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Lysine-specific gingipain K and heme/hemoglobin receptor HmuR are involved in heme utilization in *Porphyromonas gingivalis*

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We have previously reported on the identification and characterization of the *Porphyromonas gingivalis* A7436 strain outer membrane receptor HmuR, which is involved in the acquisition of hemin and hemoglobin. We demonstrated that HmuR interacts with the lysine- (Kgp) and arginine- (HRgpA) specific proteases (gingipains) and that Kgp and HRgpA can bind and degrade hemoglobin. Here, we report on the physiological significance of the HmuR-Kgp complex in heme utilization in *P. gingivalis* through the construction and characterization of a defined *kgp* mutant and a *hmuR kgp* double mutant in *P. gingivalis* A7436. The *P. gingivalis kgp* mutant exhibited a decreased ability to bind both hemin and hemoglobin. Growth of this strain with hemoglobin was delayed and its ability to utilize hemin as a sole iron source was diminished as compared to the wild type strain. Inactivation of both the *hmuR* and *kgp* genes resulted in further decreased ability of *P. gingivalis* to bind hemoglobin and hemin, as well as diminished ability to utilize either hemin or hemoglobin as a sole iron source. Collectively, these *in vivo* results further confirmed that both HmuR and Kgp are involved in the utilization of hemin and hemoglobin in *P. gingivalis* A7436.

Porphyromonas gingivalis, the etiological agent of adult periodontal disease, utilizes iron (preferably in the form of heme) for growth. The precise mechanisms employed by

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P. gingivalis to acquire heme are still not well understood. Several reports have described *P. gingivalis* genes (*ihtA, hemR, ragA, tla* and *tlr*) that exhibit homology to genes encoding putative TonB-dependent heme/hemoglobin receptors (Aduse-Opoku *et al.*, 1997; Karunakaran *et al.*, 1997; Hanley *et al.*, 1999; Dashper *et al.*, 2000; Slakeski *et al.*, 2000). We have recently reported on the identification and characterization of the outer membrane heme/hemoglobin receptor, HmuR (Simpson *et al.*, 2000; Olczak *et al.*, 2001). *P. gingivalis hmuR* mutant was shown to be defective in growth in the presence of both hemin and hemoglobin and exhibited a decreased ability to bind hemin and hemoglobin (Simpson *et al.*, 2000). Furthermore, the recombinant HmuR protein itself and *Escherichia coli* cells expressing outer membrane-associated recombinant HmuR were capable of binding hemin and hemoglobin (Olczak *et al.*, 2001).

Recent evidence indicates that *P. gingivalis* cysteine proteases (gingipains) are also involved in heme and hemoglobin binding (Kuboniwa *et al.*, 1998; Nakayama *et al.*, 1998; Okamoto *et al.*, 1998; DeCarlo *et al.*, 1999; Lewis *et al.*, 1999; Shi *et al.*, 1999; Olczak *et al.*, 2001). The arginine-specific gingipains R1 and R2 (HRgpA and RgpB) are encoded by the genes *rgpA* and *rgpB*, while the lysine-specific gingipain K (Kgp) is encoded by the single *kgp* locus (Okamoto *et al.*, 1995; Pavloff *et al.*, 1995; Okamoto *et al.*, 1996; Pavloff *et al.*, 1997; Potempa *et al.*, 1997). The *P. gingivalis* lysine-specific gingipain K, and the arginine-specific gingipain R1 (HRgpA), are purified as non-covalent complexes of the catalytic domain associated with four polypeptide chains derived from the hemagglutinin domains (Pike *et al.*, 1994; Okamoto *et al.*, 1995; Pavloff *et al.*, 1995; Okamoto *et al.*, 1996; Pavloff *et al.*, 1997). The protease regions of Kgp and HRgpA are divergent, but their hemagglutinin domains are very similar to each other (Okamoto *et al.*, 1996). Parts of the hemagglutinin domains of the *kgp* and *rgpA* genes are also encoded by *hagA* (Han *et al.*, 1996) and *tla* (Aduse-Opoku *et al.*, 1997). In contrast to Kgp and HRgpA, the second arginine-specific gingipain, RgpB, contains only a catalytic domain (Potempa *et al.*, 1998) and is not required for hemoglobin and heme utilization in *P. gingivalis*. Kgp and HRgpA have been demonstrated to bind hemin, protoporphyrin IX, and other porphyrins and metalloporphyrins (DeCarlo *et al.*, 1999; Olczak *et al.*, 2001; Paramaesvaran *et al.*, 2003), and degrade hemoglobin (Kuboniwa *et al.*, 1998; Okamoto *et al.*, 1998; Lewis *et al.*, 1999; Sroka *et al.*, 2001), as well as haptoglobin, hemopexin and transferrin (Brochu *et al.*, 2001; Sroka *et al.*, 2001). Interestingly, experiments conducted in our laboratory have revealed that recombinant HmuR can interact with Kgp and HRgpA (Olczak *et al.*, 2001). Although both proteases were found to interact with HmuR, a higher concentration of HRgpA was needed to observe this binding as compared to Kgp (Olczak *et al.*, 2001). In contrast, the binding of RgpB to HmuR was not detected, suggesting that the formation of the HmuR-Kgp and HmuR-HRgpA complexes is mediated through the hemagglutinin domains of the gingipains (Olczak *et al.*, 2001).

