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# Invasion of endothelial and epithelial cells by strains of *Porphyromonas gingivalis*

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**Abstract**

*Porphyromonas gingivalis* is a periodontal pathogen that may also be involved in the pathogenesis of coronary heart disease. This microorganism has the ability to invade several cell lines. In this study, 26 different strains of *P. gingivalis* were tested for invasion of human umbilical vein endothelial cells and KB cells, a human oral epidermoid cell line. Abilities to invade both cell lines by an individual strain were similar, and their invasion efficiencies could be assembled into four groups: high, moderate, low and non-invasive. Of the 26 strains, only *P. gingivalis* AJW4 was non-invasive. Since the fimbriae are implicated as having a key role in invasion by this species, the presence of fimbriae on strain AJW4 was investigated. Using polymerase chain reaction (PCR), strain AJW4 was found to contain the *fimA* gene. Sequence analysis revealed it to be type IV according to the typing scheme developed by Amano et al. Further, *fimA* is transcribed in this strain as demonstrated by reverse transcription PCR and is expressed on the cell surface as visualized by negative staining and electron microscopy. The adherence+invasion of strain AJW4 was 38.7% of the most invasive strain (strain 381). However, the CFU $ml^{-1}$ of strain AJW4 recovered from within cells was 2.9% of strain 381. Even though strains AJW4 and W50 have the same type IV fimbriae, strain AJW4 is 8.9-fold more adhesive yet is internalized 170-fold less. These data indicate that the invasion efficiency of *P. gingivalis* is variable among the different strains, and that the expression of FimA is not sufficient for invasion. 

*Keywords:* Invasion; Endothelial; Oral epithelial; Fimbria; *Porphyromonas gingivalis*

## 1. Introduction

*Porphyromonas gingivalis* is strongly associated with periodontal disease [1] and is thought to be a primary etiologic agent in adult periodontitis. Recent epidemiological studies have demonstrated a strong association between periodontal disease and coronary heart disease [2–6]. Additionally, *P. gingivalis* has been detected within atherosclerotic plaques by polymerase chain reaction (PCR) [7]. Therefore several studies have begun to focus on a possible relationship between *P. gingivalis* and cardiovascular tissues.

Invasion is a common strategy by a variety of pathogens to establish a privileged niche in the host [8]. *P. gingivalis* is an invasive microorganism and can invade a number of different cell lines [9–13]. Previous studies have indicated that the fimbriae of *P. gingivalis* play an essential role in adherence and invasion [14,15]. This study investigated the abilities of 26 different strains to invade human oral epithelial KB cells (ATCC CCL-17) and human umbilical vein endothelial cells (HUVEC).

## 2. Materials and methods

### 2.1. Bacterial and cell culture

*P. gingivalis* strains (Table 1) were grown on tryptic soy agar (Difco Laboratories, Detroit, MI, USA) supplemented with 5.0% sheep blood (Lampire Biological Laboratories, Pipersville, PA, USA), 0.5% yeast extract (Difco), hemin (5 μg $ml^{-1}$) and vitamin K (5 μg $ml^{-1}$). Overnight cultures of *P. gingivalis* were grown in brain heart infusion broth (Difco) supplemented with 0.5% yeast extract, 0.1% cysteine (Sigma), hemin (5 μg $ml^{-1}$) and vitamin K (5 μg $ml^{-1}$) under anaerobic conditions. *Escherichia coli* MC1061 (a gift of A.S. Bleiweis) was grown in Luria-Bertani (LB) medium consisting of Bacto tryptone (10 g $l^{-1}$;

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Difco), Bacto yeast extract (5 g $l^{-1}$) and NaCl (10 g $l^{-1}$). KB cells (ATCC CCL-17) were maintained in Eagle's minimum essential medium (EMEM; Mediatech, Herndon, VA, USA) supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA), 200 mM L-glutamine (Sigma) and 100 μg $ml^{-1}$ of penicillin–streptomycin (Sigma). HUVEC (ATCC ECV-340; a gift of M.I. Phillips) were maintained in M199 medium (Mediatech) supplemented with 10% fetal bovine serum (HyClone), 200 mM L-glutamine (Sigma) and 100 μg $ml^{-1}$ of penicillin–streptomycin (Sigma).

