Natural Competence Is a Major Mechanism for Horizontal DNA Transfer in the Oral Pathogen *Porphyromonas gingivalis*

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ABSTRACT *Porphyromonas gingivalis* is a Gram-negative anaerobe that resides exclusively in the human oral cavity. Long-term colonization by *P. gingivalis* requires the bacteria to evade host immune responses while adapting to the changing host physiology and alterations in the composition of the oral microflora. The genetic diversity of *P. gingivalis* appears to reflect the variability of its habitat; however, little is known about the molecular mechanisms generating this diversity. Previously, our research group established that chromosomal DNA transfer occurs between *P. gingivalis* strains. In this study, we examine the role of putative DNA transfer genes in conjugation and transformation and demonstrate that natural competence mediated by *comF* is the dominant form of chromosomal DNA transfer, with transfer by a conjugation-like mechanism playing a minor role. Our results reveal that natural competence mechanisms are present in multiple strains of *P. gingivalis*, and DNA uptake is not sensitive to DNA source or modification status. Furthermore, extracellular DNA was observed for the first time in *P. gingivalis* biofilms and is predicted to be the major DNA source for horizontal transfer and allelic exchange between strains. We propose that exchange of DNA in plaque biofilms by a transformation-like process is of major ecological importance in the survival and persistence of *P. gingivalis* in the challenging oral environment.

IMPORTANCE *P. gingivalis* colonizes the oral cavities of humans worldwide. The long-term persistence of these bacteria can lead to the development of chronic periodontitis and host morbidity associated with tooth loss. *P. gingivalis* is a genetically diverse species, and this variability is believed to contribute to its successful colonization and survival in diverse human hosts, as well as evasion of host immune defenses and immunization strategies. We establish here that natural competence is the major driving force behind *P. gingivalis* DNA exchange and that conjugative DNA transfer plays a minor role. Furthermore, we reveal for the first time the presence of extracellular DNA in *P. gingivalis* biofilms, which is most likely the source of DNA exchanged between strains within dental plaque. These studies expand our understanding of the mechanisms used by this important member of the human oral flora to transition its relationship with the host from a commensal to a pathogenic relationship.

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Horizontal DNA exchange between bacteria is a recognized adaptation mechanism to enhance bacterial survival during changing and stressful environmental conditions or during relocation from one distinct niche to another. Cooperatively with DNA replication errors, DNA exchange generates a "genomic cloud" of variants with phenotypic differences, which ensures survival of a portion of the population during a selection event, such as introduction of an antibiotic or a significant change in habitat conditions (1–4). The degree of genetic diversity found within a given bacterial species varies significantly, with some species exhibiting small amounts of diversity or a clonal population structure; in contrast, others are highly diverse, with a nonclonal population structure. Nonclonal populations are common to opportunistic human pathogens involved in chronic colonization and infections, with *Helicobacter pylori* and *Neisseria meningitidis* being perhaps the most notable examples (5, 6).

Porphyromonas gingivalis is a Gram-negative anaerobe that colonizes distinct niches in the human oral cavity, primarily the deep crypts of the tongue and the subgingival plaque biofilm (7). Stable colonization of the subgingival crevice by this oral pathogen is a major contributing factor in the development of host inflammatory responses and subsequent periodontal disease. *P. gingivalis* is ubiquitous within the human population and is commonly transmitted from older to younger generations within households (8–10). Successful transfer between distinct host niches likely requires this bacterium to have multiple strategies to rapidly adapt to the local environment.

There is extensive genetic variation among *P. gingivalis* strains, resulting in measurable phenotypic heterogeneity in behaviors ranging from biofilm formation (11) to virulence (12, 13). The population structure of *P. gingivalis* is nonclonal (14, 15), and it has been predicted that, as with other nonclonal opportunistic pathogens, horizontal DNA transfer is responsible for genetic variation between strains (16, 17). In a previous study using *P. gin-*

TABLE 1 Bacterial strains used in this study

Strain	Genomic marker	Mutation	Strain abbreviation	Source
P. gingivalis W83			W83	ATCC BAA-308
P. gingivalis ATCC 33277			ATCC 33277	ATCC 33277
P. gingivalis ATCC 53977			ATCC 53977	ATCC 53977
P. gingivalis W83	Rif^r		W83 Rif^r	This study
P. gingivalis W83	*PG1244* Ω*tetQ*		W83 Tet^r	This study
P. gingivalis W83	*PG1244* Ω*tetQ*	*traA–Q-mob::ermF*	W83 Tet^r Δ*tra*	This study
P. gingivalis W83	Rif^r	*traA–Q-mob::ermF*	W83 Rif^r Δ*tra*	This study
P. gingivalis W83	*PG1244* Ω*tetQ*	*comF::ermF*	W83 Tet^r Δ*comF*	This study
P. gingivalis W83	Rif^r	*comF::ermF*	W83 Rif^r Δ*comF*	This study
P. gingivalis ATCC 33277	Rif^r		ATCC 33277 Rif^r	This study
P. gingivalis ATCC 33277	*PG1244* Ω*tetQ*		ATCC 33277 Tet^r	This study
P. gingivalis ATCC 53977	Rif^r		ATCC 53977 Rif^r	This study
P. gingivalis ATCC 53977	*PG1244* Ω*tetQ*		ATCC 53977 Tet^r	This study

givalis type strains W83 and ATCC 33277 (18), we found that strain ATCC 33277, but not W83, is able to transfer plasmid DNA into *Escherichia coli*. Plasmid transfers were insensitive to treatment with DNase I, implying a mechanism most consistent with conjugation. We also documented that chromosomal DNA transfer occurs within and between *P. gingivalis* strains and hypothesized that the mechanism was similar to that of the plasmid transfer model (18).

