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Evidence of cross gene regulation of some virulence factors of *Porphyromonas gingivalis* by *Streptococcus intermedius*

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Periodontal disease has been associated with poor dental care, which promotes the accumulation of bacteria and the development of diseases of the mouth. *Porphyromonas gingivalis* are anaerobic Gram-negative bacteria found in the subgingival plaque. They are largely responsible for chronic periodontal disease. *Streptococcus intermedius* is a Gram positive coccus found in the supragingival plaque. The objective of the present work was to evaluate the expression of several virulence genes of *P. gingivalis* in a mixed culture with *S. intermedius* using qPCR and heterologous microarrays. *P. gingivalis* ATCC 33277 and W83 and *S. intermedius* ATCC 27335 strains were cultured and total RNA was extracted using the High Pure RNA isolation kit. Oligodeoxynucleotides were designed to make multiple comparisons with organisms. Microarray was performed to identify gene expression. To quantify gene expression, cDNA samples from three different *P. gingivalis*:*S. intermedius* ratios were diluted 10^{-1}, 10^{-2} and 10^{-3}. The microarray experiment indicated that in *P. gingivalis,* 29 genes were upregulated. The putative function of upregulated genes was the biosynthesis of different metabolic pathways. Heterologous microarrays are a new approach that are useful for investigating gene expression.

Key words: *Porphyromonas gingivalis, Streptococcus intermedius*, periodontal disease, virulence genes, cross gene regulation.

INTRODUCTION

There are two major diseases that develop in the oral cavity; periodontal disease and dental caries (Eke et al., 2012; Bagramian et al., 2009). The *Streptococcus anginousus* group (AGS), also known as the *S. milleri* group, have been considered opportunistic pathogens in the oral cavity where they form part of the normal oral flora in humans. *Streptococcus intermedius* is a bacteria of this group that can promote the development of deep-seated infections in the brain and liver and has been associated with periodontal disease and implantitis (Tomoyasu et al., 2010; Pecharki et al., 2008). *S. intermedius* also contributes to the formation of biofilms that act as a protective layer against antibiotics in chronic infections (Ahmed et al., 2009). *S. intermedius* secretes intermedilysin, a cytrolysin which has been considered a major virulence factor that has been determined an important factor in tissue infection and cytotoxicity in human cells (Tomoyasu et al., 2010). *Porphyromonas gingivalis*

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Table 1. General information of primers designed based on registered sequences in GenBank (http://www.ncbi.nlm.nih.gov).

Primer	Sequence (5' – 3')	Size (bp)	Accession number
PG0538F	TCTGGGAGGGCAGTACACCTATTCGC		
PG0538R	AGGCGTCCAGCGTTGTTGTCCCTCG	76	AEO15924
PG1280F	TGCCGATCAGCTCACAATCATC		
PG1280R	GCTTGGAGGGGCGATATTCTTC	147	AEO15924
PG0520F	CGCAGCCGGAAACAAAGAAGG		
PG0520R	GCAGCACCCACGTAAAGAACAG	150	AEO15924

is a Gram-negative oral anaerobe that is associated with periodontitis. It has been found associated with *Bacteroides forsythus*, *P. gingivalis* and *Treponema denticola* in bacterial complexes in subgingival plaque (Socransky et al., 1998). It is also the most-studied oral pathogen, partly because it produces several virulence factors and partly because it is relatively easy to grow and manipulate genetically (Chen et al., 2004).

Quorum sensing is a change in gene expression in response to signal molecules known as autoinductors (AI). An increase in bacterial cell population density will produce an increase in the concentration of these signal molecules. When the concentration of AI reaches a threshold level, activation of signal transduction will induce the expression or repression of target genes to produce a behavioral adaptation. Most bacteria that form biofilms use autoinductors to communicate (Williams, 2007; Atkinson and Williams, 2009).

