Concurrence between the gene expression pattern of *Actinobacillus actinomycetemcomitans* in localized aggressive periodontitis and in human epithelial cells

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*Actinobacillus actinomycetemcomitans* is a facultatively intracellular pathogen and the aetiological agent of localized aggressive periodontitis. Screening of the genome of *A. actinomycetemcomitans* for *in vivo*-induced antigen determinants previously demonstrated that the proteome of this organism differs in laboratory culture compared with conditions found during active infection. The aim of the present study was to determine whether the bacterial gene expression pattern inferred with *in vivo*-induced antigen technology (IVIAT) in human infections was consistent with the gene expression pattern occurring upon epithelial cell association. To this end, a real-time PCR method was developed and used to quantify absolute and relative bacterial gene expression of *A. actinomycetemcomitans* grown extra- and intracellularly in two human epithelial cell lines (HeLa and IHGK). The amount of template used in the assay was normalized using the total count of viable bacteria (c.f.u.) as a reference point and performed in duplicate in at least two independent experiments. Controls for this experiment included 16S rRNA and gapdh. Transcription of all eight ORFs tested increased significantly (*P* < 0.05) in HeLa and IHGK cells compared with bacteria grown extracellularly. The concurrence of gene expression patterns found in the two models suggests that these epithelial cells are valid *in vitro* models of infection for the genes tested. IVIAT is an experimental platform that can be used as a validation tool to assess the reliability of animal and other models of infection and is applicable to most pathogens.

INTRODUCTION

*Actinobacillus actinomycetemcomitans* is the primary aetiological agent of localized aggressive periodontitis (LAP) and has also been isolated in extra-oral infections (Meyer & Fives-Taylor, 1997; Offenbacher, 1996; Slots & Genco, 1984; Socransky *et al.*, 1999; van Winkelhoff & Slots, 1999; Zambon, 1985). *In vivo*-induced antigen technology (IVIAT) was previously used to identify *in vivo*-induced (*ivi*) genes in *A. actinomycetemcomitans* from LAP patients (Handfield *et al.*, 2000). Recognizing that some genes are expressed specifically *in vivo* is particularly important for understanding the virulence of *A. actinomycetemcomitans*. IVIAT identifies *ivi* genes directly in patients, following the pathogen’s natural route of infection (Handfield *et al.*, 2000). This alleviates the need for an animal model of infection or the requirement to mimic environmental conditions that are thought to exist during human colonization. The outcome of the IVIAT survey of the *A. actinomycetemcomitans* genome was a catalogue of 116 *ivi* genes (Song *et al.*, 2002).

Further characterization of these *ivi* genes was impaired because, by definition, the proteins produced during infection are not expressed during laboratory culture of this bacterium. However, it was assumed that discovery of an infection model that replicated the same gene expression pattern found with IVIAT could provide conditions from which one could characterize these *ivi* genes. Since *A. actinomycetemcomitans* is a facultatively intracellular pathogen (Meyer *et al.*, 1991, 1996, 1999; Meyer & Fives-Taylor, 1994; Rudney *et al.*, 2001; Takayama *et al.*, 2003), we hypothesized that many environmental stimuli found during human infection might also be found upon epithelial cell association. Thus, one could use an *in vitro* cell-culture system to study the function of these *A. actinomycetemcomitans* gene products in pathogenesis.

Abbreviations: IVIAT, *in vivo*-induced antigen technology; LAP, localized aggressive periodontitis.
The purpose of this study was to investigate whether the bacterial gene expression pattern observed upon epithelial cell association with HeLa and/or IHGK cells was consistent with the pattern initially established using IVIAT (Cao et al., 2004). This work offers a proof of principle that the same experimental platform could be used as a validation tool to assess the reliability of animal and other models of infection. To that end, an efficient real-time PCR method was developed to quantify the absolute and relative bacterial gene expression of A. actinomycetemcomitans grown extra- and intracellularly.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strain used in this study was A. actinomycetemcomitans strain VT1169 (a spontaneous nalidixic acid- and rifampicin-resistant variant of SUNY 465, kindly provided by Dr P. M. Fives-Taylor, University of Vermont, USA). VT1169 was grown in trypticase soy broth supplemented with 1 % yeast extract (TSBYE; Difco) in a humidified atmosphere containing 10 % CO2, with the addition of 50 µg nalidixic acid ml−1 and 50 µg rifampicin ml−1 (Sigma).

