COMPLEX HOST-MICROBE INTERACTIONS OF THE ORAL CAVITY REVEALED BY EPITHELIAL TRANSCRIPTOMICS

By

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To my family

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1KNN	1st nearest neighbor
3KNN	3rd nearest neighbors
A	absorbance units
Aa	*Aggregatibacter actinomycetemcomitans*
AFLP	amplified (restriction) fragment length polymorphism
AIDS	Acquired Immunodeficiency Virus
ATCC	American Type Culture Collection
BEC(s)	buccal epithelial cell(s)
BoP	bleeding on probing
BRB	Biometric Research Branch
BSA	bovine serum albumin
CA	California
Ca^{2+}	Calcium
CAL	clinical attachment loss
CAM	camptothecin
C	celsius
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CMV	cytomegalovirus
CO_2	carbon dioxide
COOH	carboxyl
CP	chronic periodontitis
CPS	capsular polysaccharide

CTRL	control
DAG	directed acyclic graphs
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
dPBS	Dulbecco's phosphate buffered saline
ELISA	enzyme-linked immunosorbant assay
EPEC	enteropathogenic *E. coli*
EST	expressed sequence tag
F-actin	filamentous actin
G_0	quiescence
G_1	first gap phase
G_2	second gap phase
GCOS	GeneChip operating software
GEC(s)	gingival epithelial cell(s)
GI	gastrointestinal
GO ID	gene ontology identity
GO	gene ontology
HACEK	*Haemophilus* spp. (except *H. influenzae*), *Aggregatibacter actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens*, and *Kingella kingae* complex
HG	human genome
h	hour(s)
HIGK(s)	human immortalized gingival keratinocyte(s)
HIV	human immunodeficiency virus

HPV	human papillomavirus
IADR	International Association for Dental Research
IL	interleukin
iNOS	inducible nitric oxide synthase
IVIAT	*in vivo*-induced antigen technology
k	kilo
kDa	kiloDaltons
KEGG	Kyoto Encyclopedia of Genes and Genomes
KGP	lysine specific gingipain
KSFM	keratinocyte serum free medium
LAP	localized aggressive periodontitis
LOOCV	leave one out cross validation
LPS	lipopolysaccharide
mg	milligram
mL	milliliter
M	mitosis
mM	millimolar
MN	Minnesota
MOI	multiplicity of infection
MPSS	massively parallel signature sequencing
mRNA	messenger ribonucleic acid
Nal^R	nalidixic acid resistant
NFAT	nuclear factor of activated T-cells

NH_2	amino
NIDCR	National Institute of Dental and Craniofacial Research
nm	nanometers
NY	New York
OD	optical density
ORF	open reading frame
P	probability value due to chance
PAI	pathogenicity island
PAMP(s)	pathogen-associated molecular pattern(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	pocket depth
PEDANT	protein extraction, description, and analysis tool
pH	potential of Hydrogen
PHUEC(s)	primary human urethral epithelial cell(s)
rcf	relative centrifugal force
RGP	arginine specific gingipain
Rif^R	rifampicin resistant
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
RTX	repeats in toxin

SAGE	serial analysis of gene expression
STS	staurosporine
SUNY	State University of New York
T4SS	type four secretion system
TLR	TOLL-like receptor
TNF	tumor necrosis factor
TSBYE	trypticase soy broth yeast extract
UEC(s)	urethral epithelial cell(s)
UFCD	University of Florida College of Dentistry
USA	United States of America
U	units
VT	Vermont
YoP	Yersinia outer protein
µg	microgram

Abstract of Dissertation Presented to the Graduate School
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Periodontal diseases result from bacterial infection by several pathogens combined with a destructive host immune response. Host-pathogen interactions are inherently dynamic and complex and the unique environment of the oral cavity further complicates the relationship between hosts and microbes. It is estimated that more than 700 bacterial species can inhabit the oral cavity, and a single individual may support 30 to 80 bacterial species at a given time. These bacteria interact with each other as well as with the host, and the impact of additional factors such as smoking or diabetes mellitus further influences the state of health or disease. Although there are many risk factors associated with periodontal disease, true prognostic indicators are lacking and require development. To achieve this goal, gaining an understanding of the host pathogen interactions in the oral cavity, and determining key events that shift the balance from health to illness are crucial aspects of effectively treating periodontal disease.

This study utilized transcriptional profiling to investigate the interactions between human immortalized gingival keratinocytes (HIGKs) and two oral pathogens, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. A model using HIGK cells was developed, and the most-impacted host pathways were further characterized phenotypically. Insights gained were related to tropism of oral bacteria to oral epithelia, a specific epithelial

response to different species of bacteria, and the contribution of specific bacterial components to the bacterial-host interactions. The impacts of *P. gingivalis* YPF1, *A. actinomycetemcomitans* CDT, ORF859, and Aae upon the host transcriptome were investigated. In collaboration with other researchers, the impact of *P. gingivalis* SerB was also studied. Also in collaboration, the interactions of epithelial cells to commensal *S. gordonii* and the opportunistic commensal *F. nucleatum* have been initiated.

This work focuses on the host-pathogen interplay occurring in the oral epithelium. The model system established, and the insights provided herein based on host cell global responses will lead to a greater understanding of host pathogen interactions.

CHAPTER 1
INTRODUCTION

Periodontal Disease Is a Significant Health Concern

According to the World Health Organization, Oral health means being free of chronic mouth and facial pain, oral and throat cancer, oral sores, birth defects such as cleft lip and palate, periodontal disease, tooth decay and tooth loss, and other diseases and disorders that affect the mouth and oral cavity (World Health Organization, 2007). Unfortunately, both internationally and in the United States, society is far from attaining complete oral health among its citizens. Worldwide, the most common oral diseases are dental cavities and periodontal disease. In each country, 60-90% of school children worldwide have dental cavities and 5-20% of middle-aged adults have severe periodontal disease, with rates varying across geographical regions. Incidence of oral cancer, birth defects such as cleft lip and palate, and occurrences of bacterial or fungal oral infections among immune-compromised individuals is also a significant health concern. In many high income countries, oral health accounts for 5-10% of public health expenditures. In 2006, Americans made approximately 500 million visits to dentists, and an estimated $94 billion was spent on dental services (Office of the U.S. Surgeon General, 2007). Additionally, there is a strong association between periodontal disease in pregnant women and the increased birthrate of pre-term low birth weight infants (Offenbacher *et al.,* 1996; Loesche, 1997; Dasanayake, 1998; Li *et al.,* 2000; Yeo *et al.,* 2005; Alves and Ribeiro, 2006), as well as an association between periodontal disease and cardiovascular disease (Demmer and Desvarieux, 2006; Ellis *et al.,* 2007; Tonetti *et al.,* 2007).

The Oral Cavity

The oral cavity, or cavum oris, is divided into the vestibule and the mouth cavity proper. The outer boundary of the vestibule is the area comprising the cheeks and lips, and the inner

boundary is formed by the gums and teeth. The mouth cavity proper consists of the area within the aleveolar arches and teeth, hard and soft palate, tongue and mucous membrane to the isthmus faucium just before the pharynx (Lewis and Gray, 1918). The tissues that support the teeth are known as the periodontium and consist of the cementum, alveolar processes of the maxillae and mandible, periodontal ligaments, and gingiva (SweetHaven Publishing Services, 2006).

The oral cavity is a unique and complex environment. As such, a high diversity of bacterial species exists in this microecological niche. Cloning and sequencing of 16s rRNA originally isolated from the subgingival plaque of both healthy and diseased volunteers revealed the presence of 347 species or phylotypes (Paster *et al.,* 2001). Predictive statistical methods revealed that 68 species of bacteria were not sampled, but would be found in the population at large upon 10,000 additional clones being analyzed—increasing the potential total to 415 species. The authors estimated that by including other oral surfaces—such as the cheek, tongue, and teeth—the number of bacterial inhabitants in the oral cavity was very likely to be 500 to 600 species (Paster *et al.,* 2001). Identical methods were used to assess the microbial diversity that is found colonizing the tongue dorsa of healthy individuals, and those with halitosis confirming this prediction. In the above study, 630 total bacterial species were identified from the mouths of volunteers, and estimated the total number to surpass 700 (Kazor *et al.,* 2003). An investigation of the bacterial flora found in healthy volunteers showed that a given individual is colonized by 30 to 80 of the possible 700 species at any given time (Aas *et al.,* 2005). A study performed prior to the detection of these 700 species estimated that approximately 25 species of the oral bacteria are pathogenic, or at least have association with periodontal disease (Moore *et al.,* 1982). A more recent study, which included a screen for recently discovered bacteria, has implicated several new bacteria associated with periodontal disease (Kumar *et al.,* 2003). The

estimated number of oral pathogens is very likely to increase, as new tools allowing the detection of uncultivable bacteria are applied to periodontal disease research.

The anatomy of the oral cavity has several features that promote microbial growth and survival and simultaneously present a challenge to clinicians who wish to treat microbial infections (Haffajee and Socransky, 1994). The teeth are a non-shedding foundation for bacterial colonization, which allows for the accumulation over time of bacterial biofilms in the form of plaque. A recent review summarized the general features of oral biofilms and their impact on periodontal health and disease (ten Cate, 2006).

The structure and physiology of oral biofilms resembles those found in other anatomical sites or in the external environment. Pillars of co-aggregated bacteria have been described as mushroom like, interspersed with bacteria-free channels that are probably filled with extracellular polysaccharide (EPS). These channels are a conduit for the flow of nutrients, waste products, and salivary components of the host. According to the ecological plaque hypothesis (Marsh, 1994), the composition of an oral biofilm significantly influences the development of dental caries, periodontal disease or can promote dental health. The sequence of oral plaque biofilm formation generally begins with gram positive early colonizers from the *Streptococcus* genus. *Fusobacterium nucleatum* is considered a bridge organism, which joins the plaque biofilm at an intermediate stage and then allows late colonizers to eventually attach (ten Cate, 2006). The members of the resulting bacterial community communicate through quorum sensing, and change their gene expression based on population densities. Both antagonistic and synergistic interactions occur simultaneously, and the outcome is a diverse microenvironment within the biofilm that displays variable redox potential, salivary flow, nutrient availability, and pH depending on the location. Individual bacterial species can make the environment more or

less favorable for other species through several actions, such as bacteriocin production, acid production, or secretion of EPS.

One strategy proposed in the ecological plaque hypothesis is to influence the formation of the plaque biofilm to make the conditions favorable to health-related bacteria, and simultaneously prevent biofilm communities favorable to pathogens (Marsh, 1994). Cariogenic biofilms are promoted by high-sucrose diets and other factors which lead to *mutans Streptococci* proliferation. The biofilm eventually becomes acidic, and leads to the predomination of acid tolerant bacteria that produce lactic acid and damage tooth enamel. In the periodontal disease model, the availability of oxygen decreases as the oral biofilm increases in density. Gram negative anaerobes begin to dominate and thrive, including the late colonizers *P. gingivalis* and *A. actinomycetemcomitans*. The formation of such a biofilm places the gingival and underlying tissues in danger, and the prolonged assault by bacteria upon these tissues can occur from a relatively stable "home base" which the plaque provides (Haffajee and Socransky, 1994).

The difficulty of eradicating bacteria in a biofilm is also greater than the sum of treating each strain individually. The homeostatic balance of the biofilm often induces bacteria to slow their metabolism, rendering many antibiotics that target actively growing bacteria ineffective. The EPS secreted by bacteria often binds pharmaceutical agents before they can target the bacterial cells. As a result, the required concentration of antibiotic to kill bacteria in a biofilm may be 1000 fold greater than the dose required to kill an equal number of planktonic bacterial cells. Insufficient dose leads to the development of persisting bacteria, which increases the likelihood of resistant strains evolving. The measurement Minimum Biofilm Eradication Concentration (MBEC) has replaced the Minimum Inhibitory Concentration (MIC) in many

cases, which reflects the challenge of treating bacterial infections occurring as a biofilm, such as dental plaque (ten Cate, 2006).

Multifactorial Disease

Destruction of the periodontal ligament and resorption of the alveolar bone leading to tooth loss is the hallmark of periodontal disease. Periodontal disease has recently been described as "a heterogeneous group of pathoses characterized by a predominance of specific infectious agents in the face of inadequate local host defenses" (Slots, 2000); a characterization which reflects the complexity of periodontal disease. Many risk factors for periodontal disease are well documented, and events that may contribute to periodontal disease progression are characterized. Descriptive trends have been realized for actual clinical outcomes based on these factors. However, predictable outcomes based on known virulence factors are unreliable, and the exact mechanisms of the disease process are unknown (Slots, 2000). Individuals may remain periodontially healthy for prolonged periods in the presence of these risk factors until an unknown triggering event shifts the balance towards periodontal disease.

Risk Factors

The body of evidence in periodontal disease research has provided strong support for the existence of several risk factors which increase an individual's likelihood of developing periodontal disease. A recent epidemiological review of periodontitis cases cited several major risk factors, such as cigarette smoking, poorly controlled diabetes mellitus, and infection by specific bacteria at high levels. Obesity, osteopenia/osteoporosis, socioeconomic status, and HIV infection are additional factors which tend to coincide with periodontal disease (Borrell and Papapanou, 2005). Genetic predisposition of certain individuals to periodontal disease (Kinane *et al.,* 2005; Loos *et al.,* 2005; Shapira *et al.,* 2005; James *et al.,* 2007) and the potential contribution of herpesviruses to disease (Slots, 2005; Wu *et al.,* 2006; Teughels *et al.,* 2007) are

additional factors that deserve further investigation. Therefore, a multitude of factors can influence the development and progression of periodontal disease. One goal of periodontal disease research is to determine consistent prognostic indicators from these factors to improve treatment of this complex and challenging disease.

Damage by Bacteria and Destructive Immune Response

The risk factor most implicated in periodontal disease is the infection by specific bacterial pathogens at high levels combined with a destructive host immune response (Sakamoto *et al.,* 2005; Teng, 2006a). Subgingival plaque accumulation consists of a multitude of oral bacteria allowing pathogenic bacteria to colonize the periodontium. The actions of bacterial virulence factors directly—or indirectly through the activation of the immune system—cause swelling, inflammation, and pocket formation. Eventually, this can lead to detachment of the periodontal ligament and tooth loss through resorption of supporting alveolar bone. The balance between protective and destructive immune responses is a key determinant of disease progression. This balance is strongly influenced by the host response to challenge by subgingival bacteria (Teng, 2006b). Recently, two competing models of disease progression, the Random Burst theory (Socransky *et al.,* 1984) and the Linear Progression theory were purportedly reconciled as essentially the same phenomenon (Gilthorpe *et al.,* 2003).
Linear progression held that destructive periodontal disease is a continuous phenomenon that occurs at a relatively constant pace and only varies between population groups (Loe *et al.,* 1978; Gilthorpe *et al.,* 2003). The Random Burst theory was proposed to explain findings from longitudinal studies which contradicted the linear progression model. Clinical attachment loss (CAL) and loss of alveolar bone were found to occur at rates both faster and slower than the rate predicted under the linear progression hypothesis. Periodic bursts of destruction followed by periods of remission were proposed to explain the discrepancies. To reconcile these two

theories, multilevel modeling examined the patterns of disease progression while considering several covariates in the context of a hierarchical structure (Gilthorpe *et al.,* 2003). Patterns consistent with both linear and random burst theories were uncovered, depending on the variable studied. The frequency and randomness of sampling from diseased or healthy sites was found to influence the interpretation of clinical data towards either the linear or random burst pattern of progression. The underlying phenomenon that led to both patterns previously described were cycles of damage and repair of the periodontium. These observations were found to represent real changes in the health or disease state of individual sites, and not simply a result of measurement error. Thus, the temporal component of periodontal disease progression or regression further emphasizes the dynamic nature of this disease.

Studies have been undertaken to identify the causative organisms that participate in this cycle of destruction and repair. In one study, the community associations of oral bacteria correlating to health or disease were described. Subgingival plaque samples were obtained from healthy and diseased individuals for analysis. Several statistical models were utilized to calculate relatedness coefficients, which demonstrate how frequently specific bacterial species are found complexed together in both healthy and diseased sites. The result of this study is the formulation of the Red, Orange, Yellow, Green, and Violet complexes of oral bacteria (Socransky *et al.,* 1998). The Red complex, which included *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia,* strongly correlates to Chronic Periodontal disease. *Aggregatibacter actinomycetemcomitans* complexed with other bacterial species and was not strongly correlated with CP, consistent with its association with Localized Aggressive Periodontitis. This work implicates several bacterial species for their putative roles in periodontal health or disease. Another study utilized checkerboard DNA-DNA hybridization to

assess the predominant organisms in subgingival plaque among patients from different geographical regions. The distribution of bacterial species with putative involvement in periodontal disease was investigated in plaque samples from patients in the USA, Sweden, Chile, and Brazil. These patients were also assessed clinically for severity of disease. Significant differences between the bacterial floras isolated from the participants that correlated to their country of origin. Thus, a geographical component also appears to play a role in periodontal disease (Haffajee *et al.,* 2004). A similar finding was made in a study that investigated the serum antibody levels to several strains of *A. actinomycetemcomitans* and *P. gingivalis* found in patients from the USA and Turkey. The findings demonstrated the potential for antigenic diversity among *A. actinomycetemcomitans* and *P. gingivalis* strains from different geographical regions (Celenligil and Ebersole, 1998).

The 1996 World Workshop in Periodontics Consensus Report recognized three species as causative organisms of periodontal disease (Borrell and Papapanou, 2005). These organisms are: *Tannerella forsythia* [(Maiden *et al.,* 2003), formerly *Tannerella forsythensis* (Sakamoto *et al.,* 2002) and *Bacteroides forsythus*], *Porphorymonas gingivalis*, and *Aggregatibacter* [(Norskov-Lauritsen and Kilian, 2006), formerly *Actinobacillus actinomycetemcomitans*]. However, in light of continuing efforts to identify key periodontal pathogens, and the identification of uncultivable bacteria from the oral cavity, the number of formally recognized periodontal pathogens is likely to increase (Kumar *et al.,* 2003). The following will briefly introduce *A. actinomycetemcomitans* and *P. gingivalis*, the recognized etiological agents of Localized Aggressive Periodontitis (LAP) and Chronic Periodontitis, (CP), respectively, and the model organisms for this study.

Aggregatibacter actinomycetemcomitans

General characteristics

Aggregatibacter actinomycetemcomitans was originally named *Bacterium Actinomycetem comitans* by Klinger in 1912, as a consequence of its initial isolation from oral actinomycotic lesions in combination with *Actinomyces israelii* (Kachlany *et al.,* 2001). *A. actinomycetemcomitans* has been demonstrated to support the growth of *Actinomyces* (Hoffler *et al.,* 1980), and complexes with other bacteria in subgingival plaque (Socransky *et al.,* 1998). *Actinomycetemcomitans* literally means "accompanying an actinomycete" (Quercia *et al.,* 2006). This bacterium has also been briefly referred to as *Haemophilus actinomycetemcomitans* (Potts *et al.,* 1985; Ohta *et al.,* 1986; Henderson *et al.,* 2003). The creation of a new genus to classify *A. actinomycetemcomitans* and three closely related species and subsequent name change to *Aggregatibacter actinomycetemcomitans* satisfies categorization issues with this bacterium (Norskov-Lauritsen and Kilian, 2006). The 2105 kb genome of strain HK1651 has been sequenced and is publicly available (Roe *et al.,* 2003).

A. actinomycetemcomitans is a gram negative coccobacillus that is non motile, saccharolytic, and capnophilic. Isolation of *A. actinomycetemcomitans* from within buccal epithelial cells obtained from periodontal disease patients confirms this bacterium is an invasive organism *in vivo* (Rudney *et al.,* 2001; Rudney *et al.,* 2005). The ability of this bacterium to invade several cell types *in vitro* has also been demonstrated to varying extents in several cell lines (Brissette and Fives-Taylor, 1999; Schenkein *et al.,* 2000; Handfield *et al.,* 2005). The mechanism of invasion is variously described as involving both host cell actin and microtubule rearrangement that resembles invasion strategies of enteric bacteria (Meyer *et al.,* 1996; Meyer *et al.,* 1997; Brissette and Fives-Taylor, 1999; Meyer *et al.,* 1999). *A. actinomycetemcomitans* also belongs to the HACEK group (*Haemophilus* spp. (except *H. influenzae*), *Aggregatibacter*

actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens, and *Kingella kingae*) of pathogens responsible for infective endocarditis in 2%-5% of culture-positive cases (Paturel *et al.,* 2004; Petti *et al.,* 2006). References associating *A. actinomycetemcomitans* and bacterial endocarditis can be found as early as 1966 (Overholt, 1966).

There currently are 6 recognized serotypes describing *A. actinomycetemcomitans* isolates based on the O-polysaccharide structure of the lipopolysaccharide, designated a-f (Zambon *et al.,* 1983; Saarela *et al.,* 1992; Gmur *et al.,* 1993; Kaplan *et al.,* 2001; Henderson *et al.,* 2003). Some serotypes are more prevalent in specific geographical areas or among certain ethnic groups (Henderson *et al.,* 2003). Additionally, the majority of individuals tend to be stably infected with *A. actinomycetmcomitans* from a single serotype rather than population of mixed clonality. A change in the serotype infecting an individual is a rare event, even after years of treatment directed towards eradication of *A. actinomycetemcomitans* infection (Saarela *et al.,* 1992; Saarela *et al.,* 1999). Generally, serotype b strains are most frequently isolated from localized aggressive periodontal disease patients, and serotype c strains are most often associated with healthy subjects (Asikainen *et al.,* 1991; Henderson *et al.,* 2003; Yang *et al.,* 2005). However, an association of serotype c strains among Japanese chronic and generalized aggressive periodontal patients has recently emerged (Wang *et al.,* 2005a; Thiha *et al.,* 2007).

A. actinomycetemcomitans forms rough phenotype colonies with characteristic crossed cigar morphology visible under low power magnification upon initial clinical isolation and growth on agar. This characteristic is the result of fibril formation, which are composed of Flp-1 pili and aid in the bacterial adhesion to various surfaces, including plastic and saliva coated hydroxyapatite—a condition that approximates the tooth surface. In broth culture, the rough phenotype *A. actinomycetemcomitans* autoaggregates and forms a film on exposed culture flask

surfaces. Upon subsequent subcultures, a smooth phenotype *A. actinomycetemcomitans* emerges, which grows as planktonic bacteria in liquid culture and eventually predominate over rough phenotype *A. actinomycetemcomitans*. On agar, smooth phenotype *A. actinomycetemcomitans* form colonies that are opaque and lack the crossed cigar pattern. The transition from the rough to smooth phenotype is partially based on spontaneous mutations in the *flp* operon promoter region and renders the clones deficient in fimbriae, although this is not the only mechanism of the rough to smooth conversion (Wang *et al.,* 2005b). Mutations engineered into the *tad* locus, which encodes a molecular transport system dedicated to the production of Flp pili, have been demonstrated to abrogate the formation of fibrils *in vitro*. Conceivably, similar mutations occurring spontaneously could also result in the loss of this non specific tight adherence phenotype (Schreiner *et al.,* 2003; Perez *et al.,* 2006).

Virulence factors

A. actinomycetemcomitans possesses several adhesins which allow colonization of the oral cavity in addition to Flp pili. Three nonfimbrial adhesins are currently recognized: Aae, Omp100, and EmaA. The autotransporter adhesin, Aae, confers specific adherence of *A. actinomycetemcomitans* to epithelial cells of humans and old world primates (Rose *et al.,* 2003; Fine *et al.,* 2005). The 130 kDa Aae proteins are surface expressed and possess homology to the autotransporter proteins Hap and Hia of *Haemophilus influenzae* (Rose *et al.,* 2003). Different strains of *A. actinomycetemcomitans* have one to four copies of *aae*. The number of copies can affect the binding of host defense factors, such as lactoferrin and milk whey to these strains. Strains with one or two copies of *aae* seem to escape interaction with these factors and therefore polymorphisms of *aae* may affect the pathogenic potential of some *A. actinomycetemcomitans* strains (Rose *et al.,* 2003). Omp100, or ApiA, also is important for *A. actinomycetemcomitans* binding to human epithelial cells (Yue *et al.,* 2007). This protein binds buccal epithelial cells

with lower affinity than Aae, yet demonstrates the same specificity for human epithelial cells described previously (Fine *et al.,* 2005; Yue *et al.,* 2007). The binding ability of an Aae-Omp100 double mutant was completely abrogated in a recent report (Yue *et al.,* 2007). Omp100 is a 100 kDa outer membrane protein with a role in host cell invasion in addition to adhesion (Li *et al.,* 2004). The protein also is able to induce pro-inflammatory cytokine expression in epithelial cells (Komatsuzawa *et al.,* 2002; Asakawa *et al.,* 2003). EmaA, also known as ApiB is an orthologue of *Yersinia enterocolitica* YadA, and is important in the binding of *A. actinomycetemcomitans* to collagen (Mintz, 2004; Ruiz *et al.,* 2006). EmaA is predicted to be 201 kDa, but runs aberrantly by gel electrophoresis. This protein consists of a membrane anchor region, a stalk, and a head domain (Ruiz *et al.,* 2006). ApiC is also belongs to the oligomeric coiled-coil adhesins (Oca) represented by *Y. enterocolitica* YadA and participates in collagen binding of *A. actinomycetemcomitans*. Finally, the matrix polysaccharaide PGA and glycoside hydrolase Dispersin B (DspB) play a role in the dynamics of *A. actinomycetemcomitans* biofilm formation and release, respectively (Kaplan *et al.,* 2004; Ramasubbu *et al.,* 2005).

A. actinomycetemcomitans also possesses several components which directly act upon various eukaryotic cell types and cause toxicity. The LtxA leukotoxin kills polymorphonuclear leukocytes and monocytes of humans, old world primates, and great apes (Taichman *et al.,* 1980; Diaz *et al.,* 2006) and is a member of the RTX (repeats in toxin) exotoxin family. The generic RTX toxin operon consists of four genes transcribed in the order *rtxCABD*. RtxA is generally the secreted active toxin, and the RtxB protein is generally involved in post-translational modifications which are required for the toxin to be active biologically. The C and D products appear to be involved in the transport of RtxA. RTX toxins contains a characteristic consensus amino acid sequence of GGXGXDX[L/I/V/W/Y/F]X, where X is any amino acid. This

sequence is tandemly repeated between six and 40 times, depending on the toxin (Lally *et al.,* 1999).

RTX toxins generally are believed to affect target cells in two stages. The first stage is a non-specific adsorption to the cell surface. The second phase involves membrane insertion of the toxin. According to one model of RTX toxicity, a hydrophobic region of the RtxA toxin is believed to form pores in the target cells, leading to cell death. In high concentrations, cell death is characteristic of necrosis. Low doses, however, result in apoptotic cell death. A second model proposes that the toxin incompletely inserts into the target cell membranes, and remains in the outer leaflet. By displacing lipids in the outer leaflet, pressure placed on the monolayer causes the collapse of the membrane (Kachlany *et al.,* 2000).

Leukotoxin is unique from other RTX toxins, in that the majority of LtxA produced in rough phenotype strains remains attached to the bacterial surface, although small amounts of leukotoxin are secreted by selected rough strains. This implies that direct bacteria-cell contact is necessary before the leukotoxin killing activity occurs. In contrast, the smooth phenotype *A. actinomycetemcomitans* isolates secrete large quantities of leukotoxin during early growth stages, but the amount of leukotoxin secreted decreases as cultures age. After prolonged growth, leukotoxin is apparently degraded by an unknown mechanism (Kachlany *et al.,* 2000). The expression of leukotoxin is regulated by oxygen tension, iron concentration (Spitznagel *et al.,* 1995) and pH (Kachlany et al 2000), which suggests adaptation to the environment of the oral cavity.

The specificity of *A. actinomycetemcomitans* leukotoxin to leukocytes of humans, old world primates and great apes (Taichman *et al.,* 1987), combined with the finding that these target cells uniquely produce Lymphocyte Function Associated Antigen-1 (LFA-1), led to the

discovery that LFA-1 is the specific target of leukotoxin. In 2006, Fong and colleagues demonstrated the requirement of lipid rafts for this process to occur (Fong *et al.,* 2006). Leukotoxin treatment was observed to cause elevated levels of cytosolic Ca^{2+} and the accumulation of LFA-1 clustering in lipid rafts. Dispersal of lipid rafts was found to prevent the cytotoxic effects of LtxA, which could be restored by reconstituting the lipid rafts. Consistent with the two-stage hypothesis of RTX toxin activity, the authors showed that LtxA initially signals targeted cells to release Ca^{2+}, which activated the intracellular protease calpain. Calpain cleaves talin, which anchors LFA-1 to the cytoskeleton. This cleavage event allows LFA-1 to cluster on the cell surface in the association with lipid rafts. LtxA then binds LFA-1, and the toxin moves into the lipid microdomain according to the general RTX two-stage mechanism.

Recently, CD18 was identified as the specific receptor for LtxA (Dileepan *et al.,* 2007). CD18 is the β_2 integrin subunit that combines with CD11a to form the LFA-1 complex. Using chimeric human (LtxA susceptible) and bovine (LtxA resistant) LFA-1 composed of different CD18 and CD11a fragments, the functional receptor for LtxA was mapped to specific integrin-epidermal growth factor like domains (I-EGF) 2, 3, and 4 of CD18. One advantage arising from the effects of *A. actinomycetemcomitans* leukotoxin is to enable the bacterium to escape local immune defenses and establish an infection (Narayanan *et al.,* 2002). The JP2 clone is hyper-virulent and is associated with severe forms of LAP. It possesses a 530 bp promoter deletion that confers higher leukotoxin activity, corroborating the importance of this virulence factor (Brogan *et al.,* 1994; Haubek *et al.,* 2007).

Associated with leukotoxin transport, the protein TdeA is implicated as a toxin and antibiotic exporter (Crosby and Kachlany, 2007). *A. actinomycetemcomitans* also produces a cytolethal distending toxin, Cdt, which has been implicated in causing G0/G1 or G2/M cell cycle

arrest in some oral cell types leading to apoptotic cell death (Sugai *et al.,* 1998; Mayer *et al.,* 1999; Kang *et al.,* 2005; Kanno *et al.,* 2005). Cdt holotoxin is formed by CdtA, CdtB, and CdtC subunits. CdtA is able to bind target cell membranes though lipid binding activity (Ueno *et al.,* 2006). CdtC works in concert with CdtA to deliver the B subunit into target cells. Once internalized, CdtB enters the nucleus and causes double-strand breaks through DNAse I-like activity. CDT contributes to pathogenesis by killing immune response cells, epithelial cells, and fibroblasts (Smith and Bayles, 2006). In epithelial cells, a CagE homologue induces morphological changes consistent with a pro-apoptotic phenotype (Teng and Zhang, 2005). This CagE homologue is located in the bacterial cytoplasm and is associated with a Type Four Secretion System (T4SS). Even *A. actinomycetemcomitans* LPS has a demonstrated ability to induce apoptosis in the presence of cycloheximide in human macrophages (Suzuki *et al.,* 2004; Rogers *et al.,* 2007).

This is a brief listing of the most prominent *A. actinomycetemcomitans* virulence factors that are characterized for their probable roles as direct players in the causation of tissue destruction or the ability to establish an infection. Other gene products of importance include *A. actinomycetemcomitans luxS*, which encodes an autoinducer 2 (AI-2) like molecule. AI-2 plays a role in quorum sensing and can regulate the gene expression for iron acquisition in this bacterium (Fong *et al.,* 2001; Fong *et al.,* 2003). Reviewed elsewhere, the roles of phosphorylcholine incorporation in LPS, the *hgpA* iron transport system, and the role of Type IV PilA pili in bacterial transformation are aspects of the *A. actinomycetemcomitans* lifestyle that deserve consideration for their roles in the pathogenic potential of this bacterium (Fine *et al.,* 2006).

Porphyromonas gingivalis

General characteristics

Porphyromonas gingivalis is an anaerobic, gram negative, black pigmented, asaccharolytic coccobacillus, and is an etiological agent of Chronic Periodontitis (CP). This bacterium is also implicated as a possible risk factor for the development of certain types of cardiovascular disease (Brodala *et al.,* 2005; Renvert *et al.,* 2006) and pregnancy complications (Dasanayake *et al.,* 2001; Contreras *et al.,* 2006). *P. gingivalis* was first recognized as a distinct species from *Bacteroides asaccharolyticus* in 1980 on the basis of percentage of total guanine and cytosine content, DNA-DNA hybridization assays, differences between oral and non-oral habitation, and the production of phenylacetic acid, when it was named *Bacteroides gingivalis* (Kaczmarek and Coykendall, 1980). The current name was assigned in 1988, when a new genus was proposed for several *Bacteroides* species and *P. gingivalis* became recognized as the accepted name for this oral pathogen (Shah, H.N. and Collins, M.D., 1988). The sequenced genome of *P. gingivalis* strain W83 is approximately 2343 kb (Nelson *et al.,* 2003).

In vitro growth of *P. gingivalis* is routinely performed on blood agar plates supplemented with hemin and vitamin K_1 or K_3. The colony morphology of clinical isolates has been described as rough, semi-rough, or smooth (Dahlen *et al.,* 2007). Early colonies of *P. gingivalis* can appear white or tan in color, but after several days of growth, the colonies become strongly black-pigmented. This characteristic results from the storage of acquired heme on the bacterial cell surface (Shah *et al.,* 1979; Genco, 1995; Lamont and Jenkinson, 1998) and subsequent aggregation of iron (Olczak *et al.,* 2005). *P. gingivalis* obligately requires iron for growth, and hemin satisfies this dependence (Lamont and Jenkinson, 1998). *P. gingivalis* relies on small peptide molecules for nutrition because of its inability to effectively utilize sugars as an energy source (Shah and Gharbia, 1989; Lamont and Jenkinson, 1998). The strict nutrient requirement

is suggested to place selective pressure on *P. gingivalis* to maintain several proteases, which hydrolyze proteins into small peptides and amino acids, but can simultaneously damage host tissues.

P. gingivalis has been categorized into six reactive serogroups, designated K1-K6 based on the antigenicity of its capsular carbohydrates, (Laine *et al.,* 1996). There is also a K(-) group for non reactive types (Aduse-Opoku *et al.,* 2006). Additionally, the LPS of *P. gingivalis* has given rise to three serotypes, O1 to O3. Alternate criteria have also yielded a third classification system, designating A and B serotype *P. gingivalis* (Dahlen *et al.,* 2007). As is the case with *A. actinomycetemcomitans*, individuals tend to be colonized by a single clonal type of *P. gingivalis* and thus it follows that isolates from periodontal disease patients are usually from a single K and O serotype group. Bacteria isolated from disease sites display a wide diversity of K and O antigen combinations, and CP does not appear to result from any single clone of *P. gingivalis* exclusively (Sims *et al.,* 2001; Yoshino *et al.,* 2007). Increased prevalence of some K/O serotype combinations among periodontal disease patients has been reported, which may also be specific to geographical region and ethnicity of the populations sampled (Van Winkelhoff *et al.,* 1999; Sims *et al.,* 2001; Yoshino *et al.,* 2007).

P. gingivalis can be isolated from diseased periodontal tissue (Thiha *et al.,* 2007) and has the ability to invade gingival epithelial cells (Lamont *et al.,* 1995; Tribble *et al.,* 2006) as well as vascular and cardiac endothelial cells (Deshpande *et al.,* 1998; Rodrigues and Progulske-Fox, 2005). The invasion process is not fully understood, but involves cytoskeletal rearrangement and the formation of membrane invaginations at the location of *P. gingivalis* adherence, resembling the bacterium-directed phagocytosis observed during cellular invasion by enteric pathogens (Sansonetti, 1993; Lamont and Jenkinson, 1998). The newly described haloacid dehalogenase

family phosphatase, SerB, has been demonstrated to play a direct role in the invasion process (Tribble *et al.,* 2006). This bacterium also has the ability to override host cell apoptotic programs (Nakhjiri *et al.,* 2001; Handfield *et al.,* 2005), which presumably maintains a favorable intracellular environment for bacterial survival. The diversity of strategies displayed by invasive bacteria, including *P. gingivalis*, is an interesting area of investigation for host-pathogen interaction research.

Virulence factors

The ability of a pathogen to colonize its host is considered to be a virulence factor. *P. gingivalis* produces major fimbriae which are composed of FimA fimbrillin subunits and are associated with the ability of this bacterium to adhere to various surfaces. FimA is a 67 kDa protein encoded by the *fimA* gene (Dickinson *et al.,* 1988). Six types of fimbriae have been described, designated Type I to Type V and Type Ib, based on the variable nucleotide sequence of the FimA gene from different strains of *P. gingivalis* (Amano *et al.,* 2000; Nakagawa *et al.,* 2002). *P. gingivalis* with Type II fimbriae are most often isolated from patients with CP, and Type I fimbriae have been associated with periodontal health (Amano *et al.,* 2000; Yoshino *et al.,* 2007). Different regions of *P gingivalis* fimbria have been found to interact with various substrates, such as lactoferrin, fibronectin and erythrocytes, demonstrating the dynamic range of capabilities afforded by this bacterial structure (Amano *et al.,* 1996; Lamont and Jenkinson, 1998). FimA isogenic mutant strains have impaired adherence, as well as a deficiency to invade host cells as compared with the wild type strain (Weinberg *et al.,* 1997).

The FimA(-) phenotype allowed for the discovery of shorter fimbrial structures, which have been termed minor fimbriae (Hamada *et al.,* 1996). The minor fimbriae are distinct from major fimbriae and are 67 kDa proteins encoded by the *mfa*1 gene (Hamada *et al.,* 1996). The ability of *P. gingivalis fim*A$^-$ and *mfa*1$^-$ single- and double-mutant strains to form biofilms *in*

vitro was assessed and compared to the wild type strain. The *fim*A$^-$ single mutant strain was attenuated in the ability to form a mono-species biofilm on saliva-coated plates. By comparison, the *mfa*1$^-$ strains were completely devoid of the ability to form biofilms (Lin *et al.*, 2006). These bacterial strains were also assessed for autoaggregation in broth culture. Approximately 50% of the wild type bacterial culture autoaggregated and fell out of suspension. The *mfa*1$^-$ strains did not autoaggregate to any detectable level. In contrast, the *fim*A$^-$ strain demonstrated an increased level of autoaggretation to 90% of the total culture. In the wild type *P. gingivalis*, the larger major fimbriae interfere with autoaggregation by hindering close cell-cell contact. When the major fimbriae are not expressed, the minor fimbriae are able to come into closer contact, explaining the increased autoaggregation observed in the *fim*A$^-$ strain. That deletion of *mfa*1 completely abrogates autoaggregation is additional evidence that minor fimbriae are responsible for cell-cell interactions in *P. gingivalis* (Lin *et al.*, 2006). Additionally, minor fimbriae have a demonstrated interaction with streptococcal species (Lamont *et al.*, 2002; Park *et al.*, 2005).

From these experiments, it is evident that minor fimbriae complement the adhesive characteristics afforded by *P. gingivalis* FimA fimbriae. Impacted processes are biofilm formation, autoaggregation, and microcolony formation of *P. gingivalis*. The ability of this bacterium to withstand shearing forces in the oral cavity and avoid elimination by salivary flow and swallowing is important for colonization. Therefore, both major and minor fimbriae are important *P. gingivalis* virulence factors.

As previously mentioned, the nutritional requirements of *P. gingivalis* provide selective pressure to maintain genes involved with iron and small peptide acquisition. *P. gingivalis* produces several proteinases, such as PrtT, periodontain, PrtC collagenase, Trp peptidase, and prolyl tripeptidyl peptidase (Lamont and Jenkinson, 2000). A recent review of proteolytic

activity by selected oral pathogens listed thirteen known proteinases produced by *P. gingivalis* (Potempa *et al.,* 2000). In addition to aiding this bacterium with nutritional requirements, these enzymes can play a role in processing surface components of *P. gingivalis*, or cause the destruction of host tissue (Lamont and Jenkinson, 2000).

The most infamous proteinases, are the Arg-X (Chen *et al.,* 1992; Okamoto *et al.,* 1995; Pavloff *et al.,* 1995)and Lys-X (Okamoto *et al.,* 1996; Pavloff *et al.,* 1997; Potempa *et al.,* 1997) proteases, or gingipains . The term gingipain was proposed in 1992 to describe the first Arg-X gingipain characterized, Gingipain-1 (now RgpA), in light of its specificity for arginine substrates and to reflect its source, class of proteinase, and similarity to other well characterized enzymes such as papain, clostripain, and calpain (Chen *et al.,* 1992). It is estimated that 85% of the proteolytic activity of *P. gingivalis* is attributable to gingipains (Potempa *et al.,* 1997; Imamura, 2003). *Porphyromonas gingivalis* research conducted over the last 25 years has often focused on these proteinases, and has shed considerable light on their remarkable pathogenic potential. Among other functions, the combined activities the gingipains give *P. gingivalis* the capacity to affect gingival crevicular fluid production by inducing vascular permeability, increase inflammation through activating the blood coagulation pathway, and prevent blood clotting through degradation of fibrinogen (Imamura, 2003). The binding activity of gingipains to proteins of the extracellular matrix is also thought to promote *in vivo* colonization by *P. gingivalis*, and upon proteolysis, to periodontal pocket formation and eventual tooth loss (Potempa *et al.,* 2000). Furthermore, these enzymes prevent activation of leukocytes through the degradation of macrophage CD14, a co-receptor involved in recognizing and binding bacterial LPS. Additionally, gingipains bind and lyse erythrocytes for heme acquisition (Imamura, 2003).

Porphyromonas gingivalis possesses several hemagglutinins and hemolysins. These molecules also contribute to iron acquisition through specific binding of erythrocytes and eventual lysis. Most notably, the HagA-E proteins (Progulske-Fox *et al.,* 1989; Progulske-Fox *et al.,* 1995; Han *et al.,* 1996; Lepine and Progulske-Fox, 1996; Lepine *et al.,* 1996) demonstrate this binding capability, and additional hemagglutinins have been identified in the sequenced genome (Nelson *et al.,* 2003). Iron acquisition appears to be associated with quorum sensing systems in *P. gingivalis* (James *et al.,* 2006). There are at least 51 components of *P. gingivalis* with a putative role in iron or heme utilization (Olczak *et al.,* 2005), emphasizing the importance of iron to this bacterium. Further studies dedicated to the iron uptake and acquisition components of *P. gingivalis* are likely to reveal further insights to the pathogenic potential of this organism.

Probing the Complex Interplay between Host Responses and Bacterial Virulence Factors

Clearly, periodontal disease is a complex challenge for researchers and clinicians, with a myriad of microecological, microbial, and host factors interacting to determine a healthy or pathological state. The pathogenic potential alone of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, is daunting to understand. The interrelated nature of these bacterial systems—gene regulation by iron or other environmental cues, stress responses, or production of toxins to overtly damage host tissue—is magnified and complicated when these bacteria are forced to adapt to the host response. The situation becomes inreasingly complex when the enormous numbers of possible inter-bacterial interactions between the oral cavity's 700 species are also considered.

Several methods such as *In Vivo*-Induced Antigen Technology (IVIAT) (Handfield *et al.,* 2000) have been utilized to overcome the challenge of understanding host-pathogen interactions in the oral cavity. Many interesting bacterial products with a putative role in periodontal disease

have been identified using IVIAT for both *A. actinomycetemcomitans* and *P. gingivalis* (Song *et al.,* 2002). However, this only considers the infection from the bacterial perspective. As lamented at the time this dissertation project was undertaken, little is known concerning the interplay of the host responses that regulate these pathogens (Kinane *et al.,* 1999). Investigating the host perspective provides a more complete understanding of disease pathogenesis.

The oral epithelium is now appreciated as more than a passive barrier to infection. Oral epithelial cells respond actively to microbial encounters through antimicrobial peptide production and cytokine expression in response to microbial stimulation (Dale, 2002). Paradoxically, this antimicrobial host response is not necessarily beneficial. Destructive host immune responses are largely credited for periodontal disease initiation and progression (Teng, 2006a; Teng, 2006b). In this situation, the host is perhaps just as culpable as specific bacteria for disease pathogenesis. Understanding the beneficial and harmful aspects of the host response is therefore equally important as uncovering bacterial virulence factors.