Microarrays are used to measure the expression levels of a large number of genes simultaneously. Chen et al. (2004) compared *P. gingivalis* W83 and *P. gingivalis* ATCC 33277. *P. gingivalis* ATCC 3277 was found 7% similar to virulent types W50 ATCC 53978 and W83 ATCC BAA-308. This proved the feasibility of the test with regard to previous experiments (Chen et al., 2004). The goal of this study was to use a gene microarray to identify the expression of several virulence genes of *P. gingivalis* by quorum sensing in a mixed culture with *S. intermedius* considering that when the number of cells of *S. intermedius* increases, it can trigger the expression of virulence genes in *P. gingivalis*.

MATERIALS AND METHODS

Strains and culture conditions

The strains used include *P. gingivalis* ATCC 33277 and W83 *(*kindly provided by Dr. Yumiko Hosogi of the Forsyth Institute), and *S. intermedius* ATCC 27335. Lyophilized strains were activated in tubes containing 3 mL of Trypticase Soy Broth (TBS) (Difco, Becton Dickson, Franklin Lakes, NJ) and incubated at 37°C for 48 h. After two subcultures under the same conditions, cultures were used to inoculate three 250 mL flasks containing 10 mL of TBS with different *P. gingivalis: S. intermedius* culture ratios (1:1, 1:10 and 10:1). These were determined by volume, where 1:1 was 1 mL of *S. intermedius* and 1 mL of *P gingivalis*, 1:10 1 mL of *S. intermedius* and 9 mL of *P gingivalis* and 10:1 9 mL of *S. intermedius* and 1 mL of *P gingivalis*. Cultures were incubated at 37°C and stopped at 48 h.

RNA extraction and cDNA generation

The resulting biomass of 3 mL of each culture was washed twice with Tris-HCl Buffer (10 mM, pH 8.0) and total RNA extraction was carried out using the High Pure RNA isolation kit (Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer´s recommendations. The total RNA concentration was read at 260 nm in a Smart Spect™ DNA spectrophotometer (Bio-Rad Laboratories, Philadelphia, PA). cDNA was generated using the total RNA isolated from different cultures conditions using the Transcript First Stand cDNA system kit® (Roche).

Pimer design (oligos)

In order to confirm the bacterium strains, oligodeoxynucleotides were designed based on the rDNA 16S gene of *P. gingivalis* and *S. Intermedius* in GenBank (http://www.ncbi.nlm.nih.gov/). The purpose was to make multiple comparisons with organisms involved phylogenetically and with the disease. The comparison was performed using the BLAST program (http://www.ncbi.nlm.nih.Gov/blast/) and DNAStar Primer Select software (DNASTAR, Inc., Madison, WI). In order to evaluate the effect of *S. intermedius* on *P. gingivalis* behavior, we selected the genes PG0520 (chaperonin), PG0538 (outer membrane exflux protein) and PG1280 (hypothetical protein conserved), which are differentially regulated by LuxS in *P. Gingivalis* and which could be implicated in the quorum sensing response.

Genes and primers sequence were designed as previously reported (Yuan et al., 2005) (Table 1). To assess gene expression of genes involved in quorum sensing, oligonucleotides were designed based on DNA gene sequences from GenBank. The gene encoding for glyceraldehyde 3-phosphate dehydrogenase (GADPH) was included as a housekeeping gene for qPCR, but the primer nucleotide sequence is not reported by the manufacturer (Roche). Previous to the use of the oligodeoxynucleotides with experimental cultures, their specificity was tested using a pure culture of *P. gingivalis*.

qPCR

Quantitative PCR was performed in a Light Cycler 1.5 System (Roche) using the Light Cycler® Fast Star DNA Master SYBER Green I® mix (Roche), according to the manufacturer's recommendations. Gene amplification was done in a final volume of 20 µL as follows: one denaturalization cycle at 95°C for 10 min with a ramp rate of 20°C/s; 35 cycles at 95°C for 10 s with a 20°C/s ramp rate; 60°C during 5 s with a 5°C/s ramp rate; 72°C for 10 s with a 20°C/s quantification analysis ramp rate; one 95°C melting cycle with

a 20°C/s ramp rate; 65°C for 15 s with a 20°C/s ramp rate; 95°C with a 0.1 second ramp rate and meeting curve analysis; finally, 1 freezing cycle at 40°C during 30 s with a 20°C/s ramp rate.

