**UNIVERSIDAD COMPLUTENSE DE MADRID**
FACULTAD DE ODONTOLOGÍA


# TESIS DOCTORAL

## Análisis transcriptómico comparativo de *Porphyromonas gingivalis* ATCC 33277 en diferentes estados fenotípicos

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid

**UNIVERSIDAD COMPLUTENSE DE MADRID**

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Departamento de Especialidades Clínicas Odontológicas

# ANÁLISIS TRANSCRIPTÓMICO COMPARATIVO DE *Porphyromonas gingivalis* ATCC 33277 EN DIFERENTES ESTADOS FENOTÍPICOS

**TESIS DOCTORAL**

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**Madrid, 2019**

UNIVERSIDAD COMPLUTENSE MADRID

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presentada por Doña Patricia Teresa Romero Lastra, alumna del programa de Doctorado en Ciencias Odontológicas en la Facultad de Odontología de la Universidad Complutense de Madrid.

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*ni las más rápidas,*

*ni las más inteligentes;*

*sino aquellas que se adaptan mejor al cambio.”*

Charles Darwin

A cuantos me han apoyado en este largo camino.

A mi familia.

# AGRADECIMIENTOS

Esta tesis se ha realizado en el Laboratorio de Investigación de la Facultad de Odontología de la UCM entre los años 2014 y 2019.

Muchas personas han hecho posible el desarrollo de esta investigación, tanto a nivel científico como personal. A todas ellas, desde estas líneas, les quiero expresar mi agradecimiento por su apoyo y aportación en este camino.

Quiero dar las gracias a mis directores, el Dr. Mariano Sanz y la Dra. Elena Figuero, Catedrático de Universidad y Profesora Titular de Universidad, respectivamente, del Departamento de Especialidades Clínicas Odontológicas, por la confianza y la oportunidad de poder trabajar en su grupo, por su accesibilidad, su permanente guía, ayuda y enseñanzas científicas y humanas.

Al Dr. David Herrera, por su colaboración y sugerencias científicas en todos los artículos de esta tesis, que han enriquecido este trabajo de investigación.

Muy especialmente mi reconocimiento a la Dra. María del Carmen Sánchez, por todas sus enseñanzas científicas, su permanente orientación, su continuo estímulo, guía e importantes consejos, así como su paciencia y calidez humana durante todos estos años y por transmitirme su entusiasmo por la investigación y enseñarme a ver más allá de lo que se puede observar.

A la Dra. Arancha Llama-Palacios agradezco todas sus aportaciones en los intercambios "transcriptómica-proteómica" que han ido surgiendo en la experimentación, así como por todo su apoyo y cercanía.

A la Dra. María José Marín mi gratitud por su atención y buena disposición a compartir su experiencia, que nos ha sido de vital ayuda.

A la Dra. Itziar González, a Ana O´Connor y Marta García, Facultativos y Técnicos del Laboratorio de Investigación, mi agradecimiento por la acogida y facilidades prestadas en estos años.

Al personal de la Unidad de Genómica de la Facultad de CC. Biológicas de la UCM, en especial a Sara Álvarez y Pedro Botías, a Gloria García por la ayuda imprescindible en el diseño del *microarray* y a Luis Almonacid, también Facultativos y Técnicos, en el Centro Nacional de Biotecnología (CNB-CSIC), gracias por su inestimable servicio en el desarrollo de esta investigación.

A la Profesora de Odontología Preventiva Dra. Inmaculada Casado Gómez agradezco su amable acogida, enseñanzas y gran estímulo.

A María Ángeles Lacasa Otín, Directora de la Biblioteca, a Paloma Galán Santos, Técnico del Aula del Informática y al personal de Secretaría de la Facultad de Odontología de la UCM, gracias por su gran ayuda.

A mis compañeros y amigos del Laboratorio Patricia Bermejo, Leire Virto, Oksana Potupa, Honorato Vidal, Nagore Ambrosio, por estar siempre dispuestos a ayudar y colaborar, por nuestros intercambios científicos e inolvidables congresos, por todas las pausas entre experimentos con especiales momentos, por esa parte humana tan importante y porque es un placer haber compartido esta etapa de esfuerzo y cooperación con todos, para alcanzar esta meta común.

Mi recuerdo al COIE-OPE de la UCM por la beca de formación y el tiempo compartido con el equipo de becarias Alejandra, Belén, Paula, Vicky y Diana, los "jefes" Mª Carmen y Samuel y todos los compañeros del Vicerrectorado.

Al Colegio Sagrados Corazones de Martín de los Heros, en especial a Luis Sacristán Director General, a la Dra. Carmen Soriano Directora Académica y a Pedro M. Gordillo Vicario Provincial, por su generosa comprensión y facilidades concedidas para compaginar y potenciar mi docencia con el desarrollo de esta investigación. A todos los profesores y compañeros del Centro agradezco también su gran apoyo.

A todos mis compañeros del Erasmus en Paris, *pour l´expérience inoubliable vécue à Bures-sur-Yvette* y por seguir manteniendo la amistad más de una década después.

A mis amigas Laura, Marta, Susana, Gloria, Mamen y Lucía, gracias por su cariño y tantos momentos y experiencias compartidas, por su apoyo emocional y por su constancia para buscar momentos de encuentro.

A Natalia, Ángela e Isabel, por esos recuerdos desde la infancia y por disfrutar ahora muchas tardes con "nuestro sobrino Diego", una especial inyección de energía.

A mis compañeros y amigos del Máster Interuniversitario de Genética y Biología Celular, con los que empezó toda esta "aventura doctoral", Marta, Miguel, Estela, Sergio y Hikaru, gracias por estar siempre dispuestos a compartir conocimientos, recursos y tiempo, incluso por *Skype*, y por tantos momentos inolvidables.

A Fernando, por todos sus consejos de ya Doctor y su ayuda en la presentación de comunicaciones científicas, maquetación y formato de este trabajo, ¡gracias por tu amable disposición!

A ti Hikaru, no hay palabras para agradecer tu apoyo como bioquímico, así como tu paciencia y aliento; gracias por confiar en mí e impulsarme a seguir creciendo, por tu estímulo para emprender y desarrollar este proyecto, por saber y enseñarme a esperar. Pero sobre todo gracias por tu cariño en el día a día y por el bonito camino que estamos construyendo juntos.
También a tu familia, por todo su afecto a lo largo de todos estos años.

Finalmente, mi más profunda gratitud a mi familia por su apoyo e interés durante todo este tiempo, a mis tíos Ana y Antonio y a mis primos Antonio Enrique y José Carlos, por todo el ánimo transmitido, por su cariño y todos los buenos momentos compartidos.

Y, especialmente, a mi tía Marga, mi madrina, por guiarme y darme su confianza, su apoyo y su afecto siempre. Por todo lo que he aprendido a su lado, ser un ejemplo de perseverancia, y hacerme entender que todo a lo que se aspira, con tesón, va llegando.

A mis abuelos, a los que siempre tengo presentes.

A mi queridísima hermana María Elena, por entendernos con sólo mirarnos, compartir nuestras motivaciones docentes, por su tiempo, por todo su cariño, por las vivencias que hemos tenido desde pequeñas y por todo lo que nos espera juntas.

A mis padres, José Luis y Maite por sus cuidados y haberme inculcado desde la infancia el gusto por la naturaleza y la importancia del esfuerzo personal e impulsarme siempre para continuar en cada uno de mis proyectos, pero sobre todo por darme día a día su apoyo y cariño.

A todos, muchas gracias.

# ÍNDICE

# CAPÍTULO I

# ABREVIATURAS

| | |
|---|---|
| **AI** | Autoinductores |
| **ADN** | Ácido desoxirribonucleico |
| **ADNc** | Ácido desoxirribonucleico codificante |
| **ARN** | Ácido ribonucleico |
| **ARNseq** | *ARN sequencing* |
| **AI-2** | Autoinductor-2 |
| **ATCC** | Colección americana de cultivos tipo |
| **Mb** | Megabase ($10^6$ pares de bases) |
| **CECT** | Colección española de cultivos tipo |
| **CLSM** | Microscopía laser confocal de barrido |
| **DMSZ** | Colección alemana de microorganismos y cultivos celulares |
| **EPS** | Sustancias poliméricas extracelulares |
| **FCG** | Fluido crevicular gingival |
| **FISH** | Hibridación *in situ* fluorescente |
| **HA** | Hidroxiapatita |
| **LPS** | Lipopolisacárido |
| **NCTC** | Colección nacional inglesa de cultivos tipo |
| **nm** | Nanómetros |
| **OD** | Densidad óptica |
| **OMS** | Organización mundial de la salud |
| **OMVs** | Vesículas de membrana externa, siglas del inglés *"Outer Membrane Vesicles"* |
| **PBS** | Buffer fosfato salino. siglas del inglés *"Phosphate Buffered Saline"* |
| **ROS** | Especies reactivas del oxígeno, siglas del inglés *"Reactive Oxygen Species"* |
| **RT-qPCR** | Reacción en cadena de la polimerasa con transcriptasa inversa |
| **SD** | Desviación estándar |
| **SEM** | Microscopía electrónica de barrido |
| **SNPs** | Polimorfismos de un solo nucleótido, siglas del inglés **"***Single Nucleotide Polymorphism"* |

# CAPÍTULO II

## RESUMEN

## Antecedentes y objetivos

*Porphyromonas gingivalis*, es una especie bacteriana con fuerte evidencia de asociación con la periodontitis. Esta bacteria puede presentarse de dos formas: como células que flotan libremente, planctónicas, u organizándose en estructuras tridimensionales de comunidades multimicrobianas llamadas *biofilms* que se unen a la superficie de los dientes y sobre los tejidos blandos. Lo que comúnmente se conoce como placa dental.

La actividad patogénica de estas bacterias está directamente relacionada con su presencia y la supervivencia en el *biofilm* subgingival, donde tiene que adaptarse a la vida en comunidad a través de patrones orquestados de regulación génica para desarrollar nuevas estrategias. En los últimos años, se han realizado estudios de posibles genes asociados con el desarrollo del *biofilm* de *P. gingivalis*, sin embargo, se conoce muy poco acerca de su expresión génica global y de cómo puede variar según su estado fenotípico, desarrollando mecanismos más patogénicos en estado de *biofilm*, o por su asociación con otras bacterias, formando un consorcio multiespecie.

El objetivo general de esta tesis es determinar y comparar los cambios de expresión génica global de *P. gingivalis* ATCC 33277, mediante análisis transcriptómico con el uso de *microarrays*, en diferentes condiciones *in vitro*.

Los objetivos específicos son: primero, identificar genes regulados diferencialmente por *P. gingivalis* ATCC 33277 en un estado planctónico puro y en uno en presencia de un *biofilm* monoespecie. Segundo, estudiar los cambios de expresión génica global de *P. gingivalis* ATCC 33277, en estado planctónico y en un modelo *in vitro* de *biofilm* estático monoespecie. Y tercero, estudiar y comparar los cambios de

expresión génica global de *P. gingivalis* ATCC 33277 en estado planctónico y asociado a un modelo estático *in vitro* de *biofilm* multiespecie, con el fin de ampliar el conocimiento de las bases moleculares de la formación del *biofilm* y los cambios que se producen por la interacción de este periodontopatógeno con otras especies.

## Metodología

En esta investigación se ha comparado la expresión génica global, de la bacteria *P. gingivalis* ATCC 33277 en estado planctónico puro (la condición más sencilla) con otras tres situaciones fenotípicas, de creciente complejidad: 1) Condición planctónica de *P. gingivalis* en presencia de un *biofilm* monoespecie, 2) Condición sésil de *P. gingivalis* en *biofilm* monoespecie y 3) Condición sésil de *P. gingivalis* junto con otras cinco especies para formar un *biofilm* multiespecie (*Streptococcus oralis, Actinomyces naeslundii, Veillonella parvula, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* y *P. gingivalis*) desarrollado sobre discos de hidroxiapatita de calcio cerámica. Para ello, los cultivos de cada condición se incubaron en un modelo estático de crecimiento *in vitro* en placas multipocillo a 37ºC, durante 96 horas y en anaerobiosis.

Para verificar el progreso del estado planctónico a *biofilm* y la inclusión de *P. gingivalis* en él, se utilizó microscopía electrónica de barrido (SEM) y miroscopía láser confocal de barrido (CLSM).

Después de la incubación, se recogieron las células de cada condición, se extrajo el ARN total, se trató con ADNasa I y se purificó. Tres réplicas biológicas para cada estado celular se hibridaron de forma independiente para la comparación transcriptómica aplicando la tecnología de *microarrays*.

Para estudiar la expresión diferencial y hacer el análisis de datos se aplicó el método de modelo lineal utilizando el software LIMMA, con un valor de significación $p < 0,05$ y un criterio de filtro para el cambio de expresión génica $\leq -1,5$ ó $\geq 1,5$ veces para el estudio de las dos condiciones planctónicas y $\leq -2$ ó $\geq 2$ veces para el estudio de los *biofilm*s.

La expresión diferencial fue confirmada por reacción en cadena de la polimerasa con transcriptasa inversa (RT-qPCR).

**Resultados, discusión y conclusiones**

En el primer estudio, un total de 1,5% de los genes (28/1909) de *P. gingivalis* fueron expresados diferencialmente en las células planctónicas en presencia de *biofilm* en comparación con las células planctónicas puras. Los genes encontrados se relacionaron principalmente con el metabolismo del hierro, la adhesión bacteriana, invasión o virulencia, destacando también la diferente expresión de genes de función desconocida.

En el segundo estudio, en el análisis por *microarray*, el 4,8% de los genes de *P. gingivalis* (92/1.909) fueron expresados diferencialmente al comparar el estado planctónico y el de *biofilm*. De ellos, 54 fueron sobreexpresados y 38 reprimidos en el *biofilm*. Los genes sobreexpresados están principalmente relacionados con la envoltura celular, el transporte y proteínas de unión o proteínas de membrana externa. Entre los genes reprimidos destacan las transposasas o genes implicados en el estrés oxidativo.

En el tercer estudio, los resultados transcriptómicos demostraron que, al crecer dentro del *biofilm* multiespecie, el 19,1% de los genes (365/1.909) de *P. gingivalis* se expresaban de manera diferencial (165 genes estaban sobreexpresados y 200 reprimidos) en comparación con el crecimiento planctónico. Estos genes estaban

involucrados principalmente en funciones relacionadas con el estrés oxidativo, la envoltura celular, los transposones y el metabolismo.

Este trabajo proporciona una visión de los cambios transcripcionales de *P. gingivalis* para adaptarse al estilo de vida de una comunidad y al interaccionar con otras especies bacterianas, observándose ciertos grupos de genes como los de envoltura celular, metabolismo y estrés oxidativo y virulencia de manera persistente y produciéndose cambios más significativos según va aumentando la complejidad del fenotipo de esta bacteria.

Una mejor comprensión de los genes y vías reguladoras involucradas en la transición entre estados planctónicos y *biofilm*s puede facilitar información relevante para la prevención de la formación de estas estructuras y, por consiguiente, de las enfermedades periodontales.

# CAPÍTULO III

# SUMMARY

## Background and objectives

*Porphyromonas gingivalis*, is a bacterial species with strong evidence of association with periodontal diseases. These bacteria can be found in two ways: as free-floating cells, planktonic, or organized in three-dimensional structures of multimicrobial communities called biofilms, which attach to the surface of the teeth and on the soft tissues. This is commonly known as dental plaque.

The pathogenic activity of these bacteria is directly related to their presence and survival in the subgingival biofilm, where they have to adapt to community life through orchestrated patterns of gene regulation to develop new strategies. In recent years, studies of possible genes associated with the development of the biofilm of *P. gingivalis* have been carried out, however, very little is known about the global expression of *P. gingivalis* and how it can vary according to its phenotypic state, developing more pathogenic mechanisms in a biofilm state or because of their association with other bacteria, forming a multispecies biofilm.

The general objective of this thesis is to determine and compare the global gene expression changes of *P. gingivalis* ATCC 33277, by means of transcriptomic analysis with the use of microarrays, under different conditions *in vitro*.

The specific objectives are: first: to identify genes differentially regulated by *P. gingivalis* ATCC 33277 in a pure planktonic state and in one in the presence of a monospecies biofilm. Second: to study the global gene expression changes of *P. gingivalis* ATCC 33277, in the planktonic state and in an *in vitro* model of static monospecies biofilm. And third: to study and compare the global gene expression changes of *P. gingivalis* ATCC 33277 in a planktonic state and associated with a static *in*

*vitro* model of multispecies biofilm, in order to broaden knowledge of the molecular basis of biofilm formation and the changes that are produced by the interaction of this periodontopathogen with other species.

## Methodology

In this research, the global gene expression of the bacterium *P. gingivalis* ATCC 33277 in pure planktonic state (the simplest situation) has been compared to three other phenotypic conditions of increasing complexity: 1) planktonic condition of *P. gingivalis* in the presence of a monospecies biofilm, 2) sessile condition of *P. gingivalis* in monospecies biofilm and 3) sessile condition of *P. gingivalis* together with five other species to form a multispecies biofilm (*Streptococcus oralis, Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* and *P. gingivalis*) developed on ceramic calcium hydroxyapatite discs. For this, the cultures of each condition were incubated in a static *in vitro* growth model in multi-well plates at 37ºC, for 96 hours and in anaerobiosis.

To verify the progress of the planktonic state to biofilm and the inclusion of *P. gingivalis* in it, Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Miroscopy (CLSM) were used.

After incubation, the cells of each condition were harvested, the total RNA was extracted, treated with DNase I and purified. Three biological replicates for each cell condition were hybridized independently for transcriptomic comparison using microarray technology.

To study the differential expression and make the data analysis, a linear model method was applied using the LIMMA software, with a significance value of $p < 0.05$ and a filter criterion of gene expression change of $\leq -1.5$ or $\geq 1.5$ to study the two planktonic

conditions and ≤ -2 or ≥ 2 to study biofilms. The differential expression was confirmed by polymerase chain reaction with reverse transcriptase (RT-qPCR).

**Results, discussion and conclusion**

In the first study a total of 1.5% of the genes (28/1909) of *P. gingivalis* were differentially expressed in the planktonic cells in the presence of biofilm compared to the pure planktonic cells. The genes found were mainly related to iron metabolism, bacterial adhesion, invasion or virulence, also highlighting the different expression of genes of unknown function.

In the second study a 4.8% of the genes of *P. gingivalis* (92/1909) were differentially expressed when comparing the planktonic and the biofilm state. Of these, 54 were up-regulated and 38 down-regulated in the biofilm. The over-expressed genes are mainly related to the cell envelope, transport and binding proteins or outer membrane proteins. Among the repressed genes, transposases or genes involved in oxidative stress stand out. In the third study transcriptomic results showed that when growing within the multispecies biofilm, 19.1% of the genes (365/1.909) of *P. gingivalis* were differentially expressed (165 genes up-regulated and 200 down-regulated) compared to planktonic growth. These genes were mainly involved in functions related to oxidative stress, cell envelope, transposons and metabolism.

This work provides insight into the transcriptional changes of *P. gingivalis* to adapt to the lifestyle of a community and to interact with other bacterial species, reporting in a persistent way certain groups of genes such as cellular envelope, metabolism and oxidative stress and virulence and with changes more significant as the phenotypic complexity of this bacterium increases.

A better understanding of the genes and their regulatory pathways involved in the transition between planktonic states and biofilms could facilitate relevant information for the prevention of the formation of biofilms and, consequently, of periodontal diseases.

## PREFACIO

Esta tesis doctoral está basada en los siguientes artículos originales publicados en revistas científicas:

**Artículo 1.** Sánchez MC, Romero-Lastra P, Ribeiro-Vidal H, Llama-Palacios A, Figuero E, Herrera D, Sanz M. Comparative gene expression analysis of planktonic *Porphyromonas gingivalis* ATCC 33277 in the presence of a growing biofilm *versus* planktonic cells.
BMC Microbiology (2019); 19(1):58. Epub 2019/03/15. doi: 10.1186/s12866-019-1423-9. PubMed PMID: 30866810.

**Artículo 2.** Romero-Lastra P, Sánchez MC, Ribeiro-Vidal H, Llama-Palacios A, Figuero E, Herrera D, Sanz M. Comparative gene expression analysis of *Porphyromonas gingivalis* ATCC 33277 in planktonic and biofilms states.
PLoS ONE (2017); 12(4): e0174669. https://doi.org/10.1371/journal.pone.0174669

**Artículo 3.** Romero-Lastra P, Sánchez MC, Llama-Palacios A, Figuero E, Herrera D, Sanz M. Gene expression of *Porphyromonas gingivalis* ATCC 33277 when growing in an *in vitro* multispecies biofilm.
PLoS ONE (2019); 14(8): e0221234. https://doi.org/10.1371/journal.pone.0221234

# CAPÍTULO IV

# INTRODUCCIÓN

# 1. EL *BIOFILM*

## 1.1. Características del crecimiento bacteriano

En el mundo natural, los microorganismos se organizan principalmente en dos formas de crecimiento: células individuales que flotan libremente en medios fluidos (planctónicas) o estructuras tridimensionales de agregados de diferentes comunidades bacterianas adheridas a superficies bióticas o abióticas (*biofilms*) (Marsh, 2004, Marsh, 2005), forma, ésta última, en la que se encuentran la gran mayoría de las bacterias en la naturaleza (Stoodley *et al.*, 2002, Costerton *et al.*, 1995, Donlan *et al.*, 2002, Donlan, 2002).

La definición más aceptada de *biofilm* bacteriano es la enunciada por Donlan y Costerton en 2002 como «una comunidad bacteriana inmersa en un medio líquido, caracterizada por bacterias que se hallan unidas a un substrato o superficie, o unas a otras, que se encuentran embebidas en una matriz extracelular producida por ellas mismas, y que muestran un fenotipo alterado en cuanto al grado de multiplicación celular o la expresión de sus genes» (Donlan *et al.*, 2002).

Las bacterias son capaces de adherirse, colonizar y formar *biofilms* en prácticamente cualquier superficie húmeda, como en el interior de tuberías y oleoductos y cualquier superficie costera incluso en la parte inferior de barcos, deteriorando los materiales, acelerando su corrosión o degradándolos, como ocurre, por ejemplo, en plataformas petrolíferas (Garrett *et al.*, 2008). Esto es de especial relevancia en el cuerpo humano, en estudios de otitis, enfermedades infecciosas de la cavidad bucal, afecciones cardíacas como placas de ateroma, infecciones del líquido amniótico y tejidos placentarios (Sanz *et al.*, 2013, Offenbacher *et al.*, 2006, Madianos *et al.*, 2013, Leon *et*

*al.*, 2007), o en el uso de materiales médicos como lentes de contacto, catéteres intravasculares, válvulas cardíacas y prótesis (Nakano *et al.*, 2009, Zaremba *et al.*, 2007, Figuero *et al.*, 2011) que pueden convertirse en lugar de desarrollo de *biofilms* que contengan microorganismos patógenos (Gristina, 1987, Beck *et al.*, 2000, Reyes *et al.*, 2013, Mattila *et al.*, 1989).

Las superficies que colonizan pueden ver modificadas sus condiciones fisicoquímicas y microambientales, debido al crecimiento bacteriano, a su actividad metabólica, a la producción de desechos y la síntesis de material celular y extracelular (Costerton *et al.*, 1995). Asimismo, en ellas, las concentraciones de nutrientes pueden llegar a ser mucho mayores de las que se pueden observar en el resto de la solución, facilitando así la supervivencia de estos organismos (Hamilton, 1987).

## 1.2. Estructura del *biofilm*

Una vez que la bacteria cambia de estado planctónico a *biofilm* comienza a expresar genes distintos, que cambian su comportamiento, adquiriendo mayor capacidad para adherirse al sustrato. Este sustrato o superficie puede influir en varios aspectos: la ratio de unión de las bacterias a la superficie, la influencia en el fenotipo de las mismas, o la modificación en la expresión y producción de factores de virulencia (Feng *et al.*, 2015).

Además, esta unión se facilita gracias a la síntesis de una matriz, compuesta por sustancias poliméricas extracelulares (EPS) como polisacáridos, proteínas, ácidos nucleicos, lípidos, o sales. Es el principal componente del *biofilm* y puede representar más del 75-80% del volumen. Las EPS presentan una gran variabilidad, en función de parámetros complejos como pueden ser la presencia y densidad de microorganismos, la

temperatura o las perturbaciones físico-químicas a las que se ven sometidas (Tan *et al.*, 2018).

En esta matriz quedan embebidas e inmovilizadas las bacterias **(Figura 1)**, que ocupan alrededor del 15-20% del volumen y adquieren estructuras tridimensionales con forma de torres o setas, cuyo conjunto puede ser detectable a nivel macroscópico (Donlan, 2002, Branda *et al.*, 2005, Flemming *et al.*, 2010). Estas estructuras poseen canales de agua, separados por pequeños espacios intersticiales huecos denominados canalículos, que permiten el paso de nutrientes, metabolitos y de otros agentes entre las distintas partes del *biofilm* y actúan como un "sistema circulatorio" primitivo (de Beer *et al.*, 1994).

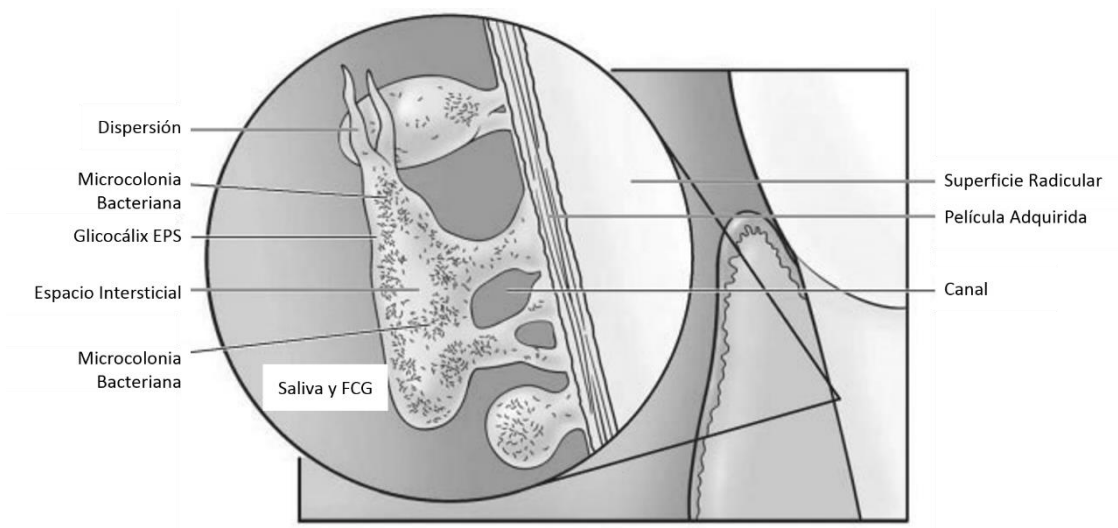


**Figura 1. Estructura del *biofilm* oral** (EPS: sustancias poliméricas extracelulares, agregados de microcolonias bacterianas, canales. FCG: fluido crevicular gingival). Figura adaptada de http://www.dent-wiki.com.

A mayor profundidad en el *biofilm*, los nutrientes, el pH, la luz, la concentración de oxígeno y el potencial REDOX tienden a ser menores. Este gradiente fisiológico determinará que las bacterias aerobias y anaerobias facultativas, de crecimiento más rápido, se congreguen en la superficie y periferia del *biofilm*, mientras que las

profundidades de la estructura se encuentren más pobladas de bacterias anaerobias gram negativas, con tasa de crecimiento más lenta. De esta manera, la placa bacteriana va incrementando en grosor, llegándose a desarrollar *biofilms* muy variados y complejos (Costerton *et al.*, 1994, Costerton *et al.*, 1995), que ofrecerían importantes ventajas a bacterias anaerobias en estado sésil, en contraposición al estado planctónico (Marsh, 2005).

## 1.3. Ventajas del *biofilm*

Los *biofilms* permiten la persistencia y aumentan la supervivencia de las bacterias en medios fluidos gracias a la estructura tridimensional que forman con la matriz y a la existencia de ADN extracelular (ADNe) que desempeña un papel crítico en la adhesión y posible almacenamiento de nutrientes, lo que le confiere una serie de ventajas adaptativas frente a sus formas libres (planctónicas) (Marsh *et al.*, 2017).

Les proporciona un medio protector que facilita el intercambio de información genética, les permite mantener la integridad actuando como amortiguador frente a perturbaciones externas (homeostasis), protegiéndolas de los microorganismos competidores y de la desecación, aumentando su tolerancia a factores de estrés ambiental como los mecanismos de defensa del hospedador, o la perfusión de sustancias potencialmente tóxicas (Marsh *et al.*, 2011, Kuboniwa *et al.*, 2010). La excreción de grandes cantidades de exopolisacárido por parte de las bacterias de los *biofilms* les hace ser notablemente resistentes, por ejemplo, a los agentes antimicrobianos, siendo capaces de sobrevivir frente a concentraciones antibióticas de 10 a 1000 veces mayores respecto a las bacterias planctónicas, una propiedad que se ha visto que mantienen incluso al liberarse de la estructura del *biofilm* (Fine *et al*., 2001, Filoche *et al.*, 2010). Además, el *biofilm*, puede ser empleado por las bacterias como concentrador y reserva de nutrientes

(los productos del metabolismo de unas pueden ser aprovechados por otras), aumentando la eficiencia y diversidad metabólicas y existiendo así una relación sinérgica entre los diferentes microorganismos y por tanto, un aumento en la capacidad de causar enfermedad (Marsh, 2004, Costerton *et al.*, 1999, Kolenbrander *et al.*, 2002).

Otra de las ventajas adaptativas del fenotipo sésil es que dificulta los mecanismos inmunitarios del hospedador como la opsonización, lisis por complemento o fagocitosis de las bacterias (Huang *et al.*, 2011, Lewis, 2001, Jenkinson *et al.*, 2005, Allaker, 2010). Estas estructuras provocan la respuesta inmunitaria celular y humoral, demostrado por la identificación de citoquinas liberadas por leucocitos expuestos a *biofilm*, dificultando el proceso de eliminación (Donlan *et al.*, 2002).

La observación de estas ventajas adaptativas ha provocado que el estudio en ámbitos sanitarios, industriales o medioambientales, de la disgregación del *glicocálix* mediante enzimas antimicrobianas (Thallinger *et al.*, 2013) y la investigación de genes diana, sea clave para poder hacer más susceptibles a las bacterias de ser atacadas por antibióticos y así poderlas eliminar de forma más efectiva (Xavier *et al.*, 2005).

## 1.4. Interacciones e intercambio de información genética entre bacterias

La proximidad de las células dentro de un *biofilm* ofrece un entorno ideal para comportamientos sociales, como ya se describió en (Parsek *et al.*, 2005) introduciendo el término *"sociomicrobiology"* para resaltar este vínculo.

Dentro de los *biofilms*, tienen lugar cambios en las condiciones locales que pueden afectar a las interacciones sinérgicas o antagónicas en estas comunidades orales:

coagregación, cometabolismo, detección de *quorum* y el intercambio genético (transferencia horizontal).

- La **coagregación y la coadhesión**, definida como el reconocimiento específico de célula a célula entre bacterias genéticamente distintas, es vital para la adherencia de células planctónicas a organismos ya adheridos en una superficie facilitando la formación de *biofilm* y promoviendo las interacciones microbianas (Rickard *et al.*, 2003).
- El **cometabolismo**, mecanismo por el cual metabolitos excretados por unas especies, pueden ser utilizados por otras como nutrientes (cooperación nutricional), y que presenta ventajas evidentes en una comunidad microbiana (Flemming *et al.*, 2010).
- El ***quorum sensing,*** es una forma de comunicación intra e interespecífica entre las bacterias mediante pequeñas moléculas difusibles llamadas autoinductores (AI), codificados por el gen *LuxS*, para detectar a microorganismos próximos, responder de forma coordinada a cambios en el medio y que la comunidad se desarrolle de forma óptima (Parsek *et al.*, 2005). Los autoinductores se acumulan a medida que aumenta la población bacteriana y al alcanzar un cierto umbral (densidad celular *quorum*), cambia la expresión génica, originando cambios fenotípicos en el desarrollo, estructura y función del *biofilm* y pudiendo activar genes relacionados con factores de virulencia (Miller *et al.*, 2001, Reading *et al.*, 2006).

  Las interacciones célula-célula aumentan las posibilidades de supervivencia de las diferentes especies bacterianas y reducen la de sus competidores, por tanto, tienen un papel fundamental en la etiopatología de las enfermedades (Campbell *et al.*, 2009).

El sistema de detección *LuxS*/*Autoinducer-2* (AI-2), descrito inicialmente en la bacteria marina *Vibrio harveyi*, es quizás uno de los sistemas de detección de *quorum* más investigados. AI-2 es sintetizado mediante un proceso que transforma S-adenosil metionina (SAM) mediante enzimas codificadas en genes expresados por *LuxS*, que a su vez regulan la expresión de genes involucrados en virulencia y metabolismo de la hemina (TonB, Rgp, Kgp).

- **La transferencia horizontal** es un proceso de intercambio genético, generalmente en forma de plásmidos (Lawrence *et al.*, 2003), que se ve facilitado por la proximidad de las células en los *biofilms* y que presenta ventajas para las especies receptoras, al aumentar la resistencia a competidores, a antibióticos y otras toxinas (Costerton, 2001, Roberts *et al.*, 2006).

  Este tipo de interacción en la que se produce intercambio genético, entre las células vecinas, puede darse por varios mecanismos, como la transducción, la conjugación y la transformación:

✓ La transducción es el proceso por el que el ADN es transferido desde una bacteria a otra mediante un fago.

✓ La conjugación se produce cuando hay un contacto directo de célula a célula o una conexión tipo puente (*pili*) que permite la transferencia de pequeños fragmentos de ADN, usualmente plásmidos o transposones que pueden portar genes de resistencia.

✓ La transformación se produce cuando tiene lugar la captación de ADN libre del medio por una célula bacteriana, lo que puede ser frecuente en los *biofilms* ya que el *glicocálix* de los *biofilm* contiene abundante ADN extracelular.

Además de los mecanismos anteriores, el ADN también puede ser transferido vía vesículas de membrana en bacterias gram negativas (Olsen *et al.*, 2013). Por tanto, el estudio de la comunicación celular y la transferencia de información genética en los *biofilms* es clave para comprender la patogénesis y persistencia de las enfermedades bacterianas, así como para diseñar tratamientos más eficaces.

## 1.5. Mecanismos para el estudio de la expresión génica: Transcriptómica-*Microarrays*

Según el dogma central de la biología molecular, el genoma compuesto por ácido desoxirribonucleico (ADN) se transcribe a ácido ribonucleico (ARN mensajero), que a su vez se traduce a proteínas (**Figura 2**).

Por tanto, existen tres niveles básicos de información biológica:

- **Genoma:** la información genética que comparten todas las células de un organismo.
- **Transcriptoma:** la parte del genoma que se expresa en una célula en unas condiciones determinadas (Lewis *et al.* (2009), Hosogi *et al.*, 2005, Lo *et al.*, 2009).
- **Proteoma:** el conjunto de proteínas sintetizadas por una célula a partir de los genes que ha expresado, cuya función caracteriza la identidad de la misma.

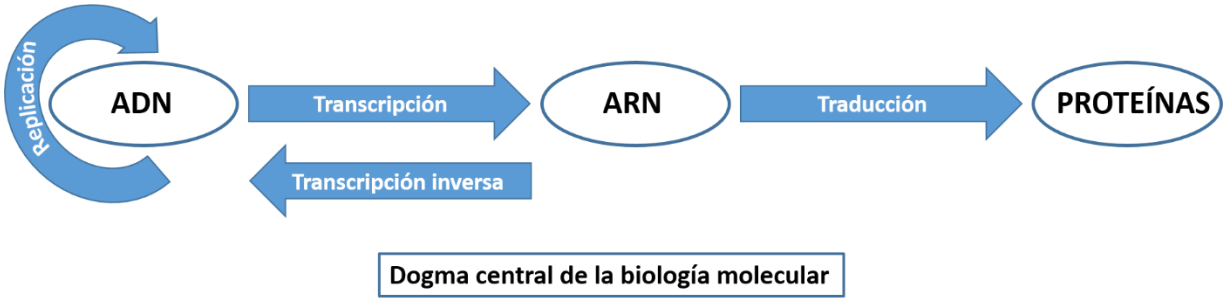


**Figura 2. Dogma fundamental de la biología molecular**. El ADN se autorreplica generando nuevas cadenas de ADN, se transcribe generando ARN mensajero que se traduce, generando la síntesis de Proteínas.

Así, el objetivo fundamental de la transcriptómica es determinar qué parte del genoma es transcrita mediante este proceso de expresión génica, ya que no todos los genes estarían expresados a la vez. Esto ha supuesto un gran avance en el estudio de comunidades de microorganismos como son los *biofilms*. Una de las tecnologías que ha permitido, desde su desarrollo a mediados de la década de los 90, disponer de datos sobre información genómica y transcriptómica a gran escala, de forma rápida y económicamente accesible, es el *microarray* o biochip (Schena *et al.*, 1995).

Ésta, junto con la técnica del ARNseq (*RNA sequencing*, por sus siglas en inglés), permite el estudio de genomas y transcriptomas completos de tejidos y organismos y han revolucionado el estudio de los seres vivos (Passalacqua *et al.*, 2009, Toledo-Arana *et al.*, 2009, Wilhelm *et al.*, 2008).