**Mammalian cell culture.** Epithelial HeLa (subline KB) cells were obtained from ATCC (CCL17-1) and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Cellgro), supplemented with 10 % fetal bovine serum and 50 U penicillin/streptomycin ml−1 (Gibco). An epithelial human papillomavirus type 16-immortalized gingival epithelial cell line (IHGK; Oda et al., 1996a, b), kindly provided by Dr D. Oda (University of Washington, USA), was used in this study. This cell line was maintained in keratinocyte serum-free medium (KSFM; Gibco) supplemented with 50 U penicillin/streptomycin ml−1 (Gibco). Cells were cultured at 37°C in a humidified 5 % CO2 atmosphere.

**Intra- and extracellular growth of bacteria.** To obtain bacteria grown intracellularly, a modified standard invasion assay was performed (Meyer et al., 1996). Prior to invasion, epithelial cell wells were washed and the medium was replaced with antibiotic-free DMEM or KSFM. A. actinomycetemcomitans was used at an m.o.i. of approximately 3000. After 2 h of infection, a 1 h incubation with gentamicin (50 µg ml−1) was performed to kill extracellular bacteria. Infected cells were washed three times prior to RNA extraction. An aliquot of the washed infected cells was lysed using 0.5 % Triton X-100 and neutralized with Dulbecco’s 1 × PBS to measure c.f.u. on TSBYE. These conditions were chosen because they consistently resulted in a level of invasion of approximately 2 × 106 c.f.u. per well (or 1 % of the inoculum), which was consistent with previously reported data (Meyer et al., 1996). To achieve the same level of background for extracellularly grown A. actinomycetemcomitans, a mock infection was designed where similar epithelial cells were prepared, lysed and neutralized as mentioned above. To compensate for the m.o.i., 2 × 106 c.f.u. per well were inoculated into the lysate and RNA was extracted immediately.

**RNA extraction and normalization.** The two RNA samples were purified at the same time with the RNeasy Mini kit and DNase I treated following the manufacturer’s recommendations (Qiagen). Each RNA sample was diluted to 50 µg ml−1 prior to cDNA synthesis.

**Quantitative real-time PCR.** A standard curve was constructed for each gene using a gene-specific amplicon (ranging from 91 to 235 bp), which was amplified from genomic DNA, gel-purified and quantified to calculate copy numbers. The real-time PCR primer sets were designed using the Beacon Designer 2.0 software (Bio-Rad). Oligonucleotide primers (Table 1) were synthesized by Integrated DNA Technologies. Both intracellular and extracellular cDNA were synthesized from normalized 10 µl samples of RNA (50 µg ml−1) and 1 µl random hexamers (30 µg ml−1). Random hexamers were used as they allowed the standardization of cDNA synthesis between experimental conditions. The Superscript II First-strand Synthesis kit (Invitrogen) was used according to the manufacturer’s protocol. To measure the amount of mRNA for each gene, 10 µl duplicates of each decimal dilution of cDNA sample were assayed with the primer sets (Table 1). Identical RNA samples and volumes were amplified with matching primers and used as controls to measure background DNA contamination. Amplicon specificities were verified by generating melting curves for each amplicon. Real-time PCR was performed in a Bio-Rad iCycler according to the manufacturer’s recommendation. PCR parameters were 95°C for 10 min, followed by 45 cycles of 95°C for 10 s and 50°C for 1 min. One hundred cycles of 10 s, with 0.4°C temperature increments from 55 to 95°C, were used for the melting curves. Samples were amplified in duplicate for two independent experiments. C_{T} was defined as the cycle at which the fluorescence became detectable above background fluorescence. This value was inversely proportional to the logarithm of the

<table>
<thead>
<tr>
<th>Table 1. Oligonucleotide primers used in this study</th>
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<td>Genes are annotated according to the PEDANT database (<a href="http://pedant.gsf.de/">http://pedant.gsf.de/</a>) as of July 2004.</td>
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<tr>
<td><strong>Gene target</strong></td>
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<tr>
<td>16S rRNA</td>
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<tr>
<td>ORF146</td>
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<tr>
<td>ORF554</td>
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<tr>
<td>ORF607</td>
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<tr>
<td>ORF791</td>
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<tr>
<td>ORF589</td>
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<tr>
<td>ORF1383</td>
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<tr>
<td>ORF1402</td>
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<tr>
<td>ORF1409</td>
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<tr>
<td>ORF1425</td>
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initial number of template molecules. A standard curve was plotted for each primer/probe set using the \( C_t \) values obtained from amplification of known quantities of DNA. To check the linearity of the detection system, decimal dilutions were performed so that a correlation coefficient could be calculated from the standard curve of \( C_t \) values. The relative number of copies for targets isolated under intracellular and extracellular growth conditions was established, after background subtraction, and normalized using c.f.u. as a standard. The significance of the differences obtained under the two experimental conditions was analysed by Student’s \( t \)-test.