Specific Aims

In light of the complex questions at hand, the goal of this study was to gain insights into host pathogen interactions that may occur in periodontal disease. This was performed with global host transcriptional profiling of oral epithelial cells responding to bacterial challenge. As reviewed in Chapter 2, microarrays have been used successfully to gain insights into epithelial cell responses to various bacteria in sites other than the oral cavity. The hypothesis driving this project was that, in contrast to the previous paradigm of a passive role of the epithelium, oral gingival epithelial cells actively and specifically respond to oral pathogens. Three Specific Aims were completed in the course of this study:

SA 1: Establish an experimental model using transcriptional profiling to study host pathogen interactions in epithelial cells. The appropriate cell line, infection conditions, and

presence of a specific response to bacterial challenge by oral epithelial cells were optimized.

SA 2: Establish the epithelial host cell baseline transcriptional profile. The uninfected transcriptome was compared to the transcriptome of epithelial cells encountering wild type pathogens to establish this point of reference.

SA 3: Assess the impact of individual bacterial factors upon the host cell transcriptome and confirm predicted phenotypes directly, such as anti- or pro-apoptotic host cell responses assessed with DNA fragmentation assays. Mutant analysis combined with transcriptional profiling of epithelial cells and subsequent phenotypic confirmations addressed the impact of specific bacterial components upon the interaction with host epithelial cells.

This work investigated the dynamic host-pathogen interplay that occurs in the oral epithelium. The insights provided herein are anticipated to further our understanding of pathogenesis by periodontal pathogens.

CHAPTER 2[1]
MICROARRAY ANALYSIS OF HUMAN EPITHELIAL CELL RESPONSES TO BACTERIAL INTERACTION[2]

Introduction

Host-pathogen interactions are inherently complex and dynamic. The recent use of human microarrays has been invaluable to monitor the effects of various bacterial and viral pathogens upon host cell gene expression programs. This methodology has allowed the host response transcriptome of several cell lines to be studied on a global scale. To this point, the great majority of reports have focused on the response of immune cells, including macrophages and dendritic cells. These studies revealed that the immune response to microbial pathogens is tailored to different microbial challenges. Conversely, the paradigm for epithelial cells has—until recently—held that the epithelium mostly served as a relatively passive physical barrier to infection. It is now generally accepted that the epithelial barrier contributes more actively to signaling events in the immune response.

The purpose of the work reported in this chapter is to address the feasibility of using epithelial cell transcriptional profiles as a reporter system and to establish the groundwork for completing Specific Aim 1: establish a model system. Reviewed here, the strategies utilized by other researchers to gain insights into host-pathogen interactions serve as a framework for the development of a microarray based model for oral epithelial cells. The diversity of experimental conditions that can be tested using microarrays is explored. Differences in host responses to live versus dead bacteria, bacteria of different strains, mutant versus wild-type comparisons, purified bacterial components and pharmacological treatments are discussed. The case for epithelial cells

[1] The following manuscript is reprinted with permission from Bentham Science Publisher Ltd. To access the definitive version, please refer to: Mans, J.J., Lamont, R.J. and Handfield, M. (2006) Microarray analysis of human epithelial cell responses to bacterial interaction. *Infect Disord Drug Targets* **6**: 299-309.

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eliciting specific pathogen-directed responses that are not simply extensions of the immune system is bolstered.

The challenges, advantages, and limitations of such a study are explored. Experiments that defined both a common core response, as well as pathogen-specific host responses are discussed. This review also summarizes the contributions that transcriptional profiling analysis has made to our understanding of bacterial physio-pathogensis of infection. This includes a discussion of how host transcriptional responses can be used to infer the function of virulence determinants from bacterial pathogens interacting with epithelial mucosa. In particular, we expand upon the lessons that have been learned from gastro-intestinal and oral pathogens, as well as from members of the commensal flora.

Class Discovery, Class Comparison, and Class Prediction Paradigms

Global gene expression data presents a unique opportunity to explore the innermost workings of cells that are not revealed by other methods. The snapshot of gene regulation afforded by microarray transcriptional profiling has been used to gain insights into cancer genetics and host pathogen interactions, among other fields. There are three primary goals of global transcriptional profiling experiments: (1) Class prediction, (2) Class comparison, and (3) Class Discovery (Simon *et al.,* 2003). Determining which of these approaches is most applicable to the questions at hand, and utilizing valid analytical methods are critical to conducting successful transcriptional profiling experiments.

The situation where the least is known about experimental samples is the class discovery analysis. Class discovery analysis is an unsupervised method that delineates the relationships between experimental samples, and often focuses on the differences between these samples. Hierarchical clustering (Eisen *et al.,* 1998) and self organizing maps (Tamayo *et al.,* 1999) are two algorithms that are useful in class discovery. Both of these algorithms employ a distance

metric, such as Euclidian distance or Pearson's correlation coefficient, to describe the relationships between samples or genes (McShane et al 2002). It is important to note that clusters or classes will always emerge in a class discovery analysis, even from random data. For this reason, one should never use classes derived from class discovery to drive a class prediction or class comparison analysis on the same dataset. This practice will lead to over-fitting the dataset and inaccurate classifiers. However, classes discovered with unsupervised methods can be further studied using class prediction methods on subsequent independent datasets (McShane et al 2002).

Class comparison analysis is used to uncover genes that are differentially regulated between two or more predefined classes. Class prediction is similar to class comparison, with the added goal of deriving classifiers that are predictive of the identities of future samples based on the expression profiles. Just as several methods exist for class discovery purposes, there are several algorithms designed to accomplish the supervised analyses of class comparison and class prediction. Diagonal linear discriminant, nearest neighbor, weighted voting method, support vector machines, and compound covariate prediction are all examples of methods designed to identify classifiers in the class prediction paradigm (Simon *et al.,* 2003; Radmacher *et al.,* 2002). The specifics of each of these methods are beyond the scope of the current work. Suffice is to say that each approach utilizes a formula that is designed to account for the variance among experimental samples, the mean expression levels for samples in the same class, and in some cases the weighted ability of a specific classifier to distinguish between classes. The class prediction paradigm is the approach undertaken in the current study, following the framework set forth by Radmacher, McShane, and Simon in 2002. The general framework of a class prediction study is to (1) determine the appropriateness for class prediction with a given dataset, (2) select a

predictive method, (3) perform cross-validated class prediction, and (4) determine the significance of the class prediction by permutation testing.

The typical outcome of a microarray experiment is the differential expression levels of thousands of genes for a handful of samples. When the number of covariates exceeds the number of classes, a situation referred to as the multiple hypothesis tests problem arises (Cheng and Pounds, 2007). A significance level of $P<0.05$ which is suitable for a small number of variables would reveal 1500 genes by chance alone in a 30,000 gene array experiment. Therefore a more stringent level of significance, such as $P<0.001$ is often used to reduce the number of false positives identified, also known as a Type I error. Most researchers want to identify genes that are truly predictors of a specific condition, and desire a target genelist with high probability of being confirmed downstream with multiple methods. As the Type I error rate declines, however, the Type II error rate increases. A Type II error is erroneously leaving a true positive out of the list of regulated genes. Conversely, decreasing the Type II error rate includes more false positives, also known as the False Discovery Rate (FDR).

There are five currently recognized methods of addressing the multiple hypothesis tests problem. These paradigms are (1) Control of the FDR, (2) estimation of the FDR, (3) significance threshold criteria, (4) control of family-wise error rate (FWER), and (5) empirical Bayesian approaches. The details of these methods are beyond the scope of this study, yet the diversity of methods available that address the multiple hypothesis tests problem demonstrates the importance of obtaining reliable genelists for further characterization.

One of the pitfalls of a class prediction analysis is over-fitting the data. As demonstrated by Radmacher and colleagues (2002), it is always possible to derive classifiers from transcriptional data, even when that data is randomly generated from the same distribution. This

occurs when the same dataset is used to build and then test the prediction model that is designed to identify classifiers. As a result of the relatively few samples compared to the number of covariates, and the power of the algorithms chosen, it is always possible for a prediction model to discriminate between two classes based on variance noise in the dataset. To avoid this situation, ideally the dataset is split evenly into a training set and a test set. The prediction model is applied to the training set and the classifiers are then applied to the test set to determine their predictive ability. Since experimental samples are often precious, an alternative to this setup is to use leave-one-out cross-validation (LOOCV).

Leave one out cross-validation approximates the situation of having a training set and a test set. With LOOCV, one experimental sample is left out of the dataset, and the rest of the samples are used to determine classifiers based on the prediction model chosen. The classifiers are then all tested for their ability to correctly predict the identity of the sample that has been left out of the training set, which is basically a one-sample test set. Each sample is left out in turn while the prediction model is built, and then cross-validated. The cross-validated misclassification rate is an indication of the predictive ability of the classifiers. This method avoids over-fitting the dataset because the test sample was not used to build the prediction model and is thus independent of the training set (Radmacher *et al.*, 2002).

LOOCV is not the final stage of a sound statistical analysis of transcriptional data. An important component of the class prediction paradigm is to test the significance of the cross-validated misclassification rate. Permutation testing is a method to compare the ability of the classifiers to correctly predict the identity of the test sample to that expected by chance alone. With permutation testing, the class labels are randomly permuted and the LOOCV analysis is repeated on the scrambled dataset. The cross-validated misclassification rate of the permuted

dataset should be higher than the misclassification rate of the true classifiers. Because every possible permutation is impossible based on current computing power, a Monte Carlo method is used to perform a user specified number of permutations on the dataset. A typical analysis will involve 2000 random permutations of the dataset. The proportion of these 2000 permuted LOOCV analyses with cross validated misclassification rates equal or lower than the misclassification rates for the classifiers tested is determined. When this proportion is less than 0.05 (or 100 per 2000 permutations), the null hypothesis is rejected (Radmacher *et al.,* 2002).

Statistical analysis of microarray experiments is a superior method to filtering candidate genes based on the simple mean fold-change of given transcript level. Simple mean calculations or visual examination of clusters do not account for the variance in a dataset, and will produce unreliable classifiers (Simon *et al.,* 2003). These methods are acceptable for class discovery projects, but not for class prediction.

Several software programs are available which apply statistical algorithms to transcriptional data. One such software program is BRB Array Tools. Rather than choosing a single statistical algorithm, BRB Array Tools is designed to implement several different hypothesis testing methods that perform well. Linear discriminant analysis, nearest neighbor, and support vector machines are examples of formulae that attempt to identify genes that are truly differentially regulated. Additionally, BRB Array Tools softare allows implementation of the cross-validation and permutation testing aspects of class prediction that are critical to obtaining classifiers with a known level of predictive ability. As bioinformatic methods improve, the reliability of microarray experiments will follow suit.

Microarrays and the Study of Host-Pathogen Interactions

The balance between health and disease is an evolutionary arms race involving complex host-pathogen interactions (Bergelson *et al.,* 2001; Woolhouse *et al.,* 2002). In recent years,

transcriptional profiling of host-pathogen interactions has increasingly been used to understand the dynamics of this race in a concerted effort to fight infectious disease. Analysis of the transcriptome changes that occur during the cross talk between host and pathogen can be accomplished through several methods, such as microarrays, or serial analysis of gene expression (SAGE). Similar techniques have been used to the same endeavor and include amplified restriction fragment length polymorphism (AFLP)-derived technique for RNA fingerprinting (cDNA-AFLP), random EST sequencing and massively parallel signature sequencing (MPSS) (Matsumura *et al.,* 2005). Host and microbe microarrays offer several advantages to other methods of transcriptional profiling, including high throughput, parallelism, miniaturization, and automation (Miller *et al.,* 2002; Bryant *et al.,* 2004; Dharmadi and Gonzalez, 2004; Kirmizis and Farnham, 2004). These advantages have been instrumental to analyze the genetic polymorphisms of pathogens that are resistant to antimicrobials, explore the distribution of genes among isolates from the same species, or to investigate the evolutionary relationships between closely related species (Kato-Maeda *et al.,* 2001). Several versions of targeted virulence factor arrays have been useful to detect the presence of pathogenic microorganisms in environmental samples (Bekal *et al.,* 2003). Transcriptional profiling has been used to evaluate the simultaneous and genome-wide response of host and pathogen cells to drug treatments or antimicrobials in order to predict the safety and efficacy of new treatments (Yowe *et al.,* 2001). The study of host-pathogen interactions has also benefited from microarray technology where the host and pathogen gene regulation patterns have been evaluated. The field of infectomics (Huang *et al.,* 2002; McManus *et al.,* 2002; Jong and Huang, 2005) or infectogenomics (Kellam and Weiss, 2006) has emerged from these seminal experiments.

Since global differential gene expression was first monitored in *Arabidopsis thaliana* (Schena *et al.,* 1995), microarray technology has advanced significantly. The potential of arrays is demonstrated by the comparison of differential gene expression that occurs between root tissue and leaf tissue with a microarray consisting of only 45 genes. In this initial report, genes that were known to be involved with photosynthesis were over-expressed in leaf tissue relative to root tissue. In addition, both direct and downstream effects of differential gene regulation were observed. This was performed through the comparison of wild-type *Arabidopsis* to a transgenic plant known to over-express the transcription factor HAT4 at the protein level. The developmental regulator, HAT4, was previously discovered in *A. thaliana*, and characterized as a homeo domain-leucine zipper protein (Schena *et al.,* 1993; Schena and Davis, 1994). The up-regulation of HAT4 was observed at the transcript level, and the subsequent cascade of gene regulation changes caused by HAT4 was noted. This pioneering report also attempted to address many challenges still relevant today, including issues with the standardization of replicates, problems with the selection of criteria to determine what gene changes qualify as significant, and how best to organize and interpret the overwhelming amount of data that can be obtained with a single experiment. Despite these challenges, global transcriptional profiling offers an unprecedented potential to further understand the fundamental basis of infectious diseases. This has been suggested to represent a milestone that will ultimately lead to the development of innovative diagnostics, prophylactics and therapeutics (Cummings and Relman, 2000; Yowe *et al.,* 2001; Huang *et al.,* 2002; Bryant *et al.,* 2004; Aldridge *et al.,* 2005; Kellam and Weiss, 2006).

Cytomegalovirus (CMV) is a virus transmitted easily at mucosal surfaces and is a critical concern for solid organ transplant and haematopoietic stem cell transplant patients. This virus

can also cause devastating neurodevelopmental sequelae in newborns (Schleiss, 2005). In 1998, the first transcriptional profiling of human cells to probe host-CMV interactions utilizing microarrays was performed. To investigate the impact of infection with this virus on host cells, the expression level of 6600 human transcripts from CMV-infected primary human foreskin fibroblasts was compared to uninfected cells. The consistency amongst biological replicates was evaluated and assessed the amount of variation that resulted from background differences. With a threshold of variation set at three-fold over background, 258 genes were found to be significant, and were further investigated (Zhu *et al.,* 1998).

In 2000, microarrays were first used to profile the host response to a bacterial pathogen, *Salmonella typhimurium* (Rosenberger *et al.,* 2000). Gastrointestinal *S. typhimurium* infection is usually non-systemic and self-limiting, but can cause debilitating enterocolitis lasting between two and three weeks. A milder gastroenteritis can last between three and five days, with symptoms for both manifestations consisting of nausea, vomiting, fever, abdominal pain, and bloody or mucous diarrhea (Hapfelmeier and Hardt, 2005). At the time of this microarray investigation, specific bacterial effectors were known to modify host cell responses at the protein level, as exemplified by Type III secretion systems (Hueck, 1998). This microarray study demonstrated that the transcriptome of the host could also be impacted by bacterial factors. In addition to the host's response to live *Salmonella*, the effect of a single bacterial component, the lipopolysaccharide (LPS), was also determined. Although LPS was known to have immune effects on host cells, the extent of these effects was not well understood at the transcriptional level. In an attempt to mimic an *in vivo* infection by using an *in vitro* model, the authors stimulated the host cells with interferon gamma (IFN-γ) and repeated their initial experiment to investigate the effect of this cytokine on the macrophage transcriptional responses. This

reconstructed some of the complex interactions that are thought to occur between host and pathogenic factors. The combination of isogenic mutant analysis in pathogens with host arrays was also proposed to investigate additional bacterial effectors whose influence on the host response may be masked by LPS-induced responses.

Following the classification of Jenner (Jenner and Young, 2005), four categories of experimental strategy have been used to dissect host-pathogen interactions. These include (1) host cells infected with a wild type pathogen, (2) host cells infected with a pathogen containing an isogenic mutation, (3) host cells exposed to purified bacterial components (such as LPS or pili), and (4) host cells exposed to a physico-chemical treatment (pH, temperature, antibiotic treatment, or pre-exposure to host-derived components). Many other exhaustive reviews have been presented in recent years that cover the transcriptional response of immune cells to infection (Cummings and Relman, 2000; Kellam, 2000; Kellam, 2001; Yowe *et al.,* 2001; Dharmadi and Gonzalez, 2004; Aldridge *et al.,* 2005; Jenner and Young, 2005).

The following is a summary of the progress made in understanding the nature of the interaction between epithelial cells and commensal or pathogenic bacteria that co-exist as part of the human microflora. Since no new drugs, vaccines, or diagnostics have yet emerged from targets found with host arrays, we will close with a speculative view of how reverse vaccinology (Rappuoli, 2001) may be applied to the results of host transcriptional profiling to develop novel diagnostics, therapeutics, or prophylactics.

Epithelial Responses to Pathogenic and Commensal Microorganisms.

Initial microarray experiments have consistently compared uninfected host cells with cells exposed to one or several pathogens to determine the baseline epithelial host response to a bacterial challenge. Table 2-1 reveals numerous reports that have exploited this strategy. In a great majority of cases, investigators have focused on host gene regulation related to a specific

class of genes—usually cytokines and chemokines—that are known to play a role during infection *in vivo*. This approach sheds some light on the alarm signal activities of epithelial cells, and has identified several novel factors involved with recruiting immune cells to the site of infection. Custom arrays, or commercially available chemokine arrays have been used in several studies to specifically investigate the host gene regulation of these factors. For example, the novel involvement of several chemokines with host response to pathogens was determined by array analysis of 277 cDNA elements in the human colorectal epithelial cell line HT-29 interacting with *Salmonella dublin* (Eckmann *et al.,* 2000). *S. dublin* naturally infects cattle, but can occasionally be spread to humans where it is highly invasive. The direct route of infection is unknown, although it is frequently isolated from blood, and can cause metastatic infections in virtually all organs. Presumably, unpasteurized cheese and milk are responsible for *S. dublin* transmission from cattle to humans (Lester *et al.,* 1995). To investigate the host response to this highly invasive pathogen, the investigators focused on genes modulated at time points that demonstrated the highest degree of up-regulation in HT-29 cells upon interaction with *Salmonella*. Two new targets of NF-κB regulation were identified by this strategy. In addition, the interaction of *Salmonella* with AGS gastric adenocarcinoma cells confirmed the role of NF-κB as a regulator of pro-inflammatory cytokines. Similarly, the activation of NF-κB has significance in SV40 transformed human colon carcinoma cells interacting with the causative agent of whooping cough: *Bordetella pertussis* (Belcher *et al.,* 2000). Besides NF-κB activation, eight of the 33 up-regulated genes in these epithelial cells were pro-inflammatory cytokines. The chemo-attractant activity of these factors is consistent with the infiltration of monocytes, neutrophils, and lymphocytes which has been observed in other models of infection (Belcher *et al.,* 2000). Likewise, clues into the mechanisms behind the clinical observations of

acute inflammation occurring in human infections by *Salmonella typhimurium* (Zeng *et al.,* 2003), *Chlamydia trachomatis* (Dessus-Babus *et al.,* 2000; Hess *et al.,* 2001), and *Neisseria gonorrhoeae* (Binnicker *et al.,* 2003) have been provided by global transcriptional profiling, revealing the specific regulation characteristics of various immuno-stimulatory factors.

A second group of host responses that has been addressed with microarrays is the perturbation of host cell apoptotic mechanisms by invasive bacteria. A study performed using a 12,626-element human array profiled primary human urethral epithelial cells (PHUECs) interacting with *Neisseria gonorrhoeae*, and demonstrated the upregulation of anti-apoptotic regulators, bfl-1, cox-2, and cIAP-2 (Binnicker *et al.,* 2003). The upregulation of antiapoptotic regulators was also shown to depend on the cell type, as RT-PCR analysis of *N. gonorrhoeae*-infected Chang/HeLa epithelial cells (American Type Culture Collection (ATCC); CCL-20.2) did not show the same up regulation of these anti-apoptotic genes. It is interesting to speculate if this dichotomy of host cell response also occurs with regard to pro-inflammatory cytokines, and if the different clinical manifestations in men and women are attributable to this host-cell based difference (Binnicker *et al.,* 2003). Further, *N. gonorrhoeae*-infected urethral epithelial cells were protected from staurosporine (STS)-induced apoptosis. This pathogen's strategy of immune system avoidance and establishment of an intracellular environment conducive to replication has been demonstrated in other bacteria.

In either epithelial cells or immune cells, *Chlamydia trachomatis* (Fan *et al.,* 1998), *Chlamydia pneumoniae* (Geng *et al.,* 2000; Fischer *et al.,* 2001), *Helicobacter pylori* (Shirin *et al.,* 2000), and *Porphyromonas gingivalis* (Hiroi *et al.,* 1998; Nakhjiri *et al.,* 2001; Handfield *et al.,* 2005a) have demonstrated anti-apoptotic activity that complements an intracellular lifestyle. Transcriptional profiling has revealed how apoptotic pathways are perturbed by *P. gingivalis*, in

Human Immortalized Gingival Epithelial cells (HIGK). *P. gingivalis*, which establishes a chronic periodontal infection (Loesche and Grossman, 2001), modulates several factors mapping to the P53 branch of the apoptotic pathway. Similar to the effect of *N. gonorrhoeae* on urethral epithelial cells, and consistent with earlier work (Nakhjiri *et al.,* 2001), phenotypic confirmation showed that *P. gingivalis* infection was protective against camptothecin-induced apoptosis in these oral epithelial cells (Handfield *et al.,* 2005a). This silencing of the apoptotic pathways in epithelial cells requires living bacteria, as RT-PCR analysis of primary gingival epithelial cells exposed to heat-killed *P. gingivalis* induced apoptosis, in contrast to the inhibition observed with live bacteria (Brozovic *et al.,* 2006). The transcriptional profiles of cells encountering killed *P. gingivalis* are consistent with the transcriptome of cells infected by other pro-inflammatory bacteria; killed *P. gingivalis* or *P. gingivalis* extracts induce NF-κB and up-regulate the innate immune response. Together, these examples further illustrate that the specificity of bacteria-epithelium interactions can be dissected with transcriptional profiling of host cells.

The strategy highlighted above is not restricted to the study of the modulation of the innate immune defenses upon bacterial challenge. Although the dissection of apoptosis and immuno-regulatory pathways has been a significant focal point, many additional cellular processes are involved during epithelium-microbe interactions. Numerous transcription factors and cell-cell communication factors, for example, have been consistently found to be differentially regulated in infected host cells compared to uninfected controls (Handfield *et al.,* 2005a; Jenner and Young, 2005). Studies of the interaction of HIGK cells with two commensal bacterial species of the oral cavity, *Fusobacterium nucleatum* ATCC 25586 and *Streptococcus gordonii* DL-1 Challis, elicit transcriptional profiles that are highly similar to each other, and far less disruptive to the baseline HIGK cell gene regulation when when compared to pathogen-

infected and uninfected cells (Hasegawa *et al.*, 2007, Appendix A). The significance of this profile is currently under investigation. Clearly, the complexity involved in predicting and confirming a testable phenotype currently hampers the downstream functional characterization of many of these genes. The ultimate validation of other factors modulated upon infection and the testing of their biological relevance in relation to disease initiation and progression compared to health remains a significant and widespread challenge.

Bacterial Mutant Analysis via Host Transcriptomics.

Combining a classical bacterial isogenic mutant analysis with host profiling has been performed with a number of human pathogens, including *Pseudomonas aeruginosa* (Ichikawa *et al.,* 2000), *Helicobacter pylori* (Chiou *et al.,* 2001; Cox *et al.,* 2001; Maeda *et al.,* 2001a; Maeda *et al.,* 2001b; Bach *et al.,* 2002; Guillemin *et al.,* 2002), *Yersinia enterocolitica* (Bohn *et al.,* 2004), *A. actinomycetemcomitans* and *P. gingivalis* (Handfield *et al.,* 2005a).

The first report describing the epithelial host cell response to *Pseudomonas aeruginosa* interaction was performed using microarray profiling of A549 human lung carcinoma pneumocytes (Ichikawa *et al.,* 2000). *P. aeruginosa* is a Gram-negative opportunistic pathogen associated, amongst other types of infection, with deteriorating lung function in cystic fibrosis patients. A microarray consisting of 1506 pneumocyte genes first established a baseline response to *P. aeruginosa* interaction at 0h and 3h post infection. The contribution of Type IV Pili-dependent bacterial adhesion to the host response was investigated using a non-piliated mutant derivative of strain PAK in parallel with the parent strain to infect epithelial cells. The baseline response to infection revealed a differential regulation of 24 genes. The regulation of 16 genes was directly attributed to Type IV pili, including the up-regulation of Interferon Regulatory Factor-1 (IRF-1). RT-PCR further characterized the factors contributing to IRF-1 activation. It was determined that pili play a critical role in this host response. Subsequent

downstream effects are also associated with adhesion and included the targeting of inducible nitric oxide synthase and certain interferon-induced immune effects (Ichikawa *et al.,* 2000). This finding determined that the cellular adhesion through these pili was directly responsible for triggering the host response, in addition to determining a physical association between the host and microbe.

Gastric epithelial cell response to *Helicobacter pylori* is frequently studied with microarray global profiling. This microorganism is the causative agent of active chronic gastritis and is associated with gastric cancer, in addition to complications resulting from pathogen-induced inflammatory damage. In particular, the Cag pathogenicity island (CagPAI) of *H. pylori* has been extensively investigated for its contribution to the NF-κB and IL-8 induction upon infection (Cox *et al.,* 2001), as well as for the inhibition of apoptosis and cell cycle interference that may lead to gastric cancer. For example, two parallel time-course studies were performed comparing gastric adenocarcinoma (AGS) epithelial cells interacting with wild type, mock-infected, and five mutant strains of *H. pylori.* These mutant strains lacked either the Vacuolating cytotoxin (VacA), or one of several structural (CagE) or effector (CagA) proteins from a type IV secretion system encoded by the CagPAI. A microarray of 22,571 elements was used and detected the differential regulation of hundreds of genes under several infection conditions. This ultimately distinguished between CagA, CagE, and CagPAI-dependent host responses. In particular, a CagA mutant strain of *H. pylori* was found to induce fewer cytoskeletal genes than the wild type strain, thus supporting the hypothesis that CagA is the bacterial component directly involved in triggering this host response. Strikingly, the deletion of the entire PAI results in a host response that closely resembles the mock infection profile, suggesting the PAI is the major contributor to the AGS cell response to *Helicobacter pylori* (Guillemin *et al.*, 2002).

Similar strategies have been utilized to study the contribution of specific putative virulence determinant of *Y. enterocolitica, A. actinomycetemcomitans,* and *P. gingivalis*. For *Y. enterocolitica*, an enteropathogen that causes self-limiting infections (Pujol and Bliska, 2005), mutant strains of the *Yersinia* virulence plasmid pYV (*inv*+/pYV-), the *Yersinia* outer protein YopP (pYV+/*yop*P-), and the double mutant for *Yersinia* virulence plasmid and invasin protein (*inv*-/pYV-) were used to infect HeLa cervical epithelial cells (Bohn *et al.,* 2004). The experimental design used uninfected HeLa cells as the baseline gene expression level, and investigated four mutant strains over three time points (1, 3 and 7 hours). A differential host response was determined for each strain using the Affymetrix Human Genome HG-U133A GeneChip, which consists of 22,283 probesets. The differential responses support the contribution of each virulence factor and established the temporal expression of host factors in response to *Yersinia* challenge.. After data filtering, two-fold changes in gene expression revealed that 193 probesets are differentially regulated between experimental conditions. The functional categories impacted included transcription factors, receptors and signaling components, growth factors, chemokines and cytokines, and cytoskeletal reorganization factors (Bohn *et al.,* 2004). Interestingly, IL-8 was highly induced in HeLa cells upon interaction with the mutants lacking YopP, yet still containing the invasin protein (Inv+/pYV+/YopP-) compared to all other conditions. This finding is consistent with other studies and supported the concept that Inv triggers IL-8 production (Schulte *et al.,* 1996), while YopP may counteract this induction (Denecker *et al.,* 2002). A comparison of the transcription profile upon infection with *Yersinia* lacking Inv to *Yersinia* lacking both Inv and the virulence plasmid identified 117 probesets. Moreover, 24 genes were coordinately induced in HeLa cells compared to uninfected

cells under these conditions. This suggested that other chromosomally encoded *Yersinia* factors were responsible for the modulation of these 24 genes.

The same strategy has been used to study the contribution of specific putative virulence determinants and extended to make inferences on the role of factors with unknown function in *A. actinomycetemcomitans,* and *P. gingivalis*. These oral pathogens are both implicated in periodontal infections (Loesche and Grossman, 2001; Sanz *et al.,* 2004). In *P. gingivalis*, the major fimbrial protein FimA is important for bacterial adhesion and necessary for effective invasion of host cells (Hamada *et al.,* 1994; Malek *et al.,* 1994; Njoroge *et al.,* 1997). An isogenic mutant strain for FimA (YPF-1) was used to profile Human Immortalized Gingival Keratinocyte (HIGK) (Oda *et al.,* 1996) cells upon a two-hour co-culture using Affymetrix HG-U133A human GeneChips (Handfield *et al.,* 2005a). The patterns of gene regulation between HIGK cells encountering wild type and mutant *P. gingivalis* revealed many genes involved with host cytoskeleton and membrane receptor activity, consistent with the observed adhesion and invasion deficiencies of this mutant. This confirmed the critical role of FimA in the adhesion and subsequent invasion events of epithelial cells by *P. gingivalis*. Further investigations are underway to determine if the observed effects are FimA dependent, or adhesion dependent, which can be accomplished by treating HIGK cells with purified FimA protein and observing the host response. The regulation of the individual genes involved in these processes can shed light on the details of the bacterial-epithelial cell interactions that result in the observed phenotypes of the host and pathogen.

Previously, an *in vivo* induced antigen technology (IVIAT) screen of human patients with localized aggressive periodontitis (LAP) (Handfield *et al.,* 2000; Cao *et al.,* 2004a) revealed the *in vivo* induction of *A. actinomycetemcomitans orf859* (PEDANT database). The bioinformatic

analysis of this gene product did not predict any significant homologies in the databases, although the gene product was highly conserved across genera. The *orf859*gene product of was further shown to be induced in plaque from infected patients (Handfield *et al.,* 2000) and in various human cell lines *in vitro* (Richardson *et al.,* 2005). Thus *orf859* is an attractive potential marker for active disease in LAP patients (Cao *et al.,* 2004b). Although expressed specifically in human LAP *in vivo*, the precise function of this bacterial gene was uncertain based on bioinformatics alone (Cao *et al.,* 2004b).

In an attempt to elucidate the role of ORF859 in host-pathogen interactions, microarray profiling and ontology analysis were performed for HIGK cells interacting with *A. actinomycetemcomitans* wild-type strain VT1169, and the isogenic mutant strain JMS04 (*orf859*$^{-}$). Specific genes were differentially regulated between HIGK cells infected by wild type and mutant strains of *A. actinomycetemcomitans*. For instance, HIGK infected with JMS04 failed to induce IL-27, which is closely related to IL-12 and has been shown to promote Th1 cell-mediated immune responses (Hunter *et al.,* 2004). Furthermore, genes involved with intermediate metabolism, signal transduction, and cytokine activity were differentially expressed in HIGK cells compared to host cells interacting with wild type *A. actinomycetemcomitans*. This suggests that the function of ORF859 is likely to be related to intracellular survival of *A. actinomycetemcomitans*, and probably contributes to immune recognition by epithelial cells (Handfield *et al.,* 2005a). The ontology analysis further revealed that the most significant variations are associated with intermediate metabolism functions, stress response, and signal transduction (Handfield *et al.,* 2005a). This is consistent with the phenotype of the mutant strain as characterized by total interaction, invasion, and competition assays with the wild-type strain (Cao *et al.,* 2004b). The product of this gene is uninvolved with adhesion, but conferred a

significant persistence defect within epithelial cells in the isogenic mutant strain. Collectively, this gene product is specifically induced intracellularly during infection and confers a selectable advantage to the microorganism in the intracellular environment. Thus, the role of a protein with unknown function has been investigated through the use of host transcriptional profiling and mutant analysis, in addition to investigating the impact of known bacterial genes.

Epithelial Cells Interacting with Purified Bacterial Products and Components.

In certain instances, directly investigating the effects of purified virulence factors on target cells has been an option (Yokoyama *et al.,* 2005). This strategy has been used to confirm a putative cause-effect function revealed by isogenic mutant analysis. As described in the previous section, *H. pylori* up-regulates several virulence factors upon interaction with gastrointestinal epithelial host cells. In particular, CagA and VacA, have substantial phenotypic effects on these cells.

The global transcriptional response of AGS human gastric epithelial cells has been investigated upon direct exposure to CagA (Yokoyama *et al.,* 2005). CagA was transfected into AGS cells with an expression vector containing either hemagglutinin-tagged CagA, or a phosphorylation-deficient CagA. CagA is normally injected into host cells through a type IV secretion system (Censini *et al.,* 1996; Akopyants *et al.,* 1998), which justified the use of the transfection method. One of the advantages of this system was the reduction of the possible noise introduced by live bacteria-cell contact. Four time points were chosen to investigate both direct and downstream events attributable to CagA. Overall, 8500 genes were analyzed using the Affymetrix Genome Focus Array. This revealed 339 up-regulated genes and 145 down-regulated genes at a late time point (24 hours). In contrast, at an earlier time point (12H), 71 genes were up regulated in gastric epithelial cells relative to AGS cells not exposed to CagA. The later inductions (24 hours) were consistent with the downstream effects of a cascade initiated at 12

hours post-infection. Of particular interest, the putative –1000 to –1 promoter regions of the activated genes were analyzed to identify cis elements from a panel of transcription factors. Binding sites for the nuclear factor of activated T cells (NFAT) family of transcription factors were over-represented among the 71 genes as compared to a pool of genes selected at random. Further investigation of these NFAT transcriptional factors determined that *Helicobacter* CagA initiates a cascade by directly stimulating PLCγ to activate calcineurin. Calcineurin is a calcium/calmodulin-dependent phosphatase, which acts upon a conserved N-terminal domain of NFAT family transcription factors (Rao *et al.,* 1997). Activated calcineurin dephosphorylates cytoplasmic NFAT, which is then translocated to the host cell nucleus and activates transcription.

When secreted VacA, was allowed to interact with the experimental system described above, it prevented NFATc3 dephosphorylation by calcineurin, inhibiting its translocation to the nucleus and antagonizing CagA. This balance of cellular state linked to specific bacterial virulence factors supports the clinically observed diverse degrees of severity of *H. pylori* infection. This is also consistent with the clonality of *Helicobacter* strains and their variable degrees of CagA and VacA expression.

Human intestinal epithelial cell line HT29 and murine RAW 264.7 macrophages have also been subjected to the *Aeromonas hydrophila* cytotoxic enterotoxin, Act. This bacterium is a water-borne pathogen of fish, amphibians, reptiles, and other cold-blooded animals. It is generally considered an opportunistic pathogen of humans, although it has been reported as a primary pathogen of healthy individuals in rare cases (Kao *et al.,* 2003). To study possible effects of *A. hydrophila* upon human cells, a sub-lethal dose of purified Act (12ng/mL) was applied to polarized and non-polarized human epithelial HT29 cells at various timepoints (0, 2,

and 12 hours). Affymetrix HU133 array GeneChips were used to profile host cells, and four different software programs were used to implement statistical algorithms designed to identify significantly regulated genes (GCOS, SAM, Spotfire 7.3, and ANOVA). All four methods consistently identified 34 genes that were induced between the 0 and 2-hour timepoints, which were investigated further using ELISA, cytokine profiles, western blot analysis, and phosphor-protein screens. Host responses related to apoptosis, cellular reorganization, cell growth/cycle/differentiation, cytokines, signal transduction, and transcription factors were identified. Calcium mobilization in human intestinal epithelial cells was found to be important, and assays confirmed this process was essential for the maximal production of IL-8 in Act-treated cells. In contrast, a different set of host response genes was up regulated in the murine RAW 264.7 macrophages upon interaction with the same toxin. This was consistent with the specialized roles that different cell types play in the encounter with microbes.

The Environmental Contribution to Host-Pathogen Interactions.

Multiple artificial treatments can be used to subject a host-pathogen system to perturbations that are thought to occur during a normal infection process. These treatments encompass perturbations such as the addition of purified host proteins, or the stimulation by IFN-γ, for instance (Rosenberger *et al.,* 2000). Another example of such treatment includes the treatment with pharmaceutical agents. In this case, the therapeutic efficacy can be assessed with the host transcriptome to confirm that the interactions between bacteria and host cell are being affected according to the drug's design. Similarly, the knockdown of host factors can also be achieved to assess their contribution to the host-pathogen interaction. The following are examples of studies that have utilized this environmental impact approach to dissect bacterial-epithelium interactions.

The experimental strategy mentioned earlier for the HT-29 human intestinal cell line and *Salmonella dublin* was modified through perturbation of the baseline system (Eckmann *et al.,* 2000). This was performed in order to dissect the specific contribution of nuclear factor-κB (NF-κB) to the induction of several genes previously revealed through the use of Gene Filter GF211 (Research Genetics Inc, Huntsville, AL) and Atlas Human Cytokine/Receptor cDNA arrays (CLONTECH, Palo Alto, CA). NF-κB is a critical transcription factor that helps coordinate the innate and adaptive immune response. Activation of NF-κB occurs through the phosphorylation of its inhibitors, such as IκB family members (Zingarelli, 2005). The authors hypothesized that NF-κB is also involved in the transcriptional regulation of the cytokine induction revealed in their microarray studies. An adenovirus-based system was used to transfect cells with a construct that expressed a mutant form of IκBα. This defective IκBα could not be phosphorylated at two positions and thus acted as a super-repressor of NF-κB activation. Infections by *S. dublin* of the super-repressor HT-29 cells were performed under conditions identical to the original array experiment, and the regulation of seven genes were characterized with RT-PCR. Although not entirely abolished, these previously induced genes were noticeably repressed. These results suggest a direct role of NF-κB in the observed HT-29 transcriptional response to *Salmonella* infection. Additionally, this approach led to the discovery of the NF-κB regulation of two host genes, EBI3 and BGT-1, as well at the confirmation of several other genes being targets of NF-κB (Eckmann *et al.,* 2000).

Another study investigating the role of NF-κB-dependent host responses to infection utilized chemical inhibitors to alter the infection system of *Bordetella pertussis* with BEAS-2B bronchial epithelial cells. The baseline microarray experiment utilizing Affymetrix HU6800 GeneChip arrays confirmed a dominant pro-inflammatory phenotype upon *B. pertussis* infection,

consistent with the clinical manifestations and *in vivo* models of whooping cough (Belcher *et al.,* 2000). The effects of NF-κB repression were characterized by an anti-inflammatory treatment of host cells with Dexamethasone and sodium salicylate prior to infection with *B. pertussis* (Belcher *et al.,* 2000). Dexamethasone is a glucocorticoid that has been suggested for therapeutic treatment of pertussis and seemed to augment IκB synthesis. Sodium salicylate inhibits NF-κB activation by preventing IκB phosphorylation. Interestingly, pre-treatment of BEAS-2B cells with these compounds prior to *B. pertussis* infection eliminated the induction of GRO-2, GRO-1, IL-8, IL-6, IL-1β, MCP-1, MIP-1α, GRO-3, CIITA, HLA-DQ, API-2/IAP and many other genes that previously were induced in the original microarray study. In addition, dexamethasone failed to block the induction of TNFAIP3 while sodium salicylate blocked induction. Dexamethasone also up regulated (4.8-fold) inducible nitric oxide synthase (iNOS), which was not observed with untreated or sodium salicylate-pretreated-infected epithelial cells. In light of this latter finding, the authors urged caution regarding the use of dexamethasone as a possible treatment for whooping cough. Potentially, the clinical condition could be exacerbated by the induction of iNOS, as collateral damage of neighboring, uninfected cells could occur through iNOS activity (Belcher *et al.,* 2000).

Similarly, dissection of pro-inflammatory gene activation was undertaken in T84 intestinal epithelial cells by exposure to purified Tumor Necrosis Factor-α (TNF-α). Parallel infection of T84 cells with various *S. typhimurium* isolates, *Escherichia coli*, *Salmonella typhi*, and treatment with TNF-α was performed and the host response at 4 hours was assessed with a custom array of >600 genes. Hierarchical cluster analysis grouped the *S. typhimurium*-infected and TNF-α treated cells together, and distinctly from the *E. coli* and *S. typhi* infected cells. This indirectly implicated TNF-α in the transcriptional profiles observed under *S. typhimurium*-

infected conditions, and led to further investigation of possible mechanisms that could lead to direct TNF-α induction by *S. typhimurium* (Zeng *et al.,* 2003).

Revised Role of Epithelial Cells in Host-Pathogen Interactions at the Mucosal Surface.

The epithelium is a critical barrier to prevent pathogen entry to the human host. As such, this first line of defense to systemic infection has been an obstacle to pathogens that provoked evolutionary changes for bacterial entry and access to underlying tissue (Eckmann *et al.,* 2000; Aldridge *et al.,* 2005). This process of pathogen entry and tissue invasion is an active process that involves signaling pathways from the pathogen and host, which differ between microorganisms (Finlay and Cossart, 1997). The role of epithelial cells has been proposed as an early warning system or sensor for infection (Eckmann *et al.,* 2000; Aldridge *et al.,* 2005). Many studies have determined that the gene regulation of host cells, in response to interaction with pathogens, is to specifically regulate pro-inflammatory and chemo-attractant signals which subsequently recruit immune effectors to the area of infection. As a result, the focus of much work has been on identifying chemokines and cytokines produced by epithelial cells. To this point, most microarray-based studies of host-pathogen interactions have focused on the primary role of the immune system in the host-pathogen encounter using dendritic cells, macrophages, and other immune cells. However, epithelial cells remain the first cell type that most mucosal pathogens and commensal organisms encounter and thus may contribute to host defense in ways not previously recognized.

Recently, the role of the epithelial layer as a sensor was emphasized with the discovery that host epithelia preemptively search for pathogenic bacteria through the monitoring for pathogen-associated molecular patterns (PAMPs) (Ayabe *et al.,* 2004). These microbe-host interactions encompass both pathogenic and commensal organisms in scope, as host epithelial

cells use TOLL-like receptors to screen for PAMPs of lipopolysaccharide (LPS), flagellins, and bacterial DNA. A detected commensal will not trigger the same host defense response that occurs when a pathogen is encountered (Akira and Hemmi, 2003). For example, the lung epithelium is markedly intolerant to bacterial colonization, whereas the gastrointestinal epithelium harbors many commensal species (Aldridge *et al.,* 2005). This situation requires the gastrointestinal host cells to de-sensitize the response to LPS, which is accomplished though the down-regulation of the LPS-sensing TOLL-like receptor (TLR)-4 (Abreu *et al.,* 2001). Additionally, TLR-5 mediated flagellin recognition is also down-regulated on the apical cell surface where commensal bacteria are known to colonize. However, TLR-5 remains active on the basolateral side where undesirable tissue invasion past the epithelial barrier would still be detected (Gewirtz *et al.,* 2001). Alternatively, induction of the mucosal inflammatory response through NF-κB activation can be accomplished by the cytosolic recognition factor Nod1. This alternative to TLR-based recognition of infection by the host is thus tolerant of commensal inhabitants, while able to trigger the immune response to invasive bacteria (Kim *et al.,* 2004). These consistent findings seem to point to a default response of epithelial cells—almost always involving NF-κB and IL-8—to recruit immune effectors to the site of infection. The observed induction of apoptotic mechanisms as an innate response to infection has also been noted, although bacteria are able to prevent this process in some instances to preserve an intracellular niche for replication and growth. These consistent findings have been broadly emphasized, but it remains unclear if this constitutes the entire scope of the epithelium's role during interaction with bacteria.

