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Short communication

Distinctive characteristics of transcriptional profiles from two epithelial cell lines upon interaction with *Actinobacillus actinomycetemcomitans*

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Transcriptional profiling and gene ontology analyses were performed to investigate the unique responses of two different epithelial cell lines to an *Actinobacillus actinomycetemcomitans* challenge. A total of 2867 genes were differentially regulated among all experimental conditions. The analysis of these 2867 genes revealed that the predominant specific response to infection in HeLa cells was associated with the regulation of enzyme activity, RNA metabolism, nucleoside and nucleic acid transport and protein modification. The predominant specific response in immortalized human gingival keratinocytes (IHGK) was 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 were significantly – yet differently – affected in both cell lines. In HeLa cells, only three regulated genes impacted the response to stress, and the response to unfolded protein was the only term that passed the ontology filters. This strikingly contrasted with the profiles obtained for IHGK, in which 61 regulated genes impacted the response to stress and constituted an extensive network of cell responses to *A. actinomycetemcomitans* interaction (response to pathogens, oxidative stress, unfolded proteins, DNA damage, starvation and wounding). Hence, while extensive similarities were found in the transcriptional profiles of these two epithelial cell lines, significant differences were highlighted. These differences were predominantly found in pathways that are associated with host–pathogen interactions.

Key words: microarray; pathogenesis

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Bacteria that colonize mucosal surfaces engage host epithelial cells in multifaceted and intimate interactions (13). For example, bacterial inhabitants of the urogenital, gastrointestinal and respiratory tracts can manipulate epithelial cell signal transduction pathways, often to direct their internalization within these otherwise non-phagocytic host cells (7). Subsequently, epithelial cells infected with bacteria can exhibit major changes in the expressed proteome and transcriptome (13, 14). As model systems in which to study the responses of epithelial cell to bacterial challenge, the HeLa cell line and its derivatives have often been used. These cells, derived from a cervical carcinoma, have generated much information concerning the pathogenic properties of organisms such as *Shigella*, *Salmonella*, *Yersinia*, enteropathogenic *Escherichia coli*, *Helicobacter* and many others (2, 11, 17). Similarly, various cell lines including KB (American Type Culture Collection, CCL-17) and HEp-2 (American Type Culture Collection, CCL-23) are often used in the study of oral periodontal pathogens such

as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (6, 21, 22). 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. (15) demonstrated that *A. actinomycetemcomitans* cytolethal distending toxin inhibits epithelial cell proliferation but does not affect fibroblasts when these cells are grown together in culture. In addition, Fine et al. (10) have shown that the *A. actinomycetemcomitans* autotransporter adhesin Aae, is the adhesin responsible for the binding to buccal epithelial cells isolated from humans and Old World primates, but not for binding to buccal epithelial cells derived from New World primates and several other mammalian species. These examples demonstrate that interactions between *A. actinomycetemcomitans* and host cells appear to exhibit specificity and tropism. However, it is unclear whether this tropism extends beyond the initial attachment of *A. actinomycetemcomitans* to oral cells and has an impact on 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 (3, 12) and the birth of preterm low-birth-weight infants (23) – 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 that are involved with bacterial interaction beyond the effects of the well-characterized adhesins and toxins. The value of this approach is especially apparent when host cell phenotypic changes are outwardly subtle and not easily observed using other methods. In this study, we have compared the transcriptional responses of two cell lines commonly used to study host–pathogen interactions – immortalized human gingival keratinocytes (IHGK) and HeLa cells – following *A. actinomycetemcomitans* co-culture. The goal was to characterize 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. This comparison will assess the consistency of interactions occurring between *A. actinomycetemcomitans* and two different epithelial cell lines.

