Influence of varying hemin concentrations on growth and physiological activity of *Porphyromonas gingivalis* strains with different *fimA* genotypes

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Contents

An article published as indicated below and new unpublished data of *Porphyrom onas gingivalis* MPWIb-01 (type Ib) and HNA99 (type V) strains.

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Abstract

Porphyromonas gingivalis requires optimal heme concentrations to grow. Heme (or hemin) has been utilized by *P. gingivalis* for several purposes including growth and defense against host immune responses which emphasizes the significance of heme for *P. gingivalis*. Moreover, heme can influence *P. gingivalis* physiology, virulence, and both transcript and protein abundances.

Heme availability and concentration in the periodontal pockets vary. Fluctuations in heme concentration may affect each *P. gingivalis* strain differently, however, this was never fully demonstrated. Here, this study elucidated the effects of varying hemin concentrations in representative *P. gingivalis* strains. Throughout this study, representative *P. gingivalis* strains [FDC381 (type I), MPWIb-01 (type Ib), TDC60 (type II), ATCC49417 (type III), W83 (type IV), and HNA99 (type V)] were used and grown in growth media under varying hemin concentrations [25ppm (5×), 5ppm (1×), 2.5ppm (0.5×), 0.5ppm (0.1×)] and time. Most *P. gingivalis* strains grew in 24 h incubation, whereas, both MPWIb-01 (type Ib) and HNA99 (type V) strains grew at 96 h and 72 h incubation time, respectively. Samples were lysed and protein standardized. Arg-gingipain (Rgp), H_2O_2, and superoxide dismutase (SOD) levels were subsequently measured. This study focused on 24 h-grown strains which excluded MPWIb-01 and

HNA99.

Rgp activity among the 4 remaining strains varied with Rgp peaking at: 1× for FDC381, 5× for TDC60, 0.5× for ATCC49417, 5× and 0.5× for W83. With regards to H_2O_2 and SOD amounts: FDC381 had similar H_2O_2 amounts in all hemin concentrations while SOD levels varied; TDC60 had the lowest H_2O_2 amount at 1× while SOD levels became higher in relation to hemin concentration; ATCC49417 also had similar H_2O_2 amounts in all hemin concentrations while SOD levels were higher at 1× and 0.5× ; and W83 had statistically similar H_2O_2 and SOD amounts regardless of hemin concentration. Results show that variation in hemin concentration affect each *P. gingivalis* strain differently.

Introduction

Porphyromonas gingivalis is a black-pigmented gram-negative oral anaerobic bacterium known to be an important pathogen among adults since it contributes to periodontal disease development [1, 2]. Periodontal disease is an inflammatory disease that destroys the periodontal tissues supporting the tooth which ultimately may lead to tooth loss. In the oral cavity, there are over 500 subgingival bacterial species and among which *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia* have been strongly related to periodontal disease infection [3].

P. gingivalis invades periodontal tissues and evades host defense mechanisms [4]. Recent reports have shown the relationship between *P. gingivalis*-related periodontal disease and systemic diseases, such as low-birth weight infants, coronary heart disease, diabetes, respiratory disease, highlighting the importance of *P. gingivalis* [2,5].

All *P. gingivalis* strains require heme to grow which is used as a virulence factor [1, 4, 6]. In humans, hemoglobin is an iron-containing cell and is a major heme source [2, 7].

Hemoglobin is a reliable heme source for *P. gingivalis* which is acquired primarily through the crevicular fluid since the composition is similar to that of serum [8]. Moreover, in a periodontal disease situation hemoglobin concentration in effect would depend on periodontal pocket bleeding associated with periodontal tissue destruction in

the host [1, 2, 9]. Heme availability in the crevicular fluid plays a major part in augmenting *P. gingivalis* growth and virulence which eventually leads to active periodontitis induction [10]. Heme is essential for *P. gingivalis* growth activities [11, 12], however, excess heme concentration can be harmful [11, 12]. It was previously shown that hemin concentration can affect *P. gingivalis* W50 physiology and virulence [13]. Several *P. gingivalis* strains exist in the human gingival pockets and each strain has varying pathogenicity attributable to fimbriae (FimA) and lipid A composition [2, 14, 15]. During a periodontal disease infection, heme concentration may fluctuate which it may affect each *P. gingivalis* strain differently [16]. However, this was never fully demonstrated.

This study elucidated the effects of varying hemin (surrogate stable form of heme) concentrations on selected *P. gingivalis* physiological activities, namely: Arg-gingipain (Rgp), hydrogen peroxide (H_2O_2), and superoxide dismutase (SOD) activities. Similarly, the potential association of varying hemin concentrations and *P. gingivalis* strain growth in a periodontal pocket during a healthy or periodontal disease condition were likewise discussed.

Materials and Methods

Growth medium and treatment conditions

Six *P. gingivalis* strains [FDC381 (type I), MPWIb-01 (type Ib), TDC60 (type II), ATCC49417 (type III), W83 (type IV), and HNA99 (type V)] classified based on their respective FimA genotype were used in this study [13, 17]. All strains were provided as a kind gift from K. Nagano (Aichi Gakuin University, Japan). Glycerol stocked *P. gingivalis* strains stored in -80 ºC were pre-incubated (OD600 = 1.0) in standard growth medium [Gifu anaerobic medium (Nissin, Tokyo, Japan) containing 0.5 μg mL^{-1} menadione and 5μg mL^{-1} hemin concentration [13]]. Similarly, this medium was used for final incubation (OD600 = 0.05) prior to growing strains in varying hemin concentrations. Strains were grown in 40 mL standard growth medium with varying hemin concentrations [25ppm (5×), 5ppm (1×), 2.5ppm (0.5×), 0.5ppm (0.1×)]. Initially, 5 μg mL^{-1} hemin concentration (1×) was set as the standard for *P. gingivalis* growth under anaerobic conditions [13]. Hemin concentration higher (5×) than the standard condition was categorized as hemin-excess while hemin concentrations lower (0.5×, 0.1×) than the standard were categorized as hemin-limited [12].