Each of the genome sequences of strains W83 and ATCC 33277 (19, 20) contain genetic loci that encode homologues of the DNA transfer apparatus of the *Bacteroides* conjugative transposons cTnDot and cTn*341* (21, 22). The *Bacteroides* DNA transfer locus contains 17 genes annotated alphabetically from *traA* to *traQ* (*traA–Q*), as well as a mobilization locus annotated as *mob*. The *Bacteroides* are the dominant anaerobes in the human intestinal flora and are close taxonomic relatives of the oral genera *Porphyromonas* and *Prevotella* (23). Extensive research has documented horizontal DNA transfer of antibiotic resistance genes throughout the human gut flora via conjugative transposons utilizing *traA–Q*-encoded transfer systems (24–28). Other members of phylum *Bacteroidetes* contain loci that encode homologues of *traA–Q*; however, outside the *Bacteroides*, the role of *traA–Q* is not defined. *P. gingivalis* type strains W83 and ATCC 33277 contain *traA–Q* regions that are genetically distinct from one another; in our previous study, we hypothesized that this sequence divergence was responsible for the differences in plasmid and chromosome DNA transfer frequencies between these two strains (18).

In this study, we examine the molecular mechanisms responsible for DNA exchange in the oral opportunistic pathogen *P. gingivalis*. Our initial working hypothesis was that *P. gingivalis* chromosomal DNA transfer was mediated by the *traA–Q* locus by a mechanism similar to chromosomal mobilization by cTns in the *Bacteroides* (29). However, in contrast to our expectations, we uncovered an efficient *comF*-dependent transformation system that is responsible for the majority of *P. gingivalis* DNA exchange.

RESULTS

***traA–Q* locus and DNA transfer.** Our initial working hypothesis was that conjugation mediated by the *traA–Q* locus was responsible for chromosomal DNA transfer in strain W83. To test this hypothesis, we created a W83 strain with a complete deletion of the locus *traA–Q* (PG1473 to PG1486) and the contiguous mobilization locus *mobABC* (PG1488 to PG1490) by allelic replace-

ment of the locus with the *ermF* marker (see Fig. S1 in the supplemental material). Assuming the conjugation hypothesis is correct, the *traA–Q-mob* locus should be required for DNA transfer, as it is for *Bacteroides* (30). To create a chromosomal marker for the comparative analysis of DNA transfer, we inserted the *tetQ* gene into the intergenic region downstream of the *PG1244* gene in wild-type W83 and in the *traA–Q::ermF* mutant, thus creating strains W83 *PG1244* Ω*tetQ* (W83 Tet^r, where Tet^r indicates tetracycline resistance) and W83 *PG1244* Ω*tetQ traA–Q-mob::ermF* (W83 Tet^r Δ*tra*). A second *traA–Q* deletion mutant was created in the W83 rifampin-resistant (Rif^r) background, resulting in W83 Rif^r Δ*tra* (Table 1).

To examine the involvement of the *traA–Q* locus in *P. gingivalis* DNA exchange, transfer assays with live strains were performed. For these assays, equal amounts of two strains (having Rif^r or Tet^r markers) were mixed, plated at a high density on agar, and incubated anaerobically for 24 h. The mixture was then collected, diluted, and plated on agar medium selective for both transfer markers (Table 2). As a result, each strain in the mixture can potentially serve as both recipient and donor of the chromosomal markers. The DNA transfer efficiencies shown are calculated as the total numbers of Tet^r and Rif^r offspring divided by the number of input cells and are the averages of results from three or more independent replicates (*n*). The DNA transfer efficiency between wild-type strains of W83 Rif^r and W83 Tet^r is 6.73×10^{-5} (Table 2, first row). The pairing of the W83 Tet^r Δ*tra* mutant and the W83 Rif^r Δ*tra* mutant resulted in an average DNA transfer efficiency of 2.02×10^{-5} (Table 2, second row), which is 3-fold lower and significantly different from the wild-type efficiency (*P* = 0.01). However, these results do not demonstrate the complete loss of DNA transfer expected if the *traA–Q-mob* locus were exclusively required for DNA exchange.

The results in Table 2 are remarkably similar to those that we previously published; in that study, the DNA transfer assays utilized erythromycin and tetracycline resistance markers (18). In that case, the DNA transfer efficiency (7.6×10^{-5} [$\pm 6.6 \times 10^{-5}$]) was not significantly different from the results shown here. To ensure that use of rifampin resistance as a marker does not result in high levels of background mutations, we tested *P. gingivalis* W83 to determine the rate of spontaneous rifampin resistance. The spontaneous mutation frequency for W83 is 2.54×10^{-9},

