

## Pathogenesis and toxins

## Changes in lipopolysaccharide profile of *Porphyromonas gingivalis* clinical isolates correlate with changes in colony morphology and polymyxin B resistance



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## ABSTRACT

Virulence factors on the surface of *Porphyromonas gingivalis* constitute the first line of interaction with host cells and contribute to immune modulation and periodontitis progression. In order to characterize surface virulence factors present on *P. gingivalis*, we obtained clinical isolates from healthy and periodontitis subjects and compared them with reference strains. Colony morphology, aggregation in liquid medium, surface charge, membrane permeability to bactericidal compounds, novobiocin and polymyxin B resistance, capsule presence and lipopolysaccharide (LPS) profiles were evaluated.

By comparing isolates from healthy and periodontitis subjects, differences in colony morphology and aggregation in liquid culture were found; the latter being similar to two reference strains. These differences were not a consequence of variations in bacterial surface charge. Furthermore, isolates also presented differences in polymyxin B and novobiocin resistance; isolates from healthy subjects were susceptible to polymyxin B and resistant to novobiocin and, in contrast, isolates from periodontitis subjects were resistant to polymyxin B and susceptible to novobiocin. These changes in antimicrobial resistance levels correlate with variations in LPS profiles, since -unlike periodontitis isolates- isolates from healthy samples synthesize LPS molecules lacking both O-antigen moieties and anionic polysaccharide. Additionally, this phenotype correlated with the absence of O-antigen ligase activity.

Altogether, our results reveal novel variations on surface components of *P. gingivalis* isolates obtained from healthy and periodontitis subjects that could be associated with differences in bacterial virulence and periodontitis progression.

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### 1. Introduction

Chronic periodontitis (CP) is an inflammatory disease resulting from the imbalance between the microorganisms of the subgingival biofilm and the host immune response. Persistent bacterial challenge in periodontal tissues can induce destruction of

periodontal connective tissue, alveolar bone and tooth loss [6]. In addition, CP has been associated with anaerobic Gram-negative bacteria such as *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* [1,14,30].

*P. gingivalis* can cause dysbiosis, inducing a microbial shift that leads to periodontitis [9,17]. Furthermore, *P. gingivalis* was the most frequent bacteria isolated from chronic periodontitis patients and has been related to disease progression [13,43].

*P. gingivalis* pathogenicity depends on surface antigens and the subsequent immune response produced against them [22,51]. Surface-related components, such as carbohydrates, determine

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differences in the morphology of colonies, hydrophobicity and adherence to human oral epithelial cells [45]. Among surface components of the bacterial envelope, polysaccharides such as K antigen (capsule) and lipopolysaccharide (LPS) are essential for *P. gingivalis* virulence [20,22].

Previous reports have indicated that capsulated *P. gingivalis* strains are more virulent than non-encapsulated strains *in vivo* [22]. K antigen is able to reduce the host immune response, thus enabling bacterial evasion of the immune system and therefore, allowing a successful long-term infection [5]. Seven serotypes of capsular polysaccharides (K1–K7) have been described in *P. gingivalis*, some of which display important differences in virulence [21,44].

On the other hand, the LPS is the major constituent of the bacterial membrane outer leaflet. It is a complex glycolipid composed of three covalently-linked domains: lipid A or endotoxin, which is embedded in the outer membrane; the central oligosaccharide core and the O polysaccharide or O antigen (OAg), which is the most external and variable part of this molecule [39]. *P. gingivalis* W50 synthesizes two different types of LPS: O-LPS, which shows a tetrasaccharide repeating unit of OAg composed of a Glc-Rha-GalNAc-Gal skeleton modified by phosphoethanolamine [35]; and A-LPS, which has a phosphorylated mannan-containing anionic polysaccharide (APS) [41].

*P. gingivalis* produces modified lipid-A molecules, as a result of the lipid-A dephosphorylation at position 4' mediated by 4' phosphatase (*PG1587* gene). This reduces the electrostatic interactions between the bacteria and cationic peptides such as polymyxin B (PMB) and therefore, increases its resistance [8].

Several reports suggest that *P. gingivalis* lipid A is relevant to PMB resistance, but little is known about the role of other LPS components (core, OAg or APS) in this process [8,15,54]. Besides, the participation of other surface virulence factors in bacterial resistance to antimicrobial compounds is poorly understood.