Several reports documenting the binding of hemin and/or hemoglobin by Kgp have employed *kgp* mutants. However, in these studies, the ability of the defined *kgp* mutants to utilize various iron sources was not examined. In addition, previous studies in our laboratory, which revealed the interaction of Kgp with HmuR, were performed using purified proteins (Olczak *et al.*, 2001). Thus, the physiological significance of the binding and degradation of hemoglobin by Kgp and the formation of the HmuR-Kgp complex in intact *P. gingivalis* cells have not been established. In this study, we report on the significance of this interaction to *P. gingivalis* by the construction and characterization of defined mutants in the *kgp* and *hmuR* genes in the A7436 strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. gingivalis* wild type A7436 strain was maintained as described previously (Simpson *et al.*, 2000) on anaerobic blood agar plates (ABA) (Remel, Lenexa, KS), and isogenic mutant strains WS1 (*hmuR* mutant), WS10 (*kgp* mutant), and WS15 (*hmuR kgp* double mutant) on ABA plates supplemented with 1 μg/ml erythromycin alone, 30 μg/ml chloramphenicol alone, or both chloramphenicol (30 μg/ml) and erythromycin (1 μg/ml). All *P. gingivalis* cultures were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, U.S.A.) with 85% N_2, 5% H_2, and 10% CO_2 for 3–5 days. Following incubation at 37°C, cultures were inoculated into Anaerobe Broth MIC (Difco, Detroit, MI, U.S.A.) and incubated at 37°C (under anaerobic conditions) for 24 h.

Construction and isolation of an isogenic P. gingivalis kgp and hmuR kgp double mutants. Both an isogenic *kgp* insertional mutant and a double *hmuR kgp* mutant were created in *P. gingivalis* A7436. To construct the *kgp* mutant (designated WS10), a *Pst*I-digested fragment of pKD362 plasmid containing the *kgp*::*cat* fusion((generously provided by Dr. Koji Nakayama, Kyushu University Faculty of Dentistry, Japan) (Okamoto *et al.*, 1998; Shi *et al.*, 1999)) was introduced into *P. gingivalis* A7436 strain. Electroporation of *P. gingivalis* WS10 with *Nde*I-linearized pWS1 ((*hmuR* mutant construct) (Simpson *et al.*, 2000)) resulted in the production of a *hmuR kgp* double mutant, designated WS15. To verify the *kgp* mutations in WS10 and WS15, Southern blot analysis was performed using the 2.45 kb *kgp*-specific probe amplified by forward 5′-GCTCAGTACATCCTGCAGAAGTTC-3′ and reverse 5′-CTATAAGAAGCCTGATTCTGAGGC-3′ primers. To verify the *hmuR* mutation, Southern blot analysis was performed using the 505 bp *hmuR*-specific probe amplified by forward 5′-ACTGGAATTCGTGTAGTAACAAAGCAG-3′ and reverse 5′-ACTGAAGCTTTGATGATATTTGATAACACC-3′ primers. To further verify the insertional mutations in *P. gingivalis* WS10 and WS15, PCR analysis was performed using *hmuR*- (5′-ACGTGAATTCGTGTAGTAACAAAGCAG-3′ and 5′-GCTGATACGCCAGTTGGCA-3′) and *kgp*-specific primers (5′-GCTCAGTACATCCTGCAGAAGTTC-3′ and 5′-CTATAAGAAGCCTGATTCTGAGGC-3′).

