INFECTION AND IMMUNITY, May 2006, p. 3002–3005 Vol. 74, No. 5
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.5.3002–3005.2006

NOTES

Role of the *Porphyromonas gingivalis* InlJ Protein in Homotypic and Heterotypic Biofilm Development

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Received 9 December 2005/Returned for modification 16 January 2006/Accepted 6 February 2006

The oral pathogen *Porphyromonas gingivalis* expresses a homolog of the internalin family protein InlJ. Inactivation of *inlJ* reduced monospecies biofilm formation by *P. gingivalis*. In contrast, heterotypic *P. gingivalis-Streptococcus gordonii* biofilm formation was enhanced in the InlJ-deficient mutant. The results indicate a nuanced role for InlJ in regulating biofilm accumulations of *P. gingivalis*.

The internalin protein family was originally identified in *Listeria monocytogenes* and is characterized by the presence of an N-terminal leucine-rich repeat (LRR) domain (1). LRRs are involved in protein-protein interactions including ligand-receptor binding (5). Indeed, internalin A (InlA) and InlB of *L. monocytogenes* are involved in adherence and invasion of the organism (1, 3). Recently, a novel internalin, InlJ, was identified in *L. monocytogenes* (12). InlJ defines a new subclass family of cysteine-containing LRR proteins. An InlJ mutant of *Listeria* was found to be significantly attenuated in virulence in mice (12). InlJ type proteins are currently identified in only six bacterial species, one of which is the gram-negative oral anaerobe *Porphyromonas gingivalis* (12). Expression of InlJ (PG0350) (http://www.lanl.gov) in *P. gingivalis* was originally detected when the organism was in contact with host epithelial cells (2). However, an InlJ mutant of *P. gingivalis* did not exhibit an invasion-related phenotype (18), as is also the case for the *Listeria* InlJ mutant (12). In addition to an intracellular location, a significant component of the *P. gingivalis* lifestyle is within the complex multispecies biofilm (dental plaque) that develops on tooth surfaces. In this study, we investigated the role of the *P. gingivalis* InlJ protein in single-species and multispecies biofilm formation by the organism. Interestingly, an InlJ-null mutant exhibited reduced monospecies biofilm development but enhanced heterotypic biofilm formation with *Streptococcus gordonii*.

An InlJ-deficient mutant was generated by insertional inactivation in *P. gingivalis* strain 33277. A central 847-bp fragment of *inlJ* (PG0350) was amplified by PCR using primers 5′-TCTTCT GCAGGGGACTATGG-3′ and 5′-TTTCCACGTGTTCGGTT GTA-3′ and subcloned into the suicide plasmid pVA3000. The recombinant plasmid was introduced into *P. gingivalis* 33277 by conjugation as described previously (10). The absence of *inlJ* transcript was confirmed by reverse transcription-PCR. As the *inlJ* gene is located between two genes transcribed in the opposite direction, the potential for pleiotropic effects of this mutation are diminished. The adjacent genes are PG0349, a putative hydrolase of the haloacid dehalogenase-like family, and PG0351, a hypothetical protein, neither of which has a documented role in biofilm biogenesis. Homotypic biofilm formation was first tested in the microtiter plate assay described previously by O'Toole and Kolter (8). Parental and mutant strains were suspended in prereduced phosphate-buffered saline, and 5×10^7 cells were incubated at 37°C anaerobically in individual wells of 96-well plates. The resulting biofilms were washed, stained with 1% crystal violet, and destained with 95%

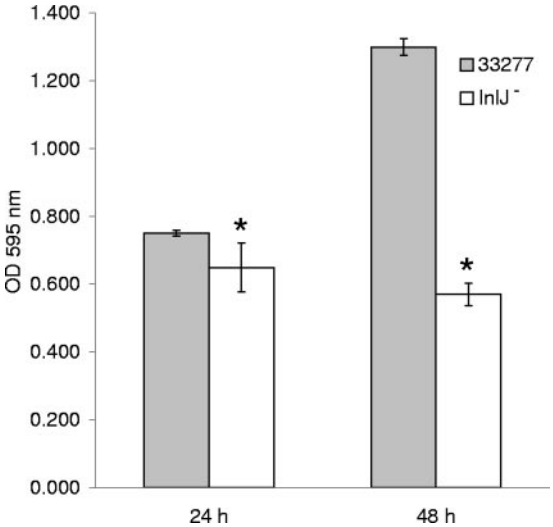

FIG. 1. Microtiter plate monospecies biofilm production by *P. gingivalis* 33277 and the InlJ mutant at 24 h and 48 h. Asterisks indicate a significant difference ($P < 0.05$, *t* test; $n = 3$) between the mutant and parental strains. OD, optical density.

