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The Periodontal Pathogen *Porphyromonas gingivalis* Induces Expression of Transposases and Cell Death of *Streptococcus mitis* in a Biofilm Model

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Oral microbial communities are extremely complex biofilms with high numbers of bacterial species interacting with each other (and the host) to maintain homeostasis of the system. Disturbance in the oral microbiome homeostasis can lead to either caries or periodontitis, two of the most common human diseases. Periodontitis is a polymicrobial disease caused by the coordinated action of a complex microbial community, which results in inflammation of tissues that support the teeth. It is the most common cause of tooth loss among adults in the United States, and recent studies have suggested that it may increase the risk for systemic conditions such as cardiovascular diseases. In a recent series of papers, Hajishengallis and coworkers proposed the idea of the "keystone-pathogen" where low-abundance microbial pathogens (*Porphyromonas gingivalis*) can orchestrate inflammatory disease by turning a benign microbial community into a dysbiotic one. The exact mechanisms by which these pathogens reorganize the healthy oral microbiome are still unknown. In the present manuscript, we present results demonstrating that *P. gingivalis* induces *S. mitis* death and DNA fragmentation in an *in vitro* biofilm system. Moreover, we report here the induction of expression of multiple transposases in a *Streptococcus mitis* biofilm when the periodontopathogen *P. gingivalis* is present. Based on these results, we hypothesize that *P. gingivalis* induces *S. mitis* cell death by an unknown mechanism, shaping the oral microbiome to its advantage.

The human oral microbiome consists of over 600 individual taxa (1, 2). Its complexity and the fact that a large fraction of species is noncultivable have hampered our knowledge of the molecular and metabolic interactions that occur between the species of the bacterial biofilm and between bacteria and host.

Periodontal disease is a polymicrobial inflammatory biofilm-mediated pathology that leads to a progressive loosening and eventual loss of teeth (3, 4), and it is responsible for half of all tooth loss in adults that occurs in moderate form in 39% of American adults and in severe form in 9% of adults. Polymicrobial diseases are increasingly being recognized as more frequent than previously thought (5). In these diseases there are complex interactions among the etiologic agents, which ultimately lead to disease. In the microbial etiology of periodontitis it is generally accepted that a consortium of bacteria, not a single microorganism, is involved in the disease. Nonetheless, it is well established that in destructive periodontitis, the "red complex" (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*) are key players in the disease process (6).

A more holistic treatment of periodontitis or any other polymicrobial disease requires a better understanding of the ecology of the microbial community and the mechanisms that cause the shift from a mature stable biofilm to a dysbiotic community responsible for disease. Maintaining a healthy biofilm would be a less invasive strategy than removing an already established pathogenic biofilm.

S. mitis is one of the most abundant organisms in healthy oral microbiomes (7). Any decrease in its numbers would have an impact in the health status of the oral biofilm. In our recent work on a multispecies biofilm model, we observed that the presence of *P. gingivalis* upregulated the expression of a large number of putative transposases in two members of the healthy biofilm model, *S. mitis* and *Lactobacillus casei* (8).

The nature of the interactions between oral streptococci and the major periodontal pathogen *P. gingivalis* is complex, demonstrating both synergistic and antagonistic interactions. Pioneer studies on coaggregation provided a foundation of knowledge regarding bacterium-bacterium interactions within the oral biofilm. Oral bacteria showed specific coaggregation patterns due to the presence of certain receptors on their surface (9). *P. gingivalis* can coaggregate with a variety of oral bacteria, such as *Fusobacterium nucleatum*, *Actinomyces viscosus*, *T. denticola*, and species of *Streptococcus*, such as *Streptococcus oralis* and *Streptococcus gordonii* (9, 10). There are also negative interactions wherein streptococci inhibit *P. gingivalis* growth and protease activity (11, 12). In a clinical study, Wang et al. have shown that there is a negative correlation of distributions of *Streptococcus cristatus* and *P. gingivalis* in subgingival plaque (13). *P. gingivalis* and *S. gordonii* do not form a mature biofilm when growing independently on coverslips coated with saliva, demonstrating synergistic interactions. However, biofilm formation occurs when *S. gordonii* cells are first deposited on the salivary pellicle. *S. gordonii* may therefore provide an attachment substrate for colonization and biofilm accretion by the potential pathogen, *P. gingivalis* (14). Moreover, Periasamy and Kolenbrander showed that although *P. gingivalis* was incapable of forming a biofilm in the presence of *S. oralis* alone, the addition of *S. gordonii* was enough to allow the establishment of a mature biofilm by *P. gingivalis* and the two streptococci (15).

As mentioned above, in a previous metatranscriptomic analysis we observed a high upregulation of a large number of putative trans-

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TABLE 1 Primers used in the experiments[a]