TABLE 2 DNA transfer assays with live strains

Strain 1 (W83) phenotype and genotype	Strain 2 (W83) phenotype and genotype	DNA transfer efficiency[a]	SD	n[b]
Rif^r	Tet^r	6.73×10^{-5}	4.65×10^{-5}	10
Rif^r Δtra	Tet^r Δtra	2.02×10^{-5}	6.83×10^{-6}	8
Rif^r	Tet^r (DNase I)	7.81×10^{-6}	7.45×10^{-6}	5
Rif^r $\Delta comF$	Tet^r	9.74×10^{-6}	9.08×10^{-6}	3
Rif^r $\Delta comF$	Tet^r $\Delta comF$	4.22×10^{-7}	3.11×10^{-8}	3
Rif^r Δtra	Tet^r Δtra (DNase I)	7.81×10^{-6}	7.45×10^{-6}	4
Rif^r $\Delta comF$	Tet^r $\Delta comF$ (DNase I)	4.43×10^{-7}	1.21×10^{-7}	4

[a] DNA transfer efficiency is calculated as the total number of Tet^r and Rif^r offspring divided by the number of input recipient cells.
[b] n is the number of replicates of each experiment.

with a standard deviation of 3.01×10^{-9}, and thus does not significantly impact our DNA transfer efficiency results.

DNA uptake via transformation. As the *traA–Q-mob* locus is not the only system responsible for *P. gingivalis* DNA transfer, we redirected our efforts to investigate the existence of other DNA exchange mechanisms in *P. gingivalis*. To determine if exogenous chromosomal DNA is involved in DNA exchange, we repeated the DNA transfer assay with wild-type cells and included 30 U of DNase I enzyme during the overnight anaerobic incubation of the cells on agar. If extracellular DNA (eDNA) is important for DNA exchange, the presence of DNase I will reduce the amount of free DNA available and cause a significant decrease in the number of recovered Tet^r and Rif^r offspring. The results from this assay show (Table 2, third row) that the DNA transfer efficiency in the presence of DNase I is reduced 7-fold relative to that in its absence (7.85×10^{-6} relative to 6.73×10^{-5}, respectively). These results indicate that the presence of extracellular chromosomal DNA is important for DNA transfer.

To further investigate if DNA can be introduced into *P. gingivalis* by a transformation-like mechanism, we modified our DNA transfer assay by incubating the donor bacteria at 80°C for 10 min to lyse the cells and release native genomic DNA. The live recipient strain was then mixed with the dead donor and treated as detailed previously. As conjugation requires both a live donor and recipient cells, conjugation would be prohibited under these conditions. The results show (Table 3, first row) that W83 is transformed by incubation with dead donor cells with an efficiency (3.17×10^{-4}) that is significantly higher than the DNA transfer efficiency in the assay with live donor cells (Table 2, first row) ($P = 0.02$). As a control, the wild-type mixture was incubated overnight with 30 U of DNase I, which reduced the DNA transfer efficiency to 7.3×10^{-8} (Table 3, second row). Taken together, these results indicate that strain W83 has an active DNA uptake system that recognizes external DNA released from donor cells and thus appears to be naturally competent.

DNA exchange by transformation occurs in other *P. gingivalis* strains. We examined two additional *P. gingivalis* strains for competence by utilizing strains ATCC 33277 and ATCC 53977. These strains are genetically distinct from each other and from W83. Most notably, ATCC 33277 has a *traA–Q* gene cluster with minimal DNA identity to the W83 *traA–Q* locus (15, 31); the ATCC 33277 *traA–Q* gene products are predicted to be more similar to those found in conjugative *Bacteroides* transposons. Previously, we demonstrated that strain ATCC 33277, but not W83, is able to transfer plasmid DNA to *E. coli* by conjugation, and this transfer was not affected by DNase I treatment (18). We created ATCC 33277 and ATCC 53977 donor strains with *PG1244* Ω*tetQ* as the transfer marker (Table 1). Based on published genome sequences (19, 20), the *PG1244* loci are 96% similar in W83 and ATCC 33277, and our PCR analysis shows it to be present in strain ATCC 53977. We then utilized the DNA transfer assay with dead donor cells to determine if strains ATCC 33277 and ATCC 53977 are competent and to compare the transformation frequencies when the DNA donor is a self or nonself strain. The results indicate that all three *P. gingivalis* strains tested could be transformed by both self- and nonself DNA (Fig. 1). However, in all cases, W83 was transformed at significantly higher frequencies (10^{-4}) than either ATCC 33277 or ATCC 53977 (10^{-6}). There was no significant difference in the transformation efficiencies between self- and nonself donors for any of the strains. Thus, our results indicated that three divergent strains of *P. gingivalis* all possess competence systems in which genomic DNA uptake shows no preference for the donor strain.

Role of *comF* in DNA exchange. The *P. gingivalis* W83 genome has genes that are predicted to be associated with competence, based on their identity to known competence genes in other bacterial species. One open reading frame carried by PG0158, here designated *comF*, is annotated as encoding an ATP-dependent DNA helicase and is predicted to possess a phosphoribosyltransferase domain, which is a distinguishing feature of ComF proteins

TABLE 3 DNA transfer assays with dead donor strains

Live recipient (W83) phenotype and genotype	Dead donor (W83) phenotype and genotype	DNA transfer efficiency[a]	SD	n[b]
Rif^r	Tet^r	3.17×10^{-4}	2.61×10^{-4}	10
Rif^r	Tet^r (DNase I)	7.30×10^{-8}	1.41×10^{-8}	3
Rif^r $\Delta comF$	Tet^r	$<10^{-8}$		8
Rif^r	Tet^r $\Delta comF$	2.02×10^{-4}	5.42×10^{-5}	4
Rif^r Δtra	Tet^r	1.92×10^{-4}	5.61×10^{-5}	5
Tet^r	Rif^r Δtra	1.61×10^{-4}	4.23×10^{-5}	4

