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Invasion of Epithelial Cells and Proteolysis of Cellular Focal Adhesion Components by Distinct Types of *Porphyromonas gingivalis* Fimbriae

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Porphyromonas gingivalis **fimbriae are classified into six types (types I to V and Ib) based on the *fimA* genes encoding FimA (a subunit of fimbriae), and they play a critical role in bacterial interactions with host tissues. In this study, we compared the efficiencies of *P. gingivalis* strains with distinct types of fimbriae for invasion of epithelial cells and for degradation of cellular focal adhesion components, paxillin, and focal adhesion kinase (FAK). Six representative strains with the different types of fimbriae were tested, and *P. gingivalis* with type II fimbriae (type II *P. gingivalis*) adhered to and invaded epithelial cells at significantly greater levels than the other strains. There were negligible differences in gingipain activities among the six strains; however, type II *P. gingivalis* apparently degraded intracellular paxillin in association with a loss of phosphorylation 30 min after infection. Degradation was blocked with cytochalasin D or in mutants with *fimA* disrupted. Paxillin was degraded by the mutant with Lys-gingipain disrupted, and this degradation was prevented by inhibition of Arg-gingipain activity by Nα-*p*-tosyl-L-lysine chloromethyl ketone. FAK was also degraded by type II *P. gingivalis*. Cellular focal adhesions with green fluorescent protein-paxillin macroaggregates were clearly destroyed, and this was associated with cellular morphological changes and microtubule disassembly. In an in vitro wound closure assay, type II *P. gingivalis* significantly inhibited cellular migration and proliferation compared to the cellular migration and proliferation observed with the other types. These results suggest that type II *P. gingivalis* efficiently invades epithelial cells and degrades focal adhesion components with Arg-gingipain, which results in cellular impairment during wound healing and periodontal tissue regeneration.**

Porphyromonas gingivalis, a gram-negative black-pigmented anaerobe, is considered a bona fide pathogen that causes several forms of severe periodontal disease (16, 22). This organism expresses a number of potential virulence factors, including fimbriae, as well as Arg-specific cysteine proteinases (gingipains A and B [RgpA and RgpB, respectively]) and Lys-specific cysteine proteinase (Lys-gingipain [Kgp]), which contribute to the pathogenesis of periodontitis (16, 33). Fimbriae reportedly mediate bacterial invasion of several human epithelial cell lines and contribute to the persistence of *P. gingivalis* at intracellular locations in vitro, which may protect the pathogen from detection by the immune system, leading to further spread into adjacent tissues (21, 27, 30, 39–41). Gingipains have also been reported to modify cellular integrity; gingival fibroblasts and epithelial cells infected with *P. gingivalis* showed reduced adhesion to the extracellular matrix, changes in morphology from spreading to rounded, and impaired motility on matrices (5, 32, 33, 35). These virulence potentials are suggested to be a pathogenetic paradigm of infection, in which *P. gingivalis* disrupts cellular integrity in periodontal tissues.

Epithelial cells form a tight barrier that prevents mucosal penetration by bacterial pathogens (21). Cellular integrins are critical molecules that mediate epithelial barrier formation, as well as cell activation, proliferation, differentiation, metabolism, and motility (10). These integrins provide a physical link, via focal adhesion, between the extracellular environment and the intracellular cytoskeleton (7). Focal adhesions are intimately involved in cellular anchorage and directed migration, as well as in signal transduction pathways, which control wound healing and regeneration, as well as tissue integrity (12). During these events, paxillin and focal adhesion kinase (FAK) play important roles. The phosphorylation of FAK is a central regulator of cell migration during integrin-mediated control of cell behavior (31). Paxillin is localized in cultured cells, primarily at sites of adhesion of cells to the extracellular matrix (i.e., focal adhesions), and activation of this molecule is a prominent event upon integrin activation for actin-cytoskeleton formation, as well as the recruitment of FAK to robust focal adhesions (25, 28). It was previously reported that *P. gingivalis* invades epithelial cells and subsequently degrades paxillin and FAK, resulting in impaired cellular function (15). These bacterial effects are suspected to be mainly due to the activities of gingipains, which follow fimbria-mediated bacterial invasion of cells.

P. gingivalis fimbriae are capable of binding specifically to components lining the oral cavity, such as salivary proteins, commensal bacteria, several types of extracellular matrices,

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TABLE 1. Efficiency of adhesion to and invasion of epithelial cells by *P. gingivalis* determined by two different methods

Strain	Assay method	Units	Amt of *P. gingivalis* added	*P. gingivalis* adhering and invading		*P. gingivalis* invading	
				Amt	%	Amt	%
ATCC 33277	Colony forming	CFU	$9.75 \times 10^6 \pm 0.45 \times 10^6$	$1.28 \times 10^6 \pm 0.34 \times 10^6$	13.1 ± 2.9	$9.30 \times 10^5 \pm 0.80 \times 10^5$	9.5 ± 2.2
ATCC 33277	^{3}H counting	cpm	$1.05 \times 10^4 \pm 0.02 \times 10^4$	$1.62 \times 10^3 \pm 0.08 \times 10^3$	15.3 ± 1.1	$9.10 \times 10^2 \pm 0.16 \times 10^2$	8.6 ± 0.2
OMZ314	Colony forming	CFU	$9.33 \times 10^6 \pm 0.42 \times 10^6$	$1.63 \times 10^5 \pm 0.54 \times 10^5$	17.3 ± 4.2	$1.33 \times 10^5 \pm 0.40 \times 10^5$	14.2 ± 4.1
OMZ314	^{3}H counting	cpm	$1.28 \times 10^4 \pm 0.07 \times 10^4$	$2.64 \times 10^3 \pm 0.15 \times 10^3$	20.3 ± 2.4	$2.11 \times 10^3 \pm 0.08 \times 10^3$	16.5 ± 0.0

and host cells, including gingival fibroblasts, epithelial cells, and endothelial cells (13). These adhesive abilities are considered to be a major pathogenic trait that causes periodontal tissue destruction. *P. gingivalis* fimbriae are classified into six types (types I to V and Ib) based on different nucleotide sequences of the *fimA* genes encoding FimA (a subunit of fimbriae) (1). Our previous epidemiological studies revealed that a majority of periodontitis patients harbored *P. gingivalis* with type II fimbriae (type II *P. gingivalis*) (2, 3, 24). However, it is not known whether there are functional differences among the six distinct types that contribute to the different pathogenicities. Since fimbriae are a type of adhesin (13), the adhesive abilities and affinities for the host of the six types may differ, and the differences could be related to distinct virulence traits. We previously found that significantly more microspheres conjugated with the recombinant protein of *P. gingivalis* type II fimbriae adhered to human epithelial cells than did microspheres conjugated with other types of fimbriae (23). Thus, variations among fimbriae may influence the bacterial invasion of epithelial cells, as well as the subsequent degradation of paxillin and FAK. In the present study, we evaluated six representative *P. gingivalis* strains with the different types of fimbriae and focused on their effects on bacterial invasion and degradation of paxillin and FAK in epithelial cells.