Strain and locus	Function[b]	Sequence (5′-3′)		Fragment size (bp)
		Forward primer	Reverse primer	
Streptococcus mitis				
SM12261_0395	LrgB family protein	TTACCAAGATTACTACCAAG	AGCAGACTACCAAAGAGA	138
SM12261_0396	LrgA family (CidA putative homolog)	ATTGTTTTGATTGTCTTTT	ATCTCCTTTCTCATAATCTC	105
SM12261_0967	Two-component sensor kinase YesM, putative (LytS putative homolog)	TGTTCCATCATTACTTTTAC	AGTCCTCAGTTTTTACTTTT	113
SM12261_0914	Membrane-bound protein LytR	AGTTTTAGGTGTGGGTGT	AATAACATTGGTTTCTTCC	100
SM12261_0124	Transcriptional regulator, LytR family	AAAAATACCAGAAAACTCA	AACATAAAAATCATACCAAA	127
SM12261_0760	Choline binding protein D	TGGGAGCCAGGAGATTGATA	TGCAGTAGACCAAGCAATGG	199
SM12261_0107	Integrase core domain, putative (putative transposase)	GGCAATACCAACACGACTCTTA	GACTCCATCATACCGTTGTCTG	103
SM12261_0212	Transposase	CTTGAGAGCGGAGAATGCCA	TACCAGTCCTTGAACAATTTCCGT	99
SM12261_0476	Transposase	TTCTCGTCTGCCTGAGATTATG	CCCTCGAGAACAGTGATGATATT	111
SM12261_1313	Transposase	TTGGGACGAGTATGCCTTTAC	ATAGCCTGTGTTCTGCCTTC	102
SM12261_1134	Transposase	GGACGAGTATGCCTTCACTAAG	TCGGATGATAGTCTGGGTTCTA	106
SM12261_0522	Endo-β-*N*-acetylglucosaminidase D (LytC putative homolog)	GACTGAAGGTGGAGAAGGTATTG	CTACCGCTTAACTGTCCAGAAG	90
SM12261_1140	Lysozyme (LytC putative homolog)	CTGACCGTGCTAAGAAGGTTAT	CGCACAATAACACCATCAACTC	103
SM12261_0045	Hypothetical protein (CibA putative homolog)	TGATGAATGTGGATGGAGGAATTA	AGCTACACCTGCACTCAAAC	92
SM12261_0414	Glyceraldehyde-3-phosphate (8)	CGTATCGGTCGTCTTGCT	GCATAACTGGATCTGTAAGGTC	106
Porphyromonas gingivalis	Arg-gingipain gene (18)	CCTACGTGTACGGACAGAGCTATA	AGGATCGCTCAGCGTAGCATT	71

[a] The annotation of all proteins is based on the RefSeq database (NCBI).
[b] In parentheses are the putative homologues based on the best blast hits of the NCBI nr-database.

posases in *S. mitis* expression profiles when *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* were added to an established healthy biofilm (8). Upregulation of putative transposases has been observed by Mitchell et al. in the periodontal pathogen *T. denticola* when growing as a biofilm but not in planktonic growth, concluding that there is a higher potential for genetic mobility in *T. denticola* biofilms (16). However, the high number of transposases upregulated simultaneously in our experiments is probably not a sign of DNA mobilization but rather of some other mechanism related to the ecology of the biofilm. Our hypothesis is that these proteins are involved in a controlled cell death process in *S. mitis*. In the present study, we address the following questions: Does the presence of *P. gingivalis* induce cell death of *S. mitis* in a two-species biofilm model? Does cell death of *S. mitis* present some of the features characteristic of controlled cell death in bacteria?

MATERIALS AND METHODS

Bacterial strains and culture conditions. The species used in the present study were: *S. mitis* (NCTC 12261), *P. gingivalis* (ATCC 33277), *P. gingivalis* (W83), *T. forsythia* (ATCC 43037), and *Escherichia coli* (ATCC 47055). *P. gingivalis* strains were grown under anaerobic conditions at 37°C for 24 h in a COY anaerobic chamber on Trypticase soy agar (TSA) plates supplemented with 5% sheep blood (Northeastern Laboratory, Waterville, ME), 1 μg of hemin/ml, and 1 μg of vitamin K/ml. *E. coli* was grown aerobically on Trypticase soy yeast blood (TSYB) at 37°C for 24 h. *T. forsythia* was grown anaerobically on TSA medium containing 5 μg of hemin/ml, 0.001% N-acetylmuramic acid, 0.1% L-cysteine, and 5% sheep blood at 37°C for 5 days. *S. mitis* was grown on TSYB anaerobically for 24 h.

Biofilm growth. Biofilms of *S. mitis* were grown on sterile hydroxyapatite discs of 7 mm x 1.8 mm (Clarkson, Inc., Chula Vista, CA) placed into each well of a 24-well cell culture plate (Nalgene Nunc International, Denmark). Wells were filled with 1 ml of the mucin growth medium (MGM) used by Kinniment et al. (17), which presents a high concentration of proteins, and supplemented with 4 ml of resazurin from a 25-mg/100 ml stock solution, 1 μg of hemin/ml, and 1 μg of vitamin K/ml. The pH was adjusted to 7.4 prior to autoclaving. In order to form the acquired pellicle, the discs were allowed to stay at least 48 h in contact with the medium under anaerobic conditions prior inoculation. This preincubation period allowed time to check for possible contamination. *S. mitis* was resuspended in MGM medium until it reached a turbidity of McFarland 3 ($\sim 10^8$ CFU/ml). Finally, 50 μl of bacterial culture was added to each hydroxyapatite disk-containing well. The plates were incubated under anaerobic conditions at 37°C. After 24 h, the old medium was replaced by new MGM, and *P. gingivalis* was added to the biofilm as described above, followed by incubation for another 6 h before sampling for analysis. As control strains, we used *S. mitis*, *E. coli*, and *T. forsythia*, which were inoculated into the biofilm and incubated as described above.

RNA extraction. Biofilms grown on hydroxyapatite disks were immediately transferred to a screw-cap tube containing lysis buffer from a mirVana miRNA isolation kit (Life Technologies, Grand Island, NY) and bead-beaten for 1 min at maximum speed. RNA was extracted with the mirVana miRNA isolation kit according to the manufacturer's instructions. Genomic DNA was removed using a Turbo DNA-free kit (Life Technologies).