Microarray analysis

Microarray were performed at the Unidad de Microarreglos de DNA (Instituto de Fisiologia Celular, UNAM).

Printing of arrays

Fifty (50) mer oligo library of the whole genome of *E. coli* from MWGBiotech Oligo Sets (http:/www.mwgbiotech.com) were resuspended to 40 µM of Micro Spotting solution (Telechem International Inc., Sunnyvale, CA). SuperAmine coated slides 25 x 75 mm (TeleChem International Inc.) were printed in duplicate, and fixed at 80°C for 4 h. For pre-hybridization, the slides were re-hydrated with water vapor at 60°C, and fixed with two cycles of UV light (1200J). After boiling for two minutes at 92°C, slides were washed with 95% ethanol for 1 min and prehybridzed in 5X SSC, 0.1% SDS and 1% BSA for 1 h at 42°C .The slides were washed and dried for further hybridization.

Probe preparation and hybridization to arrays

Total RNA (10 µg) were used for cDNA synthesis incorporating dUTP-Alexa555 or dUTP-Alexa647 using the Firs-Strand cDNA labeling kit (Invitrogen, Mexico City, Mexico). Incorporation of fluorophore was analyzed using the absorbance at 555 nm for Alexa555 and 650 nm for Alexa647. Equal quantities of labeled cDNA were hybridized using hybridization solution HybIT2 (TeleChem International Inc.). The arrays were incubated for 14 h at 42°C, and then washed three times with 1X SCC, 0.05 % SDS at room temperature.

Data acquisition and analysis of array images

Acquisition and quantification of array images was performed in ScanArray 4000 with its accompanying software ScanArray 4000 from Packard BioChips. All images were captured using 65% PMT gain, 70 to 75% laser power and 10 µm resolution at 50% scan rate. For each spot, the Alexa555 and Alexa647 density mean value and the background mean value were calculated with software ArrayPro Analyzer from Media Cibernetics.

Data analysis

An Ecoli_07_04 chip was hybridized with the sample 1:1 labeled with Alexa555 against sample 9:1 labeled with Alexa647. Microarray data were analyzed with GenArise software, developed in the Computing Unit of the Cellular Physiology Institute of the Universidad Nacional Autónoma de México (http://www.ifc.unam.mx/genarise/). The local background value was subtracted from the intensity of each spot, and normalization was applied to the slide. The normalized ratio of the expression of the experimental sample to the expression of the control was calculated with GenArise. GenArise carries out a number of transformations: background correction, lowess normalization, intensity filter, replicate analysis and selecting differentially expressed genes. The goal of GenArise is to identify which genes show good evidence of being differentially expressed. The software identifies differentially expressed genes by calculating an intensity-dependent z-score. A sliding window algorithm was used to calculate the mean and standard deviation within a window surrounding each data point, and define a z-score where z measures the number of standard deviations a data point is from the mean.

$$zi = (Ri - mean(R)) / sd(R)$$

Where, zi is the z-score for each element; Ri is the log-ratio for each element, and sd(R) is the standard deviation of the log-ratio. With this criterion, the elements with a z-score > 2 standard deviations would be the significantly differentially expressed genes.

RESULTS

Primer specificity was tested with pure cultures of *P. gingivalis*. The results of amplification (CT) of the genes PG0520, PG0530, PG1280 and GAPDH in different repetitions are shown in Figure 1. In cycle 21, the positive signal begins for the initiators PG0538, later in cycle 26 for PG1280, and finally in cycle 28 for PG0520. GAPDH showed a positive signal from cycle 35 and 37.

