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Distinct transcriptional profiles characterize oral epithelium-microbiota interactions

Martin Handfield,[1]* Jeffrey J. Mans,[1] Gaolin Zheng,[3] M. Cecilia Lopez,[2] Song Mao,[1] Ann Progulske-Fox,[1] Giri Narasimhan,[3] Henry V. Baker[2] and Richard J. Lamont[1]

[1]*Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, FL 32610-0424, USA.*
[2]*Department of Molecular Genetics and Microbiology and Department of Surgery, College of Medicine, University of Florida, Gainesville, FL 32610-0424, USA.*
[3]*BioRG, School of Computer Science, Florida International University, FL 33199, USA.*

Summary

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 *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. Consistent with their biological and clinical differences, the common core transcriptional response of epithelial cells to both organisms was very limited, and organism-specific responses predominated. A large number of differentially regulated genes linked to the P53 apoptotic network were found with both organisms, which was 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 did not appear to be Fas- or TNFα-mediated. Linkage of specific bacterial components to host pathways and networks provided additional insight into the pathogenic process. 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 help unravel the complex and dynamic interactions between host epithelial cells and endogenous bacteria that can cause opportunistic infections.

Received 6 December, 2004; revised 10 January, 2005; accepted 13 January, 2005. *For correspondence. E-mail mhandfield@dental.ufl.edu; Tel. (+352) 846 0763; Fax (+352) 392 2361.

Introduction

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. Because health is the most common state of a host, it has been speculated that the autochthonous flora has coevolved 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 are 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 epithelia cell genes involved in nutrient adsorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and maturation (Hooper *et al*., 2001). Because 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 (http://www.nidcr.nih.gov/AboutNIDCR/SurgeonGeneral/), these conditions afflict 14% of adults aged 45–54 years 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 aetiology 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 *Actinobacillus actinomycetemcomitans* and localized aggressive periodontitis (LAP) (Slots and Genco, 1984; Zambon, 1985; Offenbacher, 1996; Meyer and Fives-Taylor, 1997; Haffajee and Socransky, 1999), and between the Gram-negative anaerobe *Porphyromonas gingivalis* and severe, chronic manifestations of the disease (Slots and Genco, 1984; Slots *et al*., 1986; Haffajee and Socransky, 1999).

The initial interface between the host and 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 defence mechanisms (Kagnoff and Eckmann, 1997). Both *A. actinomycetemcomitans* and *P. gingivalis* are capable of invading gingival epithelial cells (GEC) and can remain viable intracellularly. Furthermore, epithelial cells maintain viability following intracellular penetration by either *P. gingivalis* or *A. actinomycetemcomitans* (Kato *et al*., 2000; Nakhjiri *et al*., 2001; Takayama *et al*., 2003). However, the entry mechanisms employed by these invasive organisms are distinct. *Actinobacillus 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*., 2005). 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 GEC and stimulate integrin-dependent signalling to effect invasion through both microfilament and microtubule remodelling (Yilmaz *et al*., 2002; 2003). *Porphyromonas 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 (Cummings and Relman, 2000; Kellam, 2000; 2001; Ichikawa *et al*., 2000; Manger and Relman, 2000; Kagnoff and Eckmann, 2001; Kato-Maeda *et al*., 2001; Yowe *et al*., 2001; Lory and Ichikawa, 2002; 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 coexist with each other. Consequently, the regulation of normal processes such as cell division or apoptosis may be key to maintaining a balanced long-standing 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* (Christersson, 1987a, b; 1993; Rudney *et al*. 2001). 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 human immortalized gingival keratinocytes (HIGK) 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 HIGK 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, a major pathway impacted by *P. gingivalis* and *A. actinomycetemcomitans*, was validated by phenotypic assays.