MATERIALS AND METHODS

Bacterial strains. The following *P. gingivalis* strains were used in this study: ATCC 33277, with type I *fimA* [*fimA* (I)]; HG1691, with *fimA* (Ib); OMZ314, with *fimA* (II); 6/26, with *fimA* (III); HG564, with *fimA* (IV); HNA99, with *fimA* (V); KDP150, a *fimA* mutant of ATCC 33277 [Δ*fimA* (I)] (38); and a mutant of OMZ314 with *fimA* disrupted [Δ*fimA* (II)] (26). In addition, ATCC 33277 (34), KDP129 (Δ*kgp*), KDP133 (Δ*rgpAB*), and KDP136 (Δ*rgpAB*/Δ*kgp*) mutants with gingipain genes disrupted were kindly provided by K. Nakayama (Nagasaki University, Japan). The organisms were grown anaerobically in GAM broth (Nissui, Tokyo, Japan) or on Trypticase soy agar plates (Difco, BD Diagnostics, Sparks, MD) supplemented with 5% sheep blood (Nihon Biotest, Tokyo, Japan), 5 μg/ml of hemin (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 1 μg/ml of menadione (Sigma-Aldrich, Saint Louis, MO), as described previously (23). Rgp and Kgp activities were determined using the synthetic substrates *t*-butyloxycarbonyl-L-leucylglycyl-L-arginine-4-metylcoumaryl-7-amide and *t*-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine-4-metylcoumaryl-7-amide (Peptide Institute, Osaka, Japan), respectively. The cell lysates and culture supernatants were incubated at 37°C for 1 h with the synthetic substrate (100 μM). The amount of 4-metylcoumaryl-7-amide released was determined at 460 nm with excitation at 380 nm using a fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

Cell cultures. Human cervical epithelial HeLa cells (CCL-2) were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (Invitrogen), 20 μg/ml of gentamicin (Sigma-Aldrich), and 4 mM L-glutamine (Invitrogen) at 37°C in the presence of 5% CO_2.

Adhesion and invasion assays. Adhesion to and invasion of HeLa cells by *P. gingivalis* were quantified using two different antibiotic protection assays, a colony-forming assay and a scintillation counting assay, as described previously (23).

For the colony-forming assay, *P. gingivalis* strains ATCC 33277 and OMZ314 were cultured until the optical density at 600 nm was 0.8, and then the bacterial cells were harvested and washed with prereduced sterile phosphate-buffered saline (PBS). The number of bacteria in each suspension was estimated by determining the optical density at 600 nm and extrapolating from a standard curve, as described previously (20). *P. gingivalis* cells were added to a monolayer of HeLa cells (1×10^5 cells/well) in a 24-well culture plate at a multiplicity of infection (MOI) of 200 and then incubated for 90 min at 37°C in the presence of 5% CO_2. External nonadherent bacteria were removed by washing the cells three times with PBS, after which the cells were disrupted by addition of 100 μl of distilled water and incubation at 37°C for 10 min. Serial dilutions of the disrupted mixture were plated on blood agar plates and incubated for 10 days, and the numbers of adherent and invading organisms were determined. To determine the numbers of invading bacteria, *P. gingivalis*-infected HeLa cells were incubated for 1 h with DMEM containing gentamicin (300 μg/ml) and metroimidazole (200 μg/ml; Sigma-Aldrich). The cells were washed three times with PBS, and the numbers of internalized bacteria were determined as described above.

For the scintillation counting assay, *P. gingivalis* strains with distinct types of fimbriae were incubated separately with 0.1 mCi of [*methyl*-^{3}H]thymidine for 24 h, after which the bacterial cells were harvested and washed with prereduced sterile PBS. The number of bacteria in each suspension was estimated by determining the optical density at 600 nm as described above. ^{3}H-labeled *P. gingivalis* cells (MOI, 100 to 1,000) were added to monolayers of HeLa cells as described above. External nonadherent bacteria were removed by washing the cells three times with PBS, after which the cells were disrupted by addition of 100 μl of distilled water and incubation at 37°C for 10 min. The numbers of adhering and invading organisms were determined using a liquid scintillation counter (model LSC-5100; Aloka Co., Ltd., Tokyo, Japan) and from the amounts of ^{3}H recovered from infected cells, and the results were expressed as percentages of the total number of *P. gingivalis* cells added. To determine the numbers of invading bacteria, *P. gingivalis*-infected HeLa cells were incubated for 1 h with DMEM containing antibiotics. The cells were washed three times with PBS, and the numbers of internalized bacteria were determined as described above. To inhibit actin polymerization, cytochalasin D (0.5 μg/ml; Wako) was added to the medium 30 min prior to infection.

Immunoblotting of paxillin and FAK. HeLa cells (4.0×10^5 cells/60-mm culture dish) in DMEM were incubated with *P. gingivalis* at different MOIs for various times. *P. gingivalis*-infected cells were washed with ice-cold PBS containing 10 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (Wako) and then dissolved in Triton-lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 10 mM TLCK, 6.25 mM NaF, 12.5 mM β-glycerophosphate, 12.5 mM *p*-nitrophenyl phosphate, 1.25 mM $NaVO_3$, 1% protease inhibitor cocktail [Complete protease inhibitor cocktail; Roche Diagnostics, Basel, Switzerland]). The soluble fractions were collected by centrifugation at $15{,}000 \times g$ for 5 min at 4°C, and immunoblotting was performed as described previously (18). Briefly, equal amounts of cellular proteins (20 μg) were denatured in sodium dodecyl sulfate gel loading buffer and were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were then transferred to a polyvinylidene difluoride membrane (Hybond P, Amersham Biosciences, Uppsala, Sweden) and reacted with polyclonal antisera against paxillin, phospho-paxillin, and phospho-FAK (Cell Signaling Technologies, Beverly, MA), as well as a monoclonal antibody against FAK (Transduction Laboratories, Lexington, KY). Proteins or phosphorylated proteins were detected using the ECL Plus reagent (Amersham Biosciences).