[a] DNA transfer efficiency is calculated as the total number of Tet^r and Rif^r offspring divided by the number of input recipient cells.
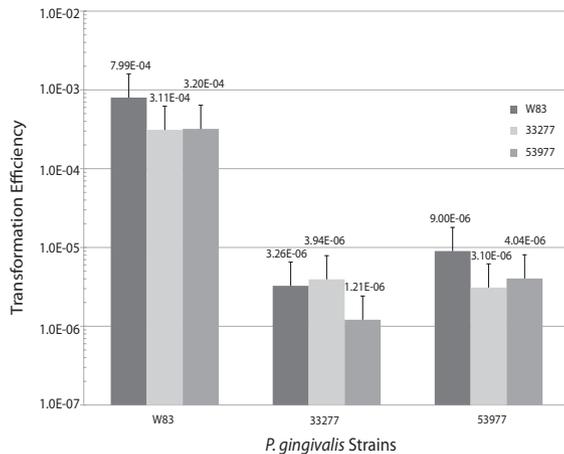[b] n is the number of replicates of each experiment.

FIG 1 DNA transfer efficiency with dead donors. The DNA uptake efficiencies of experiments with three live recipient *P. gingivalis* strains each mixed with the same three dead donor strains are shown. Transformation efficiencies are shown on the *y* axis and are calculated as the number of recovered transformants (Rif^r Tet^r) divided by the number of input recipient cells. All recipient strains (*x* axis) were rifampin resistant, and all dead donor strains contained the genomic insertion *PG1244* Ω*tetQ*.

(32–34). Another putative ComF homologue is found in the genome of strain ATCC 33277 (PGN_0270). ComF proteins are hypothesized to produce the energy required to drive translocation of DNA through the inner membrane into the cytoplasm (35). We deleted the W83 *comF* gene by allelic replacement with the *ermF* gene in the W83 Tet^r and W83 Rif^r strain backgrounds, resulting in strains W83 *PG1244* Ω*tetQ comF*::*ermF* and W83 Rif^r *comF*::*ermF*, respectively (Table 1). After confirming the mutant genotypes, we tested the phenotypes in the DNA transfer assay using a dead donor. The W83 Rif^r Δ*comF* mutant (Table 3, third row) was not able to incorporate the DNA transfer markers from the dead donor DNA into its chromosome at a detectable level, as no colonies from transfer assays grew on the agar plates with both antibiotics, even with an input of 5×10^8 donor and recipient cells. Using the W83 Tet^r Δ*comF* mutant as the dead donor strain with the W83 Rif^r recipient, there was no significant effect on DNA transfer efficiency (Table 3, fourth row). These results indicate that the *comF* gene product is critical for W83 uptake of exogenous DNA and reinforces the concept that the major DNA exchange machinery in *P. gingivalis* strain W83 is competence based.

Comparison of Δ*comF* and Δ*traA–Q-mob* mutant phenotypes. In order to delineate the relative roles of the *traA–Q-mob* locus and *comF* gene in *P. gingivalis* DNA transfer, we compared the results of DNA transfer assays with live and dead donors in both mutant backgrounds. The W83 Tet^r Δ*comF* mutant was paired with either W83 Rif^r or the W83 Rif^r Δ*comF* mutant in live donor DNA transfer assays (Table 2, fourth and fifth rows). The presence of one or two Δ*comF* mutants in the assay resulted in 7-fold and 159-fold reductions in the resulting numbers of Tet^r Rif^r colonies, respectively. This result was surprising, as the Δ*comF* mutant was completely deficient in transformation in the transfer assay with dead donors, yet two live Δ*comF* mutants were still able to exchange DNA. Thus, a low level of DNA transfer is present with a live donor and does not require *comF*.

A DNA transfer assay was then performed using the W83 Tet^r Δ*traA–Q-mob* mutant as either the dead donor or the recipient, and no significant difference from results with the wild type was found for either condition (Table 3, fifth and sixth rows). From this we conclude that, unlike *comF*, the *traA–Q-mob* locus is not involved in DNA uptake via competence. However, as two Δ*traA–Q-mob* mutants have a slight but significant reduction in DNA transfer efficiency in live-donor assays, we theorized that the Δ*traA–Q-mob* mutant phenotype may represent the loss of conjugation, leaving transformation by *comF* as the only DNA exchange process. In the reciprocal situation, the residual DNA transfer in the Δ*comF* mutant live-donor assay may result from conjugation mediated by *traA–Q-mob*.

