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RESEARCH ARTICLE

Community Development between *Porphyromonas gingivalis* and *Candida albicans* Mediated by InlJ and Als3

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ABSTRACT The pleiomorphic yeast *Candida albicans* is a significant pathogen in immunocompromised individuals. In the oral cavity, *C. albicans* is an inhabitant of polymicrobial communities, and interspecies interactions promote hyphal formation and biofilm formation. *C. albicans* colonizes the subgingival area, and the frequency of colonization increases in periodontal disease. In this study, we investigated the interactions between *C. albicans* and the periodontal pathogen *Porphyromonas gingivalis*. *C. albicans* and *P. gingivalis* were found to coadhere in both the planktonic and sessile phases. Loss of the internalin-family protein InlJ abrogated adhesion of *P. gingivalis* to *C. albicans*, and recombinant InlJ protein competitively inhibited interspecies binding. A mutant of *C. albicans* deficient in expression of major hyphal protein Als3 showed diminished binding to *P. gingivalis*, and InlJ interacted with Als3 heterologously expressed in *Saccharomyces cerevisiae*. Transcriptional profiling by RNA sequencing (RNA-Seq) established that 57 genes were uniquely upregulated in an InlJ-dependent manner in *P. gingivalis-C. albicans* communities, with overrepresentation of those corresponding to 31 gene ontology terms, including those associated with growth and division. Of potential relevance to the disease process, *C. albicans* induced upregulation of components of the type IX secretion apparatus. Collectively, these findings indicate that InlJ-Als3-dependent binding facilitates interdomain community development between *C. albicans* and *P. gingivalis* and that *P. gingivalis* has the potential for increased virulence within such communities.

IMPORTANCE Many diseases involve the concerted actions of microorganisms assembled in polymicrobial communities. Inflammatory periodontal diseases are among the most common infections of humans and result in destruction of gum tissue and, ultimately, in loss of teeth. In periodontal disease, pathogenic communities can include the fungus *Candida albicans*; however, the contribution of *C. albicans* to the synergistic virulence of the community is poorly understood. Here we characterize the interactions between *C. albicans* and the keystone bacterial pathogen *Porphyromonas gingivalis* and show that coadhesion mediated by specific proteins results in major changes in gene expression by *P. gingivalis*, which could serve to increase pathogenic potential. The work provides significant insights into interdomain interactions that can enhance our understanding of diseases involving a multiplicity of microbial pathogens.

KEYWORDS *Candida albicans*, *Porphyromonas gingivalis*, RNA-Seq, microbial communities, polymicrobial synergy

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Periodontitis is a common inflammatory disease which affects the integrity of the tissues that surround and support the teeth. Around half the adult population in the United States experiences some form of the disease, and periodontitis is the sixth most common infection worldwide (1, 2). Additionally, periodontitis and periodontal pathogens are associated with serious systemic conditions such as rheumatoid arthritis, atherosclerosis, and some forms of cancer (3–5). Periodontitis ensues from the action of complex heterogeneous microbial communities that inhabit the subgingival compartment (6). Within those communities, organisms can collectively regulate physiological activities, and microbial constituents have developed functional specialization (7–9). Keystone pathogens, such as *Porphyromonas gingivalis*, can raise community pathogenic potential (or nososymbiocity) (10, 11). Accessory pathogens such as *Streptococcus gordonii* which are considered commensal alone can increase the pathogenicity of *P. gingivalis* (11). Indeed, *P. gingivalis* and *S. gordonii* interact through physical attachment and chemical-mediated communication (12), and dual-species communities are more pathogenic in animal models of periodontal diseases than either organism is alone (13).

In addition to bacteria, fungi represent a significant component of the oral microbiome (14). *Candida* species such as *C. albicans* are common inhabitants of the oral cavity and colonize polymicrobial biofilm communities (15). Specific interactions have been identified between *C. albicans* and a range of bacteria, e.g., *Pseudomonas*, *Staphylococcus*, and *Streptococcus* (16–23). There is evidence to suggest that these interactions may modulate the clinical course of infection and have an impact on treatment regimens (19, 24–29). Furthermore, interspecies interactions are considered important in development of denture stomatitis (30) and, potentially, also periodontal disease (31, 32). In subjects with chronic periodontitis, the rate of *C. albicans* carriage can increase, together with higher isolation frequencies of periodontal bacterial pathogens such as *P. gingivalis* (32). *P. gingivalis* can increase hyphal formation by *C. albicans* (33), and the organisms can coinhabit polymicrobial biofilms *in vitro* (34, 35). In addition, *C. albicans* can enhance invasion of gingival epithelial cells by *P. gingivalis* (36). However, the nature of the interaction between *P. gingivalis* and *C. albicans* has yet to be investigated in molecular detail. In this study, we examined the bacterial and fungal adhesins that mediate coadhesion and the influence of interspecies binding on the transcriptome of *P. gingivalis*.

RESULTS

***P. gingivalis* and *C. albicans* interactions.** *C. albicans* is a persistent colonizer of the human oral cavity and a common constituent of subgingival biofilms (17, 37, 38). Therefore, we investigated the ability of *P. gingivalis* to adhere to *C. albicans* in suspension. As shown in Fig. 1A, *P. gingivalis* adheres to *C. albicans* hyphae, and approximately 80% of hyphal filaments demonstrated a binding phenotype with *P. gingivalis*. In contrast, binding of *P. gingivalis* to yeast or pseudohyphal forms of *C. albicans* was rarely observed (see Fig. S1 in the supplemental material). Since the FimA component fimbriae of *P. gingivalis* are responsible for many adhesive properties of the organism (39, 40), we next examined the involvement of FimA in *P. gingivalis* interactions with candidal hyphae. A *fimA*-deficient mutant of *P. gingivalis* did not show a reduction in binding to *C. albicans* compared to the wild-type parental strain (Fig. 1A), indicating that other surface components of *P. gingivalis* mediate interspecies adherence. Previously, we had found that the InlJ internalin-family protein is required for optimal homotypic biofilm formation by *P. gingivalis* and is also involved in heterotypic biofilm control (41); thus, we tested the involvement of InlJ in *P. gingivalis-C. albicans* coadhesion. As shown in Fig. 1A, an isogenic *inlJ* mutant of *P. gingivalis* was significantly impaired in binding to *C. albicans*. To confirm the adhesion-mediating role of InlJ, a *P. gingivalis* strain was constructed in which the *inlJ* gene deletion was complemented with the wild-type *inlJ* allele expressed in *trans* from pT-COW (strain cΔ*inlJ*). Adherence of the cΔ*inlJ* mutant to candidal hyphae was restored to wild-type *P. gingivalis* levels (Fig. 1B), verifying the role of InlJ in mediating attachment of *P. gingivalis* to the hyphae