Incubation and standardization

All bacterial strain cultures were grown at varying times and, after incubation, OD was checked to determine whether bacterial growth was enough for further downstream analyses. Representative *P. gingivalis* cultures were adjusted (OD_{600} = 1.0) using the standard growth medium and centrifuged at 1,500×g for 15 min. Bacterial pellets were processed following a previous publication [18]. Pierce® Microplate BCA Protein Assay Kit-Reducing Agent Compatible Kit (Thermo Scientific) was used to standardize protein concentrations for downstream analyses.

Rgp quantification

Rgp activity was measured following a previously published work [12]. Briefly, 0.5 mM N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA; Sigma) and enzyme activity buffer (0.2 M Tris-HCl, 0.1 M NaCl, pH 7.5) were mixed with lysed bacterial cells and incubated at 37 °C for 20 min. Subsequently, bacterial suspension was measured using a spectrometer (Abs 405 nm).

H_2O_2 and SOD measurements

Red hydrogen Peroxide Assay Kit (Enzo Life Sciences) was used to establish

bacterial H_2O_2 amounts while Superoxide Dismutase Assay Kit (Cayman Chemical Company) was used to detect bacterial SOD levels following an earlier work [12]. All kits were performed according to manufacturer's recommendation. Briefly, for H_2O_2 quantification, H_2O_2 detection reagent mix was mixed with lysed bacterial cells and incubated at room temperature for 30 minutes, protected from light. Subsequently, sample fluorescence were measured using a spectrometer at 570 nm and, afterwards, results were calculated. Similarly, for SOD measurement, two SOD assay kit components were mixed with lysed bacterial cells, mixed, and incubated in a plate shaker for 20 min at room temperature. Subsequently, absorbance was read at 405 nm using a spectrometer and calculated.

Statistical analyses

In all assays performed, the statistical significance of differences between each hemin concentration was determined by one-way ANOVA followed by a multiple comparison test (Tukey's test). Similarly, statistically significant levels were set as significance level of 95 % ($P < 0.05$).

Results

P. gingivalis strains have varying optimum incubation times

To standardize samples for this study, all bacterial strain cultures were initially grown for 24 hours. The result showed that MPWIb-01 and HNA99 were observed to have very low growth activity in all hemin concentrations (OD < 0.03) as compared to others strains (Fig. 1). Both MPWIb-01 and HNA99 grew at 96 h and 72 h incubation time, respectively (Fig. 2). As shown in Figure 2A, MPWIb-01 (type Ib) had optimal growth at 5× hemin concentrations incubated for 96 h as compared to 1× while both 0.5× and 0.1× hemin concentrations were not optimal growth conditions. Similarly and as seen in Figure 2B, HNA99 (type V) had optimal growth at 5× hemin concentration incubated for 72 h as compared to 1×. Moreover, using 0.5× hemin concentration would require more time for the strain to grow, whereas, 0.1× is not an optimal growth condition. It is worth mentioning that type Ib and type V strains were more distributed among patients with periodontitis as compared to healthy patients, however, the frequency of these 2 strains was not at par to that of type II [19]. Thus, MPWIb-01 (type Ib) and HNA99 (type V) strains preferred excess (5×) hemin concentration similar to TDC60 (type II) and, moreso, needed longer incubation time. This would imply that both MPWIb-01 (type Ib) and HNA99 (type V) strains were considered as slow-

growing strains in the periodontal pockets, as compared to demanding- and common-type *P. gingivalis* strains, whereby, both strains require high hemin condition to grow while low hemin concentration is not suitable [15, 19]. In this regard, this study omitted these two strains in further downstream analyses and focused on 24 h-grown strains.

Optimal hemin concentration differs in each *P. gingivalis* strain

Rgp is considered essential for *P. gingivalis* growth [20] making it an ideal growth marker [12]. To determine the growth pattern and optimal hemin concentration of each strain, Rgp activity was measured as previously published [12] and results showed that the *P. gingivalis* Rgp activity among the 4 strains varied (Fig. 3A-D). In particular, the peak for each Rgp activity was: 1× for FDC381 (Fig. 3A), 5× for TDC60 (Fig. 3B), 0.5× for ATCC49417 (Fig. 3C), 5× and 0.5× for W83 (Fig. 3D) which represent the optimal hemin growth condition for each strain. Rgp play an important housekeeping role in the proteolytic transaction and maturation of other surface proteins [21, 22].

Similarly, gingipain adhesion and Rgp catalytic domains mediate attachment and detachment of *P. gingivalis* to epithelial cells, respectively [23, 24]. Moreover, gingipains have an important role for hemin acquisition [25 - 27] which is consistent with the results.