To further test the theory that *comF* is involved in competence and that *traA–Q-mob* is involved in conjugation, we performed two live-donor transfer assays in the presence of DNase I. We hypothesized that two Δ*traA–Q-mob* mutants in a live-donor assay would transfer DNA only via competence mechanisms and thus that the presence of DNase I in this assay would significantly reduce the number of CFU. In a parallel experiment, two Δ*comF* mutants in a live-donor assay should exchange DNA only by conjugation; thus, the presence of DNase I should have no significant effect on DNA exchange efficiencies. The results from these assays are shown in Table 2 (sixth and seventh rows). Two Δ*traA–Q-mob* mutants in the presence of DNase I have an average DNA transfer efficiency of 7.81×10^{-6}, a 6-fold and significant reduction compared to the transfer efficiency of the non-DNase I controls ($P = 0.05$). However, the transfer efficiencies of two Δ*comF* mutants in the presence (4.22×10^{-7}) or absence (4.43×10^{-7}) of DNase I are not statistically different; these results reinforce the concept that two forms of DNA transfer occur simultaneously in *P. gingivalis*.

Induction of natural competence in *P. gingivalis*. These studies demonstrate that DNA uptake by transformation is the dominant form of DNA exchange in *P. gingivalis*; thus, we decided to further investigate the conditions that regulate DNA uptake. This point was of great interest to us, as in our previous study we found that purified chromosomal DNA was not taken up by *P. gingivalis* (18). In our DNA transfer assays with dead donor cells, native methylated DNA is released from lysed bacteria as the source of the transferred DNA. To determine if nonmethylated DNA could be transferred at similar frequencies and if this process required signaling or DNA-binding molecules from the donor lysate, a DNA transfer assay was performed with a PCR product as the source of the DNA. Specifically, 1 μg of a 3.2-kb PCR product containing the *PG1244* Ω*tetQ* marker was incubated with a *P. gingivalis* recipient, in the presence or absence of bacterial cell lysate, overnight on an agar plate surface. After recovery and enumeration of the transformants, the transformation frequencies were calculated as the total number of recovered colonies per total number of recipient bacteria, with a standardized input of 1 μg of DNA. As a baseline of comparison, 1 μg of the same PCR product was electroporated into *P. gingivalis*, and transformation frequencies were calculated. The results of these experiments are shown in Table 4.

Our first observation from this experiment was that incubation of our PCR product with recipient cells on an agar plate resulted in a high transformation efficiency (1.81×10^{-4}) and produced a significantly higher number of transformants than electroporation (180-fold). Additionally, the PCR product transferred equally

TABLE 4 DNA transfer efficiency after electroporation and transformation with a DNA fragment

| Recipient | Transfer efficiency[a] | | | |
	After electroporation	In a biofilm	In a biofilm with a cell lysate	In a biofilm treated with DNase I
W83	1.04×10^{-6} ($\pm 4.9 \times 10^{-7}$)	1.81×10^{-4} ($\pm 5.2 \times 10^{-5}$)	2.66×10^{-4} ($\pm 4.8 \times 10^{-5}$)	$<10^{-8}$
W83 $\Delta comF$	$<10^{-8}$	$<10^{-8}$	ND	ND

[a] The DNA source was a 3.2-kb PCR product containing the *PG1244* $\Omega tetQ$ gene. Standard deviations are shown in parentheses. ND, not determined.

well in the presence or absence of cell lysate. Thus, the natural competence system is tolerant to nonmethylated DNA, and there is no signal required from the donor cell lysate to activate DNA uptake. Taken together with our earlier experiments, in which high-molecular-weight chromosomal DNA was not transformative, these results suggest that the natural competence system is efficient only for uptake of low-molecular-weight DNA or requires a free 5′ DNA end.

Electroporation of the W83 Rif^r $\Delta comF$ strain compared to that of the wild type revealed a complete loss of DNA transformability in the mutant. As electroporation introduces DNA into the periplasmic space for subsequent processing and recombination, this implies that the *comF* product is critical for active transport of DNA across the highly selective inner membrane.