The aim of our study was to characterize and compare the surface properties of *P. gingivalis* isolates obtained from healthy and periodontitis individuals. This will contribute to understand which differences in bacterial surface factors could explain some important aspects of bacterial virulence and disease progression.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

*P. gingivalis* ATCC 33277 and ATCC 53978 (W50) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). *P. gingivalis* defective in OAg ligase ( $\Delta$ *PG1051*, Erm<sup>R</sup>) was kindly provided by Dr. Michael A. Curtis and Dr. Joseph Aduse-Opoku (Queen Mary University of London, Barts, and The London School of Medicine and Dentistry, Centre for Immunology and Infectious Disease, Blizard Institute, London, United Kingdom). All strains, including clinical isolates from chronic periodontitis (CP1–4) and healthy (H1–3) subjects, were grown at anaerobic atmosphere at 37 °C in blood agar (5% defibrinated sheep blood) or in brain-heart infusion broth (BHI; Oxoid), both supplemented with 5 µg/mL hemin–menadione.  $\Delta$ *PG1051* strain was grown in the presence of erythromycin (5 µg/mL).

### 2.2. Clinical isolates obtained from human subjects

Eleven periodontally healthy individuals and sixteen subjects clinically diagnosed with CP were recruited from the Dental Clinic, Faculty of Dentistry, University of Chile, Santiago, Chile under an approved protocol (Table S1), according to the selection criteria described in Table S2. For the clinical diagnosis, probing depth,

attachment level, bleeding on probing and presence/absence of plaque were recorded on six sites per tooth, excluding third molars.

Subgingival plaque samples from CP patients were collected from three periodontally affected sites. From healthy control samples were collected from the four deepest sites [27,28]. After isolating the area with cotton rolls, supragingival deposits were carefully removed and subgingival microbial samples were obtained by inserting standardized #30 sterile paper points (Maillefer<sup>®</sup>) into the periodontal pocket or periodontal crevice for 20 s. Samples from each subject were pooled in a vial containing 1 mL of cold sterilized reduced transport fluid (RTF). Vials with samples were transported at 4 °C to the Oral Microbiology Laboratory of the Faculty of Dentistry, University of Chile, and processed immediately.

*P. gingivalis* clinical isolates were obtained from dispersed subgingival plaque samples by mixing (Thermolyne maxi mix II type 37,600, IA, USA), then plated on supplemented blood agar and incubated anaerobically at 37 °C. Eight black pigmented colonies from each sample were selected under a stereoscopic microscope (Stemi 2000 C, Zeiss), tested for *Porphyromonas* genus identification by negative-fluorescence under UV light (360 nm) and finally confirmed by PCR and 16S rRNA gene sequencing. 12/16 CP and 4/11 healthy samples were positive for *P. gingivalis*. For this work, 4/12 CP and 3/11 healthy isolates (one isolate per individual) maintained viability in cultures and were used for the experiments (Table 1). Finally, a biochemical identification of the clinical isolates was performed using the RapID™ ANA II System.

### 2.3. Molecular biology procedures

To confirm *P. gingivalis* identification from clinical samples, specific primers flanking a conserved region of the gene coding for 16S rRNA were used to amplify a 404 bp fragment. PCR reactions and primers were made based in the protocol previously described [3]. Briefly, the reaction included an initial denaturation step at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 1 min and extension at 72 °C for 3 min, and then a final step at 72 °C for 10 min. The expected amplicon sizes were checked by in Gel-Red stained agarose gels under UV illumination, using a digital imaging system (KODAK Gel Logic 112). Additionally, PCR products were sequenced and compared with the fragment of 16S rRNA that corresponds to *P. gingivalis*, using Human Oral Microbiome Database (HOMD). The specificity of the primers was checked by performing PCR under the conditions described above with DNA extracted from reference and clinical isolates of *Porphyromonas endodontalis*.

PCR reactions were also performed to identify the presence of the *PG1051* gene on *P. gingivalis* clinical samples. Total DNA was

**Table 1**

Clinical characteristics of periodontitis patients and healthy individuals with *P. gingivalis* isolates (mean ± SD).

	Chronic periodontitis (n = 4)	Healthy (n = 3)
Age (mean ± SD)	40.75 ± 12.23	33.33 ± 11.37
% Females	75	66
Probing depth (mm, mean ± SD)	3.54 ± 0.74 <sup>a</sup>	2.69 ± 0.26 <sup>a</sup>
Clinical attachment level (mm, mean ± SD)	4.27 ± 0.76 <sup>b</sup>	1.70 ± 0.35 <sup>b</sup>
% sites with plaque	67.40 ± 28.60	58.24 ± 22.60
% sites with bleeding on probing	51.50 ± 22.20 <sup>c</sup>	18.40 ± 6.54 <sup>c</sup>

<sup>a</sup> Mean probing deep in periodontitis versus control group: P = 0.0426.

<sup>b</sup> Mean attachment level in periodontitis versus control groups: P = 0.0286.

<sup>c</sup> Percentage of sites with bleeding on probing in periodontitis versus control groups: P = 0.0019.

isolated from bacterial strains using a DNA extraction Kit (Favorgen). 1190 bp amplicons corresponding to the *PG1051* were obtained by using primer 1: 5'-GGCGTCAGAACGGTCTTTCTAA-3' and primer 2: 5'-GGAGAAACGGCAAATACCGAGAAC-3'. The amplicons were visualized in Gel-Red stained agarose gels under UV illumination, using a digital imaging system (KODAK Gel Logic 112).