**RESULTS AND DISCUSSION**

**Selecting an appropriate model of infection**

Choosing a suitable model to investigate a complex biological system *in vitro* can be subjective and controversial. Due to technical and ethical considerations, pathogenesis studies have often not presented definitive evidence describing causes and effects. Consequently, they have traditionally relied on inference from animal, cell-culture and other models of infection to draw reasonable conclusions. In fact, numerous examples in the literature have shown the bias that models of infection to draw reasonable conclusions. In fact, inconsistencies that have been reported in the literature (Smith, 1998). The study of most human pathogens, including *A. actinomycetemcomitans*, has been in line with this paradigm. Since the pathogenesis of *A. actinomycetemcomitans* is complex and comprises bacterial virulence factors and host susceptibilities, nearly every *in vitro* infection model used to date has been limited in that it only permits mimicking of a limited number of conditions that are thought to occur during human infection, often bypassing important steps in the infection spatial and/or temporal process. Consequently, our understanding of the pathophysiology of *A. actinomycetemcomitans* infections and of most human pathogens is incomplete.

We addressed this issue with the development of IVIAT, a novel tool that allowed us to discover genes of a pathogen that are specifically induced during human infection but not during *in vitro* growth of a pathogen (Handfield *et al.*, 2000). Obviously, no one model is expected to mimic every host–pathogen interaction perfectly. We propose that identification of an infection model that contains comparable gene expression patterns to those predicted by IVIAT provides conditions amenable to the characterization of *ivi* genes. The confirmation of a consistent gene expression pattern in a given infection model has seldom been performed to date. This may, in part, explain inconsistencies that have been reported in the literature (Smith, 1998).

We recognize that human primary cells and a plethora of various cell lines have been used to study host–*A. actinomycetemcomitans* interactions. These studies used primary gingival or buccal epithelial cells (Asakawa *et al.*, 2003; Rudney *et al.*, 2001; Teng & Hu, 2003; Uchida *et al.*, 2001), as well as established cell lines such as HaCaT (spontaneously transformed but non-malignant human skin keratinocytes; Zhang *et al.*, 2004), HeLa (HEp-2, human epithelial larynx carcinoma; DiRienzo *et al.*, 2002), HSC-2 (human oral epithelial carcinoma; Takayama *et al.*, 2003) and numerous others. The choice of cell lines

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**Table 2. Relevant characteristics of *A. actinomycetemcomitans* genes and gene products used in this study**

Genes are annotated according to the PEDANT database (http://pedant.gsf.de/) as of July 2004. Closest homologues were determined using BLAST: Aa, *A. actinomycetemcomitans*; Hi, *Haemophilus influenzae*; Pm, *Pasteurella multocida*; Rs, *Ralstonia solanacearum*; Xf, *Xylella fastidiosa*; NA, not applicable. Characteristics were deduced from the Aa HK1651 database at PEDANT and presented in Cao *et al.* (2004).

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Closest homologue</th>
<th>Putative function and relevant characteristic(s)</th>
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<tbody>
<tr>
<td><strong>Control genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rRNA</td>
<td>NA</td>
<td>rRNA. Present in six copies in the HK1651 genome</td>
</tr>
<tr>
<td>ORF1383</td>
<td>Aa <em>gapdh</em></td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12). Carbohydrate utilization</td>
</tr>
<tr>
<td><strong>Test genes</strong></td>
<td></td>
<td></td>
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<tr>
<td>ORF146</td>
<td>Xf AAF4878</td>
<td>Putative surface protein involved in long-chain fatty acid transport</td>
</tr>
<tr>
<td>ORF554</td>
<td>Pm ORF1786</td>
<td>Putative positive regulator of sigma E activity. Presents two transmembrane regions</td>
</tr>
<tr>
<td>ORF607</td>
<td>Rs ORF0205</td>
<td>Putative transcription regulator, LysR family. Possible DNA binding</td>
</tr>
<tr>
<td>ORF791</td>
<td>Aa <em>dam</em></td>
<td>DNA-adenine methyltransferase. Restriction/modification system</td>
</tr>
<tr>
<td>ORF859</td>
<td>Hi ORF0701</td>
<td>Uncharacterized protein family. Localized juvenile periodontitis patients have a significantly higher anti-ORF859 antibody titre than sera from healthy controls. Surface-localized as determined by immunofluorescence. Mutated strain JMS04 (ORF859) is defective in intracellular fitness compared with wild-type in a competition assay (Cao <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>ORF1402</td>
<td>Hi Omp1</td>
<td>Putative long-chain fatty acid transport. Localized juvenile periodontitis patients have a significantly higher anti-ORF1402 antibody titre than sera from healthy controls. Mutated strain JMS05 (ORF1402) is defective in intracellular fitness compared with wild-type in a competition assay (Cao <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>ORF1409</td>
<td>Hi ORF0864</td>
<td>Putative membrane GTPase involved in stress response</td>
</tr>
<tr>
<td>ORF1425</td>
<td>Pm HflC</td>
<td>Putative membrane protease subunits. Presents one transmembrane region</td>
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for the present study was therefore somewhat arbitrary. 
IHGK cells are thought to be an inherently more relevant 
model than the HeLa cell model due to their oral and non-
cancerous origin.