***P. gingivalis* biofilms contain eDNA.** *P. gingivalis* is a constituent of subgingival plaque biofilms and produces an extracellular matrix. Recent studies of other species have identified extracellular DNA (eDNA) as an important constituent of some biofilm matrixes (36–39). To determine if eDNA exists in *P. gingivalis* biofilms, as it may represent a potential DNA transformation substrate for uptake by *comF*, we developed a sequential biofilm-staining protocol to allow us to differentiate between eDNA (stained with GelGreen), DNA in cells with compromised membranes (stained with propidium iodide), and DNA inside live cells (stained with DAPI [4′,6-diamidino-2-phenylindole]) using epifluorescence microscopy. As shown in Fig. 2A, copious amounts of green amorphous material can be seen surrounding distinct blue- and red-staining bacteria in 24-h biofilms. Upon closer in-
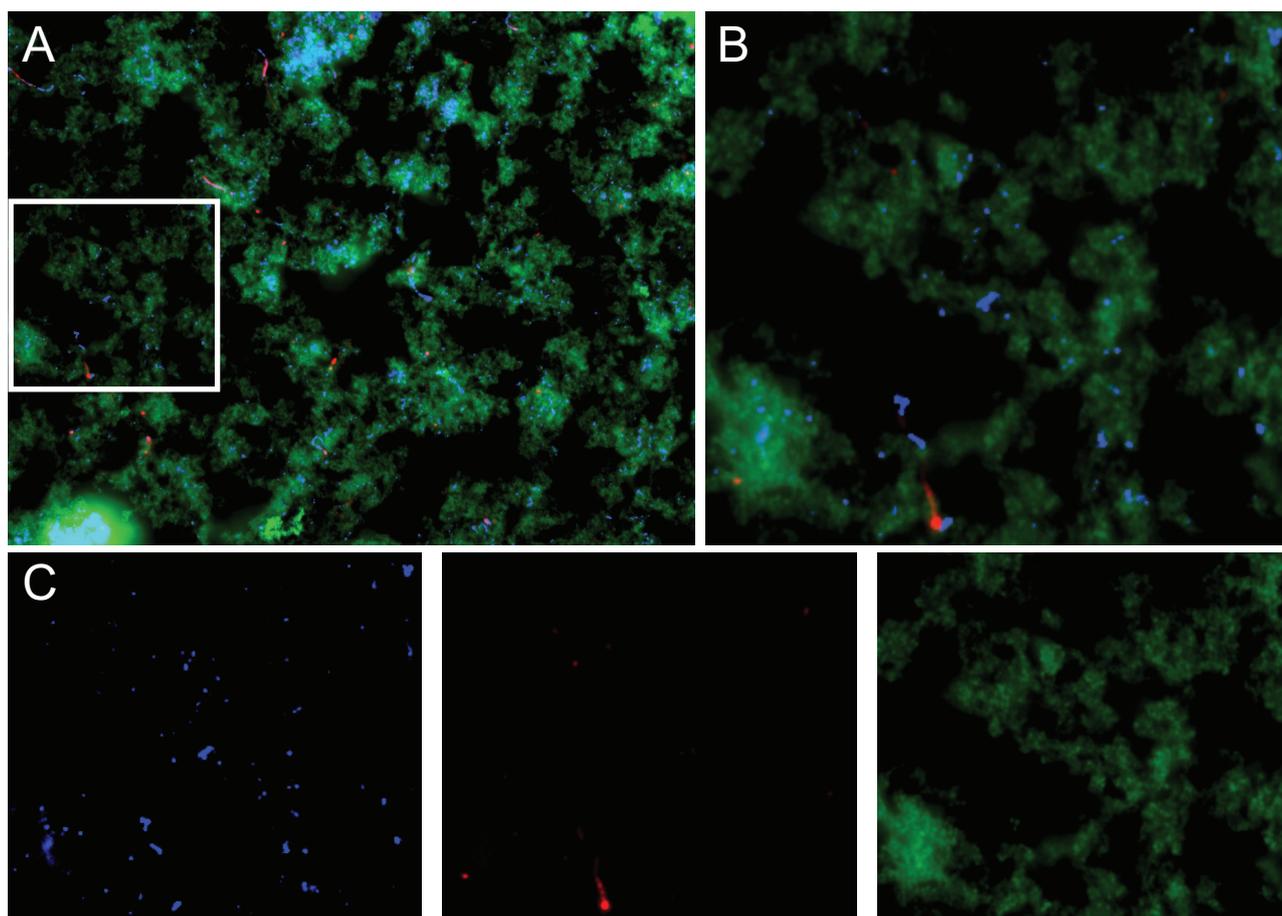

FIG 2 Fluorescence microscopy of *P. gingivalis* biofilm eDNA. *P. gingivalis* static biofilms were grown for 24 h and then stained and imaged with a fluorescence microscope. GelGreen-labeled extracellular DNA (diffuse green), propidium iodide-labeled dead bacterial cells (punctate red), and DAPI-labeled live cells (punctate blue). (A) Merged three-color image of a representative 24-h biofilm at 40× magnification. (B) The white boxed area in panel A is magnified here. (C) The blue, red, and green channels used in panel B are shown separately. The images shown are representative of three independent experiments.

spection, the green eDNA is associated with both blue- and red-staining bacterial cells (Fig. 2B). There are relatively few propidium iodide-labeled bacteria, as expected for a 24-h biofilm; the majority of bacterial cells are stained with DAPI, indicating cell viability and intact membranes (Fig. 2C). To confirm that the green-staining material is eDNA, we incubated the 24-h biofilm with and without DNase I for 4 h prior to staining (Fig. S2). Incubation of the 24-h biofilms with DNase I significantly reduced the amount of green signal, without altering the relative numbers of the blue- and red-staining bacterial cells. Although DNA released from lysed bacteria in a biofilm is a likely DNA source for uptake by the ComF system, these images indicate that active secretion of DNA by live *P. gingivalis* into the biofilm matrix may also occur.

DISCUSSION

Our results indicate that DNA transfer events in *P. gingivalis* are complex and that multiple mechanisms exist simultaneously to mediate DNA transfer. Our studies reveal that *comF*-dependent transformation by uptake of extracellular DNA is the major mechanism of horizontal DNA transfer in strain W83 and that conjugation plays a minor role in the transfer of genomic DNA. We propose that a likely extracellular DNA source is the *P. gingivalis* biofilm matrix, and we document the presence of extracellular DNA in 24-h *P. gingivalis* biofilms. The presence of redundant mechanisms to facilitate DNA exchange in this species suggests that it plays a critical role in bacterial survival in the stressful and highly competitive oral habitat.