Association of A. actinomycetemcomitans *and* P. gingivalis *with epithelial cells*

Porphyromonas gingivalis and *A. actinomycetemcomitans* demonstrate differing efficiencies of binding to, and internalization within, human immortalized gingival keratinocytes. 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 multiplicity of infections (MOIs) predicted to result in the same number of epithelial cell-associated bacteria

Table 1. Microbial-epithelial cell interaction characteristics with 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
HIGK	*P. gingivalis*	100:1	18 ± 4	24

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.

for each species. As shown in Table 1, at an MOI of 100 for *P. gingivalis* and 3000 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 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 (Sandros *et al*., 2000; Sfakianakis *et al*., 2001).

Gene expression in GEC 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 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 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 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 Fig. 1. For instance, block 1 (182 probe sets) represents genes that are downregulated 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 upregulated 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, upregulated; and block 4, downregulated). Overall, transcriptional response appeared to be diametrically opposed between the two organisms with only a small number of genes (41 probe sets) up- or downregulated by both species (blocks 3 and 6). These

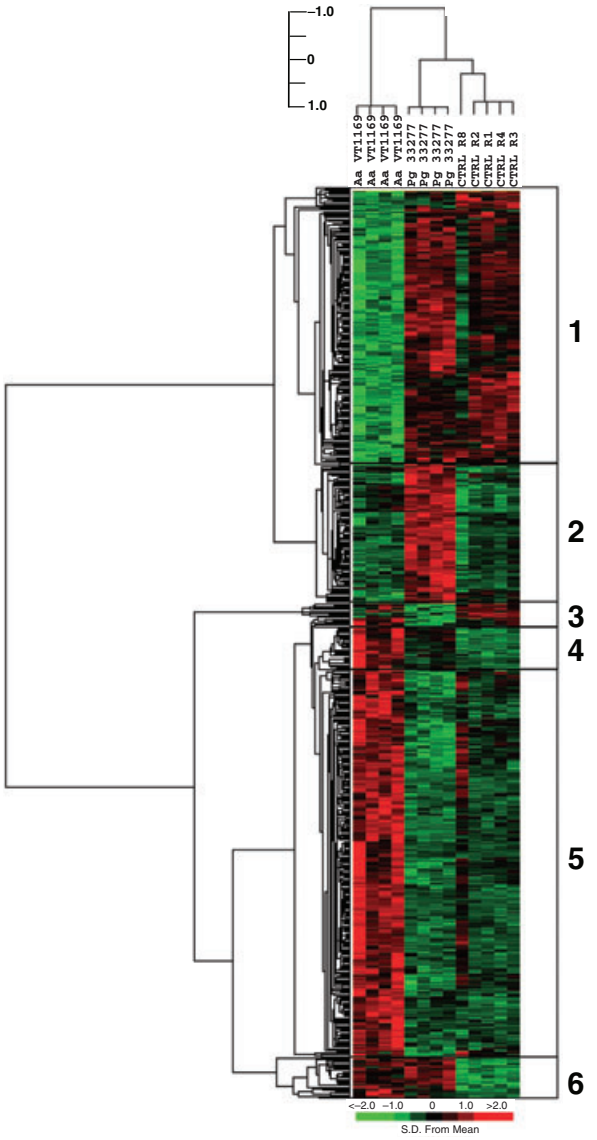

Fig. 1. Different patterns of gene expression of oral epithelial HIGK cells upon co-culture with *A. actinomycetemcomitans* or *P. gingivalis*. Hierarchical clustering of variance-normalized gene expression data from uninfected human HIGK cells and from cells in co-culture with either organism for 2 h before RNA isolation and purification. Expression and variation filters were applied to the data set before 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 VT 1169) or *P. gingivalis*-infected HIGK cells (columns Pg 33277) to the fluorescence intensity of the cRNA from mock-infected HIGK cells (columns control R1–R5). The scale adjacent to the dendrogram relates to Pearson's correlation coefficient. Highlighted blocks are described in the text.

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 $P < 0.005$ (Table 2) against the 354 different biological processes that have been identified thus far in the human GO syntax ontology database (http://obo.sourceforge.net/). Sixteen 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 Table 2.