***P. gingivalis* strains are affected differently by varying hemin amounts**

P. gingivalis Rgp activity is influenced by H_2O_2 [11]. To determine intracellular stress levels, this study measured H_2O_2 and SOD amounts. For this study, intracellular stress was classified as either tolerable (bacteria cells can grow) or toxic stress (bacteria cells die). Briefly, the peak Rgp activity concentration was considered to be the ideal growth concentration (Fig.3) while those below the peak and have SOD levels higher as compared to H_2O_2 amounts are considered unideal growth concentration. Both growth concentrations are considered under tolerable stress. In contrast, lower SOD levels compared to H_2O_2 amounts are classified toxic stress.

As shown in Figure 4A, FDC381 had similar H_2O_2 amounts in all hemin concentrations (*left panel*) while SOD levels varied [lower at 1× and 0.5×; higher at 5× and 0.1×] (*right panel*) which would that 1× and 0.5× are ideal growth concentrations, whereas, 5× and 0.1× are unideal growth concentrations. Nevertheless, all concentrations exerted tolerable stress for this strain. This would suggest that the most common type of *P. gingivalis* strain is durable since it tolerates a wide-range of hemin concentrations [28].

In Figure 4B, TDC60 had the lowest H_2O_2 amount at 1× (*left panel*) while SOD levels became higher in relation to hemin concentration (*right panel*) which may

suggest that 5× is the ideal growth concentration, whereas, 1× and 0.5× were the unideal growth concentrations. Moreover, it seems that 5×, 1× and 0.5× exerted tolerable stress while 0.1× exerted toxic stress in this strain. This would imply that this is a demanding type of *P. gingivalis* strain wherein it requires high hemins conditions to grow and, in contrast, low hemin conditions would be toxic [29].

In Figure 4C, ATCC49417 also had similar H_2O_2 amounts in all hemin concentrations (*left panel*) while SOD levels were higher at 1× and 0.5× (*right panel*) which could insinuate that 1× and 0.5× exerted tolerable stress with ideal growth concentrations while both 5× and 0.1× exerted toxic stress in this strain. This would insinuate that this is a strict type of *P. gingivalis* strain, wherein, it only grows in a specific or strict hemin condition which would explain why this strain is uncommon during periodontal disease infection [19].

In Figure 4D, W83 had statistically similar H_2O_2 and SOD amounts regardless of hemin concentration suggesting that 1×, 0.5× and 0.1× are ideal growth concentrations while 5× is considered the unideal growth concentration with both growth concentrations exerting tolerable stress in this strain. Considering the optimal growth conditions for this strain (Fig. 3D), this would suggest that this *P. gingivalis* strain is a conditional type, wherein, both hemin-excess (5×) and hemin-limited (0.5×)

concentrations are good for this strain. Coincidentally, this strain can be both pathogenic and non-pathogenic [30] which suggests that the dual function of this strain would be dependent on hemin availability and concentration.

Discussion

Heme *(in vivo* settings) or hemin (*in vitro* conditions) is essential for *P. gingivalis* growth [12], however, *P. gingivalis* strains may either be absent or present in a periodontal pocket. This can be attributed to several possible factors which include among others: *fimA* genotype; bacterial encapsulation; "red complex" formation; and heme [3, 12, 15]. Throughout this study, it was demonstrated that varying hemin amounts resulting to differing hemin availability and concentration can affect *P. gingivalis* strains differently.

Iron is utilized by *P. gingivalis* in the form of heme which in-turn plays an essential role in bacterial growth and virulence [6]. However, iron in humans is generally not in the free form [31] and the vast majority of iron is found intracellularly in the form of hemoglobin or ferritin while extracellular iron is bound by transferrin found in serum and lactoferrin present within mucosal surfaces [1]. In aqueous solution and the absence of proteins or reducing agents, the gingival crevicular fluid composed of a variety of proteins (including transferrin and hemoglobin) carry iron or heme [10, 20]. This would highlight why *P. gingivalis* preferentially grow in the periodontal pocket where the gingival crevicular fluid is found. Moreover, iron-saturated transferrin contributes to the long-term growth of *P. gingivalis* [8, 32, 33] resulting to periodontal disease.

Periodontal diseases have multifactorial characteristics and are episodic with fluctuations in bacterial infection, inflammatory response, and tissue destruction [34] that affect the protective connective tissue, the sulcular epithelium, and the supporting tissue of the teeth like the alveolar bone and the periodontal ligament [35]. Moreover, periodontal disease (in reference to periodontitis) is classified into three stages: mild, moderate, and severe [36]. In addition, periodontal disease have been associated to *P. gingivalis* infection [37]. This would suggest that heme fluctuates from a healthy to infected condition and, moreso, during periodontal disease progression which could insinuate that heme availability and concentration would be reflected at each periodontal disease stage. In this regard, heme availability and concentration between each periodontal disease stage would differ, whereby, at the mild stage of periodontal disease low heme amounts are available while at the severe stage of periodontal disease high heme amounts are available. Similarly, at the moderate stage of periodontal disease heme amounts would be higher than those detected at the mild stage but lower as compared to the severe stage putatively attributable to tissue destruction which in-turn would increase the amount of available heme. It is worth mentioning that *P. gingivalis* is composed of several virulence factors [38 - 40] and of particular importance is FimA [38, 39].