Previously, it was assumed that *P. gingivalis* is not naturally competent, as recombinant DNA has traditionally been introduced by either electroporation (40, 41) or conjugation from an *E. coli* donor (42, 43), by using genetic tools and protocols developed for *Bacteroides* (44). However, molecular investigations of natural DNA transfer events in *P. gingivalis* have only recently been initiated. Our results here indicate that the *traA–Q* locus in strain W83 is involved in low-frequency conjugal transfer of chromosomal DNA, and our previous work found that plasmids were not a substrate for the W83 transfer system (18). In contrast, the *traA–Q* locus in *P. gingivalis* ATCC 33277 can mobilize plasmids by a DNase I-insensitive mechanism, and a recent study revealed the *traA–Q*-containing element cTnPg1 to be capable of excision and conjugation (45); both behaviors are similar to those of conjugative *Bacteroides* transposons. These cTn-like elements may have been shared between the oral and intestinal *Bacteroidetes* populations at some time in evolutionary history, and similar loci are found in the genome sequences of other members of phylum *Bacteroidetes*, indicating that DNA conjugation by *traA–Q* is widespread in this taxonomic group.

In naturally competent bacteria, specialized proteins are used for the uptake of DNA. In *P. gingivalis*, we show that *comF* is critical for DNA uptake from the exogenous environment. ComF proteins are hypothesized to produce the energy required to drive translocation of DNA through the inner membrane into the cytoplasm (35). Consistent with this, our *comF* mutant cannot be transformed by incubation with exogenous DNA or by electroporation. Interestingly, our studies show no bias against natural transformation of DNA from different strains or unmethylated DNA produced by PCR. This willingness to "sample" DNA from other sources is likely a major factor in the generation of genetic diversity within this species. It may also indicate that *P. gingivalis* is able to scavenge DNA from other species as a nutritional source, as

not all DNA in a polymicrobial biofilm will be a candidate for homologous recombination into the chromosome.

From a practical perspective, *P. gingivalis* researchers should note that the introduction of genetic constructs via natural competence is more efficient than conjugation or electroporation. This is most likely due in part to the inherent degree of cell lysis resulting from the electric pulse during electroporation. An additional difference is that our competence assay mixtures mimic biofilms by incubation overnight on the surface of an agar plate, whereas cells recovered from electroporation are incubated in broth.

A recent transcriptome comparison analysis of planktonic versus biofilm-grown *P. gingivalis* revealed *comF* expression to be increased 2-fold in the biofilm cells (46). The same study showed that the expression of restriction-modification genes is decreased in biofilms. Thus, biofilm conditions appear to favor enhanced uptake of exogenous DNA. In parallel with this, we show here for the first time that *P. gingivalis* biofilms contain eDNA. As different *P. gingivalis* strains often cocolonize the same subgingival biofilm (47), it is possible that the biofilm lifestyle of eDNA release and natural biofilm competence underlies the reassortment of alleles into new, potentially better adapted strains of this oral anaerobe.

Our biofilm images indicate that *P. gingivalis* actively secretes DNA into the matrix; in other bacterial species, eDNA has been shown to represent a subset of the DNA sequences found in the genome (36–39). Secretion of select regions of DNA may represent a mechanism to control the genetic information that is shared; for example, secretion hot spots may exist near alleles that are highly variable and thus important for selection in the host environment. Future research is needed to further characterize the features of *P. gingivalis* eDNA and to identify the full complement of genes encoding the DNA uptake and eDNA release mechanisms in *P. gingivalis* biofilms.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Porphyromonas gingivalis* strains (Table 1) were grown anaerobically at 37°C in a Coy anaerobic chamber under an atmosphere of 86% nitrogen-10% carbon dioxide-4% hydrogen. The culture medium was Trypticase soy broth (TSB) supplemented with 5% yeast extract, 2% sodium bicarbonate, 7.5 μM hemin, and 3 μM menadione. TSB blood agar plates (BAP) were made with the addition of 5% sheep's blood and 1.5% agarose. *P. gingivalis* strains resistant to rifampin were selected by serial passage on BAP containing increasing amounts of the antibiotic. Selection for antibiotic-resistant *P. gingivalis* was with 30 μg/ml rifampin, 15 μg/ml erythromycin, or 1 μg/ml tetracycline. Dual resistance was selected with 30 μg/ml rifampin and 5 μg/ml erythromycin or 30 μg/ml rifampin and 1 μg/ml tetracycline.

Molecular biology. DNA cloning, sequencing, PCR amplification, *E. coli* plasmid purification, and other common molecular biology techniques were carried out by standard procedures (48). Total DNA was purified from *P. gingivalis* using the Promega Wizard genomic DNA purification kit, with further purification by phenol-chloroform extraction if necessary. DNA amplification by PCR was done using the high-fidelity ExTaq polymerase (TaKaRa Bio, Inc.). Primers were synthesized by Integrated DNA Technologies (IDT). Primers used in this study are listed in Table 1 in the supplemental material. DNA sequencing was performed by SeqWright DNA Technology Services (Houston, TX). Sequence assembly and DNA and protein analysis were done with CLC Combined Workbench, v3 (CLC bio).

Construction of *P. gingivalis* allelic-exchange mutants. Allelic-exchange mutants of *P. gingivalis* W83 were generated by PCR amplification of ~1-kb fragments upstream and downstream of the region to be

deleted and then by creating fusion PCR products with the *ermF* marker according to a protocol derived from the work of Kuwayama et al. (49). The PCR product was cloned into the pCR2-TOPO vector for DNA sequencing (Invitrogen). Confirmed constructs were then digested with SacI, and the linear DNA was electroporated into *P. gingivalis* (40). Alternatively, fusion PCR products were sequenced and electroporated directly into W83 without cloning. Transformants were selected on erythromycin and confirmed by PCR or Southern hybridization. Mutant strains used in this study are listed in Table 1, and the genetic maps for relevant wild-type and mutant loci are shown in Fig. S1.