Of particular interest to host-pathogen interactions in the oral cavity, one of the over-represented pathways was the apoptosis pathway. *Porphyromonas 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.*, 1989a,b; Ebersole *et al.*, 1990; Spitznagel *et al.*, 1995; Korostoff *et al.*, 1998; Kato *et al.*, 2000; Demuth *et al.*, 2003). 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 GEC is a CagE homologue, which encodes a component of a putative type IV secretion system (Teng and Hu, 2003). *Porphyromonas gingivalis*, by contrast, suppresses apoptosis in primary cultures of GEC. *Porphyromonas 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).

Table 2. Gene ontology[a] analysis of the transcriptome of HIGK cells infected with *P. gingivalis* or *A. actinomycetemcomitans.*

(a) Pathways common to *P. gingivalis*- and *A. actinomycetemcomitans*-infected HIGK cells.

GO ID	Term	*P. gingivalis*-infected (total change)[b]	*P*-value	*A. actinomycetemcomitans*-infected (total change)[b]	*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

(b) Pathways specific to *P. gingivalis*-infected HIGK cells.

GO ID	Term	Change	*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	0.001

(c) Pathways specific to *A. actinomycetemcomitans*-infected HIGK cells.

GO ID	Term	Change	*P*-value
8152	Metabolism	330	0.0005
30528	Transcription regulator activity	93	0
4888	Transmembrane receptor activity	26	0.004
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

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

The ontology analysis presented in Table 2a revealed that a total of 55 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, 31 were modulated only in *A. actinomycetemcomitans*, and 17 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 tumour 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 physicochemical 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 Fig. 2. *Actinobacillus actinomycetemcomitans* activated the pro-apoptotic molecules BBC3, GADD45A, E2F1 and ATM and repressed cMYC. *Porphyromonas 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 pro-apoptotic GADD45A, while SGK phosphorylates and negatively regulates the transcription factor FOXO3A that can participate in apoptosis, in part through the GADD45a protein (Brunet *et al*., 2001; Tran *et al*., 2002; Barsyte-Lovejoy *et al*., 2004). 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