DNA extraction. Standards were generated from cultures of *S. mitis* and *P. gingivalis*, whose numbers of cells were known. DNA from the standards and biofilms were extracted using UltraClean microbial DNA isolation kit (MoBio, Carlsbad, CA).

qPCR and RT-qPCR. Table 1 presents the primers used in these ex-

periments. Quantitative PCR (qPCR) was performed to quantify the relative number of *P. gingivalis* in *S. mitis* biofilms. Quantification was performed from three biological independent experiments and three technical replicates per experiment. Reverse transcription-qPCR (RT-qPCR) was performed to quantify levels of expression of the different genes assessed shown in Table 1. IQ SYBR green Supermix (Bio-Rad, Hercules, CA) was used according to the manufacturer's instructions. For RT-qPCR 100 ng of RNA was reverse transcribed with a SuperScript double-stranded cDNA synthesis kit (Life Technologies) according to the instructions provided. Real-time quantification of double-stranded DNA fragments was performed with an iCycler iQ thermal cycler (Bio-Rad). A melting-curve analysis followed the amplification. Statistical significance was tested by a Student *t* test for comparing means in R.

Confocal scanning laser microscopy of live/dead cells in the biofilm. A confocal scanning fluorescence microscope (Leica Microsystems GmbH, Germany) with lens (FV300; Olympus Co., Tokyo, Japan) was used to observe the distribution of live and dead bacteria in the biofilms. The bacteria in the biofilm were observed using a Live/Dead BacLight bacterial viability kit (Molecular Probes, Inc., Eugene, OR), according to the manufacturer's instructions. The biofilms were stained in the dark at room temperature for 15 min. An argon laser (476 nm) was used as the excitation source for the reagents, and the fluorescence emitted was collected by two separate emission filters at 500 nm (SYTO 9) and 635 nm (propidium iodide [PI]), respectively. Twenty sections per biofilm were collected, and the biovolume of live/dead biofilms was obtained using ImageJ and COMSTAT (19). Each assay was performed at least three times for each condition, capturing 20 sections for analysis. Statistical significance was tested by using the Kruskal-Wallis rank sum test in R.

DNA fragmentation assay by the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) method. Cells from the biofilm were resuspended in 50% ethanol, fixed overnight at 4°C, and washed once in phosphate-buffered saline. Permeabilization was performed by treatment with lysozyme (Sigma; 140,000 U/ml in Tris-HCl [pH 8.0]–5 mM EDTA). Afterward, an Apo-Direct kit (BD Bioscience), which uses fluorescein isothiocyanate-conjugated dUTP for staining and PI as a counterstain, was used, according to the procedure described by Dwyer et al. (20). Finally, cells were spotted on poly-L-lysine-coated slides (Polysciences, Inc., Warrington, PA) and visualized on a Zeiss Axiovert II epifluorescence microscope. We performed the experiment by triplicate, observing 20 fields in each case.

TOS assay. Oxidants present in the sample oxidize the ferrous ion-*o*-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The total oxidant status (TOS) was measured on hydroxyapatite discs of *S. mitis* biofilms grown under the conditions described previously. TOS was determined using a method described by Erel (21) and is expressed as μmol of H_2O_2 equivalent/liter. The assay was calibrated with hydrogen peroxide by linear relation. Briefly, a hydroxyapatite disc was placed for 10 min in a well of 96-well plate containing 225 μl of reagent 1 (150 μM xylenol orange, 140 mM NaCl, and 1.35 M glycerol [pH 1.75]) at room temperature. After the disc was removed with a plastic pin, the absorbance at 560 nm was recorded in a spectrophotometer Synergy (Biotek, Winooski, VT). After 11 μl of reagent 2 (5 mM ferrous ammonium sulfate and 10 mM *o*-dianisidine dihydrochloride in 25 mM H_2SO_4) was added, the sample was incubated for 4 min at room temperature, followed by an endpoint absorbance reading at same wavelength. Concentrations were calculated as described by Erel (21). The TOS was determined on *S. mitis* biofilms alone (24 h) and on *P. gingivalis* biofilm alone (6 h) as negative controls. Each test was performed in triplicate in four independent experiments. Statistical significance was tested by a Kruskal-Wallis rank sum test in R.

RESULTS

We had previously observed the upregulation of putative transposases in an S. *mitis* biofilm in a multispecies biofilm model where two periodontal pathogens (*P. gingivalis* and *A. actinomycetemcomitans*) were added simultaneously (8). We first verified the upregulation of these putative transposases when only *P. gingivalis* was added. Interestingly, when we added the periodontopathogen *T. forsythia*, no effect was observed on the expression profiles of the transposases; only SM12261_0107 was not significantly more upregulated in the presence of *P. gingivalis* (Fig. 1A). We cannot discard the possibility that with longer periods of incubation *T. forsythia* would not induce expression of those genes, but under the conditions tested it did not have a notable effect. We also observed upregulation of genes involved in cell death in *S. mitis* in our previous work when *P. gingivalis* and *A. actinomycetemcomitans* were added simultaneously (8). We selected a list of genes whose orthologues have been linked to fratricide and cell death in other organisms and performed an RT-qPCR on RNA extracted from a monospecies biofilm of *S. mitis* to which we added *P. gingivalis*. Figure 1B shows the results for these experiments and revealed upregulation of all of the genes except for *lrgA* (*cidA*), where no differences were observed.

The next series of experiments were performed to demonstrate that the presence of *P. gingivalis* in the biofilm induced cell death of *S. mitis*. As previously described, *P. gingivalis* was added and incubated only for a period of 6 h. We did not use *T. forsythia* as a control because this organism has a doubling time of 24 h (22), and it would be extremely difficult to distinguish cell death due to the presence of *T. forsythia* from cell death due to the normal evolution of an old biofilm. We showed by qPCR that the number of *S. mitis* cells was several logarithms higher than the number of *P. gingivalis* at the end of the experiment. The total numbers of cells in the biofilm for *S. mitis* and *P. gingivalis* were $1.44 \times 10^7 \pm 1.1 \times 10^7$ and $2.2 \times 10^4 \pm 9.1 \times 10^3$, respectively. Afterward, we used a live/dead stain to quantify by confocal scanning microscopy the fraction of *S. mitis* dead cells in the presence or absence of *P. gingivalis* in the biofilm. The presence of *P. gingivalis* drastically increased the fraction of *S. mitis* dead cells (Fig. 2C and D). Moreover, the addition of either *S. mitis* or *E. coli* did not have any effect on the viability of the *S. mitis* biofilm; in fact, *S. mitis* continued growing after its own addition (Fig. 2A, B, and D). The two strains of *P. gingivalis* used in these experiments (ATCC 33277 and W83) showed similar effects with a 10-fold increase in the fraction of *S. mitis* dead cells (Fig. 2D).