SDS/PAGE and Western blot analysis. The absence of the Kgp protein in WS10 and WS15 was verified by Western blot analysis. *P. gingivalis* A7436, WS1, WS10, and WS15 cultures were harvested at A_{660} = 2.0 and adjusted to an A_{660} of 1.0. One milliliter of each culture was then removed and centrifuged (1 min, 14 000 r.p.m., room temp.). The cell lysates were examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS/PAGE) on 12% gels. The proteins were transferred onto nitrocellulose membranes (BioRad, Hercules, CA, U.S.A.) and Western blotting with anti-Kgp serum (kindly provided by Dr. Jan Potempa, Jagiellonian University, Cracow, Poland) was performed as described previously (Olczak *et al.*, 2001). Polyclonal antibodies were raised against purified Kgp and IgG fraction was isolated from the resulted antiserum (Olczak *et al.*, 2001).

Arginine-X and lysine-X enzymatic assays. The arginine- and lysine-specific protease activities of *P. gingivalis* A7436, WS1, WS10, and WS15 in whole cell cultures (bacterial suspension was previously adjusted to an A_{660} of 1.0), containing cell-associated and secreted gingipains, were determined with either *N*-benzoyl-L-arginine-*p*-nitroanilide, or *N*-*p*-tosyl-glycine-proline-lysine-*p*-nitroanilide as substrates. Samples were preincubated in 200 mM Tris/HCl buffer, pH 7.6, containing 100 mM NaCl, 5 mM $CaCl_2$, and 10 mM cysteine for 5 min at 37°C and assayed for amidase activity with 0.5 mM substrates. The formation of *p*-nitroanilide was monitored spectrophotometrically at 405 nm.

Hemoglobin and hemin binding assays. To determine if WS10 and WS15 were affected in their ability to bind different iron sources, the binding of human hemoglobin or hemin to the cells was examined by a spectrophotometric assay. *P. gingivalis* wild type (A7436) and mutant (WS1, WS10 and WS15) cells were prepared as described previously (Simpson *et al.*, 2000; Olczak *et al.*, 2001). The percent binding of hemin and hemoglobin to *P. gingivalis* whole cells was determined spectrophotometrically by the decrease of absorbance at 400 nm of the supernatant of the mutant cells compared to the wild type (Simpson *et al.*, 2000; Olczak *et al.*, 2001), which was arbitrarily set at 100%.

Growth experiments. To study the ability of *P. gingivalis* to grow with different iron sources, wild type A7436 and mutant WS1, WS10, and WS15 strains were prepared and examined as described previously (Simpson *et al.*, 2000).

Statistical analysis. Data expressed as mean ± standard deviation (±S.D.) were analyzed using the Student's *t*-test; *P* values below 0.05 were considered significant.

RESULTS AND DISCUSSION

Production of an isogenic *kgp* mutant and a *hmuR kgp* double mutant

Previously (Olczak *et al.*, 2001) we showed *in vitro* using ELISA assay that recombinant HmuR interacted with purified Kgp and HRgpA. To test the importance of *P. gingivalis* HmuR-Kgp interaction *in vivo,* first we constructed and characterized a *kgp* mutant in A7436 strain. To date, *kgp* mutants were constructed in *P. gingivalis* strains other than A7436 (Nakayama *et al.*, 1998; Okamoto *et al.*, 1998; Aduse-Opoku *et al.*, 2000). This allowed us to compare the single *hmuR* mutant constructed previously (Simpson *et al.*, 2000) with the single *kgp* and double *hmuR kgp* mutants all constructed in *P. gingivalis* A7436.