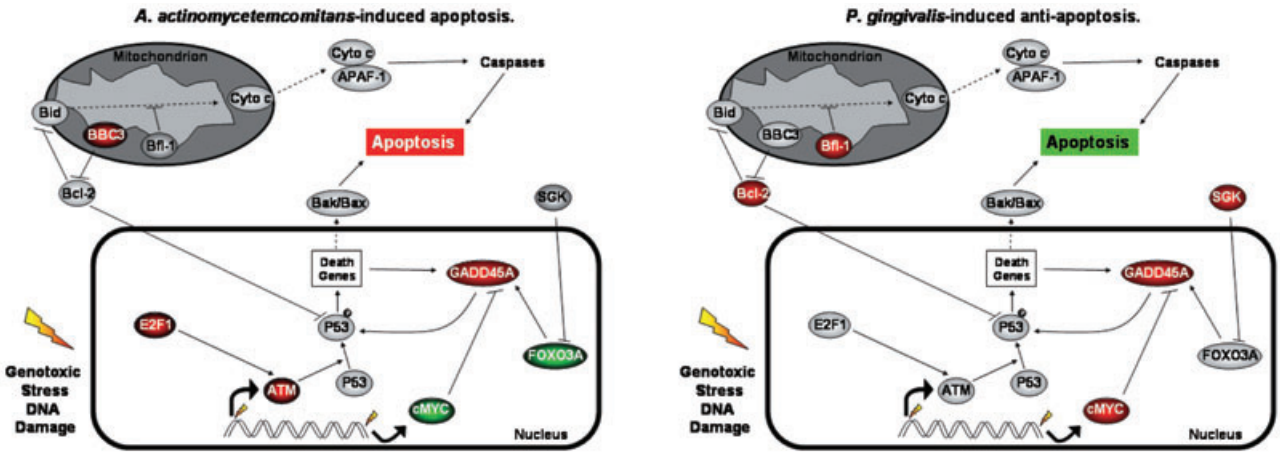

Fig. 2. Differential modulation of the p53-mediated apoptosis pathway by *A. actinomycetemcomitans* and *P. gingivalis*. Red terms are transcriptionally induced, while green terms are repressed. See text for description of individual molecules. ⟶ represents an induction mechanism with potential intermediates; - - - -▸ represents release; ⟊ represents an inhibitory mechanism.

Adams, 2002). Bfl-1 can also inhibit cytochrome *c* release and, in addition, suppresses Bid, which is an activator of the pro-apoptotic 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*, pro-apoptotic 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 tumour necrosis factor receptor superfamily member 6B precursor (TNFRSF6B or decoy receptor 3) was found to be upregulated. 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 upregulated. 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 downregulation (Skurk *et al*., 2004), was transcriptionally repressed by *A. actinomycetemcomitans*. This is consistent with the observed upregulation 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 downregulated. 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 shown to render some cells sensitive to apoptosis (Wu, 2003). 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 signalling 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 activ-

ity for normal physiologic function of epithelial cells remain to be established.

Apoptosis in GEC 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 Fig 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; Nakhjiri *et al*., 2001; Teng and Hu, 2003; 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. *Actinobacillus 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 *et al*., 2000; 2002) and in various cell lines, including HIGK cells (Richardson *et al*., 2005), 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 Fig. 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 3 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.

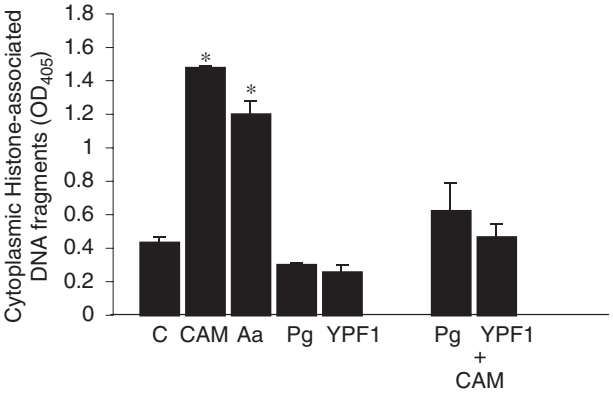

Fig. 3. Apoptotic responses of HIGK cells to *A. actinomycetemcomitans* or *P. gingivalis* by ELISA of cytoplasmic histone-associated DNA fragments. Control (C) represents HIGK under normal culture conditions. *Actinobacillus actinomycetemcomitans* (Aa) was incubated with HIGK cells at an MOI of 3000 for 4 h. *Porphyromonas gingivalis* parental (Pg) or mutant (YPF1) strains were incubated with HIGK at an MOI 100 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, $n = 3$. The asterisk denotes statistically different from control $P < 0.005$.

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 GEC 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 signalling pathways that induce remodelling 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 remodelling 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 Fig. 4B and Table 3, 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; de Hostos, 1999; Feng and Walsh, 2004). Additionally, YPF1 did not upregulate beta 3, 4 and 6 integrin, along with alpha V, 3 and 4 integrin, and CD47, an

