.

The identity of the 19 unique antigens was determined by a comparative analysis of their nucleotide sequences. The comparative analysis showed an 84% or greater homology between the sequence of clones and the sequence in the database. 18 of the 19 unique antigens showed a homology of 93% or greater. Genes ranged in length from 279 bp to 3168 bp.

Figure 9. Blast Search of *A. actinomycetemcomitans* Clone Sequence



Figure 9. Blast Search of *A. actinomycetemcomitans* Clone Sequence. This is an example of a BLAST analysis of an antigen found through tertiary screening of clones. The BLAST results show that the antigenic protein is most likely a conserved hypothetical protein that has not been studied in *A. actinomycetemcomitans*. 686 bp out of 687 bp were identical to the database's sequence of the conserved hypothetical protein. All tertiary screened clones had a homology with the database of greater than or equal to 84%.

Table 1. Antigenic Genes of *Aggregatibacter actinomycetemcomitans* Identified Through the Use of Whole Genome Protein Expression Libraries

Gene	Function	Novel*
hgpA	Hemoglobin binding protein A	No
hgpA ^b	Hemoglobin binding protein A	No
fecB	Iron (III) dicitrate-binding periplasmic	Yes
ftsA	Cell division protein A	Yes
lpcA	Phosphoheptose isomerase	Yes
acrB	Acriflavine resistance protein	Yes
atpA	ATP synthase F1, subunit alpha	Yes
mutL	DNA mismatch repair protein	Yes
amiB	N-acetylmurmoyl-L-alanine amidase II	Yes
dsbA	Thiol-disulfide interchange protein	Yes
cbiQ	Cobalt membrane transport protein	Yes
rplY	50S ribosomal protein L25	Yes
hlpB	Lipoprotein	Yes
pbpG	Penicillin-binding protein	Yes
Unknown 1	Hypothetical protein	Yes
pykA	Pyruvate kinase II	Yes
Unknown 2	Conserved hypothetical protein	Yes
hypF	Hydrogenase maturation protein	Yes
Unknown 3	Conserved hypothetical protein	Yes

Table 2. A.a. Antigen Table

Gene Number	Gene Name	Nucleotide Identity	Gaps	E Value	Definition	Gene Length	Functional Class	A. Sequences Producing Significant Alignments
AA01751	CbiK	686/687 (99%)	1/687 (0%)	0	Putative periplasmic binding protein	759	Cobalt chelatase protein Cell envelope; murein sacculus and	putative ABC-type Co ²⁺ transport
AA02101	pbpG	240/283 (84%)	4/283 (1%)	2.00E-57	Penicillin-binding protein 7	279	peptidoglycan	penicillin-binding protein 7
AA01751		213/217 (98%)	3/217 (1%)	1.00E-104	Conserved Hypothetical Protein			
AA01751		464/466 (99%)	2/466 (0%)	0	Conserved Hypothetical Protein			
AA02682	hypF	690/699 (98%)	4/699 (0%)	0	Hydrogenase maturation protein	2283	Central intermediary metabolism	Hydrogenase maturation protein
AA00763	hgpA, hgbA, hamA	269/278 (96%)	4/278 (1%)	1.00E-126	Hemoglobin binding protein A	1785	Transport and binding proteins; cations	Hemoglobin binding protein
AA01341	acrB	370/370 (100%)	0	0	Acriflavine resistance protein	3168	Drug sensitivity Cell envelope; murein sacculus and	acriflavine resistance protein
AA01588	amiB	718/722 (99%)	2/722 (0%)	0	N-acetylmuramoyl-L-alanine amidase II	1485	peptidoglycan	N-acetylmuramoyl-L-alanine amidase II
AA02099	rplY	170/178 (95%)	7/178 (3%)	4.00E-62	50S ribosomal protein L25	285	Translation	rpl25
AA01756	cbiQ	400/401 (99%)	1/401 (0%)	0	cobalt membrane transport protein	672		
AA00830	ftsA	218/221 (98%)	3/221 (1%)	1.00E-109	cell division protein A	1278	cell division	cell division protein ftsA
AA01586	mutL	469/474 (98%)	4/474 (0%)	0	DNA mismatch repair protein	1848	DNA repair	MutL
AA00795	fecB, feoB	142/147 (96%)		2.00E-64	iron (III) dicitrate-binding periplasmic protein precursor			