The specific probe used in Southern blot analysis hybridized to a 3.3 kb fragment in *Pst*I-digested genomic DNA of A7436 and WS1 strains, which contain intact *kgp* genes (not shown). In WS10 and WS15 strains, the probe hybridized to a 4.8 kb fragment encompassing the region into which the two *cat* cassettes had been inserted (not shown). In the case of the second specific probe hybridization bands of 8 kb were observed in strains A7436 and WS10. Further verification showed that the *hmuR*-specific primers amplified an 855 bp fragment from the intact *hmuR* gene of A7436 and WS10 and a 3.2 kb fragment from the disrupted *hmuR* gene in strains WS1 and WS15 (not shown). The *kgp*-specific primers amplified a 2.4 kb fragment from the intact *kgp* gene of A7436 and WS1 and a 3.9 kb fragment from the disrupted *kgp* gene of strains WS10 and WS15 (not shown).

P. gingivalis kgp and *hmuR kgp* mutant cells are devoid of Kgp expression and lysine-specific protease activity

To confirm that the disruption of the *kgp* gene resulted in translational effects, we tested WS10 (*kgp* mutant) and WS15 (*hmuR kgp* mutant) strains for Kgp protein production and lysine-specific proteinase activity. We did not observe reactivity to the hemagglutinin domains of Kgp (39, 27, 17, and 15 kDa) in WS10 and WS15 strains (Fig. 1). We did, however, detect reactivity with additional proteins in these strains, which most likely represents the cross reactive epitopes present in HRgpA and HagA (Han *et al.*, 1996; Potempa *et al.*, 1998). Interestingly, we did not observe reactivity of the Kgp-specific antibodies with the 48 kDa catalytic domain of Kgp. This finding is in accordance with previous reports (Genco *et al.*, 1995; Potempa *et al.*, 1997), which demonstrated that immunization with purified gingipains generated a major IgG response targeted to epitopes within the hemaggl-

tinin/adhesion domains, but only a very weak response against the catalytic domain. As expected, the *P. gingivalis* wild type and WS1 strains expressed proteins corresponding to the intact Kgp protein (Fig. 1).

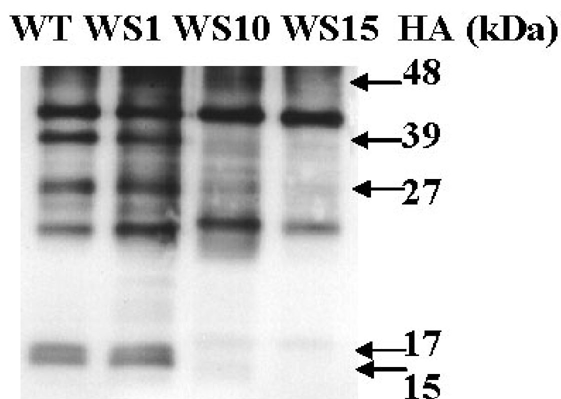

Figure 1. Kgp expression in *P. gingivalis* A7436 wild type (WT) and WS1 (*hmuR* mutant), WS10 (*kgp* mutant), and WS15 (*hmuR kgp* double mutant) strains.

After SDS/PAGE of whole *P. gingivalis* cell lysates (bacterial cells were adjusted to the same A_{660}), proteins were transferred onto nitrocellulose membrane and probed with anti-Kgp antibodies. Kgp hemagglutinin domains (HA) are shown in kDa on the right.