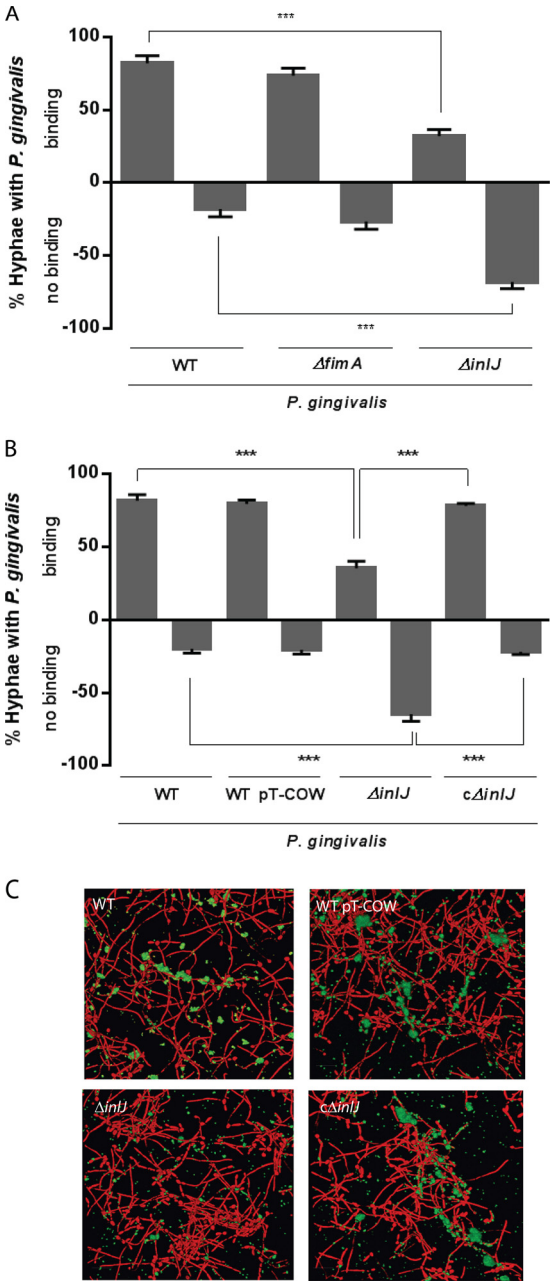


FIG 1 *P. gingivalis* interacts with *C. albicans* in an InlJ-dependent manner. (A) Percentages of total *C. albicans* SC5314 hyphae with attached cells of *P. gingivalis* 33277 (WT), Δ*fimA*, or Δ*inlJ* strains were determined on the basis of the following binding results: binding, including extensive attachment of bacteria to hyphae with bacteria clumping and bacterial cells aligned along hyphae in distinct patches, and no binding, including sparse or no interactions between bacteria and hyphae. One hundred hyphae were counted for each pairing. Results are representative of 4 independent experiments and are expressed as means ± standard deviations of the means (SD); $n = 4$. ***, $P < 0.001$ (by analysis of variance [ANOVA] with Tukey *post hoc* test). (B) Interactions of *C. albicans* SC5314 with *P. gingivalis* 33277 (WT), 33277+pT-COW (WT pT-COW), the Δ*inlJ* mutant, or the *inlJ* mutant complemented with the *inlJ* gene in *trans* (cΔ*inlJ*). Percentages of hyphae with bacteria attached were calculated on the basis of bacterial binding level as described for panel A. Results are representative of 4 independent experiments and are expressed as means ± SD; $n = 4$. ***, $P < 0.001$ (by ANOVA with Tukey *post hoc* test). (C) Fluorescence confocal micrographs of *C. albicans* SC5314 biofilms (red, stained with hexidium iodide) formed on saliva-coated glass for 3 h with attached cells of *P. gingivalis* 33277 (WT), 33277+pT-COW (WT pT-COW), the Δ*inlJ* mutant, or the *inlJ* mutant complemented with the *inlJ* gene in *trans* (cΔ*inlJ*) (green, stained with FITC). Biofilms were imaged at ×63 magnification. Merged images present projections of z-stacks obtained with Volocity software and are representative of results of 3 independent experiments.

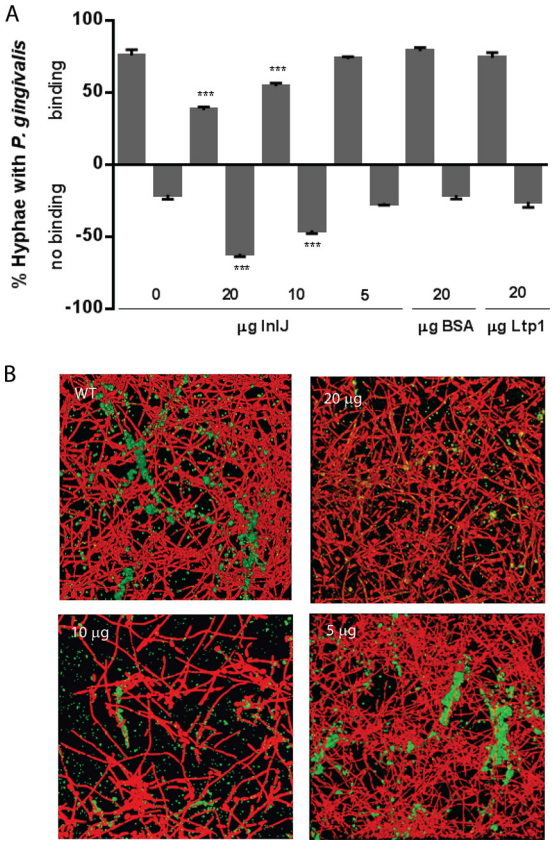


FIG 2 InlJ protein inhibits interaction of *P. gingivalis* with *C. albicans*. (A) Percentages of total *C. albicans* SC5314 hyphae with attached *P. gingivalis* 33277 in the presence of rInlJ protein at the concentrations indicated. Binding was calculated as described for Fig. 1. Recombinant *P. gingivalis* tyrosine phosphatase (Ltp1) and bovine serum albumin (BSA) (20 μg) were used as control proteins. Results are representative of 4 independent experiments and are expressed as means $\pm$ SD; $n = 4$. ***, $P < 0.001$ (compared to the control condition with no exogenous protein added by ANOVA with Dunnett's correction). (B) Fluorescence confocal microscopy projections of *C. albicans* SC5314 biofilms (red, stained with hexidium iodide) formed on saliva-coated glass for 3 h with *P. gingivalis* 33277 (green, stained with FITC) in the presence of InlJ protein at the concentrations indicated. Biofilms were imaged at $\times$63 magnification. Merged images present projections of z-stacks obtained with Volocity software and are representative of results of 3 independent experiments.

of *C. albicans*. Screening mutants of *P. gingivalis* lacking minor fimbrial adhesin Mfa1 or hemagglutinin HagB found no effect on coadhesion with *C. albicans* (not shown), implicating InlJ as the predominant *P. gingivalis* adhesin in this interaction.

Role of InlJ in heterotypic biofilm formation. As cells in biofilms can display phenotypes that are distinct from those seen with their free-living counterparts, we investigated early biofilm formation using *C. albicans* and *P. gingivalis*. *C. albicans* biofilm formation was initiated on saliva-coated glass coverslips, and, as shown in Fig. 1C, *C. albicans* cells attached to the surface and formed hyphal filaments to which *P. gingivalis* cells clearly bound. Adherence of *P. gingivalis* Δ*inlJ* to hyphal filaments was diminished, consistent with the planktonic condition. The complemented strain of *P. gingivalis*, the cΔ*inlJ* mutant, adhered to hyphal filaments under biofilm conditions at the same level as the wild type. These findings support the idea that InlJ is a mediator of *P. gingivalis* binding to *C. albicans* under both planktonic and sessile conditions.