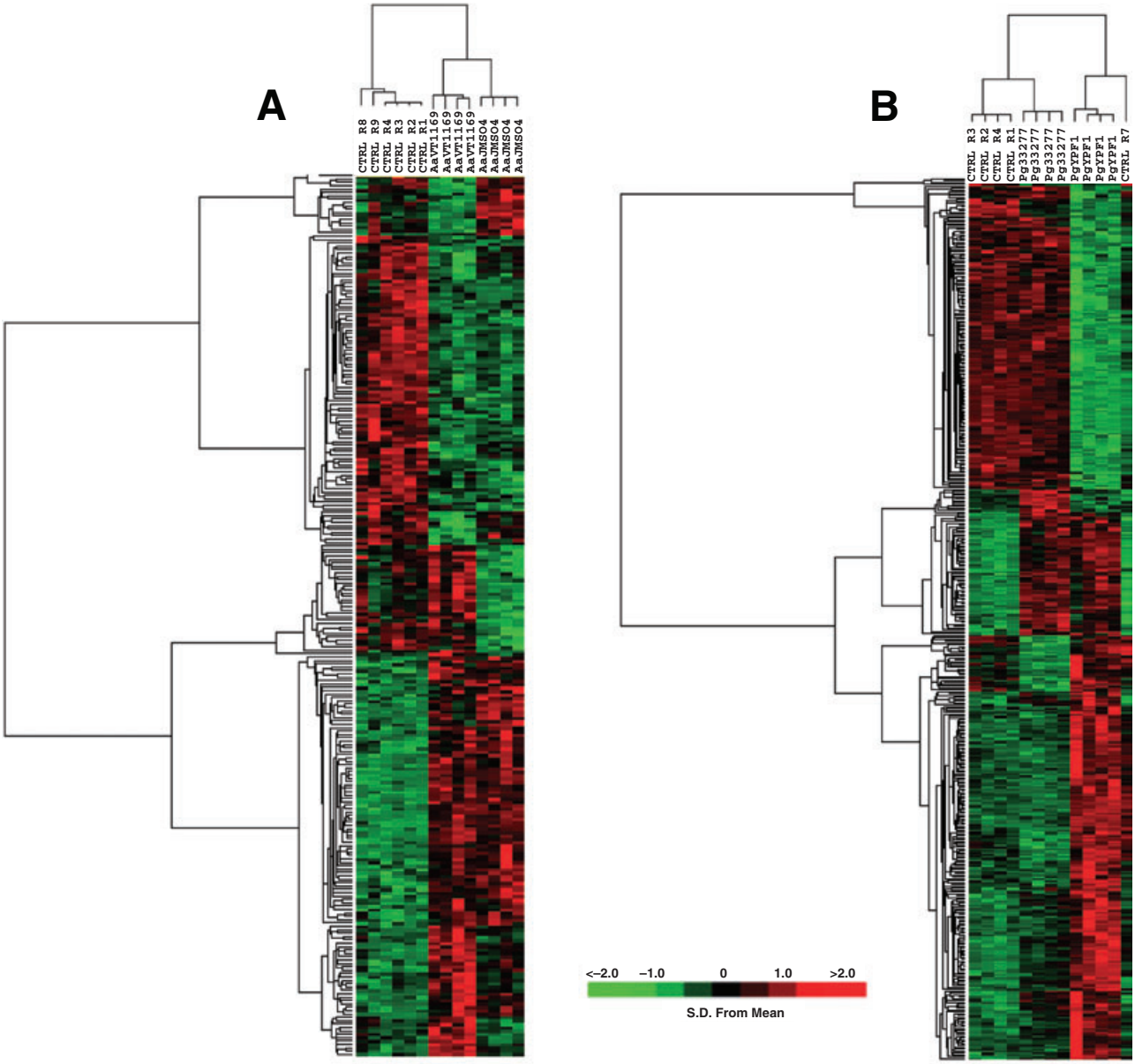

Fig. 4. Comparison of transcriptional profile of HIGK cells following co-culture with isogenic *A. actinomycetemcomitans* (A) or *P. gingivalis* (B) mutant strains. Hierarchical clustering of variance-normalized gene expression data. The expression pattern of the cRNAs analysed by microarray is represented as a supervised K-means analysis of the variance-normalized data set of differentially expressed genes with the algorithm Cluster and displayed with TreeView. Each row represents an individual cRNA element spotted on the array, and each column represents the expression states of cRNAs for the challenge condition indicated. Each expression data point represents the ratio of the fluorescence intensity of the cRNA from *A. actinomycetemcomitans*-infected [columns Aa VT1169 (parent) and Aa JMSO4 (mutant)]; or *P. gingivalis*-infected cells [columns Pg 33277 (parent) and Pg YPF1 (mutant)] to the fluorescence intensity of the cRNA from mock-treated uninfected cells (columns CTRL). 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). Leave-one-out cross validation studies were used to establish the ability of probe sets significant at the $P < 0.001$ level of significance to predict the class label of the specimen left out of the analysis.