Selection of targeted *ivi* genes

Of the 116 *ivi* genes of *A. actinomycetemcomitans* that could 
have been used (Song et al., 2002), a total of eight was selected 
for this study, in addition to two non-*ivi* controls. As shown 
Table 2, we selected genes that represented all types 
recovered with IVIAT, namely genes of known and unknown 
function associated with homeostasis, housekeeping func-
tions, regulation or host–pathogen interactions. This was to 
reduce the potential bias that could be introduced by pre-
selection of genes associated with a particular metabolic 
pathway.

Quantification of gene expression using RT-PCR

Gene regulation in prokaryotes can be studied with a 
variety of methods. Obviously, the use of different analy-
tical methods is likely to yield different quantifications of 
the absolute number of transcripts. Nevertheless, it is 
expected that the use of different methods would still yield 
similar trends, while the absolute number of copies may 
vary depending on the sensitivity, reproducibility and noise 
introduced into an assay. Choosing real-time PCR in this 
study was based on the need for a simple, sensitive and 
quantifiable method to assess the gene expression levels of 
a large number of different genes simultaneously and in a 
small sample.

The choice of appropriate controls for this study was 
problematic. The genes for 16S rRNA and *gapdh* have 
traditionally been two of the most frequently used. These 
genes are often considered to be constitutively expressed, 
offering an invariable denominator for gene expression 
studies. The use of these genes is undeniably convenient 
and a valid practice for most intended purposes. However, 
there is clear evidence that this is not a universally applicable 
solution to all experimental systems. Under many condi-
tions, the so-called 'housekeeping genes' are not constitu-
tively expressed at the transcriptional level in bacteria as 
previously assumed (Savli et al., 2003; Vandecasteele et al., 
2001; Widada et al., 2001). We showed in this study that 16S 
rRNA and *gapdh* were indeed significantly upregulated as *A. 
actinomycetemcomitans* shifted from extracellular laboratory 
growth conditions to an intracellular life style. This is 
consistent with previous observations that estimated that 
the doubling time of *A. actinomycetemcomitans* was approxi-
ately 150 min under optimal laboratory growth conditions 
but decreased dramatically to 20 min when grown intracel-
larly (Fives-Taylor et al., 1999). It is anticipated that such a 
significant increase in growth rate would be accompanied by 
increased expression of a number of genes involved in central 
metabolic functions. This variable expression level would 
preclude the use of these genes as constitutively expressed 
controls. In addition, the 16S rRNA gene is present as 
multiple copies in the *A. actinomycetemcomitans* genome, 
as opposed to IVIAT genes, which are all present as a single 
copy. Copy number divergence may result in inconsistencies 
in the data interpretation. Consequently, total viable counts 
were initially used to standardize the gene expression data. 
Besides providing a convenient means of normalizing the 
gene expression data, this method offered a simple way to 
to account for the biological variability that was observed in 
duplicate invasion experiments performed with *A. actino-
mycetemcomitans*.