Table 2. continued
A.a. Antigen Table

Gene Number LANL	Gene Name	Nucleotide Identity	Gaps	E Value	Definition	Gene Length	Functional Class	A. Sequences Producing Significant Alignments
AA00795	fecB, feoB	142/147 (96%)		2.00E-64	iron (III) dicitrate-binding periplasmic protein precursor	891	Transport and binding proteins, ABC superfamily	Beta-D-galactosidase (lacZ)
AA00764	hgpA, hamA	193/198 (97%)	4/198 (2%)	2.00E-88	Hemoglobin binding protein A	420	Transport and binding proteins; cations	TonB-dependent receptor, putative. Different LANL Gene Number than Tertiary 6
AA02214	pykA	718/735 (97%)	6/745 (0%)	0.00E+00	pyruvate kinase II	1506	Central intermediary metabolism (glycolysis & gluconeogenesis - Carbon fixation)	PykA
AA00860	lpcA, gmhA, isn	268/271 (98%)		1.00E-146	phosphoheptose isomerase	582	Cell envelope, surface polysaccharides	GmhA
AA01751		597/599 (99%)	2/599 (0%)	0.00E+00	Conserved Hypothetical Protein	759	Unknown	Same as #T1
AA02100	hlpB	558/596 (93%)	6/596 (1%)	0.00E+00	lipoprotein	663	Cell envelope; membranes	Lipoprotein HlpB
AA02613		392/396 (98%)	1/396 (0%)	0.00E+00	Conserved Hypothetical Protein	783	Unknown	Unknown
AA01748	dsbA	248/248 (0%)		1.00E-139	thiol:disulfide interchange protein	669	Translation; Protein modification	Thiol:disulfide interchange protein
AA00763	hgpA, hgbA, hamA	401/416 (96%)	8/416 (1%)	0.00E+00	Hemoglobin binding protein A			
AA00763	hgpA, hgbA, hamA	553/560 (98%)	4/560 (0%)	0.00E+00	Hemoglobin binding protein A	1785	Transport and binding proteins; cations	
AA00763	hgpA, hgbA, hamA	422/433 (97%)	4/433 (0%)	0.00E+00	Hemoglobin binding protein A			
AA01486	atpA	617/624 (98%)	6/624 (0%)	0.00E+00	ATP synthase F1, subunit alpha	1539	Energy metabolism	ATP synthase alpha chain
AA02115		378/380 (99%)		0.00E+00	Hypothetical protein (Also possible AA01289 & AA01075 hypothetical proteins with same (0) E-value)	384	Unknown	Integrase

DISCUSSION

A novel research technique was developed to discover antigenic proteins from the periodontal bacterium, *A. actinomycetemcomitans*. The technique takes advantage of the the high gene density of the bacterial genome (i.e., no introns), the efficiency of transformation by lambda vectors, and the high level of protein expression in plaques created by lambda phage. After generating *A. actinomycetemcomitans* genomic DNA expression libraries, the libraries were plated and then probed using human serum to identify periodontal antigens. Identified antigens then undergo additional screenings to confirm and define antigenicity. Bacteriophage encoding antigens were converted to plasmids for archiving and sequenced for gene identification.

The *A. actinomycetemcomitans* whole genome protein expression libraries were made using the Lambda ZapII library construction kit. Libraries consisted of random sized inserts correlating to a range of about 400 to 1400 bp in size. The key insight that allowed this project to proceed is that complex libraries can be screened from small fragments of genomic DNA with an efficiency that will theoretically provide unbiased and complete coverage of all bacterial antigens. My results demonstrated that the unbiased *A. actinomycetemcomitans* genomic DNA expression libraries expressed *A. actinomycetemcomitans* proteins at a high level that allowed subsequent screening with human serum.

Individual patient serum antibody responses to oral bacteria played a large part in the detection of bands by Western blotting. Individual serum antibody responses were highly variable and had to be taken into consideration when choosing sera for screening of expression libraries (Figure 7). Pooling of the most antigenic sera as determined by staining intensity and band dispersion provided optimum results and resulted in a greater number of positive plaques.

Immunoscreening of *A. actinomycetemcomitans* libraries allowed the identification of antigenic plaques which were subsequently isolated and sequenced. Using this technique, 26 antigens were identified, 19 of which were unique. 17 of the 19 novel antigens have not been studied in *A. actinomycetemcomitans*.