#### 2.4. Macromorphological characterization

Colony morphology of *P. gingivalis* was evaluated under a stereomicroscope (Stemi 2000 C, Zeiss) to determine the shape, color, size, elevation, convexity, surface, texture and opacity of each colony grown in blood-agar supplemented with hemin-menadione. Liquid culture color, aggregation ability and formation of a surface veil were evaluated in BHI supplemented with hemin-menadione.

#### 2.5. Bacterial growth inhibition by diffusion assay

*P. gingivalis* strains were grown anaerobically to exponential phase in supplemented BHI media. Each sample was adjusted to an  $OD_{600} = 0.3$  and aliquots were spread on blood agar plates. Filter disks impregnated with 5 mmol EDTA, 1 mg SDS, or 2.5  $\mu$ g Novobiocin were placed on the plates and incubated anaerobically for 2 days at 37 °C.

#### 2.6. Bacterial growth inhibition by dilution assay

Growth inhibition activity of PMB against *P. gingivalis* strains was evaluated using a liquid microdilution assay [2,8]. Briefly, bacterial cultures were grown to exponential phase. Multiwell plates containing serial dilutions of PMB (0–275  $\mu$ g/mL) were inoculated with an aliquot of bacterial suspension (final  $OD_{550}$  of 0.125). Plates were incubated under anaerobic conditions at 37 °C and after 48 h,  $OD_{562}$  was measured. In parallel, wells containing PMB at all concentrations used, but without the bacterial suspension, were assayed and the recorded  $OD_{562}$  values were subtracted from each concentration. Growth inhibition values for each strain at each PMB concentration were determined by:  $OD_{562}$  in the presence of antimicrobial/ $OD_{562}$  in the absence of antimicrobial, as previously described by [7].

#### 2.7. Lipopolysaccharide analysis

LPS samples were obtained by hot phenol extraction and separated on 14% acrylamide gels using a Tricine-SDS buffer system [23]. The concentration of LPS was determined by measuring 2-keto-3-deoxyoctulosonic acid (KDO) using the Purpald assay [23]. Gels were silver stained as described by [50]. Densitometric analysis was performed using the UN-SCANT-IT gel software (Silk Scientific) as described by [4].

Western blot analysis was performed as described previously [23]. The gel was transferred to a nitrocellulose membrane for 70 min at 250 mA and blocked in 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Immunodetection of APS was performed by incubation with the monoclonal antibody (MAb) 1B5 [10] and then, with a conjugated anti-mouse secondary antibody (Pierce). Detection was performed using the SuperSignal West Pico chemiluminiscent substrate (Pierce) in a Chemiluminescent Western Blot Scanner (C-DiGit™ Blot Scanner-LI-COR).

#### 2.8. Statistical analysis

The Shapiro–Wilk test was used to analyze clinical data distribution. Comparisons in age, probing pocket depth (PD), and clinical

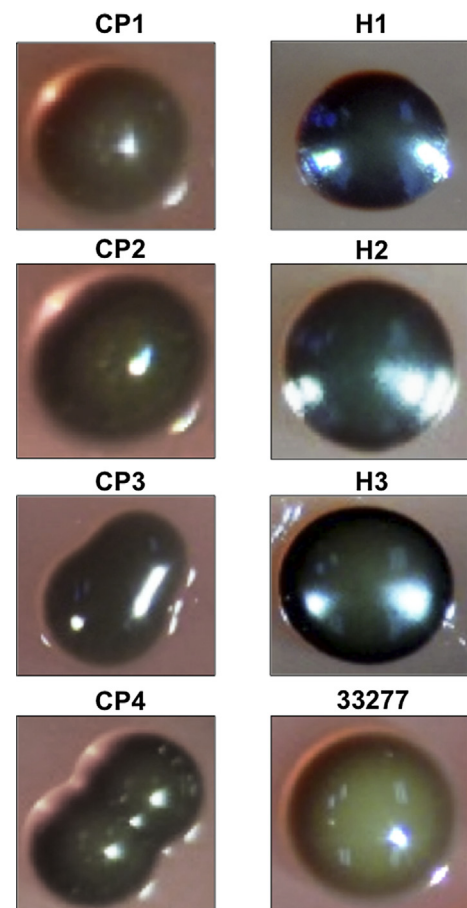
attachment loss (CAL) were made using the two-tailed unpaired Student t test, whereas for periodontal sites with supragingival bacterial plaque, bleeding on probing (BOP), and gender data, the  $\chi^2$  test was used. Experimental data were analyzed by two-tailed unpaired t-tests and one-way ANOVA test (Dunnett posttest). P-values < 0.05 were considered indicative of statistical significance (GraphPad Prism 6.0).