Materials and methods

Bacteria and cell lines

HeLa cells (KB cells; CCL-17, American Type Culture Collection, Manassas, VA) and IHGK (19, 20) were grown in Dulbecco's modified Eagle's medium and keratinocyte serum-free medium, respectively, as a monolayer to 95% confluence in an atmosphere of 5% CO_2 at 37°C (6, 19). Both cell culture media were supplemented with 50 U/ml penicillin and streptomycin (Gibco, Carlsbad, CA). The *A. actinomycetemcomitans* smooth strain VT1169 (SUNY 465 Nal^R Rif^R) (18) was grown in liquid culture at 37°C in 10% CO_2 to mid-logarithmic phase, and prepared for host cell co-culture according to standard methods (21). Briefly, epithelial cells were washed three times with 1x Dulbecco's phosphate-buffered saline (Cambrex, Walkersville, MD) to remove any residual antibiotics and waste products. In biological replicates of four per condition, epithelial cells were sham-infected with cell culture media or co-cultured for 2 h with *A. actinomycetemcomitans* resuspended in culture media, resulting in a multiplicity of infection of 1000. Previous studies in our laboratory (data unpublished) determined that 1000 was the lowest multiplicity of infection to ensure that 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 of co-culture was the time-point previously determined to display a phenotype that can be characterized in terms of host cell monolayer integrity, and was chosen to maintain consistency with previous work performed in our laboratory (13). Epithelial cells were lysed with Trizol (Invitrogen Life Technologies, Carlsbad, CA) and RNA was prepared for GeneChip hybridization as recently described (13).

Microarray analysis

Assessment of the host cellular responses to bacterial challenge was accomplished by transcriptional profiling using Affymetrix HG U133A DNA microarrays (9). Infected and uninfected HeLa cells and IHGK were tested in four independent replicates. Subsequent array analysis was performed as recently presented (13). In brief, expression filters were applied to remove Affymetrix controls and probe-sets 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 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, the half of the dataset demonstrating the most variation across samples was used to perform unsupervised hierarchical cluster analysis using CLUSTER software (8). The resulting heat-map and CLUSTER dendrograms were visualized with TREEVIEW (8) 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 probe-sets that passed the initial expression filters and were correlated using BRB Array Tools (Simon and Peng-Lam, National Cancer Institute, Rockville, MD). 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) to generate lists of class predictors whose expression state changed 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 class predictor and compared to the probability of a correct class prediction by chance alone, based on the *P*-value and total number of genes analyzed. To visualize the differentially regulated genes, MICROSOFT ACCESS database queries were used to match the subset of significantly regulated genes with their previously calculated associated variance-normalized, mean-centered signal values. 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 through NetAffx Analysis Center (Affymetrix Inc., Santa Clara, CA). Cross-validated probe-sets 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 probe-sets were visually examined via directed acyclic graphs to gain insight into the epithelial cell responses to *A. actinomycetemcomitans* co-culture. The total number of genes regulated per biological process, the percentage of total genes impacted per term, and the *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 cells and IHGK upon *A. actinomycetemcomitans* interactions.

Results and discussion

Previous work has 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 (10) and its well-characterized toxins have drastically different effects on different cell types (15, 16). Although these are clinically relevant examples of host gene modulation in response 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 ontological analysis were performed to investigate the similarities and differences between the transcriptional responses of two different lineages of epithelial cells to an *A. actinomycetemcomitans* challenge.

Initially, all samples from uninfected and infected HeLa cells and IHGK were used to determine the overall similarity of the transcriptional profiles of these two epithelial cell lines. Signal intensity data for the 14 171 probe-sets that passed initial expression filters were used to perform unsupervised cluster analysis and supervised class prediction as described in the Materials and methods. 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 14 genes would be identified as false positives. In addition, linear discriminant analysis and one-nearest-neighbor classifications were 100% accurate by LOOCV for 2000 random permutations, while nearest centroid and three-nearest-neighbor classifications were 80% accurate. Both rates of 80% and 100% are significantly more accurate than the 25% correct classification rate that would be 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 cells and IHGK, nor is it impacted significantly upon by a 2-h co-culture with *A. actinomycetemcomitans*. 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 would be 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 probe-sets 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.