Finally, we wanted to assess whether the cell death process in *S. mitis* showed some of the hallmarks of a controlled cell death mechanism described in bacteria, such as fratricide (23, 24). One of the features of antibiotic-induced cell death is the fragmentation of DNA, similar to what occurs in eukaryotic apoptosis processes (20). We assessed the possibility of DNA fragmentation in *S. mitis* chromosome during cell death by using a TUNEL assay. Cells whose DNA have been fragmented are stained green, while cells whose DNA has not been degraded are stained red. Figure 3 shows the results for the TUNEL assay when *P. gingivalis* W83 was added to the *S. mitis* biofilm (Fig. 3D, E, and F) and when *S. mitis* was added as a control to the biofilm (Fig. 3A, B, and C). The addition of *P. gingivalis* induced the fragmentation of the chromosomal DNA in *S. mitis* biofilm. *P. gingivalis* incubated under the same conditions did not show DNA fragmentation by itself (data

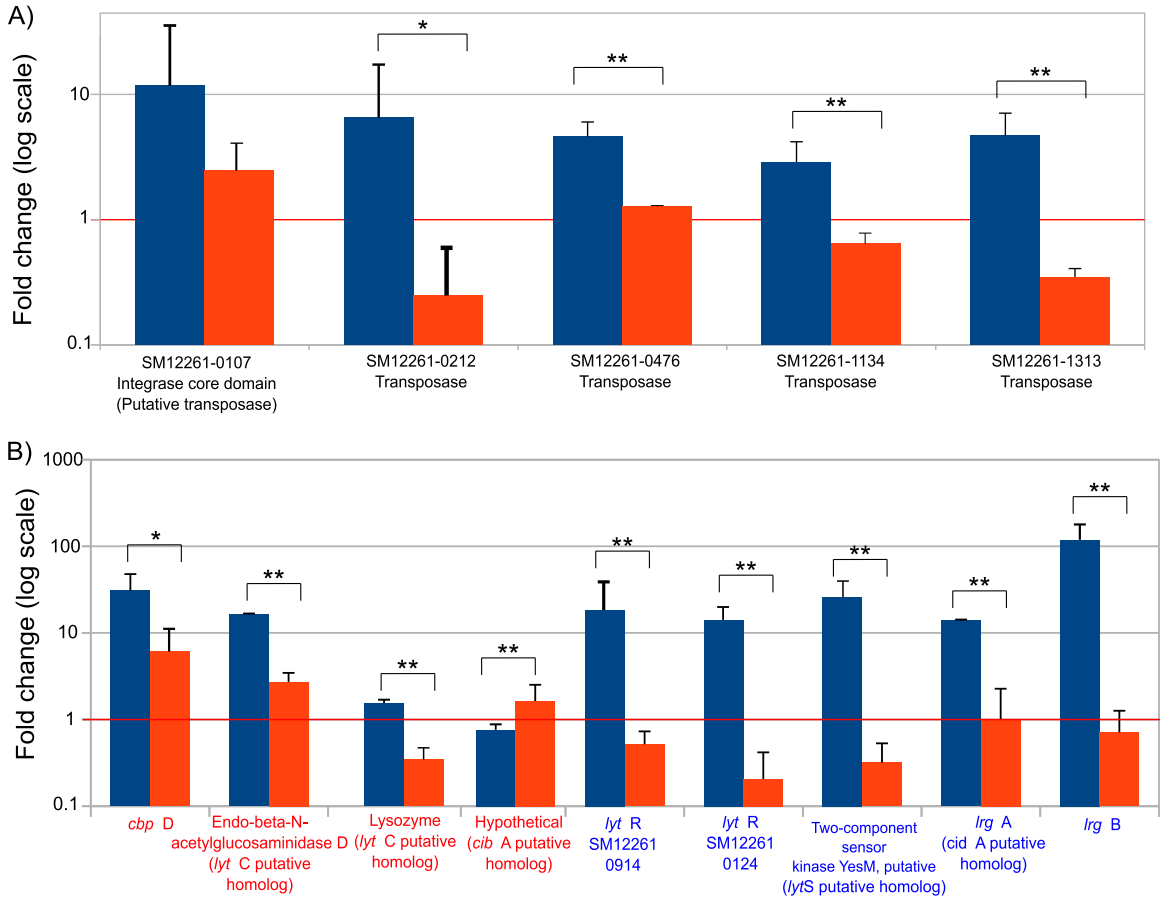

FIG 1 RT-qPCR expression results for putative transposases of *S. mitis* in the presence of different periodontal pathogens. (A) RT-qPCR expression results of putative transposases of *S. mitis* in the presence of *P. gingivalis*. To confirm the metatranscriptomic results showing an upregulation of putative transposases in *S. mitis*, we performed an RT-qPCR in another series of experiments where *S. mitis* grew as a monospecies biofilm and *P. gingivalis* (in blue) was added as described elsewhere (8). In addition, we present results for the induction of the transposases by a strain of *T. forsythia* (in orange). (B) RT-qPCR expression results of some of the genes potentially involved in *S. mitis* cell death. We selected a list of genes whose orthologues have been linked to fratricide and cell death in other organisms and performed an RT-qPCR on RNA extracted from a monospecies biofilm of *S. mitis* to which we added *P. gingivalis* and *T. forsythia*. Names in red are the genes involved in fratricide. In blue are the genes involved in PCD. In parentheses are the putative homologues based on the best blast hits of the NCBI nr-database. The red line represents no changes in expression. Columns in blue show results for the addition of *P. gingivalis*, and columns in red are for the addition of *T. forsythia*. The results are averages of three independent biological experiments ($n = 3$). Error bars show the standard deviations for biological replicate values. Significance was tested by a Student *t* test, with asterisks representing groups with significant differences at $P < 0.1$ (*) and $P < 0.05$ (**).

not shown), which confirms that the fragmentation observed in our experiments was the result of *S. mitis* DNA fragmentation. Moreover, in all of these experiments *P. gingivalis* represented a small fraction of the total cells in the communities since the incubation period of 6 h is not enough to yield a considerable amount of cells that would interfere with our observations.