Un *microarray* es un dispositivo que consiste en una superficie (*slide*) de vidrio, plástico o sílice, que cuenta con cientos de miles de compartimentos (*spots*) rellenos de sondas de ADN (oligonucleótidos) del genoma que se quiera estudiar, formando una matriz (*array*) de filas y columnas. Cada uno de los *spots* de esta matriz es capaz de

detectar la presencia de su ADN complementario de manera altamente específica en proceso de hibridación, dando lugar a la lectura de la expresión una gran cantidad de genes de forma simultánea (patrón de expresión) (Shalon *et al.*, 1996) (**Figura 3**).

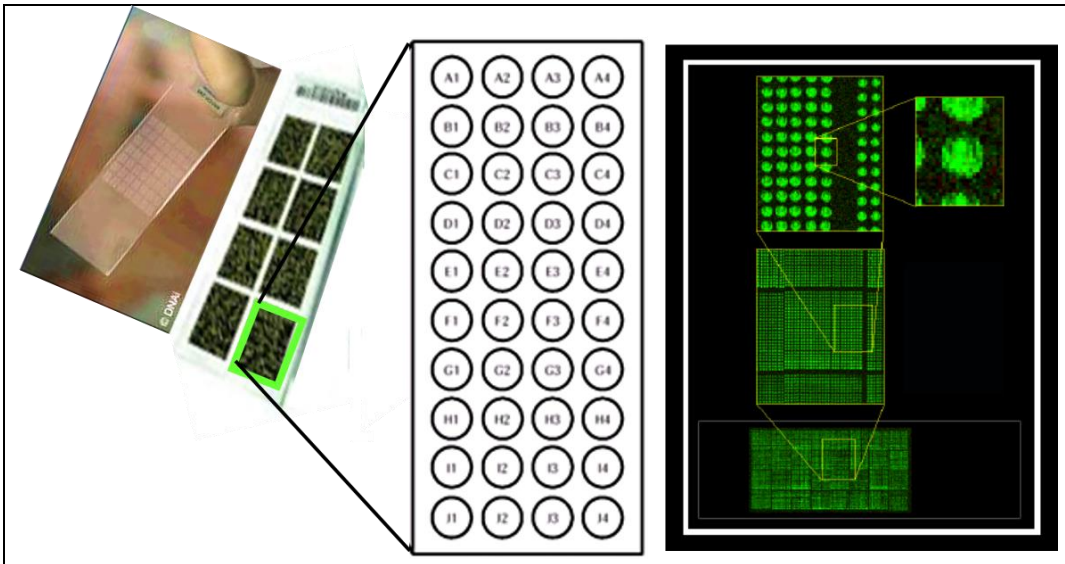


**Figura 3. Estructura de un microarray.**
Figura adaptada de https://www.agilent.com/en/product/gene-expression-microarray-platform.

Los *microarray* son ampliamente utilizados en estudios de expresión génica (estudios transcriptómicos) de organismos en condiciones distintas como pueden ser células sanas y tumorales o la forma planctónica y sésil de una bacteria. En éstos, a partir del ARN transcrito por el genoma del organismo, es necesario obtener el denominado ADN codificante (ADNc) mediante la enzima transcriptasa inversa.

Físicamente, la presencia de oligonucleótidos complementarios es detectada mediante un fluoróforo que es capaz de absorber fotones de una cierta longitud de onda y emitir con otra longitud de onda diferente al producirse la hibridación.

Esto ha permitido la investigación detallada de la relación existente entre el genoma, el transcriptoma y el estado fenotípico de las bacterias orales.

Un experimento de *microarrays* comprende básicamente 3 pasos (Churchill, 2002) (**Figura 4**):

**1) Diseño y fabricación del *microarray.***

El organismo a estudiar debe estar completamente secuenciado y en cada uno de los *spots* de un mismo compartimento, se unen oligonucleótidos correspondientes a un único gen hasta completar la totalidad del genoma.

**2) Preparación de la muestra, marcaje e hibridación.**

Una vez aislado el ARN de cada condición, se retrotranscribe a ADNc y se marca con una molécula fluorescente mediante una serie de reacciones enzimáticas.

Posteriormente, estas secuencias marcadas son incubadas sobre el compartimento correspondiente de la superficie del *slide* durante un tiempo prolongado para que tenga lugar la hibridación de las bases nitrogenadas de la sonda específica (*probe*) de manera complementaria con la molécula diana (*target*).

**3) Escaneado y análisis de los resultados.**

Tras esta combinación de procesos, se obtendrá como resultado una imagen compuesta por puntos de colores con diferentes intensidades de fluorescencia (proporcionales al número de moléculas hibridadas) sobre el *chip* de ADN, que nos va a indicar qué genes se sobreexpresan o se reprimen y en qué medida en comparación con la condición control. Esto deberá realizarse por un análisis informático de la imagen y su posterior análisis estadístico.

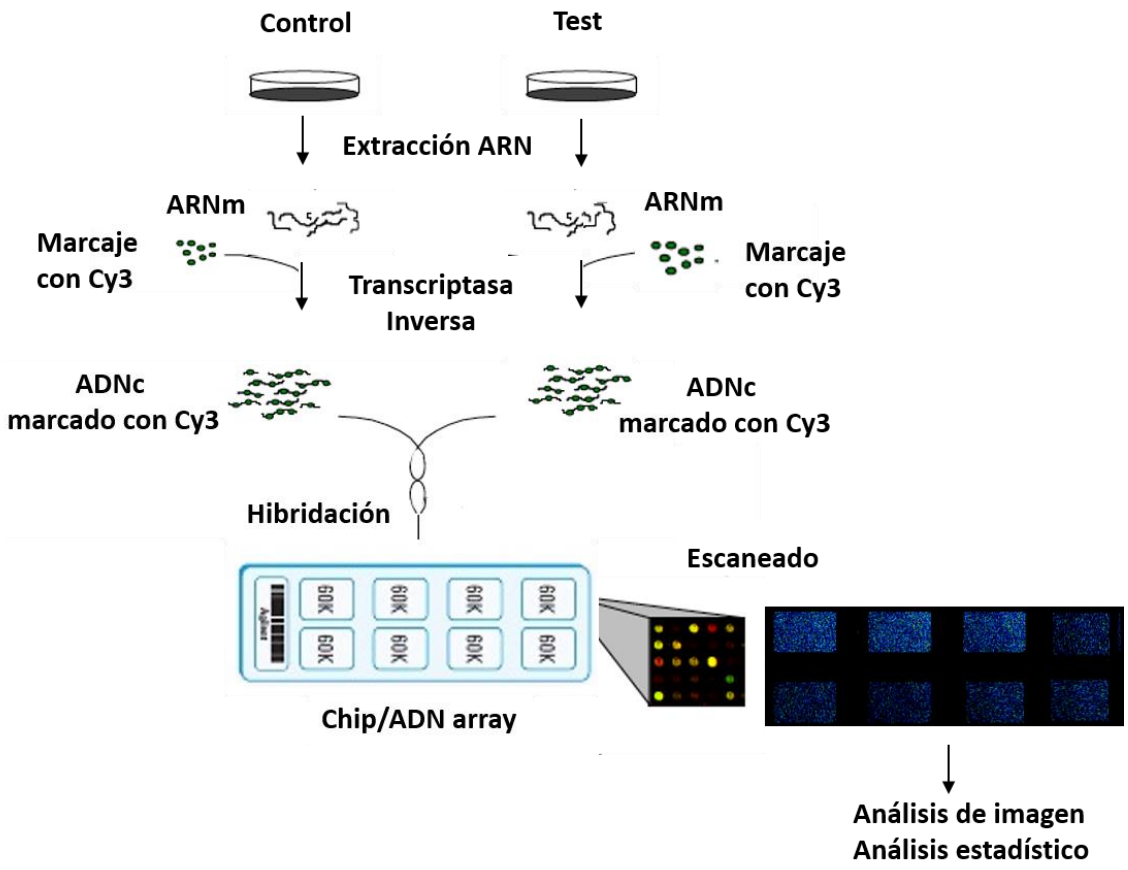


**Figura 4. Protocolo detallado de microarray de un color para comparar la expresión génica de un organismo en dos condiciones distintas (control y *test*).**
Figura adaptada de https://www.agilent.com/cs/library/usermanuals.

Esta técnica está limitada por la pequeña cantidad de ARN que se puede extraer de cada muestra, por la corta vida media del ARN procariótico y porque no sería capaz de detectar genes que no se hayan dispuesto en el *slide*. Además, al recoger la señal de la hibridación de fragmentos de un gen, esta técnica no sería sensible a mutaciones puntuales que puedan presentar los organismos estudiados (SNPs), y también hay que considerar que los niveles de ruido o fondo pueden llegar a ser elevados (Zhao *et al.*, 2014).

Sin embargo, las ventajas de su uso son múltiples: análisis simultáneo y a gran escala de miles de genes al mismo tiempo, automatización, pequeño tamaño de la muestra y fácil manipulación, permitiendo el estudio a nivel transcriptómico de las diferencias entre células planctónicas y *biofilm* (Lo *et al.*, 2009, Shemesh *et al.*, 2007).

# 2. *Porphyromonas gingivalis*

## 2.1. Clasificación

*Porphyromonas gingivalis* se clasifica en el género *Porphyromonas*, familia *Porphyromonadaceae*, orden *Bacteroidales*, clase *Bacteroidetes*, phylum *Bacteroidetes* del dominio *Bacteria* (Boone *et al.*, 2001).

Actualmente, 8 de las 19 cepas conocidas de *P. gingivalis* tienen sus genomas completamente secuenciados (Chen *et al.*, 2017):

- La cepa *P. gingivalis* W83, fue la primera secuenciada, en 2003 (Nelson *et al.*, 2003). Su genoma tiene un tamaño de aproximadamente 2,3 Mb y el 6% de este genoma está compuesto por elementos repetidos transponibles.
- La cepa *P. gingivalis* ATCC 33277, fue secuenciada en 2008. Su secuencia se comparó con la de la cepa W83, revelando importantes reordenamientos genómicos inducidos por una amplia variedad de elementos móviles (Naito *et al.*, 2008). Este genoma es del mismo tamaño que el de la variedad W83 (2,3 Mb).
- La cepa TDC60 de *P. gingivalis* se secuenció en 2011 (Watanabe *et al.*, 2011). La comparación de este genoma con las de las cepas W83 y ATCC 33277 muestra un importante reordenamiento genómico con un tamaño de genoma conservado (2,3 Mb).
- Finalmente, las cepas de *P. gingivalis* HG66, A7436, AJW4, 381 y A7A1-28, se secuenciaron a partir del año 2015 (Chastain-Gross *et al.*, 2015, Xie *et al.*, 2015, Chastain-Gross *et al.*, 2017).

## 2.2. Características bioquímicas.

Es una bacteria anaerobia, gram negativa, en forma de bastón (bacilo o cocobacilo), inmóvil, encapsulada, anaeróbica, asacarolítica y altamente proteolítica. No forma esporas, es quimioorganotrofa y negro-pigmentada. Necesita la presencia de hemina o menadiona y vitamina K para sobrevivir. Utiliza péptidos o aminoácidos como fuente de energía y carbono. Su crecimiento es óptimo a la temperatura de 37ºC y un pH entre 6,5 y 8,3.

Al crecer en un medio de cultivo adecuado como agar sangre, crecen como colonias elevadas de entre 1 y 2 mm de diámetro, lisas brillantes de color blanco a crema que se oscurecen, pigmentándose de un color rojo oscuro a negro en 4 a 8 días (How *et al.*, 2016) (**Figura 5**).

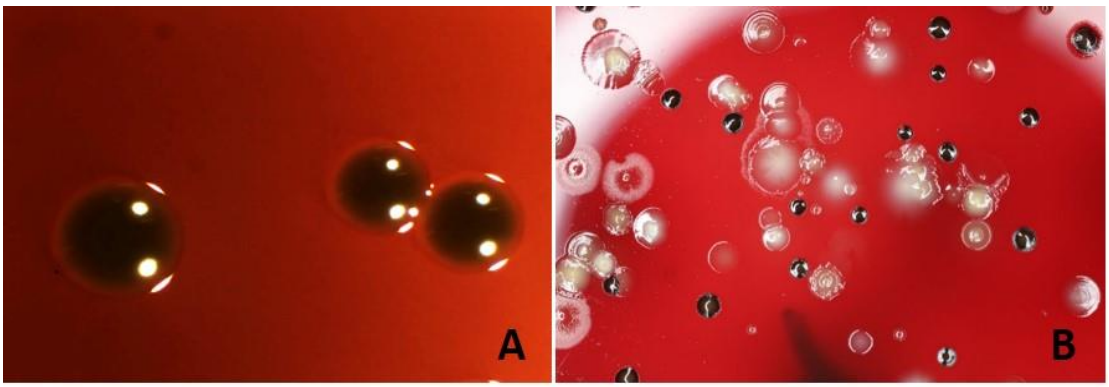


**Figura 5. Colonias de *Porphyromonas gingivalis* ATCC 33277 (en color negro) sobre medio de agar sangre**
**A)** en un cultivo puro, **B)** en un *biofilm* multiespecie.

## 2.3. Virulencia.

*Porphyromonas gingivalis* está presente principalmente en la bolsa subgingival y periodontal profunda de la cavidad oral humana. Su proceso patogénico implica adherencia y colonización de tejidos periodontales, seguido por la adquisición de nutrientes, la proliferación y la respuesta inflamatoria del hospedador, acompañada por un aumento de la temperatura del fluido gingival y la aparición de sangrado.

La asociación entre la presencia de un microorganismo específico y el desarrollo de la patología viene determinada por su virulencia. La capacidad patogénica para causar enfermedad puede depender del tipo de cepa de *P. gingivalis*, de su capacidad de infección, de la naturaleza y estado del hospedador y/o del lugar de colonización (Curtis *et al.*, 2005).

El microambiente oral es altamente dinámico y para sobrevivir en este nicho siempre cambiante, *P. gingivalis* no solo necesita detectar las condiciones ambientales tales como pH, disponibilidad de nutrientes, enzimas extracelulares, disponibilidad de temperatura y oxígeno, sino también densidad microbiana y la presencia de otros microorganismos (Inagaki *et al.*, 2006).

Produce un amplio abanico de potenciales factores de virulencia implicados en la colonización, la destrucción de tejidos, la reabsorción de hueso o la iniciación de la respuesta inflamatoria del hospedador (Bostanci *et al.*, 2012, Haffajee *et al.*, 1994, Holt *et al.*, 2005, Lamont *et al.*, 1998, Holt *et al.*, 1999):

- **Cápsula:** *P. gingivalis* posee una cápsula externa constituida por polisacáridos, que varía según la cepa. Ésta le confiere estabilidad estructural a la bacteria siendo clave en la evasión de la fagocitosis, opsonización y la acción del complemento, eludiendo la respuesta inmunitaria y actuando como antígeno (Laine *et al.*, 1998, Mysak *et al.*, 2014), siendo las cepas encapsuladas más virulentas (Singh *et al.*, 2011, Holt *et al.*, 1999).

- **Fimbrias:** Las fimbrias son estructuras filamentosas que parecen participar en muchas interacciones entre la bacteria y el hospedador, así como con otras bacterias en la adhesión para la formación de *biofilms* y colonización e internalización de tejidos periodontales del hospedador (Mao *et al.*, 2007, Lamont *et al.*, 2000), como muestra un estudio en el que la ausencia de fimbrias en *P. gingivalis* evitaría la invasión de células epiteliales (Ezzo *et al.*, 2003). Hay dos principales tipos de fimbrias que pueden ser expresadas por este patógeno, la fimbria principal larga y filamentosa (FimA I-V e Ib) y la fimbria menor (Mfa) corta y fina. La variedad de fimbria expresada parece influir en la virulencia de *P. gingivalis*, siendo la fimbria tipo II la más virulenta (Amano *et al.*, 2004) y las fimbrias tipo I e Ib más comúnmente aisladas de individuos sanos (Amano *et al.*, 1999, Fujise *et al.*, 2005, Teixeira *et al.*, 2009). Tras la internalización de *P. gingivalis* en la célula hospedadora, las principales fimbrias ven reprimidas su expresión (Xia *et al.*, 2007). Además, las fimbrias también inducen la expresión de citoquinas proinflamatorias, como las interleuquinas 1, 6 y 8 (IL- 1, IL-6, IL-8) y factor de necrosis tumoral α (TNF-α), estimulando la respuesta inmunitaria durante la infección (Feng *et al.*, 2006).

- **Lipopolisacárido:** El lipopolisacárido (LPS) de membrana externa de *P. gingivalis* es un factor clave en el desarrollo de periodontitis. Altera el equilibrio inmunológico del hospedador induciendo la producción de citoquinas proinflamatorias (IL-1β, IL-6 e IL-8), inflamación gingival y destrucción del tejido periodontal (Andrukhov *et al.*, 2015). Fracciones de lipopolisacárido de la pared celular de algunas bacterias gram negativas pueden solubilizarse y actuar como endotoxina, estimulando la respuesta inmunitaria.

- **Vesículas de membrana externa** (OMVs, del inglés Outer Membrane Vesicles)**:** Las OMVs son evaginaciones de la membrana bacteriana durante su crecimiento y división mediante las cuales las bacterias interactúan (Mug-Opstelten *et al.*, 1978, Zhou *et al.*, 1998, McBroom *et al.*, 2007).

  Suelen participar en la adherencia bacteriana, la defensa contra los factores del sistema inmunitario del hospedador, la liberación de una amplia gama de enzimas (fosfolipasa C, proteasas, fosfatasa alcalina entre otras) que producen daño celular y transportan moléculas de señalización entre bacterias (Ismail *et al.*, 2003, Klieve *et al.*, 2005, Kuehn *et al.*, 2005).

- **Hemaglutininas:** Son proteínas bacterianas de superficie capaces de mediar la adhesión no fimbrial a través de receptores de oligosacáridos a células humanas y mediar la respuesta inmunitaria (Lamont *et al.*, 2000). Adicionalmente facilitan a la bacteria la adquisición de hemina de eritrocitos (Lamont *et al.*, 1998, Ezzo *et al.*, 2003), esencial para su supervivencia (Dusek *et al.*, 1994). Varias hemaglutininas (Hag A, RgpA, Kgp) pueden ser consideradas como dianas terapéuticas, ya que se ha descrito un anticuerpo monoclonal capaz de reconocer

un epítopo común a varias de ellas, que inhibe la hemaglutinación y confiere inmunización pasiva frente a *P. gingivalis* (Frazer *et al.*, 2006, Holt *et al.*, 2005).

- **Enzimas proteolíticas:** Las bacterias que se pueden encontrar en bolsas periodontales sintetizan enzimas proteolíticas, como colagenasa, cisteín proteasas o proteasa-hemaglutinina, capaces de degradar las macromoléculas como fibronectina y fibrinógeno, a sus componentes más simples para nutrir a las bacterias. Estas enzimas destruyen el tejido periodontal, lo que activa la respuesta inmunitaria del hospedador, haciendo de las proteasas uno de los factores de virulencia más importantes (Lamont and Jenkinson, 2000).

  Estas proteasas se pueden encontrar liberadas al medio en el interior de vesículas secretoras (OMVs) o en forma libre, o adheridas a la membrana bacteriana, donde cumplirían también un papel en la maduración y activación de otras moléculas que la bacteria sintetiza como fimbrias, hemaglutininas y las proteínas receptoras de la hemoglobina (Kadowaki *et al.*, 2000).

  Entre las proteasas de membrana más importantes se encuentran las cisteín proteasas (gingipaínas), arginina RgpA, RgpB y lisina Kgp (Curtis *et al.*, 1999), que componen la mayoría de las proteasas de la superficie de *P. gingivalis*, causando una elevada respuesta inmunitaria humoral. La ausencia de gingipínas produce la reducción de los niveles de crecimiento que experimenta *P. gingivalis* (Grenier *et al.*, 2001).

  Las proteasas secretadas, además de provocar la destrucción de tejido del hospedador, pueden causar la degradación de inmunoglobulinas y proteínas del complemento, interviniendo en la evasión del sistema inmunitario y en el aumento de la proliferación bacteriana y progresión de la infección (Curtis *et al.*, 1999).

Los dominios hemaglutinina, además de ser capaces de mediar la adhesión a células tanto bacterianas como epiteliales o fibroblastos del hospedador, regulan la adquisición de hemina (Burgess *et al.*, 2002, James *et al.*, 2006), permitiendo la lisis de eritrocitos, cuyo contenido degradarán las gingipaínas hasta obtener protoporfirina IX y Fe (III), de esencial necesidad para *P gingivalis*, que acumula en su membrana; lo que provoca su característica coloración negra (Smalley *et al.*, 1998, Smalley *et al.*, 2007). Además de las proteasas endógenas, *P. gingivalis* tiene la capacidad de inducir la producción de metaloproteasas en fibrobastos, leucocitos y macrófagos del hospedador, causando una mayor degradación de la matriz extracelular (Holt *et al.*, 1999).

- **Sistema de transporte de proteínas Tipo IX (T9SS)**: Recientemente, se ha descrito PorSS como un nuevo sistema de secreción para el transporte de gingipaínas en *P. gingivalis* (Sato *et al.*, 2005, Saiki and Konishi, 2007).

## 2.4. Importancia de *P. gingivalis* en las enfermedades periodontales

La cavidad oral humana es considerada un hábitat cálido, húmedo y dinámico en el que se genera un ambiente ecológico específico y complejo de una gran diversidad, incluso siendo un nicho tan pequeño (Paster *et al.*, 2001). En ella, los microorganismos, además de encontrarse flotando libremente en un medio líquido como es la saliva, se establecen y desarrollan en *biofilms* muy complejos sobre diferentes superficies de contacto como tejidos blandos (mucosas, encía, bolsa periodontal, tejidos conjuntivos y lengua) o tejidos duros mineralizados naturales (dientes) o artificiales (prótesis) (Filoche *et al.*, 2010). Estas últimas estructuras, al ser superficies no descamativas, permiten la colonización persistente. Además, en ellas, los microorganismos se pueden distribuir de forma muy variada en dos zonas, una más en contacto con el medio externo, el área supragingival, expuesta a la saliva y a mecanismos de autolimpieza mecánica frecuentes por la lengua, las mejillas, la masticación y la deglución. Y por otro lado el área subgingival, que no es perturbada por las actividades de limpieza de la boca, creándose un ambiente relativamente estanco y favoreciendo la acumulación de microorganismos que pueden provocar la aparición de las enfermedades periodontales (Allaker, 2010, Sedlacek *et al.*, 2007, Marsh, 2005).

Estas enfermedades periodontales, de naturaleza infecciosa e inflamatoria, se han reclasificado recientemente en el *World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions* 2017, organizado por la Academia Americana de Periodoncia (AAP) y la Federación Europea de Periodoncia (EFP), como gingivitis y las periodontitis, en función del tipo de tejidos afectados (Caton *et al.*, 2018) (**Figura 6**).

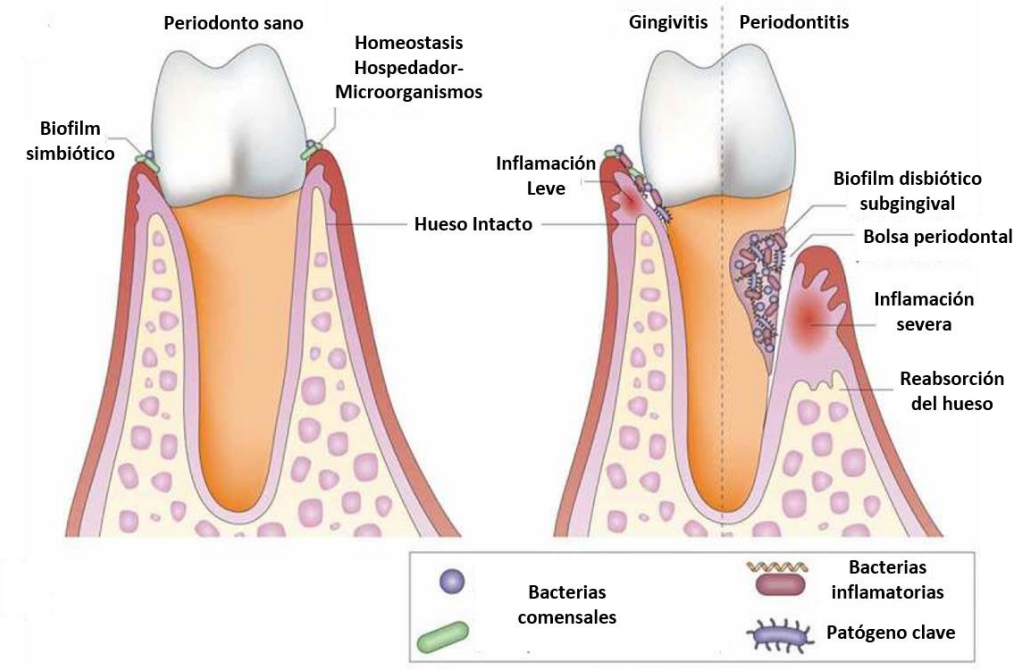


**Figura 6. Progresión en el hospedador de un estado de salud periodontal y homeostasis de microorganismos a un estado de disbiosis y enfermedad periodontal**. Gingivitis, inflamación periodontal sin pérdida ósea y Periodontitis, asociada con un *biofilm* disbiótico, formación de bolsas periodontales, inflamación y pérdida ósea. Figura adapatada de (Olsen *et al.*, 2017).

La gingivitis se define por "una reacción inflamatoria reversible de los tejidos supraalveolares ante la acumulación de placa bacteriana, sin destrucción de los tejidos de soporte" (Chapple *et al.*, 2018).

Por otro lado, la periodontitis es "una enfermedad inflamatoria crónica, progresiva y multifactorial, asociada a un proceso de disbiosis de la placa dental que se caracteriza por la pérdida del ligamento periodontal y del hueso alveolar, la presencia de bolsas periodontales y el sangrado gingival siendo la pérdida de las piezas dentales su consecuencia última" (Papapanou *et al.*, 2018).

Estas enfermedades periodontales son un importante problema de salud pública y se encuentran entre las más prevalentes a nivel mundial, siendo la periodontitis severa la sexta enfermedad más prevalente y afectando a más de un 10% de la población mundial (Tonetti *et al.*, 2017, Sheiham *et al.*, 2002, Llodra Calvo *et al.*, 2002, Loe *et al.*, 1965, Loe *et al.*, 1978), incluida la población española (Carasol *et al.*, 2016). Esta prevalencia se incrementa con la edad y tiene un gran impacto social, económico y de recursos médicos en la prestación sanitaria (Kassebaum *et al.*, 2014).

La placa dental fue el primer *biofilm* estudiado en 1684 por Antonie van Leeuwenhoek cuando observó con el primer microscopio la diversidad y alto número de "animáliculos" presentes en "raspados" tomados de alrededor de dientes y declarando en la *Royal Society* de Londres: *"The number of these animicules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom."* (Porter, 1976).

Es definida por la Organización Mundial de la Salud (OMS) como "una entidad estructural específica pero altamente variable resultante de la colonización secuencial y el crecimiento de microorganismos en las superficies de los dientes y restauraciones que consisten en microorganismos de varias cepas y especies incorporadas en una matriz extracelular de producción propia, compuesta de productos metabólicos bacterianos y sustancias del suero, saliva y sangre" (Kassebaum *et al.*, 2014).

La placa dental es por tanto, un *biofilm* en continuo cambio en sus tipos y cantidades (Sedlacek *et al.*, 2007) formado por la sucesión ordenada de más de 700 especies de bacterias identificadas (Dewhirst *et al.*, 2010), que varían según las personas y los sitios dentro de la misma boca, pudiéndose aislar en los depósitos de placa dental

de un individuo sano más de 400 diferentes (Filoche *et al.*, 2010, Dewhirst *et al.*, 2010, Mira *et al.*, 2017).

Los factores que determinan esta variación interindividual en la composición de la microbiota oral son entre otros, factores ambientales (temperatura, presión de oxígeno, pH, disponibilidad de nutrientes), factores del hospedador (salivación, fluido gingival, genética, edad, dieta, anatomía de los dientes, higiene oral) y factores microbianos (adherencia, coagregación, relaciones intra e inter específicas, heterogeneidad, mecanismos de virulencia) (Kolenbrander *et al.*, 2006, Marsh, 2005, Socransky *et al.*, 2002, Sanz *et al.*, 2017) **(Figura 7).**

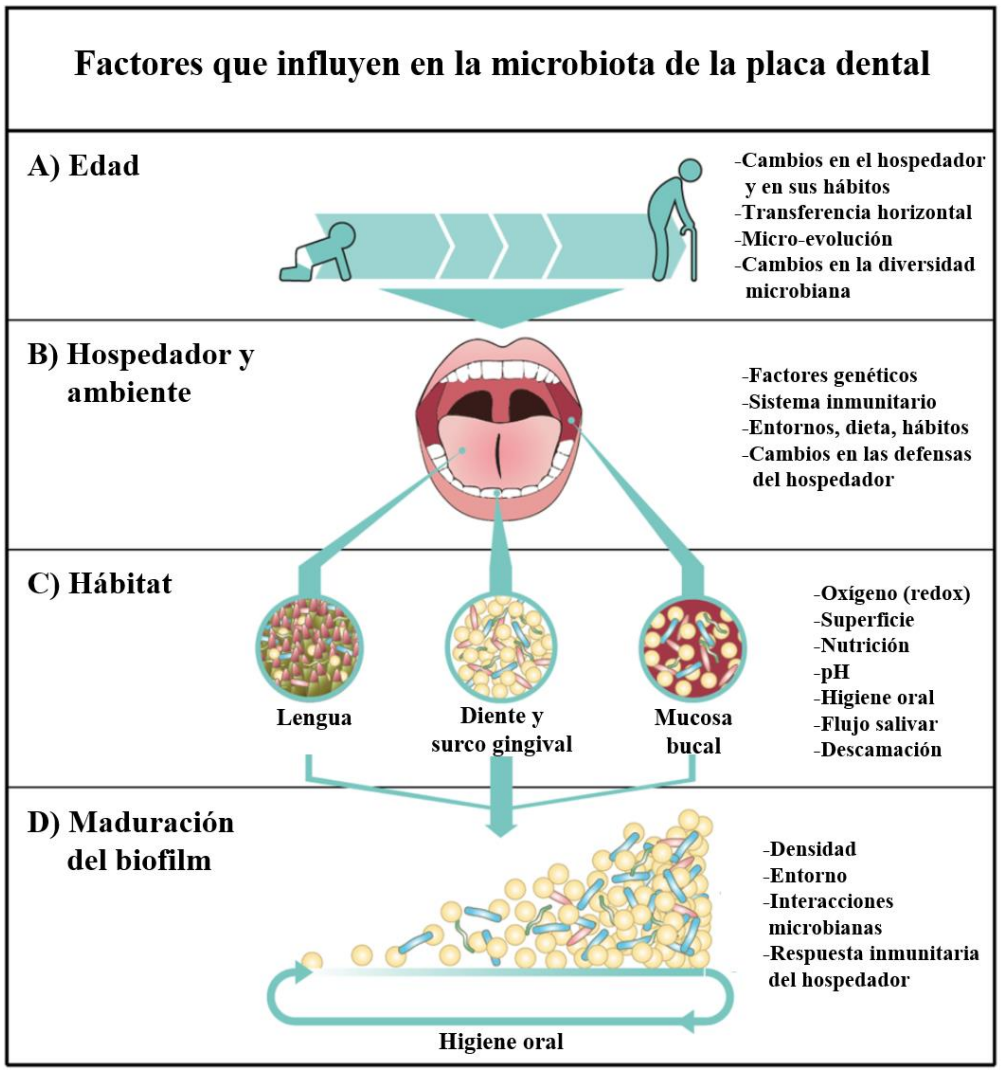


**Figura 7. Factores que determinan la composición de la microbiota oral.** Figura adaptada de (Rosier *et al.*, 2018).

Varias hipótesis han tratado de explicar el papel del *biofilm* dental como principal agente patógeno para estas enfermedades:

**1) La hipótesis de la "placa específica" (SPH)**, enunciada por Loesche en 1976, se centra en los cambios cualitativos, considera que sólo la acción de ciertas especies patógenas está implicada en el desarrollo de la enfermedad. Así, microorganismos gram positivos sacarolíticos darían lugar a caries, mientras que bacterias gram negativas y proteolíticas darán lugar a enfermedades periodontales (Loesche, 1976).

**2) La hipótesis de la "placa no específica" (NSPH)**, Theilade en 1986 reconoció la importancia de los cambios cuantitativos, las enfermedades surgirían por una cantidad excesiva de microflora total que supere el umbral de capacidad de defensa del hospedador, ya que todos los microorganismos participarían por igual en los procesos patogénicos (Theilade, 1986).

**3) La hipótesis de la "placa ecológica"**, propuesta por Marsh en 1994 unifica las ideas principales de las dos anteriores, sostiene que los organismos asociados con la enfermedad pueden estar presentes también en los sitios sanos, pero en niveles tan bajos, que no son clínicamente relevantes. Cuando se produce un cambio en el medio (pH, potencial redox, mayor o menor presencia de nutrientes…), puede producir una alteración del balance de la microflora hacia una más patogénica y favorecer la aparición de mecanismos de virulencia que desencadenen la enfermedad (Marsh, 1994).

**4) La era del *biofilm*. Grupos microbianos específicos (*clusters*).** Socransky, Haffajee y colaboradores (1998) demostraron la presencia de grupos microbianos específicos (*clusters*) que participan en la formación del *biofilm* oral tanto en individuos sanos, como en aquellos con gingivitis y periodontitis, aunque los recuentos y proporciones varíen en cada caso.

Analizaron más de 13.000 muestras de placa subgingival, procedente de 185 pacientes adultos, utilizando sondas para unas 40 especies bacterianas e identificaron seis grupos o complejos de especies bacterianas asociadas. Este hallazgo hizo que a partir de ese momento nuestra comprensión de las enfermedades periodontales no se hiciera con la descripción de bacterias individuales sino con complejos bacterianos asociados (**Figura 8**).

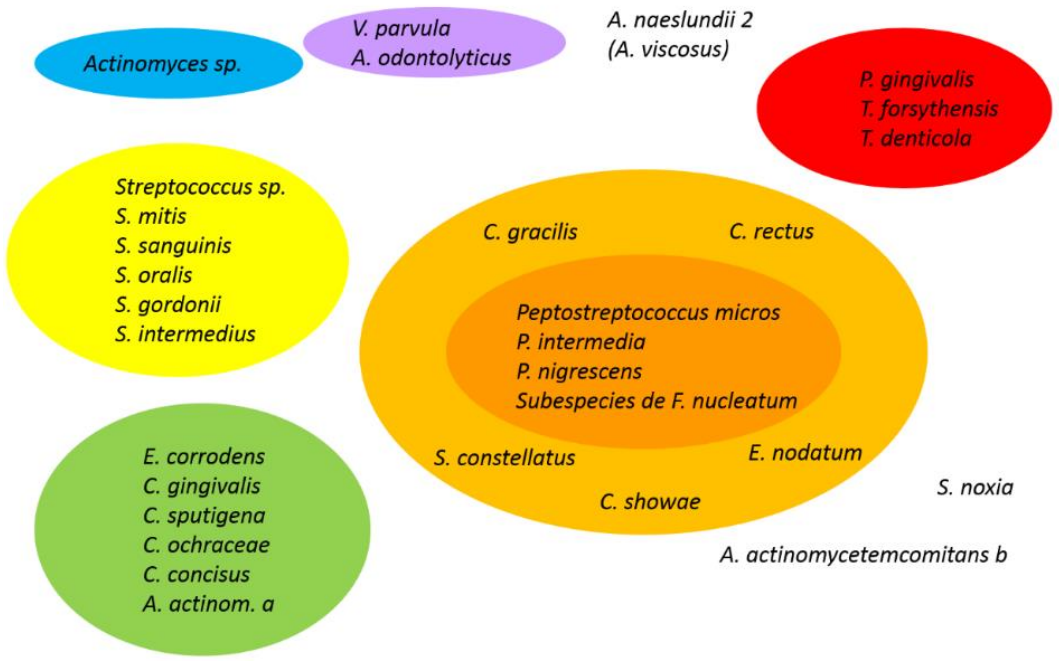


**Figura 8. Complejos microbianos subgingivales.**
Figura adaptada de (Socransky *et al.*, 1998).

Para el comienzo de las enfermedades periodontales, esta adhesión bacteriana y posterior organización espacial sobre las superficies bucales, no se produce de forma aleatoria, sino debe darse una precisa incorporación específica y secuencial de los microorganismos de los complejos bacterianos y una modificación en sus proporciones (Socransky *et al.*, 1998, Aas *et al.*, 2005, Diaz *et al.*, 2006, Kolenbrander *et al.*, 1990, Socransky *et al.*, 2002) (**Figura 9**).