### 2.2. Antibiotic protection assay

To compare the invasion efficiencies of *P. gingivalis* strains in KB cells and HUVEC, the cells were infected in parallel using an antibiotic protection assay as described previously [10,12]. Colony forming units (CFU) of invasive bacteria were then enumerated and compared. If for any reason (e.g. contamination of a well) results from the invasion of one of the cell lines tested were discarded, so were the results for the other cell line performed in parallel.

### 2.3. Adherence vs. invasion

For the adherence/invasion assay, approximately $10^5$ KB cells were seeded in parallel wells of two 24-well tissue culture plates (Sarstedt). Prior to infection, the wells were washed three times with phosphate-buffered saline (PBS). The KB cells were infected by the addition of a resuspended overnight culture of $10^7$ *P. gingivalis* AJW4, *P. gingivalis* 381, *P. gingivalis* W50 and *E. coli* MC1061 in 1.0 ml of antibiotic-free EMEM at 37°C. After 90 min of aerobic incubation, the media were removed from infected cells, and the cells were washed three times with PBS. For the cells tested for adherence, 1.0 ml of sterile distilled water was added and incubated aerobically at 37°C for 20 min. Dilutions of the cell lysates infected with *P. gingivalis* were plated in triplicate on tryptic soy agar plates supplemented with 5.0% sheep blood, 0.5% yeast extract, hemin (5 mg $ml^{-1}$) and vitamin K (5 mg $ml^{-1}$), and cultured under anaerobic conditions. The dilutions of the lysates of *E. coli* MC1061 were plated on LB agar and cultured at 37°C aerobically. The other tissue culture plate with cells to be tested for invasion followed the aforementioned antibiotic incubation before lysis and subsequent culturing as described above for the cell lysates for the adherence assay.

### 2.4. PCR

For the isolation of genomic DNA, the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) was used as specified by the manufacturer. Reactions were performed in a final volume of 50 ml containing 2.35 $MgCl_2$ (Promega), 1× *Taq* buffer (Promega), 0.4 mM of each deoxynucleotide triphosphate (dNTP), 0.3 mM of each primer, 1.25 U of *Taq* DNA polymerase (Promega) and 0.08 U of *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). All primers were synthesized by Genoysis Biotechnology, Inc. (The Woodlands, TX, USA). To amplify the entire *fimA* gene, primers $T_1$ (5′-GCGCAGCAAGGCCAGCCCGGAGCA-3′) and $M_2$ (5′-CGCGGAATTCGAGCGAACCCCGCTCCCTGTATTCCGATA-3′) were synthesized and used as described by Watanabe-Kato et al. [16]. The amplifications were performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) with an initial denaturation step at 95°C for 1 min, followed by four cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 3 min. This was followed by another 30 cycles at 95°C for 30 s, 55°C for 1 min and 72°C for 3 min with a final extension step of 2 min at 72°C. The resulting PCR products were stored at 4°C and analyzed by 1.0% TAE agarose gel electrophoresis.

For typing of the *fimA* gene, primer sets and thermal cycling were used according to the protocol of Amano et al. [17]. Primers were used for type I (5′-CTGTGTGTTTATGGCAAACTTC-3′ and 5′-AACCCCGCTCCCTGTATTCCGA-3′) and type IV (5′-CTATTCAGGTGCTATTACCCAA-3′ and 5′-AACCCGCTCCCTGTATTCCGA-3′) *fimA* genes. The amplifications were performed with an initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. The resulting PCR products were stored at 4°C and analyzed by 2.0% TAE agarose gel electrophoresis.

RNA was recovered from overnight cultures of *P. gingivalis* strain 381 and strain AJW4. Reverse transcriptase PCR (RT-PCR) was performed using the RETROscript kit (Ambion, Inc., Austin, TX, USA) as specified by the manufacturer. RT-PCR reactions were performed in a final volume of 20 μl containing 1×RT-PCR Buffer (Ambion), 2.5 mM of each dNTP, 50 μM random decamers, 10 U placental RNase inhibitor and 100 U Moloney-murine leukemia virus reverse transcriptase. The reverse transcription reaction was incubated at 42°C for 60 min followed by a 10-min incubation at 92°C. One-quarter of the reverse transcription mix was used as template for PCR amplification in a final volume of 50 μl containing 1×reaction buffer (Ambion), 2.5 mM of each dNTP, the *fimA* PCR primers (5 μM each) and 2 U of SuperTaq™ (Enzyme Technologies Ltd., UK). The amplifications were performed with an initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 20 s, 55°C for 30 s, 72°C for 40 s, and a final extension at 72°C for 5 min. The resulting PCR products were stored at 4°C and analyzed by 2.0% TAE agarose gel electrophoresis.