As shown in Table 1, WS10 and WS15 strains were found to be essentially devoid of lysine-specific proteinase activity as compared to the parental A7436 strain and to the isogenic *hmuR* mutant (WS1). Examination of WS10 and WS15 for arginine-specific activity revealed that these strains exhibited activities similar to the wild type strain. This is in agreement with results presented by Aduse-Opoku *et al.* (2000) demonstrating that a *P. gingivalis kgp* mutant made in W50 strain produced arginine-specific activity at a level comparable to the wild type strain. Our previous examination of a different *kgp* mutant, MSM-3, revealed that it exhibited increased transcription of both *rgpA* and *rgpB* (Genco *et al.*, 1995). As the *kgp* mutation in MSM-3 arose due to spontaneous mobilization of an endogenous insertion sequence element (IS*1126*) (Simpson *et al.*, 1999), it could not be definitively stated whether this increase in Rgp activity was a compensatory mechanism brought about due to the absence of Kgp from the protease population, or if a regulator of the *rgpA* and *rgpB* genes had been unknowingly affected. In contrast, studies conducted by Tokuda *et al.* (1998) showed that mutation of the *rgp*A gene resulted in a decreased transcription of the *kgp* gene.

Hemoglobin and hemin binding is diminished in *P. gingivalis* strains WS10 and WS15

Studies performed in our laboratory (Olczak *et al.*, 2001) have demonstrated that HmuR is capable of interacting *in vitro* with purified Kgp. In the current study, we have examined the biological significance of the interaction

Table 1. Arginine- and lysine-specific protease activities* of *P. gingivalis*

Strain	Lysine-specific activity	Arginine-specific activity
A7436	1.8±0.3	6.4±0.7
WS1	1.5±0.2	5.6±0.9
WS10	0.1±0.01	7.3±0.6
WS15	0.1±0.01	6.1±0.6

*Activity was determined in 1 μl of whole bacterial culture adjusted to the same absorbance and expressed as milli-*A* units per minute for each substrate. Results are representative of two separate experiments, each performed in triplicate.
A7436, wild type strain; WS1, *hmuR* mutant; WS10, *kgp* mutant; WS15, *hmuR kgp* mutant.

of Kgp with HmuR with regard to heme utilization by *P. gingivalis*. Examination of the hemoglobin binding activities of the isogenic *hmuR* (WS1) and *kgp* (WS10) mutants and the *hmuR kgp* double mutant (WS15) revealed that the removal of these proteins, either singly or in combination, significantly diminished the ability of the organism to bind both hemin and hemoglobin (Fig. 2). When

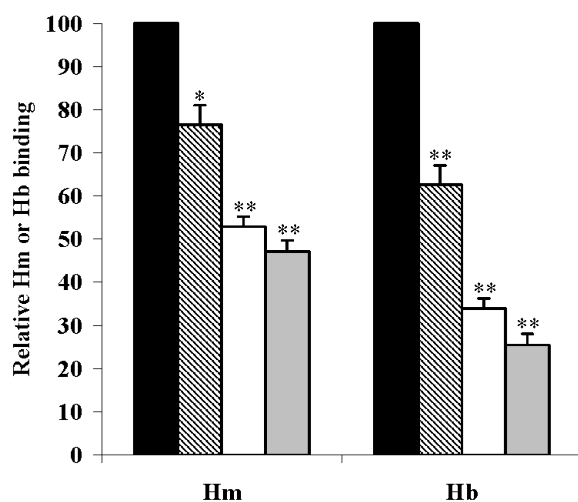

Figure 2. Binding of hemoglobin and hemin to *P. gingivalis* A7436 wild type (WT) and WS1 (*hmuR* mutant), WS10 (*kgp* mutant), and WS15 (*hmuR kgp* double mutant) strains.

P. gingivalis cells were resuspended in phosphate buffered saline, adjusted to an A_{660} of 1.0, and incubated for 1 h at room temp. with hemin (30 μM) or hemoglobin (3 μM). The percent binding of hemin and hemoglobin to *P. gingivalis* whole cells was determined by the decrease of absorbance at 400 nm of the supernatant of the mutant cells compared to the wild type strain, which was arbitrarily set at 100%. Data were analyzed using the Student's *t*-test (*P* values below 0.05 were considered significant) and are shown as the mean ±S.D. from three independent experiments, each performed in triplicate. $^*P < 0.05$, $^{**}P < 0.005$ (WS1, WS10, and WS15 mutants *versus* WT); Hb, hemoglobin; Hm, hemin; WT, black bars; WS1, striped bars; WS10, white bars; WS15, grey bars.