### 3. Results

#### 3.1. Clinical characteristics of healthy and CP subjects and isolation of *P. gingivalis*

From the sixteen subjects with clinical diagnosis of CP and eleven periodontally healthy individuals recruited, twelve (75%) and four (36%), respectively, were positive for *P. gingivalis* according to specific PCR assays. For this study, four *P. gingivalis* from CP and three from healthy individuals were isolated, successfully cultured and characterized in our laboratory.

Clinical characteristics of CP and healthy volunteers with *P. gingivalis* isolates are grouped in Table 1. According to our selection criteria, we detected significantly higher percentages of sites with BOP, CAL and PD. We also observed an average of 46 sites with deep probing greater than or equal to 5 mm in CP ( $p < 0.05$ ) when compared with healthy patient samples, where no sites with deep probing were greater than or equal to 5 mm (Table 1).



**Fig. 1.** Picture of single colonies in solid media under optical microscope of *P. gingivalis* clinical isolates from chronic periodontitis (CP), healthy (H) subjects, and 33277 reference strain.

### 3.2. Clinical isolates from healthy and CP individuals possess differences in colony morphology and aggregation in liquid culture

All the *P. gingivalis* isolates from healthy isolates (H1, H2 and H3) and from CP subjects (CP1, CP2, CP3 and CP4) were confirmed by biochemical identification and 16S rRNA gene sequencing (not shown). The colonies of all the isolates were predominantly round in shape, with an entire margin and convex elevation. The color of *P. gingivalis* colonies ranged from dark gray to black and they were also brittle in consistency (broke apart) (Fig. 1). However, CP colonies were brown-pigmented and presented butyrous consistency. CP isolates were more similar to the 33277 reference strain than the isolates from healthy subjects, although a light greenish brown color was observed in the 33277 strain (Fig. 1).

In liquid media, all isolates displayed pigmentation, although healthy isolates appeared darker than CP isolates (Fig. 2). Formation of a veil was variable: healthy (H3) and CP (CP1 and CP3) isolates were able to form it (not shown). More interestingly, healthy isolates showed stronger aggregation when compared to the CP and 33277 reference strain (Fig. 2).

Previous reports have indicated that aggregation ability in liquid culture is related to differences in bacterial surface charge [55]. To evaluate if these differences in macromorphologic characteristics between our isolates were due to differences in bacterial surface charge, cytochrome C binding affinity was measured. Results showed that binding was similar between most clinical samples and the W50 reference strain (Table S3). By contrast, 33277 strain and CP3 isolate showed a higher binding to cytochrome C,

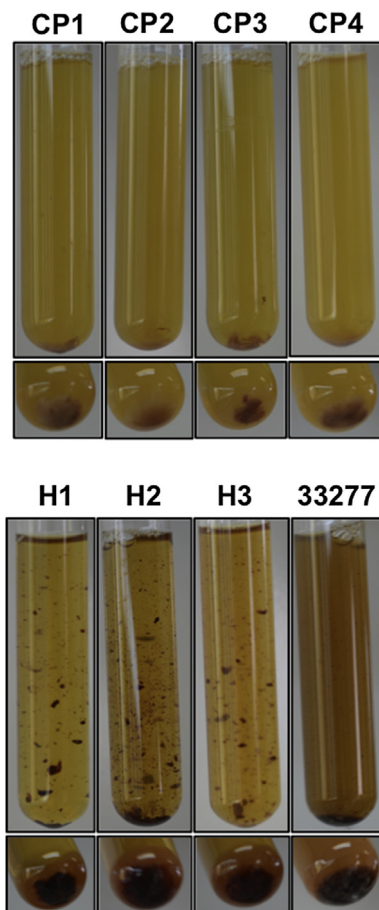


Fig. 2. Picture of cultures in liquid media of *P. gingivalis* clinical isolates from chronic periodontitis (CP), healthy (H) subjects, and 33277 reference strain.

suggesting that these strains possessed a more negatively charged surface. Hence, no correlation in macromorphological characteristics, the ability to aggregate in liquid culture, and surface charge were observed between all isolates.

In addition, no correlation was observed between capsule presence and aggregation abilities, since almost all clinical isolates (except H2) were capsulated (Fig. S2).

### 3.3. Membrane permeability is not impaired in *P. gingivalis* clinical isolates

In order to determine whether differences in colony morphology or aggregation in liquid medium could be due to differences in membrane stability, we determined susceptibility of clinical isolates to the bacteriolytic anionic detergent SDS and the divalent cationic chelating agent EDTA. Our results, obtained from diffusion assays indicate that there were no significant differences in susceptibility between healthy and CP isolates when grown in the presence of EDTA or SDS (Table 2), thus confirming that all isolates display similar membrane permeability.