FimA is important for bacterial colonization and, at present, six *P. gingivalis* FimA genotypes have been identified and structurally differentiated [17, 35]. In an earlier work [15], it was shown that among *P. gingivalis* positive healthy adults, the most detected FimA genotype is type I (50.7%) followed by type V (12.3%) [15], whereas, type II and type IV were only at 3.6% and 0.7%, respectively with a majority of periodontitis patients having type II (49.6%) and type IV (16.5%), while type III rarely occurs in healthy adults (2.2%) and periodontitis patients (5.8%). Similarly in another work, some periodontitis patients had both type Ib (17.3%) and type V (4.9%), however, healthy individuals had few of them [19].

Taken together, FimA types II, Ib, IV and V are associated with periodontal disease, whereas, type I is related to healthy gingiva and type III is uncommon [35]. This establishes the correlation between the particular *P. gingivalis* strain (based on FimA genotype) and its resulting periodontal condition. More importantly and in relation to a previous work, this may underscore how heme fluctuations ascribable to shifts in the periodontal condition (healthy to infected and vice-versa) may affect *P. gingivalis* strain growth and activities.

In this regard and in reference to the varying hemin concentrations studied, it is postulated the following: type I strains are common since this strain is durable to

varying hemin concentrations; type II strains are only found during severe periodontal disease since this strain demands high hemin concentrations; type III strains are rarely found in the gingival mucosa since this strain grown under strict hemin concentration; type IV strains have an erratic growth pattern in the gingival mucosa since this strain grows conditionally depending on hemin concentration.

Similarly, this would also explain why certain *P. gingivalis* strains do not grow concurrently in the periodontal pocket. Which may suggest that during a healthy condition durable and possibly strict strains are favored, whereas, during a periodontal disease condition: mild favors durable, conditional and possibly strict growing strains; moderate favors durable and conditional growing strains; severe favors demanding, durable, and conditional growing strains which potentially may also be ideal conditions for the rare and slow-growing strains.

Thus, these results highlight the effects of varying hemin concentration for each representative *P. gingivalis* strain and, likewise, it could be proposed that either the presence or absence of a particular *P. gingivalis* strain in a periodontal pocket can be attributed to heme availability and concentration within the periodontal pocket.

Conclusions

The purpose of this study was to elucidate the influence of varying hemin concentrations on growth and physiological activity of *Porphyromonas gingivalis* strains with different *fimA* genotypes. Thus, the following conclusions were drawn:

(1) The response to varying hemin (heme) concentrations is different in each *fimA* genotype *P. gingivalis* strains.

(2) The fluctuations in hemin concentration affect the growth and physiological activity of *P. gingivalis* strains in clinical manifestations.

(3) Changes in hemin concentration may explain the *P. gingivalis* strain transition in the periodontal pockets.

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References

1. T. Olczak, W. Simpson, X. Liu, C.A. Genco, Iron and heme utilization in *Porphyromonas gingivalis*, FEMS Microbiol. Rev. 29 (2005) 119-144.
2. J.P. Lewis, Metal uptake in host-pathogen interactions: role of iron in *Porphyromonas gingivalis* interactions with host organisms, Periodontol. 2000 52 (2010) 94-116.
3. C. Bodet, F. Chandad, D. Grenier, Pathogenic potential of *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, the red bacterial complex associated with periodontitis, Pathol. Biol. 55 (2007) 154-162.
4. J. Mysak, S. Podzimek, P. Sommerova, Y. Lyuya-Mi, J. Bartova, T. Janatova, *et al*, *Porphyromonas gingivalis*: major periodontopathic pathogen overview, J. Immunol. Res. 2014 (2014) 1-8.
5. W.S. Borgnakke, P.V. Ylostalo, G.W. Taylor, R.J. Genco, Effect of periodontal disease on diabetes: systematic review of epidemiologic observational evidence, J. Periodontol. 84 (2013) S135-152.
6. D. Grenier, V. Goulet, D. Mayrand, The Capacity of *Porphyromonas gingivalis* to multiply under iron-limiting conditions correlates with its pathogenicity in an animal model, J. Dent. Res. 80 (2001) 1678-1682.
7. P.K. Hwang, J. Greer, Interaction between hemoglobin subunits in the hemoglobin, haptoglobin complex, J. Biol. Chem. 255 (1980) 3038-3041.
8. S. Shizukuishi, K. Tazaki, E. Inoshita, K. Kataoka, T. Hanioka, A. Amano, Effect of concentration of compounds containing iron on the growth of *Porphyromonas gingivalis*, FEMS Microbiol. Lett. 131 (1995) 313-317.
9. R.J. Lamont, H.F. Jenkinson, Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*, Microbiol. Mol. Biol. Rev. 62 (1998) 1244-1263.
10. S. Mukherjee, The role of crevicular fluid iron in periodontal disease, J. Periodontol. 56 (1985) 22-27.
11. C.M. Champagne, S.C. Holt, T.E. van Dyke, B.J. Gordon, L. Shapira, Lipopolysaccharide isolated from *Porphyromonas gingivalis* grown in hemin-limited chemostat conditions has a reduced capacity for human neutrophil priming, Oral Microbiol. Immunol. 11 (1996) 319-325.
12. M.E. Cueno, M. Tamura, M. Ohya, K. Ochiai, Similar physiological effects in *Porphyromonas gingivalis* ATCC 33277 under hemin-excess and hemin-limited