In an attempt to define a conserved transcriptional response by human host cells to infection, Jenner and Young described host responses to pathogens that have been investigated

by transcriptional profiling of various cell lines (Jenner and Young, 2005). The existence of a conserved epithelial cell response to interaction with invasive pathogens was strongly suggested by early reports (Eckmann *et al.,* 2000). Included in this program were several recurring genes, such as iNOS and cyclooxygenase-2, as well as pro-inflammatory chemokines and cytokines (Finlay and Cossart, 1997; Kaufmann and Hess, 1999). This putative core response was further investigated using a wide range of cell types, which also allowed the elucidation of cell-type specific and pathogen-specific responses. To define the conserved response, a common pattern of regulation by cluster algorithms was used to overcome the differences in cell lines, time points, and pathogens used in these studies. The rationale was that the hierarchy for gene regulation would group affected genes together, and in discernable patterns, regardless of the experimental design. For all cell types, the authors determined that the core response to pathogens was the activation of 511 genes, based on their co-regulation during host-pathogen interactions (Jenner and Young, 2005). Genes that are preferentially induced by various cell types were also identified, such as immune response genes and genes for neutrophil cytosolic factor, important for skin immune responses.

Indeed, comparing host transcriptional profiles across all known arrays in search of response signatures was a tantalizing prospect. Cross-comparison has yielded useful information (Jenner and Young, 2005), but can be misleading if uniformity in the array process is lacking (Kellam, 2001). Potential pitfalls include the absence of given genes from experimental arrays, or the filtering out of these genes during the analysis being interpreted as these genes not being regulated (Kellam, 2001). These potential pitfalls appear to be in play in this instance, and suggest the description of "the core response" would more accurately be represented as "a core response." This is more than a question of semantics, as the inconsistencies between

experimental conditions potentially introduce significant variability and make the existence of other conserved host responses likely. Table 2-1 illustrates the studies that have utilized transcriptional profiling of epithelial cells to study host- bacterial interactions, which have almost exclusively relied upon different arrays to provide a core response. Further, many studies used custom arrays that were specifically targeted to study the cytokine and chemokine profiles elicited in host cells upon bacterial interaction. This strategy biases the final outcome of any attempt to define host response programs towards processes that involve innate immune defenses, such as apoptosis, cytokine/chemokine expression, and recruitment of immune cells. As these genes are reinforced for their roles in the host response, the simultaneous omission of cell-line specific and pathogen specific responses occurs. These specific responses may reveal highly diverse host strategies that are currently under-appreciated. For instance, the ability of epithelial cells to metabolize specific bacterial signaling molecules and virulence factors (Aldridge *et al.,* 2005), such as the *Pseudomonas* quorum sensing molecule 3OC12-HSL (Chun *et al.,* 2004), supports a more active role of the epithelium than simply recruiting immune cells. The preferential habitation of the mucosal layer by *Pseudomonas* in patients may arise from this metabolic activity by the epithelial layer (Doring and Worlitzsch, 2000). Additionally, the inhibition of apoptosis by invasive bacteria already been discussed is another example of an active epithelial response to bacteria. . Further investigation of this process is likely to reveal a great degree of pathogen-specific responses. Although the relationship between epithelial cells and inflammation is undeniable, additional work is necessary to reveal epithelial cell functions above and beyond the current paradigm.

Current Limitations of Microarrays and Gene Ontology Annotations

A common thread to the majority of global host transcriptional profiling experiments is the challenge of making biological sense of the hundreds of differentially regulated genes

amongst different experimental conditions. Due to data mining limitations, further confirmation steps have been largely restricted to genes that are up-regulated in infected cells as compared to the baseline uninfected controls (Eckmann *et al.,* 2000). This strategy is focused on the discovery of inducers of host response and will uncover general trends in the host response. However, down-regulated genes cannot completely be discounted in host-pathogen interactions. For example, the down-regulation of a given repressor may result in the induction of a gene/pathway. Discounting the genes that are down regulated may result in the omission of important checkpoints in response cascades. Likewise, downstream phenotypic confirmations would be limited to a small number of gene products and undermine the potential of global transcriptional profiling.

In addition to improving the accuracy of regulated genelists, methods to predict a biologically relevant outcome have emerged. One developing strategy to mine complex transcriptional datasets entails the use of gene ontology tools. Bio-ontologies have been described as formal representations of knowledge areas in which essential terms are combined with structuring rules that describe the relationships between the terms. This structured knowledge can then be linked to molecular databases (Bard and Rhee, 2004). Thus, the products of genes that are differentially regulated can be classified according to their biological process, the molecular function that they are associated with, or their cellular localization. Those individual ontology terms that are overly impacted in the output gene lists resulting from a given experimental condition are candidates for future investigation. Just as microarrays can only detect the genes that are included on the chip/membrane, gene ontology will only return functional information for genes that have been annotated. Affymetrix HG-U133A chips, for instance, have 22283 total probesets, but only 16264 of these have been annotated with a

biological process as of March 2006. Thus, approximately a third of the probesets of interest may still not be classified by ontology analysis. An additional source of complexity derives from the multifunctional nature of many genes. Hence, the final number of biological annotations may be significantly greater than the total number of probesets fed to an ontology analysis. The current genomic annotations being greatly driven by current research, a positive feedback loop remains where, in the case of host pathogen interactions, gene annotations weighted by immune responses will beget the discovery of pathways also involved with immune responses.

Another potential area of bias lies in the *in vitro* growth of the microorganism of interest for host-pathogen interaction studies. In almost all cases, bacterial strains are grown under conditions that poorly approximate the natural environment encountered at the site of infection. It is generally accepted that microorganisms modulate their gene expression profile in response to their immediate environment. Thus, critical bacterial determinants may be absent from array analysis, and consequently may not elicit all of the responses that would normally occur *in vivo* (Handfield *et al.,* 2005b; Handfield and Hillman, 2006).

Future Directions/Perspective

As predicted by Cummings *et al.,* global transcriptional profiling can potentially be applied to the paradigm of diagnosis of infectious diseases (Cummings and Relman, 2000). The hypothesis is that a pathogen expresses unique virulence factors, which will trigger a signature response in the host. This signature response will be amplified through a cascade of unique events, thus leaving a trail for investigators to trace back to the perpetrating organism (Cummings and Relman, 2000). Employing such a strategy, however, requires the characterization of the host response to hundreds of pathogens, which is no small task (Cummings and Relman, 2000).

Characterization of host responses at an actual infection site is a potentially informative exercise, although cellular heterogeneity is a potential complication (Cummings and Relman, 2000). This challenge has supported the use of cell culture models in cell lines likely to encounter a particular pathogen (Cummings and Relman, 2000). The rapid progress of microarray technology may already be on the verge of solving some of these complications. As Ichimura and colleagues demonstrated, the potential exists to combinine transcriptional profiling with histology and laser micro-dissection to address several complications of transcriptional profiling of patient samples. Once the cell type of interest has been isolated and excised, the mRNA expressed within cells can be amplified with sensitivity and fidelity acceptable for microarray analysis (Ichimura *et al.,* 2006).

A timecourse analysis of host response regulation performed in parallel with pathogen gene regulation may demonstrate temporal interplay during host-pathogen interactions. The simultaneous monitoring of the host and pathogen global transcriptomes in timecourse, or the "interaction transcriptome," would be the preferred method to demonstrate cause and effect relationships between host and pathogen gene expression (Matsumura *et al.,* 2005). Experimental conditions that include dead or inert bacteria in addition to live wild type and mutant pathogens are likely to demonstrate important differences in host cell responses to active manipulation by pathogens. A core response to dead bacteria of many types might consist of the innate immune functions consistently studied up to this point, while the live pathogen and cell type specific differences are likely to be apparent upon active alteration of the host transcriptome by bacteria, and subsequent interplay. The problems that may arise are likely to not lie in the technology or ability to perform the experiment, but again in the familiar problem of sorting through an enormous amount of data on both the host and pathogen side. Clearly, annotation of

genes and the degree of sequencing for the host cells and pathogens of interest are limiting factors that can contribute to this problem. Additionally, the familiar conundrum of choosing an appropriate experimental model is always present; the balance between accurately reproducing the conditions of actual real world infections—trending towards complexity—is paradoxically balanced by the need for simplicity in a model in order to yield understandable data. A large amount of data from inconsistent platforms, timepoints, host cell lines and other experimental conditions, is likely to lose important regulatory information and dilute the potential and resolution of arrays rather than maximizing their capacity (Jenner and Young, 2005).

Despite the current challenges associated with transcriptional profiling to study host-pathogen interactions, the bioinformatics era is anticipated to resolve biologically relevant questions. Improvements in data mining, completion of gene annotation, and a focused study of epithelial cells interacting with bacteria are expected to result in novel therapies. There is some evidence that host arrays may provide interesting candidates for vaccine development, but no new drugs, vaccines or diagnostics have yet emerged from targets found with these arrays. Attempts with "reverse vaccinology" have consisted of identifying genes of pathogens that are regulated upon interaction with a host cell. These *in vivo*-induced genes are potentially protective antigens that may be used in vaccine design. However, the specific interactions that are occurring between host and pathogen are potentially far more informative. Clearly, a more thorough investigation of the differential host responses is necessary, especially in light of differences observed between commensal and pathogenic bacteria. In this endeavor, microarray analysis will continue to be an essential tool for studying host-pathogen interactions of the epithelium.

Table 2-1. Transcriptional profiling of human epithelial cells to study host pathogen interactions [3]

Epithelial Cell Line	Bacterial species	Strain (mutant gene)	Array Type	# Genes	References
Epithelial cells interacting with wild type pathogens:					
T84	*Salmonella typhimurium*	SL1344, SL3201, KK1004, TML-83, SR11	Glass	650	(Zeng *et al.,* 2003)
	Eschericia coli	Strain 4	Glass	650	(Zeng *et al.,* 2003)
	Salmonella typhi	Clinical Isolate	Glass	650	(Zeng *et al.,* 2003)
AGS	*Helicobacter pylori*	TN2, G27	Microarray	2304; 22630	(Guillemin *et al.,* 2002; Maeda *et al.,* 2001a)
		P12, K8	Membrane	588	(Chiou *et al.,* 2001; Bach *et al.,* 2002)
HeLa	*Chlamydia trachomatis*	D/UW-3/Cx	Membrane	1176	(Hess *et al.,* 2001)
		LGV L2/434/Bu	Glass, Membrane	15000; 268	(Dessus-Babus *et al.,* 2000)
	Yersinia enterocolitica	Serotype O8 pYV+	Affymetrix HG-U133A	22283	(Bohn *et al.,* 2004)
	Actinobacillus actinomycetemcomitans	VT 1169	Affymetrix HG-U133A	22283	(Mans *et al.,* 2006)
Kato3	*H. pylori*	NCTC 11637	Membrane	136; 588; 10752; 46302	(Cox *et al.,* 2001)
IHGK	*Porphyromonas gingivalis*	33277	Affymetrix HG-U133A	22283	(Handfield *et al.,* 2005)
	A. actinomycetemcomitans	VT 1169	Affymetrix HG-U133A	22283	(Handfield *et al.,* 2005; Mans *et al.,* 2006)
	Fusobacterium nucleatum	ATCC 25586	Affymetrix HG-U133A	22283	(Hasegawa *et al.,* 2007)
	Streptococcus gordonii	DL1-Challis	Affymetrix HG-U133A	22283	(Hasegawa *et al.,* 2007)
HT-29	*Salmonella dublin*	Lane	Membrane	277	(Eckmann *et al.,* 2000)
A549	*Pseudomonas aeruginosa*	PAK	Glass	1506	(Ichikawa *et al.,* 2000)
BEAS-2B	*Bordetella pertussis*	BP536	Affymetrix Hu6800	7070	(Belcher *et al.,* 2000)
MKN45	*H. pylori*	TN2	Membrane	268	(Maeda *et al.,* 2001b)
Primary UECs	*Neisseria gonorrhoeae*	Strain 1291	Affymetrix HG-U95A	12626	(Binnicker *et al.,* 2003)

[3] Table adapted from: (Jenner and Young, 2005):

Table 2-1. Continued.

Epithelial Cell Line	Bacterial species	Strain (mutant gene)	Array Type	# Genes	References
T84	*S. typhimurium*	SL1344 (spi1-), SL2301 (flhD-, fliB-, fliC-, fliB-fliC-), KK1004 (fliD-), SR11 (invA-), TML-83 (invG-)	Glass	650	(Zeng *et al.*, 2003)
	E. coli	Strain 7	Glass	650	(Zeng *et al.*, 2003)
AGS	*H. pylori*	TN2 (cagE-)	Microarray	2304	(Maeda *et al.*, 2001a)
		(cagA-, vacA-, cagE-, cagN-, PAI-)	Microarray	22630	(Guillemin *et al.*, 2002)
		P17 (cagA-)	Membrane	588	(Bach *et al.*, 2002)
HeLa	*Y. enterocolitica*	pYV-, pYV-(inv-), pYV+ (yopP-)	Affymetrix HG-U133A	22283	(Bohn *et al.*, 2004)
Kato3	*H. pylori*	G50 (PAI-)	Membrane	136; 588; 10752; 46302	(Cox *et al.*, 2001)
IHGK	*P. gingivalis*	YPF-1 (fimA-)	Affymetrix HG-U133A	22283	(Handfield *et al.*, 2005)
	A. actinomycetemcomitans	JMS04 (orf1402-)	Affymetrix HG-U133A	22283	(Handfield *et al.*, 2005)
A549	*P. aeruginosa*	PAK, PAK-NP	Glass	1506	(Ichikawa *et al.*, 2000)
BEAS-2B	*B. pertussis*	BP356, BP-9K/129G	Affymetrix Hu6800	7070	(Belcher *et al.*, 2000)
MKN45	*H. pylori*	TN2 (cagE-)	Membrane	268	(Maeda *et al.*, 2001b)
Epithelial cells interacting with bacterial components:					
AGS	*H. pylori*	ABCCC-type CagA of NCTC 11637	Affymetrix Genome Focus 8500	8500	(Yokoyama *et al.*, 2005)
HT29	*Aeromonas hydrophila*	Cytotoxic enterotoxin Act	Affymetrix HGU133A	22283	(Galindo *et al.*, 2005)
T84	*S. typhimurium*	Flagellin of SL2301	Glass	650	(Zeng *et al.*, 2003)
BEAS-2B	*B. pertussis*	Pertussis toxin of BP536	Affymetrix Hu6800	7070	(Belcher *et al.*, 2000)

Table 2-1. Continued.

Epithelial Cell Line	Bacterial species	Strain (mutant gene)	Array Type	# Genes	References
Environmental treatments perturbing host-bacterial interactions:					
T84	N/A	TNF	Glass Affymetrix HG	650	(Zeng *et al.*, 2003)
AGS	*H. pylori*	cagA transfection	Focus	8500	(Yokoyama *et al.*, 2005)
BEAS-2B	*B. pertussis*	BP536 Sodium salicylate, BP536 Dexamethasone	Affymetrix Hu6800	7070	(Belcher *et al.*, 2000)

CHAPTER 3[4]
DISTINCTIVE CHARACTERISTICS OF TRANSCRIPTIONAL PROFILES FROM TWO EPITHELIAL CELL LINES UPON INTERACTION WITH *Aggretatibacter actinomycetemcomitans*[5]

Introduction

Utilizing several strategies introduced in Chapter 2 herein constitutes the completion of Specific Aim 1. A reporter system using epithelial cell transcriptional profiles is established, and several experimental conditions for this system are optimized. One purpose of this chapter is to demonstrate the importance of choosing a biologically relevant model system. To this point, transcriptional profiling and gene ontology analyses were performed to investigate the unique properties of two different epithelial cell lines to an *Aggregatibacter actinomycetemcomitans* challenge.

A total of 2867 genes are differentially regulated among all experimental conditions. The analysis of these 2867 genes reveals that the predominant specific response to infection in HeLa cells is associated with the regulation of enzyme activity, RNA metabolism, nucleoside and nucleic acid transport and protein modification. The predominant specific response in HIGK cells is associated with the regulation of angiogenesis, chemotaxis, transmembrane receptor protein tyrosine kinase signaling, cell differentiation, apoptosis and response to stress. Of particular interest, stress response genes are significantly, yet differently, affected in both cell lines. In HeLa cells, only three genes impact the response to stress, and the response to unfolded protein was the only term passing the ontology filters. This strikingly contrasts with the profiles

[4] This work was supported in part by NIDCR grants DE13523 (MH), DE11111 and DE14955 (RJL) and T32 training grant DE07200 (JM). Analyses were performed using BRB Array Tools developed by Dr. Richard Simon and Amy Peng Lam, National Cancer Institute

[5] The following manuscript is reprinted with permission from Blackwell Synergy ®. To access the definitive version, please refer to: Mans, J.J., Baker, H.V., Oda, D., Lamont, R.J. and Handfield, M. (2006) Distinctive characteristics of transcriptional profiles from two epithelial cell lines upon interaction with *Actinobacillus actinomycetemcomitans. Oral Microbiol Immunol* **21**: 261-267.

obtained for HIGK cells, where 61 regulated genes impact the response to stress, and constitute an extensive network of cell responses to *A. actinomycetemcomitans* interaction (response to pathogens, oxidative stress, unfolded proteins, DNA damage, starvation and wounding). Hence, the vast majority of genes and ontology terms that are currently associated with host-pathogen interactions are not common to HeLa and HIGK cells. Based on published reports of specific epithelial responses, the choice of an appropriate host cell line is important in light of bacterial tropism.

Another condition optimized is the MOI necessary to elicit a representative host response. Assessing the ratios of bacteria interacting per host cell is a useful criterion to increase the likelihood that every host cell encounters at least a single bacterium. A ratio of at least one bacterium per host cell should allow a homogeneous, representative mRNA response sampled for each treatment class. Further discussion of this hypothesis by microscopy may be found in Chapter 5. Additionally, the baseline transcriptome of HIGK cells is established for uninfected cells, and for cells interacting with *A. actinomycetemcomitans* for two hours, addressing the second Specific Aim.

Specific Host and Pathogen Interactions and the Host Transcriptome

Bacteria that colonize mucosal surfaces engage host epithelial cells in multifaceted and intimate interactions (Handfield, *et al*, 2005). For example, bacterial inhabitants of the urogenital, gastro-intestinal (GI) and respiratory tracts can manipulate epithelial cell signal transduction pathways, often to direct their internalization within these otherwise non-phagocytic host cells (Cossart and Sansonetti, 2004). Subsequently, epithelial cells infected with bacteria can exhibit major changes in the expressed proteome and transcriptome (Handfield, *et al*, 2005; Hardwidge, *et al*, 2004). As a model system to study epithelial cell responses to bacterial challenge, the HeLa cell line and its derivatives have often been utilized. These cells—derived

from a cervical carcinoma—have generated much information concerning the pathogenic properties of organisms such as *Shigella, Salmonella, Yersinia,* EPEC, *Helicobacter* and many others (Aldridge*, et al*, 2005; Garbom*, et al*, 2004; Lucchini*, et al*, 2005). Similarly, various cell lines including KB (ATCC, CCL-17) and HEp-2 (ATCC, CCL-23) are often used in the study of oral periodontal pathogens such as *P. gingivalis* and *A. actinomycetemcomitans* (Cao*, et al*, 2004; Richardson*, et al*, 2005; Sandros*, et al*, 1996). Once thought to be oral in origin, both KB and HEp-2 cells are now known to be HeLa derivatives that contaminated the original cultures (American Type Culture Collection).

Recently, Kang et al. demonstrated that *A. actinomycetemcomitans* cytolethal distending toxin (CDT) inhibits epithelial cell proliferation, but does not affect fibroblasts, when these cells are grown together in culture (Kang*, et al*, 2005). Additionally, Fine et al. have shown that the *A. actinomycetemcomitans* autotransporter adhesin Aae, is the adhesin responsible for the binding to Buccal Epithelial Cells (BEC) isolated from humans and old world primates, but not for BEC derived from new world primates and several other mammalian species (Fine*, et al*, 2005a). These examples demonstrate that interactions between *A. actinomycetemcomitans* and host cells appear to exhibit specificity and tropism. However, it is unclear if this tropism extends beyond the initial attachment of *A. actinomycetemcomitans* to oral cells and impacts the host-cell transcriptome. This question is particularly relevant in light of the increasing recognition of oral pathogens for their role in non-periodontal conditions—such as coronary artery disease (Beck*, et al*, 2005; Genco*, et al*, 2002) and birth of preterm-low birth weight infants (Scannapieco, 2005)—and subsequent interactions with different tissue types.

Undirected methods, such as DNA microarrays, can be used to survey the global transcriptional profiles of host cells in response to many different conditions. This approach is

highly useful for uncovering new processes involved with bacterial interaction beyond the effects of well-characterized adhesins and toxins. The value of this approach is especially apparent when host cell phenotypic changes are outwardly subtle and not easily observable using other methods. In this study, we have compared the transcriptional responses of two cell lines commonly used to study host-pathogen interactions—Human Immortalized Gingival Keratinocytes (HIGK) and HeLa cells—following *A. actinomycetemcomitans* co-culture.

Materials and Methods

Bacteria and Cell Lines

HeLa (KB cells; CCL-17, American Type Culture Collection) and HIGK cells (Oda, *et al*, 1996) were grown in Dulbecco's Modified Eagle's Medium (DMEM) and Keratinocyte Serum-Free Medium (KSFM), respectively, as a monolayer to 95% confluence in an atmosphere of 5% CO_2 at 37^o C (Cao, *et al*, 2004; Oda, *et al*, 1996). Both cell culture media were supplemented with 50 U penicillin/streptomycin mL^{-1} (Gibco). *A. actinomycetemcomitans* smooth strain VT1169 (SUNY465 Nal^R Rif^R) (Mintz and Fives-Taylor, 2000) was grown in liquid culture at 37^o C and 10% CO_2 to mid-logarithmic phase, and prepared for host cell co-culture according to standard methods (Richardson, *et al*, 2005). Briefly, epithelial cells were washed three times with 1X Dulbecco's Phosphate-Buffered Saline (Cambrex, Walkersville, MD) to remove residual antibiotics and waste products. In biological replicates of four per condition, epithelial cells were sham-infected with cell culture media or co-cultured with *A. actinomycetemcomitans* resuspended in culture media, resulting in a multiplicity of infection (MOI) of 1000:1. Previous studies in our lab (data unpublished) determined that 1000 was the lowest MOI ensuring every host cell encountered at least a single bacterium, resulting in a homogeneous population of infected host cells, and thus a representative mRNA sample of the infected state. Two hours co-culture was the time point previously determined to display a

phenotype that can be characterized in terms of host cell monolayer integrity, and chosen in order to maintain consistency with previous work in our lab (Handfield, *et al*, 2005). Epithelial cells were lysed with Trizol (Invitrogen Life Technologies, Carlsbad, CA) and RNA was prepared for GeneChip hybridization as recently described (Handfield, *et al*, 2005).

Microarray Analysis

Assessment of the host cellular responses to bacterial challenge was accomplished by transcriptional profiling using Affymetrix Human Genome HG U133A DNA microarrays (Feezor, *et al*, 2003). Infected and uninfected HeLa and HIGK cells were tested in four independent replicates. Subsequent array analysis was performed as recently presented (Handfield, *et al*, 2005). In brief, expression filters were applied to remove Affymetrix control oligos and probesets whose signal was undetected across all samples. The signal intensity values of the resulting dataset were variance-normalized, mean-centered, and ranked by their coefficient of variation. Normalization was performed in order to give equal weight to all probesets in the analysis, regardless of the raw signal intensity order of magnitude. To reduce the confounding effect of background signal variation on the analysis, the half of the dataset demonstrating the most variation across samples was used to perform unsupervised hierarchical cluster analysis using Cluster software (Eisen, *et al*, 1998). The resulting heat-map and Cluster dendrograms were visualized with Treeview (Eisen, *et al*, 1998) to reveal the extent of characteristic host-cell responses to each infection state, defined as identical treatments clustering together.

Following initial assessment of the host cell response to each condition, supervised analysis was performed to investigate differences in gene regulation among experimental conditions. For this analysis, the raw signal intensities were log-transformed for all probesets passing initial expression filters and correlated using BRB Array Tools (Simon and Peng-Lam). In each supervised analysis, biological replicates were grouped into classes according to host cell

type and infection state during co-culture experiments. Several methods of class prediction were utilized (compound covariate predictor, nearest neighbor predictor, and support vector machine predictor) in order to generate lists of classifiers whose expression state changes between classes at $P < 0.001$ and $P < 0.01$ levels of significance. Leave-one-out cross-validation (LOOCV) was performed to test the accuracy of each classifier and compared to the probability of a correct class prediction by chance alone, based on the P-value and total number of genes analyzed. In order to visualize the differentially-regulated genes, Microsoft Access database queries were used to match the subset of significantly regulated genes with their associated variance-normalized, mean-centered signal values calculated previously. Cluster and Treeview were used to visualize the correlations among genes and samples.

Ontology Analysis

The biological significance of the transcriptional profiles was investigated using gene ontology tools available online through NetAffx Analysis Center (Affymetrix Inc., Santa Clara, CA). Cross-validated probesets from the HG-U133A Gene Chip Arrays that were differentially expressed between classes at the $P < 0.001$ level of significance were annotated with their associated biological process ontology terms. Biological processes impacted by two or more regulated genes were visually examined via directed acyclic graphs (DAG) to gain insight of epithelial cell responses to *A. actinomycetemcomitans* co-culture. The total number of genes regulated per biological process, percentage of total genes impacted per term, and P-values calculated by NetAffx, were the criteria used to prioritize biological processes. Consistency between parent and child ontology terms was a prerequisite for additional characterization of the predicted biological response of HeLa and HIGK cells upon *A. actinomycetemcomitans* interaction.

Results and Discussion

Previous work suggested that a number of host factors are differentially expressed in response to challenges by oral pathogens. In particular, *A. actinomycetemcomitans* has been shown to display tissue tropism (Fine*, et al*, 2005b) and its well-characterized toxins have drastically different effects on different cell types (Kang*, et al*, 2005; Kanno*, et al*, 2005). Although these are clinically relevant examples of host gene modulation responding to bacterial challenge, the extent to which the transcriptome is impacted in a tissue specific manner upon direct cellular interaction with *A. actinomycetemcomitans* remains unclear. Hence, extensive transcriptional profiling and gene ontology analysis was performed to investigate the similarities and differences in the transcriptional response by two different lineages of epithelial cells to an *A. actinomycetemcomitans* challenge.

Initially, all samples from uninfected and infected HeLa and HIGK cells were used to determine the overall similarity of the transcriptional profiles of these two epithelial cell lines. Signal intensity data for the 14,171 probesets that passed initial expression filters were used to perform unsupervised cluster analysis and supervised class prediction as described in the Materials and Methods section. Unsupervised hierarchical cluster analysis revealed a characteristic host cell transcriptional profile, as biological replicates clustered together (data not shown). Class prediction at the $P < 0.001$ level of stringency revealed that 2867 genes were differentially regulated among all experimental conditions; by chance alone and with a normal distribution, one would expect that fourteen genes be identified as false positives. In addition, linear discriminant analysis and 1-nearest neighbor classifications were 100% accurate by LOOCV for 2000 random permutations, while nearest centroid and 3-nearest neighbors classifications were 80% accurate. Both rates of 80% and 100% are significantly more accurate than 25% correct classification rate expected by chance alone for class prediction using four

classes. This analysis conferred a high degree of confidence that these 2867 genes were indeed differentially regulated among all classes tested. As 2867 genes represents approximately 20% of the total genes analyzed, 80% of the transcriptome is neither significantly changed between HeLa and HIGK cells, nor is it impacted significantly upon an *A. actinomycetemcomitans* 2-hour co-culture. This level of similarity is consistent with the fact that both cell lines are epithelial in nature. Processes that are universally important to cell homeostasis would be predicted to be unaffected by bacterial challenges and thus constitutively expressed by epithelial cells regardless of their lineage. Indeed, a partial survey of the constitutively expressed biological processes revealed cellular functions related to RNA synthesis, metabolism, protein synthesis, and other generalized cellular processes (data not shown). Limitations in the algorithms used herein restricted the analysis to 1500 probesets per query, preventing an exhaustive ontology analysis for all 11,304 genes that were detected with the arrays but not differentially modulated between conditions or cell lines.

Treeview visualization of the 2867 probesets differentially expressed among all four classes (Figure 3-1) revealed interesting characteristics of HeLa and HIGK cells. The measured distance required to connect samples along the scaled dendrogram path reflects how closely related the transcriptional profiles of each sample are, based on Pearson's correlation coefficient. Thus, if a difference exists between two classes of treatments (infected *versus* uninfected, for example) replicates of a treatment will be more closely related to each other than to all other samples, as the *intra*-class distances required to connect these samples is shorter than the *inter*-class distances. This analysis also represented an indirect measure of the degree of noise introduced in that experimental system. In Figure 3-1, the major node of separation occurred between HeLa and HIGK cells, regardless of infection state. Less pronounced, yet significant,

nodes of separation could also be detected between uninfected and infected cells of the same lineage. As the differences between cell lines overshadowed the observable differences between infected and uninfected states, this dendrogram suggested a significant, lineage-based difference between the global transcriptional responses of these two epithelial cell types despite their high degree of similarity in housekeeping functions and intermediate metabolism. However, it cannot be ruled out that some differences discovered between the transcriptional profiles of HeLa and HIGK cells resulted from the different cell culture media used, differences in the growth rates of these cell lines, or differences that exist between HPV Type 16 (HIGK) and HPV Type 18 (HeLa) immortalization (Aguilar-Lemarroy*, et al*, 2001). To eliminate these variables, and further investigate the cell line-specific transcriptional profiles uncovered by our initial analysis, a comparison of the *A. actinomycetemcomitans*-infected state with the corresponding, baseline-uninfected state was performed for both cell lines independently. For these analyses, signal intensities were re-normalized across all samples, and both unsupervised and supervised analyses were repeated as presented above.

In HeLa cells 10,921 genes passed the initial expression filters, while 13,176 genes were analyzed in HIGK cells. Class prediction for HeLa cells revealed that only 67 genes were differentially expressed upon *A. actinomycetemcomitans* infection at the significance level of $P < 0.001$ (Figure 3-2A). In contrast, this analysis performed on HIGK cells yielded 625 significantly modulated genes (Figure 3-2B). LOOCV analysis for 2000 random permutations confirmed these predictors at 100% correct classification rate, using a number of analyses such as the compound covariate predictor, the diagonal linear discriminant, the 1 and 3-nearest neighbor, the nearest centroid, and the support vector machines analysis.

A directed effort was undertaken to investigate the extent of the common core transcriptional response by these two cell lines upon *A. actinomycetemcomitans* infection. Using Microsoft Access database queries, the 625 significantly-modulated genes in HIGK cells, and the 67 genes significantly-regulated in HeLa cells were correlated. Upon *A. actinomycetemcomitans* interaction, the common transcriptional response of HeLa and HIGK cells consisted of eight probe sets. Although statistically significant (P=1.4E -05), this constitutes only 1.17% of the total 684 genes regulated in both cell lines combined. In other words, a common core response to infection was found, but it was minimal and its biological significance remains uncertain. The expression patterns for seven of these eight genes showed a consistent pattern of regulation for both cell lines, six of which were up-regulated and one was down regulated (Table 1). To investigate the possible effect of sampling error on this outcome, we repeated the class prediction analysis at lower stringency of P <0.01. At that level of significance, HeLa cells modulated 404 genes and HIGK cells modulated 2011 genes (compared to 109 and 132, respectively, which would be expected by chance alone at this confidence level). Microsoft Access queries of this dataset revealed 84 genes regulated by both cell lines, representing 3.6% of the total genes modulated. This is of the same order of magnitude as the 1.17% of genes found to be in common at P <0.001 significance. Thus, this supports our contention that the low number of genes found to be modulated in both cell lines at P <0.001 was not the result of statistical error caused by the low sample number from HeLa cells.

The biological significance of this core transcriptional response to *A. actinomycetemcomitans* interaction was further investigated using the gene ontology tools as described in the Materials and Methods. The annotations were available for five of the eight genes presented in Table 1. The resulting output was 11 biological processes organized into four

main branches and associated with development (P=0.08484), morphogenesis (P=0.02327), primary metabolism (P=0.80176) and signal transduction (P=0.21298). Consistent with the stringent analysis presented above, the ontology analysis repeated for the 84 common genes identified at the P <0.01 threshold also implicated generalized cellular processes as being impacted in both cell lines. This corroborates our initial finding that approximately 80% of the transcriptome is modulated similarly between the two cell lines upon *A. actinomycetemcomitans* interaction for processes important to general homeostasis and not specifically related to host-pathogen interactions.

The biological processes that were differentially impacted in the two cell lines upon *A. actinomycetemcomitans* infection were analyzed using the same gene ontology algorithms described above. The 625 genes of HIGK cells that were found to be differentially regulated at a level of significance of P <0.001 were annotated and visualized. Using the filters described above, seven high-priority groups of host responses were identified. The biological processes identified included the regulation of angiogenesis, chemotaxis, transmembrane receptor protein tyrosine kinase signaling pathway, cell differentiation, and response to stress. Similarly, HeLa cells revealed a predominant specific response associated with the regulation of enzyme activity, RNA metabolism, nucleoside and nucleic acid transport, and protein modification. Consistent with previous reports, ontology terms related to cell death and apoptosis were uncovered in both cell lines (Handfield, *et al*, 2005).

Of immediate interest in the context of host-pathogen interactions were genes associated with stress-response. This biological process was significantly impacted in both HIGK and HeLa cells. However, a side-by-side comparison of the child ontology terms for the response to stress in HIGK (Figure 3-3A) and HeLa cells (Figure 3-3B) revealed significant differences in the

extent and nature of the transcriptional response uncovered. In HeLa cells, only three genes impacted the response to stress, and the response to unfolded protein was the only child term present that passed the ontology filters. This strikingly contrasted with the DAG obtained for HIGK cells, where 61 regulated genes impacted the response to stress, and constituted an extensive network of cell responses to *A. actinomycetemcomitans* interaction. For example, six child terms, including the response to wounding and the response to DNA damage stimulus were uncovered. These are consistent with the effect of the cytolethal distending toxin which arrests cell growth at the G2/M phase through DNA damage in immune cells and other cell types (Belibasakis, *et al*, 2005). In addition, 24 genes impacted the ontology terms associated with the response to pest, pathogen or parasite in HIGK cells. Included in this list were genes involved in the inflammatory response such as IL-1 beta and IL-6. IL-1 beta up-regulation in HIGK cells is consistent with reports documenting increased expression of this pro-inflammatory cytokine in primary GEC (Sfakianakis, *et al*, 2001) and has previously been reported (Handfield, *et al*, 2005). IL-6 stimulation in gingival fibroblasts by *A. actinomycetemcomitans* has been demonstrated in connection to CDT (Belibasakis, *et al*, 2005).

This representative example illustrated that biological processes impacted in both HeLa and HIGK cells may still be regulated differently. Consequently, solely identifying a list of differentially regulated genes between two conditions is not sufficient to predict a biologically significant outcome. Hence, the adjunction of a thorough ontology analysis favorably complements the transcriptional profile analysis and is invaluable in the context of a complex host-pathogen interaction.

The extensive transcriptional profiling and gene ontology analysis described herein did uncover a large number of common biological processes shared between both epithelial cell

lines. However, the vast majority of genes and ontology terms that are currently associated with host-pathogen interactions were not common to HeLa and HIGK cells. The relatively high number of differentially regulated genes found in oral HIGK cells (625) as compared to HeLa (67) is consistent with the tissue tropism displayed by the Aae adhesin of *A. actinomycetemcomitans* (Fine*, et al*, 2005a). The data presented here further suggested that the host transcriptional response to *A. actinomycetemcomitans* challenge is substantial. Further, in contrast to the current paradigm, the response of oral epithelial cells in host defense to infection appeared to be tailored, and have ramifications extending beyond specific toxicity and tissue tropism.

To our knowledge, this study presents the first report of the intrinsic differences that exist at the global host cellular level for two different epithelial cell lines in co-culture with the same pathogenic oral organism. By extrapolation, this study also emphasizes that caution should be exercised in the choice of epithelial cell lines or animal models of infection, regardless if the specific model behaves similarly in terms of adhesion and cytotoxicity.

Finally, the current study has evolutionary implications for the investigation of bacterial adaptation to association with host cells. Gene regulation in adhering or invading bacteria may depend not only on the presence of specific adhesins, but also to some extent, on the physiological status of the host cells. Thus, this report exemplifies that host-pathogen interaction may be more relevant if performed in the context of host cells derived from the tissue and the host of interest. The detailed analysis presented here supports the use of transcriptional profiling as a powerful tool to establish the basis of intrinsic similarities and discrepancies amongst different models of infection. This may be particularly useful to substantiate some contradictory reports in the literature pertaining to a variety of oral and other microorganisms.

Table 3-1. Transcriptional regulation of common probe sets to *A. actinomycetemcomitans*-infected HeLa (KB) and HIGK epithelial cells

Probe Set ID	HeLa	HIGK	Gene Title	Gene Symbol
202028_s_at	UP	UP	---	---
202499_s_at	UP	UP	solute carrier family 2	SLC2A3
206323_x_at	UP	UP	oligophrenin 1	OPHN1
210095_s_at	DOWN	DOWN	insulin-like growth factor binding protein 3	IGFBP3
212368_at	UP	UP	zinc finger protein 292	ZNF292
216609_at	UP	UP	---	---
221943_x_at	UP	UP	---	---
222155_s_at	DOWN	UP	G protein-coupled receptor 172A	GPR172A

Figure 3-1. Divergence of HeLa and HIGK cell transcriptional profiles. This heat map and dendrogram were constructed from 2867 probe sets differentially expressed between the four experimental classes at the significance level of $P < 0.001$. Probe set signal intensities were variance-normalized, mean-centered across samples, and subjected to hierarchical cluster analysis. Average linkage clustering by uncentered correlation was performed for genes and samples. The degree of similarity between the transcriptional profiles of each sample is expressed by Pearson's correlation coefficient distance metric, according to the adjacent scale. The expression state of each data point is represented as standard deviations from the mean expression level for that gene in all samples. Red indicates a relative increase, green indicates a relative decrease, and black indicates no relative change of mRNA transcripts for a given gene. Labels. Uninfected HIGK Cells, IHGK CTRL 01-04; *A. actinomycetemcomitans*-infected HIGK Cells, IHGK Aa 01-04; Uninfected HeLa Cells, HeLa CTRL 01-04; *A. actinomycetemcomitans*-infected HeLa Cells, HeLa Aa 01-04.

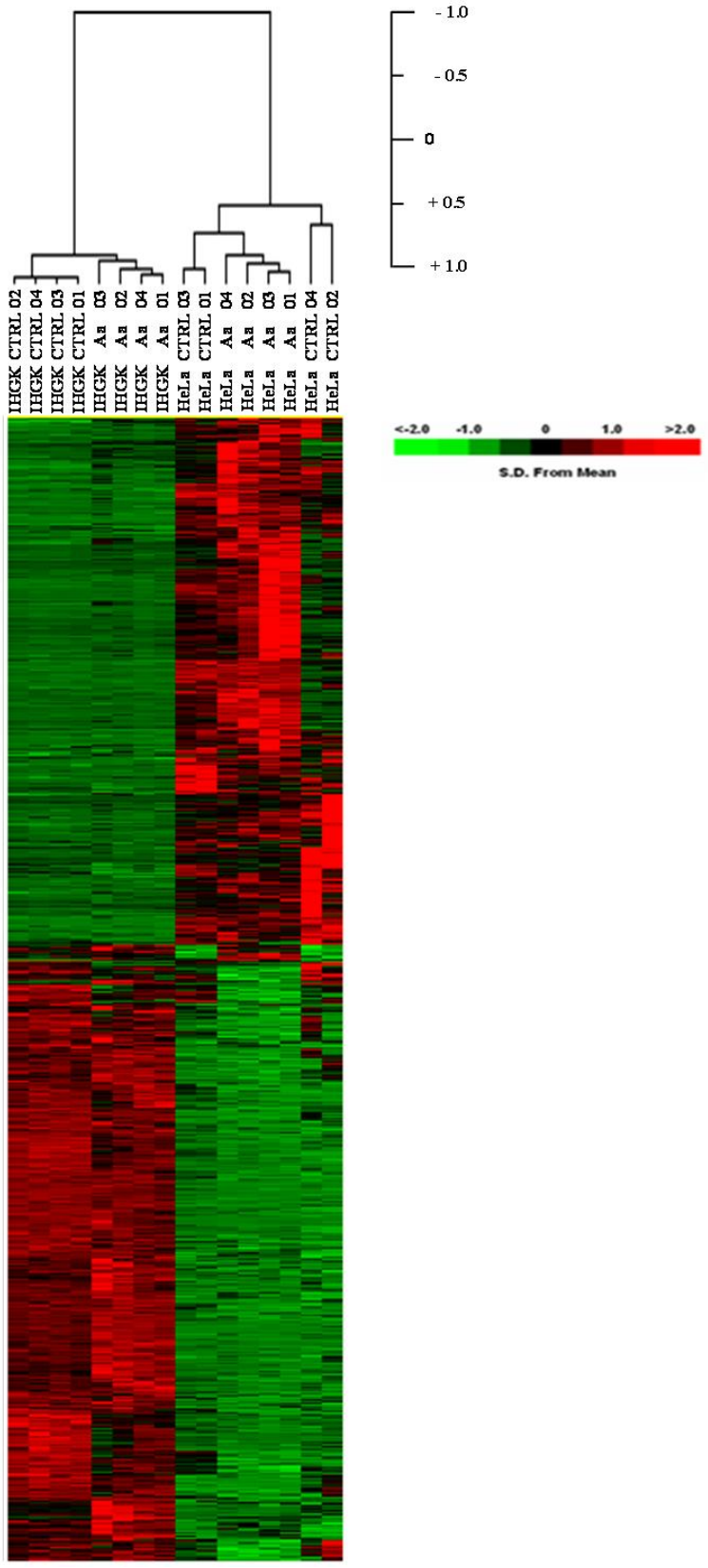

- 1.0
- 0.5
0
+ 0.5
+ 1.0
IHGK CTRL 02
IHGK CTRL 04
IHGK CTRL 03
IHGK CTRL 01
IHGK Aa 03
IHGK Aa 02
IHGK Aa 04
IHGK Aa 01
HeLa CTRL 03
HeLa CTRL 01
HeLa Aa 04
HeLa Aa 02
HeLa Aa 03
HeLa Aa 01
HeLa CTRL 04
HeLa CTRL 02
<-2.0
-1.0
0
1.0
>2.0
S.D. From Mean

Figure 3-2. Different patterns of gene expression by HeLa and HIGK cells upon co-culture with *A. actinomycetemcomitans*. RNA was isolated and purified after 2h co-culture with *A. actinomycetemcomitans* and compared to uninfected cells, for both cell lines independently. Probe set signal intensities were variance-normalized, mean-centered across samples, and subjected to hierarchical cluster analysis. Average linkage clustering by uncentered correlation was performed for genes and samples. Heat maps and dendrograms were constructed from 67 probe sets for HeLa cells (A), and 625 probe sets in HIGK cells (B), differentially expressed between uninfected and *A. actinomycetemcomitans*-infected treatments. The level of significance was $P < 0.001$. The degree of similarity between the transcriptional profiles of each sample is expressed by Pearson's correlation coefficient distance metric, according to the adjacent scale. The expression state of each data point is represented as standard deviations from the mean expression level for that gene in all samples. Red indicates a relative increase, green indicates a relative decrease, and black indicates no relative change of mRNA transcripts for a given gene. Labels. Uninfected HIGK Cells, IHGK CTRL 01-04; *A. actinomycetemcomitans*-infected HIGK Cells, IHGK Aa 01-04; Uninfected HeLa Cells, HeLa CTRL 01-04; *A. actinomycetemcomitans*-infected HeLa Cells, HeLa Aa 01-04.

