Full Length Research Paper

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 anginosus 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 cytolyisin 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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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 autoinducers (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 autoinducers 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 1:100). 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).

#### Primer 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 efflux 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 (GAPDH) 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 denaturation 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/ü/s ramp rate; 72°C for 10 s with a 20°C/ü/s quantification analysis ramp rate; one 95°C melting cycle with

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG0538F</td>
<td>TCTGGGAGGAGGGCACGTTACACCTATTCGC</td>
<td>76</td>
<td>AEO15924</td>
</tr>
<tr>
<td>PG0538R</td>
<td>AGGGTCTCGAGGTGGTGTGGTGCCTCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG1280F</td>
<td>TGCGATCAGCTCACAATCATC</td>
<td>147</td>
<td>AEO15924</td>
</tr>
<tr>
<td>PG1280R</td>
<td>GCTTGGAGGGGGCGATTTCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG0520F</td>
<td>GCAGCGCGAAAACAGAAGGG</td>
<td>150</td>
<td>AEO15924</td>
</tr>
</tbody>
</table>

The rDNA copy number of S. intermedius was calculated by the comparative Ct method and normalized to the GAPDH gene. The S. intermedius gene copy number in the subgingival plaque of healthy subjects was compared with patients suffering from gingivitis and periodontitis.
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 Microregiones de DNA (Instituto de Fisiología 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 (1200 J). After boiling for two minutes at 92°C, slides were washed with 95% ethanol for 1 min and prehybridized 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 Fire-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 Hybrid2 (TeleChem International Inc.). The arrays were incubated for 14 h at 42°C, and then washed three times with 1X SSC, 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 Cibernetica.

**Data analysis**

An Ecoll_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 Autonoma de Mexico (http://www.uic.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.

\[
zi = \frac{Ri - \text{mean}(R)}{\text{sd}(R)}
\]

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/glucoseo-genesis 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 biosynthesis, 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.

<table>
<thead>
<tr>
<th>Pg:Si ratio</th>
<th>Gene</th>
<th>PG0520</th>
<th>PG0538</th>
<th>PG1280</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td></td>
<td>1</td>
<td>1.78</td>
<td>0.87</td>
</tr>
<tr>
<td>1:1</td>
<td></td>
<td>1.42</td>
<td>6.35</td>
<td>4.01</td>
</tr>
<tr>
<td>1:10</td>
<td></td>
<td>2.5</td>
<td>7.03</td>
<td>4.08</td>
</tr>
<tr>
<td>10:1</td>
<td></td>
<td>3.1</td>
<td>8.89</td>
<td>5.02</td>
</tr>
</tbody>
</table>

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

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

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

**ACKNOWLEDGMENTS**

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