In vitro wound closure assay. HeLa cells (5.0×10^4 cells/24-well culture dish) in DMEM with 10% FCS were cultured until they were confluent. The cell layers were scratched using a plastic tip and washed three times with serum-free DMEM to remove debris, as described previously (18). HeLa cells were infected with *P. gingivalis* viable cells at an MOI of 100. The culture plates were then incubated for 24 or 48 h at 37°C in DMEM containing 10% FCS, 20 μg/ml of

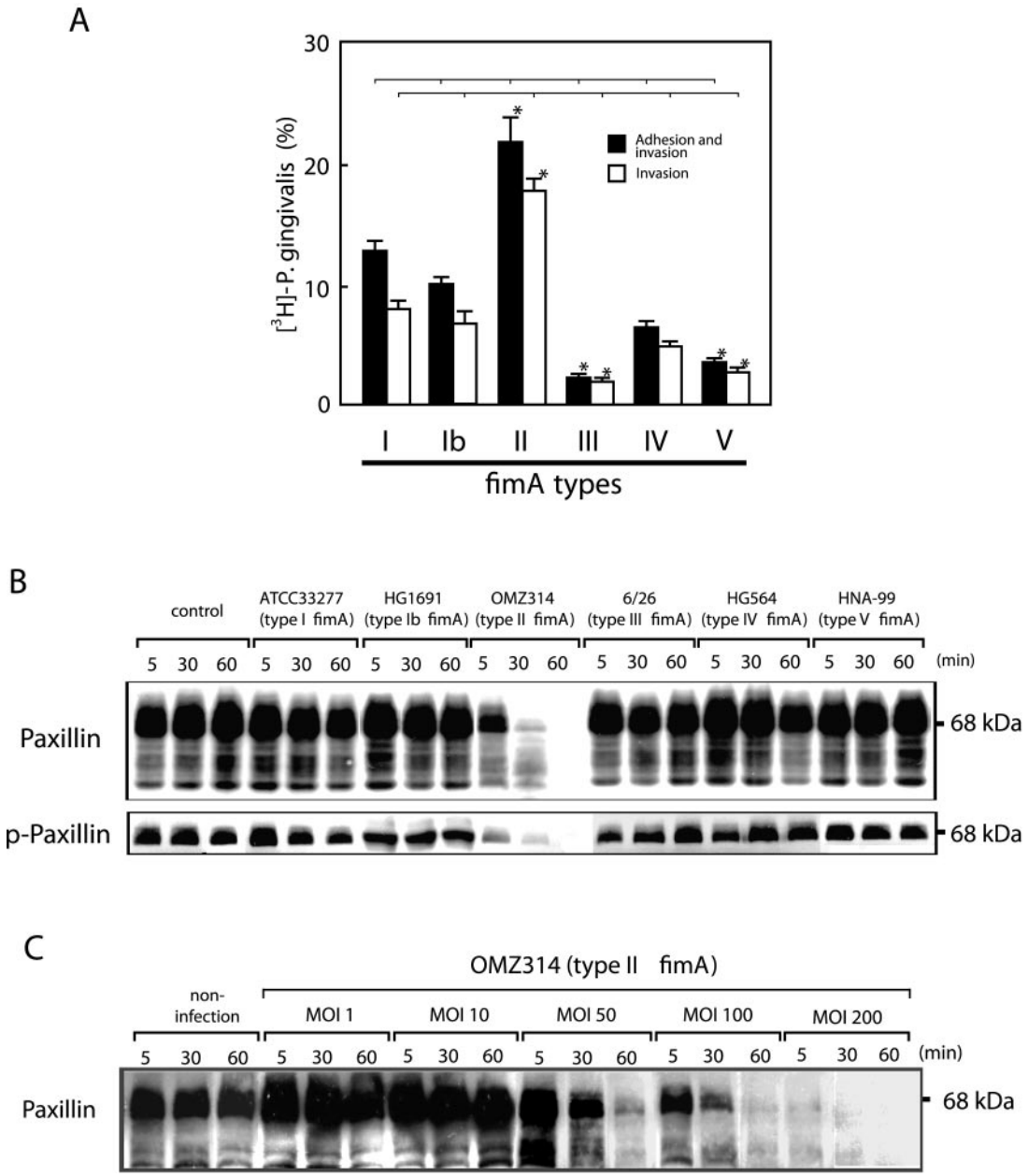

FIG. 1. Degradation of paxillin associated with adhesion to and invasion of epithelial cells by *P. gingivalis*. (A) Adhesion to and invasion of HeLa cells by *P. gingivalis* strains with distinct types of fimbriae (types I to V and Ib). HeLa cells (1×10^5 cells in a 24-well plate) were infected with [^{3}H]thymidine-labeled *P. gingivalis* cells at an MOI of 200 for 90 min. The numbers of adhering and/or invading bacteria were determined as described in Materials and Methods. An asterisk indicates that the *P* value is <0.05. Multiple comparisons of the numbers of type II *P. gingivalis* and the numbers of other strains were performed. (B) Degradation of paxillin in *P. gingivalis*-infected epithelial cells. HeLa cells (4×10^5 cells in a 60-mm dish) were infected with *P. gingivalis* with distinct types of fimbriae at an MOI of 200 for 5, 30, and 60 min. Degradation was assayed by immunoblotting the cellular lysates with specific antibodies. (C) Degradation of cellular paxillin with different numbers of type II *P. gingivalis* (strain OMZ314) cells. HeLa cells (4×10^5 cells in a 60-mm dish) were infected with *P. gingivalis* strain OMZ314 at various MOIs for 5, 30, and 60 min. Degradation was assayed by immunoblotting the cellular lysates with specific antibodies. p-Paxillin, phospo-paxillin.

gentamicin, and 4 mM L-glutamine. The rate of wound closure was determined using NIH Image analysis, as described previously (18). All assays were performed in triplicate on three separate occasions ($n = 9$).

Fluorescence analysis of paxillin. An enhanced green fluorescent protein-paxillin fusion expression vector (EGFP-paxillin) (29) was kindly provided by K. Rottner (Austrian Academy of Sciences, Institute of Molecular Biology, Salzburg, Austria). Approximately 2×10^4 HeLa cells were placed on 0.1% gelatin-coated cover glasses (Matsunami Glass, Osaka, Japan) in a 24-well culture plate. Next, the cells were transfected with the plasmid (2 μg) using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were infected with *P. gingivalis* OMZ314 or the Δ*fimA* (II) mutant for 1 h, washed extensively with ice-cold PBS three times, and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After washing with PBS, the cells were incubated with Alexa Fluor 594-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) to detect filamentous F-actin. Images were obtained with a laser scanning confocal micro-