The new antigens include a variety of functions, being involved with metabolism, in the hemoglobin binding family, responsible for drug resistance, or of unknown functions. Two of the proteins are recognized as virulence factors, pbpG, a penicillin-binding protein (Keseler, 2005) and integrase (Chen, 2009), which mediates integration of a DNA copy of the microbe genome into the host chromosome. Further, eight of the antigens are found extracellularly while eight are only found intracellularly. The presence of antibodies to cell wall antigens may indicate the individual has either current or recent active infection. The presence of antibodies to cytosolic antigens may indicate that bacteria are being effectively destroyed, making cytosolic proteins available to the immune response. The variety of different antigenic proteins suggests that a clustal analysis of antibodies as biomarkers may be a more productive approach than function-based analysis. That is, identification of a profile of multiple antibodies present in an individual may relate to the future progression of disease.

Over the last decade, approximately 23 *A. actinomycetemcomitans* antigens have been published where assignment to the bacterial gene was possible, a necessary step for identification of the antigenic protein. The novel antigens reported here have significantly increased the number of known *A.actinomycetemcomitans* antigenic genes. The limited overlap between these antigenic clones and the previously reported antigenic genes suggests that there are large numbers of uncharacterized antigens.

The novel immunoscreening technique is effective in identifying periodontal antigens. Future studies should use additional human sera to identify any other *A. actinomycetemcomitans* periodontal antigens. The identification of reactive *A. actinomycetemcomitans* bacterial antigens opens new approaches to the diagnosis and treatment of periodontal disease. Among the new approaches are vaccine candidates, pharmaceutical drug discovery assays, and serodiagnostic assays for research and clinical purposes. To attain these goals, additional studies will need to correlate the presence of specific antibodies to the progression of periodontal disease.

Identification of *A. actinomycetemcomitans* antigens may suggest vaccine candidates (Etz, 2002). For example, by identifying the antigens with the strongest association with mild disease or that protect from disease, one could develop novel vaccine candidates for immunization to prevent periodontal disease in susceptible populations (Page, 2000). Such a vaccine could reduce morbidity associated with periodontal disease and linked systemic diseases.

Additionally, the identification of reactive *A. actinomycetemcomitans* antigens may have utility as a diagnostic tool. Currently, characterization of periodontitis is predominantly based on clinical findings. However, a laboratory test has the potential to

provide a prognostic serodiagnosis predicting the probability of future severe disease or the patient's response to treatment. This would direct the clinician's treatment decisions. For example, a patient with a high probability of future severe disease may be treated more aggressively than one with a low probability of future disease.

Microarrays could potentially provide such a diagnostic tool. Protein microarrays provide a multiplex approach to detect proteins, monitor their expression levels, and investigate protein interactions and functions. Through automation, protein microarrays can carry out large numbers of determinations in parallel, achieving efficient and sensitive high throughput protein analysis. Determinations can be carried out with minimum use of materials while generating large amounts of data. Research with protein microarrays includes protein-antibody, protein-protein, protein-ligand or protein-drug, enzyme-substrate screening and multianalyte diagnostic assays.

Antigen microarrays are a subset of protein arrays that provide a rapid, high-throughput approach to serodiagnosis. Antigen array technologies enable large-scale profiling of the specificity of antibody responses against autoantigens, tumor antigens and microbial antigens. They provide insight into pathogenesis, and will enable development of novel tests for diagnosis and guiding therapy in the clinic (Robinson, 2006). Multiple putative antigen proteins are spotted on the array, and are probed with patient serum to define the patient's specific antibody profile or combinatorial pattern. Bacarese-Hamilton (Bacarese-Hamilton, 2004) found strong concordance between antigen microarrays and ELISA, the most common technique for serodiagnosis of microbial infections. This proof-of-principal for antigen microarray-based serodiagnosis of infectious disease opens up new opportunities to identify multiple specific antibodies in a patient's serum.

The identification of reactive *A. actinomycetemcomitans* bacterial antigens is a key component to developing a serodiagnostic assay for periodontal disease. This thesis research is the first step in developing such an assay. Identified antigens could be screened by microarray to compare antigen profiles of individual periodontitis patients and control subjects. Such profiles may be invaluable in the positive identification of antigens which could either confer resistance to periodontitis or contribute to disease.