Inhibition of *P. gingivalis* interaction with *C. albicans* by InlJ. To provide further insight into the role of InlJ in *P. gingivalis*-*C. albicans* coadhesion, recombinant protein was expressed as a His-tagged fusion and tested for inhibition of *P. gingivalis* binding to *C. albicans*. Figure 2A shows that recombinant InlJ (rInlJ) inhibited *P. gingivalis* binding in the suspension assay in a dose-dependent manner and that up to 65% inhibition was seen in the presence of 20 μg rInlJ. Control proteins bovine serum

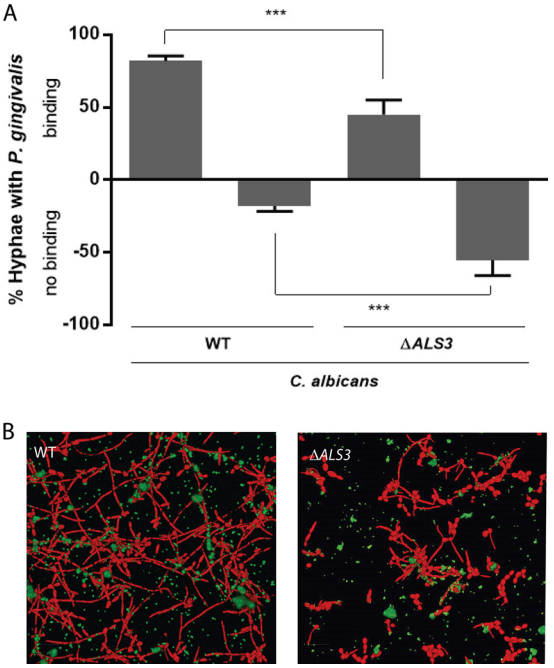


FIG 3 *C. albicans* Als3 is required for biofilm formation with *P. gingivalis*. (A) Percentages of total *C. albicans* UB1936 (WT) or mutant strain UB1930 (*als3*Δ) with attached *P. gingivalis* 33277 were calculated on the basis of bacterial binding levels as described for Fig. 1. Results are representative of 4 independent experiments and are expressed as means ± SD; $n = 4$. ***, $P < 0.001$ (by ANOVA with Tukey *post hoc* test). (B) Fluorescence confocal microscopy projections of biofilms of *C. albicans* UB1936 (WT) or mutant strain *als3*Δ (red, stained with hexidium iodide) formed on saliva-coated glass for 3 h with *P. gingivalis* 33277 (green, stained with FITC). Biofilms were imaged at ×63 magnification. Merged images present projections of z-stacks obtained with Volocity software and are representative of results of 3 independent experiments.

albumin (BSA) and rLtp1, an irrelevant phosphatase protein from *P. gingivalis* (42), did not display inhibitory activity. The same inhibitory effect was observed when dual-species biofilms were developed in the presence of rInlJ. *P. gingivalis* adherence to hyphae was reduced in a concentration-dependent manner in the presence of rInlJ protein (Fig. 2B). As relatively large amounts of soluble protein were required for inhibition, the presentation of InlJ on the bacterial surface may be necessary for the maintenance of optimal active structure. These findings support the model that InlJ mediates the attachment of *P. gingivalis* to *C. albicans*.

Als3 is necessary for *C. albicans* interactions with *P. gingivalis*. Previous studies demonstrated that the hypha-specific adhesin Als3 is important for *C. albicans* biofilm formation and for adhesion to host tissue and to the oral early plaque colonizer *S. gordonii* (43, 44). Further, Als3 is associated with hyphae (45), to which *P. gingivalis* preferentially binds. The potential involvement of Als3 in *P. gingivalis* binding was examined using an *als3*Δ *als3*Δ-*URA3* mutant (designated *als3*Δ). In the suspension assay, binding of *C. albicans als3*Δ to *P. gingivalis* decreased 58% (Fig. 3A). The *als3*Δ mutant formed sparse biofilms with few hyphae (Fig. 3B), consistent with the role of this protein in biofilm formation (46). Nonetheless, binding of *P. gingivalis* to the *als3*Δ mutant hyphae that were present was less than that seen with wild-type *C. albicans*. These results do not exclude the possible involvement of other Als proteins or, indeed, of other candidal adhesins, which may play a complementary or overlapping role in binding with Als3.

InlJ interacts with Als3. Our findings suggested that *C. albicans* Als3 can act as a component of an adhesin-receptor system with *P. gingivalis*. To investigate whether Als3 interacts directly with InlJ, we utilized *Saccharomyces cerevisiae* cells expressing *C. albicans* adhesins. In an enzyme-linked immunosorbent assay (ELISA), rInlJ bound to *S. cerevisiae* cells expressing Als3 (derived from either the large or small *ALS3* allele)

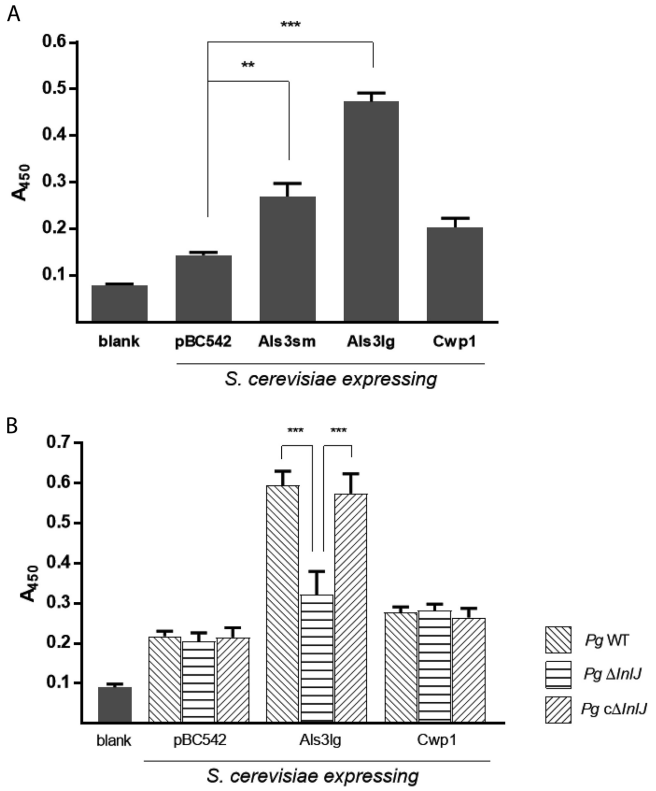


FIG 4 *C. albicans* Als3 interacts with InlJ of *P. gingivalis*. (A) Attachment of rInlJ protein to *S. cerevisiae* cells expressing candidal adhesins Als3sm, Als3lg, or Cwp1, or empty pBC542 vector was analyzed with an ELISA using His-tagged MAb (1:2,000). (B) Attachment of the *P. gingivalis* (*Pg*) WT strain, the Δ*inlJ* mutant, or the cΔ*inlJ* mutant to *S. cerevisiae* cells expressing candidal adhesins Als3lg or Cwp1, or empty pBC542 vector was analyzed with an ELISA using *P. gingivalis* antibodies (1:5,000). Results are representative of 3 independent experiments and are expressed as means ± SD; $n = 3$. **, $P < 0.01$; ***, $P < 0.001$ (by ANOVA with Tukey *post hoc* test).

but not to control cells or cells expressing *S. cerevisiae* wall protein Cwp1 (Fig. 4A). Moreover, binding of *P. gingivalis* whole cells to *S. cerevisiae* expressing Als3 occurred only in the presence of InlJ (Fig. 4B). In control experiments, all strains of *S. cerevisiae* attached to the ELISA plates to the same degree (Fig. S2). These findings support a model whereby InlJ binds to hyphal Als3 to effectuate *P. gingivalis-C. albicans* association.