### 2.5. Electron microscopy

To view the fimbriae of *P. gingivalis* AJW4, bacterial

Table 1
Bacterial strains used in this study

| Strains used | Source |
|---|---|
| *E. coli* MC1061 | A.S. Bleiweis |
| *P. gingivalis* strains | |
| 381 | SUNY-Buffalo collection[a] |
| 33277 | ATCC[b] |
| A7A128 (ATCC 53977) | B.G. Loos |
| W12 | SUNY-Buffalo collection |
| W50 | M.A. Curtis |
| W83 | SUNY-Buffalo collection |
| MB 1423 | UF PDRC collection[c] |
| MB 1432 | UF PDRC collection |
| MB 1843 | UF PDRC collection |
| 9-14k-1 | B.G. Loos |
| 17-6-1 | B.G. Loos |
| AJW1 | B.G. Loos |
| AJW2 | B.G. Loos |
| AJW3 | B.G. Loos |
| AJW4 | B.G. Loos |
| AJW5 | B.G. Loos |
| 10002 | H. Fukushima |
| 10007 | H. Fukushima |
| 10009 | H. Fukushima |
| 10011 | H. Fukushima |
| 10024 | H. Fukushima |
| 10033 | H. Fukushima |
| 10034 | H. Fukushima |
| 10044 | H. Fukushima |
| 10046 | H. Fukushima |
| 10048 | H. Fukushima |

[a]SUNY-Buffalo = State University of New York at Buffalo.
[b]ATCC = American Type Culture Collection.
[c]UF PDRC = University of Florida Periodontal Disease Research Center.

cells from an overnight culture in BHI broth were stained with methylaminetungstate for 30 s. A 5-s wash with deionized water followed staining. Samples were examined with a Hitachi 7000 transmission electron microscope.

## 3. Results

### 3.1. Invasion

The ability of individual *P. gingivalis* strains to invade HUVEC was essentially equal to their ability to invade KB cells; however, there was significant strain to strain variability (Figs. 1 and 2). Thus strains were classified into four groups based on their invasive efficiencies: high ($\times 10^5$), moderate ($\times 10^4$), low ($\times 10^3$) and non-invasive ($\leq \times 10^2$), the same level as the negative control. Most of the strains could be described as moderate, low or moderate to low. *P. gingivalis* strains 381, 33277, W12 and 10046 comprised the members of the highly invasive group. Of the 26 strains tested, only strain AJW4 was non-invasive since the CFU recovered from the cells infected with *P. gingivalis* AJW4 were less than the CFU recovered from cells infected with the non-invasive control strain, *E. coli* MC1061.

Interestingly, the CFU of the non-invasive *E. coli* MC1061 recovered from HUVEC were 16.5 times greater than the CFU recovered from infected KB cells. This difference was statistically significant as determined by the Student's *t*-test ($P < 0.002$) (Microsoft Excel, Redmond, WA, USA). The HUVEC may have a limited phagocytic capability. However, unlike *E. coli* MC1061, the non-invasive strain AJW4 was not internalized to a greater degree in the HUVEC than in the KB cells.

### 3.2. FimA analysis

Since fimbriae have been implicated as a requirement for invasion by *P. gingivalis* [14,15], we investigated the presence and expression of *fimA*, the major fimbrial protein, in strain AJW4. An explanation for the lack of invasive ability of strain AJW4 could be the absence of *fimA* in the genome or non-expression of the gene. To determine whether the *fimA* gene was present, PCR was performed

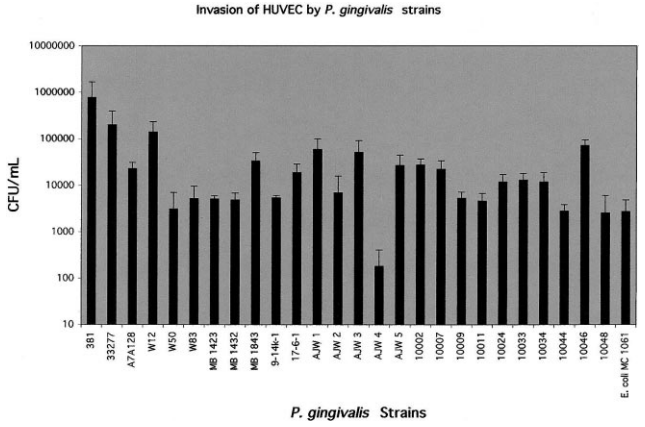


Fig. 1. Invasion of $10^5$ HUVEC by *P. gingivalis* strains at a multiplicity of infection (MOI) of 100. Data presented are averages of three separate experiments. Error bars denote standard deviation.