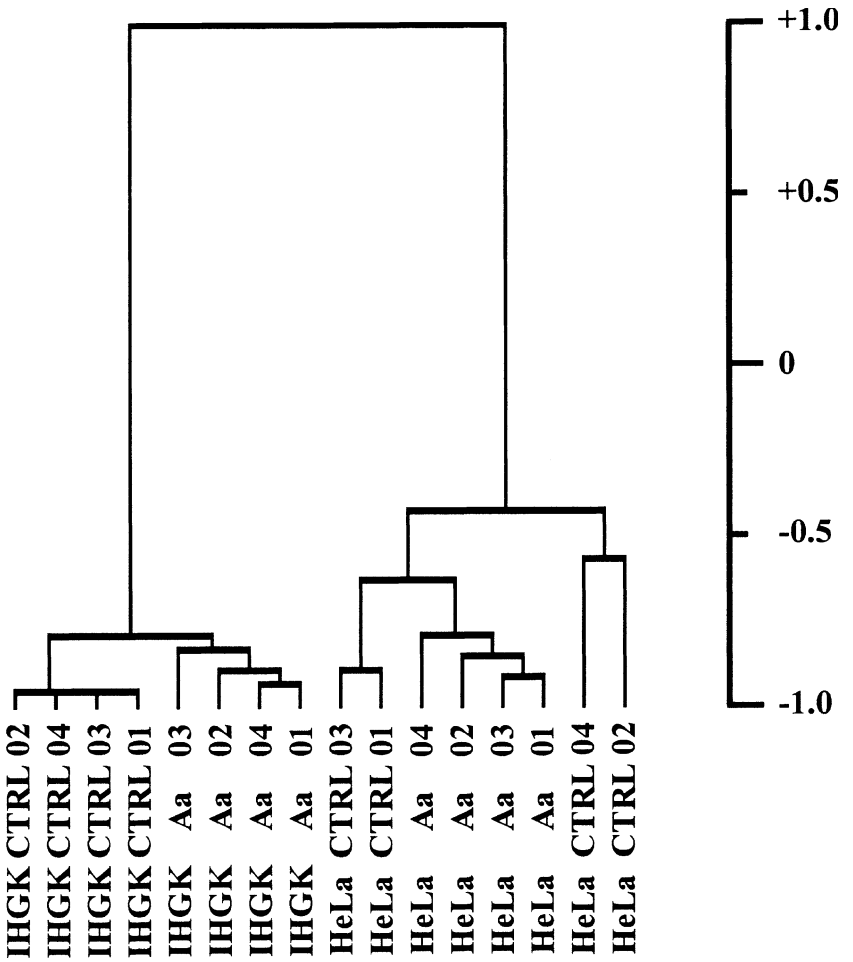
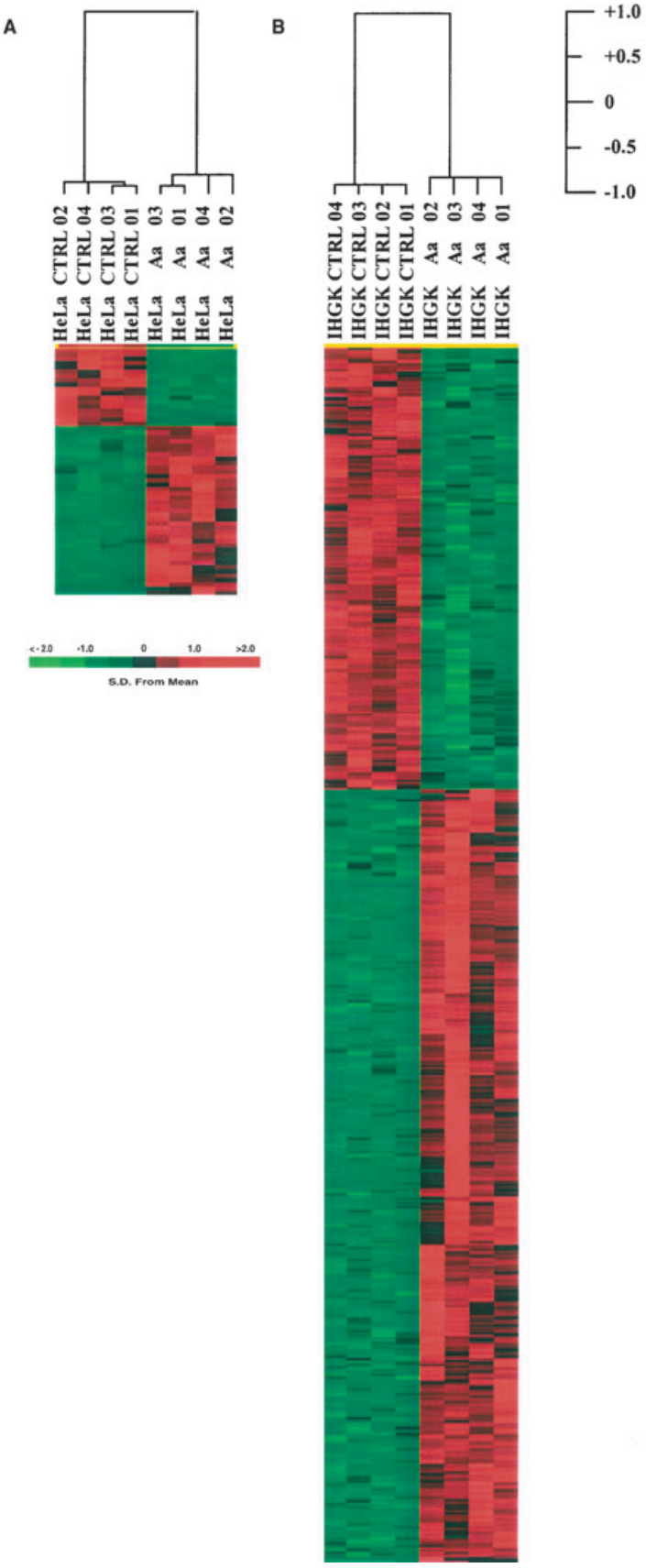