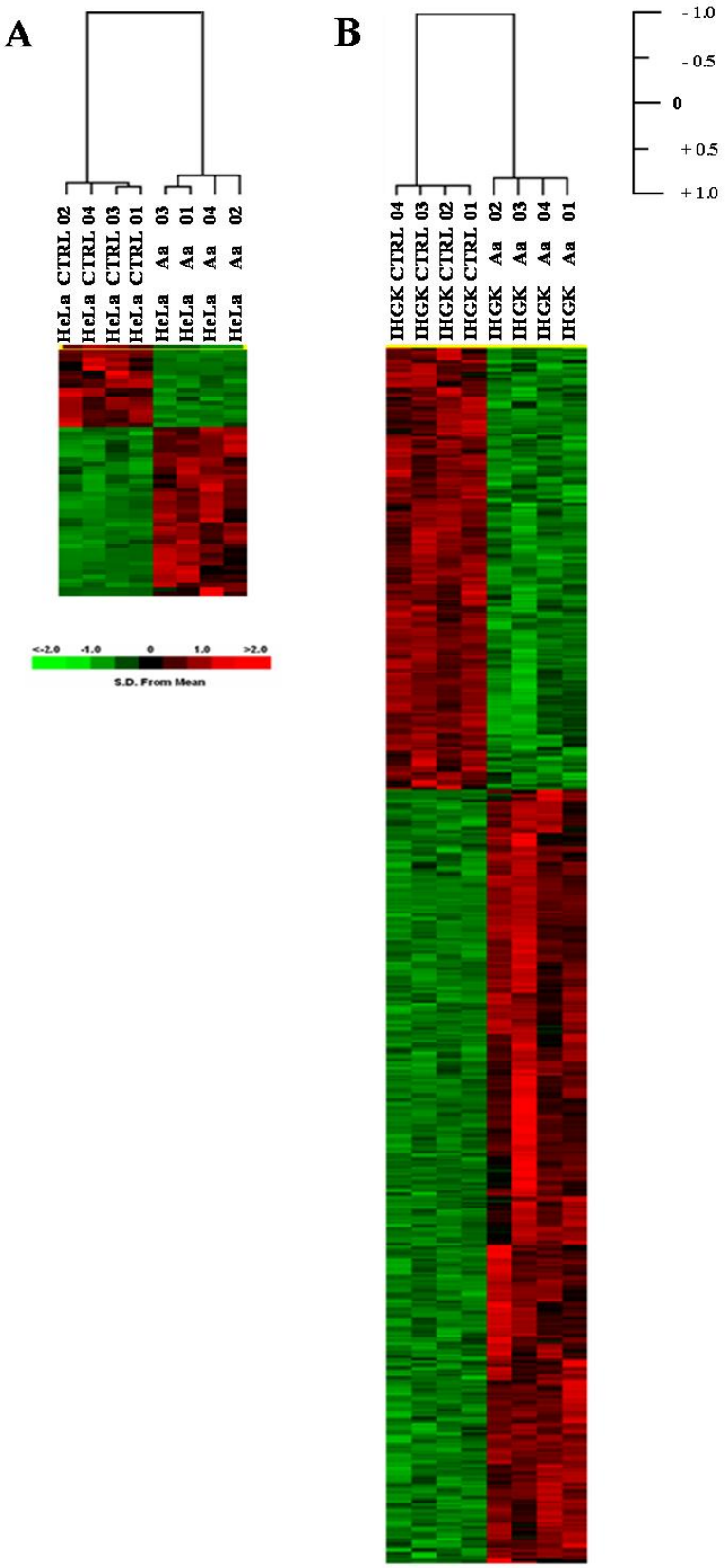
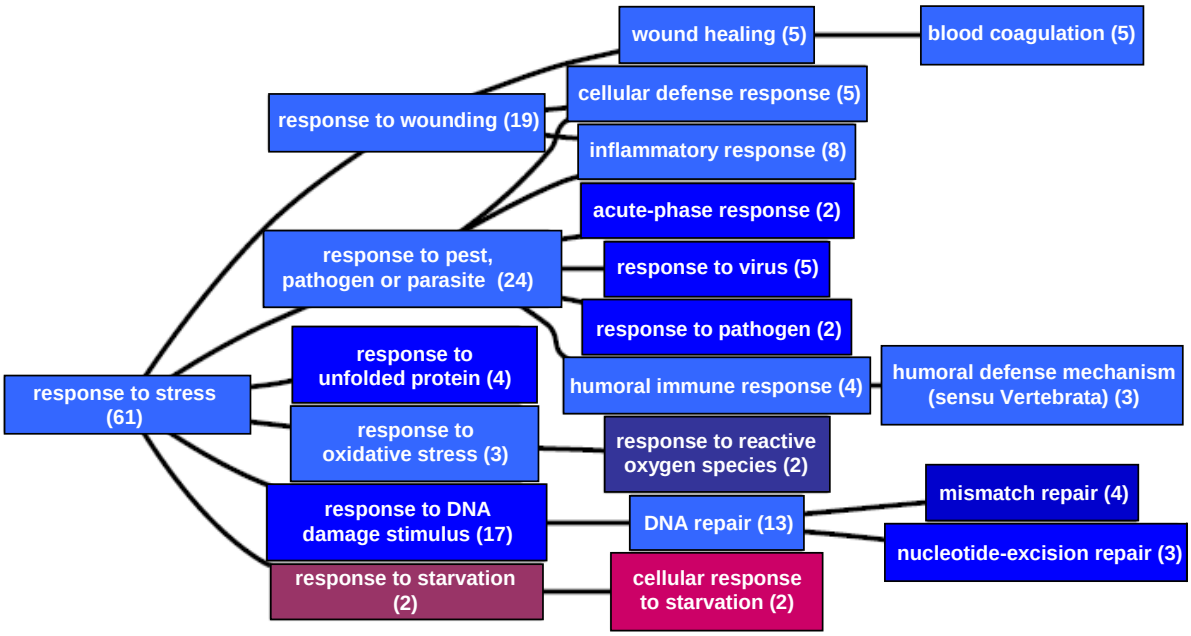
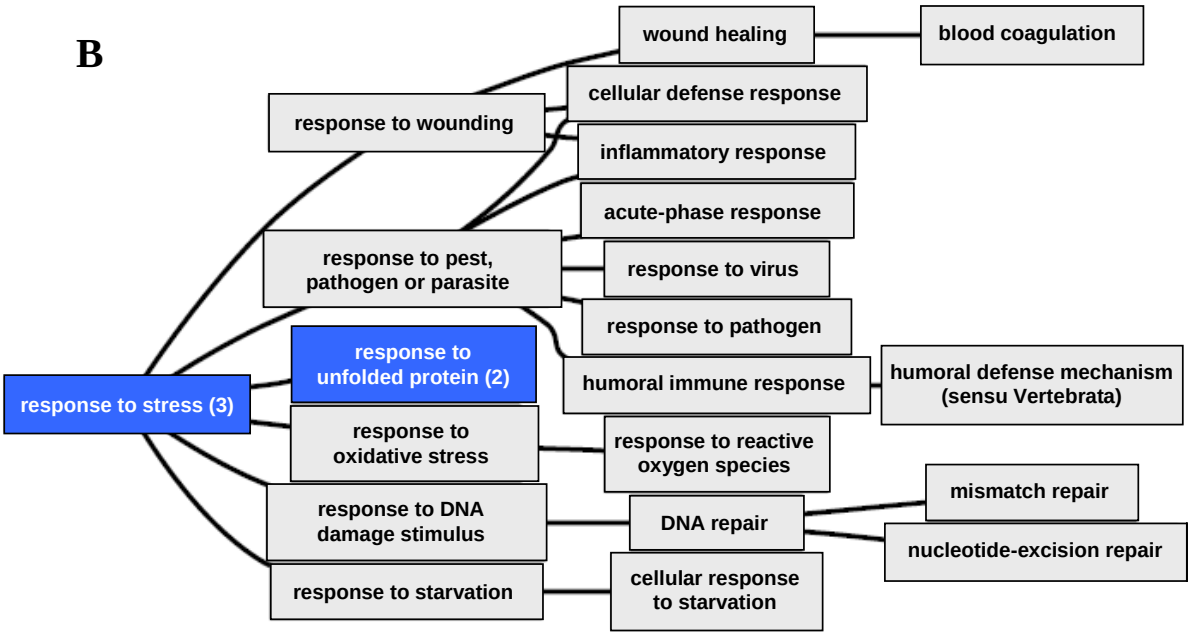

A
HeLa CTRL 02
HeLa CTRL 04
HeLa CTRL 03
HeLa CTRL 01
HeLa Aa 03
HeLa Aa 01
HeLa Aa 04
HeLa Aa 02
<-2.0
-1.0
0
1.0
>2.0
S.D. From Mean
B
IHGK CTRL 04
IHGK CTRL 03
IHGK CTRL 02
IHGK CTRL 01
IHGK Aa 02
IHGK Aa 03
IHGK Aa 04
IHGK Aa 01
- 1.0
- 0.5
0
+ 0.5
+ 1.0

Figure 3-3. Processes associated with stress response in HeLa and HIGK cells that are impacted upon *A. actinomycetemcomitans* interaction. Differentially regulated probe sets were annotated, and their associated gene ontology terms were visualized with NetAffx. Biological processes were organized by directed acyclic graphs (DAG), consisting of parent and child terms progressing from left to right. The degree of impact upon the ontology network caused by each transcriptional profile was expressed in terms of the percentage of total probe sets on the HG-U133A array. This DAG is one representative example of the total biological response to *A. actinomycetemcomitans* interaction by HIGK cells (A) and the corresponding analysis in HeLa cells (B). Individual nodes are color-coded on a spectrum of blue to red, with the latter indicating ontology terms most impacted. Biological processes missing in HeLa cells relative to HIGK cells are shown in gray.

A


wound healing (5)
blood coagulation (5)
cellular defense response (5)
response to wounding (19)
inflammatory response (8)
acute-phase response (2)
response to pest, pathogen or parasite (24)
response to virus (5)
response to pathogen (2)
response to unfolded protein (4)
humoral immune response (4)
humoral defense mechanism (sensu Vertebrata) (3)
response to stress (61)
response to oxidative stress (3)
response to reactive oxygen species (2)
mismatch repair (4)
response to DNA damage stimulus (17)
DNA repair (13)
nucleotide-excision repair (3)
response to starvation (2)
cellular response to starvation (2)

B


wound healing
blood coagulation
cellular defense response
response to wounding
inflammatory response
acute-phase response
response to pest, pathogen or parasite
response to virus
response to pathogen
response to unfolded protein (2)
humoral immune response
humoral defense mechanism (sensu Vertebrata)
response to stress (3)
response to oxidative stress
response to reactive oxygen species
mismatch repair
response to DNA damage stimulus
DNA repair
nucleotide-excision repair
response to starvation
cellular response to starvation

CHAPTER 4[6]
DISTINCT TRANSCRIPTIONAL PROFILES CHARACTERIZE ORAL EPITHELIUM-MICROBIOTA INTERACTIONS[7]

Introduction

Transcriptional profiling, bioinformatics, statistical, and ontology tools were used to uncover and dissect genes and pathways of human gingival epithelial cells that are modulated upon interaction with the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis*. The baseline transcriptome of HIGK cells is consistent with findings from the previous chapter for uninfected HIGK cells and cells interacting with *Aggregatibacter actinomycetemcomitans* for two hours at the MOI of 1000:1. Additionally, the baseline response of HIGK cells to *Porphyromonas gingivalis* interaction is determined for two hours co-culture at the MOI of 100:1. These experiments constitute the completion of Specific Aim 2.

Furthermore, the studies reported in this chapter move beyond simply establishing a model using transcriptional profiles of oral epithelial cells. Some of the anticipated insights into host-pathogen interactions are revealed. The differential regulation of pathways in epithelial cells upon bacterial interaction, such as apoptosis, is demonstrated. Consistent with their biological and clinical differences, the common core transcriptional response of epithelial cells to both organisms is limited, and organism-specific responses predominate. A large number of differentially regulated genes linked to the P53 apoptotic network were found with both

[6] This work was supported in part by NIDCR grants DE13523 (MH), DE11111 and DE14955 (RJL), T32 training grant DE07200 (JM) and funds from the Center for Molecular Microbiology (APF). The research presented here has complied with all relevant federal guidelines and institutional policies regarding the use of human subjects. We thank Dr. Dolphine Oda (University of Washington) for kindly providing HIGK cells; and Drs. K. Mintz and P. Fives-Taylor for kindly providing strain VT1169 and plasmid pVT1461. Analyses were performed using BRB Array Tools developed by Dr. Richard Simon and Amy Peng Lam. We thank Renata Salas Collazo and Paolo Rodrigues for their technical assistance.

[7] The following manuscript is reprinted with permission from Blackwell Synergy ®. To access the definitive version, please refer to: Handfield, M., Mans, J.J., Zheng, G., Lopez, M.C., Mao, S., Progulske-Fox, A., *et al*. (2005) Distinct transcriptional profiles characterize oral epithelium-microbiota interactions. *Cell Microbiol* **7**: 811-823.

organisms, which is consistent with the pro-apoptotic phenotype observed with *A. actinomycetemcomitans* and anti-apoptotic phenotype of *P. gingivalis.* Furthermore, with *A. actinomycetemcomitans*, the induction of apoptosis does not appear to be Fas- or TNFα mediated.

The limits of the reporter system are pressed by combining mutant analysis with transcriptional profiling. Linkage of specific bacterial components to host pathways and networks provides additional insight into the pathogenic process. In so doing, this chapter also addresses the third and final specific aim, the contribution of specific bacterial components to host pathogen interactions. Comparison of the transcriptional responses of epithelial cells challenged with parental *P. gingivalis* or with a mutant of *P. gingivalis* deficient in production of major fimbriae, which are required for optimal invasion, showed major expression differences that reverberated throughout the host cell transcriptome. In contrast, gene ORF859 in *A. actinomycetemcomitans,* which may play a role in intracellular homeostasis, had a more subtle effect on the transcriptome. These studies begin to unravel the complex and dynamic interactions between host epithelial cells and endogenous bacteria that can cause opportunistic infections.

Background

The human microbiota comprises a complex ecosystem characterized by the simultaneous presence of a large number of normal colonizers, associated with health and thriving in a dynamic environment. Since health is the most common state of a host, it has been speculated that the autochthonous flora has co-evolved with its host to interact in a balanced state that is beneficial to both the host and the microbiota (Galan and Zhou, 2000). There are an appreciable number of benefits to the host that the indigenous microbiota is thought to provide, including the synthesis of vitamins (B complex and K), the prevention of infection by pathogens

(by direct competition for niches or by immune cross-reactivity), and impacting the normal development of the immune system (Hooper and Gordon, 2001). Furthermore, there is an increasing realization that complex societies of indigenous microbes can influence human physiology and development. For example, in the GI tract the Gram-negative anaerobe *Bacteroides thetaiotaomicron* can modulate expression of ileal epithelial cell genes involved in nutrient adsorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and maturation (Hooper et. al., 2001). Since host and microbiota interactions are dynamic, disease may arise at the mucosal surface of a susceptible host when a perturbation occurs in the epithelial environment, for example, when the host becomes immunocompromised, or as a result of the unintended (in an evolutionary sense) consequences of bacterial activity (Galan and Zhou, 2000).

In the oral cavity, periodontal infections that affect and ultimately destroy the tissues supporting the teeth are among the most common diseases of humans. According to the 2000 Surgeons General's Report on Oral Health (NIDCR, 2000), these conditions afflict 14% of adults aged 45-54 and 23% of those aged 65-74 years. Furthermore, an epidemiological association is emerging between periodontal infections and serious systemic conditions such as coronary artery disease and preterm delivery of low birth weight infants (Scannapieco and Genco, 1999). The etiology of oral infectious diseases is complex and involves consortia of bacteria thriving in biofilms and exploiting immunological susceptibilities in the host. Despite the multifactorial nature of these diseases, there is a consistent relationship between the Gram-negative capnophile *Aggregatibacter actinomycetemcomitans* and localized aggressive periodontal disease (Slots and Genco, 1984; Zambon, 1985; Haffajee and Socransky, 1999; Offenbacher, 1996; Meyer and Fives-Taylor, 1997), and between the Gram-negative anaerobe *Porphyromonas gingivalis* and

severe, chronic manifestations of the disease (Slots *et al.,* 1986, Slots and Genco, 1984; Haffajee and Socransky, 1999).

The initial interface between the host and the potentially periodontopathic organisms, such as *P. gingivalis* and *A. actinomycetemcomitans*, is the epithelial layer that lines the subgingival crevice. Epithelial cells are both a physical barrier to infection and a component of a network that efficiently signals microbial intrusion to the immune cells to insure effective mobilization of the innate and specific defense mechanisms (Kagnoff and Eckmann, 1997). Both *A. actinomycetemcomitans* and *P. gingivalis* are capable of invading gingival epithelial cells and can remain viable intracellularly. Furthermore, epithelial cells maintain viability following intracellular penetration by either *P. gingivalis* or *A. actinomycetemcomitans* (Nakhjiri *et al.,* 2001; Takayama *et al.,* 2003; Kato *et al.,* 2000). However, the entry mechanisms employed by these invasive organisms are distinct. *A. actinomycetemcomitans* enters epithelial cells through a dynamic multistep process whereupon the organisms are first constrained in an intracellular vacuole from which they subsequently escape and spread cell-to-cell with the aid of microtubules (Meyer *et al.,* 1996, 1999). Within epithelial cells *A. actinomycetemcomitans* upregulates a distinct set of genes that facilitate adaptation to the intracellular environment (Cao *et al.,* 2004, Richardson *et al.,* 2004). Among these genes is ORF859 encoding a conserved protein of unknown function. In the case of *P. gingivalis,* the major fimbriae (comprised of the FimA protein) bind to integrins on the surfaces of gingival epithelial cells and stimulate integrin-dependent signaling to effect invasion through both microfilament and microtubule remodeling (Yilmaz *et al.,* 2002, 2003). *P. gingivalis* also impacts the MAP-kinase pathway and causes transient increases in intracellular Ca^{2+} concentrations (Watanabe *et al.,* 2001; Belton *et al.,* 2004). Both of these signal transduction pathways can converge on nuclear transcription factors

and modulate gene expression. Indeed, *P. gingivalis* has been shown to affect expression of individual genes in epithelial cells including those encoding IL-8 and Bcl-2 (Darveau *et al.,* 1998; Nakhjiri *et al.,* 2001).

Transcriptional profiling using microarrays provides a means to monitor epithelial cell responses to invading microorganisms on a global scale (Kagnoff and Eckmann, 2001; Yowe *et al.,* 2001; Kellam, 2000, 2001; Kato-Maeda *et al.,* 2001; Manger and Relman, 2000; Cummings and Relman, 2000; Lory and Ichikawa, 2002; Ichikawa *et al.,* 2000; Sepulveda *et al.,* 2002). Results from such studies suggest that the encounter between host and microbiota may involve a finely tuned set of interactions whereby both cell types adapt and co-exist with each other. Consequently, the regulation of normal processes such as cell division or apoptosis may be key to maintaining a balanced longstanding intracellular state whereby both cell types inflict a minimal degree of harm on each other. In support of this concept, epithelial cells recovered from the oral cavity show high levels of intracellular *P. gingivalis* and *A. actinomycetemcomitans* (Rudney *et al.,* 2001; Christersson *et al.,* 1993, 1987a, b). Hence an intracellular location may be a natural component of the lifestyle of these oral organisms. In this study, we have utilized human microarrays to determine the transcriptional response of gingival epithelial cells to co-culture with *P. gingivalis* or *A. actinomycetemcomitans*. Moreover, we have extended these studies to investigate the transcriptional responses of epithelial cells that are manipulated by the major fimbriae (FimA) of *P. gingivalis* and the intracellulary upregulated ORF859 of *A. actinomycetemcomitans*.

Results and Discussion

General Considerations

To investigate early events in oral infection by *P. gingivalis* and *A. actinomycetemcomitans* we analysed differential gene expression in human gingival epithelial

cells using the Affymetrix HG U133-A oligonucleotide arrays that contain over 22,000 different probe sets. In addition, isogenic mutant strains were utilized to assess the roles of specific bacterial proteins in modulation of the host cell transcriptome. Host cell apoptosis, the major pathway impacted by *P. gingivalis* and *A. actinomycetemcomitans*, was validated by phenotypic assays.

Association of *A. actinomycetemcomitans* and *P. gingivalis* With Epithelial Cells

P. gingivalis and *A. actinomycetemcomitans* demonstrate differing efficiencies of binding to, and internalization within, human immortalized gingival keratinocytes (HIGK). In order to compare epithelial cell transcriptional profiles in response to an equivalent challenge of the two organisms, we first compared adhesion and invasion at MOIs predicted to result in the same number of epithelial cell-associated bacteria for each species. As shown in Table 4-1, at a MOI of 100:1 for *P. gingivalis* and 3,000:1 for *A. actinomycetemcomitans*, the numbers of bacteria associated with the epithelial cells were of the same order of magnitude. These MOIs were then used in subsequent experiments. In contrast, the levels of invasion were significantly different; *P. gingivalis* being a considerably more efficient invasive microorganism as compared to *A. actinomycetemcomitans*. Notably, HIGK cells behaved similarly to primary gingival epithelial cells (GEC) in co-cultures with *A. actinomycetemcomitans* and *P. gingivalis*, with regard to both adhesion and invasion. Further confirmation of the relevance of the HIGK cell model was provided by the finding that the gene for IL-1 beta was upregulated in HIGK cells in co-cultures with both periopathogens (not shown). This is consistent with reports documenting increased expression of this pro-inflammatory cytokine in primary GEC (Sfakianakis *et al.,* 2001; Sandros *et al.,* 2000).

Gene Expression in Gingival Epithelial Cells Regulated by *A. actinomycetemcomitans* and *P. gingivalis*

To characterize epithelial cell responses to *A. actinomycetemcomitans* and *P. gingivalis,* and to assess the extent to which host responses may depend on the challenging organism, we used human cDNA microarrays to monitor relative abundance of HIGK cell transcripts following co-culture with *A. actinomycetemcomitans* or *P. gingivalis*. Array-to-array comparisons were carried out using unsupervised and supervised methods to assess the relatedness of the specimens (arrays) under investigation using the Cluster and TreeView Software (Eisen *et al.,* 1998). The significance level used in identifying genes that were differentially expressed was $P < 0.001$. Hierarchical clustering was first used to perform an unsupervised analysis. Visual representation of the unsupervised cluster analysis of *P. gingivalis*-infected, *A. actinomycetemcomitans*-infected, and uninfected cells was performed using Treeview software. The resulting dendrogram revealed that the array chips from each infection state clustered together (not shown). Thus, each infection state elicited a specific and distinct transcriptome in HIGK cells. This was also an indication of the quality and consistency of the hybridization procedure.

Supervised analyses were next performed to identify gene expression differences between the *P.* gingivalis-infected or *A. actinomycetemcomitans*-infected as compared to uninfected HIGK cells, at a significance level of $P < 0.001$. To test the predictive validity of the probe sets identified at this level of significance, a leave-one-out cross-validation (LOOCV) was performed with four different prediction models (linear discriminant, 1KNN, 3KNN and nearest centroid). This validation step addressed the ability of probe sets to distinguish between the different classes (i.e. infection states). Briefly, this analysis determined if the classifier performed better than one would expect by chance alone. In the present study there were three classes; on average one would expect to correctly classify the arrays by chance alone 33% of the time. Using the

gene expression classifier, the arrays were correctly classified 92% of the time. Thus, the gene expression differences significant at $P < 0.001$ can be used to distinguish between the strains and their miss-classification rate of 8% is much lower than the miss-classification rate of 67% expected by chance. Figure 4-1 shows the K-means clustering patterns of probe sets where the expression patterns were significantly different between the treatment classes. Several interesting clusters of genes are highlighted by blocks one through six in Figure 4-1. For instance, block 1 (182 probe sets) represents genes that are down-regulated in *A. actinomycetemcomitans*-infected cells, but not modulated in *P. gingivalis*-infected or control uninfected cells. Conversely, block 5 (252 probe sets) represents genes that are up-regulated in *A. actinomycetemcomitans*-infected cells, but not modulated in *P. gingivalis*-infected or control uninfected cells. These two clusters of genes may be characteristic of cellular interactions specifically associated with *A. actinomycetemcomitans*. Similarly, *P. gingivalis* elicited a transcriptional response in HIGK cells that is specific to this organism (block 2, up-regulated; and block 3, down-regulated). Overall, transcriptional response appeared to be diametrically opposed between the two organisms with only a small number of genes (41 probe sets) up- or down-regulated by both species (blocks 3 and 6). These common genes may be involved in a general host cell response to infection that may be universal for oral Gram-negative organisms or possibly even for bacterial stimulation in general. However, possibly more importantly, the data also suggest that individual organisms may have evolved to modulate a finite number of pathways that are characteristics of the genus. Moreover, host cells appear to be able to distinguish between infecting organisms and tailor transcriptional responses accordingly.

Ontology Analysis

In order to mine the array data for biologically relevant information, an ontology analysis based on relatedness to known metabolic pathways was performed. The ontology analysis was

performed at P <0.005 against the 354 different biological processes that have been identified thus far in the human GO syntax ontology database (http://obo.sourceforge.net/). 16 gene ontology pathways, including molecular functions, cellular components, and biological processes were identified as representing the canonical response to both organisms. Moreover, 21 additional gene ontology pathways were specifically found among the genes that responded to exposure to *P. gingivalis*. Similarly, a specific response for *A. actinomycetemcomitans*-infected HIGK cells resulted in modulation of 49 pathways. Those pathways with relevance to documented host-pathogen interactions are presented in Tables 4-2, 4-3, and 4-4.

Of particular interest to host-pathogen interactions in the oral cavity, one of the over-represented pathways was the apoptosis pathway. *P. gingivalis* and *A. actinomycetemcomitans* have been shown to alter cytokine expression and modulate apoptosis in various cell types. The induction of apoptosis in immune cells of the oral cavity is thought to have a significant immunomodulatory (immunosuppressive) effect and contribute to the pathogenesis of *A. actinomycetemcomitans* in periodontal diseases (Lally *et al.,* 1989ab; Ebersole *et al.,* 1990; Spitznagel *et al.,* 1995; Korostoff *et al.,* 1998, Demuth *et al.,* 2003; Kato *et al.,* 2002). Furthermore, *A. actinomycetemcomitans* can also induce apoptosis in a leukotoxin-independent manner in oral epithelial cells, periodontal ligament cells, and gingival fibroblasts (Kato *et al.,* 2000; Belibasakis *et al.,* 2002; Teng and Hu, 2003). A recent report suggests that the effector molecule associated with *A. actinomycetemcomitans* apoptosis in human gingival epithelial cells is a CagE homologue, which encodes a component of a putative type IV secretion system (Teng and Hu, 2003). *P. gingivalis*, by contrast, suppresses apoptosis in primary cultures of gingival epithelial cells. *P. gingivalis*-induced suppression of apoptosis is correlated with activation of

Bcl-2 at the transcriptional level and inhibition of cytochrome c release from the mitochondria (Nakhjiri, *et al.,* 2001; Yilmaz *et al.,* 2004).

The ontology analysis presented in Tables 4-2, 4-3 and 4-4 revealed that a total of fifty-five distinct apoptosis-associated genes were modulated upon *A. actinomycetemcomitans* or *P. gingivalis* co-cultures of HIGK cells at a significance of $P < 0.005$. Of these, eight were modulated in both organisms, thirty-one were modulated only in *A. actinomycetemcomitans*, and seventeen were differentially transcribed in *P. gingivalis* only. Interestingly, a large number of differentially regulated genes linked to the P53 network were found in both organisms. The P53 protein is a tumor suppressor gene that is positioned at a major node of a network that is involved in cell division and apoptosis. There are three major types of stress that modulate P53: aberrant growth signals, DNA damage and physico-chemical stress. The apoptosis induction by P53 can be mediated either by stimulation of Bax and Fas antigen expression, or by repression of Bcl-2 expression. A summary of the major apoptotic effector molecules impacted by the organisms is presented in Figure 4-2. *A. actinomycetemcomitans* activated the proapoptotic molecules BBC3, GADD45A, E2F1 and ATM and repressed cMYC. *P. gingivalis*, activated cMYC and SGK both of which are anti-apoptotic and play a role in cell survival and proliferation. cMYC can repress transcription of the proapoptotic GADD45A, while SGK phosphorylates and negatively regulates the transcription factor FOXO3A that can participate in apoptosis, in part through the GADD45a protein (Barsyte-Lovejoy *et al.,* 2004; Brunet *et al.,* 2001; Tran *et al.,* 2002). SGKs are related to Akt, a serine/threonine kinase that plays a crucial role in promoting cell survival and has been shown to be activated by *P. gingivalis* in primary GEC (Yilmaz et al, 2004). Most of the activity of *P. gingivalis*, however, revolved around the mitochondrial pathway, with upregulation of Bcl-2 and Bfl1. Bcl-2 inhibits release of cytochrome c from the mitochondria

and can inhibit P53 (Cory and Adams, 2002). Bfl-1 can also inhibit cytochrome c release and, in addition, suppresses Bid, which is an activator of the proapoptotic mediators Bax and Bak (Cory and Adams 2002). Thus, the transcriptional profiles are consistent with a pro-apoptotic phenotype of *A. actinomycetemcomitans* and an anti-apoptotic phenotype of *P. gingivalis.*

Besides the P53 pathway, *A. actinomycetemcomitans* and *P. gingivalis* modulated the apoptosis pathway via a number of other effectors. With *A. actinomycetemcomitans*, proapoptotic activity was restricted to the P53 pathway and multiple genes were found to be regulated in a pattern that is consistent with the repression of the Fas- and TNFα- mediated pathways. For instance, the Tumor necrosis factor receptor superfamily member 6B precursor (TNFRSF6B or decoy receptor 3) was found to be up-regulated. This factor is a soluble receptor that binds to the Fas ligand and is thought to play a regulatory role in suppression of FasL- and LIGHT-mediated cell death (Yu *et al.,* 1999). Similarly, CFLAR (CASP8 and FADD-like apoptosis regulator precursor, aka FLIP) was found to be up-regulated. This factor is a well known inhibitor of Fas and all other known human death receptors (Irmler *et al.,* 1997). FOXO3a, which is a factor that can promote apoptosis through, among other pathways, FLIP down-regulation (Skurk *et al.,* 2004), was transcriptionally repressed by *A. actinomycetemcomitans*. This is consistent with the observed up-regulation of FLIP. The programmed cell death protein 6 (PDCD6, alias ALG-2) was found to be repressed. This factor is thought to mediate calcium-regulated signals along the death pathway, and is required for Fas-induced cell death (Vito *et al.,* 1996). Similarly, RIPK1 (TNFRSF-interacting serine-threonine kinase 1) was down-regulated. This protein interacts with the death domain of FAS and TRADD and initiates apoptosis (Kreuz *et al.,* 2004). In addition, TNFAIP3 that is known to inhibit TNF-induced NF-kappa-B- dependent gene expression by interfering with a RIP- or TRAF2- mediated

transactivation signal (Ferran *et al.,* 1998), was induced by *A. actinomycetemcomitans*. Also induced was NFKB1A that inhibits NF-kappa-B by complexing with and trapping it in the cytoplasm (Haskill *et al.,* 1991). The induction of NFKB1A has also been observed with pathogenic *Pseudomonas aeruginosa* (Perez *et al.,* 2004). IER3 (alias IEX-1 or immediate early response gene X-1), upregulated by *A. actinomycetemcomitans,* is controlled by multiple transcription factors among which P53, NF-kappaB/rel, Sp1 and c-Myc play central roles. Overexpression of IER3 has been known to render some cells sensitive to apoptosis (Wu *et al.,* 2001). Receptor interacting protein-2 (RIP2) that mediates the recruitment of caspase death proteases (McCarthy *et al.,* 1998) was over expressed in response to *A. actinomycetemcomitans*, which is consistent with the activation of cell death. Collectively, these observations are consistent with the repression of the Fas and TNFα signaling pathways. Hence, in the case of *A. actinomycetemcomitans*, the transcriptional profile argues that the observed apoptotic phenotype is not Fas- or TNFα-mediated, but P53- dependant.

In common with *A. actinomycetemcomitans*, *P. gingivalis* upregulated the anti-apoptotic molecules TNFAIP3 and CFLAR. Additionally, *P. gingivalis* downregulated CDC2L2, a serine-theronine kinase that may play multiple roles in apoptosis. Moreover, CDC2L2 is deleted/translocated in neuroblastomas with MYCN gene amplification, a subset of malignant melanomas (Gururajan *et al.,* 1998.). Taken together, these data show that *P. gingivalis* can prevent the induction of apoptosis in HIGK cells at multiple levels. The long term consequences of this activity for normal physiologic function of epithelial cells remain to be established.

Apoptosis in Gingival Epithelial Cells Modulated by *A. actinomycetemcomitans* or *P. gingivalis*

The apoptotic responses of HIGK cells at the transcriptional level revealed by array analyses were verified by a phenotypic assay for apoptosis. As shown in Figure 4-3, *A.*

actinomycetemcomitans induced apoptosis in HIGK cells whereas *P. gingivalis* did not stimulate apoptotic activity. Furthermore, *P. gingivalis* cells were capable of inhibiting camptothecin-induced apoptosis in HIGK cells. These results both corroborate other reports in the literature with different epithelial cells (Kato *et al.,* 2000; Teng and Hu, 2003; Nakhjiri, *et al.,* 2001; Yilmaz *et al.,* 2004) and show that the mRNA expression levels correlate with phenotypic properties, at least with regard to some genes involved with apoptosis.

Gene Expression in Response to Isogenic Mutants

A bacterial mutant analysis was combined with host transcriptional profiling to assess the role of specific bacterial products or phenotypes on the epithelial cell gene expression programs. *A. actinomycetemcomitans* ORF859 (PEDANT database) was initially found to be induced *in vivo* in infected humans using IVIAT (Handfield *et al.,* 2000). The product of this gene was further shown to be induced in plaque from infected patients (Handfield, 2000, 2002) and in various cell lines, including HIGK cells (Richardson, 2004), and is a potential marker for active disease in LAP patients (Cao *et al.,* 2004). A bioinformatic analysis of this gene product did not reveal a predicted function, although the gene product is highly conserved across genera (Cao *et al.,* 2004). As shown in Figure 4-4a, a supervised hierarchical clustering analysis showed that several genes were differentially regulated by the ORF859 mutant strain JMS04 in comparison to the parental strain. The ontology analysis presented in Table 4-5 further revealed that the most significant and numerous variations ($P<0.001$) were associated with intermediate metabolism functions, signal transduction and cytokine activity. Interestingly, IL-27 was found to be induced by wild-type *A. actinomycetemcomitans,* but not by the JMS04 mutant (not shown). IL-27 is closely related to IL-12 in both sequence and structure (Artis *et al.,* 2004), and has been shown to promote Th1 cell-mediated immune responses (Hunter *et al.,* 2004). Together, this suggests that the product of ORF859 may be related to the intracellular adaptation and

homeostasis of *A. actinomycetemcomitans*, a process that does not impact large numbers of host cell pathways. However, upregulation of IL-27 will stimulate host cell mediated immunity and hence the ORF859 gene product may contribute to the inflammatory properties of the organism.

The *P. gingivalis* mutant (YPF1) tested is deficient in production of the major fimbrial protein, FimA, a multifunctional adhesin (Lamont and Jenkinson, 1998). FimA mediates attachment of *P. gingivalis* to gingival epithelial cells through engaging an integrin receptor on the host cell surface (Yilmaz *et al.,* 2002). Fimbrial-integrin interaction results in assembly of integrin focal adhesion complexes, and the initiation of signaling pathways that induce remodeling of cytoskeletal architecture that allows entry of the organism (Yilmaz *et al.,* 2003). The YPF1 mutant is thus significantly impaired in invasion and in cytoskeletal remodeling activity (Yilmaz *et al.,* 2002, 2003). In contrast to the profiles obtained with the *A. actinomycetemcomitans* mutant, the *P. gingivalis* mutant strain YPF1 had a transcriptional pattern strikingly divergent from the parental strain. As shown in Figure 4-4b and Table 4-6, and consistent with the phenotypic properties of the mutant, a large proportion of genes related to the cytoskeleton and to membrane and receptor activity were underrepresented in the transcriptional profile of YPF1-infected cells. For example, YPF1 failed to upregulate actin binding LIM protein 1 which may play a general role in bridging the actin-based cytoskeleton with an array of potential LIM protein-binding partners, filamin B beta (actin binding protein 278) which connects cell membrane constituents to the actin cytoskeleton, and coronin 2A another actin binding protein (Roof *et al.,* 1997.; Feng and Walsh, 2004; de Hostos, 1999). Additionally, YPF1 did not upregulate beta 3, 4 and 6 integrin, along with alpha V, 3 and 4 integrin, and CD47 an, integrin-associated signal transducer. YPF1 also demonstrated a significant inability to impinge on cell cycle and cell proliferation pathways indicating that a successful invasion event

may be necessary for *P. gingivalis* to manipulate these pathways. The apoptosis ontology pathway was not differentially influenced by YPF1, indicating that the fimbriae deficient mutant strain should capable of inhibiting apoptosis in HIGK cells to the same extent as the parental strain. This was confirmed by the phenotypic apoptosis assay (Figure 4-3) that showed YPF1 could antagonize chemically induced apoptosis to the same extent as the parental strain.

Conclusions

The transcriptional profiling presented herein begins to provide insights into both the intricate biological phenomena occurring during host-pathogen interactions and the distinct pathophysiology of *A. actinomycetemcomitans* and *P. gingivalis*. A characteristic clinical outcome is associated with infection with either organism. *A. actinomycetemcomitans*-associated disease involves acute tissue destruction in the absence of overt inflammation, whereas *P. gingivalis*-associated disease is chronic and involves inflammatory tissue destruction. Moreover, the mechanism of intracellular invasion of both organisms is distinct. Consistent with these biological and clinical differences, the common core transcriptional response of epithelial cells to these organisms is very limited, and organism-specific responses predominate. Interestingly, this contrasts with disease models in other cell types. For example, infection of dendritic cells with *Escherichia coli*, *Candida albicans*, or the influenza virus resulted in a substantial shared core response along with a pathogen-specific pattern of gene expression (Huang *et al.*, 2001). Thus oral epithelial cells, that encounter an array of microbes with varying degrees of pathogenicity, may direct a measured response that is tailored to the pathogenic potential of the infecting organism. These responses can then influence disease progression. For example induction of apoptosis in epithelial cells by *A. actinomycetemcomitans* could contribute to immunologically silent tissue destruction. Inhibition of apoptosis by intracellular *P. gingivalis*, in contrast, could contribute to bacterial persistence and chronic, slowly progressing tissue destruction. Linkage of

specific bacterial components to host pathways and networks provides additional insight into the pathogenic process. The loss of fimbriae form *P. gingivalis* retards adherence and invasion and the consequences of this reverberate throughout the transcriptome. Genes such as ORF859 in *A. actinomycetemcomitans* that appear to be involved in intracellular homeostasis have a more subtle effect on the transcriptome. Such patterns of gene expression differences in response to isogenic mutants may provide a means to evaluate the biological function of as yet undefined bacteria products.

Methods

Bacterial Strains

A. actinomycetemcomitans strain VT1169 is a nalidixic-acid and rifampin-resistant clone derived from the clinical strain SUNY465 (Mintz *et al.,* 2000). JMS04 is an isogenic mutant for ORF859 constructed in VT1169, and obtained by insertional inactivation with a spectinomycin cassette (Cao et al, 2004). *A. actinomycetemcomitans* strains were grown in Trypticase Soy Broth supplemented with 0.6% yeast extract (TSB-YE) in a humidified, 10% CO_2 atmosphere, at 37°C. *P. gingivalis* strains ATCC 33277 and its fimbriae deficient mutant YPF1 (Yilmaz *et al.,* 2002), were cultured anaerobically for 24 h at 37°C in trypticase soy broth supplemented with yeast extract (1 mg mL^{-1}), hemin (5 μg mL^{-1}), and menadione (1 μg mL^{-1}).

Eukaryotic Cell Lines

HIGK cells (human HPV-immortalized gingival keratinocyte) were originally generated by transfection of primary gingival epithelial cells with E6/E7 from HPV (Oda *et al.,* 1996;). HIGK cells are capable of normal keratin synthesis and exhibit degree of differentiation similar to parent normal cells (Oda et al. 1996). HIGK cells were cultured under 5% CO_2 in keratinocyte serum-free medium (K-SFM, Gibco/Invitrogen, Carlsbad, CA) supplemented with: 0.05 mM calcium chloride, 200 mM L-glutamine (Gibco/Invitrogen, Carlsbad, CA). Primary cultures of

gingival epithelial cells (GEC) were generated as described previously (Lamont *et al.,* 1992; Oda and Watson, 1990). Briefly, healthy gingival tissue was collected from patients undergoing surgery for removal of impacted third molars and following Institutional Review Board Guidelines. Basal epithelial cells were separated and cultured in keratinocyte growth medium (KGM; Cambrix, East Rutherford, N.J.), at 37°C in 5% CO2. GEC were used at passage four.

Microbial/Host Cell Co-Culture

Bacteria were harvested and washed by centrifugation, and resuspended in antibiotic-free K-SFM media. HIGK cells (10^5) were washed three times with phosphate-buffered saline (PBS) and incubated with bacteria at a multiplicity of infection (MOI) of 100:1 for *P. gingivalis* and 3000:1 for *A. actinomycetemcomitans* . After 2 hours at 37^0C in 5% CO_2, the cells were washed three times with PBS and lysed with Trizol (Invitrogen, Carlsbad, CA) prior to RNA extraction. In parallel, total numbers of bacteria associated with the HIGK cells, both external and internal, after 2 h incubation and washing, were determined by lysis and plate counts (Meyer *et al.,* 1996). In addition, levels of *A. actinomycetemcomitans* and *P. gingivalis* invasion were measured by antibiotic protection assays as previously described (Lamont *et al.,* 1995; Meyer *et al.,* 1996). Co-cultures were carried out in quadruplicate.

RNA Isolation, cRNA Synthesis and Chip Hybridization

Total RNA was extracted, DNAse-treated, purified and quantified according to standard methods (Qiagen and Affymetrix). cRNA synthesis was performed with 5-8 μg of total cellular RNA, based on the Affymetrix protocol. Double-stranded cDNA was synthesized according to a standardized protocol (SuperScript double-stranded cDNA synthesis kit; Invitrogen Corp., Carlsbad, Calif.). cRNA was transcribed *in vitro*, incorporating biotinylated nucleotides by using a BioArray high-yield RNA transcript labeling kit (T7) (Enzo Life Sciences, Inc., Farmingdale, N.Y.), and hybridized onto the human HG U133-A oligonucleotide arrays (Affymetrix). Each

sample was studied in parallel, and the samples were not pooled. The microarrays were hybridized for 16 h at 45°C, stained with phycoerythrin-conjugated streptavidin and washed according to the Affymetrix protocol (EukGE-WS2v4) using an Affymetrix fluidics station, and scanned with an Affymetrix scanner.

Microarray Data Analysis and Expression Filter

Probe sets that were flagged as absent on all arrays analyzed in this study by the Affymetrix GCOS software (with default settings) were removed from the datasets and were not included in the analyses. The signal intensity measurements as detected reflect the degree of hybridization of synthesized cRNA to the probe sets on the microarray chip. These probe sets represent genes or DNA sequences within genes. Some genes are represented by more than one probe set on a given microarray, and hence probe sets are not uniquely correlated to genes. However, for ease of discussion, we use the terms "probe sets" and "genes" interchangeably (Freezor *et al.,* 2003).

Variation Filter, Normalization, and Cluster Analysis

The signal intensities of the probe sets remaining after applying the expression filter were ranked according to the coefficient of variation, and 50% of the data set with the greatest coefficient of variation was then normalized to a mean of 0 and a standard deviation of 1. K-means clustering and hierarchical cluster analyses were performed with the variance-normalized data set and viewed with the algorithms in the software packages Cluster and TreeView developed by Eisen et al. (1998; Freezor *et al.,* 2003).