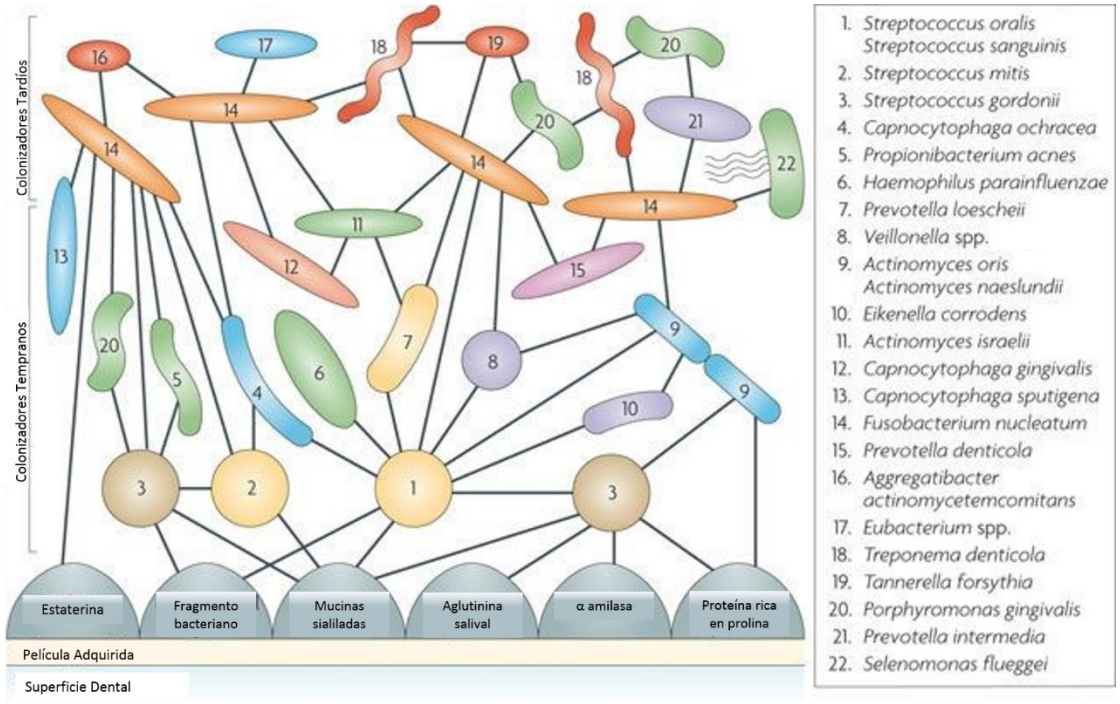


**Figura 9. Diagrama de asociación de las especies bacterianas subgingivales.**
Los diferentes complejos microbianos han sido relacionados con la secuencia de colonización de la superficie dentaria y con la gravedad de la enfermedad.
La heterogeneidad aumenta a medida que la placa envejece y madura. Como resultado de los cambios ecológicos, las bacterias gram negativas anaerobias estrictas colonizan de manera secundaria y contribuyen a una mayor patogenicidad del *biofilm*.
Figura adapatada de (Socransky *et al.*, 1998).

En primer lugar, comienza el acondicionamiento de la superficie del diente recubriéndose de glicoproteínas (mucinas) y de moléculas hidrófobas salivares, formándose lo que se conoce como "película adquirida", necesaria para la colonización por parte de las bacterias. La película adquirida atraerá a las bacterias del flujo salival (planctónicas) pasivamente y de forma reversible, mediante fuerzas fisicoquímicas débiles como la hidrofobicidad o las interacciones electrostáticas (fuerzas de *Van der Waals*), principalmente serán especies de *Actinomyces*, seguidas de un complejo amarillo compuesto por miembros del género *Streptococcus*, un complejo verde compuesto por

especies de *Capnocytophaga*, el serotipo a de *A. actinomycetemcomitans*, *Eikenella corrodens* y *Campylobacter concisus* y un complejo violeta compuesto por *Veillonella parvula* y *Actinomyces odontolyticus.* (Socransky *et al.*, 1998).

Estos grupos de especies bacterianas (complejos amarillo, verde y violeta) aparecen significativamente asociados y son los colonizadores iniciales de la superficie dentaria. Generalmente, a continuación, tiene lugar la fase de multiplicación celular de las bacterias adheridas y se empezarán a excretar sustancias extracelulares poliméricas (EPS) que formarán la matriz a la que se irán agregando distintas especies bacterianas del complejo naranja (*Campylobacter gracilis, Campylobacter rectus, Campylobacter showae, Eubacterium nodatum, Parvimonas micra, Prevotella intermedia, Prevotella nigrescens* y *Streptococcus constellatus, Fusobacterium spp.*). Estas últimas actúan como "puente" de colonizadores más tardíos, pertenecientes al complejo rojo, compuesto principalmente por bacterias gram negativas de mayor capacidad periodonto-patógena como *Tannerella forsythia, P. gingivalis* y *Treponema denticola,* originando el *biofilm* maduro y siendo consideradas un sello *hallmark* fuertemente asociado con la enfermedad periodontal (Socransky *et al.*, 1998) (Kolenbrander *et al.*, 1993, Kolenbrander *et al.*, 2002, Socransky *et al.*, 2002). El aumento de la biomasa tanto por la unión de nuevas bacterias como por la formación del *glicocálix* conduce, como hemos comentado, al agotamiento del oxígeno siendo las bacterias anaerobias las más representativas en zonas profundas, desplazando a las aerobias (Diaz *et al.*, 2006).

La comprensión de la interacción directa e indirecta entre los patógenos del complejo rojo y más en concreto el papel de *Porphyromonas* en dicha asociación puede tener efectos significativos en el manejo de las enfermedades periodontales.

**5) Simbiosis y Disbiosis: *P. gingivalis* como patógeno clave.** En la población sana, con una correcta alimentación y hábitos de higiene bucal, se establece una situación de equilibrio y de beneficio mutuo entre el microbioma oral y el hospedador (simbiosis), a pesar de las tensiones ambientales a las que es sometida regularmente, como la higiene bucal periódica, la ingesta de alimentos o los cambios diurnos en el flujo de saliva (Marsh *et al.*, 2011, Sanz *et al.*, 2017). Sin embargo, en ocasiones, el equilibrio homeostático microbiano puede romperse, produciéndose perturbaciones por cambios en el medio que superan un umbral, como aparición de sustancias tóxicas, por tabaquismo, diabetes, predisposición genética o higiene dental deficiente, lo que se conoce como disbiosis, en donde la composición cualitativa y cuantitativa de las poblaciones microbianas, asociadas a la salud, están significativamente reducidas y aumentan las proporciones de especies que adquieren propiedades potencialmente dañinas, originando el desarrollo de las enfermedades dentales (Hajishengallis, 2014, Camelo-Castillo *et al.*, 2015, Sanz *et al.*, 2017, Mira *et al.*, 2017).

Además las enfermedades periodontales pueden ser un ejemplo de "sinergismo patógeno", en el cual la enfermedad es una consecuencia de la actividad combinada de un consorcio bacteriano que de manera individual no sería tan virulento, pero que al interactuar entre sí, puede intensificar sus efectos (Marsh *et al.*, 2017, Mira *et al.*, 2017).

Aunque en ocasiones haya cambios en la estructura de estos conjuntos microbianos, esta flexibilidad es perfectamente compatible con la salud, y los individuos no desarrollan niveles similares de enfermedad oral en presencia de idénticas circunstancias.

El factor crítico diferenciador para definir la disbiosis es, por tanto, la activación del sistema inmunológico del hospedador y la producción de citoquinas y mediadores

inflamatorios, que incrementan la secreción de fluido crevicular y sangre generándose un ambiente propicio para la multiplicación y crecimiento de estas bacterias y la formación de bolsas periodontales más profundas donde su fuente principal de alimento son los restos hidrolizados por enzimas, de periodonto y sangre, de ahí que haya un mayor sangrado al sondaje y destrucción de tejidos de soporte (Offenbacher *et al.*, 1996, Socransky *et al.*, 1998, Gohler *et al.*, 2014).

El contexto ambiental y los procesos que subyacen en el desarrollo y la estabilidad de las poblaciones microbianas en la boca sana, así como las variaciones interindividuales de cada microorganismo, incluyendo la capacidad de manipular el perfil transcripcional de las células vecinas de la comunidad (Darveau *et al.*, 2012), son fundamentales para comprender cómo estas poblaciones se transforman en un estado disbiótico en la enfermedad.

Según la teoría de "*Polymicrobial Synergy and Dysbiosis* (PSD)" propuesta por (Hajishengallis *et al.*, 2012b), este cambio en el equilibrio entre los microorganismos puede causar la proliferación de bacterias patógenas clave (*keystone pathogens*), que hasta el momento estaban en menor proporción, y que sean capaces de desplazar a las demás especies y predisponer a la enfermedad, causando infecciones persistentes, como la periodontitis (Hajishengallis *et al.*, 2012a, Jenkinson *et al.*, 2005, Filoche *et al.*, 2010, Rosan *et al.*, 2000, Sedlacek *et al.*, 2007).

Dentro de estos patógenos, se considera a *P. gingivalis* como un patógeno clave especialmente relevante en el *biofilm* subgingival, cuya actividad es necesaria para el desarrollo de las enfermedades periodontales y que ve modificada su expresión génica durante la evolución de los síntomas (Hajishengallis *et al.*, 2012b, Bostanci *et al.*, 2012, Mira *et al.*, 2017). En un modelo murino se comprobó que incluso en baja abundancia

(<1% del total de la microbiota), *P. gingivalis* sería capaz de desestabilizar la función inmunitaria innata del hospedador y promover el establecimiento de una comunidad disbiótica, una respuesta inflamatoria y el desarrollo de la enfermedad (Darveau *et al.*, 2012). Además se ha demostrado en estudios de *biofilms* mixtos *in vitro* que es capaz, también, de modificar el patrón de expresión génica de los microorganismos vecinos, por lo que los grupos minoritarios de bacterias no deben ser subestimados, pues ejercen un papel clave en el desarrollo de la disbiosis (Mira *et al.*, 2017, Frias-Lopez *et al.*, 2012).

También ha sido demostrado que cuando *P. gingivalis* era introducido en un *biofilm* de especies bacterianas no patógenas, podía cambiar el perfil de expresión de la comunidad (Frias-Lopez *et al.*, 2012). Por otro lado, se ha podido detectar la presencia de *P. gingivalis* en individuos sanos, lo que parece indicar que no todas las cepas tendrían la misma capacidad patogénica, o bien como se ha comentado, que el estado del hospedador influye en el desarrollo y severidad de la enfermedad (Curtis *et al.*, 2005, Marsh *et al.*, 2017, Mira *et al.*, 2017, Sanz *et al.*, 2017).

## 2.5. Análisis transcriptómico de *P. gingivalis*.

Desde los años ochenta se están desarrollando modelos de *biofilm in vitro* de flujo continuo o dinámicos en *robbins device* (Kharazmi *et al.*, 1999, Sternberg *et al.*, 2006), o sistemas estáticos en placas multipocillo, sencillos de manejar y reproducibles, donde se pueden controlar las condiciones de la superficie, las condiciones ambientales (de accesibilidad de nutrientes, temperatura, pH, potencial redox…) y la evolución de una población definida de bacterias (Merritt *et al.*, 2005).

La mayoría de estos estudios de microorganismos se han llevado a cabo en condiciones planctónicas o utilizando una única especie bacteriana en *biofilm*, lo que permitía explorar el efecto de las variables ambientales, fisiológicas y genéticas con parámetros estrechamente controlados en sistemas de modelos *in vitro* (Liu *et al.*, 2016, Waite *et al.*, 2005, Schembri *et al.*, 2003, Whiteley *et al.*, 2001).

En relación a *P. gingivalis* la gran parte de los trabajos están realizados aplicando distintas condiciones y sobre un número limitado de genes de *quorum sensing*, de respuesta al polifosfato, de respuesta a la limitación de hemina, entre otros (Yuan *et al.*, 2005, Kiyama-Kishikawa *et al.*, 2005, Moon *et al.*, 2014).

Otros autores han comenzado a hacer estudios de la expresión génica global en la cepa W50 comparando situación planctónica y *biofilm* monoespecie, encontrando cambios significativos en grupos de genes como biogénesis, replicación de ADN, regulación transcripcional, producción de energía, biosíntesis de cofactores, y transporte (Lo *et al.*, 2009). Aún así se conoce poco acerca de los procesos regulatorios y respuesta adaptativa de la bacteria en *biofilm* y su relación con la patogenicidad. También se han llevado a cabo investigaciones, donde se detallan los cambios sufridos por la bacteria *P. gingivalis* W83 después de la inoculación en la cavidad oral de rata (Zhao *et al.*, 2015),

observándose 42 genes sobreexpresados en comparación con la cepa silvestre, en su mayoría correspondientes a proteínas hipotéticas, transposones y transposasas. En este mismo estudio 22 genes fueron reprimidos en la cepa inoculada codificando para transportadores, permeasas putativas, proteínas de unión a ATP, metabolismo de proteínas y los ácidos nucleicos entre otros.

Sin embargo, son escasos los trabajos de investigación de los patrones de expresión génica global de *P. gingivalis* con modelos de *biofilm in vitro* validados de poblaciones más complejas y heterogéneas (Kuboniwa *et al.*, 2017, Redanz *et al.*, 2011, Bao *et al.*, 2014), o con la cepa ATCC 33277, donde se reproduzca con precisión la dinámica y la secuencia de colonización bacteriana del *biofilm* subgingival multiespecie. Y donde se pueda establecer una evolución de la adaptación de la bacteria y sus respuestas desde la condición más sencilla (planctónica monoespecie) a condiciones más complejas en las que pueden estar recibiendo señales de *quorum sensing* o intercambios de ADN (transferencia génica horizontal) (Shapiro, 1998), para formar *biofilms* tanto monoespecie como multiespecie. Cuanto más completas sean las poblaciones bacterianas mejor podrán ser comparados los procesos de formación y desarrollo en las condiciones que ofrece el ambiente oral (Hall-Stoodley *et al.*, 2004, Hajishengallis *et al.*, 2012b, Comolli, 2014).

# CAPÍTULO V

# JUSTIFICACIÓN, OBJETIVOS E HIPÓTESIS

# 1. JUSTIFICACIÓN

Diversos estudios de *biofilms* revelaron que cientos de genes, entre ellos muchos genes no caracterizados, se expresan diferencialmente en el *biofilm* en comparación con el estado planctónico. Estos estudios centran la atención en determinados genes candidatos asociados con el desarrollo del *biofilm*, el análisis de los genes de detección de *quorum* regulados por su implicación en el desarrollo del *biofilm* o la expresión génica de la bacteria tras algún tratamiento concreto.

Sin embargo, se encuentra poca información que describa tanto los cambios iniciales como los que se dan posteriormente una vez *P. gingivalis* se ha incorporado al *biofilm* monoespecie y/o multiespecie, en su expresión génica global. Esto puede ser clave para entender mejor su comportamiento y sus mecanismos de acción en las enfermedades periodontales y así establecer estrategias terapéuticas adecuadas.

# 2. OBJETIVOS

## 2.1. OBJETIVO GENERAL

Determinar y comparar los cambios de expresión génica global de *P. gingivalis* ATCC 33277, mediante análisis transcriptómico en diferentes condiciones (planctónico en presencia de *biofilm* y *biofilm* tanto monoespecie (*P. gingivalis*) como multiespecie (*S. oralis, A. naeslundii, V. parvula, F. nucleatum, A. actinomycetemcomitans, P. gingivalis*).

## 2.2. OBJETIVOS ESPECÍFICOS

**1.** Estudiar la diferente expresión génica de *P. gingivalis* ATCC 33277 en dos condiciones planctónicas: medio planctónico puro y planctónico en presencia de su propio *biofilm* monoespecie.

**2.** Estudiar la diferente expresión génica de *P. gingivalis* ATCC 33277 en sus dos fenotipos de crecimiento: planctónico y en *biofilm* monoespecie, prestando especial atención a los genes implicados en la patogenicidad de esta especie bacteriana, entre ellos proteasas, adhesinas o fimbrias.

**3.** Estudiar la diferente expresión génica de *P. gingivalis* ATCC 33277 en un *biofilm* multiespecie frente al estado planctónico, para estudiar la posible influencia de otras bacterias.

# 3. HIPÓTESIS

## 3.1. HIPÓTESIS GENERAL

El perfil de expresión génica de *P. gingivalis* y su acción patogénica puede verse afectada por estado fenotípico en el que se encuentre.

## 3.2. HIPÓTESIS ESPECÍFICAS

**1.** Existe una diferente expresión génica de *P. gingivalis* ATCC 33277 en dos condiciones planctónicas: medio planctónico puro y planctónico en presencia de *biofilm* monoespecie.

**2.** Existe una diferente expresión génica de *P. gingivalis* ATCC 33277 en sus dos fenotipos de crecimiento: planctónico y en *biofilm* monoespecie.

**3.** Los cambios en la expresión génica, además de verse influidos por el estado fenotípico, están condicionados por otras variables como la presencia o asociación de otras especies bacterianas (*biofilm* multiespecie).

# CAPÍTULO VI

# MATERIALES Y MÉTODOS. RESULTADOS

Los Materiales y Métodos y los Resultados de los estudios presentados en esta tesis han sido publicados, como artículos científicos en tres publicaciones independientes, en las revistas científicas *BMC Microbiology* y *PLoS One* con las siguientes referencias:

**Artículo 1.** Sánchez MC, Romero-Lastra P, Ribeiro-Vidal H, Llama-Palacios A, Figuero E, Herrera D, Sanz M. Comparative gene expression analysis of planktonic *Porphyromonas gingivalis* ATCC 33277 in the presence of a growing biofilm *versus* planktonic cells. *BMC Microbiology.* (2019); 19(1):58. Epub 2019/03/15. doi: 10.1186/s12866-019-1423-9. PubMed PMID: 30866810.

**Artículo 2.** Romero-Lastra P, Sánchez MC, Ribeiro-Vidal H, Llama-Palacios A, Figuero E, Herrera D, Sanz M. Comparative gene expression analysis of *Porphyromonas gingivalis* ATCC 33277 in planktonic and biofilms states.
*PLoS ONE* (2017); 12(4): e0174669. https://doi.org/10.1371/journal.pone.0174669.

**Artículo 3.** Romero-Lastra P, Sánchez MC, Llama-Palacios A, Figuero E, Herrera D, Sanz M. Gene expression of *Porphyromonas gingivalis* ATCC 33277 when growing in an *in vitro* multispecies biofilm.
*PLoS ONE* (2019); 14(8): e0221234. https://doi.org/10.1371/journal.pone.0221234.

# ARTÍCULO 1

**Sánchez MC, Romero-Lastra P, Ribeiro-Vidal H, Llama-Palacios A, Figuero E, Herrera D, Sanz M. Comparative gene expression analysis of planktonic *Porphyromonas gingivalis* ATCC 33277 in the presence of a growing biofilm *versus* planktonic cells. *BMC Microbiology*. 2019;19(1):58. Epub 2019/03/15. doi: 10.1186/s12866-019-1423-9. PubMed PMID: 30866810.**

## RESUMEN

**Antecedentes:** *Porphyromonas gingivalis* es uno de los microorganismos patógenos clave en el inicio y la progresión de la periodontitis que reside en la cavidad oral dentro de complejos *biofilms* multiespecie. En este estudio *in vitro*, utilizando m*icroarrays* de ADN, se lleva a cabo el estudio de la expresión génica diferencial de *Porphyromonas gingivalis* ATCC 33277 en dos condiciones planctónicas, en ausencia o presencia de su propio *biofilm* monoespecie.

**Resultados:** Aproximadamente el 1,5% de los genes (28 de 1909 genes, con un criterio de filtro para el cambio de expresión génica mayor o igual a 1,5 veces, valor de significación $p < 0,05$) fueron expresados diferencialmente por las células planctónicas de *P. gingivalis* cuando se encontraba en presencia de un *biofilm*. Estos genes fueron predominantemente relacionados con el metabolismo del hierro, la adhesión bacteriana, la invasión, la virulencia y el sistema de detección de *quorum sensing*. Los resultados de *microarrays* fueron consistentes con los obtenidos por la. reacción en cadena de la polimerasa con transcriptasa inversa (RT-qPCR).

**Conclusión:** este estudio proporciona información sobre los cambios transcripcionales de las células planctónicas de *P. gingivalis* cuando crece en presencia de un *biofilm*, observando la importancia que el cambio de fenotipo puede tener en el transcriptoma.

Sánchez *et al. BMC Microbiology* (2019) 19:58
https://doi.org/10.1186/s12866-019-1423-9

BMC Microbiology

RESEARCH ARTICLE Open Access

# Comparative gene expression analysis of planktonic *Porphyromonas gingivalis* ATCC 33277 in the presence of a growing biofilm *versus* planktonic cells

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**Abstract**

**Background:** *Porphyromonas gingivalis*, a microorganism residing in the oral cavity within complex multispecies biofilms, is one of the keystone pathogens in the onset and progression of periodontitis. In this in vitro study, using DNA microarray, we investigate the differential gene expression of *Porphyromonas gingivalis* ATCC 33277 when growing in the presence or in absence of its own monospecies biofilm.

**Results:** Approximately 1.5% of genes (28 out of 1909 genes, at 1.5 fold change or more, $p$-value < 0.05) were differentially expressed by *P. gingivalis* cells when in the presence of a biofilm. These genes were predominantly related to the metabolism of iron, bacterial adhesion, invasion, virulence and quorum-sensing system. The results from microarrays were consistent with those obtained by RT-qPCR.

**Conclusion:** This study provides insight on the transcriptional changes of planktonic *P. gingivalis* cells when growing in the presence of a biofilm. The resulting phenotypes provide information on changes occurring in the gene expression of this pathogen.

**Keywords:** *Porphyromonas gingivalis*, Microarray hybridization, Gene transcription, Transcriptomics, Gene expression, RT-qPCR, Planktonic growth, Biofilm

## Background

*Porphyromonas gingivalis*, a Gram-negative anaerobe residing within the oral cavity, has been identified as one of the key pathogenic species implicated in the establishment and development of periodontal diseases [1–3], mainly through the expression of a broad range of virulence factors involved in tissue colonization, evasion of host defenses and stimulation of a chronic inflammatory response [3].

*P. gingivalis* is found within the oral cavity adopting a sessile biofilm lifestyle, predominately as a component of complex biofilms containing multiple microbial communities [4]. *P. gingivalis* has the capacity to adhere to mucosal and dental surfaces, including the teeth, gingiva, cheek and tongue, as well as to other oral bacteria, thus withstanding the host natural barriers, the host immune defenses and the presence of multiple antimicrobial agents [5–8]. These virulence factors are the consequence of specific gene expression, and it is, therefore, important to improve our knowledge on them in order to understand how these microorganisms can adapt to specific ecological determinants, and their role within the biofilm environment.

It is well known that microbial gene expression is significantly different within biofilms when compared to planktonic growth [9–12]. Recent investigations have studied the specific gene expression of *P. gingivalis* when growing in biofilms, and compared to planktonic growth, demonstrating the differential expression of a

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broad range of genes [13–19]. In biofilm growth, the cell replication and growth rate are decreased with repressed genes involved in cell envelope biogenesis, DNA replication, energy production, biosynthesis and phospholipid metabolism. By contrast, there is an important number of genes involved in regulatory mechanisms which are overexpressed, such as those encoding transport and binding proteins, proteins involved in signal transduction and transcriptional regulation and many others not well characterized. Within this research line, Ang et al. (2008) [20] conducted a proteomic study comparing the envelope proteins of *P. gingivalis*, either growing in planktonic or in biofilms, demonstrating an overexpression of proteins involved in hemin transport (HmuY and IhtB), in metabolic pathways, virulence factors or proteins of the cell envelope.

Other investigations have studied the overexpression of *P. gingivalis* genes affecting structural characteristics such as the presence of different fimbrial types, as well as specific polysaccharides involved in adhesion and colonization mechanisms, which may significantly contribute to biofilm formation [21–23]. Also, cell-cell signaling mechanisms has been the focus of study, and *P. gingivalis* cells have shown up-regulation of the LuxS-dependent signaling regulatory genes, involved in inter-species communication and quorum sensing but also involved in stress, protease modulation and haemagglutination or in hemin uptake [24–27].

In spite of this knowledge, the genetic and environmental determinants affecting *P. gingivalis* cells when transiting from free-floating cells to biofilm have not been fully elucidated [28–30]. It was, therefore, the purpose of this investigation to study the gene expression of free-floating *P. gingivalis* cells either growing in a pure planktonic environment or when they are placed in the presence of a *P. gingivalis* mono-species biofilm.

## Material and methods

### Bacterial strain and culture conditions

*Porphyromonas gingivalis* ATCC 33277 was used in this study and grown on supplemented blood agar plates [Blood Agar Oxoid No 2; Oxoid, Basingstoke, UK; with 5.0 mg/L hemin (Sigma, St. Louis, MO, USA), 1.0 mg/L menadione (Merck, Darmstadt, Germany) and 5% (*v*/v) sterile horse blood (Oxoid)], at 37 °C for 48 h in anaerobiosis (10% $H_2$, 10% $CO_2$, and 80% $N_2$).

### Experimental assays

Figure 1 depicts an overview of the experimental design. *P. gingivalis* ATCC 33277 was grown in modified Brain Heart Infusion (BHI) medium (Becton, Dickinson and Company, USA) in anaerobiosis at 37 °C for 24 h [13]. Bacteria were harvested in their late exponential growth phase [0.8; standard deviation (SD) =0.1 of optical density at 550 nm] and added to fresh modified BHI medium in order to obtain a pure culture containing $10^8$ colony-forming units per milliliter (CFU/mL). Two experimental groups were carried out, free-floating *P. gingivalis* cells either growing in a pure planktonic environment or when they are placed in the presence of a *P. gingivalis* mono-species biofilm. For that, the planktonic bacterial culture prepared was placed in pre-sterilized polystyrene well tissue culture plates (Greiner Bio-one, Frickenhausen, Germany) under two environmental conditions:

(1) Test group: the bacterial suspension of *P. gingivalis* was deposited in the culture plates containing sterile ceramic calcium hydroxyapatite (HA) discs [7-mm in diameter and 1.8 mm in thickness (SD = 0.2); (Clarkson Chromatography Products, Williamsport, PA, USA)].
(2) Control group: the bacterial suspension of *P. gingivalis* was deposited in the plates without HA discs.

Plates were incubated in anaerobic conditions at 37 °C for 96 h. Wells containing only culture medium were also evaluated to assure sterility and lack of contamination.

After 96 h of incubation, free-floating *P. gingivalis* cells from both groups were harvested and processed for the transcriptomic analysis, with the goal of obtaining approximately 20 μg of total RNA for each replicate. Each pooled sample corresponded to one biological replicate and was processed in the same manner.

### Confocal laser scanning microscopy (CLSM)

Test and control group samples were analyzed by confocal laser scanning microscopy (CLSM) after 96 h of growth. 100 μL of free-floating *P. gingivalis* cells from both test and control groups were deposited in a slide and were stained with the fluorochrome Syto9 (wave lengths 515–530 nm) for 9 min at room temperature to obtain an optimum fluorescence signal (Molecular Probes B. V., Leiden, The Netherlands). Samples were then covered with a coverslip, with the aim of minimizing the movement of bacteria. CLSM clearly showed the absence of sessile phenotype in the bottom of the plate of control group samples, while a sessile phenotype was developed on the surface of hydroxyapatite discs of test group. For that, after completely removing the free-floating *P. gingivalis* cells from control wells, these ones were sequentially rinsed with 2 mL of sterile PBS three times (time per rinse, 10 s), in order to remove unbound bacteria. In the same way, after completely

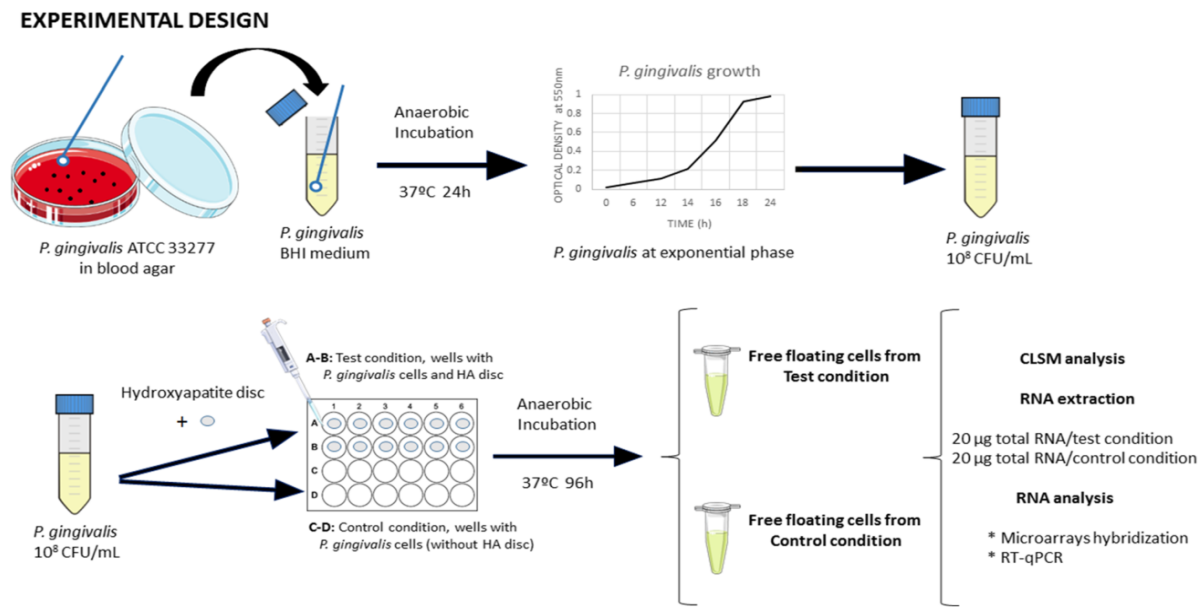


**Fig. 1** Overview of experimental design. *Porphyromonas gingivalis* ATCC 33277 strain was maintained on blood agar plates and grown in modified Brain Heart Infusion (BHI) medium. After 24 h, optical density was measured, and a pure culture containing $10^8$ colony forming units per milliliter (CFU/mL) was set. Two culture conditions were then prepared: Test cells, depositing *P. gingivalis* cells in presence of Hydroxyapatite (HA) disc, and Control cells, depositing *P. gingivalis* cells in the wells without HA discs. After 96 h of incubation of multi-well plates under anaerobic conditions, free floating *P. gingivalis* cells from both test and control condition were harvested, examined by CLSM, processed and total RNA extracted and purified. Agilent Oligo Microarrays 8x15K (074976) for *P. gingivalis* ATCC 33277 were used for hybridizations, (this slides also contents probes against the whole genome of *P. gingivalis* W83), and RT-qPCR analyses were performed to confirm the results. The experiments were repeated three times and each experimental condition was pooled into the three biological replicates and processed for the transcriptomic analysis. (Images for Fig. 1 were taken from https://smart.servier.com/ under a creative commons licence)

removing the free-floating *P. gingivalis* cells from test wells, HA discs from test group were rinsed by immersion in 2 mL of sterile Phosphate-Buffered Saline (PBS) three times (time per rinse, 10 s). Then, the bottom of the plate and the HA discs were stained with the fluorochrome for 9 min at room temperature. Samples were observed with CLSM [Ix83 Olympus® inverted microscope coupled to an Olympus® FV1200 Confocal System (Olympus; Shinjuku, Tokyo, Japan) using × 63 water-immersion lenses (Olympus) The CLSM control software was set to take a z-series of scans (xyz) of 0.5 µm thickness (8 bits, 1024 × 1024 pixels). Image stacks were analyzed with the Olympus® software (Olympus).

### Total RNA isolation

After 96 h of incubation under anaerobic conditions, free-floating *P. gingivalis* cells from both test and control groups were harvested, and total RNA was isolated using the TRIzol® Max Bacterial RNA Isolation Kit (Ambion, Life Technologies, Carlsbad, CA, USA) as detail in Romero-Lastra et al. (2017) [13]. Briefly, *P. gingivalis* cell pellets from both groups were resuspended in Max Bacterial Reagent® (Ambion) and after temperature shock treatment to help the break of the cell wall, consisting on 4 min incubation at 95 °C and cooling on ice for 10 min, TRIzol® reagent (Ambion) was added. Total RNA was extracted using the chloroform protocol and isopropanol precipitation. Isolated RNA was then washed in 75% ethanol and resuspended in 50 µl RNase-free water (Water PCR grade, Roche Diagnostic GmbH; Mannheim, Germany). To remove any contaminating DNA, samples were then treated with DNase I (Ambion), and purified using columns of RNeasy Mini kit (Qiagen) according to the manufacturer's protocol.

RNA concentration was measured by NanoDrop ND1000 spectrophotometer (NanoDropTechnologies; Thermo Scientific™, LLC, Wilmington, DE, USA), and RNA integrity was assessed using an automated electrophoresis device (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). An A260/A280 ratio of at least 2.0 was considered appropriate for the experiments.

### cDNA synthesis and transcriptomic protocol

All experiments were done in triplicates. The fluorescently labeling was performed using SuperScript Indirect

cDNA Labeling System (Invitrogen; Carlsbad, CA, USA) as described in Romero-Lastra et al. (2017) [13]. Preparation of probes and hybridization was performed as described in the manufacturer's instructions [One-Color Microarray Based Gene Expression Analysis Manual Ver. 6.5 (Agilent Technologies)].

Slides specific for the strain *P. gingivalis* ATCC 33277 [Agilent Oligo Microarrays 8x15K (074976)] were used. The array also contents the whole genome of *P. gingivalis* W83.

### Microarray and data analysis

Images from Cy3 one-color microarrays (Agilent) were taken, corrected and analyzed following the protocol detailed in Romero-Lastra et al. (2017) [13].

LIMMA language with "normexp" and loess methods were used to treat background correction and normalization [31, 32]. Log-ratio values were used for consistency among arrays [31]. Differentially expressed genes were determined using linear models and Bayes moderated t-statistic; Benjamani and Hochberg method was used to correct false discovery rate *p*-values [31, 32], and was controlled to be lower than 5% and a cutoff of fold change (increase or decrease) up to 1.5 between the two situations. Expression ratios were expressed as means of the fold changes of the three biological replicates and Standard Deviation (SD). Hybridizations and statistical analysis were performed by the Genomics Unit at the National Center of Biotechnology at the Universidad Autónoma of Madrid (Spain).

### Confirmation of microarray data by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Microarray results were confirmed by RT-qPCR selecting 8 genes differentially expressed, four genes from the up-regulated group and four from the down-regulated one. Universal Probe Library Roche software tool (Roche Diagnostics) was used to design specific primers (Table 1). *P. gingivalis* 16S rRNA gene was used as a loading control.

RT-qPCR was performed from the cDNA generated from 1 μg of total RNA from each sample, using the High Capacity cDNA Archive Kit (Applied Biosystems, ThermoFisher Scientific; MA, USA) in a final reaction volume of 10 μL. The qPCR reactions were performed in triplicate using 5 μL per well of each cDNA, and 3 μL of a mix composed by 0.4 μM of each primer, 5x HOT FIREPol1 EvaGreen1 qPCR Mix Plus (ROX), and nuclease-free water, to reach a final volume of 8 μL in 384-well optical plates and following the standards protocols provided by ThermoFisher Scientific in an Applied Biosystems ABI PRISM 7900HT apparatus (95 °C 10 min, 40 cycles of 95 °C 15 s and 60 °C 60 s, and a final standard melting curve dissociation protocol). The results of differentially expressed genes were analysed using the Expression Suite Software Version 1.1 and Comparative Ct Method (ΔΔCt) was applied [33].

## Results

By CLSM analysis it was verified that in the control group, after 96 h of incubation, free-floating *P. gingivalis* cells had not evolved to biofilm phenotype (Fig. 2 a). It could be observed that no sessile phenotype was formed on the bottom of the plate, observing only some cellular debris (Fig. 2 c). In contrast, in the test samples, free-floating *P. gingivalis* cells (Fig. 2 b) were in presence of biofilm evolved on HA disc (Fig. 2d).