### 3.4. Clinical isolates from healthy and CP individuals possess different susceptibility to novobiocin and PMB

Bacterial cell envelope stability can also be studied by using some antimicrobial compounds [12,40,56]. To evaluate possible differences in the susceptibility to antimicrobial compounds between *P. gingivalis* isolates obtained from CP and healthy individuals, we performed novobiocin and PMB susceptibility assays. Table 2 shows that healthy isolates were resistant to 2.5 µg novobiocin, in contrast to CP isolates and the reference strain, which were susceptible to this amount of antibiotic.

On the other hand, previous reports have indicated that 100 µg/mL PMB considerably inhibited the growth of the 33277 strain [8]. Our results indicated that growth of healthy isolates was significantly affected at lower PMB concentrations (<20 µg/mL) and that CP isolates were able to grow at higher concentrations (>275 µg/mL) when compared to the 33277 reference strain (Fig. 3). Notice that at all PMB concentrations the growth rates of H2 and H3 overlapped.

### 3.5. Clinical isolates from healthy individuals display altered lipopolysaccharide profiles

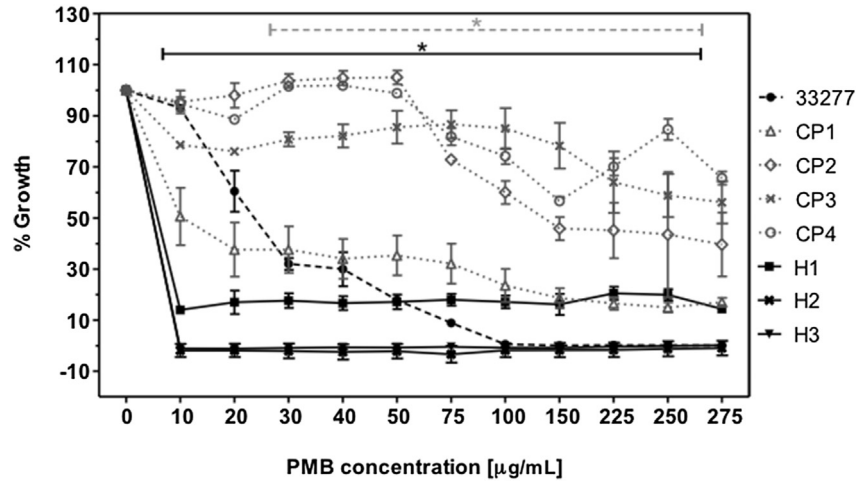
Alterations in LPS composition can induce changes in membrane permeability and, therefore, could permit the entry of antibiotics such as novobiocin [31]. Furthermore, specific *P. gingivalis*

Table 2

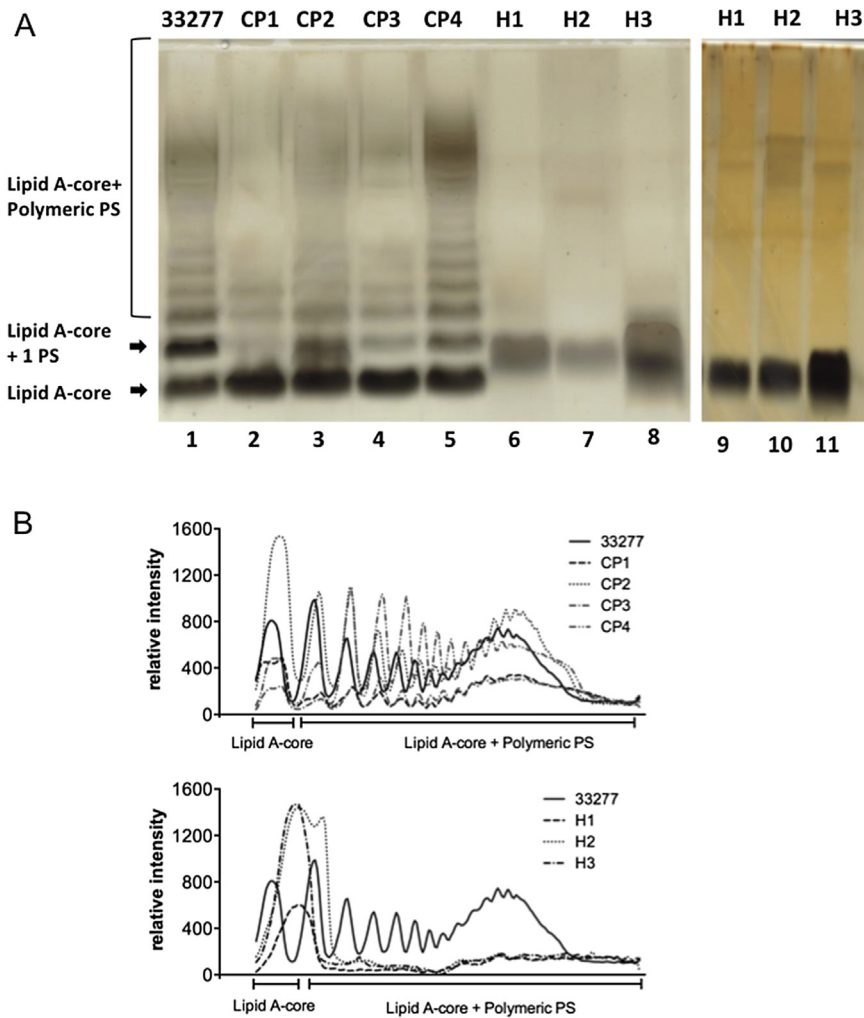
Disc diffusion assays with substances affecting the cell membrane of *P. gingivalis* clinical reference strains (33277, W50) and clinical isolates from chronic periodontitis (CP) and healthy (H) subjects.