Supervised Learning, Discrimination Analysis, and Cross Validation

The hybridization signal intensities of the genes passing the initial expression filter were analyzed (in part) with BRB Array Tools 3.01 (developed by Dr. Richard Simon and Amy Peng Lam, National Cancer Institute, Bethesda, MD) to identify genes differentially expressed among

the treatment classes: uninfected cells, cells infected with *A. actinomycetemcomitans* or mutant strain JMS04, or cells infected with *P. gingivalis* or mutant strain YPF1 ($P < 0.001$). The ability of gene identification to predict treatment class was assessed by a leave-one-out cross-validation using each of four methods of class prediction: nearest-neighbor prediction, three-nearest-neighbors prediction, linear discriminant analysis, and nearest-centroid analysis (Feezor *et al.,* 2003).

Ontology Analysis

The procedure delineated in Zheng et al. (in press) was followed to perform the ontology analysis. Briefly, sets of genes differentially expressed under experimental conditions were fed into the GoMiner software and P-values were computed for each GO term using the Fisher exact test (Zeeberg *et al.,* 2003). The Gene Ontology (GO) database organizes genes into hierarchical categories based on biological process, molecular function and subcellular location. GoMiner helps to identify all the GO-terms or categories that have been particularly enriched or depleted in the set of significantly differentiated genes (Zeeberg *et al.,* 2003).

Assessment of HIGK Cell Apoptosis

To detect fragmentation of DNA in apoptotic epithelial cells, histone associated DNA fragments were examined in a cell death detection ELISA kit (Roche, Indianapolis, IN). HIGK cytoplasmic extracts were added to wells of ELISA plates coated with monoclonal antibodies against histones. The presence of histone-associated DNA fragments was then detected in a sandwich ELISA using anti-DNA peroxidase-conjugated antibodies, with 2,2′-azino-di-[3-ethylbenzthiazoline-sulfonate] substrate. Absorbance was measured at 405 nm and background at 490 nm. As a positive control for apoptosis, HIGK cells were incubated with camptothecin (2 $\mu g\ mL^{-1}$) for 4 h.

Table 4-1 Microbial-epithelial cell interaction characteristics of human primary (GEC) and transformed (HIGK) gingival cells.

Epithelial Cells	Microorganism	MOI[a]	Total Interaction[b] (CFU/cell)	% of Interacting Bacteria that Invade[c]
GEC	*A. actinomycetemcomitans*	3000:1	40±4	<0.02
HIGK	*A. actinomycetemcomitans*	3000:1	35±25	<0.05
GEC	*P. gingivalis*	100:1	14±8	25.00
HIGK	*P. gingivalis*	100:1	18±4	24.00

[a] Multiplicity of infection (bacteria:epithelial cell). [b] Total count of adhering and invading organisms after co-culture and cell lysis at 60 min. Data are reported as mean value from two independent assays in triplicate ± the standard deviation. [c] Calculated from intracellular CFU counts after antibiotic treatment.

Table 4-2. Pathways common to *P. gingivalis*- and *A. actinomycetemcomitans*-infected HIGK cells.

GO ID	Term	*P. gingivalis*-infected (Total change)[a]	P-Value	*A. actinomycetemcomitans*-infected (Total change)[a]	P-Value
45449	regulation of transcription	49	0.0017	118	0
16021	integral to membrane	39	0.0025	96	0
5215	transporter activity	17	0.0034	55	0.0036
12501	programmed cell death	25	0	39	0.0031
17017	MAP kinase phosphatase activity	4	0.0001	6	0

Only pathways with documented relevance to host-pathogen interactions are presented. [a]The total change represents the total number of under- and over-represented genes in a particular pathway.

Table 4-3. Pathways specific to *P. gingivalis*-infected HIGK cells.

GO ID	Term	Change[a]	P-Value
7275	development	64	0.0031
9653	morphogenesis	47	0.0044
8283	cell proliferation	42	0.0009
9888	histogenesis	10	0.0004
8544	epidermal differentiation	7	0.0004
16265	death	25	0.0001
74	regulation of cell cycle	22	0.0009
5125	cytokine activity	12	0.0029
45073	regulation of chemokine biosynthesis	2	0.0034
8138	protein tyrosine/serine/threonine phosphatase activity	4	0.0048
5149	interleukin-1 receptor binding	3	0.0010

Only pathways with documented relevance to host-pathogen interactions are presented. [a]The change represents the total number of under- and over-represented genes in a particular pathway.

Table 4-4. Pathways specific to *A. actinomycetemcomitans*-infected HIGK cells.

GO ID	Term	Change[a]	P-Value
8152	metabolism	330	0.0005
30528	transcription regulator activity	93	0
4888	transmembrane receptor activity	26	0.0040
9581	detection of external stimulus	6	0.0006
4930	G-protein coupled receptor activity	5	0
15268	alpha-type channel activity	5	0.0002
5216	ion channel activity	4	0.0002
5261	cation channel activity	3	0.0025
43066	negative regulation of apoptosis	14	0.0018
3773	heat shock protein activity	8	0.0015
16337	cell-cell adhesion	3	0.0036
3786	actin lateral binding	2	0.0036

Only pathways with documented relevance to host-pathogen interactions are presented. [a]The total change represents the total number of under- and over-represented genes in a particular pathway.

Table 4-5. Gene ontology analysis for HIGK cells infected with *A. actinomycetemcomitans* mutant strain JMS04

GO ID	Under	Over	Change	P-Value	Term
6082	14	1	15	0.0001	organic acid metabolism
9451	7	0	7	0	RNA modification
5625	9	2	11	0.0001	soluble fraction
5125	5	4	9	0.0014	cytokine activity
6950	15	7	22	0.0022	response to stress
3754	7	0	7	0.0025	chaperone activity
7165	8	8	16	0.0090	signal transduction
6983	2	0	2	0.0002	response to ER-overload

Only pathways with documented relevance to host-pathogen interactions are presented. Baseline for comparison is cells infected with parental strain.

Table 4-6. Gene ontology analysis for HIGK cells infected with *P. gingivalis* mutant strain YPF1.

GO ID	Under	Over	Change	P-Value	Term
8283	112	26	138	0	cell proliferation
7049	94	16	110	0	cell cycle
166	87	29	116	0	nucleotide binding
5856	64	13	77	0	cytoskeleton
4872	31	21	52	0.0006	receptor activity
6811	6	10	16	0.0002	ion transport
16020	104	76	180	0	membrane
6974	24	6	30	0.0001	response to DNA damage stimulus
7186	15	10	25	0.0087	G-protein coupled receptor protein signaling pathway
19207	12	1	13	0.0014	kinase regulator activity

Only pathways with documented relevance to host-pathogen interactions are presented. Baseline for comparison is cells infected with parental strain.

Figure 4-1. Different patterns of gene expression of oral epithelial HIGK cells upon co-culture with *A. actinomycetemcomitans* or *P. gingivalis*. Hierarchical clustering of variance-normalized gene expression data from uninfected human HIGK cells and from cells in co-culture with either organism for 2 h prior to RNA isolation and purification. Expression and variation filters were applied to the data set prior to clustering. Probe sets giving hybridization signal intensity at or below background levels on all arrays tested were eliminated from further analysis. The resulting data set was culled by ranking on the coefficient of variation and eliminating the bottom half of the data set to remove probe sets whose expression did not vary between the treatment regimens. The gene expression observations were variance normalized to a mean of 0 and a standard deviation of 1, and this normalized data set was subjected to hierarchical cluster analysis with average linkage clustering of the nodes. The variation in gene expression for a given gene is expressed as distance from the mean observation for that gene. Each expression data point represents the ratio of the fluorescence intensity of the cRNA from *A. actinomycetemcomitans*-infected (columns Aa R1-R4) or *P. gingivalis*-infected HIGK cells (columns Pg R1-R4) to the fluorescence intensity of the cDNA from mock-infected HIGK cells (columns CTRL R1-R5). The scale adjacent to the dendrogram relates to Pearson's correlation coefficient. Highlighted blocks are described in the text.

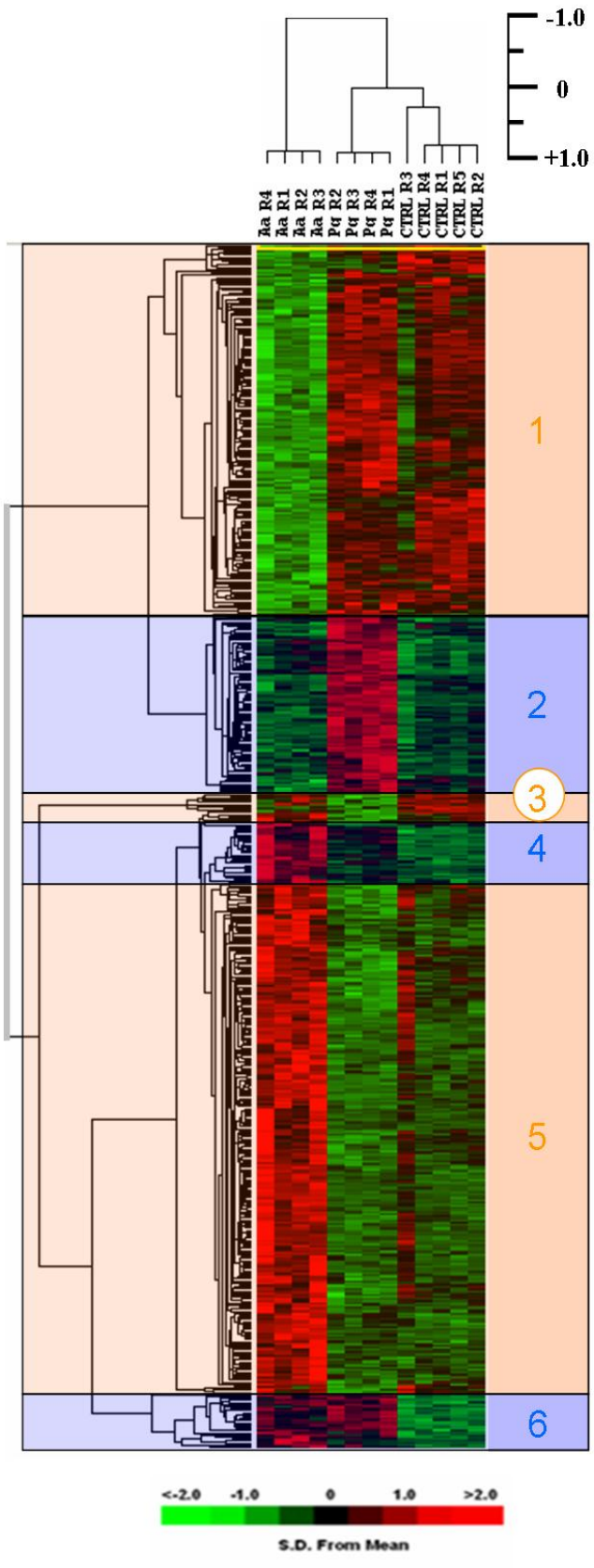
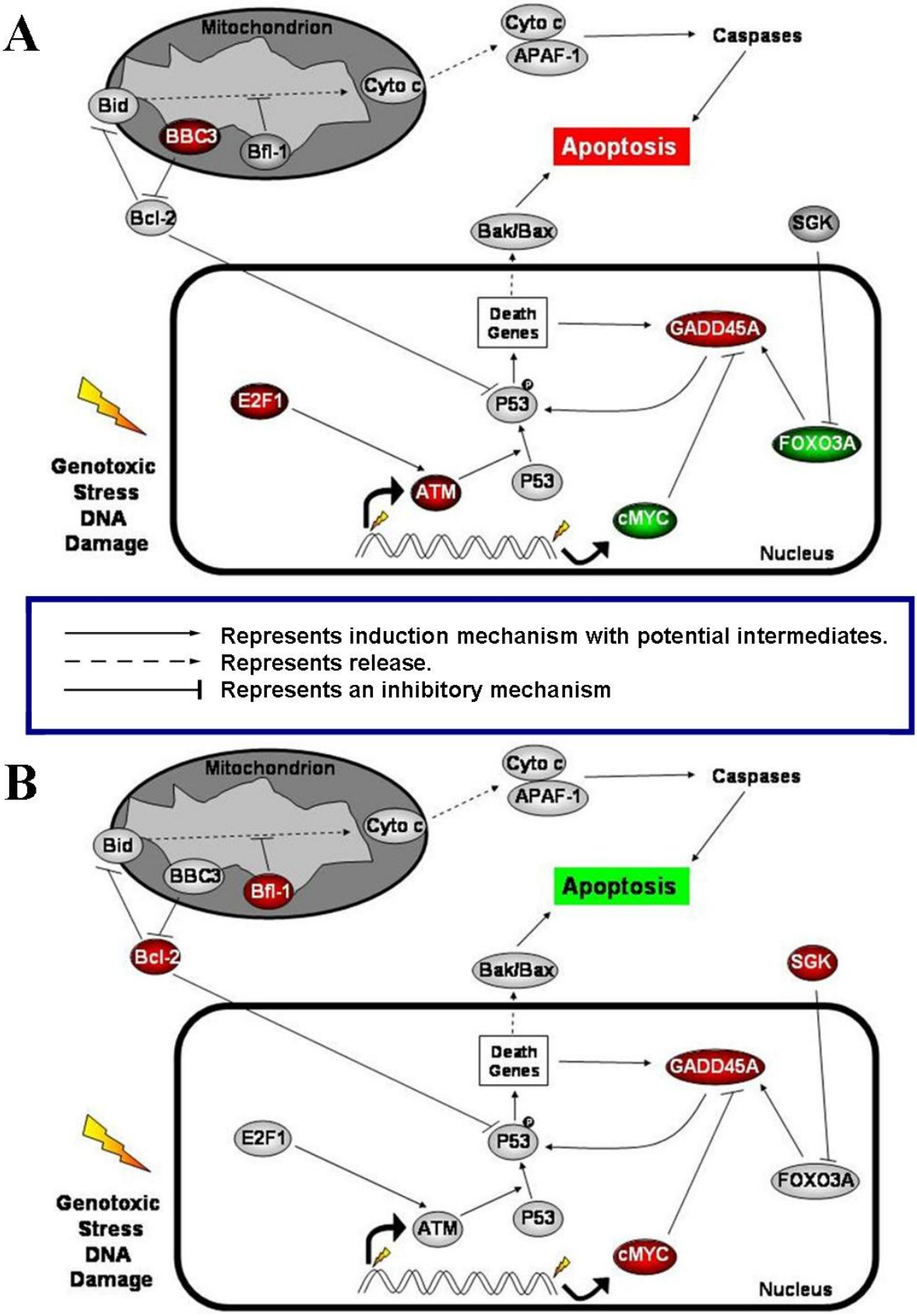

-1.0
0
+1.0
Aa R4
Aa R1
Aa R2
Aa R3
Pq R2
Pq R3
Pq R4
Pq R1
CTRL R3
CTRL R4
CTRL R1
CTRL R5
CTRL R2
1
2
3
4
5
6
<-2.0
-1.0
0
1.0
>2.0
S.D. From Mean

Figure 4-2. Differential modulation of the P53-mediated apoptosis pathway by oral bacteria. HIGK cell response to (A) *A. actinomycetemcomitans* and (B) *P. gingivalis*. Red terms are transcriptionally induced, while green terms are repressed. See text for description of individual molecules.


A
Mitochondrion
Cyto c
Bid
BBC3
Bfl-1
Cyto c
APAF-1
Caspases
Apoptosis
Bcl-2
Bak/Bax
SGK
Death Genes
GADD45A
E2F1
P53
FOXO3A
ATM
P53
cMYC
Genotoxic Stress DNA Damage
Nucleus
Represents induction mechanism with potential intermediates.
Represents release.
Represents an inhibitory mechanism
B
Mitochondrion
Cyto c
Bid
BBC3
Bfl-1
Cyto c
APAF-1
Caspases
Apoptosis
Bcl-2
Bak/Bax
SGK
Death Genes
GADD45A
E2F1
P53
FOXO3A
ATM
P53
cMYC
Genotoxic Stress DNA Damage
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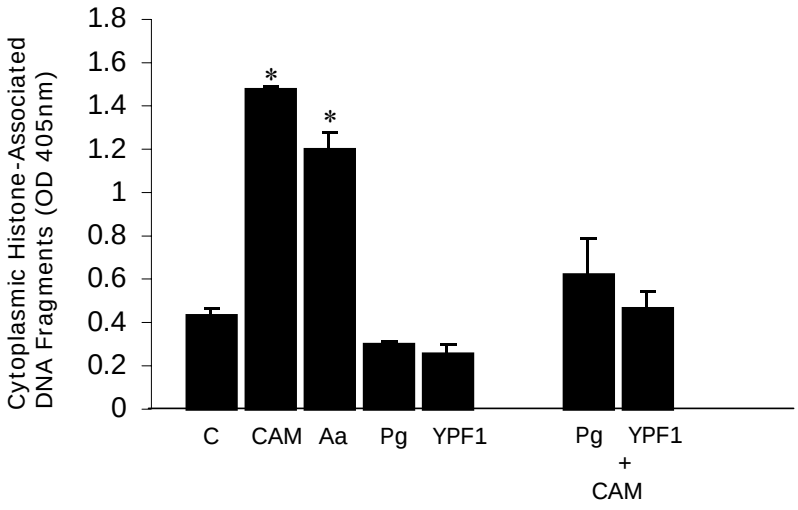
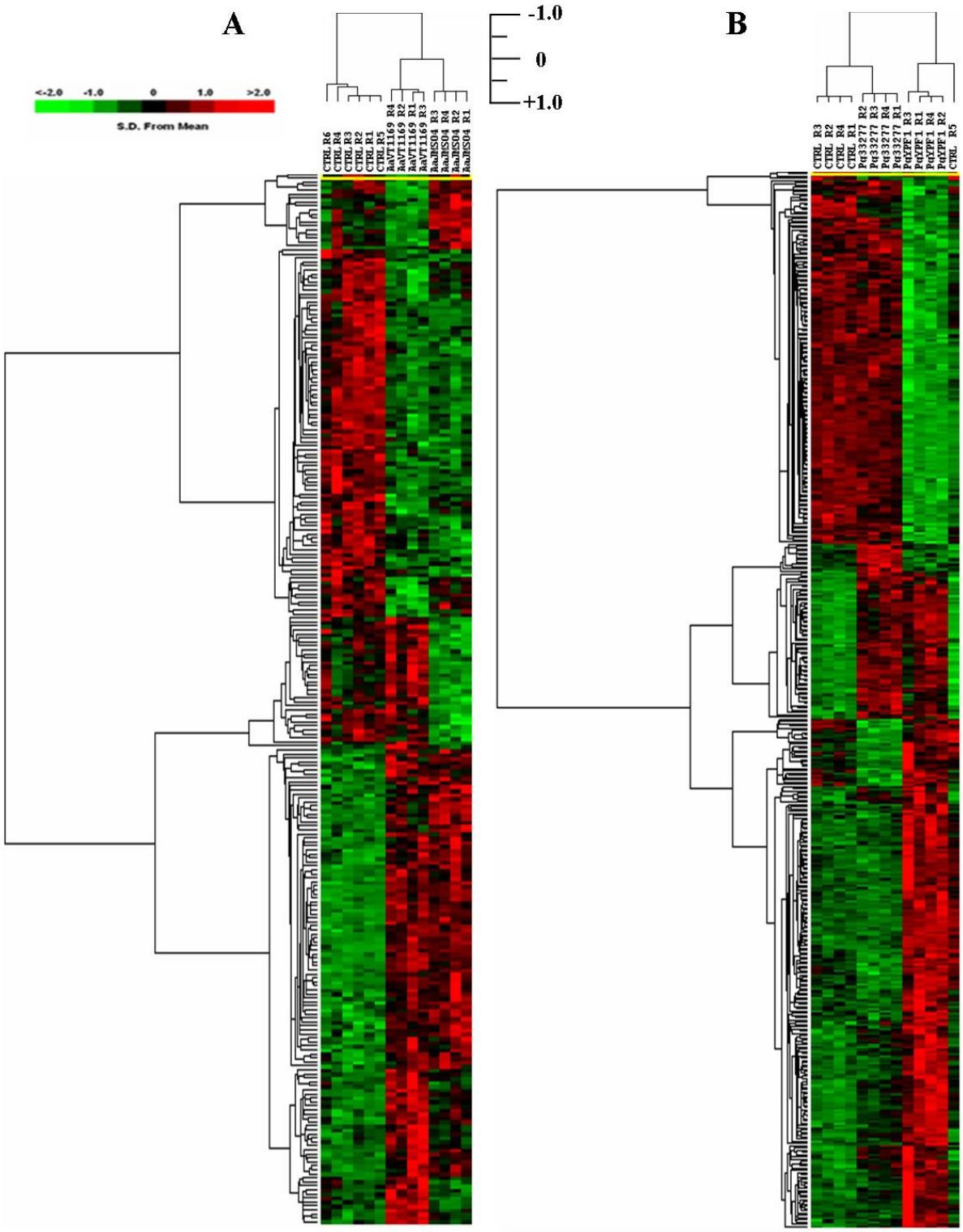


Figure 4-3. Apoptotic responses of HIGK cells to *A. actinomycetemcomitans* or *P. gingivalis* by ELISA of cytoplasmic histone-associated DNA fragments. Control (C) represents HIGK under normal culture conditions. *A. actinomycetemcomitans* (Aa) was incubated with HIGK cells at a MOI of 3000:1 for 4h. *P. gingivalis* parental (Pg) or mutant (YPF1) strains were incubated with HIGK at a MOI of 100:1 for 20 h . Camptothecin (CAM) was incubated with HIGK for 4 h. For inhibition of camptothecin-induced apoptosis, HIGK cells were incubated with *P. gingivalis* strains for 16 h followed by camptothecin for 4 h. Error bars represent standard deviation, n=3. * denotes statistically different from control $P < 0.005$

Figure 4-4. Impact of specific bacterial components upon HIGK cell transcriptomes. (A) A. actinomycetemcomitans or (B) P. gingivalis bacterial strains with an isogenic mutation were allowed to interact with HIGK cells for 2 hours. The transcriptional profiles of HIGK cells encountering mutant strains are compared to those from HIGK cells interacting with wild type parental strains or uninfected. This heat map and dendrogram were constructed from probe sets differentially expressed between experimental classes at the significance level of $P < 0.001$. Probe set signal intensities were variance-normalized, mean-centered across samples, and subjected to hierarchical cluster analysis. Average linkage clustering by uncentered correlation was performed for genes and samples. The degree of similarity between the transcriptional profiles of each sample is expressed by Pearson's correlation coefficient distance metric, according to the adjacent scale. The expression state of each data point is represented as standard deviations from the mean expression level for that gene in all samples. Red indicates a relative increase, green indicates a relative decrease, and black indicates no relative change of mRNA transcripts for a given gene. Labels. (A) CTRL R1-R6; uninfected HIGK cells, Aa VT1169 R1-R4; Wild type *A actinomycetemcomitans*, Aa JMS04 R1-R4; orf859- strain of *A. actinomycetemcomitans*. (B) CTRL R1-R5; uninfected HIGK cells, Pg 33277 R1-R4; Wild type *P. gingivalis*, Pg YPF-1 R1-R4; FimA- strain of *P.gingivalis*.


A
<-2.0
-1.0
0
1.0
>2.0
S.D. From Mean
-1.0
0
+1.0
CTRL R6
CTRL R4
CTRL R3
CTRL R2
CTRL R1
CTRL R5
AaVT1169 R4
AaVT1169 R2
AaVT1169 R1
AaVT1169 R3
AaDMSO4 R3
AaDMSO4 R4
AaDMSO4 R2
AaDMSO4 R1
B
CTRL R3
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CTRL R4
CTRL R1
Pa33277 R2
Pa33277 R3
Pa33277 R4
Pa33277 R1
PaKPF1 R3
PaKPF1 R1
PaKPF1 R4
PaKPF1 R2
CTRL R5

CHAPTER 5
IMPACT OF *Aggregatibacter actinomycetemcomitans* ADHERENCE ON GINGIVAL EPITHELIAL CELL TRANSCRIPTOME[8]

Introduction

The ability of *A. actinomycetemcomitans* to colonize the oral cavity is an important component of the disease process. Biofilm formation at disease sites can directly cause inflammation and tissue destruction, and allow pathogens to persist during the course of periodontal disease. Aae is the first adhesin of *A. actinomycetemcomitans* implicated in the specific and direct adhesion of this bacterium to oral epithelial cells (Rose *et al.,* 2003; Fine *et al.,* 2005). Although some phenotypic characterization has been performed (Rose *et al.,* 2003; Fine *et al.,* 2005), the impact on the host transcriptome by attachment of *A. actinomycetemcomitans* has not been documented. Furthermore, little is known about this newly discovered *A. actinomycetemcomitans* virulence factor. For instance, it is not known which of the documented HIGK cell responses to *A. actinomycetemcomitans* are contact-dependent and involve signaling events attributable to direct Aae binding. Conversely, it is possible that other virulence factors are responsible for the observed host responses and the binding of *A. actinomycetemcomitans* merely serve to increase the local concentration of these secreted factors. For example, CDT may reach a higher local concentration at the hose cell surface when *A. actinomycetemcomitans* adhere, compared to the levels attained by CDT secretion into the culture media by planktonic bacteria. In accordance with Specific Aim 3, the goal of this chapter is to assess the contribution of a specific bacterial component, Aae, to host-pathogen interactions.

[8] This work was supported by an NIH/NIDCR T32 Grant DE07200 (JJM) and RO1 DE16715 (MH). We thank Dr. Scott Grieshaber, University of Florida, Department of Oral Biology, for the use of his confocal fluorescent microscope.

Transcriptional profiling of HIGK oral epithelial cells combined with mutant analysis was performed to gain insights into the contribution of acherence to the global *A. actinomycetemcomitans*-HIGK interaction. A differential host response was uncovered between the experimental conditions. Among several pathways impacted by Aae, Focal Adhesion, and the Regulation of Actin Cytoskeleton were highly modulated. Although Focal Adhesion was more significantly populated, the availability of convenient methods to assess the Regulation of Actin Cytoskeleton pathway justified further investigation of this host function. The initial stages of phenotypic confirmation have been initiated using confocal fluorescence microscopy. In addition, adhesion to gingival epithelial cells by the *A. actinomycetemcomitans aae*$^-$ isogenic mutant strain VT1565 was compared to that of the parental strain SUNY465. A standard assay to enumerate viable bacteria interacting with host cells (adhesion and invasion) demonstrated a 65% reduction in *aae*$^-$ bacteria compared with the parental strain. Conversely, microscopic visualization and enumeration of bacteria interacting with HIGK cells failed to reveal a difference in the adherence of the *aae*$^-$ strain. Confirmation of this preliminary data is ongoing. If it holds, the data herein would suggest a novel function of Aae pertaining to *A. actinomycetemcomitans* viability upon host cell interaction.

This first attempt to investigate the Regulation of Actin Cytoskeleton upon infection with *aae*$^-$ or wild type bacteria failed to reveal definitive remodeling of F-actin architecture under the conditions tested. The transcriptional profile of HIGK cells suggests that other factors in the Regulation of Actin Cytoskeleton pathway may be investigated for their role in the regulation of this pathway upon Aae-mediated adherence by *A. actinomycetemcomitans*. In the absence of definitive and conclusive evidence, the confocal microscopy will be repeated using additional

markers for cytoskeletal rearrangement and Focal Adhesion amongst others, and to assess actin architecture rearrangements for earlier time points not studied in the initial experiment.

Herein, the assessment of the impact by a single bacterial component, Aae, was inconclusively attempted in completion of Specific Aim 3. Upon confirmation of this initial study, a possibly novel function for Aae may be described. The transcriptional study described here will provide the basis for future experiments investigating the contribution of *A. actinomycetemcomitans* adhesion on the pathogenic personality of this microorganism.

Background

The colonization of the periodontium by *A. actinomycetemcomitans* is mediated by several adhesins. Some of the earliest pili studied include the Flp-1 pili. Flp-1 pilin subunits are 6.5 kDa polypeptides which are secreted through a Tad secretion system and assembled into long and functional pili (Kachlany *et al.,* 2001). These pili bundle into thick fibrils, and play a significant role in autoaggregation of *A. actinomycetemcomitans in vitro* as well as nonspecific adherence to glass, plastic and saliva-coated hydroxyapatite, a surface that mimics the tooth. An animal model has also demonstrated the requirement of Flp-1 pili for colonization and pathogenesis of *A. actinomycetemcomitans* based on mutant analysis studies of *flp-1*$^{-}$ and *tadA*$^{-}$ strains (Schreiner *et al.,* 2003). *A. actinomycetemcomitans* adhesion to the extracellular matrix of connective tissue prompted the discovery of EmaA, an adhesin that attaches to collagen independently of fibrils (Mintz, 2004; Ruiz *et al.,* 2006). EmaA expression in *A. actinomycetemcomitans* results in the formation of antennae-like protrusions described as long stalks tipped by an ellipsoidal head region (Ruiz *et al.,* 2006). These non-fimbrial adhesins are believed to contribute to colonization and persistence of *A. actinomycetemcomitans* to the extracellular matrix *in vivo* (Tang *et al.,* 2007).

The first adhesin implicated in the direct attachment of *A. actinomycetemcomitans* to oral epithelial cells was Aae, an autotransporter adhesin with specificity to buccal epithelial cells of humans and old-world primates (Rose *et al.,* 2003; Fine *et al.,* 2005). Aae was discovered by computational biology and found to possess homology to the autotransporter adhesins Hap and Hia found in *Haemophilus influenzae* (Rose *et al.,* 2003).

The adhesive ability of Aae, first suggested by its homology to Hap and Hia, has been investigated in two separate studies. The original characterization was performed with KB (HeLa CCL-17) cervical epithelial cells grown in a monolayer and infected by wild type or *aae*$^-$ *A. actinomycetemcomitans* at a MOI of 100:1. The authors did not report the number of adherent bacteria per epithelial cell, but viability assays demonstrated a 70% reduction of binding by the *aae*$^-$ strain VT1565 compared to the wild type SUNY465 (smooth, serotype b). There was a similar percentage reduction in binding by a second mutant strain, VT1568, when compared to the parental strain ATCC29523 (smooth, serotype a). However, when the adhesion data of all four strains is normalized to a common denominator, SUNY465 binds epithelial cells in three to four-fold greater numbers than strain ATCC29523. Futhermore, binding of *A. actinomycetemcomitans* to epithelial cells was not completely abolished upon mutation of *aae*. This suggests an additional adhesin, and does not preclude the differential expression of this adhesin and *aae* among different strains. Additionally, lactoferrin from human milk, which cleaves Hap from *H. influenzae*, is able to cleave Aae from the ATCC 29523 strain but not from SUNY465. Taken together, these observations suggest the expression and substrate variability of this adhesin from strain to strain and further exemplifies the diversity of interaction characteristics displayed by different clinical strains of *A. actinomycetemcomitans.* Further

studies are necessary to determine the influence of post translational modifications, length polymorphism, or copy number upon the binding characteristics of different bacterial strains.

A second study further characterized Aae-mediated adhesion to host cells using a rough, serotype d strain of *A. actinomycetemcomitans* transformed with a mutated *aae* gene originally isolated from the rough, serotype f strain CU1000 (Fine *et al.*, 2005). Buccal epithelial cells (BECs) from humans, old world primates, new world primates, and non-primate mammals were exposed to *A. actinomycetemcomitans* at MOI ranging from 1000:1 to 10,000:1 for 90 minutes while suspended in liquid and under constant rotation. It is unclear if the BECs remained viable throughout the isolation and testing procedure, with implications for the host response to adherence, and subsequent pathogen-directed endocytosis events. In this assay, 210 +/- 14 wild type bacteria bound per BEC compared to14 +/- 4 for the *aae*$^-$ strain. This is approximately a 93% reduction in binding efficiency of human and old world primate buccal epithelial cells. No difference was observed between the binding of mutant and wild-type *A. actinomycetemcomitans* to rat and cow BECs. No binding was observed by wild type or *aae*$^-$ strains incubated with BECs from eleven other mammalian sources, including mouse, pig, and new world primates. Although *A. actinomycetemcomitans* have been isolated from BECs *in vivo*, the relevance of BECs to periodontal disease beyond a reservoir for bacteria is unclear. The binding efficiency of wild type or *aae*$^-$ bacteria to Ginvival epithelial cells (GECs) was not reported.

Escherichia coli transformed to express Aae on their outer membranes were also able to bind BECs in high numbers, with 10 to 100 transformed bacteria adhering per BEC. Interestingly, the only other epithelial cell type bound by Aae-transformed *E. coli* were primary gingival epithelial cells (GEC), and at the lower ratio of 1 to 3 bacteria per GEC. These GECs were also isolated using a scraping method, and the cells may not have been viable. Less than

one Aae-transformed *E.coli* bound per 100 cells during assays testing human alveolar, bronchial, palatal, tongue and cervical epithelial cells. These studies confirm the tropism of *A. actinomycetemcomitans* to BECs and to a lesser extent, GECs (Fine *et al.,* 2005).

Although the extent of Aae-specific binding appears to be lower in GECs compared to BECs, Aae-mediated binding to GECs has been demonstrated with *aae*-transformed *E. coli* (Fine *et al.,* 2005). Furthermore, gingival epithelial cells are amongst the first cell types to interact with A. actinomycetemcomitans in the oral cavity, and the outcome of this interaction is critical in periodontal disease. The initial study reporting the discovery of Aae by Rose and colleagues used cervical epithelial cells to demonstrate a 70% reduction in the binding of *aae*$^{-}$ strains compared to the parental strains. However, considering the experimental model and methods, neither study has definitively addressed the Aae-mediated binding of *A. actinomycetemcomitans* to GECs. Furthermore, beyond the phenotypic characterization of the Aae binding function for *A. actinomycetemcomitans* little is known about the effects of Aae upon host-pathogen interactions. The specificity of the Aae-mediated binding by *A. actinomycetemcomitans* to BECs and GECs prompted this study to investigate the role of Aae during adhesion to gingival epithelial cells.

Potential alternative functions for Aae besides adherence remain possible. In other bacteria, adhesion triggers a specific host response, such as an uptake signal, which can lead to pathogen-directed endocytosis. This interaction has been observed for *Shigella* spp. and *Salmonella typhimurium* (Steele-Mortimer *et al.,* 2000), and remains a likely outcome for *A. actinomycetemcomitans*. Furthermore, the role of specific adhesion is not fully understood as it relates to the delivery of bacterial effectors that are presumed to rely on contact with host cells prior to their injection through a secretion system, such as CagE (Teng *et al.,* 2003). Conversely,

it is not understood if secreted factors, such as CDT, continuously stimulate epithelial cells and override any specific response elicited by adhesion. As an initial step to address these considerations, the effect of Aae-mediated binding to human immortalized gingival keratinocytes (HIGKs) on the host transcriptome is thus examined.

Materials and Methods

Bacteria and Cell Lines

HIGK cells originally generated by transfection of primary gingival epithelial cells with E6/E7 from HPV (Oda *et al.,* 1996) were grown in Keratinocyte Serum-Free Medium (KSFM) as a monolayer to 95% confluence in an atmosphere of 5% CO_2 at 37^{o}C (Oda *et al.,* 1996; Cao *et al.,* 2004). Cell culture media contained L-glutamine and was supplemented with 50 U penicillin-streptomycin per mL (Gibco) and calcium chloride to 0.05 mM final concentration. Bacterial strains were obtained from Dr. Fives-Taylor, University of Vermont. *A. actinomycetemcomitans* SUNY465 was maintained on 1.5% agar plates consisting of 3% trypticase soy broth and 0.6% yeast extract (TSBYE). VT1565 was grown on TSBYE plates supplemented with 50μg/mL kanamycin (Rose *et al.,* 2003). Both strains were inoculated into liquid culture and grown at 37^{o} C and 10% CO_2 to mid-logarithmic phase, and prepared for host cell co-culture according to standard methods (Richardson *et al.,* 2005). Briefly, epithelial cells were washed three times with 1X Dulbecco's Phosphate-Buffered Saline (dPBS) (Cambrex, Walkersville, MD) to remove residual antibiotics, cellular waste products, and secreted toxins. In biological replicates of four per condition, epithelial cells were sham-infected with KSFM alone or co-cultured with *A. actinomycetemcomitans* resuspended in culture media, resulting in a multiplicity of infection (MOI) of 2500:1, which is the same order of magnitude compared to previous experiments (1000:1) and yielded consistent total interaction ratios for the strains being studied. This slightly increased MOI allowed the detection of a baseline level for *aae*$^{-}$ strain

interaction per host cell and subsequent calculations for the adhesion defect of the mutant strain. Previous studies in our lab (Handfield, Mans *et al.,* 2005) determined that 1000:1 was an MOI ensuring a that every host cell was likely to encounter at least a single bacterium—typically 25 to 30—, resulting in a homogeneous population of infected host cells, and thus a representative mRNA sample of the infected state. Two hours co-culture was the time point previously determined to display a phenotype that can be characterized in terms of host cell monolayer integrity, and chosen in order to maintain consistency with previous work in our lab (Handfield *et al.,* 2005). After two hours co-culture, epithelial cells were lysed with Trizol (Invitrogen Life Technologies, Carlsbad, CA) and RNA was prepared for GeneChip hybridization as recently described (Handfield *et al.,* 2005). Total RNA was extracted from Trizol-lysed cells, treated with DNase I, purified, and quantified according to standard methods (Qiagen, Valencia, CA; and Affymetrix, Santa Clara, CA). Complementary DNA (cDNA) synthesis was performed according to the Affymetrix protocol (SuperScript double-stranded cDNA synthesis kit; Invitrogen,) with 5-8 µg of total cellular RNA used as a template to amplify mRNA species for detection. Double-stranded cDNA was purified, and used as a template for labeled complementary RNA (cRNA) synthesis. *In vitro* transcription was performed using a BioArray high-yield RNA transcript labeling kit (T7) (Enzo Life Science, Farmingdale, NY), to incorporate biotinylated nucleotides. cRNA was subsequently fragmented and hybridized onto Human Genome (HG) U133 Plus 2.0 GeneChip DNA microarrays (Affymetrix) with appropriate controls. Each sample was studied in parallel, and the samples were not pooled. The microarrays were hybridized for 16 h at 45°C, stained with phycoerythrin-conjugated streptavidin and washed according to the Affymetrix protocol (EukGE-WS2v4) using an Affymetrix fluidics station, and scanned with an Affymetrix GeneChip 3000 scanner.

Microarray Analysis

Assessment of the host cellular responses to bacterial challenge was accomplished by transcriptional profiling using Human Genome (HG) U133 Plus 2.0 GeneChip DNA microarrays (Affymetrix). This generation of GeneChips allows the experimental assessment of over 47,000 human transcripts. Infected and uninfected HIGK cells were tested in four independent replicates. Subsequent array analysis was performed as recently presented (Handfield *et al.,* 2005). In brief, expression filters were applied to remove Affymetrix control oligos and probesets whose signal was undetected across all samples. The signal intensity values of the resulting dataset were variance-normalized, mean-centered, and ranked by their coefficients of variation. Normalization was performed in order to give equal weight to all probesets in the analysis, regardless of the raw signal intensity order of magnitude. To reduce the confounding effect of background signal variation on the analysis, the half of the dataset demonstrating the most variation across samples was used to perform unsupervised hierarchical cluster analysis using Cluster software (Eisen *et al.,* 1998). The resulting heat-map and Cluster dendrograms were visualized with Treeview (Eisen *et al.,* 1998) to reveal the extent of characteristic host-cell responses to each infection state, defined as identical treatments clustering together.

Following initial assessment of the host cell response to each condition, supervised analysis was performed to investigate differences in gene regulation among experimental conditions. For this analysis, the raw signal intensities were log-transformed for all probesets passing initial expression filters and correlated using BRB Array Tools (Simon and Peng-Lam). In each supervised analysis, biological replicates were grouped into classes according to infection state during co-culture experiments. Several methods of class prediction were utilized (compound covariate predictor, nearest neighbor predictor, and support vector machine predictor) in order to generate lists of class predictors whose expression state changes

significantly between classes. Leave-one-out cross-validation (LOOCV) was performed to test the accuracy of each class predictor and compared to the probability of a correct class prediction by chance alone assuming normal distribution, based on the P-value and total number of genes analyzed. In order to visualize the differentially-regulated genes, Microsoft Access database queries were used to match the subset of significantly regulated genes with their associated variance-normalized, mean-centered signal values calculated previously. Cluster and Treeview software were used to visualize the correlations among genes and samples (Eisen *et al.,* 1998).

Functional Categorization by Gene Ontology (GO) and Bioinformatics Analyses.

GO tables and KEGG pathways were populated using Pathway Express (Khatri *et al.,* 2005) available at http://vortex.cs.wayne.edu/projects.htm. This software makes pairwise comparisons of experimental conditions and populates KEGG pathways based on fold induction or decrease for each impacted gene. Therefore, the supervised analysis was repeated for two-class comparison of the HIGK response to infection at $P < 0.05$. This threshold was chosen to increase the number of genes populated for a given pathway in order to allow a phenotypic prediction based on the gene regulation observed. Three analyses were performed: (1) HIGK co-cultured with wild type SUNY465 compared to baseline uninfected controls, (2) HIGK co-cultured with *aae*$^-$ strain VT1565 compared to baseline uninfected controls, and (3) HIGK co-cultured with *aae*$^-$ VT1565 compared to wild type SUNY465-infected baseline. An arbitrary minimum threshold filter of 2-fold expression change was also applied. This additional condition increased the likelihood that expression level differences for a given gene were detectable using downstream phenotypic confirmatory methods. Gene annotations were obtained through the Genecards online database available at http://www.genecards.org/index.shtml.

Confocal Fluorescent Microscopy

HIGK cells were resuspended in KSFM without antibiotic/antimycotic and the concentration of cells was determined with a particle counter (Beckman Coulter, Inc., Miami, FL). The Lab Tek II four chamber slide system was used for microscopy experiments (Nalge Nunc International, Rochester, NY). A total of $1.25x10^5$ HIGK cells were seeded per chamber, resulting in a 70-90% confluent monolayer of cells for the duration of the infection experiment. Starting with a 95% confluent monolayer would have led to cell overgrowth for later timepoints, such as 24 H post inoculation. *A. actinomycetemcomitans* SUNY465 and VT1565 (*aae*$^-$) overnight cultures were diluted to an optical density (OD) of approximately 0.2 A at 495 nm. CellTracker Green BODIPY live dye (Invitrogen Corporation, Carlsbad, California) was added at 10 μM final concentration to VT1565 (*aae*$^-$) and SUNY465. Bacteria were incubated for 3 to 4 hours and until cultures reached mid-logarithmic growth stage (OD 495 nm, 0.6 A), in order for the bacteria to incorporate the fluorescent dye. Optimization experiments (data not shown) revealed no adverse effects on bacterial growth or viability *in vitro* due to live dye incorporation.

Bacteria were collected by centrifugation at 3000 rcf for 10 minutes, and resuspended in 1x dPBS to wash away residual dye that could be incorporated by HIGK host cells during the infection. Centrifugation was repeated, and bacteria were resuspended in antibiotic-free KSFM at a concentration to result in an MOI of 2500:1 upon infection. Prior to co-culture, host cells were washed twice with 1x dPBS to remove wastes and antibiotics, and then inoculated with fluorescent dye-labeled SUNY465, VT1565 (*aae*$^-$), or mock-infected with KSFM. One mL of inoculate was dispensed per slide chamber. Dilution plating was performed in parallel to confirm the inoculate MOI for each strain. Post-inoculation time points investigated were 20 minutes, 2 hours, 6 hours, and 24 hours.

At the 20 minute and 2 hour timepoints, HIGK cells were washed three times with 1x dPBS to remove non-tightly adherent bacteria and fixed with 4% paraformaldehyde in 1x dPBS for 30 minutes at room temperature. For 6 hour and 24 hour timepoints, HIGK cells were washed twice with 1x dPBS at 2 hours, and cells were incubated in KSFM without antibiotics for the remaining time. At 6 and 24 hours post inoculation, HIGK cells were washed once with 1x dPBS and fixed with 4% paraformaldehyde in 1x dPBS. Parallel adhesion assays were conducted at 2 hours post inoculation to maintain consistency with microarray experiments and assess the initial total interaction levels of the bacteria.