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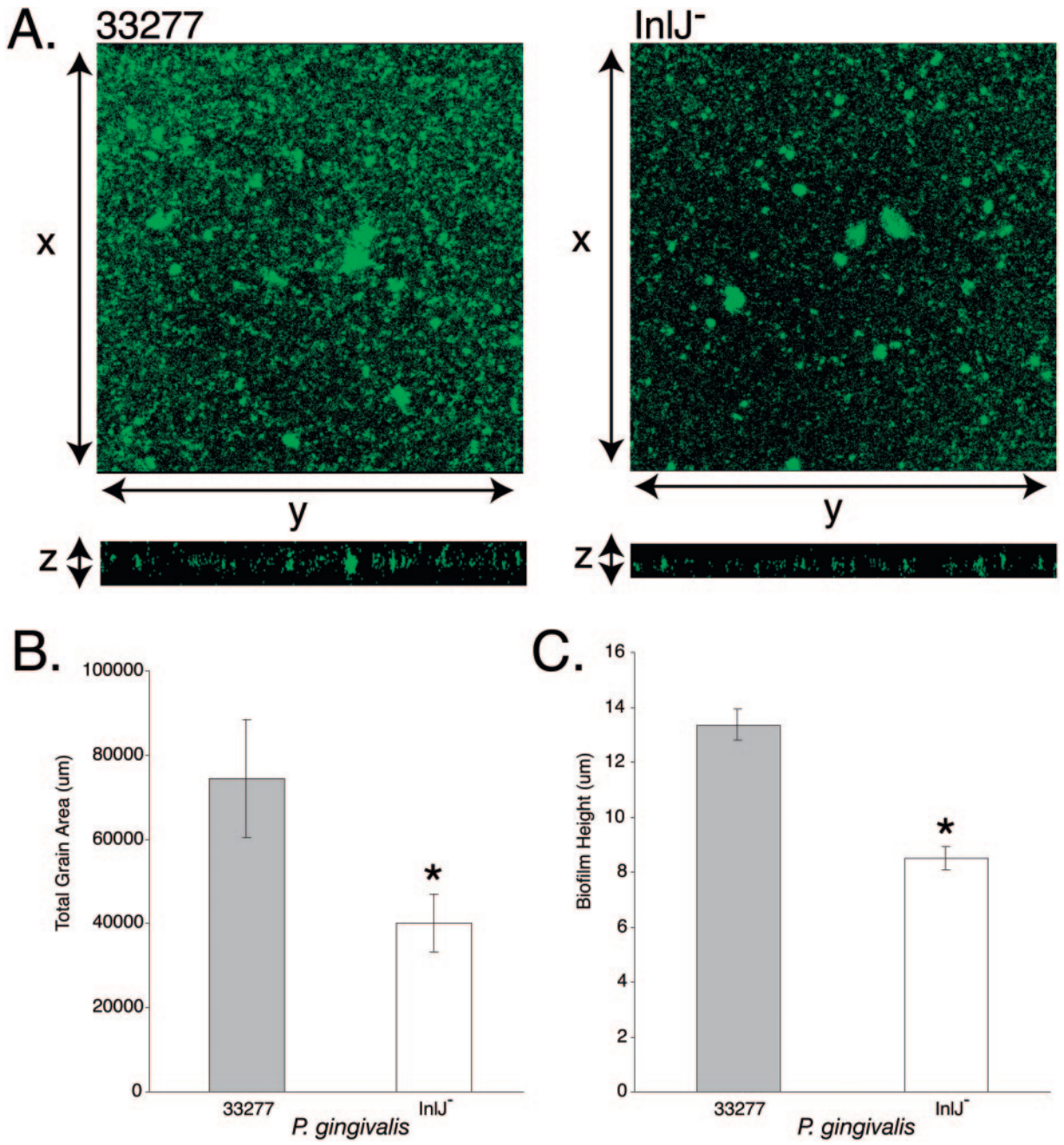

FIG. 2. (A) Confocal laser scanning microscopy projections of monospecies biofilm formation by *P. gingivalis* strains 33277 and the InlJ mutant after 24 h. Magnification, ×40. (B) Total grain area analysis of a 268.6- by 268.6-μm *x-y* section. (C) Average biofilm height of *P. gingivalis* accumulation across three random *x-z* sections. Asterisks indicate a significant difference ($P < 0.05$, *t* test; $n = 3$) between the mutant and parental strains.