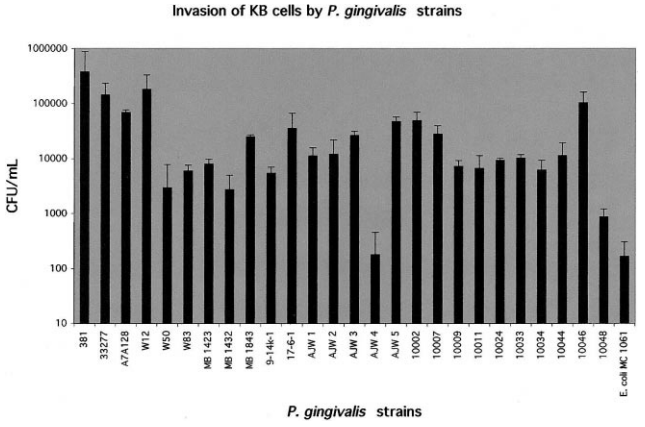


Fig. 2. Invasion of $10^5$ KB cells by *P. gingivalis* strains at a MOI of 100. Data presented are averages of three separate experiments. Error bars denote standard deviation.

on genomic DNA of AJW4. The PCR of strain AJW4 genomic DNA produced a 1.4-kb band that corresponded to the size of the *fimA* gene in strain 381 (Fig. 3A). Additional PCR analysis revealed that the *fimA* gene present in AJW4 was type IV (Fig. 3B), the same type that is present in strains W50 and W83 [17].

To rule out a translational defect, RT-PCR of mRNA transcripts from strain AJW4 was performed. This also produced the 1.4-kb fragment that corresponds to the *fimA* gene (Fig. 3C). This demonstrated that the gene was transcribed. However, the RT-PCR product from strain AJW4 in Fig. 3C was less intense than the RT-PCR product from strain 381. There may be a lower level of transcription of *fimA* in strain AJW4. Finally, cells of AJW4 were examined by electron microscopy to determine if intact fimbriae could be observed. Negatively stained preparations of *P. gingivalis* AJW4 had intact fimbriae present on their surface (Fig. 3D). Thus, *P. gingivalis* AJW4 contains a type IV *fimA* gene that is transcribed and expressed.

### 3.3. Adherence and invasion

The relative abilities of *P. gingivalis* strains 381, W50 and AJW4, and the non-invasive *E. coli* MC1061 to adhere and to invade were determined using a modified ver-