concentrations are putatively associated to different hydrogen peroxide function, Anaerobe 28 (2014) 178-181.
13. A.S. McKee, A.S. McDermid, A. Baskerville, A.B. Dowsett, D.C. Ellwood, P.D. Marsh, Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50, Infect. Immun. 52 (1986) 349-355.
14. K. Nagano, FimA fimbriae of the periodontal disease-associated bacterium *Porphyromonas gingivalis*, Yakugaku Zasshi 133 (2013) 963-974.
15. A. Amano, M. Kuboniwa, I. Nakagawa, S. Akiyama, I. Morisaki, S. Hamada, Prevalence of specific genotypes of *Porphyromonas gingivalis* fimA and periodontal health status, J. Dent. Res. 79 (2000) 1664-1668.
16. J. Ciuraszkiewicz, M. Smiga, P. Mackiewicz, A. Gmiterek, M. Bielecki, M. Olczak, *et al*., Fur homolog regulates *Porphyromonas gingivalis* virulence under low-iron/heme conditions through a complex regulatory network, Mol. Oral Microbiol. 29 (2014) 333-353.
17. M.E. Cueno, K. Nagano, K. Imai, M. Tamura, F. Yoshimura, K. Ochiai, Ab initio modeling approach towards establishing the structure and docking orientation of the *Porphyromonas gingivalis* FimA, J. Mol. Graph. Model 55 (2015) 65-71.
18. Y. Nakano, T. Takeshita, M. Yasui, Y. Yamashita, Prediction of plausible bacterial composition based on terminal restriction fragment length polymorphisms using a Monte Carlo method, Microb. Ecol. 60 (2010) 364-372.
19. J.H. Moon, Y. Herr, H.W. Lee, S.I. Shin, C. Kim, A. Amano, *et al*., Genotype analysis of *Porphyromonas gingivalis* fimA in Korean adults using new primers, J. Med. Microbiol. 62 (2013) 1290-1294.
20. M. Makela, E. Soderling, K. Paunio, J. Talonpoika, T. Hyyppa, Protein composition of crevicular fluid before and after treatment, Scand. J. Dent. Res. 99 (1991) 413-423.
21. T. Kadowaki, K. Nakayama, F. Yoshimura, K. Okamoto, N. Abe, K. Yamamoto, Arg-gingipain acts as a major processing enzyme for various cell surface proteins in *Porphyromonas gingivalis*, J. Biol. Chem. 273 (1998) 29072-29076.
22. K. Nakayama, F. Yoshimura, T. Kadowaki, K. Yamamoto, Involvement of arginine-specific cysteine proteinase (Arg-gingipain) in fimbriation of *Porphyromonas gingivalis*, J. Bacteriol. 178 (1996) 2818-2824.
23. T. Chen, K. Nakayama, L. Belliveau, M.J. Duncan, *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells, Infect. Immun. 69 (2001) 3048-3056.
24. T. Chen, M.J. Duncan, Gingipain adhesin domains mediate *Porphyromonas gingivalis* adherence to epithelial cells, Microb. Pathog. 36 (2004) 205-209.

25. A.A. DeCarlo, M. Paramaesvaran, P.L. Yun, C. Collyer, N. Hunter, Porphyrin-mediated binding to hemoglobin by the HA2 domain of cysteine proteinases (gingipains) and hemagglutinins from the periodontal pathogen *Porphyromonas gingivalis*, J. Bacteriol. 181 (1999) 3784-3791.
26. T. Olczak, D.W. Dixon, C.A. Genco, Binding specificity of the *Porphyromonas gingivalis* heme and hemoglobin receptor HmuR, gingipain K, and gingipain R1 for heme, porphyrins, and metalloporphyrins, J. Bacteriol. 183 (2001) 5599-5608.
27. M. Paramaesvaran, K.A. Nguyen, E. Caldon, J.A. McDonald, S. Najdi, G. Gonzaga, *et al*., Porphyrin-mediated cell surface heme capture from hemoglobin by *Porphyromonas gingivalis*, J. Bacteriol. 185 (2003) 2528-2537.
28. Y.S. Choi, J.H. Moon, J.H. Park, J.Y. Lee JY, Characterization of FimA in *Porphyromonas gingivalis* genotype IV, FEMS Immunol. Med. Microbiol. 65 (2012) 497-500.
29. L. Zhao, Y.F. Wu, S. Meng, H. Yang, Y.L. OuYang, X.D. Zhou, Prevalence of fimA genotypes of *Porphyromonas gingivalis* and periodontal health status in Chinese adults, J. Periodontal. Res. 42 (2007) 511-517.
30. I. Nakagawa, A. Amano, Y. Ohara-Nemoto, N. Endoh, I. Morisaki, S. Kimura, et al., Identification of a new variant of fimA gene of *Porphyromonas gingivalis* and its distribution in adults and disabled populations with periodontitis, J. Periodontal. Res. 37 (2002) 425-432.
31. J.J. Bullen, H.J. Rogers, E. Griffiths, Role of iron in bacterial infection, Curr. Top Microbiol. Immunol. 80 (1978) 1-35.
32. E. Inoshita, K. Iwakura, A. Amano, H. Tamagawa, S. Shizukuishi, Effect of transferrin on the growth of *Porphyromonas gingivalis*, J. Dent. Res. 70 (1991) 1258-1261.
33. K. Tazaki, E. Inoshita, E. Amano, T. Hanioka, H. Tamagawa, S. Shizukuishi, Interaction of *Porphyromonas gingivalis* with transferrin, FEMS Microbiol. Lett. 131 (1995) 161-166.
34. A. Salminen, U.K. Gursoy, S. Paju, K. Hyvarinen, P. Mantyla, K. Buhlin, *et al*., Salivary biomarkers of bacterial burden, inflammatory response, and tissue destruction in periodontitis, J. Clin. Periodontol. 41 (2014) 442-450.
35. S. Moreno, A. Contreras, Functional differences of *Porphyromonas gingivalis* Fimbriae in determining periodontal disease pathogenesis: a literature review, Colomb. Med. (Cali.) 44 (2013) 48-56.
36. J.A. Martin, A.C. Grill, A.G. Matthews, D. Vena, V.P. Thompson, R.G. Craig, *et al*., Periodontal diagnosis affected by variation in terminology, J. Periodontol. 84