the hemin binding capabilities were examined, it was revealed that all the mutant strains were significantly diminished in their ability to bind this iron source as compared to the wild type strain. Interestingly, the *hmuR kgp* double mutant was not completely diminished in its ability to bind hemoglobin and hemin. This indicates that the ability of *P. gingivalis* to bind hemoglobin and/or hemin is also attributed to proteins other than HmuR and Kgp. Studies in our laboratory (Olczak *et al.*, 2001; Sroka *et al.*, 2001) as well as others (Okamoto *et al.*, 1998) have indicated a role for HRgpA in this binding. Thus, HRgpA present on the outer membranes of WS10 and WS15 cells, as well as other putative hemoglobin and/or hemin binding proteins, may be involved in binding of these iron sources with lower efficiency as compared to Kgp. The probability that multiple proteins comprise the hemoglobin-binding machinery of *P. gingivalis* correlates well with the involvement of several hemoglobin binding proteins reported for other Gram-negative microorganisms (Lewis & Dyer, 1995; Lewis *et al.*, 1997; Morton *et al.*, 1999).

Growth analysis of *P. gingivalis* strains WS10 and WS15

The A7436 strain cultured in Schaedler broth plus dipyridyl supplemented with hemin, hemoglobin, and ferric chloride exhibited a typical growth pattern (Fig. 3A and 3B). In contrast, the initial growth of WS10 with hemoglobin as a sole iron source was delayed when compared to the wild type strain; however, WS10 was capable of growth at late time points. Interestingly, WS10 exhibited a diminished ability to utilize hemin as a sole iron source (Fig. 3A). Growth of the *hmuR kgp* double mutant WS15 was diminished with either hemin or hemoglobin as sole iron sources (Fig. 3B). This finding correlates well with the growth pattern of the isogenic *hmuR* mutant, strain WS1, which is deficient in its ability to utilize either hemin or hemoglobin as sole iron sources (Simpson *et al.*, 2000). These results are also in agreement with the diminished ability of another *kgp* mutant, MSM-3, to utilize hemin (Genco *et al.*, 1995). Similarly to *P. gingivalis* strain WS10, the

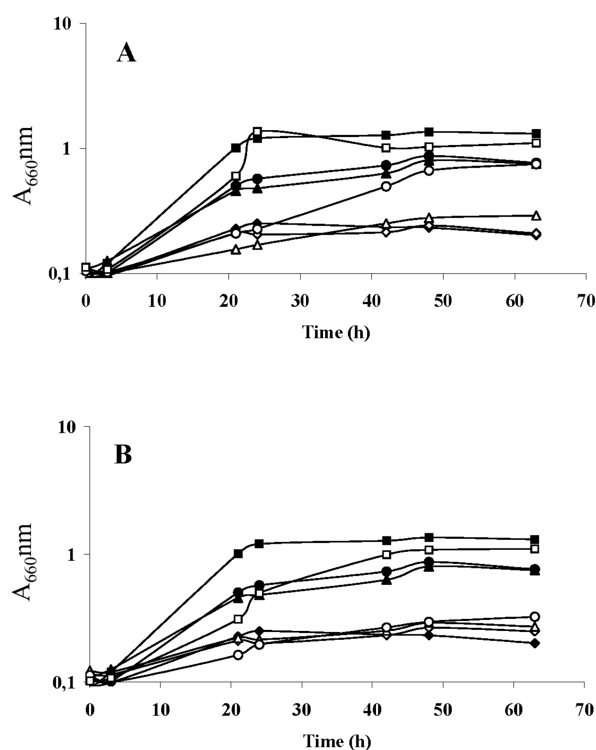

Figure 3. Growth of *P. gingivalis* with hemin, hemoglobin, and ferric chloride as sole iron sources.