Transcriptional profiling of InlJ-dependent *P. gingivalis-C. albicans* interactions. RNA sequencing (RNA-Seq) was utilized to examine the transcriptional responses of *P. gingivalis* in InlJ-dependent communities with *C. albicans* under planktonic coculture conditions. Comparing the *P. gingivalis* wild type to *P. gingivalis* Δ*inlJ* coincubated with or without *Candida*, 256 *P. gingivalis* genes in the parental strain were downregulated in the coincubation, with 96 of these being unique (i.e., not regulated without *Candida*) (Fig. 5A). Among those 96 genes, 58 had a Log2 fold change level of >1 (see Table S1 in the supplemental material). Overall, there were markedly fewer differences in expression under the coculture condition than under the monoculture condition, and there were over twice as many genes downregulated in the parental strain as in the InlJ-deficient cells (Table S1). Similarly, 125 genes were upregulated in the *P. gingivalis* wild type in the presence of *C. albicans*, with 57 of these being unique to the coincubation (Fig. 5B). Among those 57 unique genes, 21 had a Log2 fold change level of >1 (Table S1).

The 57 unique coculture InlJ-dependent upregulated genes were assigned to 31 Gene Ontology (GO) terms with an overenrichment *P* value of <0.05 (Fig. 6A). Among those 31 GO terms, biological process terms comprised 10, cellular component terms

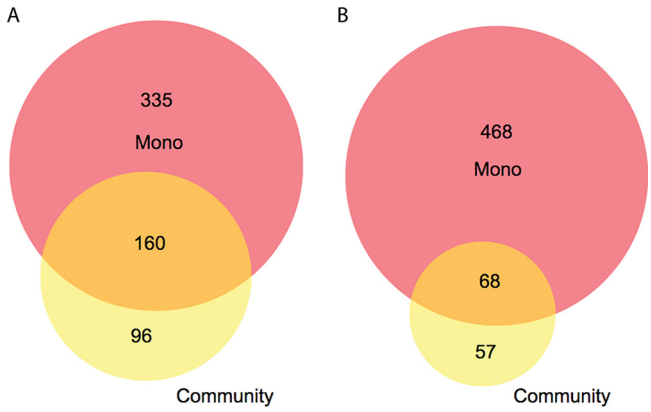
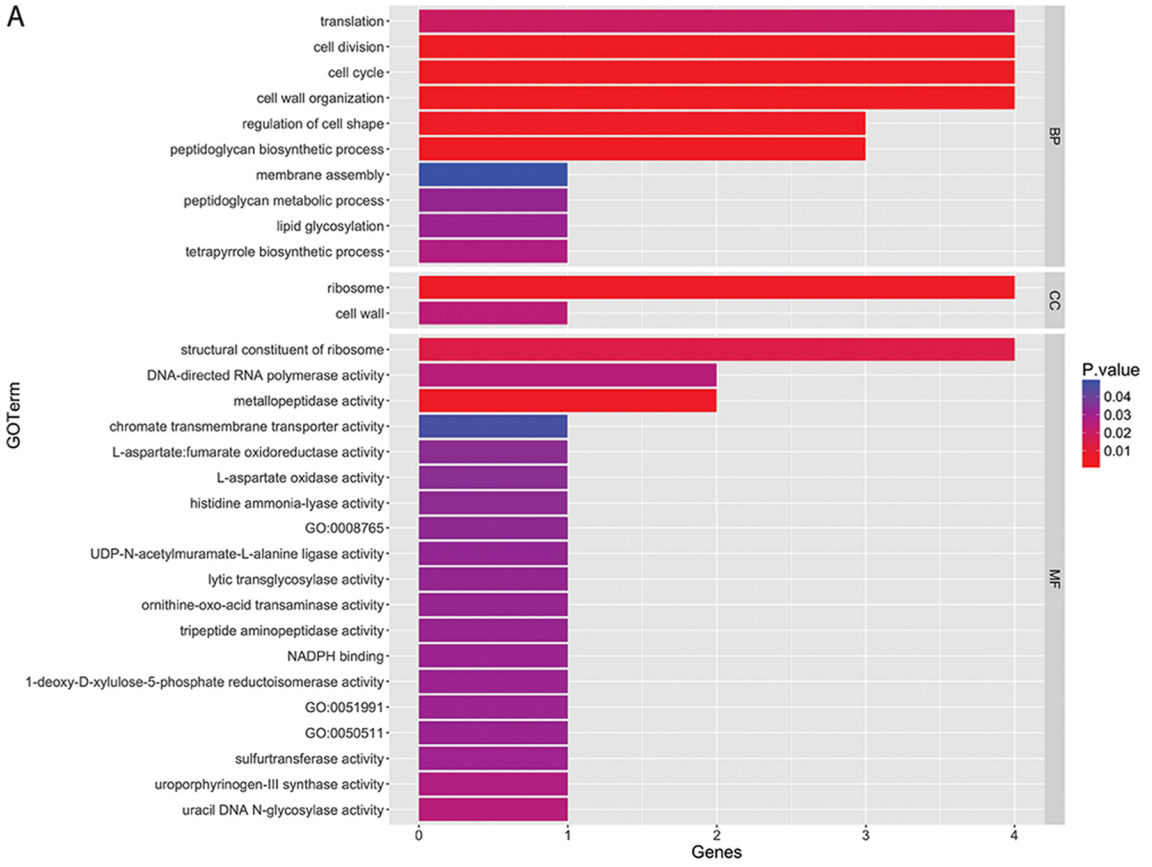


FIG 5 Venn diagram representing differentially expressed genes that were either (A) downregulated or (B) upregulated in the *P. gingivalis* 33277 WT strain compared to the Δ*inlJ* mutant, with and without coincubation with *C. albicans*. Yellow shading represents unique genes in coculture, red shading represents unique genes in monoculture (Mono), and orange shading represents genes that appeared under both conditions.

comprised 2, and molecular function terms comprised 19. Among the biological process terms, the most significantly overrepresented terms comprised cell wall organization terms, cell cycle terms, and cell division terms. The most significantly enriched cellular component terms were ribosome terms and cell wall terms, and terms corresponding to metallopeptidase activity, structural constituent of the ribosome, and uracil DNA N-glycosylase activity were those most significantly enriched among the molecular function terms. Collectively, these results suggest that InlJ-dependent association with *C. albicans* increases growth and division of *P. gingivalis*. Consistent with this, 3 genes involved in peptidoglycan biosynthesis, *murE*, *murC*, and *murG*, were upregulated with InlJ present, although only one of those genes, *murG*, had a Log2 fold change level of >1. An illustration depicting the results of a STRING network analysis (Fig. 7A) depicts genes corresponding to nodes, namely, ribosomal protein genes, peptidoglycan biosynthesis genes, and genes of the type IX secretion system (T9SS), according to k-means clustering, and also shows the potential for interactions among the products of these differentially regulated genes. There have been 18 components of the T9SS recognized in *P. gingivalis* to date, and the machinery is responsible for the translocation of over 30 proteins from the periplasm across the outer membrane (47–49). Targets of the T9SS include a number of virulence-associated proteins, including the gingipain proteases (47–49). Genes encoding 9 components of the T9SS machinery were upregulated in an InlJ-dependent manner; those genes included *porPKLMN*, representing an operon whose members are cotranscribed (Fig. 7B). The *porPKLMN* operon is controlled by the PorXY two-component system, which operates through S*igP*, an extracytoplasmic function (ECF) sigma factor. Genes encoding PorY and SigP were also upregulated by *C. albicans*. Although only two of the T9SS cargo proteins, PGN_1437 and the thiol protease PGN_0900, were upregulated (Table S1), *C. albicans* could potentially increase the pathogenicity of *P. gingivalis* indirectly through induction of type IX-dependent secretion of virulence factors, without affecting gene expression.