RT-PCR quantification of bacterial gene expression levels 
atertained the construction and optimization of absolute 
standard curves for each target amplicon. This was to 
ensure accurate reverse transcription profiles. A representa-
tive example of this process is illustrated by the standard 
curve for ORF1409 in HeLa cells and is shown in Fig. 1. In 
this example, a 149 bp gene-specific PCR fragment was 

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**Fig. 1.** Real-time PCR development of the standard curve of 
ORF1409 in HeLa cells. (a) Gene-specific fragments (149 bp) were 
amplified, gel-purified and quantified to calculate target DNA copy 
numbers. Decimal serial dilution samples were used as a template for 
the standard curve. Melting curves (inset) were used to determine 
amplification specificity. (b) Standard curves were plotted as threshold 
cycle numbers (Ct) versus log of copy numbers. Each experiment was 
performed twice. The linear equation and correlation coefficient were 
calculated to infer the linearity of the equation and the relative copy 
numbers at experimental data points, and to ensure a linear dynamic 
range.
used as the template to generate the standard curve. Melting curves, such as the one shown in Fig. 1, consistently presented unique amplification products for every amplicon tested (data not shown). The C<sub>t</sub> value was plotted against a known copy number of template DNA fragments (as shown in Fig. 1b) to derive the standard curve. In addition to providing a tool to extrapolate the amount of mRNA present in an experimental condition, linear regression of the standard curves permitted us (i) to estimate the dynamic range of the methodology, (ii) to detect differences in amplification efficiency between different target mRNAs and (iii) to estimate the relative sensitivity of individual assays. The standard curve of every targeted gene assured a linear dynamic range spanning concentrations of DNA greater than six orders of magnitude (data only shown for ORF1409 in Fig. 1). The overall mean linear correlation coefficient (r<sup>2</sup>) was 0.99 ± 0.01 for all targets tested. Differences in amplification efficiency (slope) and sensitivity (y intersect) between targets were calculated. A higher slope value denoted a greater efficiency and a lower C<sub>t</sub> value depicted higher sensitivity. As shown in Table 3, the efficiency of each reaction was similar between primer/target conditions. The most sensitive primer/probe conditions in our hands (ORF1425) allowed the detection of as few as 10 copies of mRNA per reaction containing, on average, DNA isolated from 250 c.f.u.

With the confidence that the experimental method was reliable and sensitive, the expression levels of the tested genes were assayed in bacteria grown under laboratory conditions and then compared with expression levels measured upon epithelial cell association. The laboratory conditions for bacterial growth that were used were consistent with the original conditions used in the IVIAT screening of <i>A. actinomycetemcomitans</i> (Handfield et al., 2000). As shown in Fig. 2, a marked difference in copy number could be observed for ORF1409 in HeLa cells under these conditions. These results were mirrored in IHGK cells. The raw RT-PCR data were subsequently normalized in accordance with the

### Table 3. Real-time PCR amplification characteristics

Efficiency of amplification among ORFs is defined as the slope of the standard curve. Sensitivity is defined by the position at which the standard curve intersects the y-axis.