ethanol. Absorbance at 595 nm was determined using a Benchmark microplate reader. Figure 1 shows that monospecies biofilm formation was reduced in the InlJ mutant by 13.6% after 24 h and 56.1% after 48 h. For visualization and quantification of biofilm structure, biofilms were generated in a 16-well Culture Well chambered coverglass system, stained with 5 (and 6)-carboxyfluorescein succinimidyl ester (fluorescein isothiocyanate [FITC], 4 μg ml^{-1}; Molecular Probes), and examined by confocal microscopy (Bio-Rad MRC600 confocal scanning laser microscope [Kr/Ar] system with an MS plan ×60 1.4-numerical-aperture objective). Biofilms were observed with the reflected laser light of combined 488-, 546-, and 647-nm wavelengths. The images were analyzed with Image J 1.35c and Adobe Photoshop 6.0 software. MCID-M5 5.1 software was used to determine the total grain area. Biofilm formation by the InlJ mutant was visibly more sparse than that by the parent strain (Fig. 2A), and total accumulation was reduced by 46.2% (Fig. 2B). In addition, the average height across three random

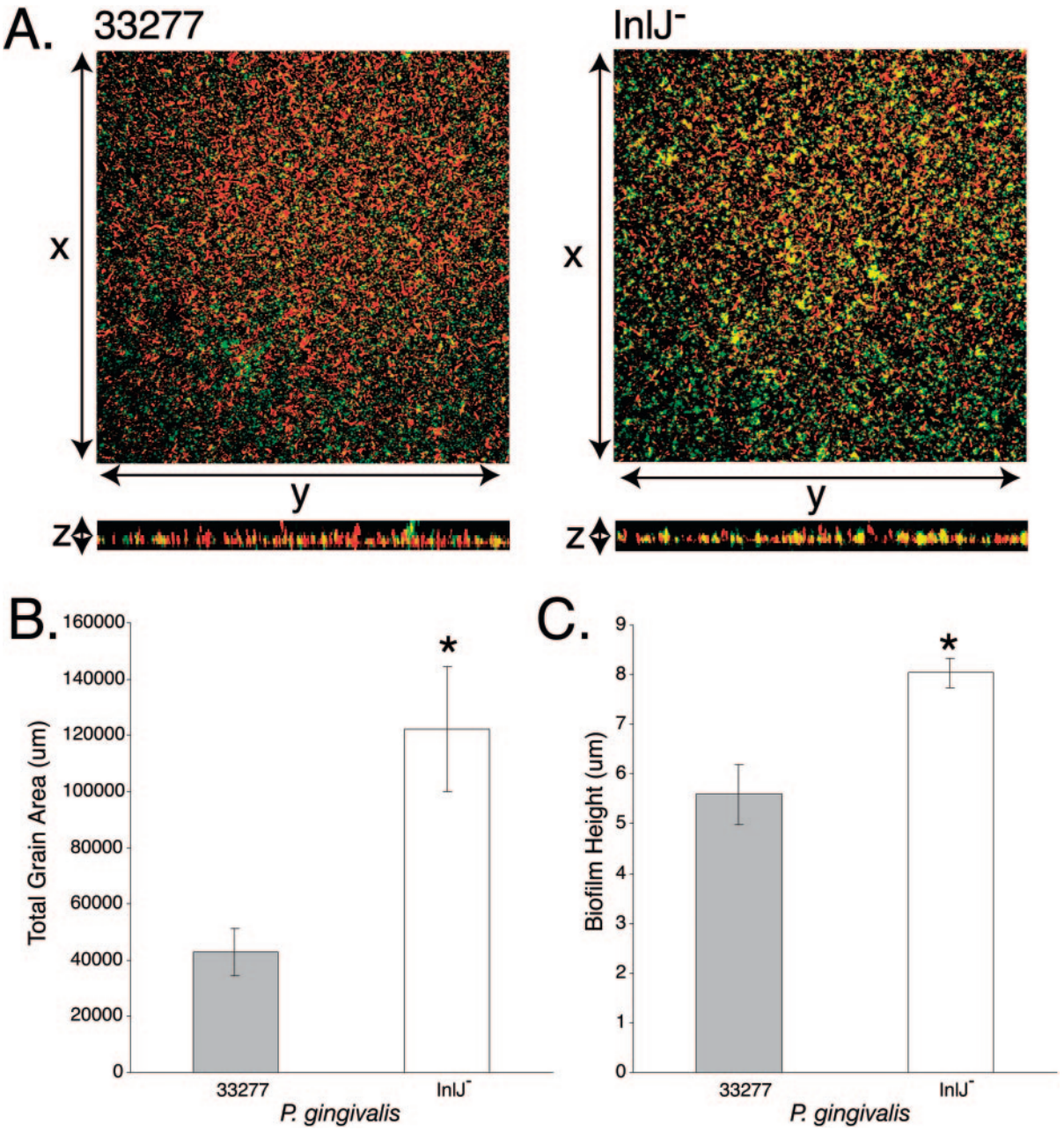

FIG. 3. (A) Confocal laser scanning microscopy projections of mixed biofilms of *S. gordonii* DL1 with *P. gingivalis* strains 33277 and the InlJ mutant after 24 h. *S. gordonii* was prestained with hexidium iodide (red), and *P. gingivalis* was prestained with FITC (green). Magnification, ×40. (B) Total grain area analysis of a 268.6- × 268.6-μm *x-y* section. (C) Average biofilm height of *P. gingivalis* accumulation across three random *x-z* sections. Asterisks indicate a significant difference ($P < 0.05$, *t* test; $n = 3$) between the mutant and wild-type strains.

x-z sections of the mutant biofilm was reduced by 36.5% compared to the parent strain (Fig. 2C). These experiments demonstrate that InlJ is required for optimal homotypic biofilm formation by *P. gingivalis*.

On the tooth surfaces, *P. gingivalis* will be in contact with the diverse species that comprise the plaque biofilm. Thus, to begin to assess the role of InlJ in heterotypic biofilms, mixed *S. gordonii-P. gingivalis* biofilms were examined. *S. gordonii* is a common component of dental plaque (11, 13, 14) and is encountered by *P. gingivalis* upon initial colonization. *S. gordonii* cells were cultured for 24 h on chambered coverglass and stained with hexidium iodide (15 μg ml^{-1}; Molecular Probes). *P. gingivalis* cells were stained with FITC as described above and reacted anaerobically with the *S. gordonii* biofilm for 24 h at 37°C in prereduced phosphate-buffered saline. After washing, accumulations of heterotypic biofilms were observed by confocal microscopy as described above. In contrast to the monospecies biofilm, the InlJ mutant formed more abundant accumulations within the mixed *P. gingivalis-S. gordonii* biofilm (Fig. 3A). This observation was supported by the total grain