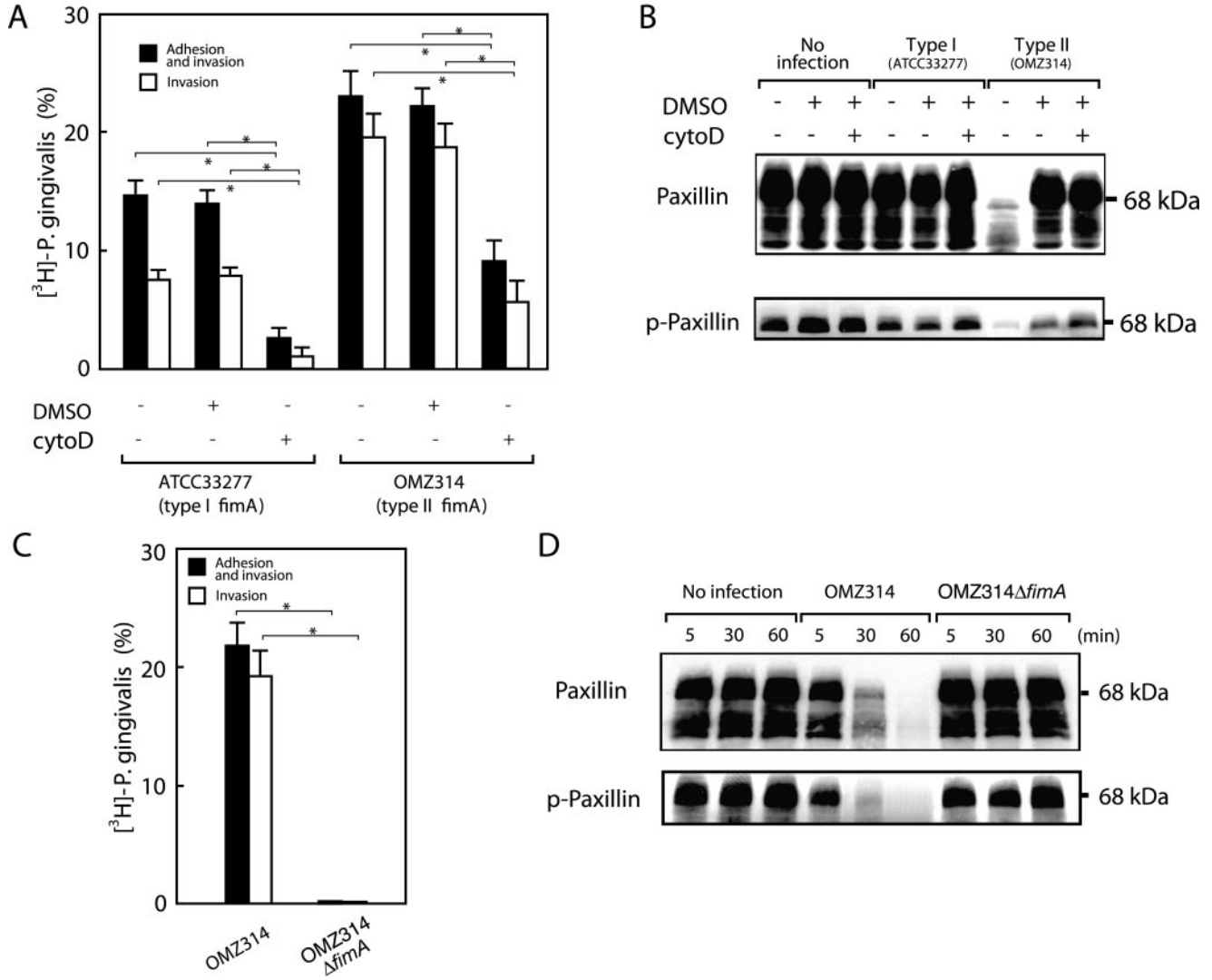

FIG. 2. Paxillin degradation is dependent on *P. gingivalis* invasion of epithelial cells. (A) Adhesion to and invasion of HeLa cells by *P. gingivalis* in the presence of cytochalasin D (cytoD). HeLa cells (1×10^5 cells in a 24-well plate) were infected with type I (ATCC 33277) and type II (OMZ314) *P. gingivalis* strains at an MOI of 200 for 90 min, similar to the method used for the experiment whose results are shown in Fig. 1. Cytochalasin D in dimethyl sulfoxide (DMSO) (final concentration, 10 μg/ml) was added to the cell culture 1 h prior to infection. Dimethyl sulfoxide (1/1,000, vol/vol) was used as a negative control. (B) Effect of cytochalasin D on paxillin degradation by *P. gingivalis*. Degradation was assayed by immunoblotting cellular lysates with specific antibodies. (C) Adhesion to and invasion of HeLa cells by *P. gingivalis* OMZ314 (type II wild type) and a mutant with *fimA* disrupted [OMZ314Δ*fimA*(II)] at an MOI of 200. An asterisk indicates that the *P* value is <0.05. (D) Paxillin degradation by *P. gingivalis* with *fimA* disrupted. HeLa cells (1×10^5 cells in a 24-well plate) were infected with *P. gingivalis* strain OMZ314 or OMZ314Δ*fimA*(II) at an MOI of 200 for 5, 30, and 60 min. p-Paxillin, phospo-paxillin.

scope (model LSM510; Carl Zeiss, Thornwood, NY). Fluorescent images were obtained at a magnification of ×630, with the laser power and irradiation time minimized to avoid photobleaching and possible photodynamic effects.

Statistical analyses. All data are expressed below as means ± standard deviations. Statistical analyses were performed using an unpaired Student's *t* test. Multiple comparisons were performed by one-way analysis of variance and Sheffe's test using the STAT View software (SAS Institute Inc., Cary, NC).

RESULTS

Degradation of paxillin associated with adhesion to and invasion of epithelial cells by *P. gingivalis*. Adhesion to and invasion of epithelial cells by *P. gingivalis* were assayed by two different methods, a colony-forming assay and a [^{3}H]thymidine counting assay. As shown in Table 1, the numbers of *P. gingivalis* cells which adhered and invaded as determined by the colony-forming assay were found to be positively correlated with the numbers of cells determined using the scintillation counting method. In the colony-forming assay intracellular survival is used as a measure of invasion, and thus the organisms have to remain viable throughout the process. This is a major limitation for studies of *P. gingivalis*, which is a strict anaerobe. Therefore, we used the scintillation counting method for all other experiments. The data for adhesion to and invasion of epithelial cells by *P. gingivalis* strains with the distinct types of fimbriae were compared. The type II *P. gingivalis* adhesion and invasion were significantly greater than the adhesion and in-

TABLE 2. Gingipain activities of *P. gingivalis* strains with distinct types of fimbriae

Type of fimbriae (strain)	Gingipain activities (Δ380+460/min/μg protein)[a]	
	Rgp	Kgp
Wild type		
I (ATCC 33277)	320.39 ± 16.21	120.06 ± 14.38
Ib (HG1691)	322.91 ± 13.67	131.83 ± 21.75
II (OMZ314)	338.67 ± 6.61	121.68 ± 11.59
III (6/26)	361.18 ± 16.85	144.70 ± 18.99
IV (HG564)	344.54 ± 9.45	165.37 ± 6.07
V (HNA99)	312.31 ± 17.36	133.00 ± 9.92
fimA mutants		
I (KDP150)	362.26 ± 18.75	136.79 ± 17.60
II (OMZ314Δ*fimA*)	347.00 ± 9.33	138.78 ± 7.20