HIGK cells were processed and stained according to the commercial protocol for staining with Texas Red phalloidin (Invitrogen Corporation, Carlsbad, California). Briefly, cells were washed with 1x dPBS once, then made permeable with 0.1% Triton x-100 in 1x dPBS, PBS-washed again, and blocked prior to staining with 1% BSA in 1x dPBS. Cells were stained with 2 Units of Texas Red phalloidin in 1% BSA-dPBS for 30 minutes per chamber. Cells were washed again, the slide chamber walls were removed, and slides were air dried and hard mounted with Vectashield HardSet antifade and mounting medium (Vector Laboratories, Burlingame, CA). Slides were dried overnight at RT in the dark, and sealed.

The F- actin cytoskeleton of HIGK cells was visualized with a spinning disk confocal microscope under oil immersion at x600 magnification. VoxCell software (VisiTech International, Sunderland, United Kingdom) and live camera images taken through an x1.5 conversion lens were used to capture a series of fluorescent optical *x-y* sections to create digitally reconstructed images (*z*-stacks) of HIGK cells interacting with *A. actinomycetemcomitans*. A collection of slices were captured for the entire height (*z*-dimenstion) of the tallest HIGK cell in the visual field at 0.2 micron steps. Bacteria labeled with Cell Tracker Green were excited with

a 491 nm laser and the emission spectrum was captured using a 525 nm narrow-pass filter. HIGK cells stained with Texas-Red phalloidin (F-actin directed) at 561 nm and the emission spectrum was captured with a 595 nm narrow pass filter. Green and Red channel images were merged using Imaris imaging software (Bitplane Inc, Saint Paul, MN), and three-dimensional reconstructions of the cells and bacteria were analyzed to measure total levels of interaction (adhesion and invasion) by *A. actinomycetemcomitans*, and to visualize HIGK cell actin cytoskeletal rearrangements.

Quantification of *A. actinomycetemcomitans* Adherence

Five to ten optical fields were selected per condition for image capture based on representative actin morphology of HIGK cells visualized by Texas Red phalloidin. In general, selected cells were not rounded and were judged to be tightly adhering to the slide surface. More than 50 total fields were analyzed to examine HIGK-bacterial interactions for both strains during four timepoints. Bacteria were not visualized until after the fields had been captured to avoid biasing the selected fields based on the numbers of bacteria associated per cell. The numbers of bacteria per cell were counted for whole cells only. Cells that were truncated from the visual field were not considered and their associated bacteria were not counted. The mean and standard deviations of bacteria observed in three dimensional Z-stacks was calculated for SUNY465 and VT1565. A two-tailed, unpaired student's t-test was performed with Microsoft Excel to determine if the mean numbers of *aae*$^-$ or wild type bacteria per cell were significantly different.

Results

A. actinomycetemcomitans Viable Counts

Viable counts of the inoculums and total interacting bacteria were measured in parallel with spectrophotometry readings to corroborate the predicted MOI and subsequent interaction ratios for a given experiment. The mean MOI for SUNY465 calculated by viable counts was

(2.8+/-0.4) x10^3. The mean MOI of VT1565 was (2.6+/-0.2) x10^3. The total interaction counts were used to confirm the apparent adhesion deficiency of the *aae*$^-$ strain VT1565 with HIGK epithelial cells. The mean total interaction count of SUNY465 was 22+/-7. The mean total interaction count for VT1565 was 8+/-3. The probability these two means are equal as determined by a two tailed, unpaired student's t-test was P=0.07. The adhesion deficiency of this strain was approximately 65% reduction from that of the parental strain by dilution plating. This is consistent with the 70% reduction observed during the initial characterization of this strain interacting with cervical epithelial cells using a slightly higher MOI and comparable methods (Rose *et al.,* 2003).

Transcriptional Profiling

Three-class supervised analysis was performed for the 30,395 probesets which passed the initial expression filters. BRB Array Tools revealed 4138 and 161 probe sets that were differentially expressed among the treatment conditions at the P <0.05 and P <0.001 levels of significance, respectively. With normal distribution, one would expect 1520 (P<0.05) and 30 (P<0.001) modulated genes by chance alone. The results of Leave-One-Out Cross-Validation (LOOCV) ranged from 67% nearest centroid correct to 75% nearest neighbor and diagonal linear discriminant correct prediction for the P <0.001 dataset. For the P <0.05 dataset, the nearest neighbor prediction and nearest centroid were also 75% and 67% correct. The diagonal linear discriminant analysis was correct 67% of the time. This is more accurate than 33% correct prediction by chance alone for LOOCV for three classes and normal distribution. The statistical significance of the cross-validated misclassification rate based on 2000 random permutations of the dataset was P=0.02 for all prediction models. Treeview visualization of significant genes by supervised analysis were organized with Cluster and revealed several distinct nodes which differentiate the infection conditions. The P <0.05 significant gene expression patterns are

shown in Figure 5-1. HIGK cells in co-culture with VT1565 (*aae*$^-$) clustered more closely with the uninfected control HIGK cells than wild type SUNY465-infected cells during all three-class analyses as determined by Pearson's correlation coefficient.

Two-class supervised analysis was performed to generate genelists for ontology analysis. At the P <0.05 level of significance, 2762 probesets were regulated in SUNY465-infected cells compared to uninfected controls. Also compared to uninfected controls, HIGK cells infected with VT1565 (*aae*$^-$) differentially regulated 3494 probesets. The comparison of HIGK cells infected with *aae*$^-$ strain of *A. actinomycetemcomitans* regulated 5147 probesets when compared to HIGK cells co-cultured with the parental strain SUNY465. LOOCV for the two-class supervised analyses ranged from 75% to 100% accurate, which is better than 50% correct classification expected by chance alone for LOOCV with two classes.

Ontology Analysis

The Pathway Express algorithm displayed many pathways of HIGK cells that were impacted upon interaction with SUNY465 and VT1565. Changing the applied level of stringency between P<0.05, P<0.01, or P<0.001 subtly changed the ranking order of implicated pathways. Yet, the overall composition of impacted pathway lists was identical regardless of the level of significance (data not shown). P <0.05 was chosen since that level of stringency allowed implicated pathways to be populated to a greater extent, which aided phenotypic predictions. At this threshold and without additional filters, the two class comparision of VT1565 to SUNY465 infected cells yielded a genelist that populated over 50% of the Rearrangement of Actin Cytoskeleton pathway. To clarify the pattern of regulation for this pathway under the three experimental conditions, a second filter was applied to reduce the number of genes considered in the phenotypic prediction (Figures 5-2 to 5-4). Genes whose expression level changed by at least two-fold and were also significantly modulated at P<0.05 were considered for additional

analysis. This filter reduced the number of total genes to 15 in the Regulation of Actin Cytoskeleton pathway (Table 5-1). This fold change filter removed statistically significant genes with expression levels that were thought to fall below the sensitivity of downstream confirmatory methods.

As shown in Table 5-1, multiple HIGK pathways were impacted by mutation in *aae*. The Regulation of Actin Cytoskeleton was consistently a top 5-impacted pathway for supervised analysis performed at $P < 0.001$, $P < 0.01$, and $P < 0.05$. When the entire $P<0.05$ genelists were used for ontology analysis, the pathway P-values for Regulation of Actin Cytoskeleton were 4.926×10^{-3}, 4.660×10^{-2}, and 1.391×10^{-4} for the wild type /Ctrl, Aae^-/Ctrl, and Aae^-/ wild type comparisons, respectively. Increasing the stringency with the additional filter applied to the $P<0.05$ dataset of at least two-fold expression change artificially increased the P-values 10 to 100 fold. This occurs because the extra filter lowers the total number of impacted genes populating each pathway and used in the pathway significance tests. Therefore, Regulation of Actin Cytoskeleton is one of the most significantly impacted pathways under the experimental conditions tested. This is consistent with early reports of a putative role for host actin rearrangement in the invasion of cultured HeLa cervical epithelial cells by *A. actinomycetemcomitans* SUNY465 (Fives-Taylor *et al.,* 1995). Therefore, this pathway was chosen for further characterization, taking into account the availability of phenotypical confirmatory assays.

Figure 5-2 shows the baseline impact of *A. actinomycetemcomitans* adhesion upon the Regulation of Actin Cytoskeleton pathway in HIGK cells. Of the genes passing filtering thresholds, ten genes were up-regulated and none were down-regulated in this pathway upon epithelial cell-*A. actinomycetemcomitans* SUNY465 interaction. Functional annotations were

obtained from the GeneCards database, accessible at http://www.genecards.org/index.shtml. The four genes PIR121, Nap125, ERM, and MyosinII were impacted exclusively in the SUNY465-infected HIGK cells. APC is involved with cell adhesion and active cell migration. This product also participates in signaling events. NWASP binds several proteins, including the regulatory GTPase, Rac. This gene product binds the Arp2/3 complex and is an effector protein that links Arp2/3 to the Rho-type GTPases. It has been reported that NWASP is important for efficient actin polymerization and regulates the structure and dynamics of the actin cytoskeleton. PIR121 (CYFIP1) also interacts with active GTP-bound Rac. PIR121 binds to F-actin and is involved in the formation of membrane ruffles and lamellipodia protrusions. Nap125 (NCKAP1) forms a lamellipodial complex with PIR121. Nap125 is also involved in Rac-dependent actin remodeling. ITG (ITGA11) is a cell surface adhesion receptor that mediates cell adhesion to extra cellular matrix and to other cells. GF (EGF) stimulates the growth of epithelial tissue. Its receptor, RTK, was also up-regulated. RTK binding to GF leads to dimerization and internalization of the GF-receptor complex. Potentially, GF-RTK mimicry by bacteria could be explored as a mechanism for pathogen-directed endocytosis. ERK is involved with the initiation and regulation of mitosis and meiosis. Bacterial stimulation could conceivably affect the host cell growth rate and explain the up-regulation of this gene. CDT is a known factor that affects the cell cycle, and this may be another avenue for *A. actinomycetemcomitans* to modulate host cells. ERM is believed to participate in connections of major cytoskeletal structures to the plasma membrane. ERM is a filopodial protein involved in cell recognition and morphological changes. MyosinII plays a role in cytokinesis and cell shape. This contractile motor protein moves towards the plus ends of actin filaments. Taken together, the up-regulation of the above

factors suggests an increase in lamellipodia and filopodia formation, and the induction of membrane ruffling upon infection with *A. actinomycetemcomitans*.

Figure 5-3 illustrates the Regulation of Actin Cytoskeleton pathway impacted by *A. actinomycetemcomitans* strain SUNY1565 (*aae*$^-$) compared to uninfected HIGK cells. Of the genes passing the selection criteria, twelve total genes were up-regulated and none were down-regulated. Six of the genes up-regulated upon SUNY465 interaction were consistently up-regulated in HIGK cells encountering SUNY1565 (*aae*$^-$) compared to the baseline uninfected transcript levels. These six genes are APC, NWASP, ITG, GF, RTK and ERK, and their functions are listed in the preceding paragraph.

Cdc42 is a GTPase which cycles between active and inactive states. When active, Cdc42 binds other effector proteins to regulate various cell responses, such as epithelial cell polarization. Another function of Cdc42 is to cause the formation of filopodia. As previously mentioned, Rac is also a GTPase which regulates many proteins in the actin rearrangement pathway. PI3K is a regulatory kinase. RhoGEF regulates RhoA GTPase. MLCP and MLCK are a phosphatase and kinase respectively that oppositely regulate MLC. The downstream function is to mediate binding to myosin and regulate myosin phosphatase activity. Since both of these opposing regulators are induced, more information is required to predict the downstream outcome of the transcriptional regulation of these genes. As was the case with wild type *A. actinomycetemtomitans*, the ontology for HIGK cells encountering the *aae*$^-$ strain SUNY1565 also predicts for the increased activity of filopodia, lamellipodia, and membrane ruffling compared to the uninfected cells. Thus, these may be adhesion-dependent responses (since no difference was observed in adhesion), but not related to Aae signaling.

Figure 5-4 illustrates the transcript levels in SUNY1565 (*aae*$^-$)-infected HIGK cells compared to the wild type-infected baseline level. This ontology analysis specifically addressed the impact of Aae on the cytoskeleton. This analysis is more sensitive than the separate comparisons of mutant and wild type infected cells to the baseline uninfected state because it is a direct comparison of the two infected conditions. However, the uninfected state is a necessary point of reference to place gene-specific differences in context. To accomplish this for genes regulated in all conditions, it is possible to extrapolate the pairwise data into a three-class comparison. For instance, ITG is up-regulated upon interaction with both SUNY465 and SUNY1565. The Aae$^-$/ wild type comparison reveals that while ITG is up-regulated upon wild type infection, it is up-regulated to an even greater extent in SUNY1565-infected HIGK cells. Eleven genes were regulated in mutant-infected cells compared to wild-type infected. Of these eleven, five genes were consistently regulated when infected HIGKs were compared to uninfected cells. The remaining six genes that were not differentially regulated when infected HIGK cells were compared to uninfected, but were significantly different in *aae*$^-$ strain infected HIGKs compared to wild type, are PIX, PAK, Ras, FN1, Mena and mDia. Only Mena and mDia were down-regulated. All other genes were up-regulated upon SUNY1565 (*aae*$^-$) infection of HIGK cells compared to infection with SUNY465. The gene product PIX interacts with PAK kinases and acts as a RAC1 guanine nucleotide exchange factor. As a result, PIX can induce membrane ruffling. PAK may act to stabilize actin filaments through the inhibition of cofilin activity. PAK plays a role in the reorganization of the actin cytoskeleton and in the formation of filopodia. Ras has GTPase activity and may transduce growth inhibitory signals. FN1 binds various cell surfaces including collagen and fibrin. FN1 is involved in cell adhesion, cell motility and maintenance of cell shape. Mena is a scaffold protein that stabilizes microtubules

and promotes cell migration. Through binding the barbed end of the actin filament, Mena slows down actin polymerization and depolymerization. The factor mDia is required for actin cables and stress fibers. This product also slows the rate of actin polymerization and depolymerization by binding to the barbed end of actin filaments. Activity by mDia also stabilizes microtubules. This is consistent with the finding that invasion by *A. actinomycetemcomitans* apprears to involve both actin and microtubules (Meyer *et al.,* 1996, Meyer *et al.,* 1999). The predicted down-regulation of in the mutant of actin-stabilizing genes Mena and mDia would result in the net increase of rearrangement activity. This is also in agreement with the up-regulation of genes that induce lamellipodia and filopodia formation. These genes were not impacted in SUNY465-infected cells compared to uninfected HIGK cells. Thus, the comparison of mutant-infected cells to wild type infected cells revealed that Aae may impact the Actin Cytoskeleton Rearrangement pathway through Mena and mDia, which was not revealed by comparing infected cells to the uninfected state. Taken together, the ontology analysis predicts that in the absence of Aae, more actin rearrangement will occur in HIGK cells, although both strains are capable of inducing actin rearrangments above uninfected levels. The predicted phenotypes would be increased numbers and/or size of filopodia, lamellipodia, and membrane ruffling in HIGK interacting with the *aae*$^-$ strain.

Preliminary Phenotypic Confirmation

Adhesion

In contrast to the data obtained from enumeration of viable colony forming units (CFU) interacting per host cell, confocal fluorescence microscopy performed on a limited number of observations and a single experiment failed to reveal a difference between the ability of the *A. actinomycetemcomitans aae*$^-$ to adhere to HIGK cells compared to the parental strain (Figure 5-5). More than 50 optical *z*-stacks consisting of merged *x*-*y* fields were analyzed. A total of 116

HIGK cells were observed independently, of which 56 were exposed to SUNY1565 (*aae*$^-$) and 60 were infected with SUNY465. The z-stacks allowed the entire cell to be visualized for interaction with *A. actinomycetemcomitans*. Thus, counts were not based on single z-sections individually, but rather the entire volume of the HIGK cell.

The first analysis examined the binding and invasion ability of the *aae*$^-$ strain compared to the wild type strain for each timepoint separately during the course of the infection. No statistically significant differences between the means were observed for any timepoint. The mean of adherent and invasive bacteria per cell for the entire timecourse covered a range with the lower limit 3.8+/-4.9 and the upper limit 5.6 +/- 4.6. The result of an unpaired, two-tailed student's t-test performed to compare the mean adhering bacteria of each strain at 20 minutes, two hours, six hours and 24 hours ranged from P=0.55 to P=0.75. These results suggest no difference in the total binding capability or the rate of binding over time for the *aae*$^-$ strain.

To increase the number of samples per condition, the total mean numbers of adherent and invading bacteria were considered for each strain separately, pooling the results from each timepoint. *A. actinomycetemcomitans* strain SUNY1565 (*aae*$^-$) interacted with HIGK cells at ratio of 4.7+/-4.3 (n=56) bacteria per cell. 5.0+/-3.9 (n=60) SUNY465 interacted per epithelial cell. Unpaired, two-tailed student's t-test revealed the probability of the two means being equal was P=0.66.

Rearrangement of Actin Cytoskeleton

The rearrangement of the actin cytoskeleton was not observed at any timepoint or condition attempted thus far (Figure 5-5). Based on the ontology analysis illustrated in Figures 5-2 to 5-4, both wild type and *aae*$^-$ *A. actinomycetemcomitans* were expected to induce membrane ruffling and increased lamellipodial protrusions through the up-regulation of PIR121 (CYFIP1) and Nap125. The anticipated actin foci beneath attached *A. actinomycetemcomitans*

previously reported in the literature were not observed (Fives-Taylor *et al.,* 1995). No obvious shortening or lengthening of filopodia was detected, and the intensity of actin staining which would indicate an increase in the amount of actin present was not observed. No changes in the localization of concentrated structures of F-actin were visible. Structures that are believed to be stress fibers were observed in all conditions, but the number or characteristics of these structures were not changed at any time point.

Discussion

Previous reports (Rose *et al.,* 2003; Fine *et al.,* 2005) and our own results using viable counts have consistently supported that *aae*$^-$ strains of *A. actinomycetemcomitans* are impaired in their ability to adhere to GECs, BECs, and HIGKs. A binding reduction ranging from 65% to 90% has been documented by this method. The current study attempted to visualize and confirm the *aae*$^-$ and wild type interaction and differential adhesion to epithelial cells using confocal fluorescence microscopy. Based on a limited number of observations and a single experiement, no difference in the ability of these strains to interact with oral epithelial cells was observed. To confirm the validity of these results, a larger sample will be taken consisting of 100 randomly-selected fields for each infection condition. Positive controls for invasion and cytoskeleton rearrangement will also be included in the analysis, such as parallel invasion of HIGK cells by *P. gingivalis*, a prototype invader that induces observable F-actin changes (Hasegawa *et al.,* 2007, submitted). Additional markers for the cell membrane of epithelial cells will be used, such as fluorescent pan-cadherin antibody (Abcam, Cambridge, UK) to more clearly delineate internalized bacteria from externally attached bacteria. Clearly the discrepancy between direct microscopic observation and plate counting requires resolution. We cannot rule out at this point that this experiment requires further optimization and calibration.

If these results are duplicated and definitively confirmed, one possible explanation for this discrepancy is a viability issue for *aae*$^-$ bacteria exposed to epithelial cells. Colony plating and spectrophotometric analysis of the inoculums have eliminated a higher MOI for *aae*$^-$ strains as a possible source of the unexpectedly high adhesion observed with microscopy. A situation where *aae*$^-$ mutant bacteria were present but non-viable would explain the differences observed. A fluorescent stain that is able to differentiate live versus dead bacteria would aid in addressing this issue, and will be explored. A product such as SYTOX Green stain (Molecular Probes Inc., Eugene, Oreg.) will be considered to enumerate dead and living bacteria under infection conditions with HIGK cells.

Ontology analysis led to predictions of increased actin rearrangement manifested in filopodia formation, lamellipodia formation, and membrane ruffling in HIGK upon interaction with *A. actinomycetemcomitans*. These events are not predicted to be Aae dependent, although absence of Aae seems to indicate an increased actin rearrangement activity above the levels observed in HIGK cells interacting with wild type bacteria. The current experiment was not able to validate these changes phenotypically using F-actin staining and fluorescence microscopy.

An explanation for the inability of the confocal fluorescence microscopy to corroborate the predicted actin cytoskeleton rearrangements is that the time points chosen may not have been able to detect a dynamic event. Previous studies using cervical epithelial cells have demonstrated *A. actinomycetemcomitans* interaction occurs within ten minutes of inoculation, and bacterial entry and exit of host cells can occur in less than 30 minutes (Fives-Taylor *et al.*, 1995). Although the 20 minute time point was chosen to account for these observations, it is possible that the rearrangement events of actin in HIGK cells occurred before the first time point was visualized microscopically. Actin foci beneath attached SUNY465 *A.*

actinomycetemcomitans have been previously demonstrated (Fives-Taylor *et al.*, 1995). Our transcriptional profiling further supports the observation that actin rearrangement does occur upon microbe-host interaction.

The discrepancies between ontology predictions, live counts and microscopic determination are under investigation. These experiments will include shorter time points and the combination of directed methods to quantify the size and numbers of filopodia and lamellipodia. ImageJ (Rasband W; NIH, Bethesda, MD) and Imaris (Bitplane Inc, Saint Paul, MN) software are capable of quantifying the size and intensity of specified features that appear in digitized images. Additional controls will also be included to assure the technical soundness of our assays, including *P. gingivalis* invasion, which can induce actin cytoskeleton rearrangements (Hasegawa *et al.*, 2007, submitted). Additionally, in light of the down regulation of the microtubule regulators, mDia and Mena, tubulin rearrangement may be a confirmable phenotype that is dependent upon Aae. Previous work has demonstrated the involvement of actin rearrangement of host cells during *A. actinomycetemcomitans* invasion which resembles pathogen-directed endocytosis of other organisms (Fives-Taylor *et al.*, 1995; Meyer *et al.*, 1996). Microtubules have been shown to play a role in the cell-to-cell spread of *A. actinomycetemcomitans*, as well as the egress of bacteria into the extracellular medium (Meyer *et al.*, 1999). The role of Aae in microtubule association and rearrangement thus warrants investigation in concert with the experiments examining actin cytoskeletal rearrangement. Chemical inhibitors of actin and tubulin assembly are a treatment which may help elucidate the effects of Aae predicted by ontology analysis. The inhibitor of actin assembly cytochalasin D, the inhibitor of microtubule polymerization colchicin, and the microtubule stabilizing agent taxol

are examples of such chemical agents that have been used previously to study *A. actinomycetemcomitans* invasion (Meyer *et al.,* 1999).

To our knowledge, this study is the first direct examination of the properties of *A. actinomycetemcomitans aae*$^{-}$ strain adhesion to gingival epithelial cells compared to wild type strains. This is also the first report of the transcriptional response by HIGKs to Aae. Previous studies have relied upon viable counts to quantify the adhesion deficiency attributed to Aae disruption in *A. actinomycetemcomitans* mutant strains (Rose *et al.,* 2003; Fine *et al.,* 2005). The only previous microscopic visual examination of Aae-mediated adhesion was performed using *E. coli* transformed to express Aae, and assessed their binding to BECs. Interestingly, Aae-transformed *E. coli* robustly adhered to BECs at the ratio of ten to 100 bacteria per BEC at the saturating MOI of 1000:1 to 10,000:1. Yet, the same assay performed using GECs found only three Aae-transformed *E. coli* adhering per cell (Fine *et al.,* 2005). Further, the adhesion assays of Aae-transformed *E. coli* and assays comparing wild type to mutant *A. actinomycetemcomitans* were performed on epithelial cells suspended in culture and under constant rotation. These conditions could create host cell binding characteristics much different from cells already adhering to a substrate and forming a monolayer, not the least of which is a greater surface area available for bacterial binding in the suspended cells. The authors' observations that BECs seem to have more binding sites for Aae than GECs also has implications in our model. If we assume each GEC has only 3 receptors for Aae-mediated binding, the microscopic observation that equal numbers of mutant and wild type *A. actinomycetemcomitans* bound HIGK may have resulted from another receptor-adhesin interaction, such as ApiA. This may have been enhanced with our infection conditions (MOI 2500:1). The original authors who discovered Aae in *A.*

actinomycetemcomitans used an MOI of only 100:1, which may have been optimal conditions to avoid non-specific interactions (Rose *et al.,* 2003).

Although Aae has been clearly demonstrated to act as an adhesin under certain conditions, it is not unreasonable to speculate this protein may have additional unforeseen functions. Aae is homologous to Hap and Hia, and subsequent characterization of this protein revealed that Aae was most closely related to the IgA1 proteases of *Neisseria* and *Haemophilus* species (Rose *et al.,* 2003). Based on this homology of Aae to IgA1 proteases and the adhesin/protease Hap of *Neisseria* and *Haemophilus*, this protein could conceivably possess proteolytic activity in *A. actinomycetemcomitans* in addition to its role as an adhesin. Re-examination of the microarray data may reveal a potential proteolytic effect of Aae transcriptionally.

Haemophilus influenzae Hap plays a role in epithelial cell binding, binding to collagen IV, fibronectin and laminin, and also plays a role in autoaggregation of *H. influenzae* (Fink *et al.,* 2003). Characteristic of autotransporter adhesins, Hap consists of a passenger domain flanked by an NH_2-terminal signal sequence and a COOH-terminal translocator domain (Henderson and Nataro, 2001). Interstingly, in *H. influenzae,* the passenger domain possesses all the adherence capability, as well as the ability to release itself into the extracellular environment through auto-proteolysis (Fink *et al.,* 2001). The proteolytic activity of the released adhesin/protease Hap has been proposed to aid initial colonization of this bacterium through proteolysis of host immune factors or epithelial components ahead of bacterial arrival, although a host substrate for this potential activity has not been identified for Hap (Fink *et al.,* 2001). Conversely, proteolysis occurring while Hap is stably adherent to an *in vivo* surface would result in the release of *H. influenzae* from its attachment site. While predictive modeling for Aae fails to predict a

proteolytic domain, assays for proteolytic activity by this protein in *A. actinomycetemcomitans* have not been conducted (Rose *et al.,* 2003) and cannot be ruled out.

The Hia protein of *Haemophilus influenzae* lacks the proteolytic activity of Hap, and has been proposed to better reflect the role of Aae in *A. actinomycetemcomitans* pathogenesis (Rose *et al.,* 2003) according to current characterizations. The lack of proteolytic activity by this protein causes Hia to remain associated with the outer membrane of *H. influenzae*, in contrast to Hap (St Geme and Cutter, 2000). Hia has been demonstrated to possess two binding domains that recognize the same receptor of human conjunctival epithelial Chang cells, and distinct from the receptor of Hap. Although the two domains individually differed in their binding affinity by 10-20 fold, both domains were demonstrated as essential for Hia binding to Chang cells to occur to the fullest extent (Laarmann *et al.,* 2002). Further differentiating Hia from Hap, the COOH-terminal domain of Hia is arranged as a trimeric translocator. This characteristic constitutes a subclass of autotransporter molecules, which appear to specialize in high affinity adhesive interactions with host surfaces (Cotter *et al.,* 2005). The IgA1 protease of *Neisseria* species is named for its ability to cleave the hinge region of IgA1 secreted by mucosal epithelium, presumably aiding bacterial colonization of this environment. Another possible role for this protein is based upon the IgA1 preotease-mediated proteolysis of lysosome/late endosome associated membrane protein, LAMP1. The intracellular lifestyle of *N. gonorrhea* has been studied, and demonstrates the survival of this bacterium within intracellular vesicles, the escape from said vescicles, and the association of *N. gonorrhea* with LAMP-1 proteins of endosomes (Hauck and Meyer, 1997). Additionally, an IgA1 protease-deficient mutant of *N. gonorrhoeae* has been reported to suffer a pronounced growth defect within epithelial cells (Lin *et al.,* 1997; Henderson and Nataro, 2001; Ayala *et al.,* 2002). Furthermore, LAMP1 proteolysis has been

proposed as a mechanism for trafficking of *N. gonorrhea* within epithelial cells. *Neisseria gonorrhea* mutants deficient in the IgA1 protease have a demonstrated impairment in their ability to traverse polarized epithelial monolayers of the T84 human colorectal epithelial cell line (Hopper *et al.,* 2000). *A. actinomycetemcomitans* has also been demonstrated to reside within a membranous vesicle following invasion of cultured epithelial cells (Meyer *et al.,* 1991). Additionally, the ontology analysis of the unfiltered dataset revealed the up regulation of PI4P5K (PIP5K3) (data not shown), which is involved in endosome related membrane trafficking. These shared characteristics between *A. actinomycetemcomitans* and *N. gonorrhea*, as well as the homology between Aae and the IgA1 protease, grant further investigation for a proteolytic activity of Aae, which may play a role in intracellular bacterial survival.

Conclusions

The deletion of *A. actinomycetemcomitans* Aae significantly impacted the transcriptome of HIGK oral epithelial cells, and revealed many pathways that appear to be involved in host-pathogen interactions. The regulation of actin cytoskeleton was one pathway highly impacted though transcriptional profiling and ontology analysis. Depending on the pairwise analysis performed, the pathway P-values ranged from 4.6×10^{2} to 1.4×10^{4}. Ontology analysis predicted increased lamellipodia, filopodia and membrane ruffling in infected cells compared to uninfected. The ontology for *aae*$^{-}$ infected HIGK predicts for a further increase of actin cytoskeleton rearrangements. Confocal fluorescence microscopy was used to assess the role of Aae on HIGK cell actin rearrangement. Under the experimental conditions used in the current study, microscopy failed to capture clear evidence of actin rearrangement. Therefore, the direct role of Aae in actin rearrangement is inconclusive. In the absence of additional controls and in a single experiment, no evidence was obtained that duplicate the co-localization of *A. actinomycetemcomitans* and host actin foci reported previously (Fives-Taylor *et al.,* 1995).

The microscopic visualization of bacteria and host cells performed did not correlate with the data obtained with viable counts, nor support primary role of Aae as an adhesin of GECs. Direct visualization methods failed to confirm the adhesion deficiency of *aae*$^-$ strains previously reported through methods relying upon viable counts. Future experiments will be conducted to reconcile these discrepancies. Additionally, actin rearrangement events that may be occurring earlier than the first time point used in this study will be further investigated. The spinning disk confocal microscope utilized for this study is capable of recording time-lapse images of bacterial and host co-cultures, and will be utilized to record the interactions of *A. actinomycetemcomitans* with HIGK cells over an extended period of time. This will potentially capture actin rearrangments that occur very quickly, and capture the adherence and invasion of *A. actinomycetemcomitans* in real time, eliminating the complication of choosing static timepoints to capture rapid and dynamic events.

Although Aae has defined capability to mediate *A. actinomycetemcomitans* adherence to the KB cervical epithelial cell line (Rose *et al.,* 2003) and to primary buccal epithelial cells (Fine *et al.,* 2005), it is reasonable to speculate that Aae possesses additional activities in oral gingival keratinocytes. As reviewed in Chapter 1, *P. gingivalis* gingipains and FimA pili are both capable of multiple interactions and functions in various models. Thus, in addition to exploring the role of Aae-mediated adhesion to gingival epithelial cells, host transcriptional profiling may yet reveal unanticipated functions for Aae. The homology of Aae to IgA1 proteases, the ontology that predicts membrane ruffling, endocytosis, and endosomal trafficking are potential clues to the function of Aae in host-pathogen interactions. Although Aae lacks a defined proteolytic motif, perhaps a proteolytic function of this component may allow the escape of *A. actinomycetemcomitans* from intracellular vacuoles following invasion, and the mutation of this

gene decreases viability of bacteria interacting with epithelial cells. The role of Hia, Hap, and IgA1 proteases are areas of interest due to their roles in pathogenesis of various bacteria and can provide clues into the functions of Aae (Henderson and Nataro, 2001).

We have previously demonstrated that the specific host response of oral epithelial cells mediated by individual bacterial components can provide insights into host pathogen interactions. Predicting phenotypic outcomes of specific pathways from transcriptional data has remains daunting, as many pathways interact in complex networks. Caution in the interpretation of transcriptomes is thus critical, and the importance of phenotypic characterizations to complement microarray studies remains invaluable. Although the particular method chosen to validate the transcriptional profiles obtained from this study was not definitive, the regulation of gingival epithelial cell cytoskeleton rearrangements will be investigated further. Experimental conditions will be optimized, including proper controls and methods of quantifying actin rearrangement definitively. The potential to identify an unknown function for Aae also has arisen from analysis of the host cell transcriptome interacting with oral pathogens and will be pursued.

Table 5-1. Gene ontology analysis (P<0.05) of the most impacted pathways caused by a mutation of *aae* (VT1565) in the parent strain *A. actinomycetemcomitans* (SUNY465).

Impact Factor[a]	Pathway Name[b]	Modulated (total) genes in pathway[c]
5.675	Regulation of actin cytoskeleton	15 (206)
5.143	Focal adhesion	18 (194)
4.376	ECM-receptor interaction	9 (87)
3.931	Jak-STAT signaling pathway	13 (153)
3.856	Adipocytokine signaling pathway	7 (69)
3.185	Leukocyte transendothelial migration	10 (117)
3.136	Cell adhesion molecules (CAMs)	10 (132)
2.856	Tight junction	10 (119)
2.581	MAPK signaling pathway	17 (273)
2.374	Cell cycle	9 (112)
2.254	Calcium signaling pathway	12 (176)
1.935	Apoptosis	4 (84)
1.769	Toll-like receptor signaling pathway	5 (91)
1.436	Cytokine-cytokine receptor interaction	14 (256)
1.247	Epithelial cell signaling in Helicobacter pylori infection	2 (46)

The epithelial cell pathways were determined by Pathway Express [a]The impact factor measures the pathways most affected by the changes in gene expression by considering the proportion of differentially regulated genes, the perturbation factors of all the pathway genes, and the propagation of these perturbations throughout the pathway. [b]According to the Kyoto Encyclopedia of Genes and Genomes pathways (http://www.genome.jp/kegg/). [c]Number of regulated genes in a pathway/total number of genes currently mapped to this pathway.

Figure 5-1. HIGK transcriptome upon VT1565-, SUNY465-, or mock-infection. RNA was isolated and purified after 2h co-culture with *A. actinomycetemcomitans* SUNY465 or VT1565 and compared to uninfected cells. This heat map and dendrogram were constructed from 4138 probe sets differentially expressed between the three experimental classes at the significance level of $P < 0.05$. Probe set signal intensities were variance-normalized, mean-centered across samples, and subjected to hierarchical cluster analysis. Average linkage clustering by uncentered correlation was performed for genes and samples. The degree of similarity between the transcriptional profiles of each sample is expressed by Pearson's correlation coefficient distance metric, according to the adjacent scale. The expression state of each data point is represented as standard deviations from the mean expression level for that gene in all samples. Red indicates a relative increase, green indicates a relative decrease, and black indicates no relative change of mRNA transcripts for a given gene. Labels. Uninfected HIGK Cells, CTRL R1-R4; *A. actinomycetemcomitans* SUNY465-infected HIGK Cells, SUNYR1-R4; *A. actinomycetemcomitans* VT1565-infected HIGK Cells, ^Aae R1-R4

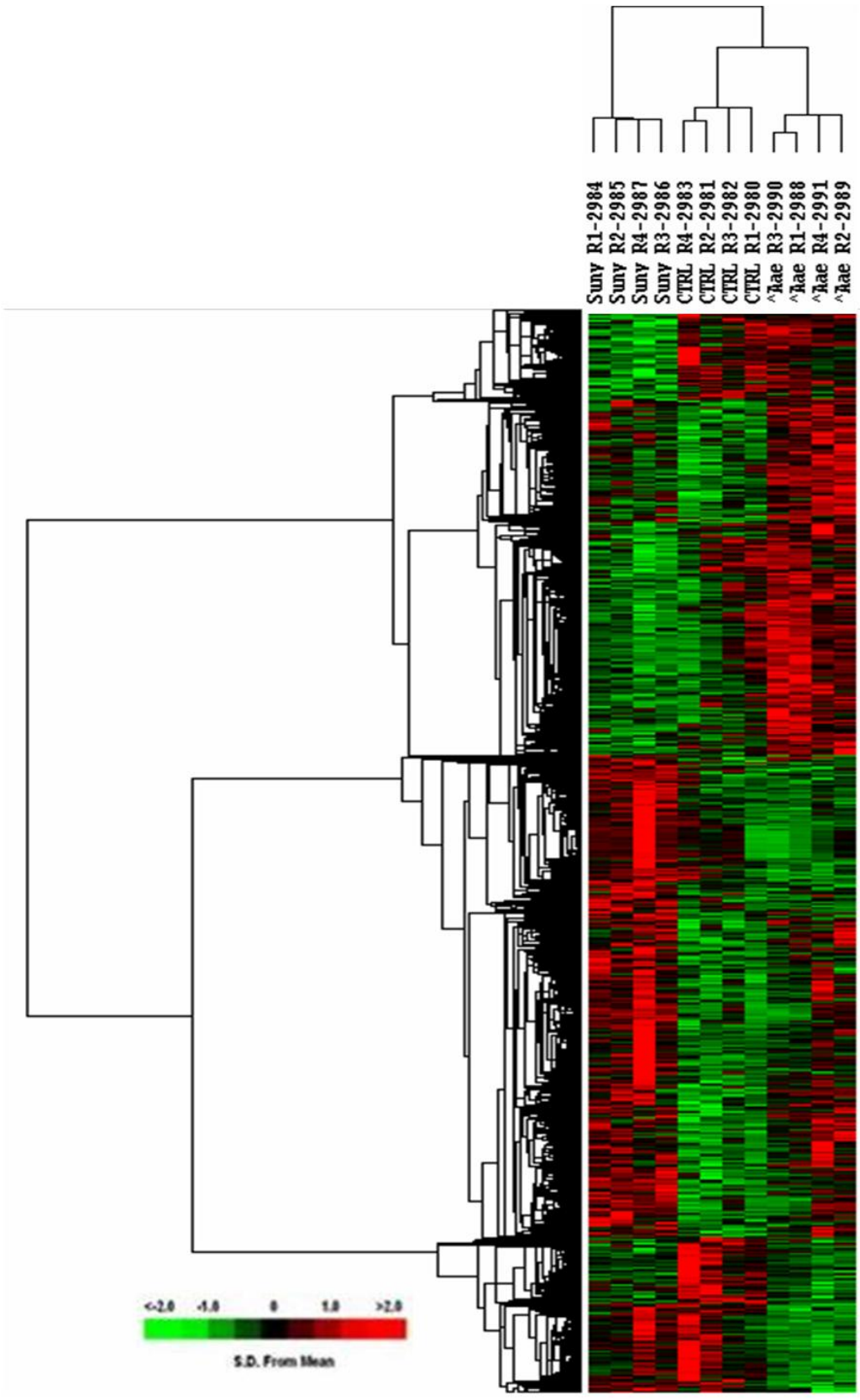

Suny R1-2984
Suny R2-2985
Suny R4-2987
Suny R3-2986
CTRL R4-2983
CTRL R2-2981
CTRL R3-2982
CTRL R1-2980
^Aae R3-2990
^Aae R1-2988
^Aae R4-2991
^Aae R2-2989
<-2.0
-1.0
0
1.0
>2.0
S.D. From Mean

Figure 5-2. Impact of *A. actinomycetemcomitans* SUNY465 interaction with HIGK cells upon the Regulation of Actin Cytoskeleton pathway. Differential modulation of pathway genes is represented as expression levels from Human Immortalized Gingival Keratinocytes upon co-culture with *A. actinomycetemcomitans* parent strain SUNY465 compared to transcript levels obtained from Uninfected HIGK cells. Red terms are transcriptionally up-regulated, while blue terms are down-regulated compared to baseline conditions. Terms in green were not significantly modulated. Modulated genes were significant at the $P < 0.05$ threshold and were changed by at least two-fold magnitude.

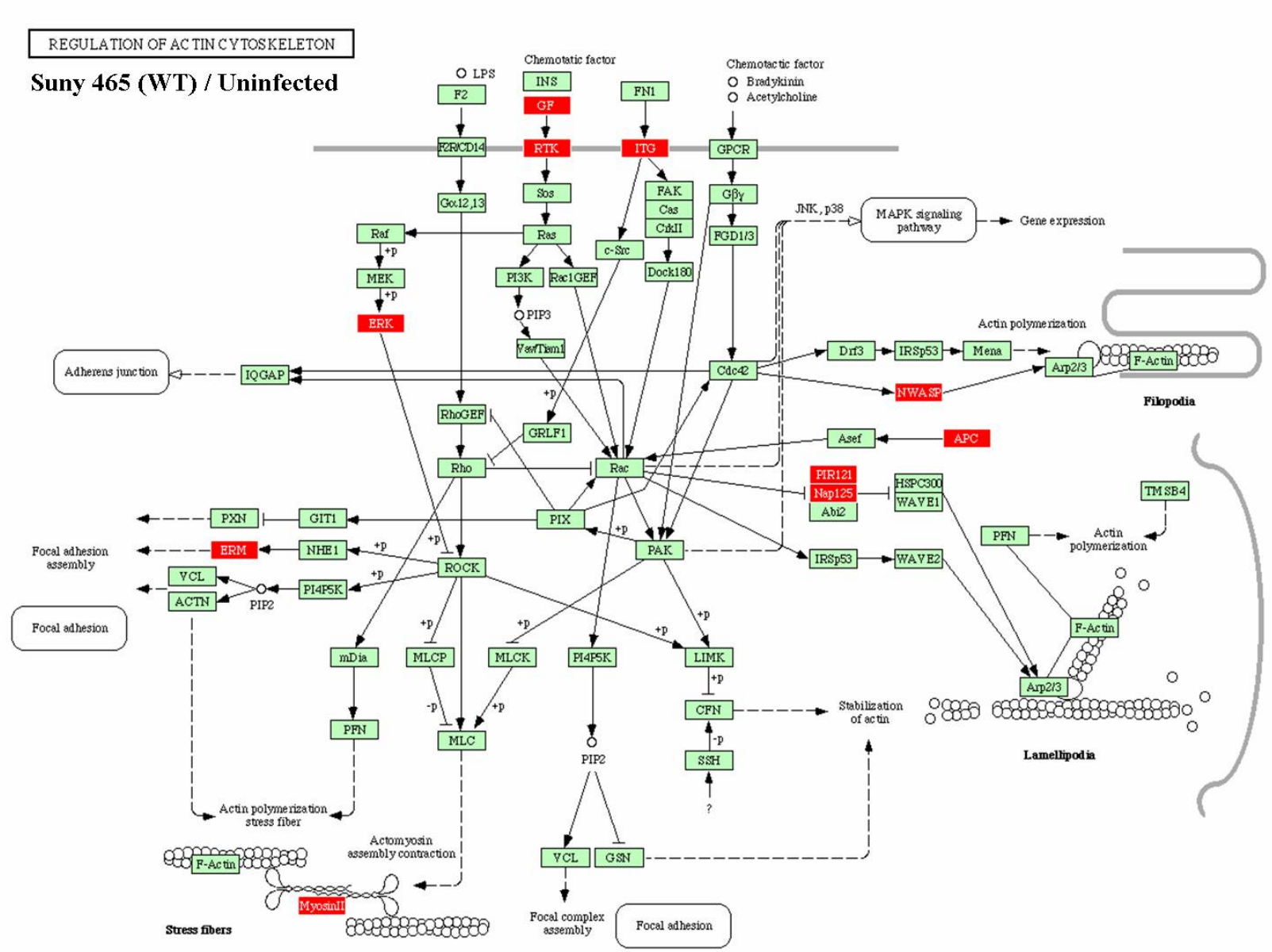

REGULATION OF ACTIN CYTOSKELETON
Suny 465 (WT) / Uninfected
LPS
Chemotatic factor
Chemotactic factor
Bradykinin
Acetylcholine
F2
INS
GF
FN1
F2R/CD14
RTK
ITG
GPCR
Gαi2,i3
Sos
FAK
Cas
CrkII
Gβγ
JNK, p38
MAPK signaling pathway
Gene expression
Raf
Ras
c-Src
FGD1/3
MEK
PI3K
Rac1GEF
Dock180
ERK
PIP3
Vav/Tiam1
Actin polymerization
Adherens junction
IQGAP
Cdc42
Drf3
IRSp53
Mena
Arp2/3
F-Actin
Filopodia
NWASP
RhoGEF
GRLF1
Asef
APC
Rho
Rac
PIR121
Nap125
Abi2
HSPC300
WAVE1
TMSB4
PXN
GIT1
PIX
PFN
Actin polymerization
Focal adhesion assembly
ERM
NHE1
PAK
IRSp53
WAVE2
VCL
ACTN
PIP2
PI4P5K
ROCK
Focal adhesion
F-Actin
mDia
MLCP
MLCK
PI4P5K
LIMK
Arp2/3
CFN
Stabilization of actin
PFN
MLC
PIP2
SSH
?
Lamellipodia
Actin polymerization stress fiber
Actomyosin assembly contraction
F-Actin
VCL
GSN
MyosinII
Stress fibers
Focal complex assembly
Focal adhesion
04810 11/15/04

Figure 5-3. Impact of *A. actinomycetemcomitans* SUNY1565(*aae*$^-$) interaction with HIGK cells upon the Regulation of Actin Cytoskeleton pathway. Differential modulation of pathway genes is represented as expression levels from Human Immortalized Gingival Keratinocytes upon co-culture with *A. actinomycetemcomitans* isogenic mutant strain SUNY1565(*aae*$^-$) compared to transcript levels obtained from Uninfected HIGK cells. Red terms are transcriptionally up-regulated, while blue terms are down-regulated compared to baseline conditions. Terms in green were not significantly modulated. Modulated genes were significant at the $P < 0.05$ threshold and were changed by at least two-fold magnitude.