Strain	EDTA 5 mmol	SDS 1 mg	Novobiocin 2.5 µg
33277	16 ± 1.00	19 ± 1.00	18 ± 3.00
W50	17 ± 1.15	18 ± 1.15	21 ± 0.58
CP4	22 ± 3.51	22 ± 2.08	28 ± 2.08
CP3	20 ± 5.00	18 ± 1.53	23 ± 2.65
CP1	20 ± 1.53	16 ± 1.00	21 ± 1.00
CP2	19 ± 4.36	20 ± 4.04	21 ± 0.58
H1	20 ± 1.15	15 ± 1.53	6 ± 0
H2	15 ± 2.65	14 ± 0.58	6 ± 0
H3	19 ± 2.08	22 ± 1.00	6 ± 0

Values correspond to diameter of inhibition (mm) ± SD. 6 mm correspond to the diameter of the disc.



**Fig. 3.** Antibacterial activity of PMB against 33277 reference strain (black dashed line), clinical isolates of *P. gingivalis* from chronic periodontitis (CP) (gray dotted lines) and healthy (H) (solid black lines) individuals. Bacterial cultures were incubated in BHI media containing 0–275 µg/mL of PMB. Final OD<sub>550</sub> was measured for each condition and the percentage of growth was expressed relative to the bacteria without polymyxin B. BHI media containing 0–275 µg/mL of PMB without bacteria were subtracted from each concentration. Results represent the means ± SE from two independent experiments in triplicate. Values that are significantly different from that of the wild-type by the one-way ANOVA test and Dunnett posttest are indicated at the top of the figure by a gray dotted line-asterisk for CP and by a solid black line-asterisk for H ( $p < 0.05$ ).



**Fig. 4.** LPS profile from *P. gingivalis* clinical isolates obtained from chronic periodontitis (CP) and healthy (H) individuals. (A) LPS was purified from CP and H isolates, and then analyzed by Tricine-SDS-polyacrylamide gel electrophoresis followed by silver staining. Gel loads were normalized by s. Lane 1: 33277 strain, lane 2–5: isolates from CP patients, lane 6–8: isolates from healthy individuals, lane 9–11 was loaded with a 5-fold excess of LPS. Different regions of LPS are indicated at the left. Polysaccharide region (PS) includes both O antigen and anionic polysaccharide. (B) Densitometric analysis of the gel lanes was performed using the UN-SCANT-IT gel software.

LPS modifications of the lipid-A region have been implicated in PMB bacterial resistance [7]. Moreover, changes in aggregation ability and colony morphology have been related with modifications in LPS of *P. gingivalis* [45,55].

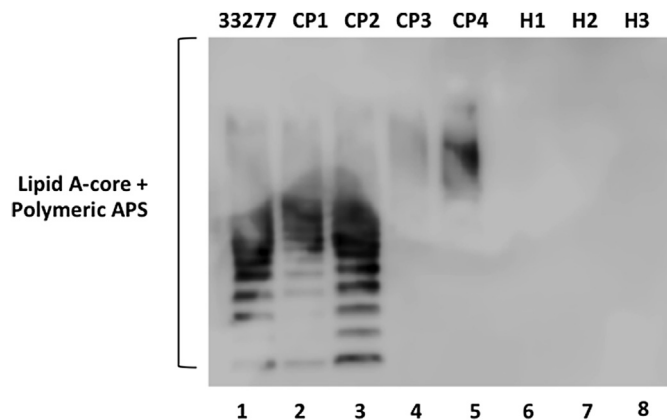
*P. gingivalis* W50 strain produces two types of LPS: O-LPS (with OAg repeating units) and A-LPS (with APS repeating units), both with different composition, but indistinguishable by silver stained SDS-PAGE [36]. Interestingly, *P. gingivalis* isolates from healthy individuals lacked high molecular weight LPS molecules (Fig. 4A, lanes 6–8). The absence of polymeric O antigen was confirmed in a gel loaded with a 5-fold excess of LPS (Fig. 4A, lanes 9–11). In contrast, high-molecular-mass LPS molecules were observed in all CP isolates (Fig. 4A, lanes 1–5). The regions of the LPS were assigned based on the structural analyses performed by [37] and by comparing the LPS profile of a mutant lacking OAg with that of the strain 33277 (Fig. S3). Densitometric quantification of lanes in the gel indicated preferred chain lengths higher than 8 PS units (Fig. 4B) for CP samples. The CP4 isolate presented a LPS with high-molecular-mass PS molecules, but with a different chain length distribution, with a higher amount of intermediate and short length LPS chains (Fig. 4A, lane 4).