Another hallmark of programmed cell death (PCD) is the production of reactive oxygen species (ROS). To test the production of these compounds, we measured the TOS of the biofilm in the presence or absence of *P. gingivalis*. *S. mitis* growing by itself under the same conditions produced low levels of ROS, while in the presence of *P. gingivalis* the production increase by up to 25-fold (Fig. 4).

DISCUSSION

In spite of our wealth of knowledge regarding the composition of the oral microbiome, we still do not completely understand the role of individual species in periodontal disease initiation and progression. Oral pathogens have been frequently identified in healthy oral samples, although in most cases at low levels (25). The fact that these pathogens are present even in healthy patients complicates the definition of what constitutes a healthy oral microbiome.

In a recent series of papers, Hajishengallis et al. and Darveau have proposed that *P. gingivalis* orchestrates the inflammatory response of the host by dysbiosis of the oral microbiome, changing the nature of the bacterium-bacterium and bacterium-host interactions (26–29). These researchers showed in an animal model that *P. gingivalis*, at low levels of colonization, was able to induce periodontitis accompanied by significant changes in the number and community organization of the oral commensal bacteria. These alterations occur soon after *P. gingivalis* colonization and precede to the onset of inflammatory bone loss, suggesting that dysbiosis is the cause of the disease. The obligatory participation of the commensal microbiota in disease pathogenesis was shown by the failure of *P. gingivalis* alone to cause periodontitis in germ-free mice, despite its ability to colonize the host (27). In a previous