Construction of *P. gingivalis* genomic markers for DNA transfer assays. For this study, we created two genomic markers to test for chromosomal DNA transfer. A *tetQ* marker is located in a large intergenic region between *PG1244* and *PG1245* and is designated *PG1244* Ω*tetQ*. We use the symbol Ω to mean a gene insertion near the indicated gene rather than in it. This gene insertion was created using a PCR fusion method as described for the mutant strains above. As our second marker, we created Rif^r strains by serial passage on agar plates containing the antibiotic. Rifampin resistance results from spontaneous mutation of the *rpoB* gene, which is gene *PG0394* in strain W83.

***P. gingivalis* DNA transfer assays with live strains.** *P. gingivalis*-to-*P. gingivalis* DNA transfer assays with a live donor were performed by combining mid-log-phase cultures (optical density at 600 nm [OD$_{600}$], 0.8 to 1.0) at equal ratios of both strains (5×10^8 cells each), pelleting the mixed cultures, resuspending the cells in 50 μl of TSB, dropping the mixture onto prereduced BAP, and incubating the resulting bacterial pellet for 24 h anaerobically at 37°C. Chimeric *P. gingivalis* offspring were selected by resuspending the bacterial pellet into TSB and incubating the recovered bacteria for 10 to 14 days anaerobically on BAP containing both rifampin and tetracycline. DNA transfer efficiencies were calculated by dividing the total number of offspring by the number of input recipient bacterial cells. Controls were individual *P. gingivalis* strains resuspended in 50 μl TSB and treated identically to the mixed-strain samples.

***P. gingivalis* DNA transfer assays with dead donor cells.** *P. gingivalis*-to-*P. gingivalis* DNA transfer assays with dead donors were performed similarly to the assays with live cells, except that the mid-log-phase donor cells were heat killed by incubation at 80°C for 10 min and were then placed on ice for 10 min prior to being mixed with live recipient cells. The mixtures were subsequently treated as in the assays with live cells. Controls were individual *P. gingivalis* strains resuspended in 50 μl TSB and treated identically to the mixed-strain samples. To confirm cell death in the dead donors, aliquots of donor controls were also plated on nonselective media. As with the assays of live cells, transformants were enumerated after dilution on selective media. DNA transfer efficiencies were calculated by dividing the total number of offspring by the number of input recipient bacterial cells.

Determination of spontaneous mutation frequency. The *P. gingivalis* strains were spread on BAP and grown for 24 h. Three tubes containing 2 ml of TSB were inoculated with one independent colony each obtained from the BAP and incubated anaerobically overnight. One hundred microliters of a 10^{-6} dilution of the overnight cultures was seeded onto BAP, and 500 μl of the overnight cultures was plated onto BAP containing rifampin (30 μg/ml). Colony counts were performed after 7 days of anaerobic incubation. Mutation frequency values are reported as the number of rifampin-resistant colonies in proportion to the total viable count, and the final value was obtained from three independent experiments.

***P. gingivalis* biofilm microscopy.** Bacterial strains were grown to early log phase and diluted to an OD$_{600}$ of 0.2 in biofilm medium (50% TSB-50% PBS). Five hundred microliters of diluted culture was added to CultureWell chambered cover glass 8-well slides (Grace Bio Labs) and incubated anaerobically and statically for 24 h at 37°C. Biofilms on the cover glass slides were stained for the detection of extracellular DNA. The supernatant was gently removed with a drawn-out Pasteur pipette, 250 μl of a 0.1× solution of GelGreen (Biotium; molecular mass [MM], >1,000 Da) in N18 solution (10 mM glucose, 10 mM HEPES, 140 mM

NaCl, 0.03 mM CaCl$_2$, 2 mM MgCl$_2$, 5 mM KCl, pH 7.5) was added to each well, and plates were incubated for 30 min with gentle rocking. An aliquot of propidium iodide (Sigma; MM = 668 Da) was then added to each well, resulting in a final 0.1× (3 μM) concentration, and plates were incubated for an additional 30 minutes. Finally, an aliquot of DAPI (Sigma; MM = 350 Da; final concentration 300 nM) was added to each well, and plates were incubated for an additional 15 minutes. The supernatant was gently aspirated, the slide chambers were removed, and the wells were mounted in PermaFluor hard-set mounting medium (Thermo Scientific). Biofilm images were recorded at a 40× magnification using a Nikon Eclipse 80i epifluorescence microscope fitted with a Nikon DS-Qi1 digital camera, using the DAPI, fluorescein isothiocyanate (FITC), and Texas Red excitation/emission filter sets and Nikon NIS-Elements imaging software. Images were processed for contrast using ImageJ (NIH). In some experiments, biofilms were incubated with 100 U of DNase I for 4 h prior to extracellular DNA staining.

Statistics. All experiments were repeated independently at least three times. Data were analyzed for statistical significance ($P \leq 0.05$) using an unpaired *t* test with Excel software (Microsoft).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00231-11/-/DCSupplemental.

Figure S1, PDF file, 0.2 MB.
Figure S2, PDF file, 1.3 MB.
Table S1, PDF file, 0.1 MB.

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