Fig. 1. Dendrogram representing the divergence of HeLa and IHGK cell transcriptional profiles. RNA was isolated and purified after a 2-h co-culture with *A. actinomycetemcomitans* and compared to uninfected cells. This dendrogram was constructed from 2867 probe-sets differentially expressed between the 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. Abbreviations used: IHGK CTRL 01-04, uninfected IHGK; IHGK Aa 01-04, *A. actinomycetemcomitans*-infected IHGK; HeLa CTRL 01-04, uninfected HeLa cells; HeLa Aa 01-04, *A. actinomycetemcomitans*-infected HeLa cells.



TREEVIEW visualization of the 2867 probe-sets differentially expressed among all four classes (Fig. 1) revealed interesting characteristics of HeLa cells and IHGK. 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 vs. 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 are shorter than the *inter*-class distances. This analysis also represented an indirect measure of the degree of noise introduced in that experimental system. In Fig. 1, the major node of separation occurred between HeLa and IHGK 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 house-

Fig. 2. Different patterns of gene expression by HeLa cells and IHGK upon co-culture with *A. actinomycetemcomitans*. RNA was isolated and purified after a 2-h co-culture with *A. actinomycetemcomitans* and was compared to that from 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 IHGK 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. Abbreviations used: IHGK CTRL 01-04, uninfected IHGK; IHGK Aa 01-04, *A. actinomycetemcomitans*-infected IHGK; HeLa CTRL 01-04, uninfected HeLa cells; HeLa Aa 01-04, *A. actinomycetemcomitans*-infected HeLa cells.

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

Probe set ID	HeLa	IHGK	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	IGFBP3 binding protein 3
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

keeping functions and intermediate metabolism.