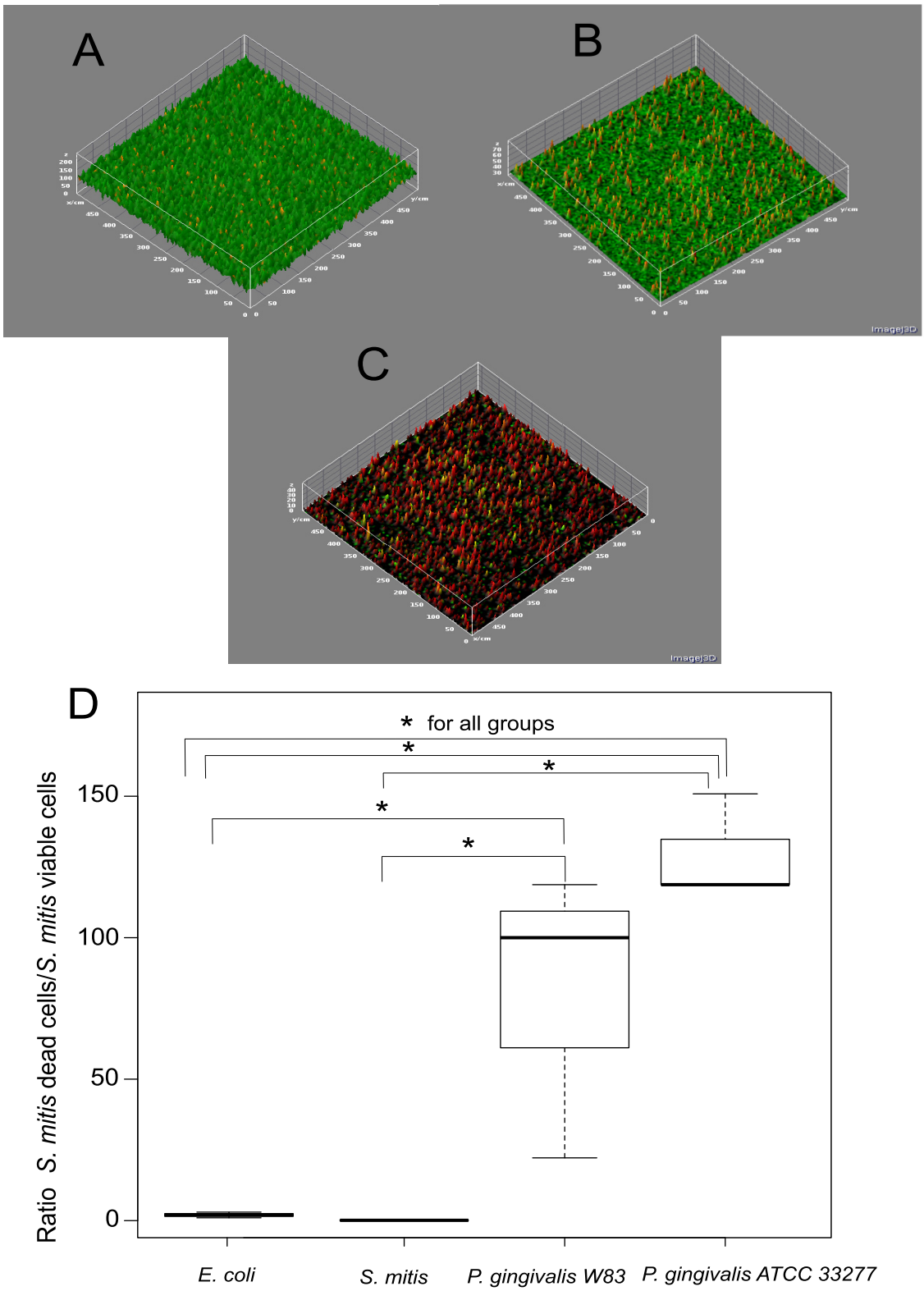

FIG 2 Live/dead bacterial viability assay by confocal microscopy. Stained in green are the viable cells, and stained in red are the dead cells. We used two control strains: *E. coli* and *S. mitis*. (A) Control *S. mitis* biofilm to which *S. mitis* cells were added. (B) Control *S. mitis* biofilm to which *E. coli* cells were added. (C) *S. mitis* biofilm to which *P. gingivalis* was added. (D) Box plot showing the results for biomass calculated using a COMSTAT v1.1 program (19). The *y* axis represents the ratio of dead to live cells after the addition of the different organisms indicated in the *x* axis. Significance was tested by a Kruskal-Willis rank sum test with an asterisk (*) representing groups with significant differences at $P < 0.05$. Analysis was performed for three independent biological experiments ($n = 3$) with a series of 20 sections. The dark line in the box represents the median. The two section in the box represent the upper and lower quartiles. Bars (whiskers) represent the maximum and minimum values in the data sets.

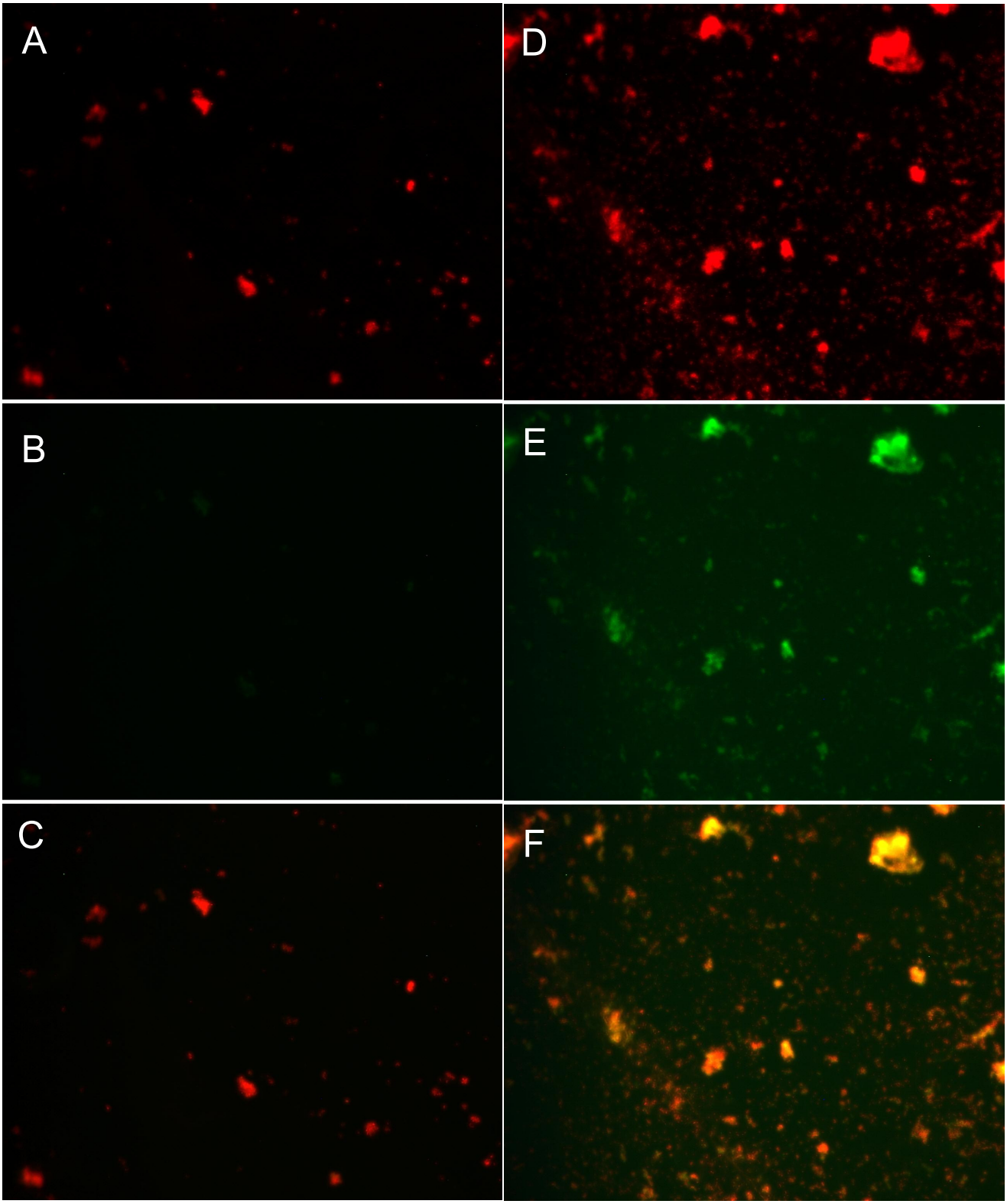

FIG 3 DNA fragmentation assay by the TUNEL method. Evidence of DNA fragmentation as measured by TUNEL was visualized via fluorescence microscopy. Cells with fragmented DNA show a green fluorescence due to the incorporation of dUTPs to the ends of the DNA fragments. We used propidium iodide (PI) as a counterstain, staining all nucleic acids in red. Original magnification, ×400. (A) *S. mitis* biofilm to which additional *S. mitis* was added stained with PI. (B) TUNEL assay on a *S. mitis* biofilm to which additional *S. mitis* was added. (C) Overlaid image of panels A and B, wherein cells positive for TUNEL are stained yellow-orange. (D) *S. mitis* biofilm to which *P. gingivalis* W83 was added, stained with PI. (E) TUNEL assay of an *S. mitis* biofilm to which *P. gingivalis* W83 was added. (F) Overlaid image of panels D and E panels, wherein cells positive for TUNEL are stained yellow-orange. We performed the experiment in triplicate ($n = 3$), observing 20 fields in each case.