In summary, we developed an *A. actinomycetemcomitans* expression library and immunoscreening method in order to identify specific bacterial protein antigens that elicit humoral immune responses during periodontal disease infection. Using this technique, 19 *A. actinomycetemcomitans* antigens recognized by some humans were identified. These novel antigens could have diagnostic utility as well as immunization potential for the diagnosis and prevention of periodontal disease.

REFERENCES

- Apatzidou, D and D Kinane (2004a). "Quadrant root planing versus same-day full-mouth root planing. I. Clinical findings." J Clin Periodontol 31(2): 132-40.
- Apatzidou, D and D Kinane (2004b). "Quadrant root planing versus same-day full-mouth root planing. III. Dynamics of the immune response." J Clin Periodontol 31(3): 152-9.
- Apatzidou, D, M Riggio and D Kinane (2004c). "Quadrant root planing versus same-day full-mouth root planing. II. Microbiological findings." J Clin Periodontol 31(2): 141-8.
- Armitage, G (1999). "Development of a classification system for periodontal diseases and conditions." Ann Periodontol 4(1): 1-6.
- Armitage, G (2003). "Position Paper: Diagnosis of periodontal disease." J Periodontol 74: 1237-47
- Armitage GC, Robertson PB (2009). "The biology, prevention, diagnosis and treatment of periodontal diseases: scientific advances in the United States." J Am Dent Assoc.: 140 Suppl 1:36S-43S.
- Armitage GC, Cullinan MP. (2010). "Comparison of the clinical features of chronic and aggressive periodontitis." Periodontol 2000. 53:12-27.
- Ausubel, FM, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith and K Struhl, Eds. (2000). Current Protocols in Molecular Biology. New York, John Wiley and Sons, Inc.
- Baelum V, Lopez R. (2003) "Defining and classifying periodontitis: need for a paradigm shift?" Eur J Oral Sci 111: 2-6.
- Burt, B (2005). "Position Paper: Epidemiology of Periodontal Diseases." J Periodontol 76: 1406-19.
- Casarin, R. C. V., Del Peloso Ribeiro, É., Mariano, F. S., Nociti Jr, F. H., Casati, M. Z. and Gonçalves, R. B. (2010), "Levels of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, inflammatory cytokines and species-specific

immunoglobulin G in generalized aggressive and chronic periodontitis." J. of Periodontal Research, 45: 635–642.

Chen C., Kittichotirat W., Si Y., Bumgarner R.(2009). "Genome sequence of *Aggregatibacter actinomycetemcomitans* serotype c strain D11S-1." J. Bacteriol. 191:7378-7379

Choi, B, S Park, Y Yoo, S Choi, J Chai, K Cho and C Kim (2000). "Detection of major putative periodontopathogens in Korean advanced adult periodontitis patients using a nucleic acid-based approach." J Periodontol 71(9): 1387-94.

Centers for Disease Control (1997). Third National Health and Nutrition Examination Survey, 1988-94. Hyattsville, MD: Public use data file no. 7-0627.

Corbet, . E. F. and Leung, W. K. (2011), "Epidemiology of periodontitis in the Asia and Oceania regions." Periodontology 2000, 56: 25–64.

Cortelli JR, Cortelli SC, Jordan S, Haraszthy VI, Zambon JJ. (2005)"Prevalence of periodontal pathogens in Brazilians with aggressive or chronic periodontitis." J Clin Periodont. 32(8):860-66.

Costa FO, Guimarães AN, Cota LOM, Pataro AL, Segundo TK, Cortelli SC and Costa JE (2009). "Impact of different periodontitis case definitions on periodontal research." J Oral Sci., 51(2):199-206

Darveau RP (2009). "The oral microbial consortium's interaction with the periodontal innate defense system." DNA Cell Biol. Aug;28(8): 389-95.

Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, Lakshmanan A, Wade WG. (2010). "The human oral microbiome." J Bacteriol. 192(19):5002-17.

Dye, B. (2012). "Global periodontal disease epidemiology." Periodontology 2000 58:1, 10-25

Ebersole, J, D Capelli and M Steffen (1995a). "Longitudinal dynamics of infection and serum antibody in *A. actinomycetemcomitans* periodontitis." Oral Dis 1(3): 129-38.