(2013) 606-613.
37. P.J. Baker, R.T. Evans, D.C. Roopenian, Oral infection with *Porphyromonas gingivalis* and induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice, Arch. Oral Biol. 39 (1994) 1035-1040.
38. A. Amano, I. Nakagawa, K. Kataoka, I. Morisaki, S. Hamada, Distribution of *Porphyromonas gingivalis* strains with fimA genotypes in periodontitis patients, J. Clin. Microbiol. 37 (1999) 1426-1430.
39. S.C. Holt, L. Kesavalu, S. Walker, C.A. Genco, Virulence factors of *Porphyromonas gingivalis*, Periodontol. 2000 20 (1999) 168-238.
40. H. Inaba, K. Nakano, T. Kato, R. Nomura, S. Kawai, M. Kuboniwa, *et al*., Heterogenic virulence and related factors among clinical isolates of *Porphyromonas gingivalis* with type II fimbriae, Oral Microbiol. Immunol. 23 (2008) 29-35.

Figures

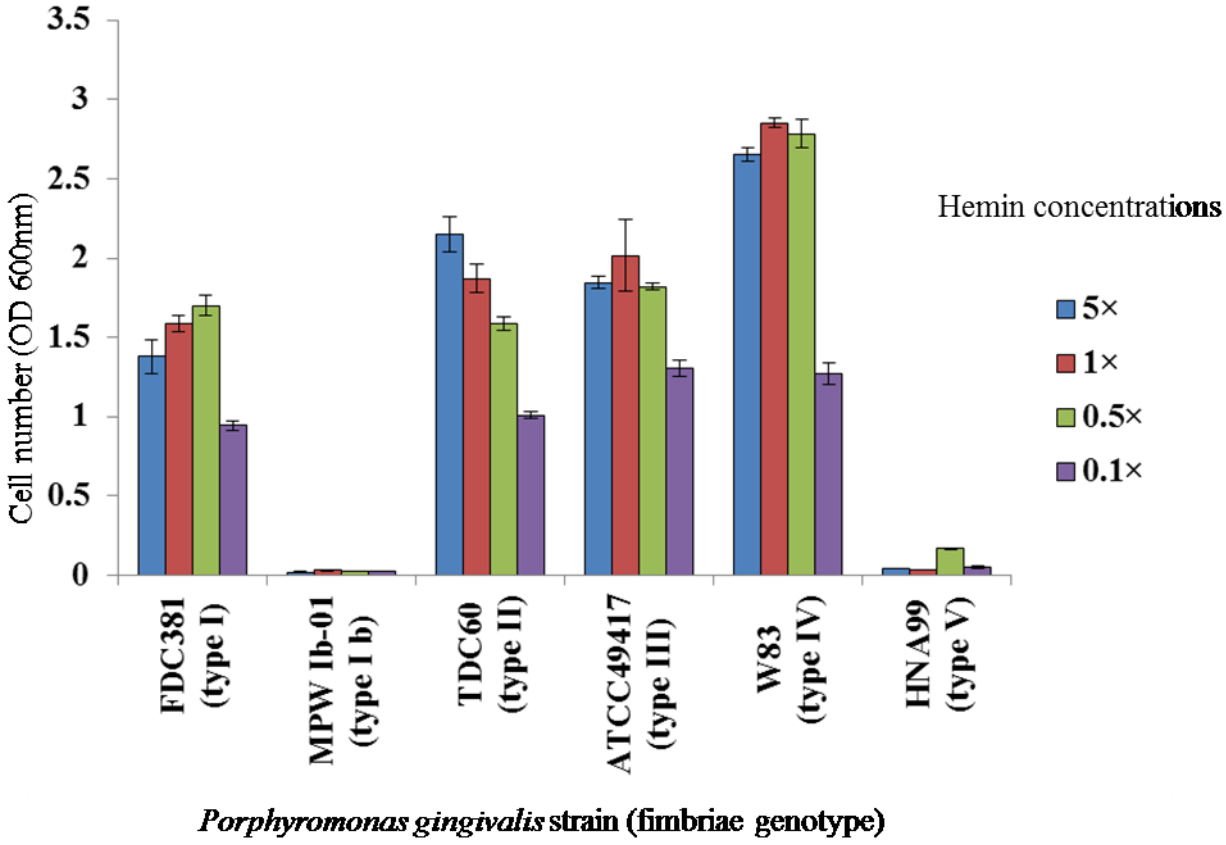

Fig. 1. *Porphyromonas gingivalis* strains after 24 h incubation. Representative strains and the strain-specific fimbriae genotype are indicated. In all assays performed, results shown are mean ± SE. Results shown correspond to n = 3 independent samples.