[a] Δ380+460, emission at 460 nm with excitation at 380 nm.

vasion observed with the other strains (Fig. 1A). Next, the effects of the various *P. gingivalis* strains on cellular paxillin (molecular mass, 68 kDa) were examined (Fig. 1B). Paxillin was markedly degraded 30 min after infection with type II *P. gingivalis*, and this was associated with the disappearance of phosphor-paxillin, whereas negligible degradation was induced by infection with the other strains of *P. gingivalis*. Furthermore, paxillin degradation was induced more when an increased number of type II *P. gingivalis* organisms had infected the cells (Fig. 1C). These results suggest that invasion by type II *P. gingivalis* is involved in the degradation of paxillin.

Degradation of paxillin is dependent on bacterial invasion. Cytochalasin D, an inhibitor of actin polymerization, is known to inhibit the invasion of epithelial cells by *P. gingivalis* (8, 42). Cytochalasin D apparently prevented bacterial adhesion and invasion, as shown in Fig. 2A, while the degradation of paxillin by type II *P. gingivalis* was also prevented (Fig. 2B). To further confirm the involvement of bacterial invasion in paxillin degradation, we employed a mutant with fimbriae disrupted, Δ*fimA*(II). When this mutant was used, there was a significant lack of adhesion and invasion (Fig. 2C), and no degradation of paxillin was observed (Fig. 2D).

Involvement of gingipains in paxillin degradation. Since it was thought that the degradation of paxillin could be related to the various activities of gingipains with the six types of fimbriae, the Rgp and Kgp activities of the strains were compared. However, there were negligible differences among the strains with the six types of fimbriae and the mutants with *fimA* disrupted (Table 2). In addition, mutants with gingipain disrupted were used to examine the involvement of gingipains in the degradation of paxillin. Since no gingipain mutants of strains with type II fimbriae were available, mutants of the type I strain (ATCC 33277) were used at an MOI of 1,000, which was a level previously shown to result in degradation of cellular paxillin with the wild-type strain (15). At an MOI that was 10-fold greater than that of the type II strain, the type I organisms degraded paxillin in a time-dependent manner (Fig. 3A). However, the mutant with *kgp* disrupted degraded paxillin to a greater degree than the wild-type strain degraded paxillin. Kgp has been shown to be not involved in paxillin degradation, whereas Rgp seemed to have paxillin degradation activity, because it was found to be overexpressed and to compensate for Kgp deficiency in a *kgp* mutant (34). The mutants with *rgp* and *fimA* disrupted exhibited markedly reduced degradation of paxillin, although mutants with *rgp* disrupted had low levels of fimbriae on their surfaces (19). Therefore, the lack of degradation seemed to be due the fact that invasion by the nonfimbriated *rgp* mutants, as well as the *fimA* mutant, was prevented. Next, TLCK, a strong inhibitor of gingipains (43), was used to confirm the involvement of Rgp in paxillin degradation. As determined by addition of TLCK, the adhesion/invasion and invasion efficiencies of *P. gingivallis* were not significantly affected (Fig. 3B), while TLCK clearly prevented paxillin degradation by type II *P. gingivalis* at an MOI of 200 (Fig. 3C). These results suggest that both bacterial invasion by the organism and Rgp of type II *P. gingivalis* are essential for paxillin degradation in infected cells.

Degradation of FAK by *P. gingivalis* strains with distinct types of fimbriae. The effects of the *P. gingivalis* strains with distinct types of fimbriae on degradation of FAK were also examined (Fig. 4). Similar to the results obtained with paxillin, FAK was swiftly degraded only by infection with type II *P. gingivalis*, which was associated with the disappearance of phosphorylated FAK. No degradation of FAK was observed following infection with strains with the other types of fimbriae.

Effect of *P. gingivalis* with type II fimbriae on focal adhesion formation by epithelial cells. Paxillin is localized in focal adhesion complexes known as macroaggregates, where it connects to actin stress fibers, which are considered to be a marker of focal adhesion (25). We evaluated the effect of type II *P. gingivalis* on focal adhesion formation (Fig. 5). In the control cells, focal adhesions (green) were localized as macroaggregates. In contrast, type II *P. gingivalis*-infected cells clearly did not exhibit aggregated expression of paxillin and showed uniform localization throughout the cells, which was associated with a rounded morphology and significant disassembly of actin fibers. However, infection with the mutant with *fimA* disrupted did not cause such changes.

Effects of *P. gingivalis* with type II fimbriae on cellular migration and proliferation. Cellular migration and proliferation are critical functions for wound healing and tissue regeneration (25, 28, 31), and *P. gingivalis* has been reported to inhibit these functions (15). Thus, we examined whether the various types of fimbriae had any influence on the effects of *P. gingivalis* with regard to the migration and proliferation of epithelial cells. Using an in vitro wound closure assay, we found that epithelial cells migrated to and filled in wound scratch areas in a time-dependent manner, and the scratched area was completely filled with the control cells within 48 h (Fig. 6). In contrast, all of the *P. gingivalis* strains tested had inhibitory effects on scratch closure, and type II *P. gingivalis* significantly impaired the cellular wound closure process, which was considered to be due to the marked degradation of paxillin and FAK. Such an inhibitory effect was not seen with the mutants with *fimA* disrupted.

DISCUSSION

We studied the effects of *P. gingivalis* strains with distinct types of fimbriae on bacterial invasion of epithelial cells and degradation of cellular focal adhesion components. Type II *P. gingivalis* had significant adhesive and invasive abilities com-