Ebersole, J, D Capelli and M Steffen (1995a). "Longitudinal dynamics of infection and serum antibody in *A. actinomycetemcomitans* periodontitis." Oral Dis 1(3): 129-38.

Ebersole, J, M Steffen and D Cappelli (1999). "Longitudinal human serum antibody responses to outer membrane antigens of *Actinobacillus actinomycetemcomitans*." J Clin Periodontol 26(11): 732-41.

- Ebersole, JL, D Cappelli, M Sandoval and M Steffen (1995c). "Antigen specificity of serum antibody in *A. actinomycetemcomitans*-infected periodontitis patients." J. Dent. Res. 74(2): 658-666.
- Etz, H, DB Minh, T Henics, A Dryla, B Winkler, C Triska, AP Boyd, J Sollner, W Schmidt, U von Ahsen, M Buschle, SR Gill, J Kolonay, H Khalak, CM Fraser, A von Gabain, E Nagy and A Meinke (2002). "Identification of in vivo expressed vaccine candidate antigens from *Staphylococcus aureus*." Proceedings of the National Academy of Sciences of the United States of America. 99(10): 6573-8.
- Flemmig, T, Selmair, I, Schmidt H and Karch H(1996). "Specific antibody reactivity against a 110-kilodalton *Actinobacillus actinomycetemcomitans* protein in subjects with periodontitis." Clin Diagnostic Lab Immunology 3: 678-681.
- Fine DH (2009). "Of mice and men: animal models of human periodontal disease." J Clin Periodontol. 36(11):913-4.
- Freire MO, Sedghizadeh PP, Schaudinn C, Gorur A, Downey JS, Choi JH, Chen W, Kook JK, Chen C, Goodman SD, Zadeh HH (2011). "Development of an animal model for *Aggregatibacter actinomycetemcomitans* biofilm-mediated oral osteolytic infection: a preliminary study." J Periodontol. 82(5):778-89.
- Keseler IM, Collado-Vides J, Gama-Castro S, Ingraham J, Paley S, Paulsen IT, Peralta-Gil M, Karp PD (2005), "EcoCyc: a comprehensive database resource for *Escherichia coli*." Nucleic Acids Res. 33(Database issue):D334-7
- Haffajee, A. D. and Socransky, S. S. (1994), "Microbial etiological agents of destructive periodontal diseases." Periodontology 2000, 5: 78–111.
- Hart, TC (1997) "Genetic factors in the pathogenesis of periodontitis." Periodontol 2000. 14:202-15.
- Henderson, B, Nair, S, Ward, J and Wilson, M (2003). "Molecular pathogenicity of the oral opportunistic pathogen *Actinobacillus actinomycetemcomitans*." Annu Rev Microbiol 57: 29-55.
- Henderson, B, Wilson, DS, Sharp, L. and Ward J (2002). "*Actinobacillus actinomycetemcomitans*." J. Med. Microbiol. 51(12): 1013-1020.
- Henderson, B and Ward, JM and Ready, D (2010). "*Aggregatibacter (Actinobacillus) actinomycetemcomitans*: a triple A* periodontopathogen?" Periodontol 2000, 54 78-105.
- Jenkinson HF, Lamont RJ. (2005). "Oral microbial communities in sickness and in health." Trends Microbiol. 13(12):589-95.