The Fig. 3 depicts the genes with differential expression in *P. gingivalis* ATCC 33277 resulting from microarray-based transcriptome analyses, when comparing the two

**Table 1** Primers used for reverse transcription quantitative polymerase chain reaction (RT-qPCR)

| Locus name | Putative identification | Primers for RT-qPCR | |
|---|---|---|---|
| PGN_0557 (*hmuR)* | TonB-dependent receptor HmuR | Forward<br>Backward | 5′-3′: TAGTCGCGACGGACAGAAAT<br>5′-3′: CTGGTGAAGATCCCACGTTT |
| PGN_1058 (*ftn)* | Ferritin | Forward<br>Backward | 5′-3′: GAAATGATCGAGGCTGTCGT<br>5′-3′: GTCCTGTGATGCCATATCTCC |
| PGN_0780 *(prtQ)* | PrtQ, protease | Forward<br>Backward | 5′-3′: CAGCTGTAAACCGCAACAAG<br>5′-3′: GGCTTGGCTCCCGTATTATC |
| PG_0437 | Polysaccharide biosynthesis/ export protein | Forward<br>Backward | 5′-3′: AGAGGGCCTTACTCGTACCG<br>5′-3′:CCACTGGAAATAATCCTCTTCTGT |
| PGN_0183 *(fimC)* | Minor component FimC | Forward<br>Backward | 5′-3′:CCTTTTCAAGAAAGAACTTGAGGA<br>5′-3′: GTCGGACTATCGGCTCGTT |
| PG_2131 | OmpA_c-like | Forward<br>Backward | 5′-3′: ACACACCCCTCTCGTCTGAG<br>5′-3′: TCCCTTCCGGATAGCTCTG |
| PGN_0181 | Fimbrillin-A associated anchor proteins Mfa1 and Mfa2 | Forward<br>Backward | 5′-3′: CCACTACGGTGTCTTTCGTG<br>5′-3′: TTAGACGCTTTGCACATTGG |
| PG_1712 | Alpha-1,2-mannosidase family protein | Forward<br>Backward | 5′-3′: GCTACGAAAGCCGTCCATC<br>5′-3′: GTACCACTCCCAACCTTTGC |

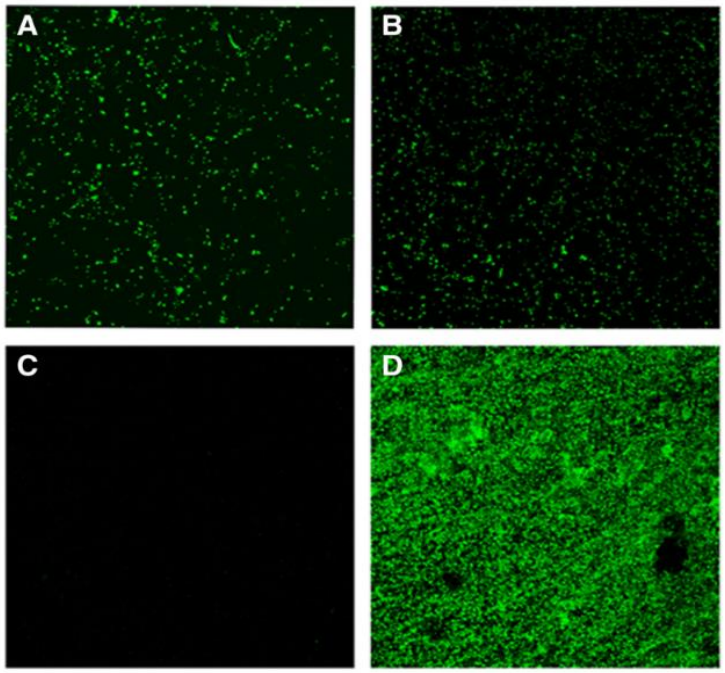


**Fig. 2** Representative confocal micrographs of *P. gingivalis* depicting 2D maximum projection images after 96 h of incubation of the two experimental groups designed, (**a**) planktonic *P. gingivalis* cells growing in a pure planktonic environment and (**b**) free-floating *P. gingivalis* cells placed in the presence of a *P. gingivalis* mono-species biofilm. Image (**c**) corroborate the absence of sessile phenotype on the bottom of the plate of control group samples, only faint debris could be observed adhered. Image (**d**) shows a sessile phenotype evolved on the surface of hydroxyapatite discs form the test group. Specimens were stained with Syto9 fluorochrome (Molecular Probes B. V., Leiden, The Netherlands)

planktonic states, either in presence or absence of a bacterial biofilm. Differentially expressed genes with 1.5 fold change (up or down) and $p$-value $< 0.05$ were plotted, X-axis represents $\log_{10}$ expression of pure planktonic state (in absence of a biofilm) and Y-axis shows the $\log_{10}$ expression genes of cells in the presence a growing biofilm.

The differentially regulated genes of *P. gingivalis* cells under both test and control groups are depicted in Table 2. Expression ratios were expressed as means of the fold changes of the three biological replicates and Standard Deviation (SD). Although the gene expression was not markedly different between both groups, a total of 28 out of 1909 genes (1.5%) were differentially expressed by free-floating *P. gingivalis* cells growing in the presence of a biofilm.

From these differentially expressed genes, 21 transcripts were found significantly increased (Table 2). These included genes related to iron acquisition and storage, among them the PGN_0557 (*hmuR)* gene, which encodes for the outer-membrane hemin utilization receptor involved in the uptake of both free hemin and heme bound to hemoproteins. Also, the gene PGN_0493 appeared overexpressed, which encodes for a receptor Hma, implicated in heme uptake. Similarly, the PGN_1494 gene, which encodes for the oxygen-independent coproporphyrinogen-III oxidase, or the genes PGN_1058 (*ftn)* and PGN_0604, which are related with intracellular iron-storage proteins, were differentially expressed. *P. gingivalis*, under these test conditions overexpressed genes encoding for transposases (PG_0009 and PG_0942), in particular the ISPg5 transposase Orf1, and the gene PGN_0780 *(prtQ)*, which encodes a protease with peptidase activity, belonging to the PrtC family of genes encoding collagenase-like proteases. The gene PGN_1312, which encodes a transcriptional regulator of arginine metabolism and the gene PG_0437 involved in the biosynthesis of polysaccharides, was also up-regulated.

Conversely, in the test conditions seven transcripts demonstrated significant down-regulation, being these genes related to adhesion or virulence (PGN_0183 (*fimC)*, PGN_0181, PG_2130, PG_2131, PG_1712) (Table 2).

RT-qPCR was used for the assessment of the microarray results from the selected genes (4 from the up-regulated and 4 from the down-regulated group). Figure 4 shows a high correlation between the $\log_2$

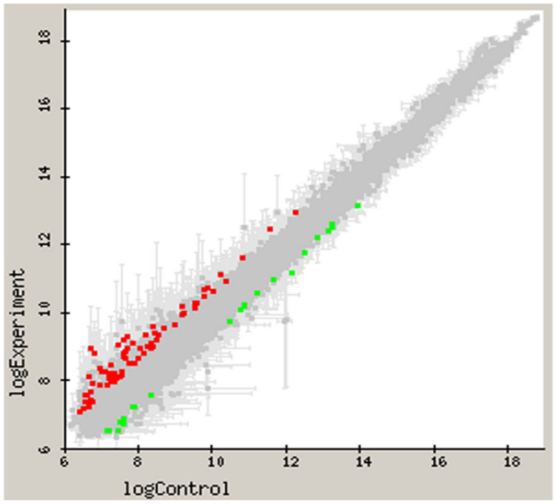


**Fig. 3** Differential gene expression by comparative microarray analyses (represented in $\log_{10}$) when comparing planktonic *Porphyromonas gingivalis* ATCC 33277 cells either in presence of a growing biofilm or in absence of a biofilm. Control planktonic cell gene expression (X-axis) is plotted against test cells (Y-axis) with a 1.5 fold change (up or down) and *p*-value < 0.05. Down regulated genes (green) and up-regulated genes (red) are shown in the figure

ratio of microarray and RT-qPCR results in the two studied conditions ($R^2 = 0.8477$).

## Discussion

This in vitro investigation has shown that a 1.5% of genes (28 out of 1909 genes, at 1.5-fold change or more, *p*-value < 0.05) were differentially expressed by *P. gingivalis* cells when in the presence of a biofilm. Several studies have investigated the differential gene expression of the periodontal pathogen *P. gingivalis* when growing under different conditions, basically under planktonic or biofilm conditions, and have reported distinct genetic expression [13–19]. Among them, our research group reported differences in gene expression when *P. gingivalis* ATCC 33277 grew either in planktonic or in biofilms states, finding that 4.8% of genes were differentially expressed when growing in biofilm. These up-regulated genes were mainly related to the cell envelope, transport, and binding or outer membranes proteins, while the down-regulated genes were mainly genes related to transposases or oxidative stress [13]. Most of the previous studies, however, have not elucidated the differential gene expression when this bacterium is in transition between both states. That is, whether planktonic *P. gingivalis* within an environment of biofilm growth, may undertake gene expression changes that would facilitate its adaptation to the developing biofilm environment.

The present study has tried to reproduce experimentally the situation in which free-floating *P. gingivalis* cells when in the presence of a *P. gingivalis* mono-species biofilm will develop a differential genetic expression when compared with similar planktonic *P. gingivalis* cells but without any biofilm influence. To define differential expression, apart from statistical significance ($p < 0.05$), a threshold of 1.5-fold (up or down) in the average expression ratio was selected, which has been previously used in microarray analysis of gene expression in *P. gingivalis* and was considered biologically relevant [14, 18, 26]. Under these experimental conditions, a total of 28 out of 1909 genes (1.5%) of *P. gingivalis* were differentially expressed when cells from the test group were compared with the control.

Among the differentially expressed genes, the gene PGN_0557 (*hmuR)* showed up-regulation [+ 1.67 (SD = 0.13)], which encodes a major hemin uptake protein, but also a potential adhesin. The role of this adhesin PGN_0557 (*hmuR*) has been demonstrated by Kuboniwa et al. (2009) [17], using an in vitro biofilm model of three bacterial species (*F. nucleatum* subsp. *nucleatum*, *P. gingivalis* ATCC 33277 and *Streptococcus gordonii* DL1), demonstrating that the lack of PGN_0557 (*hmuR)* gene in *P. gingivalis* resulted in a 70% reduction of community formation. In addition, the four genes involved in iron transport (PGN_1058 (*ftn)*, PGN_0604, PGN_1494 and PGN_0493) appeared up-regulated, what may indicate that these genes may contribute to biofilm formation by protecting *P. gingivalis* from oxidative stresses generated by intracellular free iron. One of these genes, PGN_0493, encodes for the receptor Hma, which has also been implicated in heme uptake. Hagan and Mobley (2009) [34] demonstrated that iron acquisition, mediated by specific outer membrane receptors, was critical for the colonization of the urinary tract by *Escherichia coli*. Similarly, heme acquisition facilitated by the receptor Hma was a pre-requisite for the development of kidney infection by *E. coli*. Therefore, the results obtained may indicate that *P. gingivalis* would use similar colonization mechanisms to develop biofilm state.

Under these experimental conditions, the gene PG_2094 was up-regulated [+ 1.91 (SD = 0.32)]. It is classified as a hypothetical protein but with certain homology with the LuxR family transcriptional regulator of *P. gingivalis* SJD2, involved in the quorum sensing system in bacteria [35]. This might suggest a promotion of cell communication in *P. gingivalis* when these cells are in the presence of a biofilm. However, nowadays, although the homology with this domain is preserved, a plausible function was assigned: translocation/assembly module TamB. Bacteria export proteins across the cell envelope using diverse systems. The secretion mechanisms fulfill general cellular functions but are also

**Table 2** Differentially expressed genes in free-floating *Porphyromonas gingivalis* ATCC 33277 cells either in presence of a growing biofilm or in pure planktonic growth (cutoff ratio ± 1.5-fold change, p-value < 0.05) for the microarray analysis

| Open reading frame[a] | Gene[a] | Protein or function | Expression ratio by Microarray[b] (SD) |
|---|---|---|---|
| PGN_0181 | | Fimbrillin-A associated anchor proteins Mfa1 and Mfa2 | −1.60 (0.09) |
| PGN_0183 | *fimC* | Minor component FimC | −1.76 (0.16) |
| PGN_0493 | | Heavy-metal-associated domain (Hma) | + 1.56 (0.03) |
| PGN_0495 | | Conserved hypothetical protein | + 1.73 (0.15) |
| PGN_0529 | *batA* | Aerotolerance-related membrane protein BatA | −1.62 (0.02) |
| PGN_0557 | *hmuR* | TonB-dependent receptor HmuR | + 1.67 (0.13) |
| PGN_0604 | | Ferritin | + 1.78 (0.08) |
| PGN_0649 | | Conserved hypothetical protein | + 1.78 (0.18) |
| PGN_0780 | *prtQ* | PrtQ, protease | + 1.84 (0.41) |
| PGN_0787 | | Conserved hypothetical protein | + 1.61 (0.06) |
| PGN_1058 | *ftn* | Ferritin | + 1.74 (0.12) |
| PGN_1093 | | Conserved hypothetical protein | −1.61 (0.14) |
| PGN_1206 | *folD* | Methylenetetrahydrofolate dehydrogenase/ cyclohydrolase | + 1.86 (0.25) |
| PGN_1312 | | Probable transcriptional regulator as Arg-repressor | + 1.85 (0.47) |
| PGN_1494 | | Putative oxygen-independent coproporphyrinogen III | + 2.02 (0.02) |
| PGN_1534 | | Hypothetical protein | + 1.95 (0.28) |
| PGN_2071 | *topA* | DNA topoisomerase I | + 2.26 (0.18) |
| PG_0009 | | ISPg5 transposase Orf1 | + 1.74 (0.16) |
| PG_0437 | | Polysaccharide export protein, BexD/CtrA/VexA family | + 1.89 (0.42) |
| PG_0718 | | Conserved hypothetical protein | + 1.99 (0.38) |
| PG_0942 | | ISPg5 transposase Orf1 | + 1.59 (0.01) |
| PG_1169 | | Hypothetical protein | + 1.93 (0.28) |
| PG_1403 | | Rhomboid family protein | + 1.70 (0.09) |
| PG_1712 | | Alpha-1,2-mannosidase family protein | −1.52 (0.02) |
| PG_1979 | | Hypothetical protein | + 1.82 (0.26) |
| PG_2094 | | Conserved domain protein | + 1.91 (0.32) |
| PG_2130 | | Hypothetical protein | −1.58 (0.09) |
| PG_2131 | | OmpA_C-like | −1.61 (0.04) |

[a]Putative identification from Genebank. [b] Results of three biological replicates. Expression ratio by Microarray indicates the mean fold expression (SD) of that gene

essential for pathogenic bacteria during the interaction with the host cells. Selkrig et al. (2012) described a new translocation and assembly module (TAM) that promotes efficient secretion of autotransporters in proteobacteria. Functional analysis of the TAM in several bacteria, *Salmonella enterica*, *Citrobacter rodentium* or *E. coli* demonstrated that TamB is an integral inner membrane protein that forms the translocation and assembly module or TAM complex [36] with the outer membrane protein, TamA, an Omp85-family protein. The discovery of the TAM provides a new target for the development of therapies to inhibit colonization by bacterial pathogens [37].

It must be noted that, involved in inter-species communication and quorum sensing, LuxS-dependent signaling regulatory genes have been also related to stress, protease modulation and haemagglutination or in hemin uptake [24–27, 38]. Studies using LuxS-deficient mutants of *P. gingivalis* have reported an altered expression of genes involved in hemin uptake, specifically up-regulation of the genes for a TonB-linked hemin binding protein, HmuR and the iron storage protein ferritin [27, 39, 40]. In the present study, and under these experimental conditions, genes related with the synthesis of both proteins were up-regulated [PGN_0557 (*hmuR)* [+ 1.67 (SD = 0.13)], PGN_0604 [+ 1.78 (SD = 0.08)] and PGN_1058 (*ftn)* [+ 1.74 (SD = 0.12)]. In low level conditions of hemin and iron, HmuR production is suppressed by LuxS signaling, and thus the requirement of ferritin for iron storage should be reduced [40, 41]. Instead, when *P. gingivalis* has availability of heme/iron, as occurred in the conditions used in vitro with 5.0 mg/L

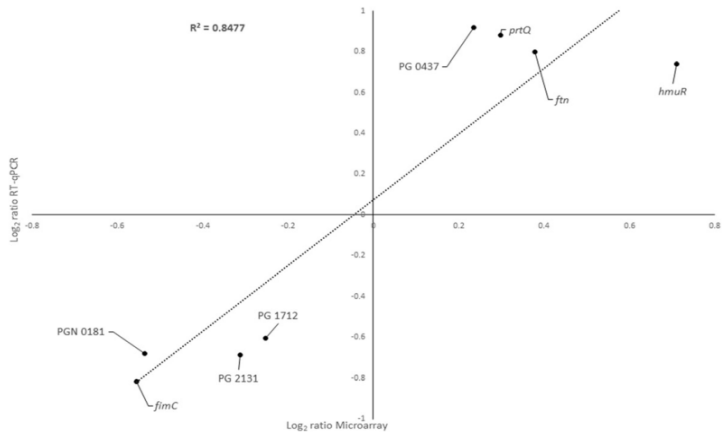


**Fig. 4** Graph depicting the correlation between the $\log_2$ ratio Microarray and Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) gene expression ratios when test and control conditions were compared. The RT-qPCR $\log_2$ values were plotted against the microarray data $\log_2$ values ($R^2 = 0.8477$)

hemin concentration or in vivo when the established bacterial community starts to destroy the periodontal tissue, reduced Autoinducer-2 (AI-2) expression, removes the repression of LuxS over *hmuR* gene, and subsequently of ferritin, which facilitate tight control and ensure adaptability to environmental conditions [40–42].

Free-floating *P. gingivalis* cells in the presence of a biofilm also overexpressed the genes PG_0009 and PG_0942, which encode for transposases, in particular the insertion sequence (IS) elements and ISPg5 transposase Orf1. These results are in agreement with those reported by Califano et al. (2000) [43], that described how ISPg5 and others IS elements could contribute to the diversity of *P. gingivalis* strains, as a mode of adapting to specific ecological determinants. This differential regulation in transposases genes and transposon functions has also been reported by our research group [13], in the comparative gene expression analysis of *P. gingivalis* ATCC 33277 in biofilm versus planktonic cells, and has been attributed to an adaptation to the changes to the new phenotypic state. The results from this current investigation could indicate that *P. gingivalis* begins to adapt to different environmental conditions, and may gradually adopt a sessile phenotype growth.

Among other genes related to a biofilm formation, PG_0437 showed + 1.89 fold changed expression (SD = 0.42). This gene encodes a polysaccharide outer membrane protein exporter, which is involved in polysaccharide biosynthesis. Haft et al. (2006) reported that the overexpression of these proteins occurred preferentially in bacteria from sediments, soils and biofilms [44], so that these results may imply that, under the experimental conditions used, *P. gingivalis* cells would use this system when adapting to evolve to a biofilm state.

The genes PGN_1312 and PGN_0780 *(prtQ)* encoding proteases were also up-regulated in the test group. *P. gingivalis* cells in the presence and, possibly evolving into a biofilm, may develop peptidase activity, with the purpose of inactivating host defense mechanisms, what may be relevant when this pathogen is forming a biofilm [45, 46].

Conversely, several genes encoding proteins involved in bacterial adhesion, invasion or virulence, were identified as down-regulated in free-floating *P. gingivalis* cells in the presence of a biofilm (PGN_0183 (*fimC)*, PGN_0181, PG_2130, PG_2131, PG_1712). In fact, *P. gingivalis* fimbriae have been identified as one of its major colonization factors [47–53]. Although these results demonstrating the down-regulation of the genes encoding for the formation of fimbriae may appear contradictory, these genes encode only minor components of the fimbriae proteins FimA and Mfa1. In the comparative gene expression analysis of *P. gingivalis* ATCC 33277, in planktonic versus mature biofilms states, carried out by our research group, it was reported that *fimD*, one of the minor components of the fimbriae A, appeared down-regulated in biofilm state [13]. Similarly, Krogfelt and Klemm (1988) [54] showed that a clone of *E. coli* lacking the genes encoding these minor component proteins did produce fimbriae consisting of pure Fim A protein, indicating that these minor protein components were not necessary for the structural integrity of the fimbriae. These results are also coincident with those reported by Nagano et al. (2012), which demonstrated that despite a lack of fimC, the FimA protein was still produced and polymerized to form fimbriae

[53]; likewise Nishiyama et al. (2007) reported that the complete deletion of PGN_0181 did not affect the formation of FimA fimbriae by *P. gingivalis* ATCC 33277 [55]. Similarly, previous works demonstrated that *P. gingivalis* autoaggregation, and its subsequent biofilm initiation, are probably due to FimA [56, 57], this autoaggregation is intensified by a loss of short fimbriae, however, other authors claim that Mfa fimbriae is essential in the process of colony formation on solid surfaces [57, 58]. These reports appear to indicate the assay- and context-dependency in assessing the role of each fimbrial type.

PG_1712 gene encoding the protein alpha-1,2-mannosidase was also down-regulated in test group. This protein belongs to the glycosidic hydrolase family 92, cleaving mannose, which is an important sugar in the synthesis of glycoproteins by Gram-negative bacterium as *P. gingivalis*. Five genes hydrolyzing mannan have been reported in *P. gingivalis* (PG_0032, PG_0902, PG_0973, PG_1711, and PG_1712), although the resulting enzymes have not been well characterized, thus making it difficult to interpretate these results. Rangarajan et al. (2013) reported that α- and β-mannosidases from *P. gingivalis* did not have an effect on the biosynthesis of O-LPS and A-LPS or in the secretion of Arg-gingipains [59].

## Conclusions

This in vitro investigation has demonstrated that 28 genes (1.5%) were differentially expressed (up-regulated or down-regulated) when comparing free-floating *P. gingivalis* placed in the presence of a *P. gingivalis* mono-species biofilm versus cells growing in a pure planktonic environment. Most of these genes are related to the metabolism of iron, bacterial adhesion, invasion, virulence and quorum-sensing system. Although the differential gene expression between *P. gingivalis* planktonic cells growing under the test or control conditions may seem limited, the thorough understanding of the genes and its regulatory pathways involved in the transition between planktonic and biofilm states may provide important insights in the prevention of biofilm formation and consequently of periodontal diseases.

### Abbreviations

BHI: Brain Heart Infusion culture medium; CFU/mL: Colony-forming units per milliliter; CLSM: Confocal Laser Scanning Microscopy; DNA: Deoxyribonucleic acid; HA: Hydroxyapatite; RT-qPCR: Reverse Transcription Quantitative Polymerase Chain Reaction; SD: Standard deviation

### Acknowledgements

Project was supported by Extraordinary Chair of DENTAID-UCM. We thank G. García and L. Almonacid from the Centro Nacional de Biotecnología (Universidad Autónoma, Madrid, Spain) for performance of hybridizations and statistical analysis of the microarrays and RT-qPCR. We thank C. Pérez from the Centre of Microscopy and Cytometry from Universidad Complutense (Madrid, Spain).

### Funding

This work was funded by the Cátedra Dentaid-UCM. None of the funders had any role in designing and/or conducting of the study; collection, management, analysis and interpretation of the data; and preparation, review or approval of the manuscript.

### Availability of data and materials

The datasets generated and analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository, using the accession GSE122623.
URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122623

### Authors' contributions

Conceptualization: MCS PR-L EF DH MS. Funding acquisition: DH MS. Investigation: PR-L MCS HR-V AL-P. Methodology: MCS PR-L HR-V AL-P. Supervision: MCS EF DH MS. Writing - original draft: MCS PR-L. Writing - review & editing: EF DH MS. We confirm that the final version manuscript has been read and approved by all named authors.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



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Received: 21 October 2018 Accepted: 19 February 2019
Published online: 12 March 2019

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# ARTÍCULO 2

**Romero-Lastra P, Sánchez MC, Ribeiro-Vidal H, Llama-Palacios A, Figuero E, Herrera D, Sanz M. (2017) Comparative gene expression analysis of *Porphyromonas gingivalis* ATCC 33277 in planktonic and biofilms states. PLoS ONE 12(4): e0174669. https://doi.org/10.1371/journal.pone.0174669.**

## RESUMEN

**Antecedentes y objetivo:** *Porphyromonas gingivalis* es un patógeno clave en el inicio y la progresión de la periodontitis. Su patogenicidad se ha relacionado con su presencia y supervivencia dentro del *biofilm* subgingival. El objetivo del presente estudio fue comparar las actividades transcriptómicas de todo el genoma de *P. gingivalis* en *biofilm* y en crecimiento planctónico, utilizando la tecnología de *microarrays*.

**Material y métodos:** *P. gingivalis* ATCC 33277 se incubó en placas de cultivo multipocillo a 37 °C durante 96 horas bajo condiciones anaeróbicas usando un modelo estático *in vitro* para desarrollar tanto el estado planctónico como el de *biofilm* monoespecie sobre discos cerámicos estériles de hidroxiapatita cálcica (HA). El desarrollo del *biofilm* fue comprobado por microscopía láser confocal de barrido (CLSM) y microscopía electrónica de barrido (SEM). Después de la incubación, se recogieron las células bacterianas y se extrajo y purificó el ARN total. Tres réplicas biológicas para cada estado celular fueron hibridadas independientemente para comparaciones transcriptómicas. Se utilizó un modelo lineal para determinar genes expresados diferencialmente y la reacción en cadena de la polimerasa con transcriptasa inversa (RT-qPCR) para confirmar la expresión diferencial. Como criterios de filtro se seleccionaron valores de cambio en la expresión de genes $\leq -2$ ó $\geq 2$ veces y valores significativos de $p < 0,05$.

**Resultados:** Un 4,8% de los genes (92 de 1.909 genes) fueron expresados diferencialmente por *P. gingivalis* en crecimiento en *biofilm* comparado con el fenotipo planctónico. De ellos, 54 genes fueron sobreexpresados en el *biofilm*, principalmente relacionados con la envoltura celular, el transporte, unión o proteínas de las membranas externas.

Y 38 genes mostraron expresión disminuida, principalmente relacionados con transposasas o estrés oxidativo.

**Conclusión:** La respuesta adaptativa de *P. gingivalis* a crecer en fenotipo de *biofilm* monoespecie demostró un aumento de la expresión génica diferencial con respecto a su crecimiento en forma planctónica.

PLOS ONE

RESEARCH ARTICLE

# Comparative gene expression analysis of *Porphyromonas gingivalis* ATCC 33277 in planktonic and biofilms states

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**Citation:** Romero-Lastra P, Sánchez M., Ribeiro-Vidal H, Llama-Palacios A, Figuero E, Herrera D, et al. (2017) Comparative gene expression analysis of *Porphyromonas gingivalis* ATCC 33277 in planktonic and biofilms states. PLoS ONE 12(4): e0174669. https://doi.org/10.1371/journal.pone.0174669

**Editor:** Roy Martin Roop, II, East Carolina University Brody School of Medicine, UNITED STATES

**Received:** December 28, 2016

**Accepted:** March 13, 2017

**Published:** April 3, 2017



**Data Availability Statement:** Gene expression data can be found at the Gene Expression Omnibus (GEO) repository using the accession GSE96756. Please see GEO data at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE96756.

**Funding:** The authors received no specific funding for this work.

## Abstract

### Background and objective

*Porphyromonas gingivalis* is a keystone pathogen in the onset and progression of periodontitis. Its pathogenicity has been related to its presence and survival within the subgingival biofilm. The aim of the present study was to compare the genome-wide transcription activities of *P. gingivalis* in biofilm and in planktonic growth, using microarray technology.

### Material and methods

*P. gingivalis* ATCC 33277 was incubated in multi-well culture plates at 37˚C for 96 hours under anaerobic conditions using an *in vitro* static model to develop both the planktonic and biofilm states (the latter over sterile ceramic calcium hydroxyapatite discs). The biofilm development was monitored by Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM). After incubation, the bacterial cells were harvested and total RNA was extracted and purified. Three biological replicates for each cell state were independently hybridized for transcriptomic comparisons. A linear model was used for determining differentially expressed genes and reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to confirm differential expression. The filtering criteria of $\geq$ ±2 change in gene expression and significance p-values of <0.05 were selected.

### Results

A total of 92 out of 1,909 genes (4.8%) were differentially expressed by *P. gingivalis* growing in biofilm compared to planktonic. The 54 up-regulated genes in biofilm growth were mainly related to cell envelope, transport, and binding or outer membranes proteins. Thirty-eight showed decreased expression, mainly genes related to transposases or oxidative stress.

**Competing interests:** The authors have declared that no competing interests exist.


## Conclusion

The adaptive response of *P. gingivalis* in biofilm growth demonstrated a differential gene expression.


## Introduction

Human dental plaque is a complex and dynamic biofilm attached to tooth surfaces, where microbial communities are embedded in a matrix of bacterial extracellular polymeric substances (EPS), proteins, salivary peptides and food scraps [1, 2]. The differential activity of these microbial communities within the dental biofilm may have profound implications in the onset and progression of periodontitis, one of the most prevalent chronic inflammatory diseases affecting humans [3]. *Porphyromonas gingivalis*, a Gram-negative and black-pigmented anaerobic bacterium is one of the keystone pathogens associated with the etiology of periodontitis. Its main ecological niche is the oral microbiome [4] and its pathogenic activity has been directly related to its relative high numbers and proportions within the subgingival biofilm, as well as the expression of virulence factors that facilitate its colonization within the periodontal tissues and its resistance from the host inflammatory and immune responses. [5–7].

Virulence factors in periodontal pathogens have been attributed to either presence of highly pathogenic strains or to the up- and down- regulation of a number of genes due to the specific ecological conditions of the bacterial communities within the biofilm. In fact, several transcriptomic studies have been conducted to elucidate the behavior of different pathogenic bacteria growing in biofilm [8–11]. Whiteley *et al.* [10] reported that about 1% of the genes from *Pseudomonas aeruginosa* had shown differential expression when growing in biofilm compared with planktonic. Liu *et al.* [9] reported that 16.2% of the genes from *Clostridium acetobutylicum* were differentially expressed in biofilm growth, mainly up-regulation of genes involved in amino acid biosynthesis, sporulation, extracellular polymer degradation and other various metabolic processes, what indicated that *C. acetobutylicum* had a distinct phenotype when growing in a biofilm.

Similarly, transcriptomic studies have reported that approximately 18.0% of the W50 genome of *P. gingivalis* was differentially expressed in biofilms [8]. These studies have shown down-regulation of genes encoding for cell envelope biogenesis, DNA replication, energy production and biosynthesis of co-factors and up- regulation of genes involved in transport and binding proteins. Some of these studies have focused specifically on LuxS-dependent signaling and quorum-sensing-regulated genes since they play an important role in the physiology of these micro-organisms, their communication with other bacteria, and their adaptation to the biofilm environment [12–14]. Yamamoto *et al.* [15] reported that an increase of more than 1.5-fold in the number *P. gingivalis* (ATCC 33277) genes differentially regulated during the biofilm growth (312/2,090 genes, 155 genes were up-regulated and 157 genes were down-regulated).

In spite of these studies, our understanding of the regulatory processes and interactions, which allow *P. gingivalis* to grow within the biofilm and to develop its virulence is still limited. It is, therefore, the aim of this study to assess the differential expression of *P. gingivalis* genes under two different physiological states, planktonic and biofilm growth, using transcriptomic analysis in an *in vitro* static model.

## Material and methods

### Bacterial strain

Standard reference strain *P. gingivalis* ATCC 33277 was selected for the present study. Bacteria were grown on blood agar plates (Blood Agar Oxoid No 2; Oxoid, Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5.0 mg/L hemin (Sigma, St. Louis, MO, USA) and 1.0 mg/L menadione (Merck, Darmstadt, Germany) in anaerobic conditions (10% $H_2$, 10% $CO_2$, and balance $N_2$) at 37˚C for 72 hours.

### Bacterial growth and experimental assays

Planktonic cultures of *P. gingivalis* were grown anaerobically at 37˚C for 24 h in a protein-rich medium containing brain-heart infusion (BHI) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 2.5 g/L mucin (Oxoid), 1.0 g/L yeast extract (Oxoid), 0.1 g/L cysteine (Sigma), 2.0 g/L sodium bicarbonate (Merck), 5.0 mg/L hemin (Sigma), 1.0 mg/L menadione (Merck) and 0.25% (v/v) glutamic acid (Sigma). Upon reaching late-exponential phase [$10^9$ colony forming units (CFU)/mL, as measured spectrophotometrically by optical density at 550 nm], the cells were diluted in modified BHI medium to obtain a final concentration of $10^8$ CFU/mL.

In order to study the gene expression of *P. gingivalis*, in biofilm or planktonic growth, under the same culture conditions, a volume of 1.5 mL of *P. gingivalis* inoculums was placed in pre-sterilized polystyrene 24-well tissue culture plates (Greiner Bio-one, Frickenhausen, Germany) with or without the presence of sterile ceramic calcium hydroxyapatite discs (HA) [7-mm diameter (standard deviation, SD = 0.2) and 1.8 mm thickness] (Clarkson Chromatography Products, Williamsport, PA, USA). To carry out the experiment, a total of 45 multiwell plates were used. In each plate 19 wells were filled with disk to develop the biofilms (each of the aggregates in each hydroxyapatite disk is considered as a biofilm) and the other five wells were used to analyze the planktonic state without hydroxyapatite disk.

Plates were incubated in anaerobic conditions at 37˚C for 96 h. Wells containing only culture medium were also incubated to verify sterility and the possible contamination of bacteria growing in both planktonic and biofilm growth was frequently checked.

### Confocal Laser Scanning Microscopy (CLSM) analysis to monitor *P. gingivalis* biofilm development

To ensure the change of *P. gingivalis* phenotype, from planktonic to biofilm, its growth was studied by CLSM when the biofilm reached a mature state (from 24 to 96 h). To confirm the reproducibility of the biofilm-growth, three independent experiments using trios of biofilms were carried out for each incubation time (a concentration of $10^8$ CFU/mL *P. gingivalis* cells in planktonic culture were placed on sterile hydroxyapatite discs). Before the CLSM analysis, the discs were rinsed in 2 mL of sterile Buffer Phosphate Saline (PBS) three times (10 sec of immersion time per rinse), in order to remove non-adherent bacteria. Non-invasive confocal imaging of fully hydrated biofilms was carried out using a fixed-stage Ix83 Olympus inverted microscope coupled to an Olympus FV1200 confocal system and with a ×63 water-immersion lens (Olympus; Shinjuku, Tokio, Japan). Specimens were stained with LIVE/DEAD® BacLightTM Bacterial Viability Kit solution (Molecular Probes B. V., Leiden, The Netherlands) at room temperature. A 1:1 fluorocromes ratio and 9±1 min of staining time was used to obtain the optimum fluorescence signal at the corresponding wave lengths (Syto9: 515–530 nm; PI: >600 nm). At least three separate and representative locations on the HA discs covered with biofilm were selected for the study. The CLSM control software was set to take a

z-series of scans (xyz) of 0.5 μm thickness (8 bits, 1024x1024 pixels). Image stacks were analyzed with the proprietary Olympus® software (Olympus).

## Scanning Electron Microscopy (SEM) analysis

Before SEM analysis, three hydroxyapatite discs covered with biofilms grown *in vitro* for 96 h were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde for 4h at 4˚C. Then, the discs were washed twice in PBS and sterile water (immersion time 10 min) and then, dehydrated through a series of graded ethanol solutions (50, 60, 70, 80, 90 and 100%; immersion time per series, 10 min), Then, the samples were critical point dried, sputter-coated with gold and analysed with an scanning electron microscope JSM 6400 (JSM6400; JEOL, Tokyo, Japan) equipped with back-scattered electron detector and with an image resolution of 25 KV.

## Harvesting of planktonic and biofilm cells for gene expression analysis

After 96 h of incubation, *P. gingivalis* planktonic and biofilm cells were harvested (three biological replicates of each state) for independent hybridization.

For planktonic cells 1 mL was recovered from 15 diskless well. In the same experiments a set of 300 biofilms were harvested independently, then added to 1 mL of sterile PBS, disaggregated by vortexing during 3 min. In both cases the samples were recovered as partial plucks by centrifugation at 9,000 rpm at 4˚C during 5 min, in order to obtain a final 10 μg of total RNA for each replicate in each state. To preserve the bacterial total RNA intact during the time taken for the procedures, the work has always been in cold conditions.

In all cases, after the incubation period, an aliquot of each sample and 1 to 3 discs were used as quality control. They were cultivated on supplemented blood agar plates under anaerobic conditions at 37˚C during two weeks to assure the absence of contamination.

## Total RNA extraction

Total RNA was extracted from the harvested samples using the TRIzol® Max Bacterial RNA Isolation Kit (Ambion, Life Technologies, Carlsbad, CA, USA). Briefly, pools from planktonic and biofilm growth samples were suspended in 200 μL of preheated Max bacterial Reagent® (Ambion), incubated at 95˚C for 4 min and then chilled on ice for 10 min. After, 1 mL of TRIzol® reagent (Ambion) was added to lysate the cells, incubating them at room temperature 5 min. After that, 200 μL of cold chloroform was added and incubated at room temperature for 3 min. The mixtures were then centrifuged at 13,000 rpm for 15 min at 4˚C. RNA colourless aqueous phase (~ 600 μL) was collected, augmented with 0.5 mL of cold isopropanol, mixed by inversion, and incubated at room temperature for 10 min. After centrifugation at 13,000 rpm for 10 min at 4˚C, the pellet of RNA was suspended in 1 mL of cold 75% ethanol, centrifuged at 9,000 rpm for 5 min, air-dried and suspended in 50 μL of RNase-free water (Roche Diagnostics, Mannheim, Germany). The samples were then treated with DNase I (Ambion, NY, USA) to remove any contaminating DNA (set of RNase-free DNase; Qiagen, CA, USA) and purified using columns of RNeasy Mini kit (Qiagen) according to the manufacturer's protocol.

RNA quantity was measured by NanoDrop ND1000 spectrophotometer (NanoDrop-Technologies; Thermo Scientific™, LLC, Wilmington, DE, USA). RNA quality was monitored by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All the samples used in this study exhibited an A260/A280 ratio of at least 2.0.

## cDNA synthesis and transcriptomic analysis

Three biological replicates were independently hybridized for each transcriptomic comparison. Fluorescently labeled cDNA for microarray hybridizations was obtained by using the SuperScript Indirect cDNA Labeling System (Invitrogen). In brief, 5 μg of total RNA was transformed to cDNA with Superscript III reverse transcriptase using random hexamers as primers and with aminoallyl-modified nucleotides in the reaction mixture. After cDNA purification, the Cy3 fluorescent dyes (Amersham Biosciences) were coupled to the amino-modified first-strand cDNA. Labelling efficiency was assessed using a NanoDrop ND1000 spectrophotometer (NanoDropTechnologies).