The results of gene expression of the three genes normalized to GAPDH are shown in Table 2. In order to quantify gene expression, cDNA samples from three different *P. gingivalis*: *S. intermedius* (Pg:Si) ratios (1:0/Pg alone) were diluted 10^{-1}, 10^{-2} and 10^{-3} and gene amplification was run. We presented representative results obtained with the 10^{-1} dilution (Table 2). These results show that in pure culture (Pg), the level of gene expression of PG0520 was the same as that of GAPDH; gene PG0538 is upregulated and gene PG1280 is downregulated. When *P. gingivalis* was cultured with *S. intermedius* in different ratio conditions (1:1, 1:10 and 10:1), all the genes were upregulated.

The microarray data were analyzed with GenArise software. The genes that up/downregulated were identified in a 9:1 sample. The genes that up/downregulated in *P. gingivalis* are shown in Table 3. Annotation of the identified genes was performed with DAVID Functional Annotation Bioinformatics Microarray Analysis.

The microarray experiment indicated that in *P gingivalis,* 29 genes were upregulated (Table 3) while 12 genes were downregulated (Data not shown). These genes or ORFs were categorized by their differential regulation. PG0310 is expressed on the ribosome large subunit. The putative function of upregulated genes was the biosynthesis of different metabolic pathways; in the case of downregulated genes, their functions were cell division, protein synthesis, DNA repair, and glycolysis/gluconeogenesis metabolism. Other down/upregulated gene functions are not listed in the table. These genes were mainly involved in microbial metabolism in diverse environments, purine metabolism, peptidoglycan biosynthesis, amino sugar and nucleotide sugar metabolism, methane metabolism, RNA degradation, and lysine byosinthesis, among others.

DISCUSSION

Gene expression of the PG0520, PG0538 and PG1280 loci was evaluated with qPCR. Values were compared with those obtained with pure cultures of *P. gingivalis* and