However, it cannot be ruled out that some differences discovered between the transcriptional profiles of HeLa cells and IHGK were the result of the different cell culture media used, of differences in the growth rates of these cell lines, or of differences that exist between human papillomavirus type 16 (IHGK) and human papillomavirus type 18 (HeLa) immortalization (1). 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 independently for both cell lines. 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 IHGK. Class prediction for HeLa cells revealed that only 67 genes were differentially expressed upon *A. actinomycetemcomitans* infection at the significance level of $P < 0.001$ (Fig. 2A). In contrast, this analysis performed on IHGK yielded 625 significantly modulated genes (Fig. 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 one- and three-nearest-neighbor, the nearest centroid, and the support vector machines analyses.

A directed effort was made to investigate the extent of the common core transcriptional response by these two cell lines to *A. actinomycetemcomitans* infection. Using MICROSOFT ACCESS database queries, the 625 significantly modulated genes in IHGK, and the 67 genes significantly regulated in HeLa cells were correlated. Upon *A. actinomycetemcomitans* interaction, the common transcriptional response of HeLa cells and IHGK consisted of eight probe-sets. The expression patterns for seven of these eight genes showed a consistent pattern of regulation for both cell lines: six were up-regulated and one was down-regulated (Table 1), which 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.

To investigate the possible effect of sampling error on this outcome, we repeated the class prediction analysis at the lower stringency of $P < 0.01$. At that level of significance, HeLa cells modulated 404 genes and IHGK modulated 2011 genes (compared to 109 and 132, respectively, that 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 a significance of $P < 0.001$. 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 IHGK 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 IHGK host responses were identified. The biological processes identified included the regulation of angiogenesis, chemotaxis, the 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 (13).

Of immediate interest in the context of host-pathogen interactions were the genes associated with the stress response. This biological process was significantly impacted in both IHGK and HeLa cells. However, a side-by-side comparison of the child ontology terms for the response to stress in IHGK (Fig. 3A) and HeLa cells (Fig. 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 contrasted strikingly with the directed acyclic graphs obtained for IHGK, 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 G_2/M phase through DNA damage in immune cells and other cell types (5). In addition,