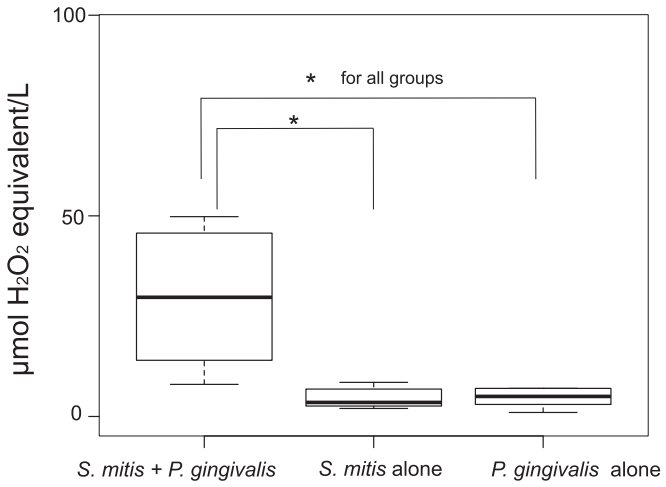

FIG 4 Effect of *P. gingivalis* on the total oxidant status (TOS) of the *S. mitis* biofilm. *P. gingivalis* induced the production of oxidant species in an *S. mitis* biofilm. The results show the average values of four independent biological experiments ($n = 4$), following the protocol described in Materials and Methods. The *P. gingivalis* biofilm was incubated for 6 h before measurement of the TOS. Three replicates were measured for each sample. Significance was tested by using the Kruskal-Wallis rank sum test with an asterisk (*) representing groups with significant differences at $P < 0.05$. The dark line in the box represents the median. The two sections in the box represent the upper and lower quartiles. Bars (whiskers) represent the maximum and minimum values in the data sets.

work, we observed that the addition of the periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans* to a healthy biofilm in a multispecies biofilm model caused a complete rearrangement of the expression profiles of the commensal microbiota (8).

One surprising observation of our previous study was that a large number of putative transposases were highly upregulated in *L. casei* and *S. mitis* when two periodontal pathogens were added to the biofilm. A high upregulation of transposases has been described in a previous report on the periodontal pathogen *T. denticola*. When this organism was growing as a biofilm, this upregulation was observed, but it did not happen during planktonic growth. Mitchell et al. concluded that there is a higher potential for genetic mobility in *T. denticola* biofilms than in suspension (16). In a very recent study, Kleiner et al. reported abundant transposase expression in bacterial symbionts of a gutless marine worm, and they concluded that their results reflect expression under natural environmental conditions (30). Furthermore, we observed that the effect was specific of the species added to the *S. mitis* biofilm. When *T. forsythia* was added to the biofilm, we did not observe any upregulation of the same transposases that were highly transcribed in the presence of two different strains of *P. gingivalis*. However, due to the slow growth of *T. forsythia*, we cannot discard the possibility that this organism would have an influence in *S. mitis* cell death when they are together for long periods of time.

Streptococci are considered the main group of early colonizers in the oral biofilm, making up over 80% of the initial plaque. Their early attachment determines the composition of late colonizers in the oral biofilm and impacts the health or disease status of the host (9, 41). Members of the *S. mitis* group (*S. oralis*, *S. mitis*, and *S. sanguinis*) are considered part of these early colonizers and have been frequently associated with oral health (7, 31). It is the uncontrolled growth of the Gram-negative component of dental plaque that leads to periodontitis. Therefore, during progression of periodontitis, there is a reduction in the fraction of streptococci present in the biofilm.

We found that the addition of at least two different strains of *P. gingivalis* to an *S. mitis* biofilm induced cell death by a still-uncharacteristic molecular mechanism. Two different mechanisms of controlled cell death have been described in bacteria: fratricide, a killing mechanism used by competent cells to acquire DNA from noncompetent sibling cells, and PCD as part of biofilm development in which a subfraction of the population commits altruistic "suicide" with the subsequent release of extracellular DNA that stabilizes the biofilm using a toxin-antitoxin system (40).

Fratricide has been extensively studied in *Streptococcus pneumoniae* (24), while cell death in biofilm maturation has been studied in several bacteria, including the oral pathogen *Streptococcus mutans* (32). Essential proteins involved in those mechanisms are CbpD, CibAB, LytA, and LytC in fratricide and CidABC, LytR, LrgAB, LytSR, and CidR in PCD (24, 33). We found that a large number of orthologues for these genes were upregulated in *S. mitis* biofilms when *P. gingivalis* was present.

In *S. pneumoniae* choline-binding murein hydrolases, CbpD, LytA, and LytC, constitute the lysis mechanism of fratricide. CbpD is the key component of that mechanism and also involved in competence, while LytA and LytC play auxiliary roles (24). Other proteins involved in this process are the lytic factors CibA and CibB (CibAB), which presumably constitute a two-peptide bacteriocin, and the immunity factor CibC (23). *S. mitis* NCTC 12261, the strain used in our experiments, contains an almost identical orthologue of CbpD in its genome, two orthologues of LytC and one orthologue of CibA, annotated as hypothetical protein in the National Center for Biotechnology Information (NCBI) database, but it does not have orthologues of LytA, CibB, and CibC proteins. Claverys et al. have identified a region that potentially could encode *cib* genes upstream of the *comAB* operon, but it has not been proven they do indeed encode those proteins (23). We observed a high upregulation after the addition of *P. gingivalis* in our metatranscriptome and RT-qPCR assay of the gene for the key protein in fratricide, *cbpD*. One orthologue of LytC (SM12261_0522) was also upregulated in both the metatranscriptome and the RT-qPCR results. However, the other orthologue of *lytC* (SM12261_1140) and *cibA* did not show an increase in expression after the addition of *P. gingivalis*. Interestingly, *comGA*, another gene associated with competence, was also highly upregulated in our metatranscriptomic analysis.