- Kinane, D and Lappin D, (2001a). "Clinical, pathological and immunological aspects of periodontal disease." Acta Odontol Scand 59(3): 154-60.
- Kinane, D, Mooney, J and Ebersole, J (1999). "Humoral immune response to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in periodontal disease." Periodontol 2000 20: 289-340.
- Kinane, D, M Podmore, M Murray, P Hodge and J Ebersole (2001b). "Etiopathogenesis of periodontitis in children and adolescents." Periodontol 2000 26: 54-91.
- Kinane, DF (2000a). "Causation and pathogenesis of periodontal disease." Periodontology 25: 8-20.
- Kinane, DF, J Mooney and JL Ebersole (2000b). "Humoral immune response to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in periodontal disease." Periodontology 20: 289-340.
- Kornman, KS (1997) "The host response to the microbial challenge in periodontitis: assembling the players." Periodontol 2000. 14:33-53.
- Kuboniwa, M, A Amano, K Kimura, S Sekine, S Kato, Y Yamamoto, N Okahashi, T Iida and S Shizukuishi (2004). "Quantitative detection of periodontal pathogens using real-time polymerase chain reaction with TaqMan probes." Oral Microbiol Immunol 19(3): 168-76.
- Kuramitsu HK, He X, Lux R, Anderson MH, Shi W. (2007). "Interspecies interactions within oral microbial communities." Microbiol Mol Biol 71(4):653-70.
- Loesche, WJ and NS Grossman (2001). "Periodontal Disease as a Specific, albeit Chronic, Infection: Diagnosis and Treatment." Clin. Microbiol. Rev. 14(4): 727-752.
- Lovegrove, J (2004). "Dental plaque revisited: Bacteria associated with periodontal disease." J. New Zealand Soc. Periodontol. 87: 7-21.
- Meisel P, Kocher T. (2009) "Definitions of periodontal disease in research: an alternative view." J Clin Periodontol 36: 411-12.
- Mombelli, A, F Casagni and P Madianos (2002). "Can presence or absence of periodontal pathogens distinguish between subjects with chronic and aggressive periodontitis? A systematic review." J Clin Periodontol 29 Suppl 3: 10-21; discussion 37-8.
- Ng SK, Keung Leung W. (2006) "A community study on the relationship between stress, coping, affective dispositions and periodontal attachment loss." Community Dent Oral Epidemiol 34: 252-266.

- Nibali L, Donos N, Henderson B. (2009) "Periodontal infectogenomics." J Med Microbiol. 58:1269-74.
- Norskov-Lauritsen, N., Kilian, M. (2006) "Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates." Int. J. Syst. Evol. Microbiol., 56, 2135-2146.
- Oz HS, Puleo DA (2011). "Animal Models for Periodontal Disease," J. of Biomedicine and Biotechnology, vol. 2011, 8 pages.
- Page, RC (1997) "Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions." Periodontol 2000. 14:216-48.
- Paster, BJ, Boches, SK, Galvin, JL, Ericson, RE, Lau, CN, Levanos, VA, Sahasrabudhe, A and Dewhirst, FE (2001). "Bacterial Diversity in Human Subgingival Plaque." J. Bacteriol. 183(12): 3770-344.
- Picolos, DK, et al. (2005). "Infection patterns in chronic and aggressive periodontitis." J Clin Perio 32:1055–61
- Rams TE, Listgarten MA, Slots J. (2006) "*Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* subgingival presence, species-specific serum immunoglobulin G antibody levels, and periodontitis disease recurrence." J Periodontal Res 41:228–234.
- Robinson, W (2006) "Antigen arrays for antibody profiling." Current Opinion in Chemical Biology 10:67–72.
- Rodrigues, R, C Goncalves, R Souto, E Feres-Filho, M Uzeda and A Colombo (2004). "Antibiotic resistance profile of the subgingival microbiota following systemic or local tetracycline therapy." J Clin Periodontol 31: 420-427.
- Rylev M, Kilian M. (2008) "Prevalence and distribution of principal periodontal pathogens worldwide." J Clin Periodontol. 35(8):346-61.
- Sambrook, J, Fritsch E and Maniatis T Molecular Cloning; A laboratory Manual.
- Sandholm, L, Tolo K and Olsen I (1987). "Salivary IgG, a parameter of periodontal disease activity? High responders to *Actinobacillus actinomycetemcomitans* Y4 in juvenile and adult periodontitis." J Clin Periodontol 14(5): 289-94.