(**A**) Growth of *P. gingivalis* A7436 and WS10 (*kgp* mutant) strains. Cultures were initially starved in Schaedler broth containing 200 μM dipyridyl (SB+dip) overnight, then used to inoculate SB+dip alone (black rhombs, A7436; open rhombs, WS10), SB+dip supplemented with 10 μM hemin (black triangles, A7436; open triangles, WS10), SB+dip supplemented with 4 μM hemoglobin (black circles, A7436; open circles, WS10), or SB+dip supplemented with 50 μM ferric chloride (black squares, A7436; open squares, WS10). (**B**) Growth of *P. gingivalis* A7436 and WS15 (*hmuR kgp* double mutant) strains. The legend is the same as in (A). Five independent experiments were performed and representative growth curves are shown.

MSM-3 strain was capable of utilizing hemoglobin as a sole iron source (Genco *et al.*, 1995). We demonstrated that both WS10 and WS15 strains grew with ferric chloride, similarly to the wild type and WS1 mutant strains (Fig. 3A and B). This indicates that the uptake of this iron source in *P. gingivalis* occurs *via* a mechanism(s) which is independent of either Kgp or HmuR. In conclusion, our results indicate that the formation of the Kgp-HmuR complex might increase the overall efficiency of heme acquisition in *P. gingivalis*.

We observed that following prolonged growth on blood agar plates *P. gingivalis hmuR* mutant is characterized by higher pigmentation than the wild type A7436 strain (Simpson *et al.*, 2000). This effect may be due to excessive heme storage on the cell surface, likely trough Kgp, and an inability to uptake the heme moiety into the cell due to the absence of HmuR. *P. gingivalis kgp* mutant was non-pigmented, as it was shown for the *kgp* mutants constructed in other *P. gingivalis* strains (Nakayama *et al.*, 1998; Okamoto *et al.*, 1998; Aduse-Opoku *et al.*, 2000). *P. gingivalis hmuR kgp* double mutant was also non-pigmented, suggesting that these cells cannot store and/or use heme due to the absence of Kgp and HmuR. Based on the results presented here it is likely that Kgp is mostly involved in hemoglobin and/or hemin binding and delivering heme to HmuR, and HmuR is engaged in heme transport into the cell.

CONCLUDING REMARKS

Based on the results presented in this report we propose that HmuR alone might be sufficient for the binding and internalization of hemin and hemoglobin. Kgp has been demonstrated to bind (Kuboniwa *et al.*, 1998; Nakayama *et al.*, 1998; Okamoto *et al.*, 1998; DeCarlo *et al.*, 1999; Lewis *et al.*, 1999; Shi *et al.*, 1999; Olczak *et al.*, 2001) and degrade hemoglobin (Lewis *et al.*, 1999; Sroka *et al.*, 2001), a process by which heme can be liberated. We assume that the uptake of heme in *P. gingivalis* may require the binding of heme to Kgp, and the subsequent binding of Kgp by the receptor HmuR. These collective findings led us to hypothesize that soluble Kgp and also in lesser degree HRgpA could function as heme scavengers or hemophore-like proteins, similarly to the hemophore HasA protein secreted by *Serratia marcescens* (Letoffe *et al.*, 1994; Ghigo *et al.*, 1997). HasA is capable of

extracting heme from heme-hemopexin or hemoglobin and delivering it to an outer membrane receptor, HasR, through a specific interaction (Ghigo *et al.*, 1997; Letoffe *et al.*, 1999). Ghigo *et al.* (1997) have shown that HasR alone is sufficient for hemoglobin/heme utilization, yet more efficiently utilizes heme from hemoglobin *via* HasR–HasA cooperation. The HmuR system in *P. gingivalis* may function in an analogous manner with Kgp and HRgpA. The presented data, although not numerous, demonstrate the role of the receptor–protease complex in heme utilization in *P. gingivalis*.

In conclusion, our results indicate that both Kgp and HmuR are required for heme utilization in *P. gingivalis*. Our future studies are focused on examination of the influence of amino-acid point mutations in the HmuR and Kgp proteins on hemin and hemoglobin binding and *P. gingivalis* growth in the presence of these iron sources.

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