Preparation of probes and hybridization was performed as described (One-Color Microarray Based Gene Expression Analysis Manual Ver. 6.5, Agilent Technologies). Briefly, for each hybridization, 600 ng of Cy3 probes were mixed and added to 5 μL of 10x Blocking Agent and Nuclease free water in a 25 μL reaction. Then, 25 μL from 2x GExHybridization buffer was added and mixed carefully. The samples were placed on ice and quickly loaded onto arrays, hybridized at 65˚C for 17 h and then washed once in GE wash buffer 1 at room temperature (1 min) and once in GE Wash Buffer 2 at 37˚C (1 min).

Slides corresponded to Agilent *P. gingivalis* Oligo Microarrays 8x15K (074976), a genome annotation specific for strain ATCC 33277 and W83. For each culture pair, three technical replicates of array hybridizations were performed.

## Microarray and data analysis

Images from Cy3 channel were equilibrated and captured with a high-resolution scanner (Agilent) and spots quantified using Feature Extraction software (Agilent). Background correction and normalization of data expression were performed using LIMMA [16, 17]. LIMMA is part of bioconductor, an R language project [18]. For local background correction and normalization, the methods "normexp" and loess in LIMMA were used, respectively [16]. To ensure similar distribution across arrays and to achieve consistency among arrays, log-ratio values were scaled using the median-absolute-value as scale estimator [17].

Linear model methods were used for determining differentially expressed genes. Each probe was tested for changes in expression over replicates by using an empirical Bayes moderated t-statistic [17]. To control the false discovery rate *p-values* were corrected by using the method of Benjamani and Hochberg [16, 17]. The expected false discovery rate was controlled to be less than 5% and a filtering criterium of increase/decrease up to 2-fold differential expression between states was selected.

The National Center for Biotechnology (Genomics Unit) at Universidad Autónoma, Madrid (Spain) performed the hybridizations and statistical analysis.

## Assessment of microarray data by Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

To confirm the microarray results using RT-qPCR, nine genes differentially expressed between both situations were selected, four genes from the up-regulated group and five from the down-regulated one. Specific primers were designed using the Universal Probe Library Roche software tool (Roche Diagnostics) (Table 1). All quantifications were normalized to the *P. gingivalis* 16S rRNA gene.

To carry out the Reverse Transcription-qPCR, cDNA was generated from 1 μg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, ThermoFisher Scientific) in a 10 μL of final reaction volume. After that, quantitative PCR reactions were performed

**Table 1. Primers used for Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR).**

| LOCUS NAME | PUTATIVE IDENTIFICATION | PRIMER SEQUENCES |
|---|---|---|
| **porP** | Porins | Forward 5´-3´: GGGTAGTGACCGAAACGAGA |
| | | Backward 5´-3´: GAAGGCATATTGCCCCATC |
| **PGN 0319** | Probable RNA polymerase sigma-70 factor ECF subfamily | Forward 5´-3´: CGTCTGGTGGAAGCTGCTAT |
| | | Backward 5´-3´: CAGCCGGAAAGTCATTCG |
| **PG 0215** | Hypothetical protein | Forward 5´-3´: GCCTTCGATGCTGTATCCAT |
| | | Backward 5´-3´: TCAAAGGTCGAAAAGCTCCT |
| **PGN 0320** | Hypothetical protein | Forward 5´-3´: GCCTTCGATGCTGTATCCAT |
| | | Backward 5´-3´: TCAAAAGGTCGAAAAGCTCCT |
| **PG 2130** | Hypothetical protein | Forward 5´-3´: TTCGAATGTGCCAAGTGC |
| | | Backward 5´-3´: TCGTCACACCGAAGTAGTCG |
| **PGN 0575** | Transposase in ISPg1 | Forward 5´-3´: AGACAATCGGAGCGAGGAG |
| | | Backward 5´-3´: TTTACGCYGACGGACAACCT |
| **PGN 1925-Cas1** | Mobile and extrachromosomal element functions | Forward 5´-3´: GAGCCTCTCTCCAACGCTATC |
| | | Backward 5´-3´: GCCCTCCGCTATGGGTAT |
| **PG 0619** | Alkyl hydroperoxide reductase, F subunit | Forward 5´-3´: CTGCAGCCATCYATTCTGCTC |
| | | Backward 5´-3´: CTACCCGTTCGGCTACGAT |
| **vimF** | Virulence modulating gene F | Forward 5´-3´: CCGAAATTCTCCGCCATAG |
| | | Backward 5´-3´: CTCCGGGCTTCTCTGTGTT |

https://doi.org/10.1371/journal.pone.0174669.t001

in triplicate by using 5 μL per well of each cDNA, and 3 μL of a mix composed by 0.4 μM of each primer, 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX), and nuclease-free water, to reach a final volume of 8 μL in 384-well optical plates. PCR reactions were run in an Applied Biosystems ABI PRISM 7900HT machine with SDS v2.4 software and standard protocol from Applied Biosystems (95˚C 10 min, 40 cycles of 95˚C 15 sec and 60˚C 60 sec, and a final standard dissociation protocol). The results were analysed with the Comparative Ct Method (ΔΔCt) [19].

## Results

CLSM and SEM confirmed that *P. gingivalis* ATCC 33277 changed its phenotypic state, from planktonic to a mono-species biofilm. Fig 1 shows representative CLSM (depicting viable bacteria as green and nonviable as red stained cells.) and SEM images of the obtained biofilms at 96 h of incubation,

With the use of the filtering criteria threshold of two-fold change in differential expression (up or down) of the contained in *P. gingivalis* ATCC 33277 arrays, a total of 92 out of 1,909 (4.8%) genes were differentially expressed in the biofilm phenotype compared to planktonic growth. These differences were statistically significant ($p<0.05$).

Fig 2 shows the genes differentially expressed in *P. gingivalis* ATCC 33277 biofilms compared to planktonic cells. From the identified genes, the 54 up-regulated genes in the biofilm were mainly related to cell envelope, transport and binding proteins, outer membranes proteins, DNA repair enzymes, ribosomal proteins, or genes related to transcription initiation. Conversely, the 38 genes that were down-regulated in biofilm cells were mostly genes encoding proteins related to transposases, the CRISPRs system (cluster regularly inter-spaced short palindromic repeats) or oxidative stress.

In Table 2, these genes are grouped by functional categories, such as the genes encoding for the cationic outer membrane proteins (OmpH-1 and OmpH-2, PG_0448, and PG_0987), which have shown up-regulated expression in this model of *P. gingivalis* biofilm. These genes

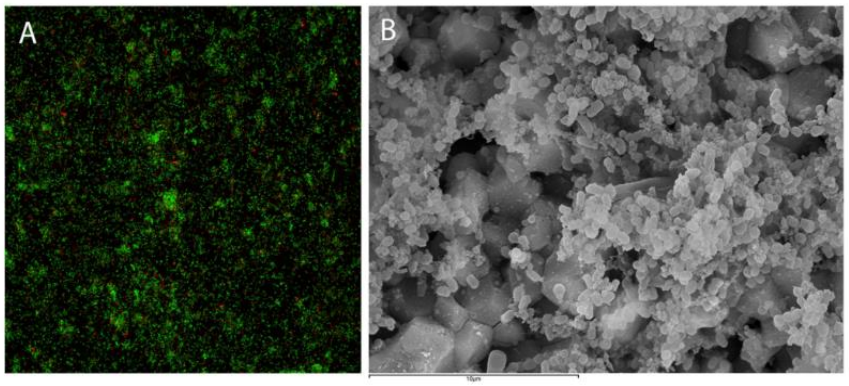


**Fig 1. Representative confocal (A) and scanning electron (B) micrographs representing *Porphyromonas gingivalis* ATCC 33277 biofilm after 96h of growth.** BacLight Live/Dead strain was used to assess the viability of cells in CLSM distinguishing viable bacteria depicted as green and non-viable as red stained cells.

https://doi.org/10.1371/journal.pone.0174669.g001

codifying proteins located specifically in the outer membrane vesicles, have been recognized as important virulence factors of *P. gingivalis*. Moreover, the gene coding lipoprotein PGN_0151 appeared up regulated by a factor of 3.16 (SD 0.28) compared to planktonic state (Table 2). Similarly, the genes related with the Por Secretion System (PorSS) (*porP*, PGN_1514 and PG_0448), involved in the biosynthesis of cell surface polysaccharides and implicated in the translocation of gingipains were up-regulated in biofilm growth. These proteins are well known virulence factors and serve as anchors for Rgp, Kgp, hemagglutinins, and the hemoglobin receptor protein. Only one gene, implicated in predicted exporter proteins (PGN_0946) was found significantly down regulated.

An additional group of genes related to oxidative stress and metabolism was differentially expressed in *P. gingivalis*, as shown in Table 2. This group of genes, represented by PGN_2076 and PG_2213, are involved in oxidative and/or regulatory mechanisms, as Nitric oxide (NO) stress resistance and were significantly suppressed in biofilm growth. These genes enable bacteria to survive within the inflammatory microenvironment of the periodontal pocket. Similarly, alkyl hydroperoxidase reductase subunits genes (AhpC-F (PG_0618, PGN_0660, PG_0619 and PGN_0661) were down regulated in *P. gingivalis* biofilms. These genes are involved in the primary defense against reactive oxygen species (ROS), and therefore affecting the bacterium aero-tolerance. In fact, PG_0619 was the gene most differentially suppressed (-13.13 (SD 0.70)). On the other hand, the putative genes related to metabolism NADPH-NAD transhydrogenases (PGN_1120, PGN_1122 and pntB) were up-regulated.

The genes involved in transposon functions, demonstrated heterogeneous results (Table 2). While genes corresponding to partial transposase in IS*Pg1* (PGN_0219, PGN_0575, PGN 1216 and PGN_1420) and PGN_0579 were down-regulated, genes belonging to the partial transposase in IS*Pg4* (PGN_0478) and PGN_0578 were up-regulated.

Genes related to the CRISPRs and associated CAS proteins system (CRISPR/Cas), like (PGN_1924-Cas2, PGN_1925-Cas1) were down-regulated in biofilm growth, while the gene PGN_1286, thought to be a lysozyme, was up regulated.

Among the genes related to fimbriae, only one gene, *fimD*, one of the minor components of the fimbriae A, appeared down-regulated by a factor of -2.30 (SD 0.26) in biofilm *versus* planktonic cells.

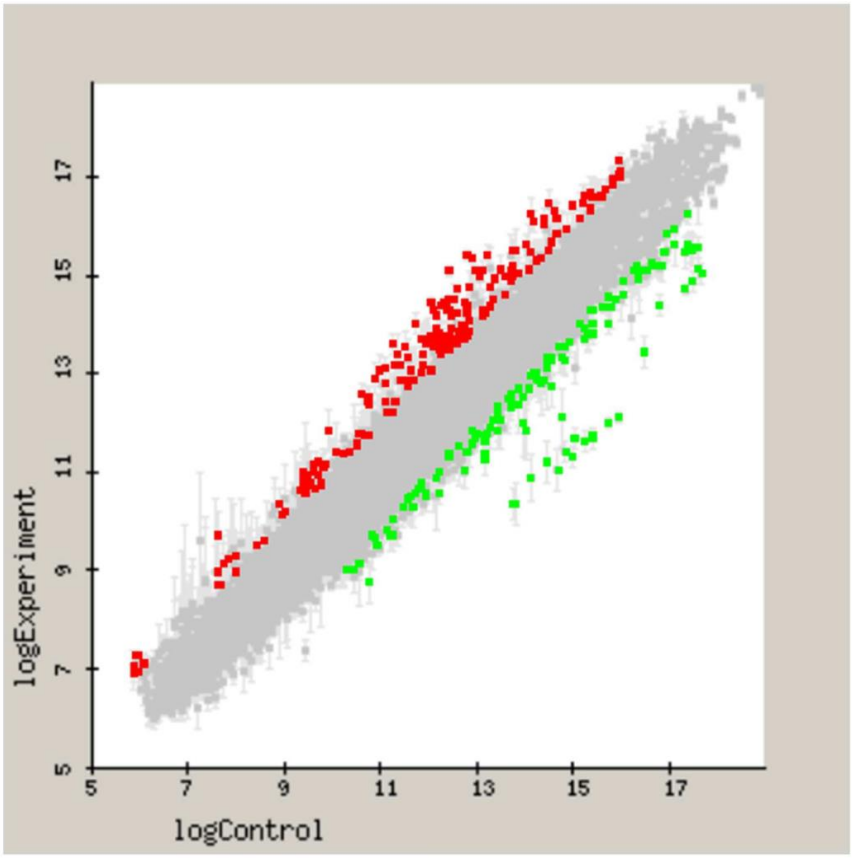


**Fig 2. Differential gene expression in *Porphyromonas gingivalis* ATCC 33277 biofilm as opposed to planktonic cells.** Differentially expressed genes with 2.0 fold change (up or down) and p-value < 0.05 were plotted. X-axis presents fold difference between log expression of planktonic, and y-axis shows the log expression of biofilm. Up-regulated genes (over-expressed in biofilm) were represented as red color and down-regulated genes were colored in green.

https://doi.org/10.1371/journal.pone.0174669.g002

Among the genes involved in the biogenesis of components of ribosomal subunits, the genes *rpmH*, *rpsF* and *rpII* were up-regulated while *KsgA* were down-regulated when in comparing biofilm with planktonic growth.

The array data (Table 2) indicated that several RNA polymerase sigma factors of the σ70 family (PG_0214, PG_0985, PGN_0319, PGN_0450, PGN_0970), involved in the regulation of biofilm formation and diverse physiological processes, particularly virulence, were up-regulated in biofilm *versus* planktonic cells. On the other hand, PGN_0082, a probable transcriptional regulator in the AraC family, was down-regulated in biofilms cells.

The riboflavin-related gene encoded to the 3,4-dihydroxy-2-butanone 4-phosphate synthase/ GTP cyclohydrolase II protein (PGN_0643) was found up-regulated in *P. gingivalis* biofilm. This gene has been implicated in quorum sensing signaling and extracellular electron transfer. On the contrary, the gene VimF was down-regulated. This gene has been involved in the maturation/activation/anchorage of gingipains and other virulence factors of *P. gingivalis*.

**Table 2. Genes differently expressed in *Porphyromonas gingivalis* ATCC 33277 biofilm (cutoff ratio ≥ ±2.0 fold change, p-value < 0.05) for the microarray analysis, grouped by functional role categories.**

| LOCUS NAME | PUTATIVE IDENTIFICATION[A] | AVG RELATIVE FOLD CHANGE (SD)[B] |
|---|---|---|
| **1. GENES RELATED TO CELL ENVELOPE** | | |
| **ompH-1** | Cationic outer membrane protein OmpH | 2.38 (0.37) |
| **ompH-2** | Cationic outer membrane protein OmpH | 2.23 (0.09) |
| **PG 0987** | | 2.85 (0.12) |
| **PGN 0301** | | 2.17 (0.08) |
| **PGN 0968** | | 3.29 (0.12) |
| **PGN 0151** | Lipoprotein | 3.16 (0.28) |
| **PGN 0946** | Predicted exporter protein | -2.29 (0.23) |
| ***porP*** | Porins | 2.43 (0.27) |
| **PGN 1514** | Conserved hypothetical proteinporins | 2.09 (0.09) |
| **PG 0448** | Porins | 2.54 (0.35) |
| **2. GENES RELATED TO OXIDATIVE STRESS AND METABOLISM** | | |
| **PGN 2076** | Bacterioferritin-associated ferredoxin proteins | -2.46 (0.33) |
| **PG 2213** | Bacterioferritin-associated ferredoxin proteins | -3.50 (0.39) |
| **PG 2029** | Metalloprotease | 2.22 (0.16) |
| **PGN 1120** | Putative NADPH-NAD transhydrogenase | 2.26 (0.05) |
| **PGN 1122** | NADPH-NAD transhydrogenase beta subunit | 2.43 (0.30) |
| ***pntB*** | NAD(P) transhydrogenase, beta subunit | 2.35 (0.28) |
| **PG 0618** | Alkyl hydroperoxide reductase, C subunit | -5.52 (1.98) |
| **PGN 0660** | Putative alkyl hydroperoxide reductase C subunit | -4.93 (1.21) |
| **PG 0619** | Alkyl hydroperoxide reductase, F subunit | -13.13 (0.70) |
| **PGN 0661** | Alkyl hydroperoxide reductase F subunit | -11.59 (1.16) |
| **3. GENES RELATED TO TRANSPOSON FUNCTIONS** | | |
| **PGN 0219** | Partial transposase in ISPg1 | -2.78 (0.29) |
| **PGN 0575** | Transposase in ISPg1 | -2.50 (0.35) |
| **PGN 1216** | Transposase in ISPg1 | -2.43 (0.26) |
| **PGN 1420** | Transposase in ISPg1 | -2.47 (0.16) |
| **PGN 0478** | Partial transposase in ISPg4 | 2.16 (0.14) |
| **PGN 0578** | Conserved hypothetical protein found in conjugate transposon | 2.12 (0.14) |
| **PGN 0579** | Conserved hypothetical protein found in conjugate transposonTra related domains | -2.83 (0.55) |
| **4. GENES RELATED TO CRISPR** | | |
| **PGN 1924-Cas2** | Mobile and extrachromosomal element functions | -2.10 (0.06) |
| **PGN 1925-Cas1** | Mobile and extrachromosomal element functions | -2.53 (0.42) |
| **5. GENES RELATED TO LYSOZYMES** | | |
| **PGN 1286** | Probable lysozyme | 2.63(0.28) |
| **6. GENES RELATED TO FIMBRIA** | | |
| ***fimD*** | Minor component FimD | -2.30 (0.26) |
| **7. GENES RELATED TO RIBOSOME** | | |
| ***rpmHrpmH*** | 50S ribosomal protein L34 ATCCRibosomal protein L34 W83 | 2.41 (0.26) 2.47 (0.34) |
| ***rpsF*** | 30S ribosomal protein S6 | 2.32 (0.22) |
| ***rplI*** | 50S ribosomal protein L9 | 2.18 (0.09) |
| ***KsgA*** | Dimethyladenosine transferase | -2.24 (0.06) |
| **8. GENES RELATED TO TRANSCRIPTION INITIATION RNA POLYMERASE SIGMA-70 FACTOR, ECF SUBFAMILY** | | |
| **PG 0214** | RNA polymerase sigma-70 factor, ECF subfamily | 4.37 (0.18) |
| **PG 0985** | RNA polymerase sigma-70 factor, ECF subfamily | 3.81 (0.60) |
| **PGN 0319** | Probable RNA polymerase sigma-70 factor ECF subfamily | 5.50 (0.87) |

(*Continued*)

**Table 2.** (*Continued*)

| LOCUS NAME | PUTATIVE IDENTIFICATION[A] | AVG RELATIVE FOLD CHANGE (SD)[B] |
|---|---|---|
| **PGN 0450** | Putative RNA polymerase sigma-70 factor ECF subfamily | 2.88 (0.01) |
| **PGN 0970** | Putative RNA polymerase sigma-70 factor ECF subfamily | 3.19 (0.23) |
| **PGN 0082** | Probable transcriptional regulator AraC family | -2.37 (0.33) |
| **9. GENES RELATED TO RIBONUCLEOSIDE TRIPHOSPHATE REDUCTASE** | | |
| **PG 1260** | Anaerobic ribonucleoside triphosphate reductase | -2.54 (0.14) |
| **PGN 1396** | Anaerobic ribonucleoside triphosphate reductase | -2.28 (0.28) |
| **10. GENES RELATED TO RIBOFLAVIN** | | |
| **PGN 0643** | 3,4-dihydroxy-2-butanone 4-phosphate synthase | 2.11(0.10) |
| **11. OTHER** | | |
| ***ung*** | Uracil-DNA glycosylase | 2.11 (0.06) ((0.06) |
| ***vimF*** | Virulence modulating gene F | -2.36 (0.25) |
| **PGN 1914** | Carboxyl-terminal processing protease | 2.74 (0.23) |
| **PGN 1156** | Glycerol-3-phosphate dehydrogenase | -2.19 (0.06) |
| **PGN 0906** | Probable dihydoorate dehydrogenase electron transfer subunit | -2.26 (0.30) |
| **12. GENES RELATED TO HYPOTHETICAL PROTEIN** | | |
| **PG 0100** | Hypothetical protein | 2.78 (0.27) |
| **PG 0161** | Hypothetical protein | 2.50 (0.49) |
| **PG 0215** | Hypothetical protein | 4.72 (0.60) |
| **PG 0216** | Hypothetical protein | 3.67 (1.14) |
| **PG 0217** | Hypothetical protein | 3.01 (0.37) |
| **PG 0218** | Hypothetical protein | 2.82 (0.41) |
| **PG 0323** | Conserved hypothetical protein | 3.94 (0.45) |
| **PG 0606** | Hypothetical protein | 2.43 (0.20) |
| **PG 0621** | Conserved hypothetical protein | -2.60 (0.24) |
| **PG 0622** | Hypothetical protein | -2.39 (0.12) |
| **PG 0986** | Hypothetical protein | 2.99 (0.83) |
| **PG 1152** | Hypothetical protein | 3.28 (0.95) |
| **PG 1267** | Hypothetical protein | 2.46 (0.13) |
| **PG 1634** | Hypothetical protein | 2.58 (0.68) |
| **PG 1675** | Hypothetical protein | 2.55 (0.53) |
| **PG 1908** | Hypothetical protein | -2.08 (0.07) |
| **PG 2130** | Hypothetical protein | -2.50 (0.21) |
| **PG 2212** | Hypothetical protein | -9.49 (0.66) |
| **PG 2224** | Hypothetical proteinmembrane protein, putative | -2.84 (0.56) |
| **PGN 0052** | Hypothetical protein | 2.33 (0.29) |
| **PGN 0078** | Hypothetical protein | -2.47 (0.25) |
| **PGN 0178** | Conserved hypothetical protein | -2.42 (0.31) |
| **PGN 0320** | Conserved hypothetical protein | 4.11 (0.50) |
| **PGN 0321** | Conserved hypothetical protein | 3.66 (0.28) |
| **PGN 0322** | Conserved hypothetical protein | 3.31 (0.99) |
| **PGN 0323** | Conserved hypothetical protein | 3.81 (0.20) |
| **PGN 0332** | Conserved hypothetical protein | 2.33 (0.14) |
| **PGN 0486** | Conserved hypothetical protein | 2.28 (0.12) |
| **PGN 0588** | Conserved hypothetical protein | -2.49 (0.35) |
| **PGN 0663** | Conserved hypothetical protein | -2.72 (0.26) |
| **PGN 0664** | Conserved hypothetical protein | -2.51 (0.58) |
| **PGN 0797** | Conserved hypothetical protein | 2.15 (0.06) |

(*Continued*)

**Table 2.** (*Continued*)

| LOCUS NAME | PUTATIVE IDENTIFICATION[A] | AVG RELATIVE FOLD CHANGE (SD)[B] |
|---|---|---|
| **PGN 0837** | Conserved hypothetical protein | -2.30 (0.31) |
| **PGN 0907** | Conserved hypothetical protein | -2.91 (0.63) |
| **PGN 0969** | Conserved hypothetical protein | 2.87 (0.23) |
| **PGN 1083** | Hypothetical protein | 2.23 (0.09) |
| **PGN 1385** | Hypothetical protein | 2.24 (0.05) |
| **PGN 1400** | Conserved hypothetical protein | 2.65 (0.22) |
| **PGN 1639** | Conserved hypothetical protein | 3.48 (0.46) |
| **PGN 1992** | Conserved hypothetical protein | -2.24 (0.22) |
| **PGN 2087** | Conserved hypothetical protein | -2.29 (0.12) |
| **uvrAII** | Conserved hypothetical protein | -6.89 (2.97) |

[A] Putative identification from Genebank.

[B] Results of three biological replicates.

https://doi.org/10.1371/journal.pone.0174669.t002

Lastly, 45% of the 92 differentially regulated *P. gingivalis* genes were of unknown or poorly characterized functions, most of them encoding unknown proteins.

The microarray results were validated by RT-qPCR on four of the genes from the up-regulated group and five from the down-regulated group. Fig 3 illustrates the high correlation between the gene expression of logarithm-transformed of RT-qPCR plotted against the average $\log_2$ ratio values obtained by microarray analysis (R = 0.9716).

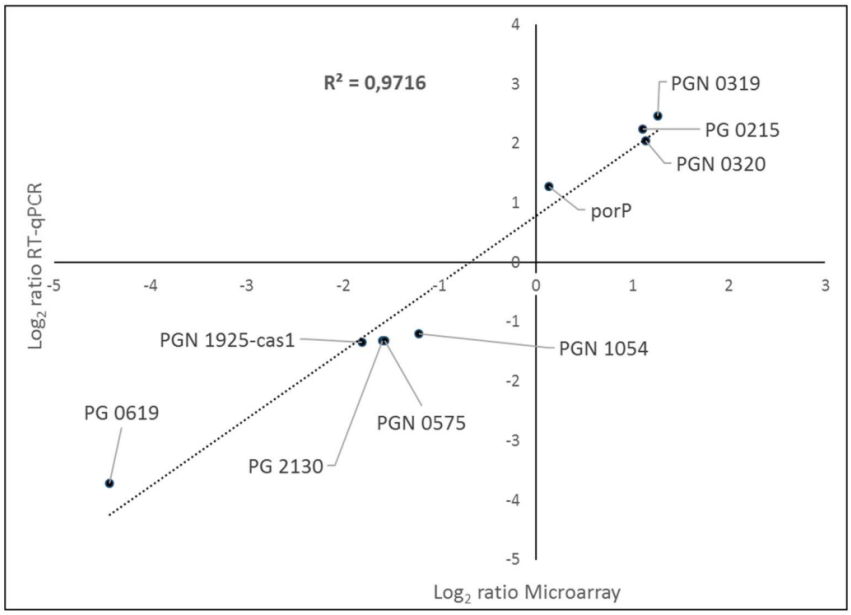


**Fig 3. Correlation between microarray and Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) gene expression ratios determined for biofilm *versus* planktonic cells.** The RT-qPCR $\log_2$ values were plotted against the microarray data $\log_2$ values ($R^2$ = 0.9716).

https://doi.org/10.1371/journal.pone.0174669.g003

## Discussion

This microarray-based comparative transcriptomic study has shown the up- and down- regulation of specific genes of *P. gingivalis* during the early stages of biofilm maturation (96 h of incubation). These gene expression patterns showed that 4.8% (92/1,909) of the genes of *P. gingivalis* significantly changed in biofilm, when compared to planktonic growth. Although this does not represent a huge difference between the two lifestyles [10, 20, 21], small changes in the level of expression of one gene can be amplified through regulatory networks and result in significant phenotypic alterations [22–24]. These results are in agreement with previous reports on other pathogens, such as *P. aeruginosa* or *Escherichia coli* grown under similar differential growth conditions, in which less of 5% of differential expression was demonstrated [10, 11, 25, 26].

When assessing the different functional categories affected by the differentially regulated genes, a wide diversity was observed, which may indicate that the adaptation of *P. gingivalis* to a community lifestyle required a broad-based transcriptional modulation. This adaptation involved different virulence factors, as proteins codifying for outer membrane proteins or for fimbriae. Outer membrane vesicles (OMVs) of *P. gingivalis*, which are formed by "blebbing" portions of their outer membrane, have been recognized as important virulence factors of this pathogen in relation to periodontitis [6]. These vesicles contained specific proteases, termed gingipains (Arg-gingipain [Rgp] and Lys-gingipain [Kgp]) [5] associated with the capacity of *P. gingivalis* to invade host epithelial cells [27, 28]. This transcriptomic study has revealed four genes, which codify proteins located in the OMVs of *P. gingivalis* being over-expressed (OmpH-1 and OmpH-2, PG_0448, and PG_0987). This finding was already described by Veith *et al.* (2014) [29]. Similarly, Kuboniwa *et al.* (2009) [30] using proteomic technology studied *P. gingivalis* in biofilm growth and reported significantly increased cell envelope proteins, such as OmpH protein PGN_0301, whose encoding gene has been shown over expressed in this investigation.

The up-regulation of these proteins in biofilm *versus* planktonic state has also been reported in others studies demonstrating that OMVs and related genes play an important role in bacterial co-aggregation [31] and attachment to epithelial cells [32]. Although differential expression of genes has been shown at *in vivo* polymicrobial biofilms (Díaz and Kolenbrander [33], this study has confirmed that up-regulation could also occur when growing in an *in vitro* mono-species *P. gingivalis* biofilm.

Fimbriae of *P. gingivalis* have also been recognized as a major virulence factor, since they mediate in cell adhesion and may facilitate their capacity to invade periodontal tissues [34–38]. Only one gene, *fim D*, was found down-regulated in this study. This gene is a minor component of a seven gene cluster, *fimX*, *pgmA and fim ABCDE*, which encode type 1 fimbriae, and it is characterized by mannose-sensitive hemagglutination and being assembled via the chaperone/usher pathway [39, 40]. These genes participate in the biogenesis of the fimbriae, regulating their number and length, as well as their adherence function [41, 42]. Nevertheless, Krogfelt and Klemm (1988) observed that a clone of *E. coli*, not containing the genes encoding the minor component proteins, still produced fimbriae consisting of pure Fim A protein, (main structural component of the fimbriae type I), indicating that, at least in the case of *E. coli*, the minor components were not necessary for the structural integrity of the fimbriae, although these fimbriae were non-adhesive and did not confer hemaglutination [42–45]. Similarly, Whiteley *et al.* (2001) suggested that these appendages may not be required at the later stages of biofilm formation for maintenance of a mature biofilm, since fimbria, pili or flagella were only involved at initial steps of attachment [10, 15].

The lipoprotein-related gene PGN_0151 was over-expressed in biofilm. Hirano *et al*. (2013) reported that a mutant of this gene was reduced in its ability to form biofilms compared to wild type [46] what suggests that these genes were significantly involved in the biofilm lifestyles of *P.gingivalis*. In regards to those genes involved in the adaptation to new local environmental conditions, this investigation showed a differential expression of those genes involved in the transposition system (PGN_0219, PGN_0575, PGN 1216, PGN_1420, PGN_0579, PGN_0478 and PGN_0578), some of them codifying insertion sequences (IS). Since transposition is generally known to be triggered by cellular stress [47–49], this finding suggests that these transposable elements, moving from one site within the genome to another, could have an important role in the genomic re-arrangement and recombination in *P. gingivalis* growing in biofilm. This adaptation to stressful local environmental conditions has been previously reported [7, 50–53]. Furthermore, the CRISPR-Cas and associated CAS proteins system represents a unique system that provides prokaryotic cells, as *P. gingivalis*, adaption and protection from host defenses [54, 55]. Down-regulation of the genes PGN_1924-Cas2, PGN_1925-Cas1 may suggest a decrease in the defensive capability of *P. gingivalis* ATCC 33277 when growing as single-species biofilm *in vitro* or its adaptation to an environment without competing species.

Gene PG_2213, encoding a putative nitrite reductase-related protein and implicated in nitric oxide (NO) stress resistance was repressed in *P. gingivalis* biofilm growth [56, 57]. The ability to down-regulate nitrite reduction [58], involves the expression of several genes known to be induced by nitrogen oxides and low oxygen tension [59, 60]. Whether *P. gingivalis* PG_2213 has a similar role is unknown. Boutrin et al. (2012) suggested that NO stress resistance in *P.gingivalis* was facilitated by a complex and tightly regulated network of genes involved in multiple pathways, including, energy metabolism, gene regulation, detoxification, and virulence [56].

Furthermore, although *P. gingivalis* seems to lack a protective NADH oxidase, Alkyl hydroperoxide reductase (genes PG_0618, PGN_0660, PG_0619 and PGN_0661), C subunit (AhpC), have been reported to be involved in *P. gingivalis* aero-tolerance processes. The up-regulation of genes related to NADPH-NAD transhydrogenases (PGN_1120, PGN_1122, *pntB*) suggests that *P. gingivalis* growing in biofilm has elevated metabolic activities, as shown with *C. acetobutylicum*, by Liu *et al*. (2016) [9]. In this investigation, several genes related to ribosome function (*rpmH*, *rpsF*, *rpII* and *KsgA)* were over expressed in the biofilm, what may indicate that the metabolic increase was associated to ribosome function, that may require up to 40% of the cell's energy in growing bacteria [52].

The observed differential up regulated expression of sigma factors in biofilm cells (PG_0214, PG_0985, PGN_0450, PGN_0970, PGN_0319) might indicate that these genes are important regulators of *P. gingivalis* during biofilm growth [8]. Similar results have been reported for *E.coli* [61]. Besides, members of the AraC family of transcriptional regulators (PGN_0082), with decreased expression in the biofilm, have been shown to be important in carbon metabolism (degradation of sugars such as arabinose), stress response to virulence in other species [62], and in the regulation of quorum sensing signaling in *P. aeruginosa* [63]. Also, related to quorum sensing signaling, the up regulated gene PGN_0643, has been involved in the biosynthesis of riboflavin, a substance associated in a number of extracellular processes by bacteria, especially Gram-negative organisms [64–66].

There are, however, important limitations associated to this study, since the biofilm used was an *in vitro* single-species model. The obtained results, however, may serve as a resource for future studies in oral biofilms aimed to further understand the genetic basis of the regulatory mechanisms of *P.gingivalis* and other pathogenic bacteria involved in subgingival biofilm growth and maturation.

## Conclusions

By means of transcriptomic analysis, this study has shown that 4.8% of the *P. gingivalis* ATCC 33277 genome exhibited differential expression profiles when grown in biofilm. In such biofilm growth, the up-regulated genes were mainly those related to the cell envelope, as the genes encoding for the cationic OMPs or gene PGN_0151, which appear as a novel *P. gingivalis* gene that seems to have a role in the biofilm state. Also, the genes implicated in PorSS system and RNA polymerase sigma factors of the σ70 family, which are genes related to virulence/proliferation factors were up-regulated. On the contrary, the expression of most of the genes involved in oxidative stress or CRISPRs system were suppressed.

Therefore the adaptive response of *P. gingivalis* in biofilm growth demonstrated changes in gene expression profiles.

## Acknowledgments

We thank G. García and L. Almonacid from the Centro Nacional de Biotecnología (Universidad Autónoma, Madrid, Spain) for hybridizations and statistical analysis of the microarrays and RT-qPCR performed by the Genomics Facility. We thank C. Pérez from the Centre of Microscopy and Cytometry from Universidad Complutense (Madrid, Spain). And also AM. Vicente for her technical microscopy services at the ICTS National Centre of Electron Microscopy, Universidad Complutense (Madrid, Spain). And finally we want to express our gratitude to Dr. Vanessa Blanc y Rubén León from the company DENTAID (Cerdanyola del Vallés, Barcelona, Spain).

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# ARTÍCULO 3

**Romero-Lastra P, Sánchez MC, Llama-Palacios A, Figuero E, Herrera D, Sanz M. (2019) Gene expression of *Porphyromonas gingivalis* ATCC 33277 when growing in an *in vitro* multispecies biofilm. PLoS ONE 14(8): e0221234. https://doi.org/10.1371/journal.pone.0221234.**

## RESUMEN

**Antecedentes y objetivo:** *Porphyromonas gingivalis* es un microorganismo oral que reside en el *biofilm* subgingival y puede ejercer diferente patogenicidad dependiendo de la presencia de factores de virulencia específicos, pero el estudio de su expresión génica al estar acompañada de otros microorganismos, no se ha establecido completamente. Esta investigación tiene como objetivo comparar el perfil transcriptómico de este patógeno cuando crece dentro de un *biofilm* multiespecie *in vitro* con respecto a su estado planctónico.

**Materiales y métodos:** *P. gingivalis* ATCC 33277 se cultivó en anaerobiosis en placas de cultivo multi-pocillo a 37ºC en dos condiciones: a) muestras planctónicas (sin discos de hidroxiapatita) y b) acompañada de *S. oralis*, *A. naeslundii*, *V. parvula*, *F. nucleatum* y *A. actinomycetemcomitans* sobre discos de hidroxiapatita para formar un *biofilm* multiespecie. La microscopía electrónica de barrido (SEM) y la microscopía láser confocal de barrido (CLSM) combinadas con la hibridación *in situ* fluorescente (FISH) se utilizaron para verificar la formación del *biofilm* y la presencia de *P. gingivalis* en él. El ARN total se extrajo de ambas muestras, planctónicas y *biofilm* multiespecie, se purificó y, con el uso de un *microarray*, se analizó la expresión génica diferencial de *P. gingivalis* ATCC 33277. Se utilizó un modelo lineal para determinar los genes expresados diferencialmente utilizando un criterio de filtro para el cambio de la expresión génica de al menos $\leq -2$ ó $\geq 2$ veces con respecto a la condición control y un valor significativo $p < 0,05$. La expresión diferencial se confirmó mediante la reacción en cadena de la polimerasa con transcriptasa inversa (RT-qPCR).

**Resultados:** Mediante SEM se verificó el desarrollo del *biofilm* multiespecie y la técnica de FISH confirmó la incorporación de *P. gingivalis*. El *microarray* demostró que *P. gingivalis* dentro de un *biofilm* multiespecie expresa de manera diferencial el 19,1% de sus genes (165 genes sobreexpresados y 200 reprimidos) en comparación con la condición planctónica. Estos genes estaban involucrados principalmente en funciones relacionadas con el estrés oxidativo, la envoltura celular, los transposones y el metabolismo. Los resultados del *microarray* fueron confirmados por la reacción en cadena de la polimerasa con transcriptasa inversa (RT-qPCR).