The 96 unique coculture InlJ-dependent downregulated genes were assigned to GO terms (Fig. 6B). Among those 96 genes, 25 showed overrepresentation (*P* value of <0.05). Of the 25 genes, 9 were part of the biological process GO category, with the term ″pathogenesis″ being the most highly represented followed by ″cell redox homeostasis″ and then ″metal ion transport.″ Note that the data corresponding to the term ″pathogenesis″ were not *P. gingivalis* specific but were assembled from a variety of organisms. Overpopulation among the members of this category is derived from the genes for the fimbrial adhesins (*fimA* and *mfa1*) and the *hagA* gene encoding a hemagglutinin adhesin. Expression of these adhesion-associated genes may be up-


A
translation
cell division
cell cycle
cell wall organization
regulation of cell shape
peptidoglycan biosynthetic process
membrane assembly
peptidoglycan metabolic process
lipid glycosylation
tetrapyrrole biosynthetic process
ribosome
cell wall
structural constituent of ribosome
DNA-directed RNA polymerase activity
metallopeptidase activity
chromate transmembrane transporter activity
L-aspartate:fumarate oxidoreductase activity
L-aspartate oxidase activity
histidine ammonia-lyase activity
GO:0008765
UDP-N-acetylmuramate-L-alanine ligase activity
lytic transglycosylase activity
ornithine-oxo-acid transaminase activity
tripeptide aminopeptidase activity
NADPH binding
1-deoxy-D-xylulose-5-phosphate reductoisomerase activity
GO:0051991
GO:0050511
sulfurtransferase activity
uroporphyrinogen-III synthase activity
uracil DNA N-glycosylase activity
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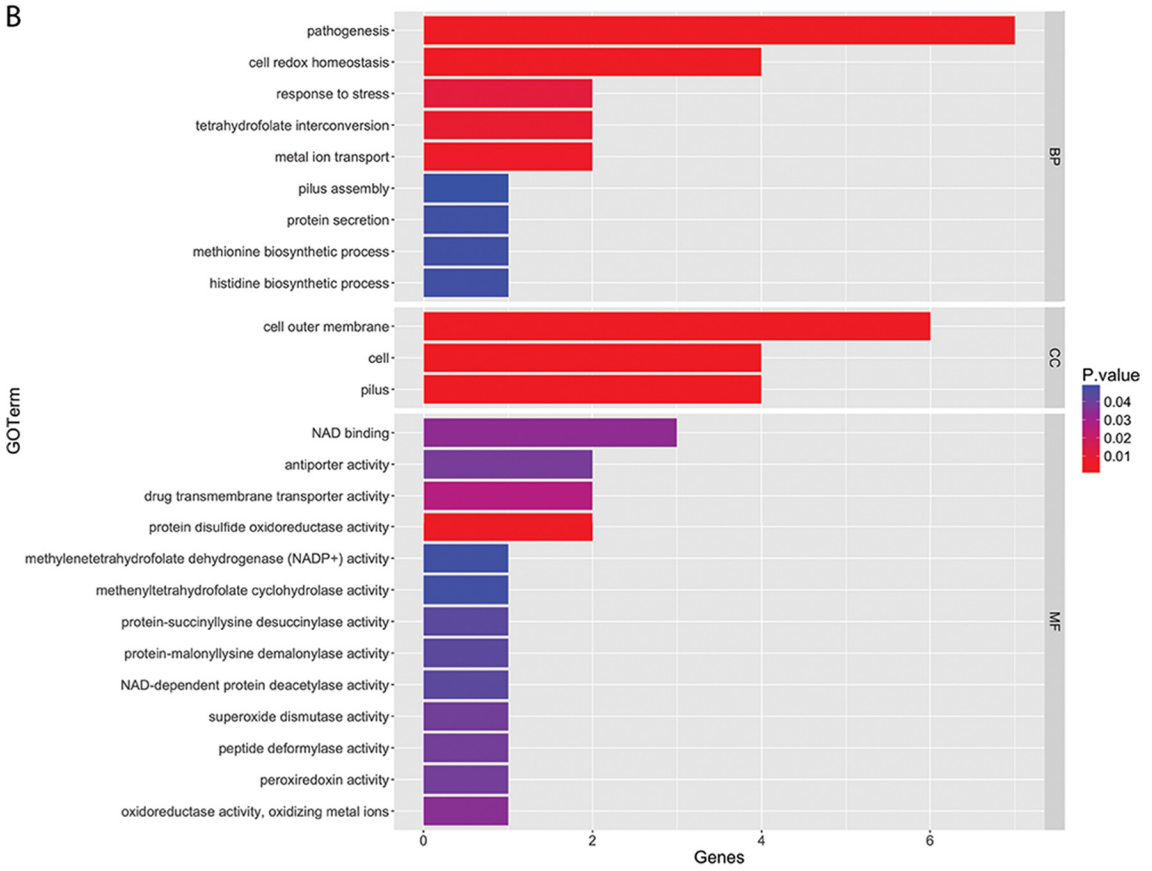
B
pathogenesis
cell redox homeostasis
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tetrahydrofolate interconversion
metal ion transport
pilus assembly
protein secretion
methionine biosynthetic process
histidine biosynthetic process
cell outer membrane
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pilus
NAD binding
antiporter activity
drug transmembrane transporter activity
protein disulfide oxidoreductase activity
methylenetetrahydrofolate dehydrogenase (NADP+) activity
methenyltetrahydrofolate cyclohydrolase activity
protein-succinyllysine desuccinylase activity
protein-malonyllysine demalonylase activity
NAD-dependent protein deacetylase activity
superoxide dismutase activity
peptide deformylase activity
peroxiredoxin activity
oxidoreductase activity, oxidizing metal ions
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2
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Genes
P.value
0.04
0.03
0.02
0.01

regulated in the mutant to compensate for the loss of InlJ. Data corresponding to the term "tetrahydrofolate interconversion" also showed significant regulation, and this pathway has been found to have a significant impact on the pathogenicity of *P. gingivalis–S. gordonii* dual-species communities (50). Within the cellular component category, 3 terms were significantly enriched from the upregulated genes, namely, the terms "cell outer membrane," "cell," and "pilus." The majority belonged to the molecular function category, with 13 terms being enriched. The term "protein disulfide oxidoreductase" was shown to be the most significantly overrepresented term, followed by "drug transmembrane transport activity."

DISCUSSION

C. albicans is an opportunistic fungal pathogen which colonizes the gut, genital tract, and oral cavity of healthy individuals. Disruption of immune surveillance or broad-spectrum antibiotic therapy can allow overgrowth and realization of pathogenic potential by the organism. *C. albicans* can cause superficial infections, such as oropharyngeal candidiasis (thrush) and vulvovaginal candidiasis, and also life-threatening systemic infections (17, 51, 52). In addition, *C. albicans* biofilm infections are common on prosthetic devices such as urinary or intravascular catheters, artificial joints, and voice boxes (53). *C. albicans* is a pleiomorphic fungus and can transition among three distinct morphological states: yeast cells, pseudohyphae, and filamentous hyphae. Different morphological states are associated with colonization and growth, and the hyphal form enables biofilm formation (54). Several virulence properties contribute to the pathogenic potential of the organism. Expressed cell wall adhesins, including the members of the agglutinin-like sequence family (Als) and hyphal wall protein (Hwp1), are crucial for *C. albicans* attachment to host tissue and for multispecies biofilm formation (44, 55, 56). *C. albicans* secretes a number of hydrolytic enzymes, such as lipases, esterases, and secreted aspartyl proteinases (Saps), that affect a variety of processes, including biofilm formation with streptococci, tissue invasion, and immune evasion (57–59). A newly reported candidalysin, a cytolytic peptide toxin secreted by *C. albicans* hyphae, causes damage to oral epithelial cells by intercalation, permeabilization, and calcium influx; triggers a proinflammatory signaling pathway response; and activates epithelial immunity (60).