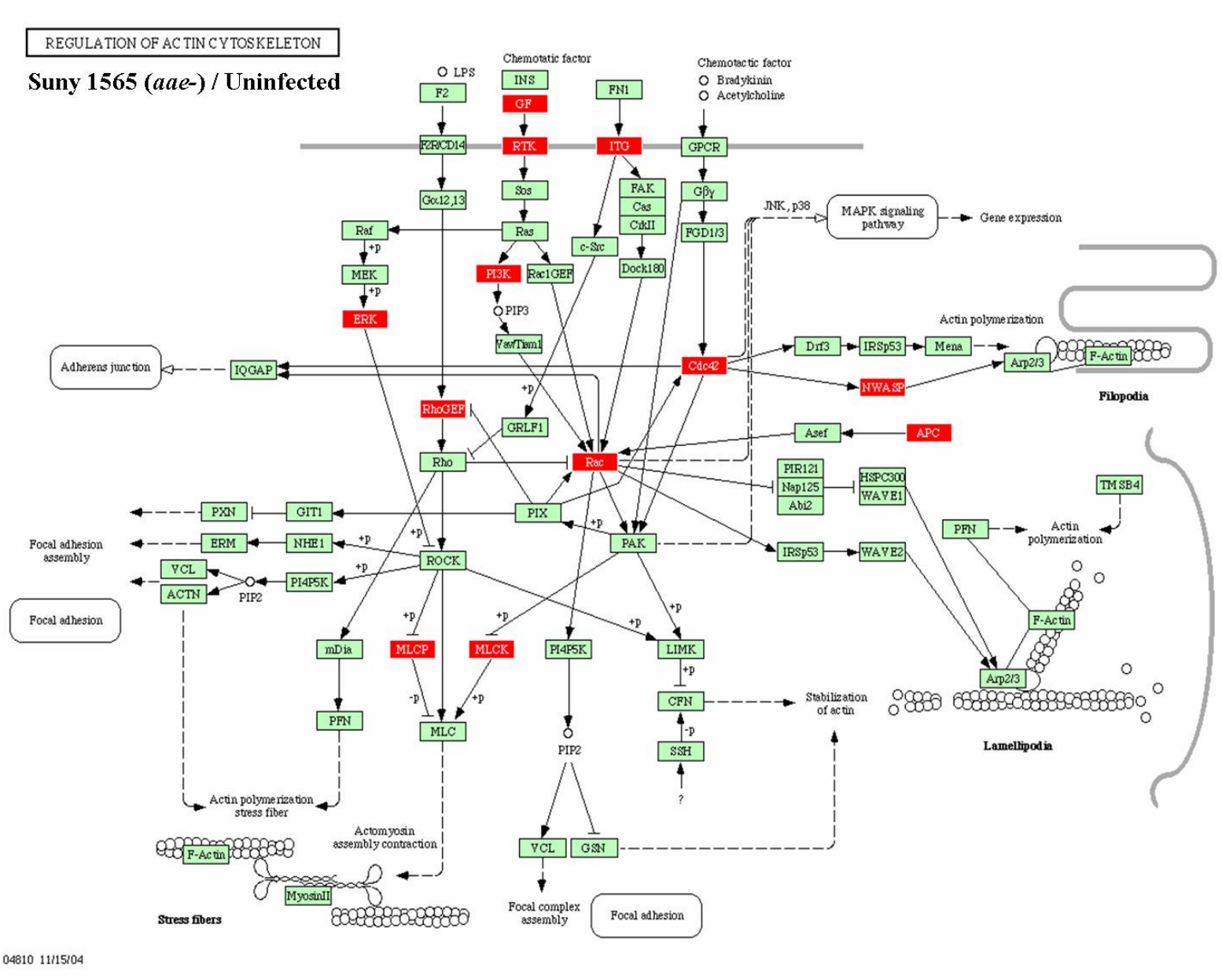

REGULATION OF ACTIN CYTOSKELETON
Suny 1565 (aae-) / Uninfected
LPS
Chemotatic factor
Chemotactic factor
Bradykinin
Acetylcholine
F2
INS
GF
FN1
FZR/CD14
RTK
ITG
GPCR
Go.12,13
Sos
FAK
Cas
CdkII
Gβγ
JNK, p38
MAPK signaling pathway
Gene expression
Raf
Ras
c-Src
FGD1/3
+p
MEK
PI3K
Rac1GEF
Dock180
ERK
PIP3
Vav/Tiam1
Actin polymerization
Adherens junction
IQGAP
Cdc42
Drf3
IRSp53
Mena
Arp2/3
F-Actin
NWASP
Filopodia
RhoGEF
GRLF1
Asef
APC
Rho
Rac
PIR121
Nap125
Abi2
HSPC300
WAVE1
TMSB4
PXN
GIT1
PIX
PFN
Actin polymerization
Focal adhesion assembly
ERM
NHE1
PAK
IRSp53
WAVE2
VCL
ACTN
PIP2
PI4P5K
ROCK
F-Actin
Focal adhesion
mDia
MLCP
MLCK
PI4P5K
LIMK
Arp2/3
CFN
Stabilization of actin
PFN
MLC
PIP2
SSH
?
Lamellipodia
Actin polymerization stress fiber
Actomyosin assembly contraction
F-Actin
MyosinII
Stress fibers
VCL
GSN
Focal complex assembly
Focal adhesion
04810 11/15/04

Figure 5-4. Specific contribution of Aae to the HIGK cell Regulation of Actin Cytoskeleton pathway. Differential modulation of pathway genes is represented as expression levels from Human Immortalized Gingival Keratinocytes upon co-culture with *A. actinomycetemcomitans* isogenic mutant strain SUNY1565(*aae*$^{-}$) compared to transcript levels obtained from HIGK cells interacting with *A. actinomycetemcomitans* parental strain SUNY465. Red terms are transcriptionally up-regulated, while blue terms are down-regulated compared to baseline conditions. Terms in green were not significantly modulated. Modulated genes were significant at the $P < 0.05$ threshold and were changed by at least two-fold magnitude.

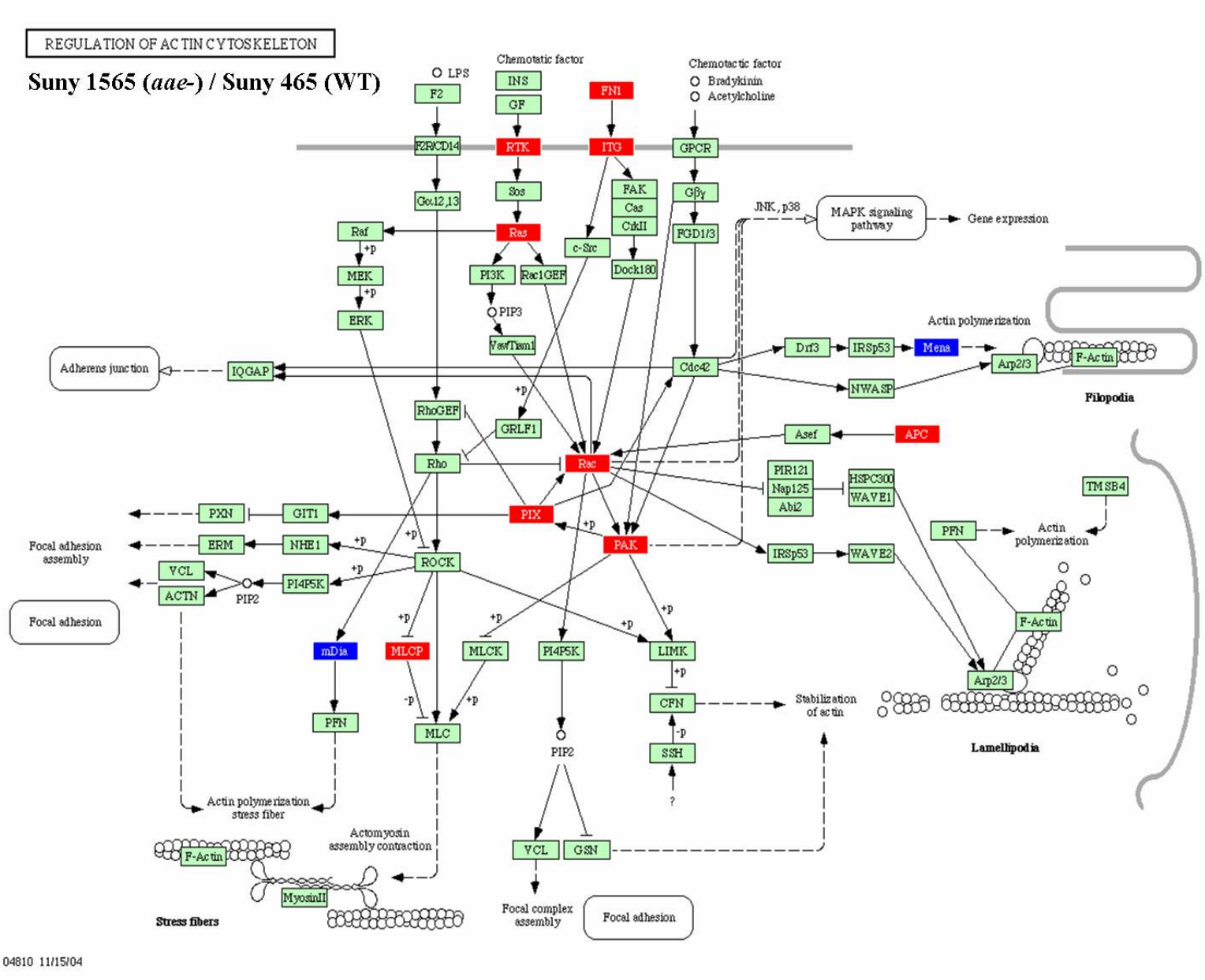
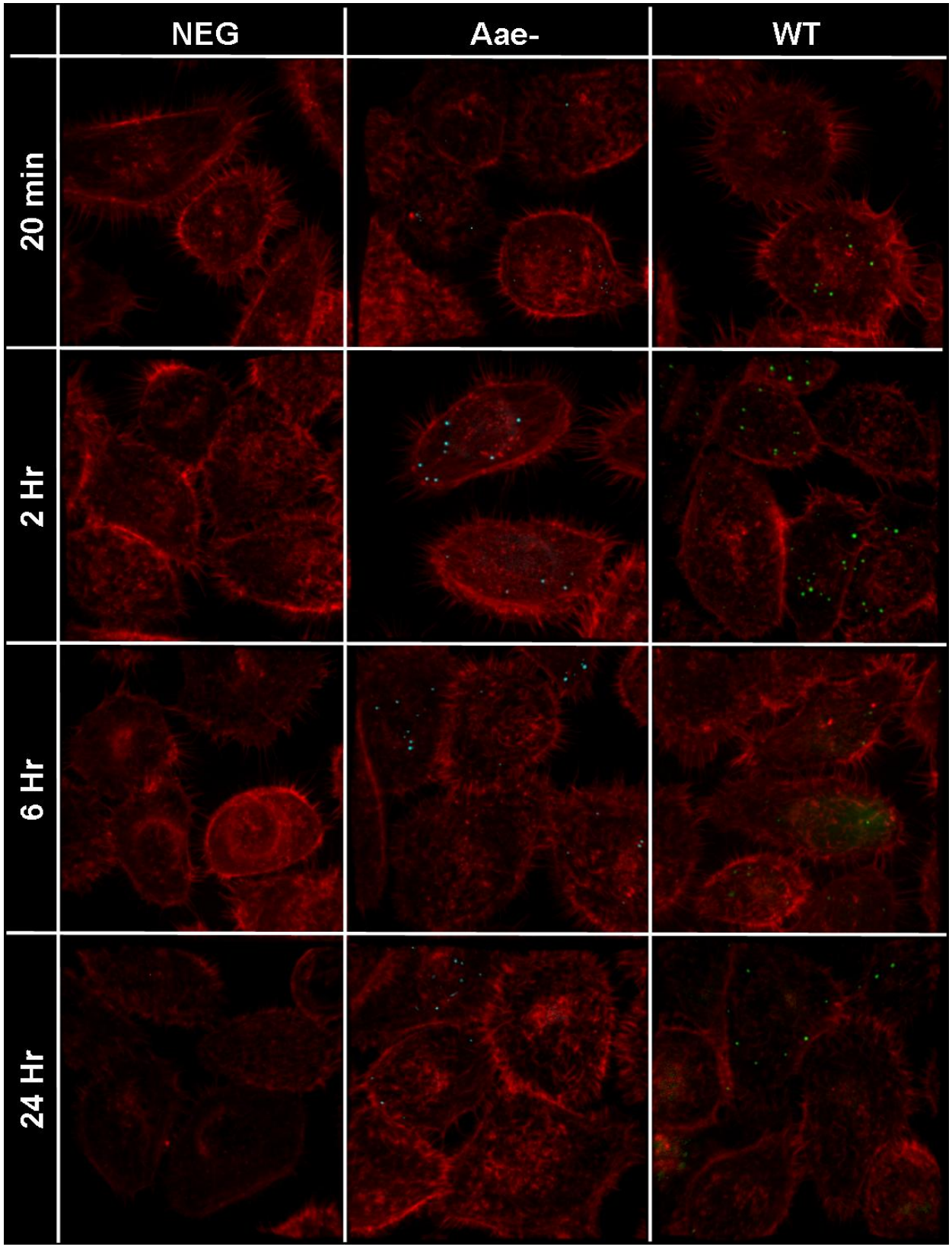

REGULATION OF ACTIN CYTOSKELETON
Suny 1565 (aae-) / Suny 465 (WT)
LPS
Chemotatic factor
Chemotactic factor
Bradykinin
Acetylcholine
F2
INS
GF
FN1
FZR/CD14
RTK
ITG
GPCR
Gα12,13
Sos
FAK
Cas
CrkII
Gβγ
JNK, p38
MAPK signaling pathway
Gene expression
Raf
Ras
c-Src
FGD1/3
MEK
PI3K
Rac1GEF
Dock180
ERK
PIP3
Vav/Tiam1
Actin polymerization
Adherens junction
IQGAP
Cdc42
Drf3
IRSp53
Mena
Arp2/3
F-Actin
NWASP
Filopodia
RhoGEF
GRLF1
Asef
APC
Rho
Rac
PIR121
Nap125
Abi2
HSPC300
WAVE1
TMSB4
PXN
GIT1
PIX
PAK
PFN
Actin polymerization
Focal adhesion assembly
ERM
NHE1
ROCK
IRSp53
WAVE2
VCL
ACTN
PIP2
PI4P5K
Focal adhesion
mDia
MLCP
MLCK
PI4P5K
LIMK
F-Actin
Arp2/3
PFN
MLC
CFN
Stabilization of actin
Lamellipodia
PIP2
SSH
Actin polymerization stress fiber
Actomyosin assembly contraction
VCL
GSN
F-Actin
MyosinII
Stress fibers
Focal complex assembly
Focal adhesion
04810 11/15/04

Figure 5-5. HIGK cells interacting with *Aggregatibacter actinomycetemcomitans*. Human Immortalized Gingival Keratinocytes were visualized by Texas Red phalloidin staining of host cell actin cytoskeleton (red). *A. actinomycetemcomitans* (green or turquoise dots) were stained with CellTracker Green BODIPY live dye prior to co-culture experiments and allowed to interact for the indicated times. Imaris software was used to digitally recolor signal channels. *A. actinomycetemcomitans*, VT1565 (*aae*$^{-}$) are shown in turquoise and SUNY465 (wild type) are shown in green. Columns shown from left to right are the three experimental conditions, Uninfected HIGK cells, VT1565-infected HIGK cells, and SUNY465-infected HIGK cells. Rows from top to bottom are the four timepoints chosen to investigate, 20 minutes, Two Hours, Six Hours, and 24 Hours post-innoculation.


NEG
Aae-
WT
20 min
2 Hr
6 Hr
24 Hr

CHAPTER 6
DISCUSSION AND PERSPECTIVES

The fundamental questions that arise in this work are not only "What are pathogens doing to cause disease?" and "How can we promote health?" but "What is health besides the absence of disease?" From the microecological standpoint, is health simply a microbial imbalance with constant turnover and the inability for a single bacterium to set up residence and cause tissue damage? Is health the opposite situation, the ability of truly commensal bacteria to stably colonize the oral cavity and prevent pathogens from ever occupying the niche that the "good" flora occupies? The intestinal flora has been shown to positively benefit the host in several instances, such as maintenance of homeostasis and prevention of injury in the gut through TLR signaling (Rakoff-Nahoum *et al.,* 2004), nutrient absorption (Hooper *et al.,* 2001; Backhed *et al.,* 2004), mucosal barrier fortification, and angiogenesis (Hooper *et al.,* 2001). While this study cannot answer all these questions—however—it does lay the groundwork for investigating these complicated issues at a future time.

In light of the complex questions at hand, the goal of this study was to gain insights into host-pathogen interactions that may occur in periodontal disease. This was performed with global host transcriptional profiling of oral epithelial cells. The hypothesis driving this project was that oral epithelial cells actively and specifically respond to oral pathogens, and can thus serve as a reporter system to shed insight on bacterial pathogenesis. To accomplish this endeavor, three specific aims were completed:

SA-I Establish a periodontal disease experimental model of epithelial cells and transcriptional profiling

SA-2 Establish the epithelial host cell baseline global transcriptional profiles for uninfected and infected conditions under investigation

SA-3 Assess the impact of individual bacterial factors upon the host cell transcriptome and confirm biologically relevant predicted outcomes phenotypically

Additional Collaborative Work

In addition to the work presented as the focus of this study, the strategies developed herein have been applied to address additional questions addressing host-pathogen interactions likely to occur in the oral cavity that did not pertain directly to our specific aims. Through collaborations between the laboratory of Dr. Martin Handfield, as well as the lab of Dr. Richard Lamont, a wealth of transcriptional data has been collected. As these tools continue to be applied to various organisms or bacterial components, a broader perspective is to be gained. Afterall, with *P. gingivalis* and *A. actinomycetemcomitans* studies well underway, at least 698 other oral microbes await further study!

In collaboration with Dr. Yoshiaki Hasegawa (Lamont Lab), the baseline transcriptional profiling for the commensal bacterium *Streptococcus gordonii*, and the opportunistic commensal *Fusobacterium nucleatum* was performed. The host epithelial response to these organisms was compared to the baseline previously determined for *P. gingivalis* and *A. actinomycetemcomitans* (Chapter 4). One finding was a common response shared by all four of these microbes, as presented in Appendix A (Hasegawa *et al.,* 2007). Also uncovered, were various epithelial responses which were completely unique to each organism. These unique responses are under investigation for possible implications in actual human oral infections.

The impact of specific bacterial components was extended to study the effect of *A. actinomycetemcomitans* CDT on epithelial host cell transcriptomes in collaboration with Dr. Mounia Alaoui El Azher (Handfield Lab). As described for studies of *A. actinomycetemcomitans* ORF859 and *P. gingivalis* FimA, mutant analysis was combined with transcriptional profiling to reveal the CDT effects on HIGK cells. Consistent with the putative

activity of CDT, the Cell Cycle and Apoptosis pathways were both implicated. The dissection of these pathways was enabled through microarray analysis, and the involvement of specific host genes in the epithelial response was further confirmed by Western analysis (Appendix B, Alaou-El-Azher). The effects of interfering with the host response were studied with RNA interference, and the results have implications for the specific activity and targets of CDT (Alaoui El Azher *et al.,* unpublished). Mutant analysis of another *P. gingivalis* component, SerB, has also been conducted (Hasegawa *et al.,* submitted). Although the specific findings are beyond the scope of this project, these examples illustrate how the epithelial host transcriptome has broadly been applied to study oral microbes.

Lessons Learned

Specific Aim 1: Establish the Epithelial Transcriptome Experimental Model

As reviewed in Chapter 2, prior to this study, global transcriptional profiling with DNA microarrays had been utilized successfully to gain insights of host-pathogen interactions, primarily between gut epithelium and enteric pathogens. In particular, the study of *Helicobacter pylori* which incorporated mutant analysis and assessment of the host transcriptome (Guillemin *et al.,* 2002) provided an encouraging basis to attempt a model of epithelial cell interactions for the oral cavity. Prior to executing a similar model in the oral epithelium, however, several factors had to be addressed.

Choosing the microarray and data analysis platform

In contrast to other works at the time, the current project was designed to reveal genes whose modulations were statistically different between classes—not simply those that met a threshold for fold induction or decrease. Using statistical methods to find genes that are differentially regulated provides a measurable probability that uncovered genes are truly predictors of a given experimental condition. Additionally, as replicates are added to a statistical

analysis, variance is reduced and the differences between mean values become more clearly defined. This strategy allowed genes to be uncovered during the analysis which would normally be missed due to the lack of power by other filtering methods, such as those based on fold-change or visual interest in clusters of like-regulated genes. Further, the variability of a given gene's expression within samples of the same class was considered in statistical methods, which reduced false positives with large fold-expression changes but even larger standard deviations between samples. The advantage of the significance threshold is a reliable method to anticipate the quality of the dataset under study.

In addition to utilizing statistical analysis software, the use of commercially available, annotated microarrays were an important feature of this study. The annotations for impacted genes were readily available online (Affymetrix), and ontology tools were available to organize impacted genes into biologically relevant pathways. The use of these tools has been an often underappreciated aspect of the current study, but a critical component of interpreting the transcriptional data. The advent of Pathway Express has been a welcome improvement to the bioinformatic aspect of the process, which automatically generates populated KEGG pathway maps that previously had to be populated manually. These considerations did not directly impact the biological aspects of our experiments, yet their importance to successful array experiments cannot be overlooked.

Determining standard infection conditions

The actual benchwork that comprises a transcriptional profiling experiment was refined through trial and error until the experimental conditions that came to define a standard array experiment were developed. The growth stage of the infecting bacteria to use for co-culture experiments, the extent of monolayer confluence, and even the appropriate cell line to use had to be optimized. The first microarray experiment was conducted using the MOI of 100:1 for *A.*

*actinomycetemcomitan*s and *P. gingivalis* in parallel with uninfected cells. An interesting finding was the close clustering of *A. actinomycetemcomitans*-infected epithelial cells with uninfected cells under these conditions. Review of the phenotypic data, which showed the ratio of adherent *A. actinomycetemcomitans* to host cells was less than one per cell, suggested a majority of the cells were profiling as uninfected. This led to the hypothesis that the MOI had to be high enough for every host cell to encounter a single bacterium in order for a homogenous mRNA pool reflective of infection to result. The next round of experiments incorporated this change, and a robust host response to *A. actinomycetemcomitans* was observed. The importance of including proper controls to monitor the actual co-culture interactions was reinforced by this experience in addition to establishing an MOI of 1000:1 to 2500:1 as the target for *A. actinomycetemcomitans*. The ratio of total interacting bacteria thus became the benchmark for a successful co-culture experiment, rather than the initial MOI. More aggressive bacteria, such as *P. gingivalis* thus required fewer initial bacteria to obtain the same level of interaction with host cells as compared to *A. actinomycetemcomitans*. Interstingly, the latest confocal microscopy experiments demonstrate that not every host cell in fact encounters at least a single bacterium. This raises the possibility that a dose response between the MOI and host transcriptional profiles exists, possible dependent on secreted factors.

Effect of host cell lineage on transcriptional response

Reliance upon the KB (HeLa CCL-17) cell line was widespread upon the onset of this study, because KB cells were believed to descend from an oral carcinoma, and *in vitro* invasion assays between these cells and oral pathogens could successfully replicate the clinical observations of an invasive bacterial phenotype. The agreement of phenotypic characteristics during these assays between KB cells and primary oral epithelial cells also supported their use. The discovery that KB cells were actually HeLa cervical epithelial cells that contaminated the

original cultures combined with the realization that epithelial cells are able to respond actively and specifically to infection raised questions regarding the relevance of using KB cells in periodontal research. Thus, in what is believed to be the only study of its kind, two different epithelial cell lines—KB and HIGK— were co-cultured with *A. actinomycetemcomitans* in parallel, and the transcriptomes were analyzed. Not surprising in relation to the hypothesis that epithelial cells mount an active a specific response to infection (Dale, 2002), significant differences were found between the responses of these two cell lines to the same bacteria. As detailed in Chapter 3, a large degree of similarity was shared for many genes that involve homeostasis and other functions not within the host-pathogen interaction realm. This was not surprising since both cell lines were epithelial cells and shared some major characteristics. However, the differences in Response to Pest, Pathogen, and Parasite pathways and others with documented relevance to host-pathogen interactions revealed specific interactions that could be attributed to bacterial tropism. Establishing this cell-line based specificity under the optimized infection conditions already discussed was an important aspect of completing Specific Aim 1, as well as establishing the transcriptional model for probing host-pathogen interactions of the oral epithelium. This lesson also cautions that although a high degree of similarity has been shown between HIGK cells and primary GECs in terms of their behavior during invasion assays and other phenotypic responses to oral bacteria, differences in the transcriptional responses of these cells will also likely exist. One potential source of differences is the HPV immortalization of HIGK cells (Oda *et al.,* 1996). Over the course of 350 passages, several chromosomal abnormalities were detected, including a mutation in P53 (Oda *et al.,*1996b). Although the impact on cellular regulatory pathways, such as cell cycle and apoptosis, were not severe enough to cause these cells to become tumorigenic, the overall effects of HPV immortalization are

unknown as related to HIGK responses to bacterial interaction. Therefore, this may be a limitation of the current model.

Specific Aim 2: Establish the Baseline Oral Epithelial Transcriptome Upon Infection *P. gingivalis* and *A. actinomycetemtomitans*-specific responses

Now established, the epithelial transcriptional model system began to provide insights into the effects of *A. actinomycetemcomitans* and *P. gingivalis* upon the epithelium. The experiments described in Chapter 4 which compared co-culture of wild type bacteria and HIGK cells to uninfected HIGK cells revealed both common and exclusive pathways for a given experimental treatment. Many pathways await further investigation into their potential role in health or disease of the oral epithelium. Notably, the different impact of *P. gingivalis* and *A. actinomycetemcomitans* upon HIGK apoptotic pathways was revealed using transcriptional profiling. The subsequent ontology analysis and phenotypic confirmations demonstrated the ability of *P. gingivalis* to actively prevent apoptosis in these cells, and the pro-apoptotic phenotype initiated by *A. actinomycetemcomitans*. These findings were presented to the 83rd General Session of The IADR in a symposium (Appendix B, Mans). The different host apoptotic response to these two pathogens could help explain why LAP progression is rapid, and CP is a chronic infection, as the host tissue is prevented from undergoing cell suicide in one case, and promoted in the other situation. Further dissection of the *A. actinomycetemcomitans*-impacted apoptotic pathway was performed as a direct result of the transcriptional findings. The induction of several apoptosis proteins were studied using Western blot analysis, and were also presented to the 83rd General Session and Exhibition of the IADR (Appendix B, Naselsker).

Baseline host response to *S. gordonii* and *F. nucleatum*

Although not a specific component of the current study, the methods developed herein have been used in collaboration with others (Appendix A). As a result, the baseline

transcriptome was also established for HIGK cells interacting with the commensal *Streptococcus gordonii*, and the opportunistic commensal *Fusobacterium nucleatum*. When compared to the transcriptional profiles and ontology established for *A. actinomycetemcomitans* and *P. gingivalis* interaction, core pathways were found for all four infection conditions. The specific manner in which each bacterium impacts these common pathways is currently under investigation. Also in support of an active response to infection by oral epithelial cells were the pathways that were distinct for each bacterial species. These pathways will be examined for their potential to promote health or disease, and may also serve as markers of active bacterial infection compared to generalized colonization or mere presence of bacteria in the oral cavity.

A more detailed analysis of the HIGK response to *S. gordonii* and *F. nucleatum* have been completed, and the findings were presented to the 84th General Session and Exhibition of the IADR (Appendix B, Hasegawa) and later published in the journal Infection and Immunity (Appendix A). The baseline HIGK response to infection yielded useful insights which allowed species-specific outcomes to be studied, as presented, such as cytokine regulation. Of great interest, although *F. nucleatum* increased the expression of the pro-inflammatory cytokines Interleukin-6 (IL-6) and IL-8, *S. gordonii* actually repressed these cytokines below levels observed in uninfected HIGK cells. This finding suggests that *S. gordonii* may function to counteract a destructive immune response caused by other bacteria and has implications in periodontal disease that will be explored futher. Additionally, the baseline response to interaction with wild type bacteria became the foundation of completing the third and final specific aim, the contribution of specific bacterial components.

Specific Aim 3: Investigate the Impact of Individual Bacterial Components on the Host Cell Transcriptome.

Impacts of *P. gingivalis* fimbriae and *A. actinomycetemcomitans* ORF859

The combination of host transcriptional profiling with a mutant analysis strategy allowed the completion of Specific Aim 3: assess the contribution of individual bacterial components on host-pathogen interactions. As first performed in Chapter 4, the host response to *P. gingivalis* YPF-1, a strain deficient in major fimbriae, and *A. actinomycetemcomitans* JMS04, as strain with a mutation in the *ORF859* gene implicated by IVIAT as an *in vivo* induced gene in actual human LAP infections, were compared to the baseline host responses of HIGK cells to the wild type parental strains. Strain JMS04 was characterized prior to this array study, and mutation of the *ORF859* gene was found to confer a defect in intracellular survival by *A. actinomycetemcomitans* encountering epithelial cells (Cao *et al.*, 2004). Interestingly, the impact of this bacterial product upon the HIGK transcriptome was found to be minimal, and may reflect a lack of interaction between this bacterial protein and the host cell. This is consistent with a role for ORF859 in bacterial homeostasis and that this component is not a toxin. In contrast, the mutation in strain YPF-1, which abrogated the expression of functional major fimbriae, was shown to highly impact the HIGK transcriptome. Previous work had shown a diversity of functions for major fimbriae, as reviewed in Chapter 1, and the extent of interaction between this bacterial component and host cells was reflected by the transcriptional profile. This work was also presented to the 83rd General Session and Exhibition of the IADR.

***A. actinomycetemcomitans* CDT and Aae impact on host pathogen interactions**

The contributions by two additional *A. actinomycetemcomitans* components to host-pathogen interactions have been investigated. As introduced in Chapter 1, the Cytolethal Distending Toxin (CDT) and autotransporter adhesin Aae are important virulence factors of *A.*

actinomycetemcomitans. CDT is known to cause cell cycle arrest leading to apoptosis in several cell types, and Aae is currently credited for specific adhesion to oral epithelial cells. To probe the transcriptional impact upon the host cells of these functions, the array analysis, ontology, and phenotypic assays were performed in collaboration with Dr. Alaoui-El-Hazer. Not surprisingly, CDT impacted the Apoptosis and Cell Cyle pathways significantly in HIGK cells. Importantly, the transcriptional profiling not only confirmed the reported function of this exotoxin, it also revealed the manner in which cell cycle arrest was perturbed at the level of individual genes of the cell cycle pathway. Although not a specific aim of the current project, development of Specific Aims 1 and 2 directly led to progress in the study of CDT. The results of this collaboration were presented to the 84th General Session and Exhibition of the IADR during a symposium (Appendix B, Mans). Additional work to dissect the cell cyle pathway was performed using RNAi, and was also presented to the IADR (Appendix B, Alaoui-El-Azher).

As discussed in Chapter 5, mutation of the *aae* gene conferred an adherence deficiency by viable counts, as previously determined by two other groups (Rose *et al.,* 2003; Fine *et al.,* 2005). In addition, transcriptional profiling revealed a significant impact on HIGK regulation of actin cytoskeleton among the most significantly impacted pathways. This transcriptional data was presented to the 85th General Session and Exhibition of the IADR (Appendix B, Comerford). Confocal fluorescence microscopy failed to conclusively and definitively reveal actin rearrangement at several timepoints and after repeated co-culture experiments. This situation has several possible explanations. The phenotypic prediction could have been incorrect, as the result of an interpretation error of the resultant effect on the pathway based on the transcriptional profiles obtained. Alternatively, the timepoints chosen to confirm actin rearrangement may have been inadequate to capture a fleeting event. The possibility also exists that this pathway is

simply a false positive, as the stringency for this particular experiment would predict 5% of the total genes are called significant by chance alone. To investigate these questions, other proteins in the pathway will be chosen to confirm the predicted phenotype arising from the transcriptional profile and ontology analysis.

Microscopic visualization of a single experiment suggested that an equal number of wild type and mutant bacteria adhered to and invaded host cells. Further confirmation experiments will be performed with additional controls to conclusively investigate these initial findings. If confirmed, the data reported here would suggest a novel function for Aae that may have been discovered through the use of transcriptional profiling. A possible explanation based on the homology of Aae to other bacterial components is a defect in intracellular viability by *A. actinomycetemcomitans* Aae mutants. *Neisseria gonorrhea* deficient in the close homolog of Aae, IgA1 protease, is less viable than the parental strain in trans-endothelial migration assays. The discrepancy found in the mutant bacteria VT1565 between the adherence defects demonstrated by indirect colony counts compared to direct visualization requires resolution. If nothing else, this situation emphasizes the critical importance of phenotypic confirmations of transcriptional profile data.

Advantages and Limitations of the Current Epithelial System

Advantages

The obvious advantage and primary reason for conducting global transcriptional profiling is the enormous amount of data that can be collected from a single experiment. Microarray experiments are time consuming to optimize, but once the experimental system is established, throughput of samples becomes highly efficient. Additionally, the use of a microarray for all known human genes avoids biasing the lists of significant genes to reflect known paradigms of host-pathogen interactions, such as concentrating on immune effectors and signaling molecules

as reviewed in Chapter 2. The global screening afforded by this technology facilitates the discovery of new host responses, as is potentially the case for Aae. Once biological pathways have been implicated through transcriptome and ontology analysis, more traditional assays can be used to validate the findings phenotypically. This strategy quickly focuses investigations to a manageable experimental question that is highly likely to provide meaningful data. A single array experiment can provide a workable dataset for confirmation that will drive laboratory research for many years after the initial experiment. As the case with the current model, only the most relevant impacted pathways are typically confirmed immediately following a successful array experiment. Although lower priority at first glance, the numerous pathways that are first passed over for phenotypic confirmation still yield very valuable information. Datasets can be revisited as ontology tools improve, and new discoveries can come from old data in this manner.

Limitations

The most glaring limitation of the current strategy is the exorbitant costs associated with transcriptional profiling of human host cells. Roughly $800 to $900 dollars must be spent per sample, which limits the possible experiments that can be reasonably run. The total cost of the project described herein, not including collaborations, was estimated at $45,000. Since the current strategy relies on biological replicates of four per condition, financial costs limit the number to between two and four experimental conditions per microarray experiment.

Another limitation lies in the infection model used for host profiling. Quite clearly, cells grown in tissue culture are different from cells in the mouth of a human host. The intercellular interactions that occur in differentiated tissue are different from the interactions between cell monolayers and plastic. Additionally, the grown media provides different nutrients than what are found in the oral cavity. As IVIAT work previously demonstrated (Song *et al.,* 2002; Handfield *et al.,* 2000), bacteria grown in liquid culture also express different genes compared to

those found in an actual infection. The MOI used to elicit a representative response may also be different than normal *in vivo* infection conditions in order for the transcriptional profiling to reveal a consistent pattern of gene expression. Thus, any findings made using the current *in vitro* model have to be accepted for their limitations as a model of periodontal disease and not periodontal disease proper. Confirming the hypotheses from *in vitro* models in a clinical setting is of course a necessary exercise to fully advance the understanding of periodontal disease pathogenesis.

Future Endeavors

Revisit the Database Periodically.

Including recent collaborations, 123 GeneChips for the Human Genome have been assayed for ten separate transcriptomic studies directed towards host-microbe interactions of the oral cavity. The approximate costs of these studies was $105,000. This wealth of information reveals the global status of oral epithelial cells in the presence of 4 bacterial species, three isogenic mutant strains of *A. actinomycetemcomitans* and two mutant strains in *P. gingivalis.* As gene ontology and pathway mapping tools improve, this information will be invaluable to unraveling the complex interplay between host and pathogen. The current study presents a first level of the interpretation and validation of the transcriptional profiles already obtained. Phenotypic confirmations for a handful of predicted phenotypes have been performed, but potentially many more experiments can be based on the data already on hand. It is becoming increasingly clear that the interrelatedness of the pathways implicated tell an important story. Transcriptome wide reverberations attributable to single bacterial components have provided a glimpse of how host cell pathways are all intertwined at some level. As pathways become better characterized, identifying the connections will become more manageable.

Uncharacterized Bacterial IVIAT Genes

A major motivating factor to developing the current experimental system of host transcriptional profiling was the prospect to assign probable functions to bacterial genes with no known homology or function in current databases. In Vivo Induced Antigen Technology (Handfield *et al.,* 2000; Rollins *et al.,* 2005) has identified 116 *A. actinomycetemcomitans* and 144 *P. gingivalis* sequences specifically induced in human periodontal infections (Song *et al.,* 2002). Many of these sequences have no known homology or biological function. Proof of principle was established when the *A. actinomycetemcomitans* strain JMS04 isogenic mutant for ORF859 was studied using the current transcriptome model. A biological impact upon the host cell was uncovered, although the total numbers of impacted genes was lower than what was observed for known virulence determinants. Although expensive to characterize over 200 IVIAT genes in this manner, using the host transcriptome as a reporter system seems to be the most efficient method available to assign functions to these uncharacterized genes. Thus, mutants for IVIAT genes with unknown functions should be investigated using the current strategy. A phenotypic screen prior to the array experiment to identify prospective clones with an observable impact upon the host cells would help identify bacterial genes that are likely to yield a definitive result and formal assignment of function in pathogenesis.

Complex Flora

As reviewed prior to this project being started, one strategy to prevent periodontal disease may be to control key members of the oral cavity that disrupt the microbial ecological balance from a symbiotic and healthy host-pathogen relationship towards pathogenesis and disease (Kinane *et al.,* 1999). Studies attempting to understand the outcome of inter-bacterial encounters and their subsequent effects on host cells have already been undertaken in another collaboration study performed during the course of this study (Dr. Kate Von Lackum, Hanfield Lab).

Transcriptome analysis presented to the 85th General Session of the International Association for Dental Research (IADR) (Appendix B, Von Lackum) investigated the differences in epithelial cell responses to a mixed *S. gordonii*-*P. gingivalis* culture compared to HIGK cells interacting with each bacteria alone or uninfected. This four-class analysis revealed transcriptional differences between classes, and gene ontology work is underway to predict the biological outcome of these interactions. It will be interesting to see if *S. gordonii* is able to confer a protective effect to gingival epithelial cells during mixed co-culture experiments, when *P. gingivalis* infection alone modulates host cells in a manner that is presumed to directly cause a chronic infection. Pathways already demonstated to be affected by *P. gingivalis*, such as apoptosis as presented in Chapter 4, will be especially interesting under mixed culture conditions. Another antagonistic relationship that may exist *in vivo* was discovered from the transcriptome of HIGK cells interacting with *P. gingivalis* and *A. actinomycetemtomitans* and mentioned previously. The anti-apoptotic phenotype promoted by *P. gingivalis* and pro-apoptotic phenotype caused by *A. actinomycetemcomitans* infection would presumably counteract in epithelial cells encountering both bacteria simultaneously. The outcome of a mixed species co-culture to investigate the resultant apoptotic state of the cell is one example of additional host-microbe interactions worth future study.

Time course of Infection and Parallel Host and Pathogen Array Analysis

A tantalizing prospect introduced in Chapter 2 is to study the host transcriptome and pathogen transcriptome simultaneously. A timecourse experiment monitoring the dynamics of gene expression by both participants in the host-pathogen interplay would truly reveal point-counterpoint modulations that occur as both entities try to cope with the other. The progression of the responses conceivably would reveal cause-and effect relationships between the actions of host and pathogen.

As presented during the 2007 UFCD Research Day (Appendix C) a timecourse analysis of the host transcriptome is challenging and more complex than using static "snapshots" of an interaction. The wavelike modulation of genes is difficult to resolve with basic statistics, and complicated models must be applied to interpret the data. Based on this initial experiment, biological replicates for each timepoint under investigation would help resolve expression patterns, while unfortunately increasing the financial cost of an experiment. However, the statistical tools are available to undertake such an experiment and a parallel profiling of host and pathogen transcriptomes over time potentially could yield highly informative data that can not be obtained any other way.

Improvements to the Transcriptome Reporter System Model

Adapting the current *in vitro* model to more accurately reflect the oral cavity is a goal that co-culture assays using mixed bacterial infections could bring closer to reality. Another option is to use a more complicated host tissue model to probe the host response more deeply. Although the specific responses of epithelial cells in a monolayer have been studied successfully, how a more complex model that more closely mirrors multi layered host tissue would react is unknown. Previously, such an endeavor was limited by the inability to separate homogenous populations of cells from mixed models. As mentioned in Chapter 2, laser microdissection (Schutze *et al.,* 2007) and improvements in the amplification of minute quantities of mRNA (Viale *et al.,* 2007) make complicated host model systems feasible.

Of course, an alternative to complex *in vitro* models, whether cell lines are derived from primary cells, immortalized gingival tissue, or differentiated tissue models, is to study the transcriptome of diseased tissue sites from actual patients with LAP or CP. An initial concern with this strategy is the patient-to-patient genetic variability that could overshadow differences between healthy and diseased tissue. Additionally, the complexity of periodontal disease

discussed in Chapter 1 introduces numerous variables into a transcriptomic analysis that would add noise to the analysis. The number of matched samples that would need to come from healthy and diseased sites from the same patient is likely to incur a significant cost of materials. This type of experiment would cost more than the *in vitro* studies described herein, and would present several technical challenges, such as obtaining a homogenous cell type from the tissue samples in order to obtain representative transcriptional profiles. A pilot study to investigate transcriptional profile signatures that differentiate CP and LAP patients was recently performed using 35 arrays covering 14 total patients (Papapanou *et al.,* 2004). No significant differences were found between transcriptional profiles from CP and LAP patients. However, a new grouping of patients was uncovered by microarray analysis of patient-isolated tissue. Although all patients had similar clinical presentation, the profiles of these patients separated into two groups along the lines of antibody titers to several periodontal pathogens. This study indicates a larger sample size of patients is necessary to uncover additional gene expression differences in LAP or CP patients. Two other recent studies that involved clinical patient samples under microarray analysis utilized 312 (Buness *et al.,* 2007) and 1422 individual samples (Brodsky *et al.,* 2006)!