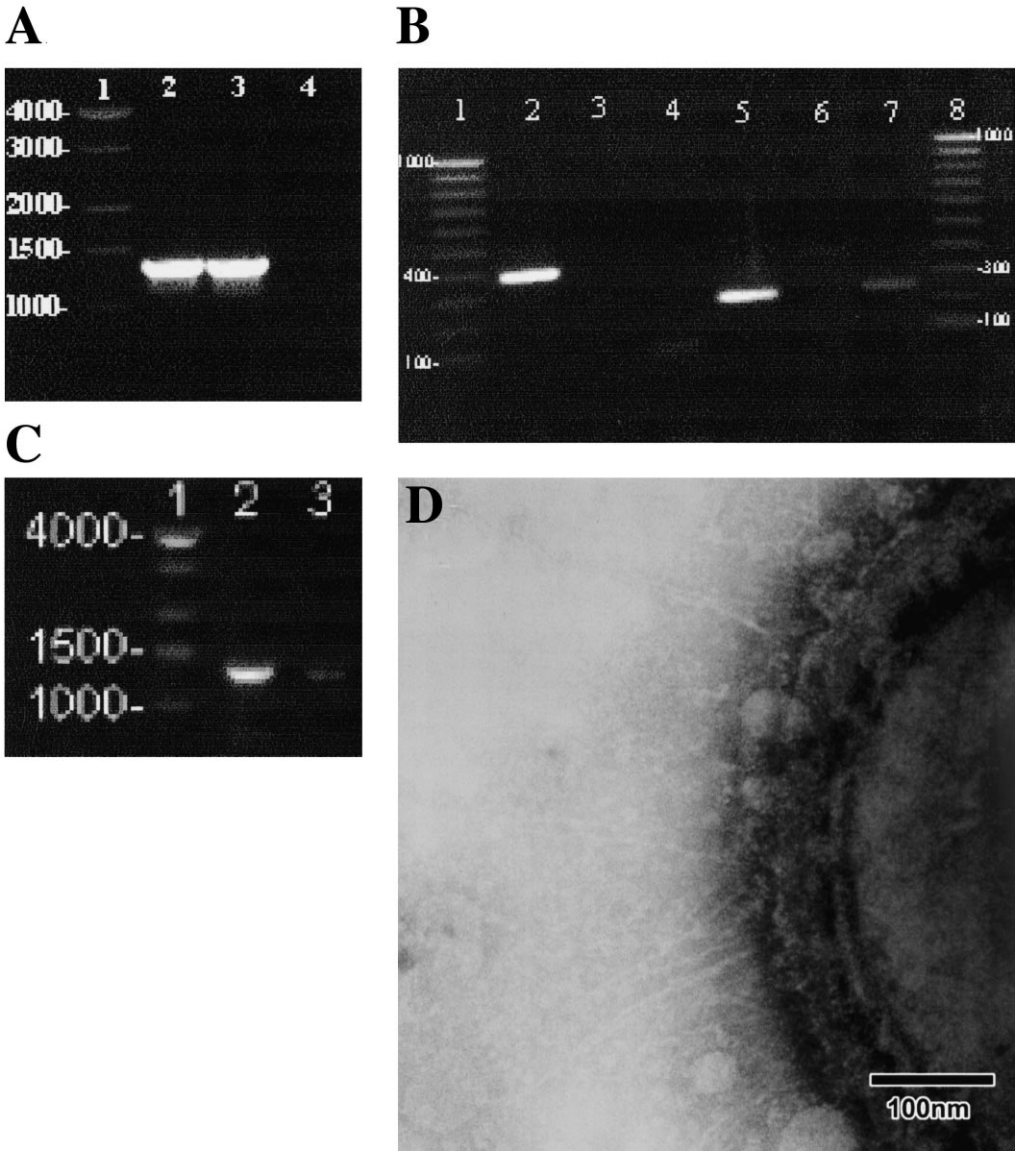


Fig. 3. Analysis of *fimA*. (A) PCR analysis of genomic DNA using *fimA* primers. Lane 1, Novagen perfect marker (Novagen, Inc., Madison, WI, USA); lane 2, *P. gingivalis* 381; lane 3, *P. gingivalis* AJW4; and lane 4, *E. coli* MC1061. (B) PCR analysis of *fimA* subtype. Lane 1, FMC 100-bp ladder (FMC BioProducts, Rockland, ME, USA); lane 2, *P. gingivalis* 381 with type I primers; lane 3, *P. gingivalis* 381 with type IV primers; lane 4, *P. gingivalis* W50 with type I primers; lane 5, *P. gingivalis* W50 with type IV primers; lane 6, *P. gingivalis* AJW4 with type I primers; lane 7, *P. gingivalis* AJW4 with type IV primers; and lane 8, FMC 100-bp ladder. (C) Analysis of *fimA* mRNA by RT-PCR with *fimA* primers. Lane 1, Novagen perfect marker; lane 2, *P. gingivalis* 381; and lane 3, *P. gingivalis* AJW4. (D) Negative stain electron micrograph of *P. gingivalis* AJW4.