analysis of *P. gingivalis* accumulation (Fig. 3B). In addition, measurement of average biofilm height across three random *x-z* sections showed higher vertical accretion of the InlJ mutant than the parental strain (Fig. 3C). Hence, in the absence of InlJ, more luxuriant heterotypic biofilms are formed by *P. gingivalis*.

Biofilm accumulation proceeds through a series of developmental steps involving attachment of bacterial cells to a surface; accumulation by the recruitment of additional cells and proliferation; and, in certain cases, inclusion of additional species. Defined genetic profiles are considered important for distinct phases of biofilm development (7, 15). Regulation of biofilm development can be hypothesized to involve mechanisms that both stimulate an increase in biomass and limit or stabilize accumulation according to environmental constraints. For example, in *Pseudomonas aeruginosa*, biofilm depth is reduced by the transcription factor RpoS (4, 17). However, RpoS mutants of *P. aeruginosa* form biofilms of greater depth under flowing conditions (17). RpoS production is regulated at multiple levels, including transcription, translation, and proteolysis, in response to different stress conditions such as nutrient limitation (16). The results of the current study indicate that the InlJ protein of *P. gingivalis* is exploited to perform roles both in the stimulation of biofilm accumulation on abiotic surfaces and in biofilm control in the more complex and in vivo-relevant situation where other organisms are present. Such a multifunctional role is not inconsistent with the structure and properties of LRR proteins. Internalins of *Listeria* are involved in adherence, and adhesive activity mediated by InlJ may be important for biofilm initiation on abiotic surfaces. InlJ does possess a signal peptide and is therefore likely to be present on the surface of *P. gingivalis*. Adherence of *P. gingivalis* to *S. gordonii*, however, is mediated through the long and short fimbriae (6, 9) and thus may not require the presence of InlJ. LRRs can also be involved in signal transduction through their capacity to provide a versatile structural framework for the formation of protein-protein interactions. Such a role for InlJ may be important in constraining biofilm growth, possibly to avoid excessive exposure to oxygen in the oral cavity. As a strict anaerobe, *P. gingivalis* is likely to favor an existence deep within the plaque biofilm. Alternatively, restriction of biofilm development may be important to maintain the integrity of channels that allow nutrient penetration into the biofilm.

This work was supported by NIDCR DE12505 and DE11111.

Editor: V. J. DiRita

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