The oral carriage rate of *C. albicans* in healthy subjects ranges from 25% to 60% (43). Successful colonizers of the oral microbiota generally interact synergistically with one another (61). *P. gingivalis*, for example, can attach to *S. gordonii* and accumulate into heterotypic communities, a process facilitated by several adhesin-receptor interactions and phosphotyrosine-dependent signaling within *P. gingivalis* (12, 62). Similarly, *C. albicans* is usually present in heterotypic communities (16) and interacts synergistically with a variety of other organisms, including the oral streptococci (17, 19, 63). *C. albicans* can be isolated from periodontal pockets along with *P. gingivalis* (64, 65). In this study, we found that *P. gingivalis* and *C. albicans* can coadhere, both in suspension and in sessile communities, and that interspecies binding in both contexts is mediated by the InlJ internalin-family protein on the surface of *P. gingivalis* interacting with the candidal Als3 hyphal protein.

Internalins belong to a multigene family characterized by variable numbers of leucine-rich repeats (LRRs). The internalins InlA and InlB in *Listeria* are major virulence factors and mediate attachment and bacterial uptake by nonprofessional phagocytic epithelial cells (66). InlJ comprises a distinct class of internalins, and the LRR consensus sequence contains 21 leucine residues, compared to the standard 22 residues found in other internalins. In addition, a hydrophobic residue in one of the LRRs is replaced by a cysteine in InlJ, and InlJ thus possesses a total of 14 cysteine residues (67). In *Listeria*,

FIG 6 Distribution of genes that were significantly upregulated (A) and significantly downregulated (B) in the *P. gingivalis* 33277 (WT) strain relative to the Δ*inlJ* mutant in communities with *C. albicans*, grouped into the following Gene Ontology (GO) categories: biological process (BP), cellular component (CC), and metabolic function (MF). All terms have a *P* value of <0.05 based on results of the GOSeq hypergeometric distribution test.

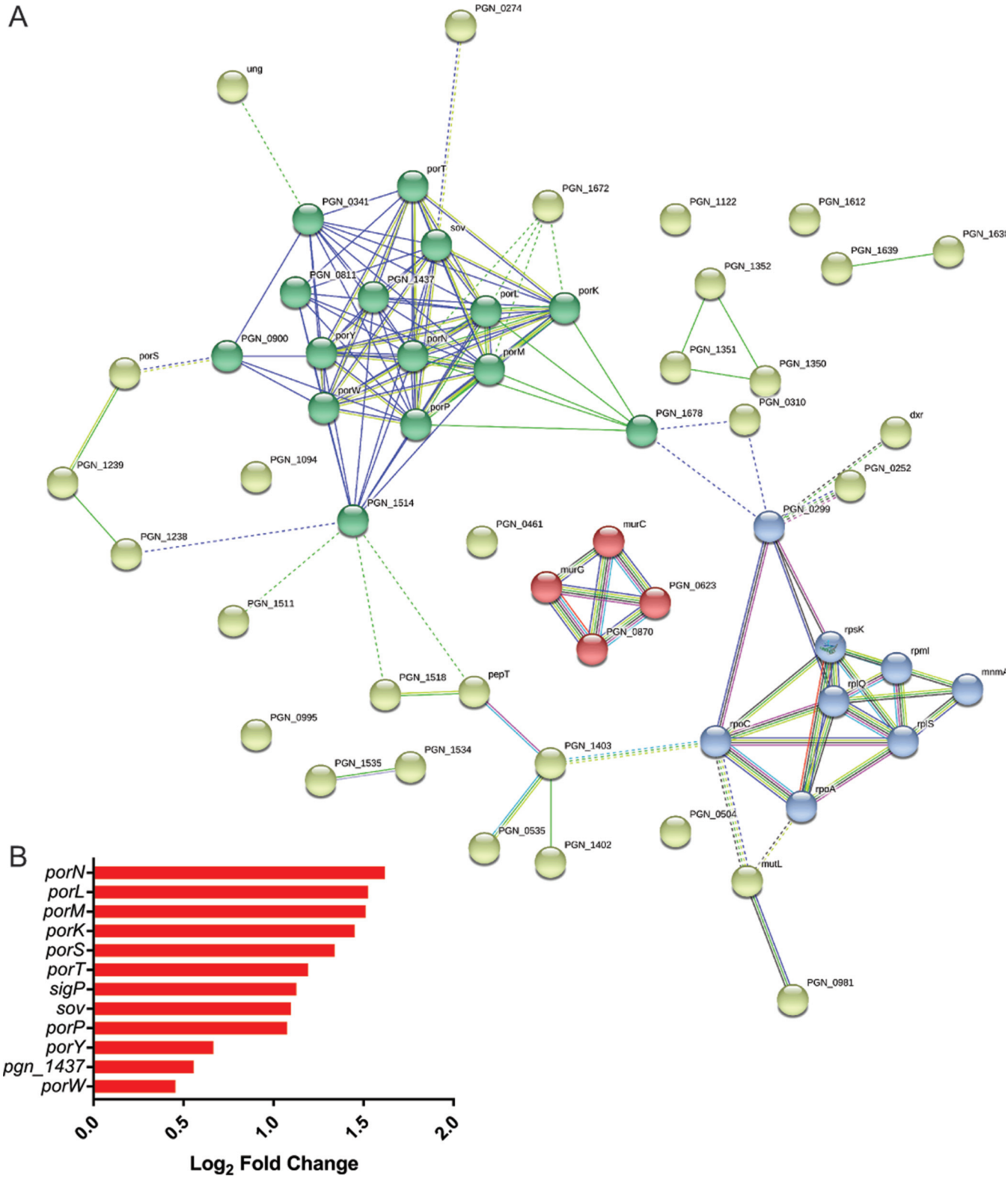

FIG 7 (A) STRING analysis of network of 3 groupings of related genes: "por" (genes corresponding to secretion systems), "rp" (genes corresponding to ribosomal proteins), and "mur" (corresponding to peptidoglycan biosynthesis). The genes indicated are those upregulated in *P. gingivalis* with InlJ in the context of a community shared with *C. albicans*. Edges between nodes represent protein interactions between nodes. The greater the number of edges, the larger the evidence base for identification of a functional link. These edges are drawn from curated databases (light blue), from experimental data (purple), and from predicted interactions (green, red, and blue). Other edges are also drawn and are from text mining, coexpression, and protein homology data. (B) Differential expression of T9SS genes in the *P. gingivalis* 33277 (WT) strain relative to the Δ*inlJ* mutant in response to *C. albicans* expressed as Log2 fold change. See Materials and Methods for statistical thresholds.

InlJ is a sortase-LPXTG anchored adhesin upregulated during infection *in vivo* (68). Listerial InlJ can bind to MUC2 (the major component of intestinal mucus) and to a variety of human cells *in vitro* (68, 69), and oral infection with an *inlJ* mutant results in reduced *Listeria* levels in the intestine, mesenteric lymph nodes, liver, and spleen (70). In *P. gingivalis*, the InlJ homologue is upregulated following contact with gingival epithelial cells (71), and an *inlJ* mutant is deficient in homotypic biofilm formation by *P. gingivalis* (41). In the current study, we found that InlJ was required for maximal attachment of *P. gingivalis* to the hyphae of *C. albicans*. In addition, soluble recombinant InlJ protein was able to compete with whole *P. gingivalis* cells for attachment to *Candida*. These results establish a new role for InlJ of *P. gingivalis* as an adhesin mediating attachment to hyphae of *C. albicans*. In addition, an interdomain binding function extends the repertoire of internalin-family activities.