**Conclusión:** Se produjeron cambios transcripcionales significativos en *P. gingivalis* al crecer en un *biofilm* multiespecie en comparación con el estado planctónico.

PLOS ONE

RESEARCH ARTICLE

# Gene expression of *Porphyromonas gingivalis* ATCC 33277 when growing in an *in vitro* multispecies biofilm

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OPEN ACCESS

**Citation:** Romero-Lastra P, Sánchez MC, Llama-Palacios A, Figuero E, Herrera D, Sanz M (2019) Gene expression of *Porphyromonas gingivalis* ATCC 33277 when growing in an *in vitro* multispecies biofilm. PLoS ONE 14(8): e0221234. https://doi.org/10.1371/journal.pone.0221234

**Editor:** Roy Martin Roop, II, East Carolina University Brody School of Medicine, UNITED STATES

**Received:** May 14, 2019

**Accepted:** August 1, 2019

**Published:** August 22, 2019



**Data Availability Statement:** Gene expression data can be found at the Gene Expression Omnibus (GEO) repository using the accession GSE132157. Please see GEO data at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132157.

**Funding:** This study was partially supported by research funds from "The DENTAID - UCM Extraordinary Chair in Periodontal Research". This financial support had no influence in the study

## Abstract

### Background and objective

*Porphyromonas gingivalis*, an oral microorganism residing in the subgingival biofilm, may exert diverse pathogenicity depending on the presence of specific virulence factors, but its gene expression has not been completely established. This investigation aims to compare the transcriptomic profile of this pathogen when growing within an *in vitro* multispecies biofilm or in a planktonic state.

### Materials and methods

*P. gingivalis* ATCC 33277 was grown in anaerobiosis within multi-well culture plates at 37˚C under two conditions: (a) planktonic samples (no hydroxyapatite discs) or (b) within a multi-species-biofilm containing *Streptococcus oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* deposited on hydroxyapatite discs. Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) combined with Fluorescence *In Situ* Hybridization (FISH) were used to verify the formation of the biofilm and the presence of *P. gingivalis*. Total RNA was extracted from both the multispecies biofilm and planktonic samples, then purified and, with the use of a microarray, its differential gene expression was analyzed. A linear model was used for determining the differentially expressed genes using a filtering criterion of two-fold change (up or down) and a significance p-value of <0.05. Differential expression was confirmed by Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR).

### Results

SEM verified the development of the multispecies biofilm and FISH confirmed the incorporation of *P. gingivalis*. The microarray demonstrated that, when growing within the multispecies biofilm, 19.1% of *P. gingivalis* genes were significantly and differentially expressed (165 genes were up-regulated and 200 down-regulated), compared with planktonic growth. These genes were mainly involved in functions related to the oxidative stress, cell envelope, transposons and metabolism. The results of the microarray were confirmed by RT-qPCR.

design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Conclusion

Significant transcriptional changes occurred in *P. gingivalis* when growing in a multispecies biofilm compared to planktonic state.

## Introduction

The oral cavity is a unique ecological environment colonized by more than 500 bacterial species [1–3]. These bacteria are part of the oral microbiome and may be floating freely or within structured bacterial communities being part of complex biofilms, which provide bacteria protection against shear forces and host immune responses [4–6]. If these biofilms are not allowed to grow and mature, mainly through effective oral hygiene practices, these stable communities develop immune tolerance and may remain in symbiosis with the oral tissues. However, if they increase in mass or there are relevant changes in the local environment that favors the growth of pathobionts (dysbiosis), the immunological tolerance will be surpassed leading to inflammation [7, 8]. Among these pathobionts, *Porphyromonas gingivalis* has shown the expression of virulence factors to evade the host responses and to favor its colonization and spread within the tissues [9, 10].

Several studies have shown that when *P. gingivalis* grows within a biofilm, specific genes will become differentially regulated [11–14]. These genes may be relevant in promoting phenotypic adaptations of this pathogen, what may facilitate its infective potential [15–17], mostly by evading the immune response and promoting non-resolving chronic inflammation resulting in soft and hard tissue destruction, which are the key pathological features of periodontitis [5, 9, 18–20]. There is, however, scarce transcriptomic information of *P. gingivalis* and most available knowledge on the gene expression comes from *in vitro* monospecies biofilm models [21, 22]. Our research group has recently reported significant differences of gene expression when *P. gingivalis* was growing within a monospecies biofilm, mainly in those genes related to cell envelope, transport, outer membrane proteins, transposases and oxidative stress genes [14]. Furthermore, significant differences were encountered in those genes related to metabolism, adhesion, invasion, virulence and quorum sensing, when a growing monospecies biofilm was in the presence of planktonic *P. gingivalis* [23]. However, within the oral cavity, symbionts and pathobionts colonize as multispecies biofilms [7, 19, 24–26], and these bacteria will be faced with diverse DNA exchanges (horizontal gene transfer) and to multiple stressors, but only those bacteria with expressed genes that will enable them to colonize or resist host defenses, will be able to survive and predominate [2, 3, 27–30]. It was, therefore, the purpose of this *in vitro* study to compare the gene expression of *P. gingivalis* when growing within a multispecies oral biofilm with its growth in planktonic conditions.

## Material and methods

### Bacterial strains and culture conditions

Methodology for developing the multispecies biofilm was similar to that previously reported from our research group [14, 23]. Briefly, reference strains of *P. gingivalis* ATCC 33277, *Streptococcus oralis* CECT 907T, *Actinomyces naeslundii* ATCC 19039, *Veillonella parvula* NCTC 11810, *Fusobacterium nucleatum* DMSZ 20482 and *Aggregatibacter actinomycetemcomitans* DSMZ 8324 were used. Each bacterial strain was grown on blood agar plates (blood agar Oxoid no. 2; Oxoid, Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood

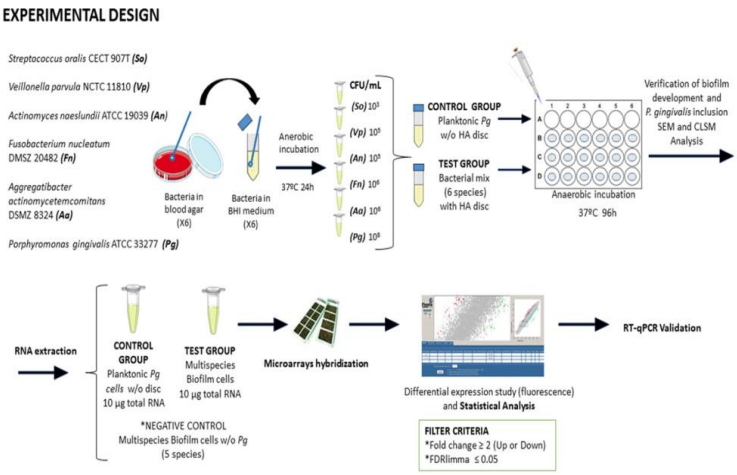


**Fig 1. Overview of experimental design.** *P. gingivalis* ATCC 33277 was incubated in anaerobiosis at 37˚C for 96 h and grown under planktonic conditions (control group) and within a multispecies-biofilm on hydroxyapatite (HA) discs, also containing the other described five bacterial species (test group). Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) were used to verify the multispecies-biofilm development and the presence of *P. gingivalis* in it. Total RNA was extracted, purified and the differential gene expression was analyzed by microarray [Agilent *P. gingivalis* Oligo Microarrays 8x15K (074976)] with a filtering criterion of two-fold change (up or down) and significance p-value <0.05. Differential expression was confirmed by Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR). (Images for Fig 1 were taken from https://smart.servier.com/ under a creative commons license).

https://doi.org/10.1371/journal.pone.0221234.g001

(Oxoid), 5.0 mg/L hemin (Sigma, St Louis, MO, USA) and 1.0 mg/L menadione (Merck, Darmstadt, Germany) under anaerobic conditions (10% $H_2$, 10% $CO_2$ and 80% $N_2$) at 37˚C for 24–72 h.

## Experimental assays

Fig 1 depicts the experimental design of this investigation. Planktonic cultures of each reference strain were grown anaerobically at 37˚C for 24 h in a protein-rich medium containing Brain-Heart Infusion (BHI) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 2.5 g/L mucin (Oxoid), 1.0 g/L yeast extract (Oxoid), 0.1 g/L cysteine (Sigma), 2.0 g/L sodium bicarbonate (Merck), 5.0 mg/L hemin (Sigma), 1.0 mg/L menadione (Merck) and 0.25% (v/v) glutamic acid (Sigma). By means of spectrophotometry, the late exponential phase growth was verified (optical density at 550 nm). Due to the fact that the bacteria used have different growth rates, and that this biofilm model is static we have adjusted the inocula of the different bacteria as was previously developed and validated by Sánchez *et al*., [31], in order to avoid the overgrowth of certain species and an excessive accumulation of waste products, obtaining by dilution in fresh modified BHI medium the following final concentrations:

- $10^3$ colony forming units (CFUs)/mL for *S. oralis*,
- $10^5$ CFUs/mL for *V. parvula* and *A. naeslundii*,
- $10^6$ CFUs/mL for *F. nucleatum* and *A. actinomycetemcomitans*,
- $10^8$ CFUs/mL for *P. gingivalis*.

Using pre-sterilized polystyrene 24-well tissue culture plates (Greiner Bio-one, Frikenhausen, Germany), two types of growing conditions were developed:

1. The test group (*P. gingivalis* growing within a multispecies biofilm). In each well of the plate, 1.5 mL of the mix solution containing the six reference strains, at the above referred concentrations, were deposited together with a sterile ceramic calcium hydroxyapatite discs (HA) [7-mm diameter (standard deviation, SD = 0.2 mm) and 1.8 mm thickness (Clarkson Chromatography Products, Williamsport, PA, USA)];
2. The control group (*P. gingivalis* growing planktonically). In each well, a volume of 1.5 mL of pure culture of *P. gingivalis* ($10^8$ CFUs/mL) was deposited in the absence of discs.

The plates were then incubated in anaerobiosis at 37˚C for 96 h. To rule out any possible contamination, a set of wells within the same plate were incubated with only culture medium.

## Monitoring of biofilm development: Scanning electron microscope and confocal laser scanning microscopy

In order to verify the multispecies-biofilm development on discs, Scanning Electron Microscope (SEM) was used. Three HA discs covered with biofilms grown for 96 h were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde for 4h at 4˚C, then washed twice in phosphate-buffered saline (PBS) and sterile water (immersion time 10 min) and dehydrated through a series of graded ethanol solutions (50, 60, 70, 80, 90 and 100%; immersion time per series, 10 min). Then, critical point drying and sputter-coating with gold was carried out before analyzing the samples with a scanning electron microscope JSM 6400 (JSM6400; JEOL, Tokyo, Japan) equipped with back-scattered electron detector and with an image resolution of 25 KV.

In addition, in order to ensure the incorporation of *P. gingivalis* from planktonic to biofilm, Confocal Laser Scanning Microscopy (CLSM) combined with Fluorescence *In Situ* Hybridization (FISH) was used. Three HA discs covered with multispecies biofilms, grown *in vitro* for 96 h, were incubated for 18 h with 40 μg/ml in hybridization buffer of the 16S rRNA *P. gingivalis* ALEXA Fluor 488 probe [5´-3´: CACTGAACTCAAGCCCGGCAGTTTCAA; Life Technologies Invitrogen (Carlsbad, CA, USA)]. Stained biofilms were washed for 15 min in a wash buffer [0.1 M Tris-HCl [pH 7.2], 0.18 M NaCl, 0.05 M EDTA and 0.005% sodium dodecyl sulfate [wt/vol]), and then exposed to 1 μg/mL of DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride; Thermo Fisher Technologies, Life Technologies Corporation, Carlsbad, CA, USA) for 5 min. Specimens were then washed 10 min with wash buffer and examined with a fixed-stage Ix83 Olympus inverted microscope coupled to an Olympus FV1200 confocal system (Olympus; Shinjuku, Tokio, Japan). The objective lens was a ×63 water-immersion lens (Olympus) and image stacks were acquired with a z-step size of 0.5 μm thickness (8 bits, 1024x1024 pixels). ALEXA Fluor 488 signals were detected with a PMT detector using a 405 nm laser and an emission range of 647–665 nm, together with DAPI (PMT detector / 552 nm laser / 350–470 nm emission range). Image analysis was performed with Imaris Biteplane software (Belfast, UK).

## Harvesting of planktonic and biofilm cells for gene expression analysis

After 96 h incubation in anaerobiosis at 37˚C, samples from both groups, test and control, were harvested and pooled into three biological replicates of each condition for independent hybridization. In the control group, a total of 5 mL were recovered from the wells of the culture plates and sequentially centrifuged to pool them into a single pellet in order to obtain 10 μg of RNA. In the test group, a set of 100 discs per biological replicate were harvested independently in 1 mL of sterile PBS and disaggregated by vortex during 3 min. The disaggregated multispecies biofilms were then pooled in a single sample, to obtain at least 10 μg of total RNA per biological replicate.

Both samples groups (planktonic and multispecies biofilms) were processed in the same manner, centrifuged at 9,000 rpm at 4˚C during 5 min and pooled as a single pellet for each biological replicate.

In all cases, an aliquot of each sample was used as quality control. For that, these aliquots were cultured on supplemented blood agar plates under anaerobic conditions at 37˚C for two weeks to control for the presence of each intended bacteria and the absence of contamination.

## Total RNA extraction

The three biological replicates of each condition, test and control, were suspended in 1 mL of TRIzol reagent (Ambion, NY, USA) to lysate the cells and to extract the total RNA [TRIzol Plus RNA Purification Kit (Invitrogen)]. Then, 200 μL of cold chloroform was added to separate its hydrophobic and hydrophilic content. The mixtures were then centrifuged at 13,000 rpm for 15 min at 4˚C, and the RNA phase collected. Nucleic acids were then precipitated with ~ 500 μL of cold 70% ethanol and centrifuged at 11,300 rpm for 15 seconds at room temperature. Pellets were then suspended in 50 μL of RNase-free water (Roche Diagnostics, Mannheim, Germany). To ensure the absence of any contaminating DNA, DNase I (Ambion, NY, USA) was added to the samples (set of RNase-free DNase; Qiagen, CA, USA) and purified using columns of RNeasy Mini kit (Qiagen) following the manufacturer's protocol.

RNA quantity and quality were measured with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). An A260/A280 ratio of at least 2.0 was reached for all the samples used in this study.

## cDNA synthesis, labeling and hybridization

The three biological replicates were independently hybridized for transcriptomic comparison of the test and control groups. The fluorescent labeling was performed using SuperScript Indirect cDNA Labelling System (Invitrogen; Carlsbad, CA, USA). Preparation of probes and hybridization were performed following the manufacturer's instructions [One-Color Microarray Based Gene Expression Analysis Manual Ver. 6.5 (Agilent Technologies)]. A slide specific for the strains of *P. gingivalis* ATCC 33277 and W83 was used [Agilent *P. gingivalis* Oligo Microarrays 8x15K (074976)].

As negative control, two replicates of multispecies biofilm without *P. gingivalis* (5 species-biofilm) were prepared and loaded onto the same microarray to rule out any possible gene cross-hybridization from the other bacteria.

## Microarray and data analysis

Images from Cy3 channel were equilibrated and captured with a high-resolution scanner (Agilent) and spots quantified using Feature Extraction software (Agilent) following a similar protocol as in the previous published investigations from our research group [14, 23]. Background correction and normalization of data expression were performed using LIMMA [32–34]. For local background correction and normalization, the methods "normexp" and loess in LIMMA were used, respectively [32]. To ensure consistency and similar distribution across arrays, log-ratio values were scaled using the median-absolute-value as scale estimator [33]. Linear model methods were used for determining differentially expressed genes. Each probe was tested for changes in expression over replicates by using an empirical Bayes moderated t-statistic [33]. To control for false discovery rates, p-values were corrected using the Benjamani and Hochberg method [32, 33]. We selected an expected false discovery rate of less than 5% and a filtering criterium of increase/decrease up to 2-fold differential expression between the two conditions, as used in other similar studies [14, 17]. Expression ratios were expressed as means of the fold changes of the three biological replicates and SD.

**Table 1. Primers used for Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR).**

| LOCUS NAME | PUTATIVE IDENTIFICATION | PRIMERS FOR RT-qPCR | |
|---|---|---|---|
| PG_2131 | OmpA_c-like | Forward<br>Backward | 5´-3´: ACACACCCCTCTCGTCTGAG<br>5´-3´: TCCCTTCCGGATAGCTCTG |
| PGN_0183<br>*(FimC)* | Minor component FimC | Forward<br>Backward | 5´-3´: CCTTTTCAAGAAAGAACTTGAGGA<br>5´-3´: GTCGGACTATCGGCTCGTT |
| PG_1712 | Alpha-1,2-mannosidase family protein | Forward<br>Backward | 5´-3´: GCTACGAAAGCCGTCCATC<br>5´-3´: GTACCACTCCCAACCTTTGC |
| PGN_1058<br>*(Ftn)* | Ferritin | Forward<br>Backward | 5´-3´: GAAATGATCGAGGCTGTCGT<br>5´-3´: GTCCTGTGATGCCATATCTCC |
| PGN_0033 | Thioredoxin | Forward<br>Backward | 5´-3´: CAACATTTGACGGCTTGGTA<br>5´-3´: CCATGTAGCCCAGAAATCCA |
| PGN_1208 *(ClpB)* | ClpB protein | Forward<br>Backward | 5´-3´: ACAAGGGGCATGTGGTAAAC<br>5´-3´: AACCGAGGTTCGACGTCAT |

https://doi.org/10.1371/journal.pone.0221234.t001

The National Center for Biotechnology (Genomics Unit) at Autónoma University of Madrid (Spain) performed the hybridizations and statistical analysis.

The transcriptomic results were inspected manually using different Internet platforms KEGG (Kyoto Encyclopedia of Genes and Genomes) and UniProt.

## Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

To validate microarray results, RT-qPCR of selected genes was performed, as described in our previous investigations [14, 23]. Three genes were selected from the up-regulated group and other three from the down-regulated group. Specific primers were designed using the Universal Probe Library Roche software tool, Roche Diagnostics (Table 1). *P. gingivalis* 16S rRNA gene was used as a reference gene for normalization of the RT-qPCR.

The cDNA was generated from 1 μg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, ThermoFisher Scientific) in a 10 μL of final reaction volume. Then qPCR reactions were performed in triplicate by using 5 μL per well of each cDNA, and 3 μL of a mix composed by 0.4 μM of each primer, 5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX), and nuclease-free water, to reach a final volume of 8 μL in 384-well optical plates. PCR reactions were run in an Applied Biosystems ABI PRISM 7900HT machine with Software Defined Storage (SDS) v2.4 and standard protocol from Applied Biosystems (95˚C 10 min, 40 cycles of 95˚C 15 sec and 60˚C 60 sec, and a final standard melting curve dissociation protocol).

All the RT-qPCR measurements were performed in triplicate and the results were analysed with the comparative cycle threshold method (ΔΔCt) [35]. The transcriptional $\log_2$ ratio from RT-qPCR analysis was plotted against the average $\log_2$ ratio values obtained by microarray analysis.

## Results

A mature multispecies-biofilm was confirmed by SEM. Fig 2A depicts this multispecies-biofilm, where most of the bacteria were organized in clusters with *F. nucleatum* acting as the backbone inter-connecting among the other bacterial morphotype. The CLSM combined with FISH was applied to detect the presence of *P. gingivalis* within the mature multispecies-biofilm. Fig 2B depicts the presence of *P. gingivalis* highlighted with a fluorescent stain in purple among the rest of bacterial species stained nonspecifically with DAPI in blue.

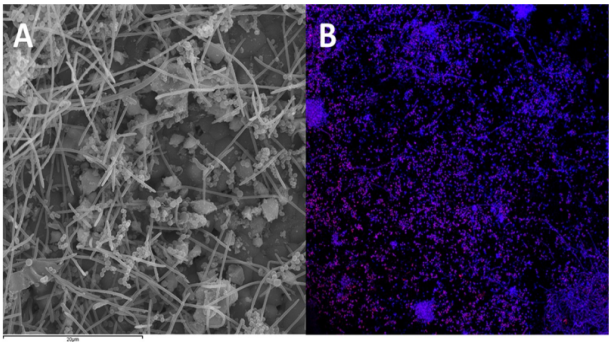


**Fig 2. Micrographs representing the multispecies-biofilms after 96 hours of growth.** (A) Scanning Electron Microscopy (SEM) depicting the structure of the biofilm. Note the microcolonies organized in clusters with *F. nucleatum* connecting them; (B) Confocal Laser Scanning Microscopy combined with Fluorescence *In Situ* Hybridization (CLSM-FISH). The ALEXA Fluor 488 probe detected the 16S rRNA *P. gingivalis* (cells in purple) within the multispecies-biofilm (other bacterial species in blue stained with DAPI).

https://doi.org/10.1371/journal.pone.0221234.g002

Fig 3 depicts the scanning glass slide of the microarray [Agilent *P. gingivalis* Oligo Microarrays 8x15K (074976)] and the fluorescence intensity measured after the hybridization of the three experimental replicates per group. Positive fluorescence was observed for *P. gingivalis* in planktonic growth (control group) (Fig 3 A1-A3) as well as within the multispecies biofilm (test group) (Fig 3 B1-B3). In contrast, there was no fluorescence in the negative control group, when *P. gingivalis* was not part of the multispecies biofilm (Fig 3 C1-C2), which confirmed the high specificity of the microarray without any sign of cross-hybridization.

Fig 4 depicts the *P. gingivalis* gene differential expression generated by the microarray (expressed as $\log_{10}$ of fluorescence), when growing in planktonic (X-axis, control) or within a multispecies biofilm (Y-axis, test). Using a linear model (LIMMA) with a filtering criterion of two-fold change (up or down) and significance p-value $<0.05$, a total of 365 out of 1,909 genes (19.1%) were found to have a differential expression when the two growing conditions were compared (S1 Table).

The *P. gingivalis* differentially expressed genes could be categorized in functional groups as depicted in Fig 5.

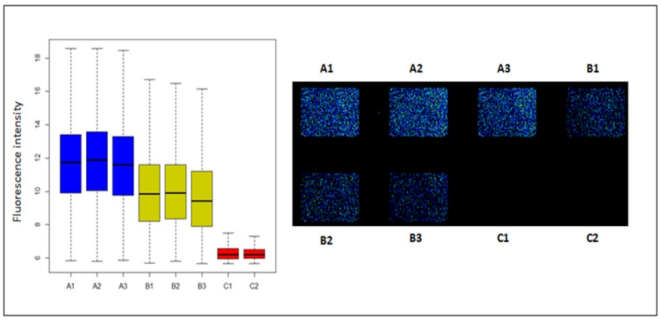


**Fig 3. Fluorescence signal of hybridization conditions on the microarray represented in boxplot (left) and the microarray slide (right).** Control group (A1, A2 and A3 in both cases) corresponds to the three experimental replicates of planktonic *Porphyromonas gingivalis* ATCC 33277. Test group (B1, B2 and B3 in both cases) represents the multispecies biofilm including *P. gingivalis*. And the negative control group (C1 and C2 in both cases) shows the multispecies-biofilm without *P. gingivalis*.

https://doi.org/10.1371/journal.pone.0221234.g003

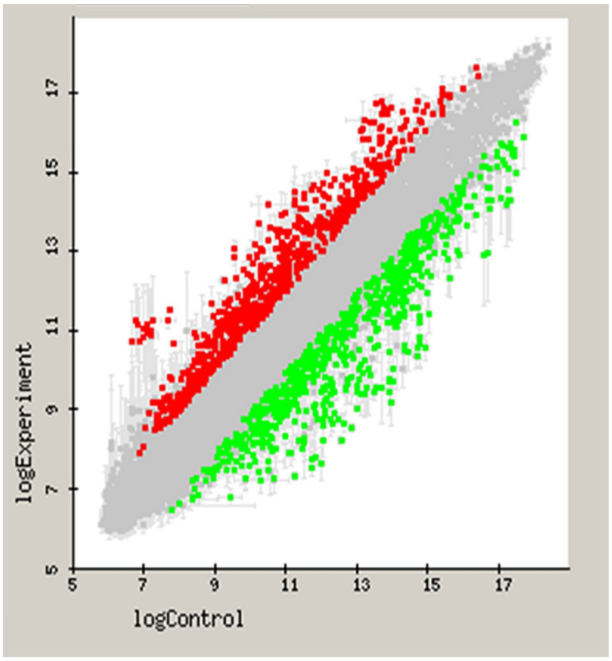


**Fig 4. Microarray-based comparative transcriptome demonstrating the gene expression (represented in $log_{10}$) for *Porphyromonas gingivalis* ATCC 33277 when growing in a multispecies biofilm compared to planktonic growth.** X-axis depicts the fold difference in gene expression of *P. gingivalis* in planktonic growth, and the Y-axis the gene expression of *P. gingivalis* inside a multispecies-biofilm. Up-regulated genes (over-expressed in multispecies-biofilm) were represented as red color and down-regulated genes were colored in green.

https://doi.org/10.1371/journal.pone.0221234.g004

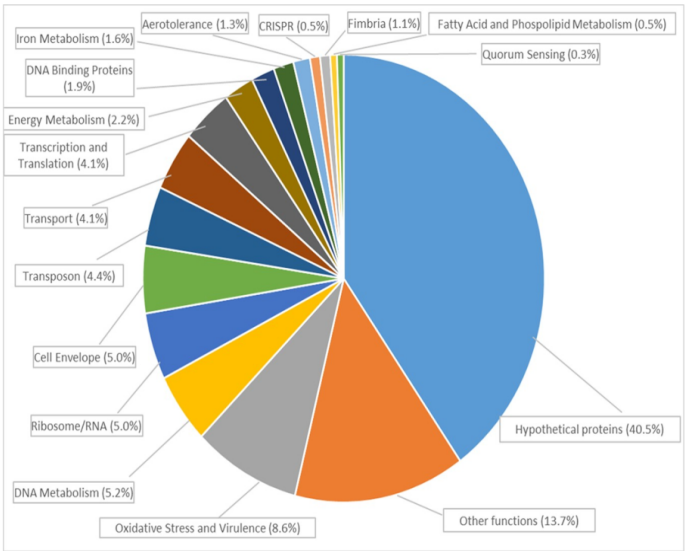


**Fig 5. Distribution in functional categories of the differentially regulated genes of *Porphyromonas gingivalis* ATCC 33277 in planktonic cells compared to *P. gingivalis* within a multispecies-biofilm.**

https://doi.org/10.1371/journal.pone.0221234.g005

The complete list of 365 genes can be found in S1 Table. Of them, when *P. gingivalis* grew within a multispecies biofilm (test group), 165 genes were identified as up-regulated. These genes were mainly related to:

- **oxidative stress protection and secretion of virulence factors**, such as *SodB* superoxide dismutase [+5.71 (SD = 2.33)], thioredoxin system PGN_0033 [+7.33 (SD = 1.03)] and PG_0275 [+3.54 (SD = 1.11)], thiol peroxidases PG_1729 [+5.04 (SD = 0.74)] and PGN_0388 [+4.75 (SD = 1.01)], heat shock proteins as PGN_0041 [+2.58 (SD = 0.25)] and *Clp* system PGN_1208 [+8.31 (SD = 1.75)] and PGN_0008 [+3.01 (SD = 0.24)], chaperones *GroES* PGN_1451 [+7.93 (SD = 0.94) ] and *GrpE* PGN_1715 [+2.95 (SD = 0.50)];
- **cell–cell communication**, such as the gene encoding for quorum sensing S-adenosylmethionine synthase (PGN_1827) [+2.55 (SD = 0.26)];
- **iron metabolism**, as ferritin PGN_1058 [+6.11 (SD = 1.48)] and PGN_0604 [+7.28 (SD = 1.54)] and ferrodoxin PG_1813 [+2.25 (SD = 0.24)], *HmuY* [+3.29 (SD = 0.49)], PGN_0741 [+2.41 (SD = 0.38)];
- **ribosomes**, as *RpsA*, *RpsP*, *RpsT*, *RpmF*, *RpmH*, *RpmL*, *RplQ*, *RplT*, PG_0627 [+2.47 (SD = 0.05)] and PGN_0668 [+2.42 (SD = 0.17)];
- Other important functional genes, like the **transposon genes** that allow for adaptation to life in communities (*TraA-Q*) and CRISPR (*Cas*2-2 PGN_1959 [+4.80 (SD = 0.50)]).

When *P. gingivalis* grew within a multispecies biofilm (test group), 200 genes were down-regulated (S1 Table). These genes were mainly related to:

- **cell envelope**, as membrane proteins PG_0922 [-7.29 (SD = 2.61)], PG_1180 [-2.48 (SD = 0.59)], PG_2224 [-5.33 (SD = 0.13)], PGN_1020 [-4.36 (SD = 0.83)];
- **lipoproteins**, as PG_0180 [-3.44 (SD = 0.56)], PG_0399 [-2.34 (SD = 0.29)], PG_2133 [-14.42 (SD = 3.64)], PG_0924 [-5.03 (SD = 0.80)];
- **transport**, as ABC transporters PG_0912 [-2.36 (SD = 0.05)], PGN_1898 *MgtE* [-2.24 (SD = 0.27)], PGN_1876 [-2.41 (SD = 0.18)], PG_1010 [-2.57 (SD = 0.23)], PGN_1343 [-2.81 (SD = 0.71)], PGN_1734 [-3.09 (SD = 0.43)];
- **aerotolerance**, as (*BatA-E*), PGN_0529 [-5.86 (SD = 1.09)], PGN_0528 [-5.15 (SD = 1.70)], PGN_0527 [-5.25 (SD = 0.65)], PGN_0526 [-4.74 (SD = 1.14)], PGN_0525 [-8.28 (SD = 0.63)]
- **fimbria**, as *FimA* [-11.03 (SD = 1.71)], *FimC* [-17.73 (SD = 2.28)], *FimD* [-9.65 (SD = 1.85)].

Of the 365 differentially regulated *P. gingivalis* genes, 40.5% encoded for unknown function or hypothetical proteins (S1 Table).

RT-qPCR confirmed the microarray results in three of the highly up-regulated genes and three of the highly down-regulated. Fig 6 depicts the $\log_2$ expression ratios for each technique demonstrating a high correlation between both ($R^2 = 0.9785$).

## Discussion

The results from this *in vitro* investigation have revealed significant transcriptional changes when *P. gingivalis* grew within a multispecies biofilm, compared to planktonic growth, with 19.1% of *P. gingivalis* genes differentially expressed (165 genes were up-regulated and 200 down-regulated). The complete list of 365 genes can be found as Supporting Information file (S1 Table). In a previous report [14], we showed that gene expression of *P. gingivalis* changed

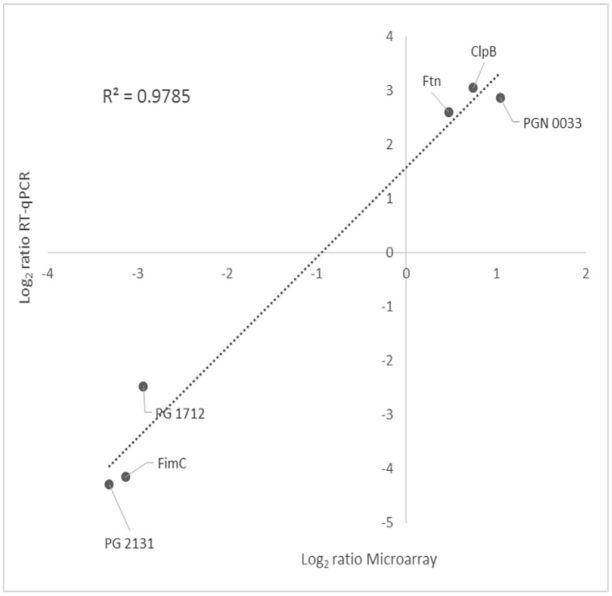


**Fig 6. Correlation between Microarray and Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) gene expression ratios when comparing *Porphyromonas gingivalis* ATCC 33277 growing within a multispecies-biofilm *versus* planktonic growth.** RT-qPCR $\log_2$ values were plotted against the microarray data $\log_2$ values ($R^2 = 0.9785$).

https://doi.org/10.1371/journal.pone.0221234.g006

from free floating to sessile, but the present study demonstrates that when biofilm conditions become more complex, as it is a multispecies microbial community, gene expression changes significantly enhanced inside the functional categories. In agreement with previous reports [9, 36], this polymicrobial synergy may increase the ability of *P. gingivalis* to colonize/predominate in the presence of other bacteria and thus increase its pathogenicity [22].

When compared to planktonic growth, *P. gingivalis* within a multispecies biofilm had significant differential expression in relevant groups of genes related to different functions and pathogenic pathways:

## Oxidative stress and virulence

The gene encoding the enzyme superoxide dismutase (*SodB)* was significantly up-regulated. This enzyme is responsible for transforming Reactive Oxygen Species (ROS) into peroxides, produced even in anaerobic conditions as a result of its own metabolism [37–39]. After that, peroxides are then generally disposed of by the enzyme catalase [40]. However, *P. gingivalis* cannot synthetize catalase and usually eliminates peroxides through the enzyme alkyl hydroperoxide reductase (*Ahp C–F*) [41]. Even though *AhpF* (PGN_0661) was down-regulated in the present study, the thioredoxin system (PGN_0033, PG_0275) and thiol peroxidase-encoding genes (PG_1729, PGN_0388), which are other pathways of peroxide removal [42, 43], were up-regulated. This finding has also been previously reported [44].

It was also significant with the up-regulation of genes involved in protein regulatory systems, such as heat shock proteins (Hsp), Clp proteolytic system *(ClpB*, *ClpC)* and the chaperones *(GroES*, *GrpE)*, which are usually expressed during stresses situations [45]. Clp proteases and chaperones are secreted by many pathogenic bacteria [45, 46] and they are involved in processes of colonization and adaptation to stress conditions. In fact, different bacterial species

have shown attenuated virulence, reduced adhesion and biofilm formation when Clp proteins were mutated [47–53].

The complex (GroEL/GroES) is also relevant in terms of virulence, since these proteins can mediate in adhesion and attachment to the host cells [54–56]. Moreover, diverse inhibitors of GroEL/GroES are currently being tested as broad-spectrum antimicrobial agents [57, 58]. Hosogi and Duncan (2005) showed that GroEL and other Hsp mediated the entry of *P. gingivalis* into host epithelial cells [59]. *Porphyromonas gingivalis* GroEL was inhibited by immunization, which significantly reduced levels of alveolar bone loss in experimental animal model [60]. Llama-Palacios *et al.*, in a proteomic study of *A. actinomycetemcomitans* biofilms, also reported increased expression in GroEL, and sera from patients with periodontitis were shown to be immunoreactive against GroEL [61].

Another potential pathogenic mechanism of GroEL proteins is their structural similarity with human Hsp. When overexpressed, GroEL may cause cross-reaction against human Hsp, causing autoantibodies and leading to chronic inflammation [53, 62].

Another important group of genes also found overexpressed in our study were those encoding for proteases, (PG_1060, PGN_1914 and PGN_0952) and peptidases (PG_0088, PGN_2035, PGN_1103, PGN_0788 and PG_1313). These proteases have been related to virulence, since they are able to degrade antibodies and, thus, to evade host tissue defenses and also to be involved in periodontal tissue destruction [63].

When the transcriptomic profile of *P. gingivalis* was studied when growing in a monospecies biofilms [14], only PGN_1914 was up-regulated, which may indicate that the up-regulation of the rest of the described proteases is mainly driven by the presence of other species within the biofilm.

## Cell envelope

The present transcriptomic study has revealed differential expression of those genes involved in cell envelope (PG_0679, PG_0922, PG_1039, PG_1180, PG_2224 and PGN_1020). PG_0679 was significantly up-regulated, what is in agreement with previous reports [13]. This gene is associated with antimicrobial resistance in multispecies biofilms, since efflux transporters pump out antimicrobial molecules [64].

However, *P. gingivalis* within a multispecies biofilm showed most of these genes down-regulated, which contrasts with previous reports studying *P. gingivalis* in monospecies biofilms [11, 14]. For example, the putative membrane protein gene PG_1180 and the putative epithelial cell attachment gene PG_2224, were significantly down-regulated.

Although the function is putative or not so concise, in general, the down-regulation of many genes involved in cell envelope biogenesis, taken together, with the down-regulation of metabolism genes involved in energy production or DNA replication, suggest a down-turn in cell replication and a reduced growth rate in biofilm. It has been previously attributed to restricted penetration of nutrients and helps explain the relative resistance of biofilms to antibiotics targeting growth [11]

Genes related with cell envelope lipoproteins, such as PG_0180, PG_0399, PG_1767, PG_1828, PG_2105, PG_2133 and PG_0924, were also differentially expressed. Among them, PG_1828 has been described as a strong cell activator, being major virulence factor for enhancing inflammatory responses [65]. In fact, inhibition of its activity by a deficient mutant suggests a direct link of PG_1828 in the pathogenesis of periodontitis [65].

## Quorum sensing

The gene PGN_1827 (*MetK)* demonstrated a significantly higher expression in *P. gingivalis* when growing in a multispecies biofilm. This gene encodes a protein related to the radical S-

adenosyl-l-methionine (SAM) superfamily, the universal signal for quorum sensing (QS). These proteins are responsible of the biosynthetic pathways leading to autoinducer-2 (AI-2) production, which are key in cell-cell communication what affects different bacterial functions related to virulence, such as motility, nutrition, phenotype expressions and modulation, stability and composition of the biofilms [9, 66–69].