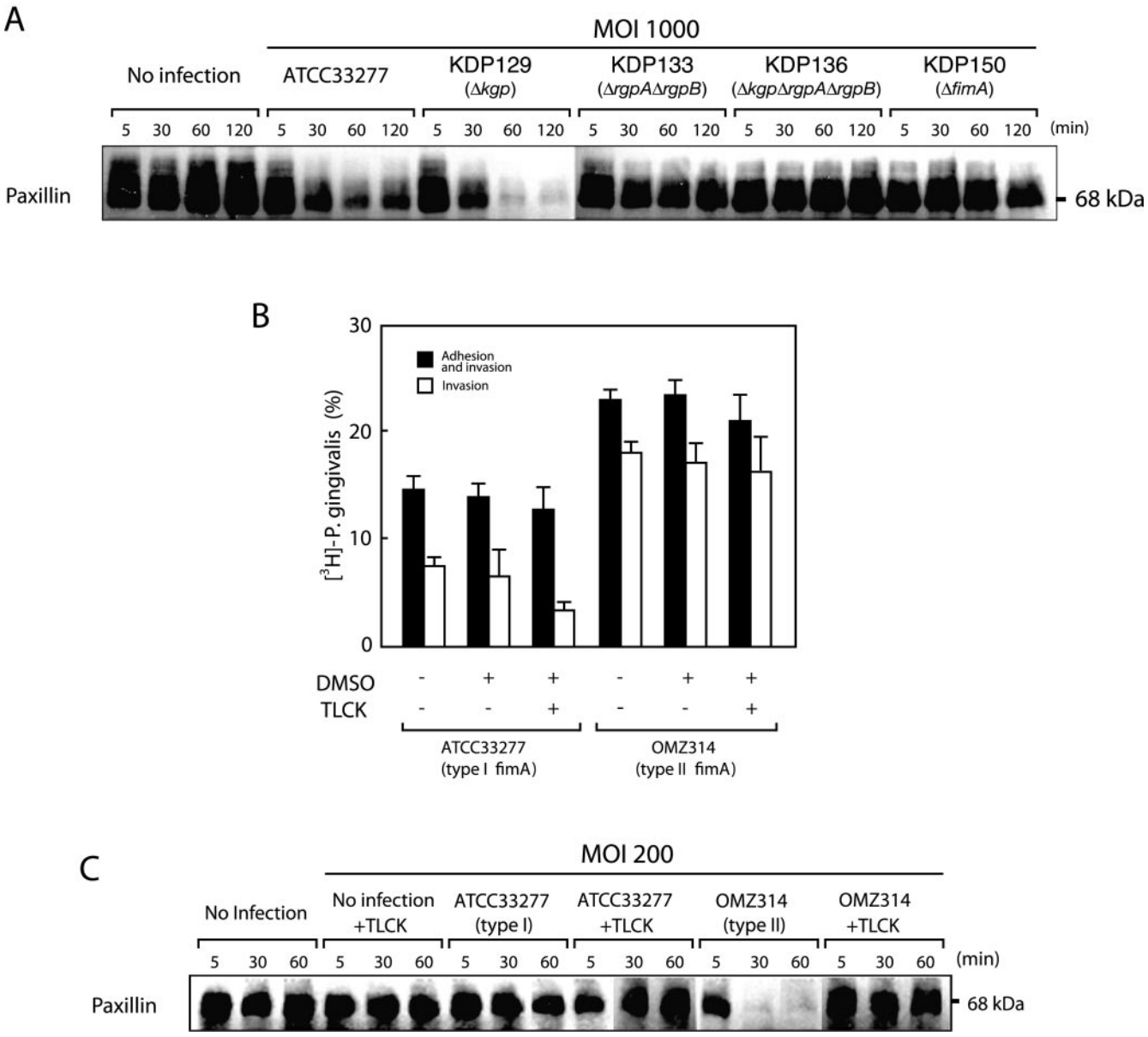

FIG. 3. Involvement of Rgp in paxillin degradation in *P. gingivalis*-infected epithelial cells. (A) HeLa cells (1×10^5 cells in a 24-well plate) were infected with *P. gingivalis* ATCC 33277 (type I fimbriae), its isogenic mutant with *kgp* disrupted (KDP129), a mutant with both *rgpA* and *rgpB* disrupted (KDP133), a mutant with *rgpA*, *rgpB*, and *kgp* disrupted (KDP136), and a mutant with *fimA* disrupted (KDP150) for 5, 30, and 60 min at an MOI of 1,000. Cellular lysates from the *P. gingivalis*-infected cells were analyzed by Western blotting using antipaxillin antibodies. (B) Effect of TLCK on invasion of HeLa cells by *P. gingivalis*. HeLa cells (1×10^5 cells in a 24-well plate) were infected with [^{3}H]thymidine-labeled *P. gingivalis* cells at an MOI of 200 for 90 min. TLCK (10 mM) in dimethyl sulfoxide (DMSO) (final concentration, 0.1%) or 0.1% dimethyl sulfoxide (negative control) was added to the culture 30 min prior to infection. The numbers of adherent and/or invading bacteria were determined as described in Materials and Methods. Statistical analyses were performed by multiple comparisons. (C) HeLa cells (1×10^5 cells in a 24-well plate) were infected with *P. gingivalis* strain ATCC 33277 (type I) or OMZ314 (type II) at an MOI of 200 for 5, 30, and 60 min with or without TLCK (10 mM). Paxillin degradation was analyzed by Western blotting.

pared to the other strains (Fig. 1). In addition, apparent degradation of paxillin by type II *P. gingivalis* was observed, which was dependent on swift invasion of epithelial cells and was mediated by fimbriae (Fig. 2), as well as proteolysis by Rgp (Fig. 3). FAK was also swiftly degraded by type II *P. gingivalis* and not by any of the other organisms tested (Fig. 4). The degradation of focal adhesion components clearly influenced cytoskeletal morphology, as well as cellular migration and proliferation (Fig. 5 and 6). Since paxillin and FAK are critical regulators of wound healing and regeneration of periodontal tissue (25, 28, 31), these virulence traits of type II *P. gingivalis* likely contribute to the development of periodontitis and the associated deterioration. Furthermore, the efficient invasion mediated by type II fimbriae observed in this study may permit sufficient intracellular localization of the pathogen, which might be related to its pathogenicity. In fact, in our previous

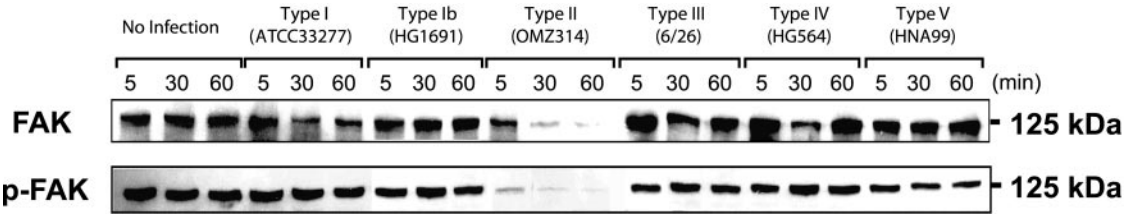

FIG. 4. Degradation of FAK by *P. gingivalis* strains with distinct types of fimbriae. HeLa cells were infected with the *P. gingivalis* strains at an MOI of 200 for 5, 30, and 60 min. Cellular lysates of *P. gingivalis*-infected cells were analyzed by Western blotting using anti-FAK antibodies or anti-phosphorylated FAK (p-FAK) antibodies.

epidemiological study we showed that 60% of periodontitis patients carried type II *P. gingivalis*, while 90% of the patients with advanced periodontitis harbored type II organisms (2, 3). Accumulated evidence shows that various *P. gingivalis* strains have different heterogenic virulence potentials; however, the factor(s) regulating the differences has not been clearly elucidated (1). The present results suggest that the expression of heterogenic virulence properties by various *P. gingivalis* strains is dependent to some extent on the clonal diversity of fimbriae.