In order to identify the *C. albicans* receptor for *P. gingivalis*, we first tested the involvement of the Als3 hyphal protein. *C. albicans* defective for Als3 showed a significantly reduced ability to bind *P. gingivalis*. The ability of recombinant InlJ and of *P. gingivalis* expressing InlJ to bind to *S. cerevisiae* strains expressing Als3 corroborated the role of Als3 in mediating attachment through interactions with InlJ. Als3 is able to bind extracellular matrix (ECM) proteins and epithelial and endothelial cells (44, 72–74), induce endocytosis through adherence to E- or N-cadherins (75), and mediate trafficking to the brain (76). Als3 also mediates attachment to *S. gordonii* through binding to the SspA/B streptococcal surface proteins, and this interaction stimulates the development of a mixed bacterium-fungus community with a potentially increased risk for candidiasis (43, 77). Interestingly, *P. gingivalis* also bound to the SspA/B proteins of *S. gordonii* (78–80), raising the possibility that *P. gingivalis* and *C. albicans* could compete for binding to a streptococcal substratum.

To further delineate the role of the *P. gingivalis* InlJ in the interaction with *C. albicans*, a global transcriptional approach was undertaken. Here, 381 *P. gingivalis* genes were shown to be InlJ regulated, among which 153 (79 with a Log2 fold change >1) were unique to coincubation with *C. albicans*. While this level of community-dependent regulation is similar to data reported for *S. gordonii* in association with *C. albicans* (81), the differentially expressed genes were functionally different, indicating organism-specific responses of bacteria to *Candida*. Overall, GO analysis indicated that InlJ may play a role in increased growth and cellular division during coculture. Indeed, these analyses showed that peptidoglycan biosynthesis potential was increased during coculture, a characteristic demonstrated within complex polymicrobial biofilms from periodontitis patients (82). Alternatively, the close association between the organisms mediated by InlJ may facilitate generation of an anaerobic environment by *C. albicans* which enhances the growth of *P. gingivalis*, as has been demonstrated with *Candida* and *Bacteroides* species (83). Perhaps the most notable cluster of coassociated subnetworks of genes that were uniquely upregulated in coculture were those from the T9SS, which is widely distributed in the *Fibrobacteres-Chlorobi-Bacteroidetes* superphylum and secretes cargo proteins that are often cell associated and possess a conserved C-terminal domain (48). Many of the substrate proteins are considered major virulence factors in *P. gingivalis*, including the gingipains and other proteases; peptidylarginine deiminase (PAD), which catalyzes the conversion of peptidylarginine to peptidyl citrulline; and InlJ itself. Upregulation of T9SS components in *P. gingivalis-C. albicans* communities is thus consistent with elevated community pathogenicity, or nososymbiocity (11). Similarly, communities of *P. gingivalis* with the accessory pathogen *S. gordonii*, which are synergistically pathogenic (13), show an increase in expression of genes encoding T9SS components (84). Periodontal diseases are polymicrobial infections, and it is the heterotypic community that is considered the fundamental unit of pathogenicity (61). As an inhabitant of these complex multispecies biofilms, *P. gingivalis*, which is a keystone pathogen, may thus have evolved mechanisms to sense the community environment and respond through upregulation of the secretion system which can modulate virulence potential.

TABLE 1 Microbial strains used in this study

Strain	Characteristic[a]	Source or reference
P. gingivalis		
33277	Wild type	Laboratory collection
33277 Δ*fimA*	*fimA*-deficient mutant *fimA*::*tet*	87
33277 Δ*inlJ*	*inlJ*-deficient mutant *inlJ*::*erm*	41
33277 cΔ*inlJ*	*inlJ*-deficient mutant *inlJ*::*erm* with plasmid Pt-COW:*inlJ* expressing InlJ protein	This study
33277+pTCOW	*P. gingivalis* 33277 with pT-COW plasmid	This study
C. albicans		
SC5314	Wild type	88
UB1936	*iro1-ura3*Δ::λ*imm*^{434}/*iro1*Δ-*ura3*Δ::λ*imm*^{434}/Clp10; CAI-4/Clp10 parent strain	90
UB1930	*als3lg*Δ/*als3sm*Δ-*URA3*; deficient in Als3	74
S. cerevisiae		
UB2155	pB542; BY4742 containing Gateway destination vector pBC542 (8.3 kb; Ap^r; pMB1 *ori*)	89
UB2156	pBC542-*als3sm*; BY4742 expressing *C. albicans* Als3sm (small allele)	89
UB2157	pBC542-*als3lg*; BY4742 expressing *C. albicans* Als3lg (large allele)	89
UB2161	pBC542-*cwp1*; BY4742 expressing *S. cerevisiae* Cwp1	89
E. coli		
TOP10	F$^-$ *mrcA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen

[a]Ap^r, ampicillin resistance; Str^r, streptomycin resistance.

MATERIALS AND METHODS

Microbial strains and growth conditions. The bacterial and yeast strains used in this study are listed in Table 1. *P. gingivalis* strain ATCC 33277 and its isogenic mutants Δ*inlJ*, Δ*fimA*, 33277+pT-COW, and cΔ*inlJ* (see below) were cultured in Trypticase soy broth (TSB) supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml) and menadione (1 μg/ml) (TSBHM). Erythromycin (5 μg/ml) or tetracycline (1 μg/ml) were incorporated into the medium for the growth of strains Δ*inlJ*, Δ*fimA*, cΔ*inlJ*, and 33277+pT-COW as appropriate. *C. albicans* strains were maintained aerobically on Sabouraud dextrose agar at 37°C, and broth cultures were grown in YPD broth (1% yeast extract, 2% neopeptone, 2% glucose) at 37°C with shaking. YPT medium (yeast nitrogen base, 10 mM NaH_2PO_4 buffer [pH 7.0], 0.05% Bacto tryptone) supplemented with 0.4% glucose (YPT-Glu) was utilized to support *C. albicans* biofilm formation and induction of hyphae. *S. cerevisiae* cells were cultured with shaking at 30°C in complete synthetic medium (CSM) supplemented with 0.67% yeast nitrogen base and 2% glucose (CSM-Glu). *Escherichia coli* strains were grown aerobically with shaking at 37°C in Luria-Bertani broth supplemented with ampicillin (100 μg/ml) when required.

Complementation of strain Δ*inlJ*. For complementation of the Δ*inlJ* mutant, the DNA sequence containing the promoter and the coding region of *inlJ* was amplified from *P. gingivalis* 33277 chromosomal DNA using primers F1 (AATAGGATCCGTCCCGACTTTCCGATATATAAG) (containing a BamHI restriction site) and R2 (AATAGTCGACTTACGGCATCGCGGTTTTG) (containing a SalI restriction site). The shuttle vector pT-COW plasmid was digested with the appropriate restriction enzymes to allow cloning of the amplified PCR product into the *tetC* region. The resulting plasmid, pT-COW:*inlJ*, was transformed into *E. coli* TOP10 and selected with ampicillin. Purified pT-COW:*inlJ* was introduced into the Δ*inlJ* strain by conjugation as described previously (85). The presence of the pT-COW:*inlJ* plasmid and of the *ermF* gene on the chromosome of the transconjugants was confirmed by PCR and sequencing. The resulting strain was designated cΔ*inlJ*. As determined by quantitative reverse transcription-PCR (qRT-PCR), the expression levels of the *inlJ* gene were similar ($P > 0.05$) in strains cΔ*inlJ* and 33277.