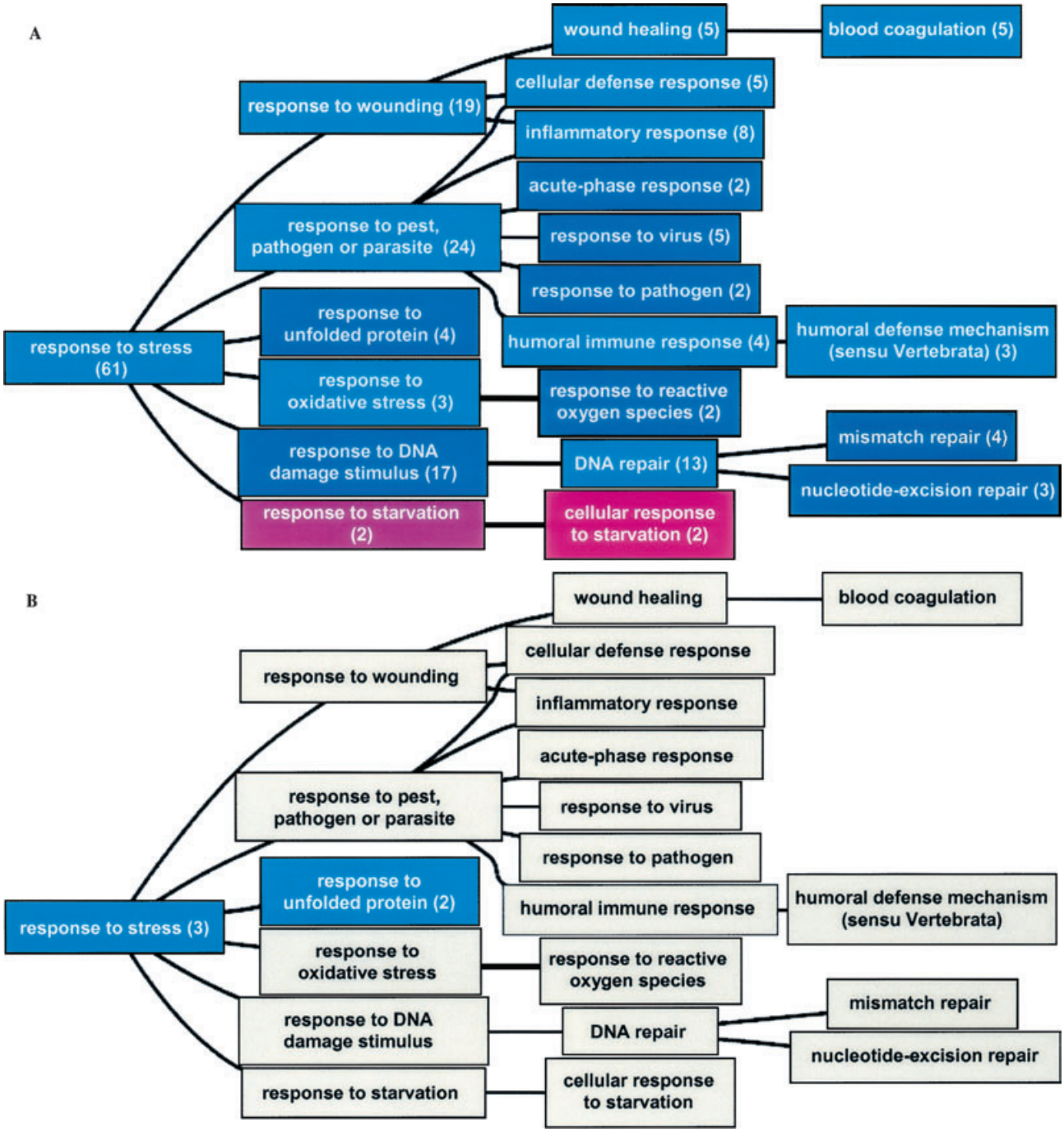


Fig. 3. Processes associated with the stress response in HeLa cells and IHGK that are impacted by *A. actinomycetemcomitans* interaction. Differentially regulated probe sets were annotated, and their associated gene ontology terms were visualized with NetAffx. Biological processes were organized by directed acyclic graphs, 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 directed acyclic graph is one representative example of the total biological response to *A. actinomycetemcomitans* interaction by IHGK (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 the most impacted ontology terms. Biological processes missing in HeLa cells relative to IHGK are shown in gray.

24 genes impacted the ontology terms associated with the response to pest, pathogen or parasite in IHGK. Included in this list were genes involved in the inflammatory response such as interleukin-1β and interleukin-6. Interleukin-1β up-regulation in IHGK cells is consistent with reports documenting increased expression of this pro-inflammatory cytokine in primary gingival epithelial cells (24) and has been reported previously (13). Interleukin-6 stimulation in gingival fibroblasts by *A. actinomycetemcomitans* has been demonstrated in connection with cytolethal distending toxin (4).

This representative example illustrated that biological processes impacted in both HeLa cells and IHGK may still be regu-

lated 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 cells and IHGK. The relatively high number of differentially regulated genes found in oral IHGK (625) as compared to HeLa cells (67) is consistent with the tissue tropism displayed by the Aae adhesin of *A. actinomycetemcomitans* (10). The data presented here further suggested that the host transcriptional response to *A. actinomycetemcomitans* challenge is substantial. Furthermore, in contrast to the current paradigm, the response of oral epithelial cells in host defense to infection appeared to be tailored, and to have ramifications extending beyond specific toxicity and tissue tropism.

To our knowledge, this study represents 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 of whether the specific models behave 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 in substantiating some contradictory reports in the literature pertaining to a variety of oral and other micro-organisms.

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