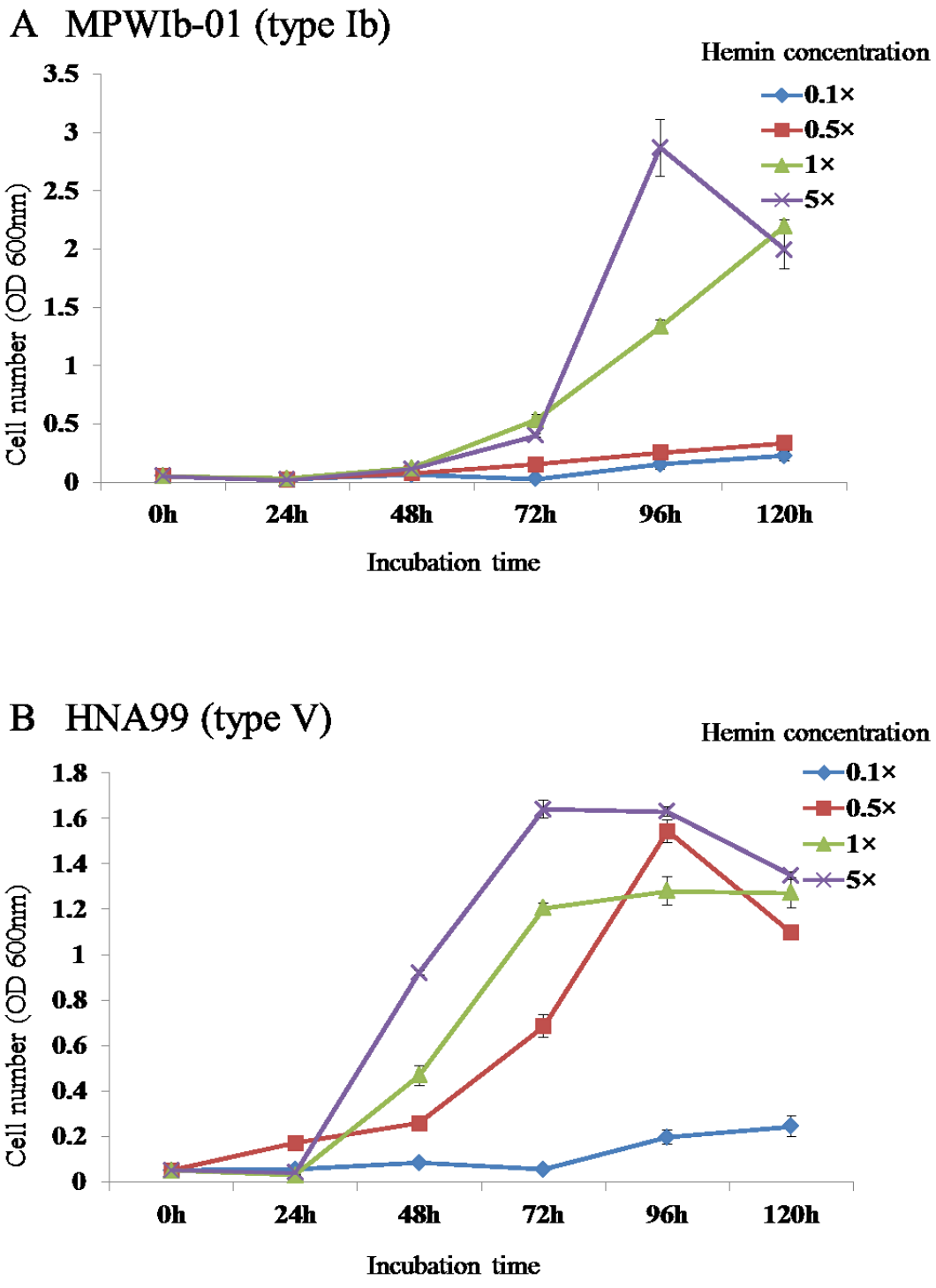

Fig. 2. 120 h incubation of (A) MPWIb-01 (type Ib) and (B) HNA99 (type V) *Porphyromonas gingivalis* strain under varying hemin concentrations. Representative strains and the strain-specific fimbriae genotype are indicated. In all assays performed, results shown are mean ± SE. Results shown correspond to n = 3 independent samples.