### 3.6. Clinical isolates from healthy individuals lack APS

Since APS contains phosphorylated branched mannan repeating units, differences in its phosphorylation status may determine its interaction with cationic peptides. We therefore evaluated the presence of APS in the clinical isolates by Western blot. No APS was observed in healthy clinical isolates (Fig. 5, lanes 6–8) compared with CP samples or the reference strain (Fig. 5, lanes 1–5), indicating that this structure was either not produced or lost in the healthy *P. gingivalis* isolates evaluated in this study. Additionally, CP3 and CP4 isolates only presented high molecular weight APS molecules, compared with CP1, CP2 and the 33277 strain. These three strains also showed low molecular weight APS (Fig. 5, lanes 4–5).

### 3.7. *P. gingivalis* OAg ligase gene was not detected in clinical isolates from healthy individuals

OAg and APS covalently bound to the Lipid A-core portion of the LPS is produced by the OAg ligase enzyme coded by the *PG1051*



**Fig. 5.** Identification of APS in *P. gingivalis* clinical isolates obtained from chronic periodontitis (CP) and healthy (H) individuals. LPS purified from different isolates was analyzed by tricine-SDS-polyacrylamide gel electrophoresis followed by Western blotting, and probed with MAB 1B5 to visualize APS. Gel loads were normalized by the bacterial density at the starting point of the LPS preparations. Lane 1: 33277 strain, lane 2–5: isolates from CP patients, lane 6–8: isolates from healthy individuals. Polymeric APS is indicated at the left.

gene [37,41]. Since, *P. gingivalis* healthy isolates lacks both high molecular OAg and APS molecules, we evaluated the presence of the *PG1051* gene in all clinical strains. We only observed PCR amplification products corresponding to the *PG1051* gene in CP isolates and the 33277 reference strain (Fig. 6) and no amplification was observed in H isolates or in the  $\Delta$ *PG1051* strain used as a negative control [41].

## 4. Discussion

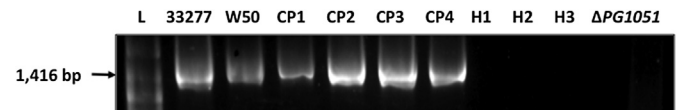
This study reveals significant differences in the composition of some virulence factors among clinical isolates of *P. gingivalis* derived from healthy and periodontitis subjects. These findings contribute to our understanding of the changes in specific species involved in the development of this disease.

After comparing isolates from healthy donors and CP subjects, our results indicated differences in colony color, consistency, and also in auto aggregation ability in liquid medium (Figs. 1 and 2). Previous reports indicated that differences in pathogenicity of *P. gingivalis* strains and isolates from CP patients are related to variations in color, morphology and hydrophobicity of the colonies [26,42,45]. These differences have been attributed to the presence of the capsule, LPS composition, gingipain proteases activity, auto aggregation ability, and biofilm formation ability [24,55]. Additionally, characteristics of colony morphology are associated with the presence of surface-related components (carbohydrates) involved in resistance to antimicrobial compounds [11,19] and adherence to human oral epithelial cells [45], among others.

Some previous reports have demonstrated that a highly negative bacterial charge increases susceptibility to cationic peptides [18,52]. However, other factors should be involved since some species have a low net charge and are also highly susceptible to human beta defensins (hBDs) and other cationic peptides, such as LL-37 [34]. In healthy and CP samples we did not observe a correlation between bacterial charge and susceptibility: in fact, most of the strains showed a similar charge and some resistant strains (CP3 and 33277) showed a low charge. These results indicate that factors other than surface charge could be involved in the differences in macromorphology of the *P. gingivalis* colonies and PMB resistance.

Because of its cationic nature, PMB would interact with the negative charges of lipid A and, subsequently, induce bacterial cell death [8]. In this context, lipid-A desphosphorylation could decrease the electrostatic interactions between LPS and cationic peptides, increasing bacterial resistance to PMB and also to other physiologically relevant cationic peptides, such as hBDs [15,53]. However, we cannot discard that other genes, different from the OAg ligase, could be absent in H isolates, and that some of these genetic differences could indirectly affect the phosphorylation of the lipid A-region. Currently, we are doing additional studies to determine lipid-A phosphorylation levels and also other genetic modifications in the *P. gingivalis* isolates used in this study.