## Iron metabolism

The present study has shown a significant up-regulation of genes as PGN_0741, a TonB-dependent outer membrane receptor important for iron transportation [70] and *HmuY*. *Hmu* family proteins are important for hemin acquisition, which is key for *P. gingivalis*. This bacterium has an absolute growth requirement for hemin, which provides them with iron and protoporphyrin IX that cannot be synthesize by itself [41, 71]. *Hmu* family genes has also been implicated as a virulence factor in promoting mononuclear cell-mediated inflammatory responses [72, 73]. The up-regulation of these genes, and in this case *HmuY*,can influence the ability of *P. gingivalis* to promote biofilm formation, as was seen in our previous study, in which the gene *HmuR* was up-regulated when growing in planktonic form in presence of a growing monospecies biofilm [23].

There was also a significant up-regulation of the genes coding for ferritin and ferrodoxin (*Ftn*, PGN_0604, PG_1813). Ferritin has been shown to be a requirement for *P. gingivalis* to grow under iron-depleted conditions and peroxide stress [42, 74].

## Transposons and CRISPR

*P. gingivalis* growing within a multispecies biofilm demonstrated up-regulation of several transposons genes (*TraA*, *TraF*, *TraG*, *TraI*, *TraJ*, *TraK*, *TraM*, *TraN*, *TraO*, *TraP*, *TraQ*, PGN_0058, PGN_0056, PG_1061, PGN_0954 and PGN_1912), suggesting that *P. gingivalis*, when growing among competing species, develops horizontal DNA transfer, which may facilitate its adaptation to different micro-environments. This up-regulation has also been reported in other studies [75–77]. Again, these genes were also up-regulated when planktonic *P. gingivalis* was in the presence of a growing biofilm, what may indicate the importance of DNA transfer to allow for survival in different environments and to adapt to an evolving biofilm [23].

Similarly, the genes of the CRISPR system: *Cas*2-2 and PGN_1959 were up-regulated in the test group. These genes encode for a CRISPR protein related to CAS2 family, which play broad roles in controlling bacterial pathogenesis, gene regulation and physiology [78], and protect its genome against other surrounding microorganisms and mobile foreign genetic elements, in particular plasmids and transposons [79–81].

Among other functional groups, genes related to ribosome, as *RpsA*, *RpsP*, *RpsT*, *RpmF*, *RpmH*, *RpmL*, *RplQ*, *RplT*, PG_0627 and PGN_0668, were up-regulated in the test group, what presumably indicates increased translation and higher protein synthesis. The fimbria genes *FimA*, *FimC*, *FimD* were down-regulated in *P. gingivalis* within the multispecies biofilm. This fact has also been reported when *P. gingivalis* grew in monospecies biofilm [14], or when planktonic *P. gingivalis* grew in the presence of a monospecies biofilm [23]. Other authors have reported that FimA and Mfa1 were not required for the development of pathogenicity in biofilms [82, 83]. A large proportion of the genes in (S1 Table) of the Supporting Information, demonstrating differential expression when comparing both groups, were however related to proteins of unknown function (40.5%), what indicates the need to further research to understand the functionality of these genes.

This study has clear limitations, such as its *in vitro* nature, the limited number of bacterial species used to develop the biofilms or the lack of influence of the patient's immune response

as well as its physiological condition, which would also influence the gene expression of *P. gingivalis*. Hence, further studies are needed to ascertain the pathogenic capability of *P. gingivalis* in the initiation and progression of periodontitis, as well as the transcriptomic changes that *P. gingivalis* could suffer accompanied by the other species when growing also in planktonic state.

## Conclusions

This study has shown that 19.1% of the *P. gingivalis* genome was differentially expressed when it grew within a multispecies biofilm, in comparison with monospecies planktonic growth. Within the biofilm, *P. gingivalis* has shown increased expression of virulence factors and antioxidant enzymes, especially Hsp proteins and several proteases. The identification and quantification of these known genes and other related to unknown proteins may provide new knowledge on the virulence and pathogenicity of this important periodontal pathogen, *P. gingivalis*.

## Supporting information

**S1 Table. Differentially expressed genes in *Porphyromonas gingivalis* ATCC 33277 in planktonic condition and within a multispecies-biofilm (cutoff ratio $\geq \pm 2$; p-value $<0.05$), grouped by functional categories.** Microarray data indicate the mean expression fold change and Standard Deviation (SD) of each gene.
(DOCX)

## Acknowledgments

This investigation was supported by Dentaid Extraordinary Chair for Periodontal Research (Cátedra Extraordinaria Dentaid en Investigación Periodontal), Complutense University of Madrid, Spain. We thank I. López and L. Almonacid from the Centro Nacional de Biotecnología (Autónoma University of Madrid, Spain) for hybridizations and statistical analysis of the microarrays and RT-qPCR performed by the Genomics Facility. We thank C. Pérez from the Centre of Microscopy and Cytometry from Complutense University of Madrid, Spain.

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**Validation:** Patricia Romero-Lastra.

**Writing – original draft:** Patricia Romero-Lastra, María C. Sánchez.

**Writing – review & editing:** Elena Figuero, David Herrera, Mariano Sanz.

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## SUPPORTING INFORMATION

**S1 Table. Differentially expressed genes in *Porphyromonas gingivalis* ATCC 33277 in planktonic condition and within a multispecies-biofilm (cutoff ratio of 2; p-value <0.05), grouped by functional categories. Microarray data indicate the mean expression fold change and Standard Deviation (SD) of each gene.**

| OPEN READING FRAME | LOCUS NAME | PUTATIVE IDENTIFICATION | AVERAGE RELATIVE FOLD CHANGE (SD) |
|---|---|---|---|
| **GENES RELATED TO OXIDATIVE STRESS AND VIRULENCE** | | | |
| **PGN_0564** | *SodB* | superoxide dismutase Fe-Mn | +5.71 (2.33) |
| **PGN_1055** | *VimE* | virulence modulating gene E | -2.50 (0.64) |
| **PGN_0033** | *Trx* | Thioredoxin | +7.33 (1.02) |
| **PG_0275** | | thioredoxin family protein | +3.54 (1.11) |
| **PG_1729** | | thiol peroxidase | +5.04 (0.74) |
| **PGN_0388** | | putative thiol peroxidase | +4.75 (1.01) |
| **PGN_0661** | | alkyl hydroperoxide reductase F subunit | -3.89 (0.55) |
| **PGN_1286** | | probable lysozyme | +3.02 (0.37) |
| **PGN_0055** | | probable lysozyme | +2.45 (0.23) |
| **PGN_0290** | | immunoreactive 32 kDa antigen | -3.55 (0.02) |
| **PG_0181** | | immunoreactive 32 kDa antigen PG49 | -3.46 (0.52) |
| **PGN_0152** | | immunoreactive 61 kDa antigen | -8.51 (3.46) |
| **PG_2102** | | immunoreactive 61 kDa antigen PG91 | -6.44 (1.06) |
| **PG_0234** | | immunoreactive 23 kDa antigen PG66 | +2.38 (0.29) |
| **PGN_0482** | | probable immunoreactive 23 kDa antigen | -3.07 (0.85) |
| **PGN_0041** | *HtpG* | heat shock protein HtpG | +2.58 (0.25) |
| **PGN_1208** | *ClpB* | ClpB protein | +8.31 (1.75) |
| **PGN_0008** | *ClpC* | ATP-dependent Clp protease ATP-binding subunit ClpC | +3.01 (0.24) |
| **PGN_1550** | *ClpX* | ATP-dependent Clp protease, ATP-binding subunit ClpX | -2.43 (0.70) |
| **PGN_1451** | *GroES* | chaperonin GroES | +7.93 (0.94) |
| **PGN_1715** | *GrpE* | putative chaperone protein GrpE | +2.95 (0.50) |
| **PG_1060** | | carboxyl-terminal protease | +8.08 (4.41) |
| **PGN_1914** | | carboxyl-terminal processing protease | +5.99 (0.39) |
| **PGN_0952** | | carboxyl-terminal processing protease | +5.53 (1.84) |
| **PG_0088** | | peptidase, M16 family | -2.87 (0.24) |
| **PGN_2035** | | putative peptidase | -2.74 (0.55) |
| **PGN_1103** | | Dipeptidase | +8.89 (2.53) |
| **PGN_0788** | | peptidyl-dipeptidase | +2.35 (0.26) |
| **PG_1313** | | dipeptidase-related protein | +9.78 (4.24) |
| **PG_1597** | | DnaK suppressor protein, putative | +2.32 (0.19) |
| **PG_0256** | | CvpA family protein | -3.60 (0.49) |
| **GENES RELATED TO AEROTOLERANCE** | | | |
| **PGN_0525** | *BatE* | probable aerotolerance-related exported protein BatE | -8.28 (0.63) |
| **PGN_0526** | *BatD* | aerotolerance-related exported protein BatD | -4.74 (1.14) |
| **PGN_0527** | *BatC* | probable aerotolerance-related exported protein BatC | -5.25 (0.65) |
| **PGN_0528** | *BatB* | putative aerotolerance-related exported protein BatB | -5.15 (1.70) |
| **PGN_0529** | *BatA* | aerotolerance-related membrane protein BatA | -5.86 (1.09) |

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| | | | |
|---|---|---|---|
| **GENES RELATED TO CELL ENVELOPE** | | | |
| **PG_0180** | | lipoprotein, putative | -3.44 (0.56) |
| **PG_0399** | | lipoprotein, putative | -2.34 (0.29) |
| **PG_0679** | | outer membrane efflux protein | +2.27 (0.08) |
| **PG_0922** | | membrane protein, putative | -7.29 (2.61) |
| **PG_0924** | | 5'-nucleotidase, lipoprotein e(P4) family | -5.03 (0.80) |
| **PG_1039** | | integral membrane protein | +2.41 (0.30) |
| **PG_1180** | | membrane protein, putative | -2.48 (0.59) |
| **PG_1587** | | PAP2 superfamily protein | -8.08 (2.56) |
| **PG_1711** | | alpha-1,2-mannosidase family protein | -7.43 (1.60) |
| **PG_1712** | | alpha-1,2-mannosidase family protein | -5.53 (1.34) |
| **PG_1767** | | lipoprotein, putative | +2.85 (0.57) |
| **PG_1828** | | lipoprotein, putative | +4.57 (0.29) |
| **PG_2105** | | lipoprotein, putative | +3.08 (0.25) |
| **PG_2133** | | lipoprotein, putative | -14.42 (3.64) |
| **PG_2224** | | membrane protein, putative | -5.33 (0.13) |
| **PGN_0081** | | putative Na driven multidrug efflux pump | -2.36 (0.18) |
| **PGN_0405** | | alpha-1,2-mannosidase family protein | -5.85 (0.31) |
| **PGN_1020** | | probable ATP/GTP-binding transmembrane protein | -4.36 (0.83) |
| **GENES RELATED TO QUORUM SENSING** | | | |
| **PGN_1827** | *MetK* | S-adenosylmethionine synthase | +2.55 (0.26) |
| **GENES RELATED TO TRANSPORT** | | | |
| | *MgtE* | magnesium transporter | -2.24 (0.27) |
| **PG_0091** | | transporter, putative | +2.87 (0.71) |
| **PG_0321** | | LAO/AO transport system ATPase | -3.00 (0.13) |
| **PG_0646** | | iron compound ABC transporter, ATP-binding protein | -3.16 (0.62) |
| **PG_0647** | | iron compound ABC transporter, permease protein | -4.43 (0.27) |
| **PG_0648** | | iron compound ABC transporter, periplasmic iron compound-binding protein, putative | -2.42 (0.34) |
| **PG_0912** | | polysaccharide transport protein, putative | -2.36 (0.05) |
| **PG_1010** | | ABC transporter, ATP-binding protein | -2.57 (0.23) |
| **PGN_0685** | | putative iron compound ABC transporter ATP-binding protein | -2.67 (0.05) |
| **PGN_0686** | | putative iron compound ABC transporter permease protein | -4.35 (1.44) |
| **PGN_1343** | | probable ABC transporter ATP-binding protein | -2.81 (0.71) |
| **PGN_1641** | | arginine/ornithine transport system ATPase | -3.77 (0.06) |
| **PGN_1734** | *NupG* | nucleoside permease NupG | -3.09 (0.43) |
| **PGN_1876** | | putative ABC transporter membrane protein | -2.41 (0.18) |
| **PGN_1898** | | probable transport protein | -2.93 (0.49) |
| **GENES RELATED TO IRON METABOLISM** | | | |
| | *HmuY* | hmuY protein | +3.29 (0.49) |
| **PG_0668** | | TonB-dependent receptor | -2.27 (0.17) |
| **PG_1813** | | ferredoxin, 4Fe-4S | +2.25 (0.24) |
| **PGN_0604** | | Ferritin | +7.28 (1.54) |
| **PGN_0741** | | TonB-dependent receptor | +2.41 (0.38) |
| **PGN_1058** | *Ftn* | Ferritin | +6.11 (1.48) |
| **GENES RELATED TO TRANSPOSON FUNCTIONS** | | | |
| **PG_1061** | | ISPg6, transposase | -2.71 (0.91) |
| **PGN_0056** | | probable conserved protein found in conjugate transposon | +3.76 (0.54) |
| **PGN_0057** | *TraP* | probable conserved protein found in conjugate transposon TraP. | +3.99 (0.70) |
| **PGN_0058** | | probable conserved protein found in conjugate transposon | +5.26 (0.67) |

2

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|---|---|---|---|
| **PGN_0059** | *TraN* | conserved protein found in conjugate transposon TraN | +2.54 (0.36) |
| **PGN_0060** | *TraM* | putative conserved protein found in conjugate transposon TraM | +2.82 (0.52) |
| **PGN_0062** | *TraK* | putative conserved protein found in conjugate transposon TraK | +2.94 (0.58) |
| **PGN_0063** | *TraJ* | conserved transmembrane protein found in conjugate transposon TraJ | +2.68 (0.63) |
| **PGN_0064** | *TraI* | putative conserved protein found in conjugate transposon TraI | +3.11 (1.07) |
| **PGN_0065** | *TraG* | conserved protein found in conjugate transposon TraG | +3.49 (0.39) |
| **PGN_0066** | *TraF* | probable conserved transmembrane protein found in conjugate transposon TraE | +2.31 (0.10) |
| **PGN_0069** | *TraA* | probable conserved protein found in conjugate transposon TraA | +3.01 (1.04) |
| **PGN_0592** | *TraQ* | putative conserved protein found in conjugate transposon TraQ | +3.02 (0.84) |
| **PGN_0954** | | partial transposase in ISPg6 | -3.33 (0.40) |
| **PGN_1283** | *TraO* | conserved protein found in conjugate transposon TraO | +5.20 (0.20) |
| **PGN_1912** | | partial transposase in ISPg6 | -3.42 (0.40) |
| **GENES RELATED TO CRISPR** | | | |
| | *Cas2-1* | CRISPR-associated protein Cas2 | -2.93 (0.81) |
| **PGN_1959** | *Cas2-2* | CRISPR-associated protein Cas2 | +4.80 (0.50) |
| **GENES RELATED TO RIBOSOME/ RNA** | | | |
| | *RpmI* | probable 50S ribosomal protein L35 | +2.87 (0.68) |
| | *RnpA* | ribonuclease P protein component | -2.66 (0.27) |
| | *RbfA* | ribosome-binding factor A | -7.81 (1.48) |
| | *PyrC* | Dihydroorotase | -2.81 (0.53) |
| | *KsgA* | dimethyladenosine transferase | -2.53 (0.24) |
| **PG_0075** | | phosphoribosylformylglycinamidine cyclo-ligase, putative | -2.33 (0.28) |
| **PG_0627** | | RNA-binding protein | +2.47 (0.05) |
| **PGN_0167** | *RpsP* | 30S ribosomal protein S16 | +2.38 (0.25) |
| **PGN_0188** | *RpmF* | 50S ribosomal protein L32 | +2.66 (0.36) |
| **PGN_0394** | *RpsT* | probable 30S ribosomal protein S20 | +2.72 (0.41) |
| **PGN_0668** | | RNA-binding protein | +2.42 (0.17) |
| **PGN_0694** | *RpmH* | 50S ribosomal protein L34 | +4.37 (0.59) |
| **PGN_0761** | | ribosomal large subunit pseudouridine synthase | -3.11 (0.26) |
| **PGN_0965** | *RplT* | putative 50S ribosomal protein L20 | +2.94 (0.56) |
| **PGN_1024** | | putative ribosome-binding factor A | -6.88 (1.16) |
| **PGN_1088** | *RpsA* | 30S ribosomal protein S1 | +2.80 (0.18) |
| **PGN_1840** | *RplQ* | 50S ribosomal protein L17 | +2.62 (0.38) |
| **PGN_1871** | *RpsG* | 30S ribosomal protein S7 | -2.57 (0.41) |
| **GENES RELATED TO FIMBRIA** | | | |
| **PGN_0180** | *FimA* | FimA type I fimbrilin | -11.03 (1.71) |
| **PGN_0181** | | Fimbrillin-A associated anchor proteins Mfa1 and Mfa2 | -8.41 (2.84) |
| **PGN_0183** | *FimC* | minor component FimC | -17.73 (2.28) |
| **PGN_0184** | *FimD* | minor component FimD | -9.65 (1.85) |
| **GENES RELATED TO TRANSCRIPTION AND TRANSLATION** | | | |
| | *NrdG* | anaerobic ribonucleoside-triphosphate reductase activating protein | -8.42 (1.42) |
| **PG_0020** | | transcriptional regulator, MarR family | +2.43 (0.52) |
| **PG_0997** | | transcriptional regulator, putative | +2.50 (0.31) |
| **PG_1260** | | anaerobic ribonucleoside-triphosphate reductase, putative | -12.50 (4.82) |
| **PG_2000** | | signal peptidase-related protein | -2.45 (0.35) |
| **PGN_0082** | | probable transcriptional regulator AraC family | -2.99 (0.59) |
| **PGN_0319** | | probable RNA polymerase sigma-70 factor ECF subfamily | +2.66 (0.23) |
| **PGN_0355** | | translation initiation factor IF-2 | -3.00 (0.41) |
| **PGN_0782** | | putative tRNA pseudouridine synthase A | +2.70 (0.52) |

3

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|---|---|---|---|
| **PGN_0945** | | putative TetR family transcriptional regulator | -2.36 (0.08) |
| **PGN_0970** | | putative RNA polymerase sigma-70 factor ECF subfamily | +4.06 (0.70) |
| **PGN_1226** | | ribonucleotide reductase | -2.91 (0.52) |
| **PGN_1312** | | probable transcriptional regulator as Arg-repressor | +2.32 (0.05) |
| **PGN_1395** | | putative anaerobic ribonucleoside-triphosphate reductase activating protein | -5.47 (0.52) |
| **PGN_1396** | | anaerobic ribonucleoside-triphosphate reductase | -14.51 (2.00) |
| **GENES RELATED TO FATTY ACID AND PHOSPOLIPID METABOLISM** | | | |
| **PG_1155** | | ADP-heptose--LPS heptosyltransferase, putative | -6.31 (1.56) |
| **PGN_1255** | *Rfa* | putative heptosyltransferase | -4.81 (2.33) |
| **GENES RELATED TO DNA METABOLISM** | | | |
| | *XseA* | exodeoxyribonuclease VII, large subunit | -2.60 (0.37) |
| | *Tmk* | thymidine kinase | -2.67 (0.56) |
| | *RecJ* | single-stranded-DNA-specific exonuclease RecJ | -2.92 (0.23) |
| | *PurB* | adenylosuccinate lyase | +2.53 (0.37) |
| | *GuaB* | inosine-5'-monophosphate dehydrogenase | +2.80 (0.68) |
| **PG_0030** | | cytidine deaminase | +3.77 (0.32) |
| **PG_0174** | | pyridine nucleotide-disulphide oxidoreductase family protein | -2.72 (0.43) |
| **PG_1038** | | ATP-dependent DNA helicase UvrD/PcrA/Rep Family | -9.02 (4.61) |
| **PGN_0001** | *DnaA* | chromosomal replication initiator protein DnaA | +2.12 (0.08) |
| **PGN_0026** | | putative cytidine deaminase | +4.05 (0.41) |
| **PGN_0084** | | DNA topoisomerase I | +6.92 (0.11) |
| **PGN_0923** | | putative DNA primase | -2.62 (0.25) |
| **PGN_1022** | | putative thymidine kinase | -3.94 (0.40) |
| **PGN_1027** | | Dihydroorotase | -3.26 (0.72) |
| **PGN_1225** | | probable exodeoxyribonuclease VII large subunit | -2.91 (0.25) |
| **PGN_1314** | | ATP-dependent DNA helicase | -8.90 (4.14) |
| **PGN_1449** | | inosine-5'-monophosphate dehydrogenase | +3.36 (0.34) |
| **PGN_1567** | *RecF* | recF protein | -2.69 (0.33) |
| **PGN_1992** | | putative helicase | -2.34 (0.08) |
| **GENES RELATED TO ENERGY METABOLISM** | | | |
| | *PckA* | phosphoenolpyruvate carboxykinase (ATP) | +2.27 (0.22) |
| | *HprA* | glycerate dehydrogenase | +2.54 (0.32) |
| **PG_1834** | | glycogen synthase-related protein | -3.24 (1.07) |
| **PG_2171** | | D-isomer specific 2-hydroxyacid dehydrogenase family protein | -2.36 (0.23) |
| **PG_2213** | | nitrite reductase-related protein | -2.76 (0.18) |
| **PGN_1120** | | putative NADPH-NAD transhydrogenase | +3.08 (1.09) |
| **PGN_1736** | | putative glycogen synthase | -3.51 (0.31) |
| **PGN_1746** | *NrfA* | cytochrome c nitrite reductase, catalytic subunit NrfA | -2.92 (0.64) |
| **GENES RELATED TO DNA BINDING PROTEINS** | | | |
| | *Hup-2* | DNA-binding protein HU | +2.85 (0.46) |
| **PG_0254** | | N utilization substance protein A, putative | -4.15 (0.89) |
| **PG_0555** | | DNA-binding protein, histone-like family | +3.76 (0.50) |
| **PG_2040** | | DNA-binding protein, histone-like family | +4.06 (0.85) |
| **PGN_1393** | | putative DNA-binding protein HU | +2.97 (0.80) |
| **PGN_1415** | | DNA-binding protein histone-like family | +3.90 (0.42) |
| **PGN_1986** | | DNA-binding protein, histone-like family | +4.28 (0.61) |
| **GENES RELATED TO OTHER FUNCTIONS** | | | |
| | *LysC* | aspartate kinase | -2.59 (0.25) |
| | *LysA* | diaminopimelate decarboxylase | -2.46 (0.32) |

4

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|---|---|---|---|
| | *KdsA* | 2-dehydro-3-deoxyphosphooctonate aldolase | +2.44 (0.32) |
| | *FolP* | dihydropteroate synthase | +2.35 (0.21) |
| | *Dxr* | 1-deoxy-D-xylulose 5-phosphate reductoisomerase | -4.08 (0.32) |
| **PG_0079** | | abortive infection protein, putative | +4.64 (1.48) |
| **PG_0199** | | TatD family protein | -3.78 (0.11) |
| **PG_0226** | | transglutaminase-related protein | +2.41 (0.29) |
| **PG_0917** | | GtrA family protein | -3.25 (0.60) |
| **PG_0920** | | glycosyl transferase, group 2 family protein | -3.63 (1.90) |
| **PG_1014** | | TPR domain protein | -2.58 (0.37) |
| **PG_1840** | | conserved domain protein | -3.63 (0.47) |
| **PG_1963** | | Sua5/YciO/YrdC/YwlC family protein | +2.20 (0.11) |
| **PG_2028** | | ebsC protein | -2.62 (0.47) |
| **PG_2131** | | 60 kDa protein/OmpA_C-like | -19.57 (6.37) |
| **PGN_0082** | *AroC* | chorismate synthase | -3.39 (0.73) |
| **PGN_0094** | | putative bacteriophage integrase | +2.78 (0.57) |
| **PGN_0101** | | putative 1,4-dihydroxy-2-naphthoate octaprenyltransferase | -3.50 (0.69) |
| **PGN_0179** | | 60 kDa protein | -17.32 (2.32) |
| **PGN_0232** | | probable glycosyl transferase family 2 | -2.52 (0.43) |
| **PGN_0243** | | phosphoglycerate mutase | +2.50 (0.19) |
| **PGN_0248** | | putative dimethyladenosine transferase | -2.71 (0.24) |
| **PGN_0285** | | pyridine nucleotide-disulphide oxidoreductase | -2.71 (0.60) |
| **PGN_0354** | | putative nitrogen utilization substance protein A | -4.30 (0.98) |
| **PGN_0406** | | conserved hypothetical protein with glycosyl hydrolase family 92 domain | -6.40 (2.32) |
| **PGN_0466** | | putative cardiolipin synthetase | +2.30 (0.13) |
| **PGN_0518** | | putative ribulose-phosphate 3-epimerase | +2.94 (0.52) |
| **PGN_0522** | | putative dihydropteroate synthase | +2.51 (0.16) |
| **PGN_0524** | | lipid A 4'-phosphatase | -8.40 (1.72) |
| **PGN_0531** | | putative von Willebrand factor type A | -3.09 (0.77) |
| **PGN_0571** | | putative undecaprenol kinase | -3.25 (0.11) |
| **PGN_0743** | | probable FKBP-type peptidyl-prolyl cis-trans isomerase FkpA | +2.50 (0.29) |
| **PGN_0753** | | probable two component system response regulator | +2.87 (0.33) |
| **PGN_0917** | | tyrosine type site-specific recombinase | -4.39 (1.10) |
| **PGN_0975** | *MenA* | 1,4-dihydroxy-2-naphthoate octaprenyltransferase | -3.11 (0.31) |
| **PGN_1023** | | acid phosphatase OlpA | -5.16 (1.03) |
| **PGN_1089** | | probable methyltransferase | -4.30 (0.85) |
| **PGN_1104** | | chorismate synthase | -3.68 (0.50) |
| **PGN_1151** | | 1-deoxy-D-xylulose-5-phosphate reductoisomerase | -3.93 (0.87) |
| **PGN_1206** | | putative methylenetetrahydrofolate dehydrogenase | +3.08 (0.68) |
| **PGN_1209** | | probable flavodoxin | +2.82 (0.20) |
| **PGN_1220** | | adenylosuccinate lyase | +2.53 (0.42) |
| **PGN_1221** | | probable ATP:corrinoid adenosyltransferase | +3.98 (1.69) |
| **PGN_1272** | | putative diaminopimelate decarboxylase | -2.53 (0.24) |
| **PGN_1273** | | probable 1,4-dihydroxy-2-naphthoate octaprenyltransferase | -3.23 (0.67) |
| **PGN_1706** | | probable phosphoribosylglycinamide formyltransferase | +3.37 (0.53) |
| **PGN_1748** | | putative cytochrome c biogenesis protein CcsA | -2.82 (0.44) |
| **PGN_1975** | | putative regulatory protein | -2.59 (0.60) |
| **PGN_1985** | | probable N-acetylmuramoyl-L-alanine amidase | +3.52 (0.11) |
| **PGN_2026** | | putative abortive infection protein | +4.40 (0.71) |

5

| GENES RELATED TO HYPOTHETICAL PROTEIN | | |
|---|---|---|
| **PG_0031** | hypothetical protein | +3.28 (0.22) |
| **PG_0039** | hypothetical protein | -2.24 (0.10) |
| **PG_0161** | hypothetical protein | +2.74 (0.39) |
| **PG_0164** | conserved hypothetical protein | -3.24 (0.68) |
| **PG_0179** | hypothetical protein | -3.48 (1.00) |
| **PG_0204** | hypothetical protein | +2.47 (0.30) |
| **PG_0229** | hypothetical protein | -2.69 (0.42) |
| **PG_0286** | hypothetical protein | +3.57 (0.53) |
| **PG_0323** | conserved hypothetical protein | +3.10 (0.02) |
| **PG_0404** | hypothetical protein | +4.53 (0.48) |
| **PG_0409** | hypothetical protein | +2.24 (0.09) |
| **PG_0421** | hypothetical protein | +5.43 (1.62) |
| **PG_0447** | conserved hypothetical protein | +2.99 (0.27) |
| **PG_0448** | hypothetical protein | +3.08 (0.34) |
| **PG_0547** | conserved hypothetical protein | -2.71 (0.41) |
| **PG_0612** | hypothetical protein | -2.60 (0.21) |
| **PG_0613** | hypothetical protein | -2.83 (0.52) |
| **PG_0883** | hypothetical protein | -2.52 (0.75) |
| **PG_0898** | conserved hypothetical protein | +2.56 (0.27) |
| **PG_0918** | hypothetical protein | -2.89 (0.64) |
| **PG_0926** | hypothetical protein | -4.78 (0.59) |
| **PG_0927** | conserved hypothetical protein TIGR00150 | -5.33 (0.60) |
| **PG_0929** | hypothetical protein | +2.48 (0.32) |
| **PG_0994** | hypothetical protein | -3.70 (1.33) |
| **PG_1085** | hypothetical protein | +2.96 (0.40) |
| **PG_1257** | hypothetical protein | +3.03 (0.24) |
| **PG_1300** | conserved hypothetical protein | -2.99 (0.93) |
| **PG_1388** | hypothetical protein | -2.65 (0.27) |
| **PG_1398** | hypothetical protein | -2.05 (0.05) |
| **PG_1546** | hypothetical protein | +3.91 (0.56) |
| **PG_1661** | hypothetical protein | -2.50 (0.32) |
| **PG_1715** | hypothetical protein | -3.66 (0.31) |
| **PG_1817** | conserved hypothetical protein | -3.20 (1.17) |
| **PG_1818** | hypothetical protein | -2.63 (0.37) |
| **PG_1819** | hypothetical protein | -4.50 (0.89) |
| **PG_1908** | hypothetical protein | -3.20 (0.83) |
| **PG_1966** | conserved hypothetical protein | -3.24 (0.59) |
| **PG_1997** | hypothetical protein | +2.41 (0.32) |
| **PG_2031** | hypothetical protein | -2.79 (0.24) |
| **PG_2037** | hypothetical protein | +2.53 (0.71) |
| **PG_2101** | hypothetical protein | -8.79 (2.11) |
| **PG_2106** | hypothetical protein | +3.07 (0.66) |
| **PG_2130** | hypothetical protein | -17.17 (3.46) |
| **PG_2139** | hypothetical protein | +2.86 (0.18) |
| **PG_2204** | hypothetical protein | +3.88 (0.36) |
| **PG_2212** | hypothetical protein | -7.57 (0.89) |
| **PG_2225** | conserved hypothetical protein | -3.22 (0.49) |
| **PG_2226** | hypothetical protein | -3.50 (0.68) |
| **PGN_0029** | conserved hypothetical protein | +2.74 (0.35) |

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|---|---|---|
| **PGN_0053** | conserved hypothetical protein | +2.31 (0.19) |
| **PGN_0061** | hypothetical protein | +3.42 (0.79) |
| **PGN_0068** | hypothetical protein | +2.99 (0.16) |
| **PGN_0070** | hypothetical protein | +2.37 (0.05) |
| **PGN_0072** | hypothetical protein | +3.77 (1.28) |
| **PGN_0083** | conserved hypothetical protein | +4.85 (0.80) |
| **PGN_0090** | hypothetical protein | +2.71 (0.52) |
| **PGN_0091** | hypothetical protein | +3.78 (1.17) |
| **PGN_0092** | conserved hypothetical protein | +3.40 (0.10) |
| **PGN_0093** | conserved hypothetical protein | +5.14 (0.24) |
| **PGN_0110** | hypothetical protein | +2.73 (0.21) |
| **PGN_0124** | hypothetical protein | -3.68 (0.57) |
| **PGN_0154** | conserved hypothetical protein | +3.66 (0.07) |
| **PGN_0156** | conserved hypothetical protein | +2.97 (0.36) |
| **PGN_0164** | conserved hypothetical protein | -3.69 (0.26) |
| **PGN_0178** | conserved hypothetical protein | -16.48 (1.52) |
| **PGN_0182** | conserved hypothetical protein | -16.16 (2.92) |
| **PGN_0187** | conserved hypothetical protein | +2.85 (0.68) |
| **PGN_0273** | conserved hypothetical protein | +3.09 (0.32) |
| **PGN_0288** | conserved hypothetical protein | -3.09 (1.08) |
| **PGN_0289** | conserved hypothetical protein | -4.02 (0.76) |
| **PGN_0307** | conserved hypothetical protein | -3.45 (0.70) |
| **PGN_0312** | hypothetical protein | +2.39 (0.29) |
| **PGN_0332** | conserved hypothetical protein | -2.42 (0.40) |
| **PGN_0356** | conserved hypothetical protein | -3.79 (0.60) |
| **PGN_0400** | conserved hypothetical protein | -5.05 (1.30) |
| **PGN_0404** | conserved hypothetical protein | -3.56 (0.33) |
| **PGN_0481** | hypothetical protein | -4.01 (1.08) |
| **PGN_0511** | conserved hypothetical protein | +2.42 (0.11) |
| **PGN_0530** | conserved hypothetical protein | -4.40 (0.41) |
| **PGN_0538** | conserved hypothetical protein | -2.72 (0.11) |
| **PGN_0562** | conserved hypothetical protein | +2.53 (0.35) |
| **PGN_0578** | conserved hypothetical protein found in conjugate transposon | +2.39 (0.23) |
| **PGN_0583** | conserved hypothetical protein | -2.52 (0.39) |
| **PGN_0586** | conserved hypothetical protein | -2.63 (0.48) |
| **PGN_0589** | conserved hypothetical protein | -2.67 (0.08) |
| **PGN_0654** | conserved hypothetical protein | -2.50 (0.36) |
| **PGN_0655** | conserved hypothetical protein | -2.56 (0.27) |
| **PGN_0656** | conserved hypothetical protein | -2.67 (0.40) |
| **PGN_0752** | hypothetical protein | +3.92 (0.35) |
| **PGN_0794** | conserved hypothetical protein | -2.42 (0.26) |
| **PGN_0797** | conserved hypothetical protein | +2.53 (0.09) |
| **PGN_0835** | conserved hypothetical protein | -3.19 (0.57) |
| **PGN_0874** | conserved hypothetical protein | -2.98 (0.33) |
| **PGN_0978** | hypothetical protein | -2.59 (0.34) |
| **PGN_1017** | conserved hypothetical protein | -3.99 (0.36) |
| **PGN_1021** | hypothetical protein | -5.92 (0.75) |
| **PGN_1025** | conserved hypothetical protein | -8.12 (2.36) |
| **PGN_1028** | conserved hypothetical protein | -2.96 (0.20) |
| **PGN_1029** | conserved hypothetical protein | -3.28 (0.36) |

| | | |
|---|---|---|
| **PGN_1081** | conserved hypothetical protein | +2.50 (0.33) |
| **PGN_1159** | conserved hypothetical protein | -2.35 (0.21) |
| **PGN_1182** | conserved hypothetical protein | +2.84 (0.27) |
| **PGN_1254** | hypothetical protein | -2.46 (0.37) |
| **PGN_1256** | conserved hypothetical protein | -3.86 (0.26) |
| **PGN_1306** | hypothetical protein | -5.89 (2.38) |
| **PGN_1307** | hypothetical protein | -8.18 (1.61) |
| **PGN_1313** | conserved hypothetical protein | +3.23 (0.49) |
| **PGN_1337** | conserved hypothetical protein | -2.37 (0.21) |
| **PGN_1340** | conserved hypothetical protein | -2.62 (0.15) |
| **PGN_1380** | hypothetical protein | +3.53 (0.63) |
| **PGN_1385** | hypothetical protein | +3.63 (0.48) |
| **PGN_1392** | conserved hypothetical protein | +2.89 (0.50) |
| **PGN_1419** | conserved hypothetical protein | -2.65 (0.68) |
| **PGN_1435** | hypothetical protein | +2.30 (0.18) |
| **PGN_1436** | conserved hypothetical protein | +2.66 (0.43) |
| **PGN_1438** | hypothetical protein | -2.17 (0.13) |
| **PGN_1476** | conserved hypothetical protein | +3.53 (0.09) |
| **PGN_1496** | hypothetical protein | +3.77 (0.56) |
| **PGN_1509** | conserved hypothetical protein | +2.26 (0.25) |
| **PGN_1515** | conserved hypothetical protein | +2.65 (0.33) |
| **PGN_1547** | conserved hypothetical protein | +6.85 (1.74) |
| **PGN_1561** | conserved hypothetical protein | +5.07 (1.10) |
| **PGN_1591** | conserved hypothetical protein | +2.38 (0.49) |
| **PGN_1609** | hypothetical protein | +2.46 (0.28) |
| **PGN_1639** | conserved hypothetical protein | +3.43 (0.49) |
| **PGN_1661** | conserved hypothetical protein | +2.44 (0.23) |
| **PGN_1678** | conserved hypothetical protein | +3.55 (0.35) |
| **PGN_1731** | conserved hypothetical protein | -3.23 (0.87) |
| **PGN_1739** | conserved hypothetical protein | +3.97 (0.61) |
| **PGN_1747** | conserved hypothetical protein | -3.05 (0.58) |
| **PGN_1837** | conserved hypothetical protein | -2.90 (0.20) |
| **PGN_1878** | conserved hypothetical protein | +2.62 (0.37) |
| **PGN_1889** | conserved hypothetical protein | +2.36 (0.19) |
| **PGN_1920** | conserved hypothetical protein | -2.40 (0.06) |
| **PGN_1923** | hypothetical protein | -3.06 (0.68) |
| **PGN_1924** | conserved hypothetical protein | -2.85 (0.57) |
| **PGN_1942** | hypothetical protein | +2.24 (0.07) |
| **PGN_1965** | hypothetical protein | -2.64 (0.30) |
| **PGN_1966** | conserved hypothetical protein | -2.70 (0.54) |
| **PGN_1984** | hypothetical protein | +2.28 (0.22) |
| **PGN_2000** | hypothetical protein | -2.32 (0.21) |
| **PGN_2004** | conserved hypothetical protein | -2.46 (0.17) |
| **PGN_2038** | conserved hypothetical protein | +3.54 (0.37) |
| **PGN_2070** | conserved hypothetical protein | +3.29 (0.32) |
| **PGN_2076** | conserved hypothetical protein | -8.19 (0.74) |
| **PGN_2088** | conserved hypothetical protein | -5.95 (0.59) |
| **PGN_2089** | conserved hypothetical protein | -3.46 (0.65) |
| **PGN_2090** | conserved hypothetical protein | -3.35 (0.63) |

# CAPÍTULO VII

# DISCUSIÓN

Los estudios transcriptómicos de *P. gingivalis* ATCC 33277, realizados en esta investigación revelan que esta bacteria modifica su expresión génica global, en relación con su estado planctónico, según sea su fenotipo de crecimiento: planctónico en presencia de *biofilm* y *biofilm* tanto monoespecie (*P. gingivalis*) como multiespecie (*S. oralis, A. naeslundii, V. parvula, F. nucleatum, A. actinomycetemcomitans, P. gingivalis*). Siendo esta última condición, la más propicia para mayores interacciones de comunicación molecular y de intercambio metabólico y genético entre las distintas bacterias. Además, se observa un incremento en el patrón de expresión génica, y un aumento de su capacidad patogénica cuando forma parte de un *biofilm* multiespecie con respecto a las células planctónicas (Liu *et al.*, 2016, Whiteley *et al.*, 2001, Waite *et al.*, 2005, Prigent-Combaret *et al.*, 1999, Schembri *et al.*, 2003).