<table>
<thead>
<tr>
<th>Target amplicon</th>
<th>HeLa</th>
<th>IHGK</th>
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<tbody>
<tr>
<td></td>
<td>Efficiency</td>
<td>Sensitivity</td>
</tr>
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<td><strong>Control genes</strong></td>
<td></td>
<td></td>
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<tr>
<td>16S</td>
<td>-3.3861</td>
<td>36.741</td>
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<tr>
<td>ORF1383</td>
<td>-3.4980</td>
<td>37.659</td>
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<tr>
<td><strong>Test genes</strong></td>
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<td></td>
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<tr>
<td>ORF146</td>
<td>-3.3908</td>
<td>36.468</td>
</tr>
<tr>
<td>ORF554</td>
<td>-3.1911</td>
<td>39.614</td>
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<tr>
<td>ORF607</td>
<td>-3.1082</td>
<td>34.861</td>
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<td>ORF791</td>
<td>-3.4399</td>
<td>36.990</td>
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<tr>
<td>ORF859</td>
<td>-3.4943</td>
<td>37.926</td>
</tr>
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<td>ORF1402</td>
<td>-2.9946</td>
<td>34.897</td>
</tr>
<tr>
<td>ORF1409</td>
<td>-3.5654</td>
<td>38.408</td>
</tr>
<tr>
<td>ORF1425</td>
<td>-3.3282</td>
<td>34.769</td>
</tr>
<tr>
<td><strong>Means</strong></td>
<td>-3.4 ± 0.2</td>
<td>38 ± 3</td>
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</tbody>
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Fig. 2. Gene induction of ORF1409 in HeLa cells. Bacteria grown under standard laboratory conditions (extracellular) and bacteria recovered from infected HeLa cells after invasion (intracellular) were used for RNA extraction. RNA samples were DNase I-treated, purified and normalized relative to the recovered c.f.u. in the infected cells. Reverse transcription was carried out using random hexamer primers. cDNA samples were assayed in duplicate using specific IVIAT primers for real-time PCR. Amplicon specificity was demonstrated by generating a melting curve for each amplicon (inset).
number of c.f.u. present in each sample. This step was essential to standardize the data to a common denominator. This procedure was repeated for all other IVIAT genes and controls in both HeLa and IHGK cell lines. As shown in Fig. 3(a, b), the relative copy numbers of target per c.f.u. did vary for both controls used in this experiment (16S rRNA and gapdh). The increase in intracellular expression averaged 114-fold for these ‘housekeeping’ controls. Similarly, every IVIAT gene tested showed a significant ($P < 0.05$) increase in gene expression upon epithelial cell association of A. actinomycetemcomitans with IHGK and HeLa cells (Fig. 3a, b). For these genes, the increase in intracellular expression was on average 2811-fold. The data were also normalized based upon housekeeping gene expression levels (Fig. 3c, d). In IHGK cells, the intracellular relative induction for the tested genes averaged 28.8-fold (normalized against 16S) and 21.4-fold (normalized against gapdh) above housekeeping controls, which further confirmed the significant increase in transcription levels for all IVIAT genes tested in that cell line. This also suggested that the increased levels of transcription were the result of specific induction, as opposed to a general increased level of expression. This would be expected if the growth rate alone were at the origin of the observed transcriptional variations. It cannot be ruled out that some of the expression changes that were observed and that were ascribed to cellular interaction might also relate to exposure to, or growth in, tissue culture media during the infection process. Indeed, tissue culture media are known to contain substances not routinely found in common bacterial growth media (such as hormones, serum and growth factors). The nature of the environmental determinant that may be found fortuitously in cell culture media and that may trigger the induction of certain IVIAT genes is currently under study. In addition, it will be pertinent to verify whether the gene pattern observed here correlates with host–cell contact and/or invasion.

The elevated expression of the bacterial genes tested so far which occurred upon epithelial cell association suggested that the environmental conditions within these infection models were consistent with those found in patients, at least at some point during the course of the infection. This is in line with the fact that A. actinomycetemcomitans is found intracellularly in gingival and buccal cells isolated from periodontitis patients. The chronic nature of LAP may result in persistent activation of the immune system,

![Graphs showing IVIAT gene induction in epithelial cells.](image-url)

**Fig. 3.** IVIAT gene induction in epithelial cells. Bacteria grown under standard laboratory conditions and bacteria recovered from infected HeLa cells (a, c) or IHGK cells (b, d) after invasion were used for RNA extraction. RNA samples were DNase I-treated and normalized based on the recovered c.f.u. in the infected cells as well as the total RNA quantification. Reverse transcription was carried out using random hexamer primers. The cDNA sample was assayed in duplicate and for two independent experiments, using specific IVIAT primers for real-time PCR. Amplon specificity was demonstrated by generating a melting curve for each amplicon. mRNA copy numbers that were obtained upon infection of HeLa cells or IHGK cells are shown for each gene (a, b); the fold induction data resulting from 16S rRNA normalization (open bars) or gapdh normalization (filled bars) are also shown (c, d).
which, in turn, may lead to a preferential stimulation by antigens induced intracellularly. Further studies will be required to demonstrate that this pattern is conserved for the remaining genes initially found with IVIAT. Additional studies will also be needed to confirm that the genes transcriptionally induced in epithelial cells are indeed expressed at the protein level. Although not definitive, the approach used here still presents a first line of evidence that this panel of genes were induced at the transcriptional level. Many products from the genes studied here have already shown serological evidence that they are undoubtedly expressed preferentially in LAP patients when compared with healthy donors (Cao et al., 2004). Additionally, at least one of the genes tested above has previously been identified as being specifically induced at the protein level in biological specimens from LAP patients (Handfield et al., 2000, 2003).

The experimental evidence presented above suggests that the IVIAT genes investigated to date can now be characterized in greater depth in various epithelial cells by classical methods with the a priori confidence that they are induced in these models. Recent evidence further indicates that IHGK cells are indeed particularly suitable to study relevant host–pathogen interactions for A. actinomycetemcomitans (Handfield et al., 2005). We propose that other currently used infection models may be more compelling if the bacterial gene expression pattern found in that particular model is consistent with the gene expression pattern found in patients (as determined with IVIAT) for that particular organism.

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