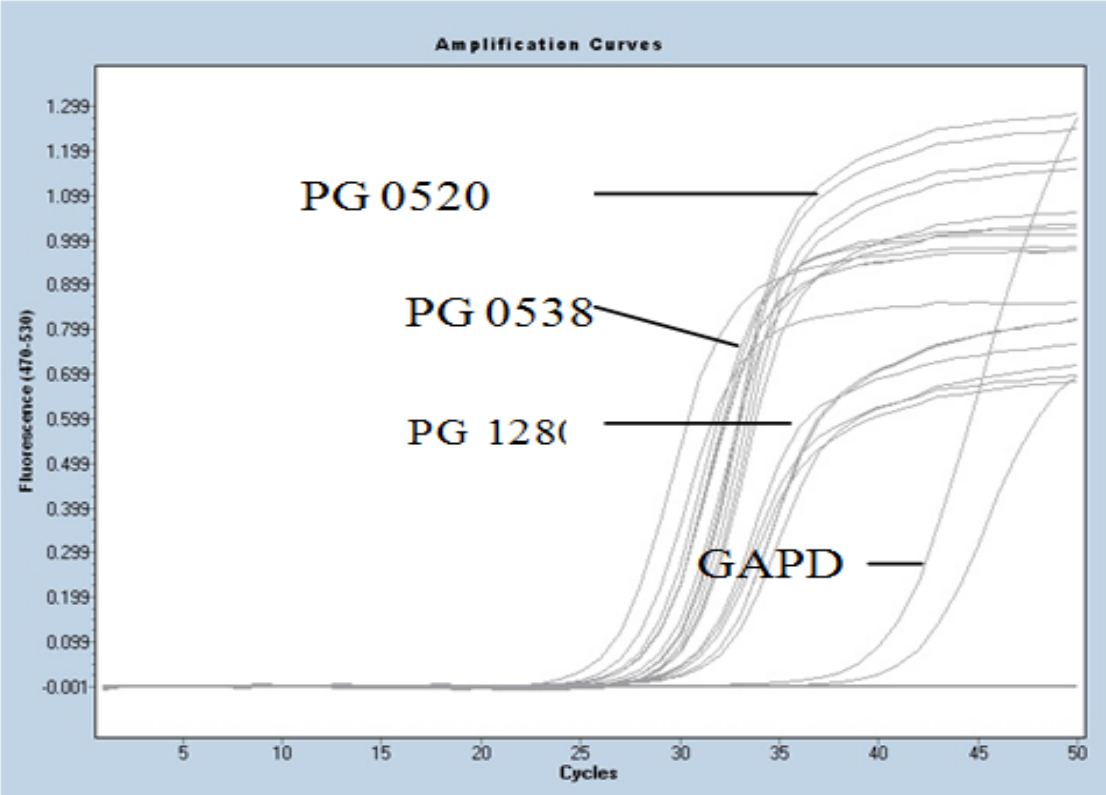

Figure 1. Amplification curves of the *P. gingivalis* genes PG0520, PG0530, PG1280 and housekeeping gene GAPD.

Table 2. Gene expression results of selected genes using the cDNA dilution 10^{-1}. Gene expression was normalized with GAPD.

Pg:Si ratio	Gene		
	PG0520	PG0538	PG1280
1:0	1	1.78	0.87
1:1	1.42	6.35	4.01
1:10	2.5	7.03	4.08
10:1	3.1	8.89	5.02

Pg, *P. gingivalis*; Si, *S. intermedius*

Table 3. Genes or ORFs differently regulated in *P gingivalis.*

Gene name	Locus	Putative function
Upregulated genes		
Ribosomal protein 50s	PG0310	Ribosome unit
Ribosomal protein 30s	PG0310	Ribosome unit
Methionyl-tRNA formyltransferase	PG00670	One carbon pool by folate
serS	PG0316	Aminoacyl-tRNA biosynthesis
PG1239	PG1239	Fatty acid biosynthesis
PG0520	PG0520 molecular chaperone GroEL	Chaperonin
PG0538	PG0538 outer membrane efflux protein	Iron ion transmembrane transporter activity
PG1280	PG1280 hypothetical protein	N-acetylglucosamine 1-carboxyvinyltransferase activity

the different culture ratio conditions of *P. gingivalis*:*S. intermedius*. Regulation of PG0520, PG0538, and PG1280 (virulence genes) showed different expression levels in pure culture. This is in accordance with the study of Yuan et al. (2005) who found the same expression levels for the three genes assessed in the present study. Gene regulation was clear when *P. gingivalis*: *S. intermedius* had a ratio of 1:10. Here the PG0538, PG0520 and PG1280 loci were upregulated. However, when the PG: SI ratio was 10:1, the real value remained relatively unchanged. These results agree with the study of Yuan et al. (2005) who suggest that conditions of the environment may influence gene regulation. It is well known that many microorganisms live in the oral cavity and their cell concentrations and cell ratio are in constant fluctuation. Early reports have suggested that *S. intermedius* are associated with periodontitis (De La Garza-Ramos et al., 2008). On the other hand, Dennis et al. (2002) used a mixed culture system for waste water cleaning, a condition similar to saliva, which suggests that it is possible to find different activities of microorga-nisms through mixed cultures.

The use of heterologous microarrays is a new approach for the investigation of gene expression (Buckley, 2007). In some cases, low specificity may affect the obtained biological results: an effect of low specificity at lower levels accumulates and influences the results at higher levels. For example, a single spot, which contains multiple probes, may simultaneously possess probes that are perfectly hybridized to the target sequence, probes that are partially hybridized, for example by cross-hybridization to nontarget molecules, and probes that are not hybridized to a target molecule. Hence, a combination of high- and low-specificity hybridizations may compose a spot signal, leading to a poor reflection of the target molecule amount (Koltai et al., 2008).

With the aim of understanding and quantifying microarray-hybridization specificity, studies have either modeled various effectors or used empirical means to quantify them. Other studies have applied data filtration, based on some specificity effectors, for increased specificity of the obtained results (Koltai et al., 2008). Thus, with these primary results, the present study shows how different genes are expressed in mixed culture. However, to obtain more information of these interactions, microarrays of native cultures are necessary.

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