## 1. DIFERENCIAS CUANTITATIVAS EN EL NÚMERO DE GENES CON EXPRESIÓN DIFERENCIAL

En esta tesis se ha utilizado un modelo estático de *biofilm* subgingival *in vitro* en placa multipocillo reproducible puesto a punto en el Laboratorio de Investigación de la Facultad de Odontología de la Universidad Complutense de Madrid, desarrollado sobre discos de hidroxiapatita (Sanchez *et al.*, 2011). Se ha llevado a cabo una evaluación de la expresión génica global, mediante técnicas basadas en la biología celular (transcriptómica), de la bacteria *P. gingivalis* ATCC 33277 en estado planctónico puro (la situación más sencilla) comparándose con otras tres situaciones fenotípicas, de creciente complejidad:

- Estudio 1: Condición planctónica de *P. gingivalis* en presencia de un *biofilm* monoespecie.
- Estudio 2: Condición sésil de *P. gingivalis* en *biofilm* monoespecie.
- Estudio 3: Condición sésil de *P. gingivalis* junto con otras cinco especies (*biofilm* multiespecie).

Primero, se detectó una diferencia en el transcriptoma de *P. gingivalis* ATCC 33277 entre dos estados planctónicos diferentes, uno en un medio planctónico puro y el otro en presencia de un *biofilm* monoespecie en crecimiento, del que puede estar recibiendo señales de *quorum sensing* para favorecer la agregación. Se trata de una condición de transición entre ambos estados, donde se obtuvo una expresión diferencial en el 1,5% de los genes (28/1909). De ellos, 21 genes fueron sobreexpresados relacionados con el metabolismo del hierro y la recepción de hemina, transposasas y proteasas, el sistema de detección de *quorum* y 7 resultaron reprimidos relacionados principalmente con la adhesión bacteriana, la invasión o la virulencia (Sanchez *et al.*, 2019).

Segundo, se compararon los cambios en la expresión génica de *P. gingivalis* ATCC 33277 de la condición planctónica pura con la condición de *biofilm* monoespecie, obteniéndose una expresión diferencial en el 4,8% de los genes (92/1909). De los cuales, 54 de ellos fueron sobreexpresados y principalmente relacionados con la envoltura celular, el transporte, las proteínas de la membrana externa y 38 reprimidos relacionándose con funciones de transposasas, sistema CRISPR y los genes de estrés oxidativo (Romero-Lastra *et al.*, 2017).

Tercero, *P. gingivalis* ATCC 33277 en presencia de otras cinco especies bacterianas, *S. oralis, A. naeslundii, V. parvula, F. nucleatum* y *A. actinomycetemcomitans*, formando un *biofilm* multiespecie, expresa de manera diferencial el 19,1% de los genes (365/1909) en comparación con el fenotipo planctónico. Estos genes, 165 sobreexpresados y 200 reprimidos, estaban involucrados principalmente en funciones relacionadas con el estrés oxidativo, la envoltura celular, los transposones, CRISPR o metabolismo (Romero-Lastra *et al.*, 2019).

En la **Figura 10** se puede observar una comparativa de los perfiles transcriptómicos de *P. gingivalis*, entre los dos estados planctónicos, y cuando se cultivó como *biofilm* monoespecie o multiespecie, mostrando diferencias significativas entre cada condición estudiada. Se aprecia un claro aumento en el número de genes con expresión diferencial a medida que avanzamos en la complejidad de los fenotipos comparados en la condición experimental.

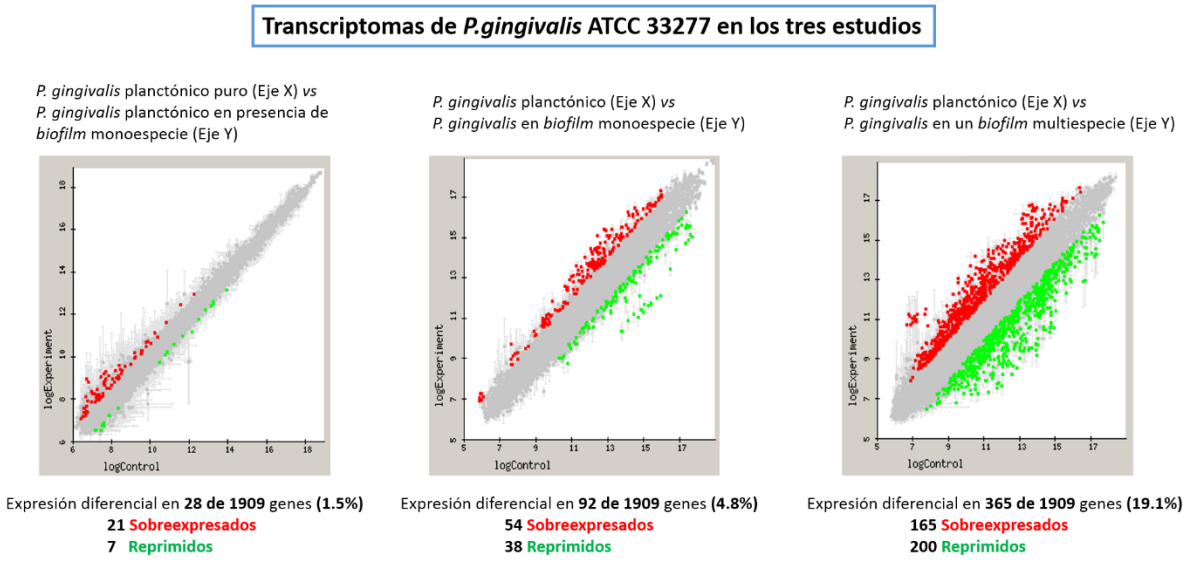


**Figura 10. Comparación de los transcriptomas de *Porphyromonas gingivalis* ATCC 33277 en cada uno de los tres estudios:** 1º) planctónico (control) con planctónico en presencia de *biofilm* monoespecie (*test*), 2º) planctónico (control) con *biofilm* monoespecie (*test*) y 3º) planctónico (control) con *biofilm* multiespecie (*test*).

El rango de genes diferencialmente expresados en los resultados de los dos primeros trabajos en condiciones monoespecie, coinciden con los hallados por otros autores en investigaciones de otros patógenos, como *P. aeruginosa* o *Escherichia coli* cultivados en condiciones de crecimiento similares, y en las que se demostró menos del 5% de la expresión diferencial (Whiteley *et al.*, 2001, Waite *et al.*, 2005, Schembri *et al.*, 2003, Beloin *et al.*, 2004).

Se sigue una tendencia similar en cuanto a la expresión de los genes en estas condiciones monoespecie, de los grupos funcionales tales como aerotolerancia, *quorum sensing*, transporte, fimbrias y metabolismo, entre otros. Es en el tercer estudio, donde además de tener la variable cambio de estado fenotípico (planctónico o *biofilm*), contamos con la presencia de otras especies bacterianas (desarrollo de un *biofilm* multiespecie), cuando se observan los mayores cambios en la expresión génica, obteniéndose un notable reajuste transcriptómico en la adaptación de *P. gingivalis* a un estilo de vida comunitario multiespecie, aumentando el número de genes en cada una de las categorías funcionales **(Tabla 1).**

**Tabla 1. Comparación del número de genes sobreexpresados o reprimidos en cada uno de los tres estudios por grupo funcional.**

| GRUPOS FUNCIONALES | EXPRESIÓN | *P. gingivalis* planctónico *versus* planctónico en presencia de *biofilm* monoespecie | *P. gingivalis* planctónico *versus* *biofilm* monoespecie | *P. gingivalis* planctónico *versus* *biofilm* multiespecie |
|---|---|---|---|---|
| **Estrés oxidativo y virulencia** | Sobreexpresados | **1** | **4** | **20** |
| | Reprimidos | 0 | **7** | **11** |
| **Envoltura celular** | Sobreexpresados | 0 | **9** | **5** |
| | Reprimidos | **2** | **1** | **13** |
| **Aerotolerancia** | Sobreexpresados | 0 | 0 | 0 |
| | Reprimidos | **1** | 0 | **5** |
| ***Quorum sensing*** | Sobreexpresados | 0 | 0 | **1** |
| | Reprimidos | 0 | 0 | 0 |
| **Transporte** | Sobreexpresados | **1** | 0 | **1** |
| | Reprimidos | 0 | 0 | **14** |
| **Transposones** | Sobreexpresados | **2** | **2** | **13** |
| | Reprimidos | 0 | **5** | **3** |
| **Sistema CRISPR** | Sobreexpresados | 0 | 0 | **1** |
| | Reprimidos | 0 | **2** | **1** |
| **Fimbria** | Sobreexpresados | 0 | 0 | 0 |
| | Reprimidos | **2** | **1** | **4** |
| **Ribosomas/RNA** | Sobreexpresados | 0 | **8** | **10** |
| | Reprimidos | 0 | **2** | **8** |
| **Transcripción y Traducción** | Sobreexpresados | **1** | 0 | **6** |
| | Reprimidos | 0 | **2** | **9** |
| **Metabolismo del hierro** | Sobreexpresados | **4** | 0 | **5** |
| | Reprimidos | 0 | 0 | **1** |
| **Metabolismo de ácidos grasos y fosfolípidos** | Sobreexpresados | 0 | 0 | 0 |
| | Reprimidos | 0 | **1** | **2** |
| **Metabolismo energético** | Sobreexpresados | 0 | 0 | **3** |
| | Reprimidos | 0 | 0 | **5** |
| **Metabolismo de ADN** | Sobreexpresados | **1** | 0 | **7** |
| | Reprimidos | 0 | 0 | **12** |
| **Proteínas de unión de ADN** | Sobreexpresados | 0 | 0 | **6** |
| | Reprimidos | 0 | 0 | **1** |
| **Otras funciones** | Sobreexpresados | **4** | **4** | **19** |
| | Reprimidos | 0 | **2** | **31** |
| **Proteínas hipotéticas** | Sobreexpresados | **7** | **26** | **68** |
| | Reprimidos | **2** | **16** | **80** |
| **TOTAL GENES** | | **28** | **92** | **365** |

## 2. DIFERENCIAS CUALITATIVAS POR GRUPOS FUNCIONALES

En la **Figura 11** se pueden ver los grupos funcionales más representativos, así como su tendencia de expresión en cada uno de los estudios que componen esta tesis.

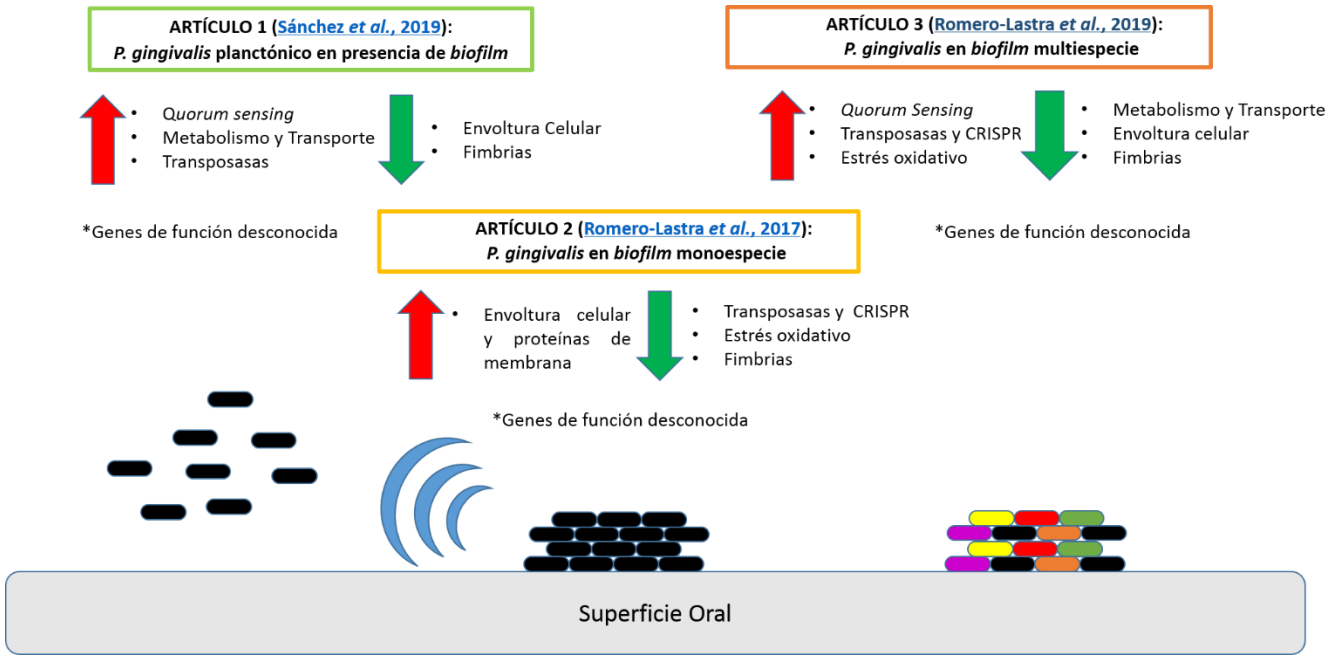


**Figura 11. Grupos funcionales más representativos en cada uno de los tres estudios.**

En un análisis detallado de cada uno de los grupos funcionales que aparecen, observamos que existe un gran número de ellos que son comunes a los tres estudios, encontrándose 23 genes presentes en al menos dos de las tres condiciones estudiadas **(Tabla 2).**

**Tabla 2. Genes diferencialmente expresados [sobreexpresados (+) o reprimidos (-)] comunes a cada uno de los artículos clasificados por grupo funcional.**

| GRUPOS FUNCIONALES | GENES | FUNCIÓN CELULAR | *P. gingivalis* planctónico *versus* planctónico en presencia de *biofilm* monoespecie Cambio (SD) | *P. gingivalis* planctónico *versus* *biofilm* monoespecie Cambio (SD) | *P. gingivalis* planctónico *versus* *biofilm* multiespecie Cambio (SD) |
|---|---|---|---|---|---|
| Estrés oxidativo y virulencia | PGN_0661 | Alkyl hydroperoxide reductase F subunit | | -11,59 (1,16) | -3,89 (0,55) |
| | PGN_1914 | Carboxyl-terminal processing protease | | +2,74 (0,23) | +5,99 (0,39) |
| | PGN_1286 | Probable lysozyme | | +2,63 (0,28) | +3,02 (0,37) |
| Envoltura celular | PG_2131 | OmpA_C-like | -1,61 (0,04) | | -19,57 (6,37) |
| | PG_1712 | Alpha-1,2-mannosidase family protein | -1,52 (0,02) | | -5,53 (1,34) |
| Aerotolerancia | PGN_0529 | Aerotolerance-related membrane protein BatA | -1,62 (0,02) | | -5,86 (1,09) |
| Fimbria | PGN_0181 | Fimbrillin-A associated anchor proteins Mfa1 and Mfa2 | -1,60 (0,09) | | -8,41 (2,84) |
| | PGN_0183 | Minor component FimC | -1,76 (0,16) | | -17,73 (2,28) |
| | PGN_0184 | Minor component FimD | | -2,30 (0,26) | -9,65 (1,85) |
| Ribosomas/ARN | *rpmH* | 50S ribosomal protein L34 ATCC | | +2,41 (0,26) | +4,37 (0,59) |
| | *KsgA* | Dimethyladenosine transferase | | -2,24 (0,06) | -2,53 (0,24) |
| Transcripción y Traducción | PGN_0970 | Putative RNA polymerase sigma-70 factor ECF subfamily | | +3,19 (0,23) | +4,06 (0,70) |
| | PGN_0319 | Probable RNA polymerase sigma-70 factor ECF subfamily | | +5,50 (0,87) | +2,66 (0,23) |
| | PG_1260 | Anaerobic ribonucleoside triphosphate reductase | | -2,54 (0,14) | -12,50 (4,82) |
| | PGN_1312 | Probable transcriptional regulator as Arg-repressor | +1,85 (0,47) | | +2,32 (0,05) |
| | PGN_1396 | Anaerobic ribonucleoside triphosphate reductase | | -2,28 (0,28) | -14,51 (2,00) |
| Metabolismo del Hierro | PGN_0604 | Ferritin | +1,78 (0,08) | | +7,28 (1,54) |
| | *Ftn* | Ferritin | +1,74 (0,12) | | +6,11 (1,48) |
| | PG_2213 | Bacterioferritin-associated ferredoxin proteins | | -3,50 (0,39) | -2,76 (0,18) |
| Metabolismo energético | PGN_1120 | Putative NADPH-NAD transhydrogenase | | +2,26 (0,05) | +3,08 (1,09) |
| Otras funciones | PGN_1206 | Putative methylenetetrahydrofolate | +1,84 (0,04) | | +3,08 (0,68) |
| | PGN_0082 | Probable transcriptional regulator AraC family | | -2,37 (0,33) | -2,99 (0,59) |
| Proteínas hipotéticas | PG_2130 | Hypothetical protein | -1,58 (0,09) | -2,50 (0,21) | -17,17 (3,46) |

## 2.1. Estrés oxidativo y virulencia

En cuanto al grupo de genes de estrés oxidativo y virulencia se puede observar una clara progresión en el número de genes tanto sobreexpresados como reprimidos. Algunos de los genes son coincidentes, entre la condición de *biofilm* monoespecie y multiespecie, como PGN_0661 (*Alkyl hydroperoxide reductase F subunit*), PGN_1914 (*Carboxyl-terminal processing protease*) y PGN_1286 (*Probable lysozyme*), manteniendo la misma tendencia de expresión. Como tendencia general, aumenta la expresión de genes de virulencia según aumenta la complejidad del fenotipo/la comunidad y disminuye la expresión de los genes de resistencia al estrés oxidativo. Estos resultados reflejan la ventaja con la que cuentan las bacterias en *biofilm* multiespecie a la hora de suponer una amenaza patogénica para el hospedador.

Aunque en general los genes de resistencia al estrés oxidativo están reprimidos, se sigue conservando la expresión de algunos de estos genes como *Sod*, tiorredoxina (PGN_0033, PG_0275) o tiol peroxidasa (PG_1729, PGN_0388), que ayudarían en la eliminación de las especies reactivas de oxígeno (ROS), como ya se ha descrito en trabajos de otros autores (Lewis *et al.*, 2009, Moon *et al.*, 2014).

Además, en condiciones de *biofilm* se detecta una expresión al alza de los genes involucrados en los sistemas reguladores de proteínas, como las proteasas o las chaperonas, que generalmente se expresan durante procesos de colonización y adaptación al estrés (Capestany *et al.*, 2008). Esta sobreexpresión es más evidente en el *biofilm* multiespecie, donde se ve favorecida por la presencia de otros microorganismos. Las proteasas sintetizadas, favorecerían la virulencia de la bacteria, participando en la degradación de los anticuerpos del sistema inmunitario del hospedador y en la destrucción del tejido periodontal (Kadowaki *et al.*, 2000). Esto contrasta con el primero de los

estudios, en el que se comparan las dos condiciones planctónicas, donde las diferencias sufridas por la bacteria *P. gingivalis* serían menores, al ser estados similares (Sanchez *et al.*, 2019).

Todos estos resultados apuntarían a que *P. gingivalis*, al encontrarse en los estratos más profundos del *biofilm* multiespecie, recibiría parte de los nutrientes presentes en el medio a través de los canales del *biofilm*, pero además para obtener más cantidad, tendría que atacar activamente los tejidos del hospedador, mediante la sobreexpresión de los genes de virulencia. Por otro lado, el acceso a éstos conllevaría la necesidad de aumentar la expresión de los genes relacionados con estrés oxidativo, como *SodB* o la familia de las tiorredoxinas, por provenir de ambientes aerobios.

## 2.2. Envoltura celular

Los genes de envoltura celular, que codifican las proteínas de membrana externa (OMPs) o lipoproteínas, se considera que cumplen funciones de mantenimiento de la integridad de la membrana y transporte de moléculas como por ejemplo la secreción de sustancias antimicrobianas (Kamaguchi *et al.*, 2003). En la presente tesis se ha observado un gran cambio en la expresión de estos genes, estando reprimidos sólo dos de ellos en el paso de la condición planctónica hacia su transición a *biofilm* monoespecie, y sobreexpresándose nueve de ellos, en el *biofilm* monoespecie maduro.

Sin embargo, el *biofilm* multiespecie (Romero-Lastra *et al.*, 2019) contrasta con el caso anterior ya que aparece un menor número de genes sobreexpresados y muchos más subexpresados, que en el biofim monoespecie (Romero-Lastra *et al.*, 2017), como ya describieron también otros autores (Lo *et al.*, 2009, Yamamoto *et al.*, 2011, Hovik *et al.*, 2012).

Sólo dos genes son coincidentes en dos de las diferentes condiciones PG_2131 (*OmpA_C-like*) y PG_1712 (*Alpha-1,2-mannosidase family protein*), estando reprimidos en el fenotipo planctónico en presencia de un *biofilm* monoespecie y cuando *P. gingivalis* se encuentra incluido en un *biofilm* multiespecie, aumentando más incluso su represión en este caso.

Además, en esta última condición, se pueden encontrar, también, genes sobreexpresados, como PG_0679 (*outer membrane efflux protein*), que está asociado con la resistencia antimicrobiana por ser capaz de bombear estas moléculas fuera de la bacteria (Rahman *et al.*, 2017).

## 2.3. Aerotolerancia

En cuanto a los genes relacionados con la aerotolerancia únicamente se encontró PGN_0529 (*aerotolerance-related membrane protein BatA*) como gen común presente en los estudios donde comparamos las dos condiciones planctónicas y en el *biofilm* multiespecie, encontrándose en ambos casos subexpresado. Además de *Bat A*, cuando *P. gingivalis* se encuentra rodeada de otras cinco especies e inmersa en el *glicocálix*, reprime de manera diferencial otros cuatro genes (*Bat B-C-D-E*). En esta estructura, *P. gingivalis* podría encontrarse protegido contra los oxidantes del ambiente, lo que podría explicar la represión de la transcripción de estos genes de aerotolerancia en comparación con el estado planctónico. Dado que en todos los estudios se trabajó en condiciones de anaerobiosis, los resultados que indican un aumento en el número de genes de aerotolerancia reprimidos, no serían únicamente debidos a la adaptación al nuevo ambiente, sino también como consecuencia de cambios en otras vías de regulación génica.

## 2.4. *Quorum sensing*

Aunque se podría esperar un aumento de los genes de *quorum sensing* en la condición planctónica en presencia de *biofilm* por las señales químicas que ahora estarían afectándola, no se puede apreciar una variación en estos genes. A pesar de esto, el gen sobreexpresado PG_2094, aunque catalogado como proteína hipotética, presenta cierta identidad compartida con la proteína SDJ2, gen codificante de un factor de transcripción de la familia luxR, principal factor responsable del *quorum sensing* (Fuqua *et al.*, 1994).

Sin embargo, en el *biofilm* multiespecie, se puede observar un aumento de la expresión del gen PGN_1827 (*MetK*) que codifica para S-adenosil-metionina sintetasa, responsable de la síntesis de un importante precursor de la principal molécula señalizadora del *quorum sensing*, autoinductor-2 (AI-2) (Miller *et al.*, 2001, Parveen *et al.*, 2011). Esto reflejaría que la regulación para adaptarse a otras formas de crecimiento no sería únicamente por un aumento en la capacidad de recepción por parte de las células planctónicas, sino también por el incremento de la síntesis de señalizadores por parte del *biofilm*.

## 2.5. Transporte

*Porphyromonas gingivalis* mostró niveles de expresión reducidos en catorce genes de proteínas transportadoras dentro de una comunidad multiespecie, cinco de estos (PG_0646, PG_0647, PG_0648, PGN_0685, PGN_0686) son transportadores ABC de hierro. Este nivel reducido de expresión puede indicar que los microorganismos como *P. gingivalis* en condiciones de *biofilm*, tienen una actividad metabólica limitada pero más específica, o que, al extraer nutrientes de los tejidos como se describió en apartados anteriores, el acceso a medios abundantes en hierro, como puede ser la sangre, no haga

tan necesarios los transportadores relacionados con este elemento y de hecho requieran la subexpresión de algunos, para evitar una cantidad exacerbada de estos compuestos que pueden ser oxidantes.

## 2.6. Transposones y Sistema CRISPR

En las condiciones monoespecie, ya sea en la comparación de las dos situaciones planctónicas o en la comparación con el *biofilm*, se puede observar una sobreexpresión de dos de los genes codificantes para transposones. Cuando *P. gingivalis* forma parte de un *biofilm* multiespecie, la relación competitiva/colaborativa que se establece y la presencia de material genético de otras especies, que favorece la transferencia horizontal y su adaptación, produce un notable aumento en el número de genes con expresión diferencial y especialmente en los que incrementan su expresión génica. Además, se ha descrito que la transposición está causada por estrés celular (Zhang *et al.*, 2011, Wheeler, 2013, Arnault *et al.*, 1994), por lo que este aumento en los genes sobreexpresados nos indica que la adaptación a condiciones de *biofilm* multiespecie estaría generando más estrés que en monoespecie (Tribble *et al.*, 2013, Hendrickson *et al.*, 2009).

Este hecho también puede ser observado en el sistema CRISPR, ya que en condiciones monoespecie, o bien no encontramos diferencias de expresión (planctónico en presencia de *biofilm*) o aparecen reprimidos (*biofilm* monoespecie), mientras que su expresión se iría incrementando en entornos multiespecie. Esto parece indicar que estos genes de adaptación y protección contra elementos genéticos móviles (plásmidos y transposones) de microorganismos circundantes (Barrangou *et al.*, 2007, Marraffini *et al.*, 2008, Garneau *et al.*, 2010), van a sobreexpresarse cuando *P. gingivalis* se tenga que adaptar a un entorno con especies competidoras (*biofilm* multiespecie).

## 2.7. Fimbrias

A medida que aumenta la complejidad del fenotipo, aumenta también ligeramente el número de genes reprimidos que codifican para fimbrias. Esto podría parecer poco intuitivo, ya que han sido reconocidas como un importante factor de virulencia, en la adhesión celular y capacidad para invadir los tejidos periodontales (Yoshimura *et al.*, 2009, Weinberg *et al.*, 1997, Malek *et al.*, 1994). Sin embargo, se ha descrito cómo estos genes sólo serían necesarios para las primeras fases de formación y adhesión de las fimbrias y no para el mantenimiento de éstas (Whiteley *et al.*, 2001, Yamamoto *et al.*, 2011). Además, otros autores demostraron que estos genes que codifican para componentes menores [PGN_0181, PGN_0183 (*FimC*), PGN_0184 (*FimD*)] no eran necesarios para la integridad estructural y funcional de las fimbrias (Minion *et al.*, 1986, Krogfelt *et al.*, 1988, Klemm *et al.*, 1987, Nagano *et al.*, 2012, Nishiyama *et al.*, 2007). Este aumento en el número de genes reprimidos en *P. gingivalis* dentro de un *biofilm* multiespecie podría estar causado precisamente porque la bacteria ya estaría incluida de manera estable dentro de los *biofilm*s y podría reducir los recursos dedicados a la producción de estos genes.

## 2.8. Ribosomas/RNA, Transcripción y Traducción

En general, a medida que avanza la complejidad del fenotipo de la bacteria, el número de genes ribosómicos sobreexpresados o reprimidos aumenta. Este aumento indica una regulación de la transcripción y la traducción cuando *P. gingivalis* se encuentra dentro del *biofilm* tanto monoespecie como multiespecie, llevada a cabo por PGN_0970 (*Putative RNA polymerase sigma-70 factor ECF subfamily*), PGN_0319 (*Probable RNA polymerase sigma-70 factor ECF subfamily*) y PG_1260 (*Anaerobic ribonucleoside triphosphate reductase*) comunes en ambas condicones experimentales. La síntesis y

mantenimiento de ribosomas supone un gasto energético elevado, por lo que su regulación debe estar muy controlada y podría explicar que en ninguna de las condiciones veamos una clara deriva hacia la sobreexpresión o subexpresión (Moon *et al.*, 2014).

## 2.9. Metabolismo

Al encontrarse dentro de un *biofilm* multiespecie, *P. gingivalis* subexpresa mayor número de genes de los que sobreexpresa en su metabolismo, comparado con su condición planctónica. Estos cambios no se aprecian en la condición de *biofilm* monoespecie, por lo que estas variaciones en la transcripción pueden atribuirse a la presencia de diferentes especies bacterianas. Por el mecanismo del cometabolismo la existencia de las otras especies supondría quizá que *P. gingivalis* pueda disponer de sustratos y nutrientes de más fácil asimilación que le puedan permitir no tener todas las funciones activas y entrar en "modo de ahorro de energía".

Esto tiene implicaciones en la duplicación bacteriana, como podemos observar en la expresión disminuida de los genes de replicación del ADN (xseA, tmk, recJ, PG_1038, PG_0174, PGN_1992, PGN_1314, PGN_1225) y el aumento de la expresión de los genes de proteínas de unión al ADN (PGN_1415, hup-2, PG_2040, PG_0555, PGN_1986, PGN_1393). Estos resultados parecerían indicar que, en esta situación de *biofilm* multiespecie, por una parte habría menor replicación y metabolismo y por otra, que los genes que sí se expresen se emplearían en la secreción de fragmentos de ADN, como parte de los mecanismos de intercambio genético.

Nuestros resultados en cuanto a los niveles de expresión génica, coinciden con los mostrados por otros autores a nivel proteómico (Mohammed, 2018), en donde *P. gingivalis* ve reducida la expresión de muchas de sus proteínas implicadas en

metabolismo de lípidos o ácidos nucleicos al comparar su crecimiento planctónico, *biofilm* monoespecie o en *biofilm* dual, acompañado por *F. nucleatum*.

## 2.10. Genes de función desconocida

En todos los casos en los que se ha comparado un estado planctónico con uno de *biofilm* el grupo más numeroso con cambios transcriptómicos ha sido el de las proteínas hipotéticas, de las que se desconoce su función. Esto pone de manifiesto que todavía hay un gran número de genes sin identificar y que parecerían tener un papel relevante en el desarrollo del *biofilm* y en los factores de virulencia de *P. gingivalis.* La identificación y cuantificación de estos genes pueden proporcionar candidatos potenciales que podrían ser objetivo para el control de la inclusión o el crecimiento de *P. gingivalis* en un *biofilm*.

## 3. VENTAJAS Y LIMITACIONES DEL MODELO

Hay, sin embargo, algunas limitaciones asociadas a este estudio, ya que al utilizar un modelo estático no puede simular las condiciones de flujo a las que están sometidas las bacterias en la cavidad oral (flujo salival y crevicular), que pueden condicionar las propiedades estructurales y fisiológicas de los *biofilm*s y modificar su expresión génica (Vaughan *et al.*, 2010, Drescher *et al.*, 2011).

Además, trabajar con muestras orales de pacientes, o de entrada con un gran número de especies bacterianas *in vitro* es complicado, por lo que este estudio se ha llevado a cabo sobre la base de un análisis comparativo de la expresión génica de *P. gingivalis* en condiciones planctónicas o de *biofilm* monoespecie (Sanchez *et al.*, 2019, Romero-Lastra *et al.*, 2017). Para posteriormente emplearse un modelo *in vitro* de consorcio multiespecie (Romero-Lastra *et al.*, 2019), incorporando a las bacterias de *P. gingivalis,* otras cinco especies diferentes implicadas en el desarrollo del *biofilm* subgingival en las enfermedades periodontales, para estudiar su expresión génica global.

El *biofilm* multiespecie utilizado incluye representantes de los diferentes complejos de la placa subgingival y es un modelo de uso en el laboratorio donde se estandarizan tratamientos, implantes, materiales, etc., por lo que constituye una excelente plataforma para analizar la posible influencia en la expresión génica de *P. gingivalis* de las especies acompañantes en el *biofilm*. En este modelo de *biofilm* de seis especies hemos encontrado ya un número importante de diferencias en la expresión génica, por lo que cabría esperar que hubiera diferencias aún mayores si se aumentara el número de especies cohabitantes.

En la literatura científica hay muy pocos estudios que aborden los perfiles de expresión de patógenos periodontales en *biofilms* multiespecie (Park *et al.*, 2014, Redanz *et al.*, 2011), por lo que nuestra comprensión de los procesos regulatorios e interacciones, que permiten a *P. gingivalis* crecer dentro de estas estructuras y desarrollar su virulencia todavía es limitada.

## 4. LÍNEAS FUTURAS

Actualmente nos encontramos en los primeros pasos para comprender el funcionamiento de estas comunidades, por tanto, el conocimiento de la expresión de los genes identificados y la investigación con genes de los que aún se desconoce su función (que codifican para proteínas hipotéticas), nos permitirá comprender mejor el proceso regulatorio que acontece en la compleja y diversa comunidad microbiana oral.

La aplicación de nuevas tecnologías para el estudio genético, junto con métodos para la integración de grandes conjuntos de datos de expresión génica a nivel de la comunidad microbiana, tanto en condiciones simbióticas como disbióticas y su importancia funcional, permitirá una visión global y holística de la etiología multifactorial de las enfermedades periodontales. Combinando la contribución no sólo de las poblaciones microbianas, sino también del hospedador y el ambiente.

La comprensión de los factores y el proceso que se requiere para mantener los estados simbióticos y disbióticos, así como los factores estresantes que conducen de un estado a otro, podría constituir una potente herramienta para entender mejor la formación del *biofilm*, desarrollar estrategias para poder desorganizarlo y que sea más susceptible de ser atacado, pudiendo prevenir, en cierto modo, el desarrollo de enfermedades periodontales.

# CAPÍTULO VIII

# CONCLUSIONES

## CONCLUSIÓN GENERAL

El perfil de expresión génica global de *P. gingivalis* ATCC 33277 se ve afectado por el estado fenotípico en el que se encuentre, planctónico puro, planctónico en presencia de *biofilm* o *biofilm* monoespecie o multiespecie. La complejidad del fenotipo adoptado por la bacteria *P. gingivalis* ATCC 33277 incrementa el número de genes con expresión diferencial en su transcriptoma.

## CONCLUSIONES ESPECÍFICAS

**1.** En la comparación de dos condiciones planctónicas similares (planctónica pura o planctónica en presencia de un *biofilm* monoespecie), *Porphyromonas gingivalis* ATCC 33277 expresa de manera diferencial el 1,5% de sus genes (28/1909).

**2.** *Porphyromonas gingivalis* ATCC 33277 en sus dos fenotipos de crecimiento monoespecie (planctónico y *biofilm*) expresa de manera diferencial el 4,8% de sus genes (92/1909).

**3.** *Porphyromonas gingivalis* ATCC 33277 en presencia de otras cinco especies bacterianas (*S. oralis, A. naeslundii, V. parvula, F. nucleatum* y *A. actinomycetemcomitans)* formando un *biofilm* multiespecie, expresa de manera diferencial el 19,1% de sus genes (365/1909) en comparación con el fenotipo planctónico.

Por tanto, los estudios *in vitro* de los cambios de estado bacterianos entre planctónico y biofilm, así como las interacciones que pueden ocurrir entre las distintas especies, son necesarios para obtener resultados más completos de los diferentes grupos funcionales.

# CAPÍTULO IX

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