- Savage, A (2009). "A systematic review of definitions of periodontitis and methods that have been used to identify this disease." J Clin Periodontol. 36(6):458-67.
- Savitt, E and Kent, R (1991). "Distribution of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis by subject age." J. Periodontol 62(8): 490-494.
- Schaudinn, C, Gorur A, Keller D, Sedghizadeh PP, Costerton JW (2009). " Periodontitis: An Archetypical Biofilm Disease." J Am Dent Assoc, Vol 140, No 8, 978-986.
- Schenkein, H "The American Academy of Periodontology. Proceedings of the World Workshop in Clinical Periodontics." J Periodontol 70:457-470.
- Sixou, M, D Duffaut-Lagarrigue and J Lodter (1991). "The distribution and prevalence of Haemophilus actinomycetemcomitans in the oral cavity." J. Biol. Buccale 19(3): 221-228.
- Slots, J (1999). "Update on Actinobacillus Actinomycetemcomitans and Porphyromonas gingivalis in human periodontal disease." J Int Acad Periodontol 1(4): 121-6.
- Slots, J, D Feik and T Rams (1990). "Actinobacillus actinomycetemcomitans and Bacteroides intermedius in human periodontitis: age relationship and mutual association." J Clin Periodontol 17(9): 659-62.
- Socransky, S. S., Smith, C. and Haffajee, A. D. (2002), "Subgingival microbial profiles in refractory periodontal disease." Journal of Clinical Periodontology, 29: 260–268.
- Song, H, E Kozarov, S Walters, S Cao, M Handfield, J Hillman and A Progulsk-Fox (2002). "Genes of Periopathogens Expressed During Human Disease." Ann. Periodontol 7: 38-42.
- Van Dyke TE, Sheilesh D (2005). "Risk factors for periodontitis." J Int Acad Periodontol.J;7(1):3-7.
- Yang, H, S Asikainen, B Dogan, R Suda and C Lai (2004). "Relationship of Actinobacillus actinomycetemcomitans serotype b to aggressive periodontitis: frequency in pure cultured isolates." J Periodontol 75(4): 592-9.
- Zambon, J (1996). "Periodontal diseases: microbial factors." Ann Periodontol 1(1): 879-925.

CURRICULUM VITA

Gerald Bernard Pevow

Experience	2000–2006	Valen Biotech, Inc.	Atlanta, GA	
	President and CEO			
	<ul style="list-style-type: none">▪ Founded molecular diagnostic company focused on Oncology and Infectious Disease▪ Successfully raised \$500,000 in seed capital▪ Patented method for recombinant protein purification▪ Developed key partnerships to grow revenue			
	1998–1999	Abgenix, Inc.	Fremont, CA	
	Business Development Manager			
<ul style="list-style-type: none">▪ Negotiated licensing, non-disclosure, and material transfer agreements▪ Conducted and managed pharmaceutical market research▪ Developed company literature and promotional materials▪ Performed financial modeling				
	1994–1998	Becton Dickinson, Clontech Laboratories	Palo Alto, CA	
	Market Manager/Product Manager			
	<ul style="list-style-type: none">▪ Successfully managed a large product portfolio, launching 20 products annually▪ Created and implemented marketing plans▪ In-licensed new technologies and products▪ Conducted market research, developed forecasts and prepared budgets			
	Full Time 1999–2000 Part Time 1994-1998	The Mendel Group	Alpharetta, GA	
	Pharmaceutical Marketing Consultant			
<ul style="list-style-type: none">▪ Conducted consulting projects in pharmaceutical business development, marketing, strategic planning, and competitive intelligence▪ Managed East Coast pharmaceutical accounts▪ Negotiated, wrote, and closed project proposals ranging from \$10,000 - \$300,000				
	1992–1994	Lederle Laboratories	Salt Lake City, UT	
	Oncology Sales Representative			
	<ul style="list-style-type: none">▪ Marketed and successfully sold oncology products to physicians, hospitals, and clinics▪ Effectively managed the territory of Montana, Utah, Idaho, Nevada, and Washington			
	Education	2006 – Present	Univ. of Louisville School of Dentistry	Louisville, KY
		<ul style="list-style-type: none">▪ Pursuing joint D.M.D./M.S. Oral Biology: Expected graduation date May 2012▪ Part-time research assistant, identifying periodontal antigens by immunoscreening▪ 2006 Research Louisville		
1989–1991		Tulane – A.B. Freeman School	New Orleans, LA	
<ul style="list-style-type: none">▪ M.B.A, Marketing and International Business▪ Awarded A.B. Freeman Merit Fellowship▪ A.B. Freeman Study Abroad Program, Haute Etudes Commerciale▪ Internships: IBM (Budapest, Hungary) Vitkovice (Ostrava, Czech Republic)				
1984–1988		The University of Texas at Austin	Austin, TX	
<ul style="list-style-type: none">▪ B.S, Molecular Biology, Chemistry concentration▪ Dean's List - College of Natural Sciences				

Activities
Skills &
Interests

Louisville American Student Dental Association, American Marketing Association,
Licensing Executive Society, Society of Competitive Intelligence Professionals
Proficient in Spanish
Biotechnology, Dentistry, Basketball, Electronics, Painting, Skiing, and Fishing