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 be capable of inhibiting apoptosis in HIGK cells to the same extent as the parental strain. This was confirmed by the phenotypic apoptosis assay (Fig. 3) that showed YPF1 could antagonize chemically induced apoptosis to the same extent as the parental strain.

Table 3. Gene ontology[a] analysis for HIGK cells infected with *A. actinomycetemcomitans* or *P. gingivalis* mutants.

GO ID	Under	Over	Change	*P*-value	Term
(a) JMS04[b]					
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	0.009	Signal transduction
6983	2	0	2	0.0002	Response to ER-overload
(b) YPF1[b]					
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 signalling pathway
19207	12	1	13	0.0014	Kinase regulator activity

a. Only pathways with documented relevance to host-pathogen interactions are presented.
b. Comparison to infected with 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. *Actinobacillus actinomycetemcomitans*-associated disease involves an 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 *P. gingivalis* fimbriae 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 changes in response to isogenic mutants may provide a means to evaluate the biological function of as yet undefined bacteria products.

Experimental procedures

Bacterial strains

Actinobacillus actinomycetemcomitans strain VT1169 is a nalidixic-acid and rifampin-resistant clone derived from the clinical strain SUNY 465 (Mintz *et al*., 2002). JMS04 is an isogenic mutant for ORF859 constructed in VT1169, and obtained by insertional inactivation with a spectinomycin cassette (Cao *et al*., 2004). *Actinobacillus 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. *Porphyromonas 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}), haemin (5 μg ml^{-1}), and menadione (1 μg ml^{-1}).

Eukaryotic cell lines

Human immortalized gingival keratinocytes cells (human HPV-immortalized gingival keratinocyte, or HIGK) were originally generated by transfection of primary GEC with E6/E7 from HPV (Oda *et al*., 1996;). Human immortalized gingival keratinocytes cells

are capable of normal keratin synthesis and exhibit degree of differentiation similar to parent normal cells (Oda *et al*., 1996). Human immortalized gingival keratinocytes 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 GEC were generated as described previously (Oda and Watson, 1990; Lamont *et al*., 1995). 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, NJ), at 37°C in 5% CO_2. Gingival epithelial cells 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. Human immortalized gingival keratinocytes cells (10^5) were washed three times with phosphate-buffered saline (PBS) and incubated with bacteria at an MOI of 100 for *P. gingivalis* and 3000 for *A. actinomycetemcomitans*. After 2 h at 37°C in 5% CO_2, the cells were washed three times with PBS and lysed with Trizol (Invitrogen, Carlsbad, CA) before 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 10 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, Carlsbad, CA). cRNA was transcribed *in vitro*, incorporating biotinylated nucleotides by using a BioArray high-yield RNA transcript labeling kit (T7) (Enzo Life Sciences, Farmingdale, NY), 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 analysed in this study by the Affymetrix GCOS software (with default settings) were removed from the data sets 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 (Feezor *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 were 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; Feezor *et al*., 2003).

Supervised learning, discrimination analysis, and cross validation

The hybridization signal intensities of the genes passing the initial expression filter were analysed (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-neighbour prediction, three-nearest-neighbours prediction, linear discriminant analysis, and nearest-centroid analysis (Feezor *et al*., 2003).

Ontology analysis

The procedure delineated in Zheng and colleagues (2005) 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). Human immortalized gingival keratinocytes 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 μg ml^{-1}) for 4 h.

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