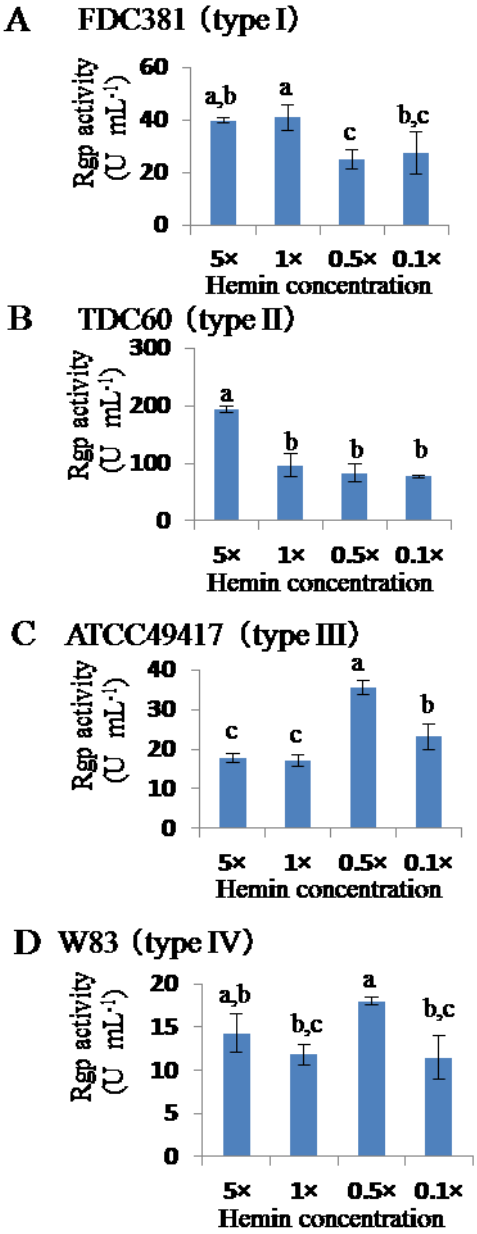

Fig. 3. Arg-gingipain activity of each *Porphyromonas gingivalis* strain under varying hemin concentrations. (A) FDC381 (Type I), (B) TDC60 (Type II), (C) ATCC49417 (Type III), and (D) W83 (Type IV) strains and the strain-specific fimbriae genotype (indicated in parenthesis) are shown. Hemin concentrations are indicated. In all assays performed, results shown are mean ± SE. Results shown correspond to n = 4 independent samples. Statistical analyses were performed by one-way ANOVA followed by Tukey's test. Letters a, b, and c represent different statistical groups and hemin dilutions with the same letter indicates no significant difference at $P < 0.05$.

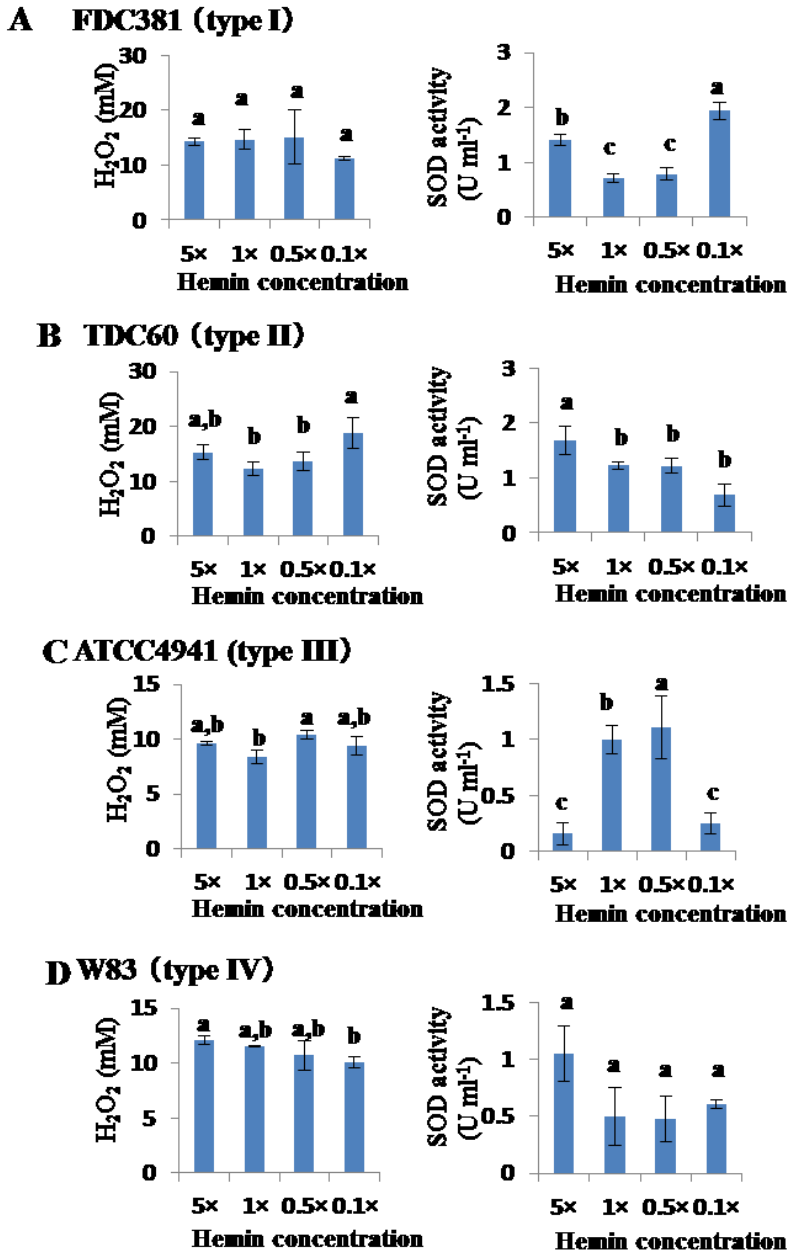

Fig. 4. H_2O_2 and SOD amounts of each *Porphyromonas gingivalis* strain under varying hemin concentrations. (A) FDC381 (Type I), (B) TDC60 (Type II), (C) ATCC49417 (Type III), and (D) W83 (Type IV) strains and the strain-specific fimbriae genotype (indicated in parenthesis) are shown. Hemin concentrations are indicated. In all assays performed, results shown are mean ± SE. Results shown correspond to n = 4 independent samples. Statistical analyses were performed by one-way ANOVA followed by Tukey's test. Letters a, b, and c represent different statistical groups and hemin dilutions with the same letter indicates no significant difference at $P < 0.05$.