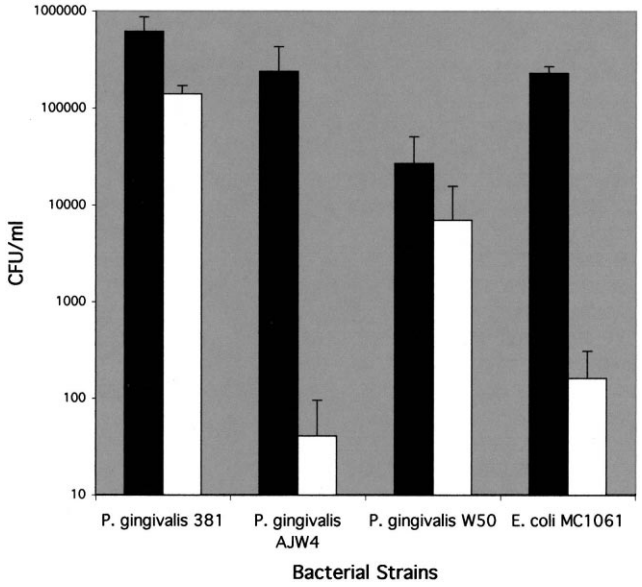


Fig. 4. Adherence and invasion of KB cells by *E. coli* MC1061, *P. gingivalis* strains 381, AJW4 and W50. Adherence+invasion (■) was measured by lysing the KB cells prior to the antibiotic incubation; whereas invasion alone (□) was measured by lysing the KB cells after application of the antibiotics for 1 h. Data presented are averages of three separate experiments. Error bars denote standard deviation.

sion of the antibiotic protection assay [14]. To this end, KB cells were lysed following 90 min of infection with no antibiotic. Therefore, the CFU recovered are the number of bacteria that adhered and/or invaded the KB cells. The CFU recovered from cells in parallel wells to which antibiotic was added represent only those bacteria that were internalized. Therefore the difference in the two quantities is a measure of the number of bacteria that adhered but did not invade. *P. gingivalis* 381 adhered in the greatest numbers (Fig. 4). Strain AJW4 adhered slightly less, but nearly as well as *P. gingivalis* 381. Strain W50 adhered the least, about 10-fold less than strain AJW4. The invasive abilities of these strains in this assay were similar to the values in Fig. 2. In this set of experiments, 22.6% of *P. gingivalis* 381 and 25.9% of strain W50 that adhered also invaded as compared to only 0.02% of strain AJW4. The adherence+invasion of strain AJW4 was 38.7% of strain 381. However, strain AJW4 invasion of KB cells was 2.9% of strain 381. Strain W50 invaded KB cells 170 times greater than strain AJW4, even though strain AJW4 adhered 8.9 times more than strain W50. Thus invasion does not necessarily correlate with adherence or with presence and expression of the *fimA* gene.

## 4. Discussion

Invasion by *P. gingivalis* has been proposed as a possible mechanism of pathogenesis in periodontal [18] and cardiovascular diseases [10,19]. Previous invasion studies have used *P. gingivalis* strains 381 and 33277, which are genetically the same. We explored the heterogeneity of invasive abilities within this species using a variety of *P. gingivalis* strains. Twenty-six different strains of *P. gingivalis* exhibited a range of invasion efficiencies. Only one of the 26 strains did not invade HUVEC or KB cells.

Since previous reports indicated that fimbriae encoded by *fimA* are necessary for adherence and invasion by *P. gingivalis*, we investigated the presence and expression of *fimA* in strain AJW4. The 1.4-kb *fimA* gene was present within this strain, and encodes type IV fimbriae. The *fimA* gene was transcribed and expressed by strain AJW4 as analyzed by RT-PCR and negative stain microscopy.

Strain AJW4 was also tested for its ability to adhere to KB cells. A previous study by Duncan et al. [11] demonstrated that strain W50 adherence to immobilized KB cells was low as compared to strain 33277. This most likely accounts for strain W50 being classified in the low invasive group. If a bacterium cannot adhere, it cannot invade. Adherence of strain AJW4 was 38.7% of the highly invasive strain 381. However the CFU of strain AJW4 recovered following the antibiotic incubation, which selects for intracellular bacteria, were 2.9% of strain 381. Strain W50 was tested for its adherence and invasion since this strain also possesses the same type IV class of the *fimA* gene. The adherence of strain W50 was 8.9-fold lower, but invasion was 170-fold greater than the invasion by strain AJW4. Thus, the type of fimbriae cannot solely explain the relative and absolute values of adherence and invasion.

In conclusion, strains of *P. gingivalis* exhibit a range of invasion efficiencies of human oral epithelial KB cells and HUVEC. Of 26 strains tested, only strain AJW4 was non-invasive. A type IV *fimA* gene was present, transcribed and expressed in strain AJW4. Strain AJW4 had a decreased ability to adhere to KB cells as compared to strain 381, but not to strain W50. Even though strain W50 was less adherent than strain AJW4, the ability of strain W50 to be internalized was 170 times greater than strain AJW4. Therefore, *fimA* may be necessary but is not sufficient for invasion of host cells by *P. gingivalis*.

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