The cost of materials alone would currently preclude a 100 sample microarray experiment with our current level of understanding the host response to specific bacteria and specific bacterial components. A study of this nature would also address different questions that are generally more descriptive and predictive of periodontal disease outcome, in contrast to the current study which investigated specific host-pathogen interactions. Until the current model is no longer yielding results, and the targeted investigations of specific virulence factors is no longer worth the cost, the *in vitro* system developed during this project will be highly beneficial.

However, as the questions being asked increase in complexity, and the utility of epithelial cell monlayers wanes, the necessity to conduct transcriptional profiling experiments from actual patients will become pressing. At some point, these experiments will simply have to be conducted, despite the financial cost. Perhaps the cost of materials will decrease as array technologies improve to aid in this transition.

Summary

Altogether, the current project has successfully developed a model that used the transcriptome of oral epithelial cells as a reporter system to dissect host-pathogen interactions. In addition to establishing a platform technology that will provide useful data for years to come, the development process itself has yielded insights into the interplay between oral epithelial cells and several oral microbes. A host response that is specific to bacterial species was uncovered, and the degree of specificity also allowed the impact of individual bacterial products to be assessed. Improvements to the established model will allow more complex questions to be addressed, shedding light on the amazing array of interactions that occur in the oral cavity and result in a healthy or diseased outcome. Indeed the paradox is true, "the more we learn, the less we know." However the insights gained herein set the stage for exciting discoveries in the future of periodontal disease research.

CHAPTER 7
GENERAL CONCLUSIONS

The work described herein has demonstrated the successful development and application of a powerful strategy to probe the interactions between gingival epithelial host cells of the oral cavity and oral microbes. The specificity of the host response to bacterial challenge has proven useful, and subsequently the cells themselves have served as a reporter system to reveal previously unknown genome-wide reverberations caused by whole bacteria and specific bacterial components. This reporter system has been completely conceived and developed within the scope of the current research project, and the collaborations making this work possible represent the only instances of host transcriptional profiling currently used in oral biology to study disease pathogenesis.

In parallel with the development of the current model to study host-pathogen interactions, insights into the pathogenesis of species such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* have been gained. The host regulation of many biological processes, such as Apoptosis and Regulation of Cell Cycle have been distinct upon exposure to these pathogens, and many more candidate processes await phenotypic confirmation. A specific response has also been demonstrated by epithelial cells encountering an isogenic mutant for specific bacterial components when compared to the baseline response to a parental strain. The specificity of the host response has been demonstrated as dependant upon the origins of the epithelial cell reacting to bacterial challenge, as the transcriptome from cervical and oral epithelial cells were distinct.

Many lessons have been learned and the void in understanding the complex interplay between oral bacteria and host cells has begun to fill, if only slightly. This project has established a solid foundation for future investigations of the factors contributing to LAP and CP

progression, from both the standpoint of a reliable model for host-pathogen interactions, as well as a wealth of transcriptional data that will help unravel the complications of periodontal disease.

APPENDIX A
GINGIVAL EPITHELIAL CELL TRANSCRIPTIONAL RESPONSES TO COMMENSAL AND OPPORTUNISTIC ORAL MICROBIAL SPECIES[9]

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Transcriptional profiling and ontology tools were utilized to define the biological pathways of gingival epithelial cells modulated by coculture with the oral commensal *Streptococcus gordonii* and the opportunistic commensal *Fusobacterium nucleatum*. Overall, *F. nucleatum* and *S. gordonii* perturbed the gingival epithelial cell transcriptome much less significantly than the oral pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* perturbed the transcriptome, indicating that there was a greater degree of host adaptation by the commensal species (M. Handfield, J. J. Mans, G. Zheng, M. C. Lopez, S. Mao, A. Progulske-Fox, G. Narasimhan, H. V. Baker, and R. J. Lamont, Cell. Microbiol. 7:811–823, 2005). The biological pathways significantly impacted by *F. nucleatum* and *S. gordonii* included the mitogen-activated protein kinase (MAPK) and Toll-like receptor signaling pathways. Differential regulation of GADD45 and DUSP4, key components of the MAPK pathway, was confirmed at the protein level by Western blotting. Modulation of the MAPK pathway is likely to affect host cell proliferation and differentiation. In addition, both the MAPK and Toll-like receptor pathways ultimately converge on cytokine gene expression. An enzyme-linked immunosorbent assay of secreted interleukin-6 (IL-6) and IL-8 demonstrated that *F. nucleatum* induced production of these cytokines, whereas *S. gordonii* inhibited secretion from the epithelial cells. Stimulation of secretion of proinflammatory cytokines from epithelial cells may reflect the invasive phenotype of *F. nucleatum* and contribute to the greater pathogenic potential of *F. nucleatum* than of *S. gordonii*.

Humans are hosts of a complex and abundant population of microorganisms that colonize the mucosal membranes and skin. This commensal microbiota is an integral component of a complex homeostasis mechanism that impedes the activity of pathogenic microorganisms (6, 24). At optimal composition, commensals can prevent both the attachment and into the multiplication of pathogens and their invasion of epithelial cells and into the circulation. In addition, commensals supply essential nutrients, regulate epithelial development, and contribute to the maturation and maintenance of the immune system (5, 11, 25, 43). Thus, commensal inhabitants have become host adapted during a long evolutionary relationship. Under certain conditions, however, a subset of species can escape host restraint mechanisms and initiate disease, and these species have been called opportunistic commensals. There is increasing evidence that the innate immune system may discriminate among commensals, opportunistic commensals, and overt pathogens, and it has been suggested that this discrimination controls the balance between microbial intrusion and host integrity (21, 40, 50, 51, 55).

More than 700 species, or phylotypes, of bacteria can inhabit the oral cavity (1). Temporally distinct patterns of microbial colonization result in biofilm formation on all surfaces in the oral cavity. On the tooth surfaces, the initial colonizers of the dental plaque biofilm are principally oral streptococci and actinomyces. Establishment of these organisms facilitates the subsequent colonization of additional actinomyces and related gram-positive rods along with gram-negative bacteria, such as *Fusobacterium nucleatum*. Further maturation is characterized by colonization by gram-negative anaerobes, such as *Porphyromonas gingivalis* (34, 52). Once colonization of the subgingival area has occurred, organisms shed from the plaque biofilm can interact with host epithelial cells that both have a barrier function and act as sensors of microbial infection (28). While many common oral organisms can adhere to gingival epithelial cells, only a subset of these organisms, including *F. nucleatum*, *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, and *P. gingivalis*, can invade host cells (22, 23, 39, 59). Although it is well established that the bacterial inhabitants of the subgingival crevice are direct precursors of periodontal disease, the oral microbiota includes a spectrum ranging from commensals, such as *Streptococcus gordonii*, to aggressive pathogens, such as *P. gingivalis*. Some species, such as *F. nucleatum*, are located near the center of this spectrum as opportunistic commensals and are frequently found in individuals with good oral health but are also potentially able to contribute to disease (4, 15, 17, 44, 58, 62).

Transcriptional profiling using microarrays provides a way to monitor host cell responses to colonizing microorganisms on a global scale (8, 41). Numerous innate immune factors, for example, have been consistently found to be differentially regulated in host cells infected with pathogenic organisms compared to the regulation in uninfected controls (23, 29, 42). In

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† Supplemental material for this article may be found at http://iai.asm.org/.

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the oral cavity, the pathogens *P. gingivalis* and *A. actinomycetemcomitans* induce widespread changes in the gingival epithelial cell transcriptome that are largely organism specific (23). There have been only a limited number of studies of the transcriptional responses to commensal organisms, and in these studies the focus is almost exclusively on nonoral mucosal ecosystems. For example, a study of the gastrointestinal tract commensal *Bacteroides thetaiotaomicron* showed it can modulate expression of genes involved in several important intestinal functions, including nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation (25). Commensal bacterial reconstitution of germfree mice has been shown to up-regulate expression of colonic epithelial cell genes associated with growth, apoptosis, and immune responses (19). In contrast, genes that may participate in extracellular oxidant defense and cellular metabolism were down-regulated by a nonpathogenic bacterial challenge (19). Hence, there is evidence that the host cell transcriptional response can be specific for the infecting organism and that commensals and pathogens can regulate distinct physiological functions in host cells.

In this study we utilized expression microarrays to investigate the transcriptional responses of oral epithelial cells to challenge with the commensal *S. gordonii* and the opportunistic commensal *F. nucleatum*. The transcriptional responses induced by these organisms were very similar to each other yet significantly different than the responses reported previously for oral pathogens (23). Among the biological processes altered most significantly in the host cells was the signal transduction pathway associated with mitogen-activated protein kinase (MAPK) and downstream effector molecules, including interleukins. Understanding how the host has adapted to commensals and how barrier cells respond to limit the impact of commensals should provide a mechanistic biological basis for health in the mixed bacterium-human ecosystem of the oral cavity.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *F. nucleatum* ATCC 25586 was cultured anaerobically at 37°C in Trypticase soy broth supplemented with yeast extract (1 mg ml^{-1}), hemin (5 μg ml^{-1}), and menadione (1 μg ml^{-1}). *S. gordonii* DL-1 was cultured anaerobically at 37°C in Todd-Hewitt broth with 0.5% yeast extract and glucose (0.5%).

Epithelial cells. Human immortalized gingival keratinocytes (HIGK) were originally generated by transfection of primary gingival epithelial cells with E6/E7 from human papillomavirus (45). HIGK were cultured in the presence of 5% CO_2 in keratinocyte serum-free medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 0.05 mM calcium chloride, 200 mM L-glutamine, and an antibiotic-antimycotic (Invitrogen).

Microbe-host cell coculture. Bacteria in the mid-log phase were harvested and washed by centrifugation and then resuspended in antibiotic-free keratinocyte serum-free medium. HIGK (10^7 cells) were cocultured with bacteria to obtain a total association (adherent plus invading bacteria) of approximately 100 bacteria per epithelial cell. The numbers of adherent and invading organisms were confirmed in parallel experiments by plate counting (23). After 2 h of incubation at 37°C in the presence of 5% CO_2, the HIGK were lysed with Trizol (Invitrogen) prior to RNA extraction. Cocultures were carried out in quadruplicate.

RNA isolation, cDNA-cRNA synthesis, and chip hybridization. RNA isolation, cDNA synthesis, labeled cRNA synthesis, and chip hybridization were performed as previously described (23). Briefly, total RNA was extracted from Trizol-lysed cells, treated with DNase I, purified, and quantified by using standard methods (QIAGEN, Valencia, CA, and Affymetrix, Santa Clara, CA). cDNA was synthesized by using the Affymetrix protocol (SuperScript double-stranded cDNA synthesis kit; Invitrogen); 5 to 8 μg of total cellular RNA was used as a template to amplify mRNA species for detection. Double-stranded cDNA was purified and used as a template for labeled cRNA synthesis. In vitro transcription was performed using a BioArray high-yield RNA transcript labeling kit (T7; Enzo Life Science, Farmingdale, NY) to incorporate biotinylated nucleotides. cRNA was subsequently fragmented and hybridized on Genechip human genome U133-A oligonucleotide arrays (Affymetrix) with proper controls. RNA samples were not pooled. The microarrays were hybridized for 16 h at 45°C, stained with phycoerythrin-conjugated streptavidin, and washed using the Affymetrix protocol (EukGE-WS2v4) with an Affymetrix fluidics station, and then they were scanned with an Affymetrix GeneChip 3000 scanner.

Microarray data analysis. Microarray data analysis was performed as previously described (23, 41). Briefly, expression filters were applied to remove Affymetrix controls and probe sets whose signals were not detected in all samples. The signal intensity values in the resulting data set were variance normalized, mean centered, and ranked by their coefficients of variation. Normalization was performed to give equal weight to all probe sets in the analysis, regardless of the order of magnitude of the raw signal intensity. To reduce the confounding effect of background signal variation on the analysis, only the half of the data set exhibiting the most variation across samples was used to perform an unsupervised hierarchical cluster analysis using the Cluster software (16). The resulting heat map and cluster dendrograms were visualized with the Treeview software (16) to determine the extent of characteristic host cell responses to each infection state, defined as identical treatments clustering together. Additional quality control data for the arrays are provided in the supplemental material.

Following the initial assessment of the host cell response to each condition, a supervised analysis was performed to investigate differences in gene regulation among experimental conditions. For this analysis, the raw signal intensities were log transformed for all probe sets that passed the initial expression filters and were correlated using BRB Array Tools (R. Simon and A. Peng-Lam, National Cancer Institute, Rockville, MD). In each supervised analysis, biological replicates were grouped into classes based on their infection states during coculture experiments, and probe sets significant at the $P < 0.001$ level for the class were identified. To test the abilities of the significant probe sets to truly distinguish between the classes, leave-one-out cross-validation (LOOCV) studies were performed. In these LOOCV studies each array was left out in turn and a classifier was derived for the three groups by selecting probe sets significant at a P value of <0.001. The significant probe sets were then used with several prediction models (compound covariate predictor, nearest-neighbor predictor, and support vector machine predictor) to predict the class identity of the array that was left out and not included when the classification model was constructed. The ability of the classifier to correctly predict the class identity of the array that was left out was estimated using Monte Carlo simulations with 2,000 permutations of the data set.

Functional categorization by gene ontology and bioinformatics analyses. Gene ontology trees were populated using Pathway Express (33), available at http://vortex.cs.wayne.edu/projects.htm.

Immunoblotting. HIGK were infected with *F. nucleatum* or *S. gordonii* as described above, using a time course of 1, 2, or 6 h. Cells were washed three times with phosphate-buffered saline and lysed in radioimmunoprecipitation buffer with proteinase inhibitors (Sigma, St. Louis, MO). Twenty-five micrograms of protein was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and blocked with 5% skim milk in Tris-buffered saline–0.1% Tween 20. The membranes were incubated for 1 h with primary antibodies to GADD45α (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), GADD45β (1:200; Santa Cruz Biotechnology), DUSP4 (1:2,000; Abcam, Cambridge, MA), and β-actin (1:10,000; Abcam). After three washes in Tris-buffered saline–0.1% Tween 20, each membrane was reacted with the species-appropriate peroxidase-coupled secondary antibody (1:1,000). Visualization was performed with the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom). Band intensities were scanned and quantified using the Kodak 1D image analysis software (v.3.6.1).

Detection of cytokines. Supernatants of HIGK infected with *F. nucleatum* or *S. gordonii* were collected and filter sterilized. Interleukin-6 (IL-6) and IL-8 concentrations were determined by an enzyme-linked immunosorbent assay (Quantikine, Minneapolis MN) performed according to the manufacturer's protocol. Experiments were conducted in triplicate.

Microarray data accession numbers. The array results have been deposited in the GEO repository (http://www.ncbi.nlm.nih.gov/geo/index.cgi) under accession numbers GSM159477 to GSM159488, series GSE6927.

RESULTS

Association of *F. nucleatum* and *S. gordonii* with HIGK. *F. nucleatum* and *S. gordonii* exhibited different capacities to invade HIGK. At a concentration of approximately 100 organisms per epithelial cell, 55% of *F. nucleatum* cells were recovered intracellularly, whereas *S. gordonii* remained essentially extracellular (99.5%).

Comparison of the transcriptional profiles of HIGK infected with *F. nucleatum* and HIGK infected with *S. gordonii*. All samples of uninfected HIGK, *F. nucleatum*-infected cells, and *S. gordonii*-infected cells were used to determine the overall similarity of the transcriptional responses. After elimination of probe sets whose signals were not greater than background levels on all arrays, signal intensity data for the 12,125 probe sets that passed the initial expression filters were included in an unsupervised cluster analysis and supervised class prediction analysis. The unsupervised hierarchical cluster analysis revealed an infection state-dependent host cell transcriptional profile, as biological replicates clustered together (data not shown). In a supervised analysis based on the infection state, at a significance level of $P < 0.001$, 240 probe sets were differentially expressed. Assuming normality of the data set, the 240 significant genes is 20-fold higher than the 12 probe sets that would be expected by chance at a significance threshold of $P < 0.001$, given that 12,125 probe sets passed the expression filter. Treeview visualization of the 240 probe sets differentially expressed in the three classes is shown in Fig. 1. The major separation node occurred between uninfected cells and infected organisms, independent of bacterial species, and the differences between the uninfected and infected states exceeded the observable differences between *F. nucleatum* and *S. gordonii*, although organism-specific gene signatures could be discerned. Overall, 84% of the probe sets were regulated similarly by *F. nucleatum* and *S. gordonii*, which may reflect the degree of adaptation to the host of both these species.

Ontology analysis. While it is becoming increasingly clear that the predictive power of regulation of individual genes is limited due to the extensive interconnectivity among regulatory networks, the assembly of regulated genes into biologically relevant pathways by ontology analysis has greater biological resolution. Therefore, in order to mine the array data for biologically relevant information, an ontology analysis of known metabolic pathways was performed using individual comparisons of the *F. nucleatum*-infected state or the *S. gordonii*-infected state with the corresponding baseline uninfected state. A pairwise comparison was performed by using the ontology algorithms described above. For this analysis, signal intensities were renormalized across all samples, and supervised analyses were repeated as described above. In *F. nucleatum*-infected HIGK, 11,909 genes passed the initial expression filters, while 11,835 genes were analyzed for *S. gordonii*-infected cells. At a significance level of $P < 0.001$, 145 genes were differentially expressed in the *F. nucleatum*-infected cells compared to the expression in uninfected controls, whereas 268 genes were differentially expressed in the *S. gordonii*-infected cells. At a less stringent significance level, $P < 0.05$, class prediction analysis revealed 1,917 *F. nucleatum*-regulated genes and 2,910 *S. gordonii*-regulated genes. The abilities of probe sets with significance at $P < 0.001$ or at $P < 0.05$ to correctly identify treatment groups were confirmed by LOOCV analysis. The classifiers performed flawlessly and correctly predicted the treatment group with 100% accuracy. To populate biological pathways to the maximal extent and thus enhance their predictive power, probe sets at a significance level of $P < 0.05$ were analyzed by the Pathway Express algorithm (12–14, 31–33). Table 1 shows the most impacted epi-

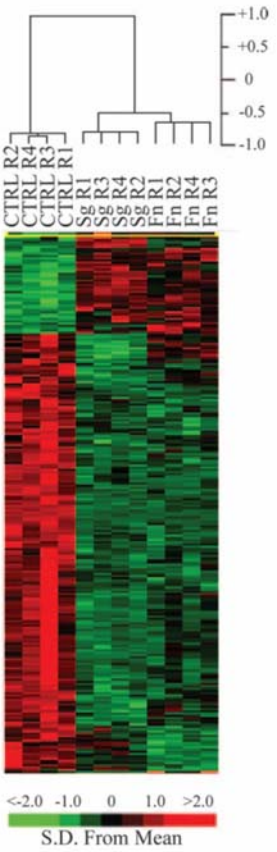

FIG. 1. Hierarchical clustering of variance-normalized gene expression data. The expression pattern of the cRNAs analyzed by using microarrays was determined by a supervised analysis of the variance-normalized data set of differentially expressed genes ($P < 0.001$, BRB ArrayTools) with the algorithm Cluster and was displayed with Treeview. Each row represents an individual gene element on the array, and each column represents the expression states of cRNAs for the challenge condition indicated. Each expression data point represents the relative fluorescence intensity of the cRNA from *F. nucleatum*-infected cells (columns Fn R1 to Fn R4) or *S. gordonii*-infected cells (columns Sg R1 to Sg R4) to the fluorescence intensity of the cRNA from uninfected cells (columns CTRL R1 to CTRL R4). The distance matrix used to determine the relatedness of samples through gene expression space was 1 − Pearson's correlation coefficient. The cluster is subdivided into three groups consisting of genes that were repressed (green), genes that were induced (red), and genes whose expression did not change (black). The variation in gene expression for a given gene is expressed as the distance from the mean observation for that gene according to the color scale below the heat map.

TABLE 1. Ontology analysis of epithelial cell pathways impacted by infection with *F. nucleatum* or *S. gordonii*[a]

Impacted pathway[b]	Impact factor[c]	No. of input genes/no. of pathway genes[d]
F. nucleatum-infected cells		
Phosphatidylinositol signaling pathway	178.648	11/79
MAPK signaling pathway	173.395	43/273
Toll-like receptor signaling pathway	105.509	13/91
Regulation of actin cytoskeleton	91.253	31/206
Cell cycle	75.558	20/112
Wnt signaling pathway	69.173	26/147
Cytokine-cytokine receptor interaction	61.258	24/256
Focal adhesion	55.455	25/194
S. gordonii-infected cells		
MAPK signaling pathway	248.404	57/273
Toll-like receptor signaling pathway	179.365	20/91
Phosphatidylinositol signaling pathway	172.608	9/79
Cell cycle	95.198	30/112
Regulation of actin cytoskeleton	93.062	39/206
Wnt signaling pathway	74.227	31/147
Cytokine-cytokine receptor interaction	72.52	29/256
Focal adhesion	72.117	37/194
Tight junction	64.433	21/119
Jak-STAT signaling pathway	56.993	25/153
Apoptosis	52.94	20/84

[a] The epithelial cell pathways were determined by Pathway Express (33).
[b] Kyoto Encyclopedia of Genes and Genomes pathways (http://www.genome.jp/kegg/).
[c] The impact factor measures the pathways most affected by changes in gene expression by considering the proportion of differentially regulated genes, the perturbation factors of all pathway genes, and the propagation of these perturbations throughout the pathway. Only pathways with an impact factor greater than 50 are included.
[d] Number of regulated genes in a pathway/total number of genes currently mapped to this pathway.

thelial pathways generated in this analysis in order of their impact factors. Pathways impacted by *F. nucleatum* or *S. gordonii* showed considerable overlap, and the MAPK pathway and the Toll-like receptor signaling pathway were two of the top three most affected pathways for both organisms. Thus, recognition of and response to these oral commensals by gingival epithelial cells involve the "classical" system of microbe-associated molecular pattern-pattern recognition receptor binding and signaling propagation through MAPK pathways. Coupled with the absence of a danger signal (46), this interaction may maintain a physiologic balance between the host and the organism.

Correlation between mRNA and protein levels in the MAPK pathway. As the MAPK pathway was significantly ($P < 0.001$) impacted at the transcriptional level by both organisms, we investigated the correlation between mRNA and protein levels for three of the most statistically significantly regulated genes. Genes encoding GADD45α and GADD45β were transcriptionally up-regulated following *F. nucleatum* infection ($P = 0.0005$ and $P < 10^{-6}$, respectively), whereas expression of the gene encoding DUSP4 (one of several MAPK phosphatase family members) was down-regulated ($P < 10^{-6}$). *S. gordonii* up-regulated GADD45β gene expression ($P < 10^{-6}$), down-regulated DUSP4 gene expression ($P < 10^{-6}$), and had no detectable effect on GADD45α gene expression. The protein expression data obtained by Western blotting (Fig. 2) were consistent with the transcriptional data. Both *F. nucleatum* and *S. gordonii* decreased the levels of DUSP4; *F. nucleatum* up-regulated GADD45α and GADD45β expression; and *S. gordonii* infection resulted in an increase in the GADD45β level. In general, the amount of regulated protein continued to change over a 6-h period, corroborating the predictive power of the transcriptional "snapshot" for the longer-term phenotype of the epithelial cells, at least for these genes. The roles of these genes in signal transduction through the MAPK pathway and their impact on cell physiology are shown diagrammatically in Fig. 3.

Correlation between mRNA and secreted cytokines. Both the MAPK and Toll-like pathways can converge on expression of cytokines. While recognizing that control of cytokine secretion is hierarchical and can occur at the transcriptional and posttranscriptional levels, we assessed the phenotypic significance of the ontology analysis by directly assaying at the protein level the proinflammatory cytokines IL-6 and IL-8. Secre-

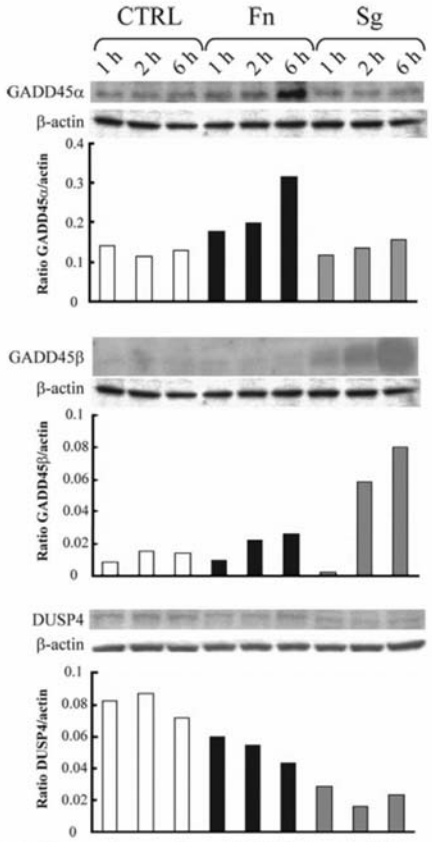

FIG. 2. Western immunoblots of HIGK infected with *F. nucleatum* (Fn) or *S. gordonii* (Sg) and uninfected controls (CTRL) for 1, 2, or 6 h. The blots were probed with antibodies to GADD45α (upper panel), GADD45β (middle panel), and DUSP4 (lower panel) and then stripped and reprobed with antibodies to β-actin. The graphs show the results of densitometric analyses of the ratio of test protein band intensity to β-actin band intensity.

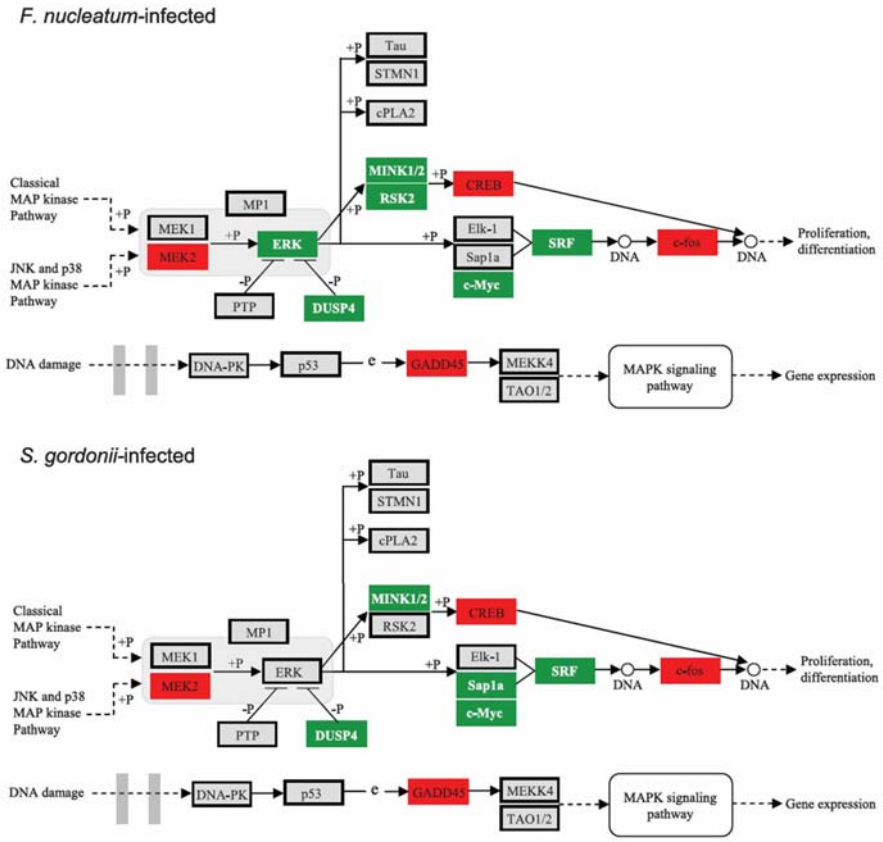

FIG. 3. MAPK-related pathways containing *F. nucleatum* and *S. gordonii* differentially regulated genes at $P < 0.05$, adapted from Pathway Express and using the Kyoto Encyclopedia of Genes and Genomes nomenclature (see text for details). Red indicates up-regulation, green indicates down-regulation, and gray indicates no change in expression. +P indicates phosphorylation, and −P indicates dephosphorylation. An arrow indicates a molecular interaction resulting in activation, and a line without an arrowhead indicates a molecular interaction resulting in inhibition. e, expression.

tion of IL-6 and IL-8 following infection with *F. nucleatum* or *S. gordonii* was monitored over time (Fig. 4). After 4 h of coculture, *F. nucleatum* stimulated secretion of IL-8, which is consistent with previous reports (9, 26, 36), and of IL-6. In contrast, *S. gordonii* reduced the levels of secreted IL-6 and IL-8 compared to the levels in uninfected control cells over an 8-h incubation. Inhibition of proinflammatory cytokine secretion by *S. gordonii* may eliminate initiation of a potentially destructive immune response and contribute to the maintenance of oral health.

DISCUSSION

The epithelial cells that line the gingival crevice form the initial interactive interface between the host and subgingival bacteria. The bacterial inhabitants of this area are diverse and have a spectrum of pathogenic potentials, ranging from commensal (such as *S. gordonii*) through opportunistic commensal (such as *F. nucleatum*) to overtly pathogenic (such as *P. gingivalis* and *A. actinomycetemcomitans*). The outcome of the bac-

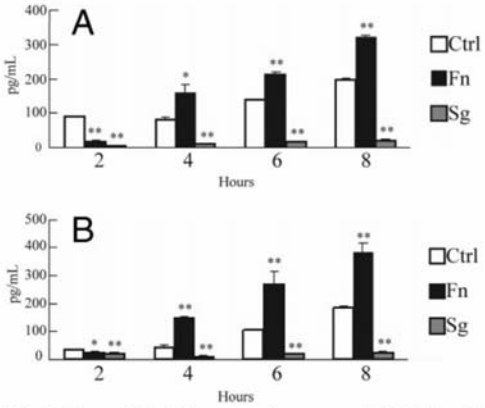

FIG. 4. Enzyme-linked immunosorbent assay of IL-6 (A) and IL-8 (B) accumulation in HIGK supernatants following coculture with *F. nucleatum* or *S. gordonii* for 2, 4, 6, or 8 h. Ctrl, uninfected control. The error bars indicate standard deviations ($n = 3$). One asterisk, $P < 0.05$; two asterisks, $P < 0.001$ (as determined by a *t* test).

terium-epithelial cell interaction, in which the host cells distinguish the infecting bacteria and tailor a response while the bacteria attempt to manipulate host cell responses, is an important component in establishing pathogenic potential. Transcriptional profiling provides insights into these extremely complex systems. In a previous study Handfield et al. (23) reported that HIGK responses to the pathogens *P. gingivalis* and *A. actinomycetemcomitans* reverberated throughout the transcriptome and impacted biological pathways, including cell development and morphogenesis pathways. In contrast, the HIGK response to the less pathogenic organisms *F. nucleatum* and *S. gordonii* was much more restrained. The similar transcriptional responses to *F. nucleatum* and *S. gordonii* provide experimental support for the concept that commensals and opportunistic commensals have developed a more balanced evolutionary relationship with the host than pathogens have developed (17, 24). Future studies will be directed toward trying to identify host cell genes and pathways that can be used to predict a commensal or pathogenic challenge. Such genes and pathways could be used as markers to assess the pathogenic potentials of the hundreds of species or complex mixtures of subgingival bacteria for which no virulence information or culture methods are available (38). An additional distinction between the gingival cell responses to more pathogenic and less pathogenic organisms was that the core common transcriptional responses to *P. gingivalis* and *A. actinomycetemcomitans* were very limited (around 15% of the regulated genes), and organism-specific responses predominated (23). However, the core transcriptional responses to *F. nucleatum* and *S. gordonii* were more extensive, with 84% of the genes similarly regulated by these two organisms, including the genes phenotypically validated and described here. Considering that *F. nucleatum* possesses an outer membrane while *S. gordonii* does not and that *F. nucleatum* is orders of magnitude more invasive than *S. gordonii*, it appears that transcriptional responses are not strictly dependent on the physical nature of the bacterial microbe-associated molecular patterns or on the extra- or intracellular location of the bacteria.

One pathway that was significantly impacted by *F. nucleatum* ($P = 0.00825$) and *S. gordonii* ($P = 0.00437$) involves MAPK signal transduction. This signaling pathway, which is evolutionarily conserved, connects cell surface receptors to regulatory targets within cells. MAPK signaling is involved in a vast array of physiological processes, including cell growth, migration, proliferation, differentiation, survival, development, and innate immunity (37, 49, 64). Consistent with these diverse roles, a variety of stimuli can activate MAPK pathways; these stimuli include growth factors, cytokines, ligands for G protein-coupled receptors, transforming agents, environmental stress, and viral and bacterial ligands. In the MAPK pathway, expression of GADD45α and GADD45β was up-regulated by *F. nucleatum* infection, whereas expression of DUSP4 was down-regulated. Similarly, *S. gordonii* up-regulated GADD45β expression and down-regulated DUSP4 expression. Regulation occurred at both the mRNA and protein levels. The GADD45 gene was originally identified as a gene that is rapidly induced by agents that cause DNA damage (18, 48). Transcriptional regulation of the GADD45 gene is mediated by both p53-dependent and p53-independent mechanisms (56), and GADD45 family members (α, β, and γ) are involved in the activation of the p38 and JNK pathways through MEKK4. Up-regulation of GADD45 expression may ultimately converge on growth arrest and on the activation of the nuclear transcription factor NF-κB (47, 56, 57, 63). The dual-specificity phosphatase DUSP4 is involved in the inactivation of the MAPKs by dephosphorylating both Thr and Tyr residues of ERK1 and ERK2 (30). One role of the DUSP family of phosphatases may be in the postinduction repression of MAPK activity (60). Hence, both up-regulation of GADD45 expression and down-regulation of DUSP4 expression by *F. nucleatum* or *S. gordonii* engender the concordant phenotype of enhanced information flow through the MAPK pathway. Together, these results provide a mechanistic framework for previous reports which showed that *F. nucleatum* and *S. gordonii* activated MAPK and NF-κB in gingival epithelial cells (7, 9, 26, 27, 35, 61).

One of the downstream targets of MAPK signaling is the production of cytokines. *F. nucleatum* induced IL-6 and IL-8 secretion, whereas *S. gordonii* repressed secretion of both of these proinflammatory cytokines. Differences in the cytokine secretion profiles may be related to differential regulation of individual components of the MAPK pathway, such as the ability of *F. nucleatum* to up-regulate both GADD45α expression and GADD45β expression. In addition, *F. nucleatum*, but not *S. gordonii*, down-regulated expression of RSK2, whereas *S. gordonii*, but not *F. nucleatum*, down-regulated expression of Sap1a. RSK2 is a serine/threonine kinase that may play a role in mediating the growth factor- and stress-induced activation of the transcription factor CREB. SAP1a is a nuclear protein that stimulates transcription via the c-*fos* serum response element and also via an Ets binding site independent of the serum response factor. However, although RSK2 and Sap1a are involved in distinct aspects of MAPK signaling, their precise physiological roles are not known. The induction of expression of proinflammatory cytokines in gingival tissues and the subsequent inflammatory tissue damage are considered contributory factors in the pathogenesis of periodontal disease (10, 20, 53). Hence, stimulation of IL-6 and IL-8 by *F. nucleatum* may be one property that increases the pathogenicity of this organism compared to the pathogenicity of *S. gordonii*. Indeed, a general hyporesponsiveness to commensals may be advantageous in order to limit tissue destruction that might occur if a strong proinflammatory response were induced. Furthermore, it has been proposed that commensal species can "program" host cells to limit subsequent responses to more pathogenic organisms (2, 54) Conversely, in gingival epithelial cells, prior infection with the pathogen *P. gingivalis* can paralyze the local chemokine response to *F. nucleatum* (9). The extent to which challenge with *S. gordonii* can modulate subsequent host cell responses to other organisms is currently being investigated. Of possibly greater in vivo relevance is the potential of a complex microbial community to impact the transcriptional profile and phenotypic responses of host cells in a manner distinct from the summed activities of its constituents. Such community-based responses could partially explain apparent discrepancies that are observed between certain oral clinical manifestations and the putative pathogenic potentials of causative microbial species. For example, it is generally recognized that clinical cases of localized aggressive periodontitis that are associated with proapoptotic and proinflammatory *A. actinomycetemcomitans* do not result in significant inflammation or

gingival destruction (3). It is tempting to speculate that the anti-inflammatory nature of certain commensal species has the potential to restrain the proinflammatory capability of pathogenic species.

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Editor: J. B. Bliska

APPENDIX B
INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH (IADR) ABSTRACTS RESULTING FROM TRANSCRIPTOMICS PROJECT[10]

83rd General Session and Exhibition of the IADR, Baltimore 2005

Seq#66, Thursday 10 March 2005, 10:45 AM - 12:45 PM Oral, Room 330
Microbiology/Immunology and Infection Control - Proteomics/Genomics
0328 12:15 Transcriptional Profiling of Epithelial Cells Interacting with Oral Pathogens.
J J. Mans*, H.V. Baker, G. Narasimhan, A. Progulske-Fox, R.J. Lamont, M. Handfield

Seq# 246, Friday 11 March 2005, 2;00 PM - 4:00 PM, Poster, Exhibit hall E-F
Microbiology/Immunology and Infection Control- Microbiology/Immunology of Periodontal Diseases
2244 Modulation of Apoptosis-Associated Proteins of Gingival Cells by *Actinobacillus actinomycetemcomitans*.
D Naselsker*, M. Nolin, J.J. Mans, M Handfield

84th General Session and Exhibition of the IADR, Brisbane 2006

Seq #113, Thursday 29 June 2006, 3:30 PM - 4:30 PM, Poster, Exhibit Hall 1
Microbiology/Immunology and Infection Control- Clinical Oral Microbiology
1374 Insight into Bacteria-Epithelium Interactions from Transcriptional Profiling.
Y. Hasegawa*, J.J. Mans, H.V. Baker, R.J. Lamont, M Handfield

Seq# 163, Friday 30 June 2006, 1:30 PM - 3:30 PM, Oral, Room M4
Microbiology/Immunology and Infection Control - Oral Microbiology and Immunology
1988 ATM Pathway Involvement in *Actinobacillus actinomycetemcomitans* Induced-Apoptosis of IHGK Cells
M. Alaoui-El-Azher*, N. Comerford, A. Progulske-Fox, R.J. Lamont, and M. Handfield

Seq# 163, Friday 30 June 2006, 1:30 PM - 3:30 PM, Oral, Room M4
Microbiology/Immunology and Infection Control - Oral Microbiology and Immunology
1990 Dissecting Specific *Actinobacillus actinomycetemcomitans*-Epithelial Cell Interactions with Transcriptional Profiling.
J.J. Mans*, W. Chen, C. Chen, H.V. Baker, R.J. Lamont, M. Handfield

85th General Session and Exhibition of the IADR, New Orleans 2007

Seq #268 Saturday, 24 March 2007, 10:45 AM-12:00 PM, Poster, Ernest N. Morial Convention Center Exhibit Hall I2-J
AADR/Pfizer Hatton Awards
2574 Characterization of Epithelial Responses to a Complex Microbiota Challenge
K Von Lackum*, J.J. Mans, J. Van Puymbrouck, R.J. Lamont, and M. Handfield

[10] *Presenting author

Seq #89 Thursday, 22 March 2007, 2:00 PM-3:15 PM, Poster, Ernest N. Morial Convention Center Exhibit Hall I2-J
Microbiology / Immunology and Infection Control-*Aggregatibacter actinomycetemcomitans* II
0614 Effect of *Aggregatibacter actinomycetemcomitans* Adhesion on the Epithelial Transcriptome
N.P. Comerford*, J.J. Mans, P. Fives-Taylor, R.J. Lamont, and M. Handfield

APPENDIX C
TEMPORAL VARIATION OF THE TRANSCRIPTOME OF GINGIVAL CELLS INTERACTING WITH *Aggregatibacter actinomycetemcomitans*[11]

J.J. Mans*, H.V. Baker, R.J. Lamont and M. Handfield

Fifth annual UFCD Research Day. Friday, April 13, 2007.
1:30-3:30, Founders Gallery
PhD/Post-doc Division, Abstract 30

*Presenting author (poster)

We have previously described how *Aggregatibacter actinomycetemcomitans (A.a.)* can modulate the transcriptome of epithelial cells upon interaction. Further, this method has been useful in determining impacted biological pathways, and in predicting downstream phenotypic changes resulting from the host-pathogen encounter. **Objective**: The purpose of this study was to expand the predictive power of transcriptional profiling by adding a temporal component to our previously established model of host-pathogen interaction. **Methods**: Human immortalized gingival keratinocytes (HIGK) were grown in vitro following standard procedures and co-cultured with A.a. VT1169, a nalidixic acid/rifampicin resistant spontaneous mutant derived from a serotype b clinical isolate, A.a. SUNY465 (smooth phenotype). Host cell total RNA was extracted for seven timepoints at 20-minute intervals for two hours. The RNA was purified, quantified, and reverse transcription was performed to generate complementary-RNA. This cRNA and relevant controls were labeled and used to probe the Affymetrix HG-U133A human microarrays according to the manufacturer's recommendations. Expression patterns were analyzed with bio-informatic, statistical and gene ontology tools. Antibiotic protection assays were performed in parallel to measure total levels of bacterial interaction. **Results**: At 20 minutes post infection, A.a. interacted with HIGK cells at a ratio of 19+/-3 A.a. per gingival

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epithelial cell. The interaction peaked at 40 minutes post infection, with 36+/-10 A.a. per HIGK cell at this timepoint, and gradually declined to 18+/-2 per HIGK cell at two hours. The unsupervised microarray analysis by Cluster revealed two distinct clusters of up- and down-regulated genes over the course of infection. Performance of supervised analysis with Edge revealed genes that were significantly modulated over time in a non-linear manner, demonstrating the dynamics and complexities of the HIGK transcriptional profile as it impacts biological response pathways. Pairwise ontology analysis of each timepoint relative to time 0 revealed biological functions most highly impacted in HIGK cells upon infection, and how they were modulated temporally. The 20 biological functions with the highest impact factors ranged from phosphatidylinisitol signaling (10.02) and cell cycle (4.5), to actin cytoskeleton rearrangement (2.5). Predicted phenotypes are currently under investigation. **Conclusion**: Combining transcriptomic, ontology analysis and temporal methods constitute a predictive tool to study host pathogen interactions. Phenotypic confirmations of the transcriptomic data generated here will provide further insights and definitive evidence of the contribution of novel host pathogen interactions in the oral cavity.

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BIOGRAPHICAL SKETCH

Jeffrey Jay Mans was born January 22, 1979 to Glenn and Judy Mans of Gainesville, FL. He is the grandson of Dr. Rusty and Beulah Mans of Gainesville, FL and Donald and Marilyn McMillan of Nashville, TN.

A lifelong Floridian, Jeff attended Gainesville High School (GHS) where he competed in junior varsity soccer and varsity baseball for GHS, and American Legion baseball for Gainesville Post 16. Advanced Placement biology taught by Mrs. Nancy Smith was the most influential class in Jeff's high school education, and impacted Jeff's decision to pursue a biology degree after his graduation from GHS in 1997.

Jeff was awarded the Presidential Scholarship to attend the University of North Florida (UNF). While a student at UNF, Jeff was actively involved in the Honors Program and earned High Pass distinction for his Honors portfolio, as well as University Honors for his undergraduate research, "Prevalence of Lyme Disease Bacteria in Northeast Florida" conducted under the guidance of Dr. Kerry Clark. The Honors Program and mentoring of Dr. Clark also allowed Jeff to complete summer coursework abroad at the University of Cambridge in Cambridge, UK. He graduated from UNF Cum Laude with his Bachelor of Science degree for biology in 2001.

After graduation, Jeff worked as a Nurse Tech I in Labor, Delivery, Recovery and Postpartum at North Florida Regional Medical Center under guidance of many wonderful nurses and Mrs. Beverly Griseck. Jeff later worked in the laboratory of Dr. Bill Castleman at the University of Florida, College of Veterinary Medicine, Department of Pathobiology before his acceptance into the University of Florida Interdisciplinary Program (IDP). Jeff joined the lab of Dr. Martin Handfield in 2002 to complete his Doctoral research. Jeff is 4 years happily married to Lori K. Mans of Jacksonville, FL and UNF sweetheart.