To determine if *P. gingivalis* LPS regions, other than lipid A, are related with PMB resistance, we analyzed LPS profiles of clinical isolates obtained from healthy and CP individuals. CP isolates



**Fig. 6.** Identification of the *PG1051* gene in clinical isolates of *P. gingivalis* from chronic periodontitis (CP) and healthy (H) individuals. Lanes contain PCR products of *P. gingivalis* clinical isolates, using specific primers flanking the *PG1051* gene. The expected amplicon size of the *PG1051* gene corresponds to 1416 pb. Positive and negative controls were wild-type *P. gingivalis* strain ATCC 33277 and the isogenic mutant in the *PG1051* gene, respectively.

produced LPS with high molecular OAg and APS molecules, similar to the reference strain, but isolates from healthy donors produced a rough LPS, lacking both OAg and APS. To our knowledge, this is the first report that show the presence of PS lacking isolates in healthy individuals and that this phenotype correlates with higher sensitivity to PMB. These results are in agreement with those previously reported by [38]; showing that in *Salmonella* Typhimurium strains producing a smaller amount of OAg molecules and OAg polymers with a shorter length, are more susceptibility to PMB [38]. Additionally, the LPS electrophoretic profile of healthy samples exhibited a different mobility pattern of lipid-A-core region, as compared to the reference strain, suggesting that other modifications should exist in the LPS.

Modifications in LPS structure that were initially detected by alterations in the SDS-PAGE electrophoretic mobility have been previously described [32]. In *Burkholderia cenocepacia*, the lack of a predicted glycosyltransferase gene produced a LPS with truncated OAg subunits. Interestingly, this phenotype was related to impaired ability to survive in the presence of serum complement. In the same way, modifications in the lipid A region of *Shigella flexneri* and *Salmonella* Typhimurium, are induced by moderated acidic pH [16,25]. In these studies, the main modification that was reported increased resistance to extreme acid conditions. Moreover, in *S.* Typhimurium, these modifications could confer resistance to cationic antimicrobial peptides by masking negative phosphate groups with positively charged moieties [16]. To evaluate if the differences in electrophoretic mobility of the LPS samples from H isolates are due to chemical modifications of the LPS, we are currently studying the LPS chemical structure and its implications in pathogenicity.

Interestingly, strains lacking high molecular weight OAg and APS molecules probably have differences in the DNA sequence of the OAg ligase gene (*PG1051*), a result that could explain the absence of PCR amplification and the lack of OAg ligase activity. This strongly suggests that the *PG1051* gene is related with the different LPS profiles observed in healthy and CP samples, with the latter possessing a different genetic background than those isolated from healthy donors. This idea is supported by the macromorphological variations that we observed in the phenotype of the samples used in this work. In this regard, an extensive genetic heterogeneity has been observed in species of *P. gingivalis*, which has been mainly attributed to chromosomal DNA transfer between *P. gingivalis* strains [49]. This heterogeneity within the same species has been linked to differences in pathogenicity, since genetic variations have been reported for many of the major bacterial virulence factors related to the onset of disease [48].

The presence and proper distribution of OAg and APS chains are required for membrane integrity and resistance to complement and antimicrobial compounds in many Gram-negative species [29,33,47]. However, the sensitivity to toxic compounds affecting the bacterial cell membrane, such as EDTA or SDS- was similar between samples obtained from healthy and CP donors, suggesting that the absence of high molecular weights OAg or APS molecules did not affect bacterial permeability completely. Moreover, PS presence is specifically related to morphologic differences and PMB resistance. Interestingly, we found that healthy samples were resistant to novobiocin, which is in agreement with previous reports, which indicated that a complete OAg region is necessary for penetration of novobiocin to cells [46].

## 5. Conclusions

In conclusion, we showed that clinical isolates obtained from healthy and CP subjects display differences in macromorphologic characteristics related with surface virulence factors. Remarkably,

healthy isolates showed a LPS lacking high molecular weight PS, probably due to the absence of OAg ligase activity. These isolates were more susceptible to PMB, a model for cationic peptides, suggesting that high molecular weight OAg molecules and APS are relevant to the resistance of *P. gingivalis* to cationic peptides. Therefore, it may also be important for resistance to other more physiologically relevant cationic peptides, and hence, to bacterial virulence. These findings broaden the current understanding about virulence profiles of *P. gingivalis* from healthy and periodontitis subjects. And thus, allows us to understand which of these virulence factors would be key to a successful colonization.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anaerobe.2015.01.009>.

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