PCD in other organisms follows a different pathway (33–35, 42). The current accepted hypothesis is that when a biofilm matures, a subsection of the population activates a controlled process of cell death that releases nutrients and DNA which stabilizes the rest of the population in the biofilm by maintaining the biofilm structure (34, 40). The process follows a very tightly regulated pattern of expression. In *Staphylococcus aureus*, a well-studied Gram-positive model, *cidA* and *lrgA* genes encode orthologues hydrophobic proteins that are believed to function as a holin and antiholin, respectively. In *S. mitis* NCTC 12261 the gene *lrgA* is the homolog for *cidA*. The actual function of this open reading frame in *S. mitis* is still unknown, and in our experiments its expression was highly upregulated in the presence of *P. gingivalis*. In *S. aureus cidB* and *lrgB* genes also encode hydrophobic proteins whose functions are unknown (33). Interestingly, the putative homolog

for *lrgB* in *S. mitis* was highly upregulated in the presence of *P. gingivalis*.

S. mitis does not have orthologues for all proteins linked to PCD. Bayles indicates that *S. mitis* has orthologues to CidAB but not to the rest of proteins involved in the PCD regulatory network (33). However, the RefSeq annotation from the NCBI of *S. mitis* NCTC 12261 shows that it indeed has orthologues to LytR and LrgB but does not have orthologues to CidB or LytS. However, we found that one protein, annotated LrgA, has high homology to CidA proteins and a two-component sensor kinase YesM has high homology to LytS proteins. The gene *lrgB* and homologs to *cidA* and *lytS* were highly upregulated in the presence of *P. gingivalis* both in our metatranscriptomic results (8) and by RT-qPCR analysis. The two homologs to *lytR* genes were not detected in the metatranscriptome results, but our RT-qPCR analysis showed the upregulation of those two genes. If *lytR* and *lytS* homologs are indeed orthologues of *lytSR*, they do not belong to the same operon, in contrast to what happens in *S. pneumoniae*.

Using two different techniques, we observed DNA fragmentation in *S. mitis* biofilms when *P. gingivalis* was added but not when *S. mitis* or *E. coli* were present in the biofilm. Chromosomal DNA fragmentation is a hallmark of cell death both in eukaryotes during apoptosis and in prokaryotes in the presence of antibiotics (20, 43). Another hallmark of PCD in bacteria is the production of ROS (20, 36). We observed that the presence of *P. gingivalis* in the biofilm led to the production of ROS by *S. mitis*, which supports the idea that *P. gingivalis* is directly involved in the induction of cell death in *S. mitis* biofilms.

It has been suggested that passive DNA fragmentation is likely to occur in microorganisms during PCD (37). However, other authors have proposed the role of specific nucleases as BapE in the process of DNA fragmentation during PCD (34). Transposases have the ability to cut DNA at specific sites, resulting in double-stranded fragments of the nucleic acid. We believe that the upregulated putative transposases may be involved in the process of DNA fragmentation during PCD in *S. mitis*. In *E. coli* the overexpression of Tn*5* transposase results in filamentation and ultimately cell death (38). We would expect that a large number of simultaneously upregulated transposases would result in cell death in *S. mitis* as we observed. Whatever their role, these putative transposases seem to be important players in the process of *S. mitis* cell death. From these results, it seems as though *S. mitis* is using a mixed mechanism of cell death involving proteins associated with both fratricide and PCD. We do not know of any report showing DNA fragmentation as a part of the fratricide process, although it is a common feature of cell death phenotype in a variety of organisms. During fratricide DNA is released to the biofilm (23), but it is not known whether it is fragmented before releasing. Hakansson et al. showed that in an apoptosis-like death LytA plays a major role in autolysis of *S. pneumoniae*, and it is accompanied by DNA fragmentation; however, none of the other proteins described as important in the canonical fratricide process seemed to play a role (39).

In summary, we have shown that the addition of the periodontal pathogen *P. gingivalis* to an S. *mitis* biofilm model induced cell death of *S. mitis*, as well as the upregulation of a large number of transposases. The detailed mechanisms of cell death or competence-induced cell lysis of *S. mitis* and the environmental signals that trigger them are still unknown, but we have identified genes that could be involved in the process. More importantly, to our knowledge there are no reports showing that either PCD or fratricide is induced by the presence of another microorganism. These may be internal cellular processes caused by stresses not yet completely understood. Furthermore, we hypothesize that the upregulated proteins annotated as transposases are involved in cell death processes rather than in DNA mobilization. In support of this idea, we could not identify other proteins of transposon origin in the vicinity of the putative transposases in *S. mitis*, and no other nucleases were upregulated during cell death.

A genetic approach would give us direct evidence of the role of the different proteins in induced cell death of *S. mitis*. Nonetheless, the genetic manipulation of the *S. mitis* strain that we are using in our experiments has been challenging, and we are in the process of developing the molecular techniques needed to target these genes.

Unraveling the molecular mechanism by which *P. gingivalis* induces death on *S. mitis* will facilitate developing novel targeted approaches to prevention of oral diseases by maintaining a healthy biofilm. The potential impact extends beyond the study of the oral cavity because the same principles and methods potentially can be applied to other diseases where streptococci are key players that could be targeted by using the mechanisms that *P. gingivalis* used here to initiate cell death in an *S. mitis* biofilm. The impact will be significant in our understanding of the mechanisms by which *P. gingivalis* modifies the behavior of the oral biofilm and in designing new strategies to treat streptococcal infections.

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