In the present experiments, type II *P. gingivalis* significantly inhibited cellular migration and proliferation during the wound closure process (Fig. 6), and it eliminated macroaggregates associated with focal adhesions (Fig. 5). These observations are consistent with the phenotype of paxillin-deficient cells, which exhibit delayed spreading and migration and do not form macroaggregates even when they are cultured on fibronectin-coated dishes (11). Furthermore, the focal adhesion dynamics and organization of the membrane cytoskeletal structures are impaired in paxillin-deficient cells (15). These findings suggest that paxillin degradation by type II *P. gingivalis*

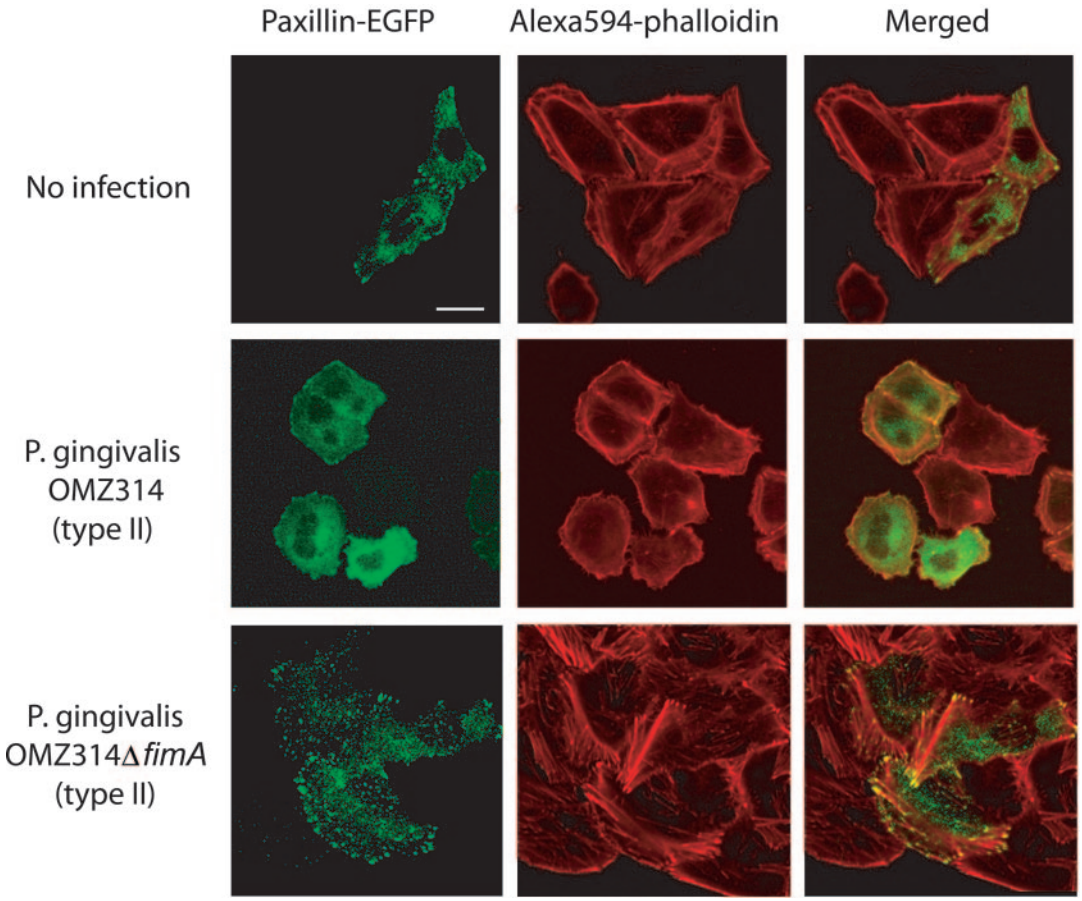

FIG. 5. Effect of *P. gingivalis* with type II fimbriae on formation of focal adhesions by epithelial cells. An enhanced green fluorescent protein EGFP-paxillin expression vector (Paxillin-EGFP) was transfected into HeLa cells, and then the cells were infected with type II *P. gingivalis* (OMZ314) and OMZ314Δ*fimA*(II) for 1 h. The cells were fixed with 4% paraformaldehyde–PBS and stained with Alexa Fluor 594-conjugated phalloidin. Fluorescent images were obtained with a laser scanning confocal microscope at a magnification of ×630. Red, actin; green, paxillin. Bar = 10 μm.