Expression of recombinant InlJ protein (rInlJ). InlJ protein was expressed as a His-tagged fusion protein using an Expressway cell-free *E. coli* expression system (Invitrogen, Carlsbad, CA). Briefly, the entire coding region of *inlJ* (PGN_1611) was amplified from a *P. gingivalis* 33277 genomic template using primers F1 (ATGAAAAGAAAACCGCTATTCTCAG) and R1 (TTACGGCATCGCGGTTTTGATCG), cloned into pEXP5-NT/TOPO, and transformed into *E. coli* TOP10 cells. Following confirmation by sequencing, soluble His-tagged protein was obtained using MagneHis particles (GE Healthcare, Pittsburgh, PA). The purity of the resulting protein was verified by SDS-PAGE electrophoresis.

***C. albicans-P. gingivalis* interactions in the planktonic phase.** Binding interactions between *C. albicans* and *P. gingivalis* in suspension were measured essentially as described previously (43). *C. albicans* cells were grown for 16 h in YPD medium, harvested by centrifugation (4,000 × *g*, 10 min), washed twice with YPT medium, and suspended at an optical density at 600 nm (OD_{600}) of 1.0 (~1 × 10^7 cells/ml). Aliquots (0.2 ml) of cell suspension were then incubated in YPT-Glu (1.8 ml) at 37°C for 2 h with shaking to induce formation of hyphae. *P. gingivalis* was cultured for 16 h in TSBHM medium, harvested by centrifugation (4,000 × *g*, 10 min), washed with phosphate-buffered saline (PBS), suspended in 1.5 mM fluorescein isothiocyanate (FITC) solution, and incubated at 20°C for 30 min. After two washes with PBS to remove excess FITC, cells were suspended in YPT-Glu supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) (YPT-GluHM) at an OD_{600} of 0.5. FITC-labeled bacteria were then added to the yeast cell suspension and incubated for 1 h at 37°C with shaking. Samples (50 μl) of the

suspension were applied to microscope slides and visualized by light and fluorescence microscopy. Images were analyzed using Zeiss Zen imaging software. Attachment of *P. gingivalis* to *C. albicans* was categorized as representing either "binding" (extensive attachment of bacteria to hyphae with bacterial clumping and bacterial cells aligned along hyphae in distinct patches) or "no binding" (sparse or no interactions between bacteria and hyphae) (see Fig. S3 in the supplemental material). The numbers of hyphae within these categories were expressed as the percentages of the total number of hyphae counted from 4 independent experiments. One hundred hyphal cells were counted for each assay. For inhibition assays, *C. albicans* was incubated with rInlJ or control protein at 37°C for 2 h, prior to addition of *P. gingivalis*.

Dual-species biofilm formation. Biofilm formation by *C. albicans* and *P. gingivalis* was assayed as described previously (43). Sterile glass coverslips were incubated with filter-sterilized 10% saliva for 16 h at room temperature and washed twice with PBS. *C. albicans* cells were grown for 16 h in YPD medium, harvested by centrifugation (4,000 × *g*, 10 min), washed twice with YPT medium, and suspended to an OD_{600} of 1.0. Cells (1×10^6) were added to wells of 12-well plates containing saliva-coated coverslips and YPT-Glu medium (0.9 ml) and were incubated at 37°C for 2 h with gentle shaking to induce formation of hyphae. The YPT-Glu medium was replaced with YPT-GluHM, and FITC-labeled *P. gingivalis* cells (5×10^6) were added. Dual-species cultures were incubated for a further 1 h at 37°C with gentle shaking (50 rpm). Unbound bacteria in suspension were removed, and 1 ml of YPT medium containing hexidium iodide (Sigma-Aldrich, St. Louis, MO) was added for 5 min to fluorescently stain *C. albicans*. Coverslips were washed twice with PBS, mounted with Prolong Gold (Invitrogen), and imaged with a Leica SP8 confocal microscope. Images were analyzed using Volocity 6.3 software (PerkinElmer, Waltham, MA). For inhibition assays, *C. albicans* was incubated with rInlJ or control protein at 37°C for 2 h, prior to addition of *P. gingivalis*.

Whole-cell enzyme-linked immunosorbent assay (ELISA). *S. cerevisiae* cells were grown for 16 h in CSM-Glu medium, harvested by centrifugation (4,000 × *g*, 10 min), washed twice in PBS, and suspended at an OD_{600} of 1.0. Microtiter plates were coated with 100 μl of cell suspension at room temperature for 1 h. The coated plate was washed twice with 0.1% Tween–PBS followed by blocking performed for 1 h with 100 μl of 10% skim milk–PBS and was further washed as described above. *S. cerevisiae* cells were then reacted for 1 h with either rInlJ (5 μg) or *P. gingivalis* cells (1×10^7). After a washing step, bound rInlJ protein was detected using a 1:2,000 dilution of His-tagged monoclonal antibody (MAb) (Cell Signaling, Inc., Danvers, MA) and *P. gingivalis* was detected with *P. gingivalis* whole-cell antibodies (1:10,000). After 1 h of incubation, reactions were developed with goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (Cell Signaling) and TMB substrate (Invitrogen). The reaction was stopped with 100 μl of 1 N HCl, and the OD value was determined at 450 nm.

RNA sequencing (RNA-Seq). *C. albicans* was induced to form hyphae in YPT-GluHM for 2 h, as described above. Equal numbers of *P. gingivalis* and *C. albicans* cells (5×10^8) were then incubated together in the planktonic phase in YPT-GluHM for 1 h. The two species were treated identically in monocultures in separate experiments. Cells were harvested by centrifugation and suspended in ice-cold RLT buffer (Qiagen, Manchester, United Kingdom) containing 2-mercaptoethanol. Acid-washed Biospec glass beads (0.6 ml) were added, and cells were disrupted by alternating shaking (30 s) using a FastPrep-25 bead beater (MP Biomedicals, Santa Ana, CA) and incubating for 1 min on ice (repeated 3 times). RNA was extracted and purified using an RNeasy minikit (Qiagen) with on-column DNase digestion (Qiagen). rRNA was depleted with a RiboZero Magnetic Gold kit (Epicentre, Illumina Inc., Madison, WI), and Illumina sequencing libraries were prepared using ScriptSeq v2 (Epicentre) with 10 cycles of PCR amplification. Paired-end sequencing of 100 bp was undertaken using a HiSeq 2500 system (Illumina) in high-output mode with Truseq v3 reagents.

FASTQ data were filtered using the fastq-mcf command from the EA-UTILS suite to remove adapter sequences and low-quality bases (86). Filtered data were aligned against the reference using Bowtie v.2.2.6. The resulting aligned reads were processed with SAMtools (v0.1.19), and gene features were counted using SAM files and the function htseq-count from Python package HTSeq v0.9.1 (https://pypi.python.org/pypi/HTSeq). The DESeq2 package was then used to apply a negative binomial model to account for dispersion between samples, before assessing differential expression between variables. *P* values were calculated using DESeq2, and Benjamini-Hochberg adjusted *P* values of <0.05 were considered significant. Following identification of differentially expressed *P. gingivalis* genes, unique genes (with an adjusted *P* value of <0.05) that were differentially expressed only during the coincubation with *C. albicans* were discerned. *P. gingivalis* genes were annotated with their associated Gene Ontology identifiers from UniProt (http://www.uniprot.org/). GO enrichment/overrepresentation analysis was performed by the use of the R GOSeq package, which implements a Wallenius hypergeometric distribution to account for bias based on gene length. Protein interaction networks were drawn from significantly differentially expressed genes using STRING (https://string-db.org/cgi/input.pl).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00202-18.

FIG S1, EPS file, 1.7 MB.

FIG S2, EPS file, 1.2 MB.

FIG S3, EPS file, 1.8 MB.

TABLE S1, XLSX file, 0.3 MB.

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