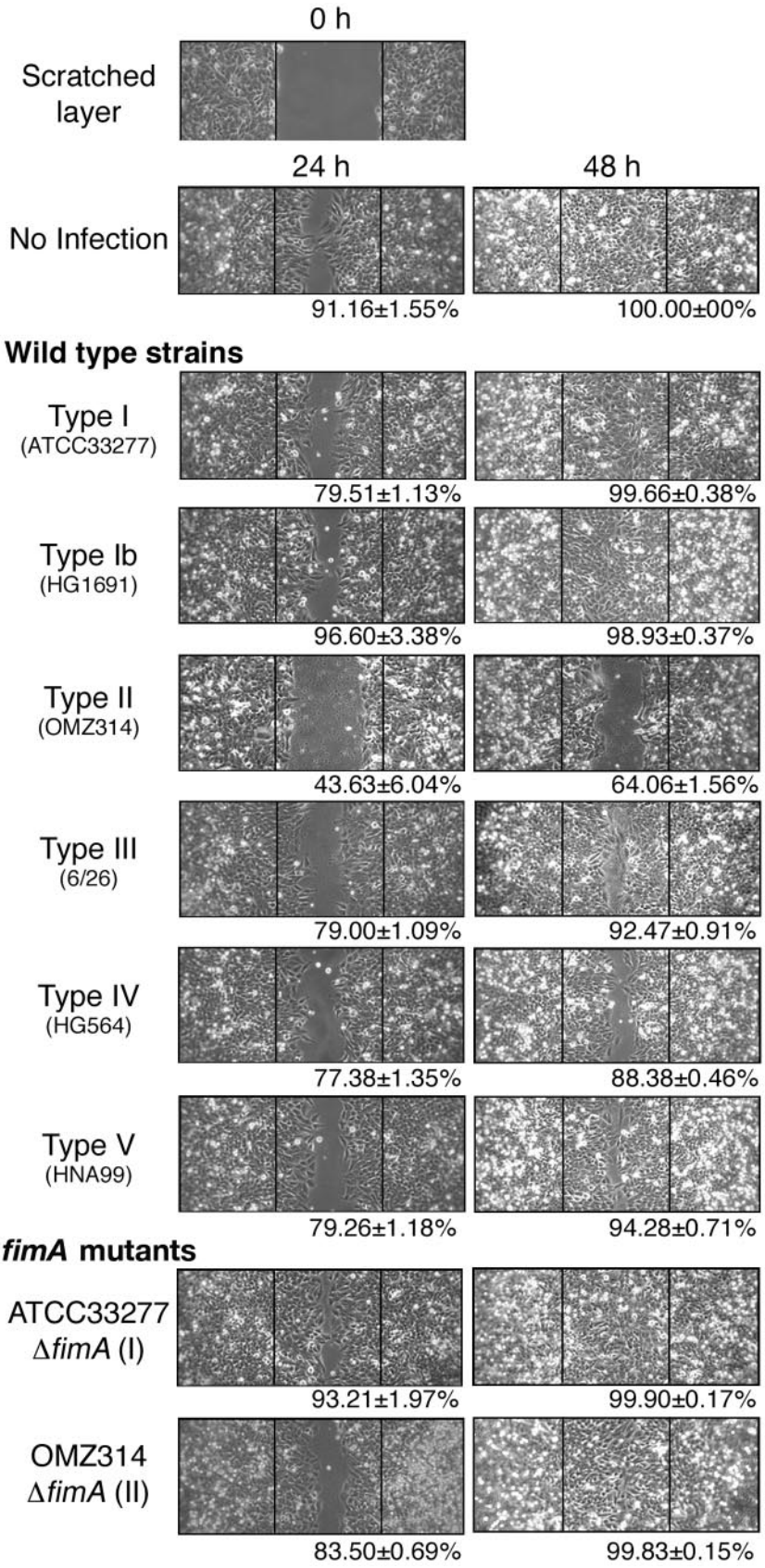

FIG. 6. Microscopic views of wound closure by HeLa cells infected with *P. gingivalis* strains with distinct types of fimbriae. Confluent HeLa cell layers were scratched with a plastic tip. The cells were infected with *P. gingivalis* with distinct types of fimbriae at an MOI of 100, after which the cellular migration and proliferation to the scratched areas were analyzed at 37°C for 24 and 48 h. The images show the scratched wound regions at zero time and 24 and 48 h, and the rates of wound closure, indicated under the images, were determined by assays performed in triplicate on three separate occasions ($n = 9$), as described in Materials and Methods.

causes serious damage, which makes it difficult for the host cells to retain the functions involved in tissue wound healing and regeneration. No biological explanation for the significant adhesive/invasive capacities of type II fimbriae is available. However, type II fimbriae may have a marked affinity with integrin α5β1, which is a receptor molecule for fimbriae (23, 41). It is also possible that other factors influence the proteolytic efficiencies of the focal adhesion components in the six types of fimbriae, such as the varied affinities of the six different strains for paxillin and FAK molecules. Additional study is necessary to examine these possibilities.

The invasive efficiency of *P. gingivalis* seems to be dependent on the ability of fimbriae to adhere to the cell surface and on the numbers of intracellular bacteria. In this study, we observed paxillin degradation with type II *P. gingivalis*-infected cells only at an MOI of 100, while no degradation occurred at an MOI of 10 (Fig. 1C). Similarly, the type I strain (ATCC 33277) failed to degrade paxillin at an MOI of 100, while degradation occurred at an MOI of 1,000. In another study the workers found cellular paxillin degradation and morphological changes caused by infection with strain ATCC 33277 at an MOI of 1,000 but not by infection with strain W50 (type IV) (15), which is a sparsely fimbriated strain and is far less adhesive and invasive than ATCC 33277 (17). However, at an MOI of 100, strain ATCC 33277 failed to degrade paxillin in gingival epithelial cells (41). Together, these findings suggest that infection with a greater number of bacteria (i.e., at a 10-fold-greater MOI) allows effective degradation of paxillin, even by less adhesive strains. The adhesion/invasion level of type I *P. gingivalis* was about one-half that of type II *P. gingivalis*, while the efficiency of degradation of paxillin by type I *P. gingivalis* was found to be much lower than the efficiency of degradation of paxillin by type II *P. gingivalis*. Although we have no convincing explanation for this difference, it might be dependent on the dynamics of *P. gingivalis* after internalization. Recently, several reports have indicated that *P. gingivalis* cell or vesicle internalization is mediated by clathrin-independent processes (35, 37). In addition, it has also been reported that *P. gingivalis* ATCC 33277 has remained within late endosomes with autophagosomal markers (8). These observations indicate that *P. gingivalis* that is internalized in host cells remains in membrane-bound vacuoles, such as endosomes. In contrast, another reports showed that *P. gingivalis* ATCC 33277 localized in the perinuclear region of the gingival epithelial cells after it escaped from the membrane-bound vacuoles (4). Organisms such as *Shigella* and *Listeria* rapidly gain access to the cytoplasm and can subsequently spread to adjacent cells (9). Therefore, we speculated that *P. gingivalis* is able to escape from membrane-bound vacuoles after internalization and that type II *P. gingivalis* can escape from the vacuoles more quickly than other strains. However, more detailed studies are required to substantiate this hypothesis.

P. gingivalis-infected cells were previously reported to lose the ability to adhere to the culture dish and to float in the culture medium without serum components, which was shown to be due to the activity of gingipains (5, 18, 33, 36). In this study, the epithelial cells did not float in the medium containing 10% FCS for 48 h after infection (Fig. 6). Serum components, provided via the capillary blood vessels in various tissues, are necessary to maintain a relative consistency in epithelial cells (12). Thus, an experimental cell culture system containing serum would be appropriate to test for a cellular response to bacterial infection.

In our previous study with a mouse abscess model, type II strains caused the most significant induction of acute general inflammation among the six types of strains, while type II mutants with fimbriae disrupted clearly had lost the ability to infect (26). The present findings also support the notion that variations in fimbriae have effects on the expression of virulence by *P. gingivalis*. In addition, invasion of host cells by *P. gingivalis* has been reported to have a great effect on gene expression by the host cells, as cellular expression profiling using a microarray analysis demonstrated that the fimbria-mediated invasion by *P. gingivalis* directly accelerates cellular inflammatory responses (6) and also apparently influences the expression of various genes regulating the cell cycle, proliferation, and the cytoskeleton (14). Thus, the invasive efficiency of type II *P. gingivalis* may disable various cellular functions, resulting in chronic and destructive periodontal inflammation.

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