Characterization of the hemin-binding property of *Porphyromonas endodontalis*

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Porphyromonas endodontalis, found in the root canal of teeth, requires iron for growth. However, the mechanism of iron uptake in *P. endodontalis* remains unclear. The ability of bacteria to utilize heme compounds to acquire iron for growth has been reported in some pathogenic bacteria. In the present study, we analyzed the ability of *P. endodontalis* to obtain iron from heme compounds. Further, we investigated the hemin-binding characteristics of *P. endodontalis* and the relationship between hemin binding and Congo red binding. To confirm the bacterial growth in hemin-supplemented medium, iron was removed from the medium with an iron-chelator, and hemin was supplemented to an iron-free medium. The hemin-binding characteristics of *P. endodontalis* were analyzed by incubating bacteria with hemin and measuring the optical density of the supernatant obtained via centrifugation, using hemin concentration standard curve. Although growth of *P. endodontalis* was not observed in the iron-depleted medium, it was observed in a hemin-supplemented medium. Further, hemin binding was dependent on the concentrations of hemin and bacteria. Hemin binding proceeded quickly in *P. endodontalis*, and the incubation temperature had no effect on this binding. Similar to hemin binding, Congo red binding of *P. endodontalis* was dependent on Congo red and bacterial concentrations. In addition, Congo red binding of *P. endodontalis* was inhibited by hemin prebinding. Hemin-agarose beads and SDS-PAGE were used to identify a 40-kDa protein that could be involved in hemin binding. The results showed that *P. endodontalis* could bind to and use hemin to obtain the iron required for growth.

Key Words: Congo red; Hemin; Hemin binding protein; *Porphyromonas endodontalis*

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Introduction

Iron is a nutrient essential for the growth of bacteria and is required for various biochemical and physiological responses [1]. Growth of pathogenic bacteria observed in the infected areas of animals [2] or humans [3] was severely limited when the availability of iron was limited. Therefore,

the ability of bacteria to obtain iron in the host is likely to be involved in the survival of bacteria.

Pathogenic bacteria have diverse and sophisticated systems to obtain the iron required for growth, three mechanisms are known [4]. First, the iron transport system is the mechanism using siderophore. Many microorganisms not only synthesize the iron-chelator siderophore but also have

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a specific transport system for transporting the ferric-siderophore complex (siderophore-Fe³⁺) into the cell [4–6]. As a second iron transport system, some bacteria can acquire iron from human iron binding proteins by specific recognition and transport systems. Iron binding proteins that can be used by pathogenic bacteria include transferrin found in serum and lactoferrin present on mucosal surface [7]. Transferrin or lactoferrin, coupled with iron, can transport iron into bacteria by binding to iron-inducible outer membrane proteins (OMPs) present in bacterial membranes. The third iron transport system uses heme and heme compounds. Heme and heme compounds can provide enough iron for the growth of bacteria [8]. Heme compounds include hemoglobin, haptoglobin, and hemopexin, which can supply iron to bacteria at low concentrations (<10 μ M) [8]. *Porphyromonas gingivalis* [8], *Treponema denticola* [9] and *Aggregatibacter actinomycetemcomitans* [10] are known to acquire iron by using heme compounds. In addition to oral bacteria, bacterial species such as *Helicobacter pylori* [11] and *Vibrio cholera* [12] are known to obtain iron using heme compounds.

Porphyromonas endodontalis is a black anaerobic coccobacillus suspected to play an important role in endodontic infection of teeth [13]. For cultivation of *P. endodontalis*, amino acids, reducing agents and heme compounds are required in a medium [13]. In addition, *Porphyromonas* spp. accumulate high concentrations of hemin to the extent that colony appears black in blood agar.

Previous studies have shown that the ability to bind to hemin in certain pathogenic bacteria is strongly associated with their ability to bind Congo red [14–16]. In a study of oral spirochetes (*Treponema denticola*, *Treponema vincentii*, and *Treponema socranskii*), the hemin binding property of bacteria was reported to be associated with Congo red binding [15].

In this study, we investigated whether the mechanism of *P. endodontalis* to acquire iron includes hemin binding properties and whether hemin binding is related to Congo red binding.

Materials and Methods

Bacterial strains and growth conditions

P. endodontalis ATCC 35406 was cultured for 48 hours in Brain Heart Infusion (BHI) medium supplemented with hemin (Sigma-Aldrich, Saint Louis, MO, USA) (5 mg/L), menadione (Sigma-Aldrich) (10 mg/L) and yeast extract (Becton Dickinson biosciences, Franklin Lake, NJ, USA) (5 g/L) at 37°C under an anaerobic condition (Bactron Anaerobic Chamber, Sheldon Manufacturing Inc., Cornelius, OR, USA) with 90% N₂, 5% CO₂, 5% H₂.

Growth tests

In order to investigate the use of hemin for *P. endodontalis* growth, the growth of bacteria was investigated by adding hemin to the iron-free medium. Bacterial growth experiments were carried out using the procedures described by Eichenbaum et al. [17]. BHI medium containing menadione (10 mg/L), 5% yeast extract and 0.1 M Tris HCl was adjusted to pH 7.3. The iron in the medium was depleted by the addition of 30 mM nitrilotriacetic acid trisodium salt (NTA) as an iron chelator. 1 mM each of MgCl₂, CaCl₂, MnCl₂ and ZnCl₂ was added to the medium. In addition, hemin (10 μ g/mL or 25 μ g/mL) was added to NTA-treated medium. As a control, BHI medium (pH 7.3) containing menadione (10 mg/L) and yeast extract (5 g/L) prepared using 0.1 M Tris-HCl without addition of NTA and hemin, was used. *P. endodontalis* was cultured in each medium at 37°C under an anaerobic condition (Bactron Anaerobic Chamber) with 90% N₂, 5% CO₂, and 5% H₂. OD values were measured at 660 nm at 0, 9, 24, 33, 48, 57, 72, 81, and 96 hours after incubation.

Hemin binding and Congo red binding analysis

To analyze the hemin binding of *P. endodontalis*, bacterial pellets were collected by centrifugation (12,000 $\times$ g, 4°C, 10 minutes) after cultivating the bacteria for 48 hours. The bacterial pellet was washed twice with phosphate buffered saline (PBS) and then resuspended in PBS (OD₆₆₀=1.5; [1 $\times$ 10¹⁰ cells/mL]). 1 mL of the bacterial suspension was in-

cubated with 0.5 mL of hemin (final concentration 40 µg/mL) (Sigma-Aldrich) for 30 minutes in a 37°C water bath. The optical density (OD) of the supernatant was measured at 400 nm after centrifugation (12,000 ×g, 4°C, 10 minutes) [18]. The amount of hemin remaining in the supernatant was calculated from the concentration standard curve. The amount of hemin bound to the bacteria was calculated as the difference between the amount of hemin initially added and the amount remaining in the supernatant after the reaction [18].

In order to investigate the characteristics of hemin binding of *P. endodontalis*, hemin binding was analyzed under various conditions. First, to examine whether the hemin binding of *P. endodontalis* is dependent on the incubation temperature, hemin binding assay was performed at the incubation temperature of 4°C, 25°C and 37°C. Second, to investigate whether the hemin binding of *P. endodontalis* varies with the incubation time, the hemin binding was analyzed by varying the incubation time to 1, 10, and 30 minutes. Third, the experiment was conducted in various concentrations of *P. endodontalis* ($[0-20] \times 10^9$ cells/mL) to investigate whether the hemin binding of *P. endodontalis* was dependent on the concentration of bacteria. Finally, the experiment was performed in various hemin concentrations (final concentration 0–80 µg/mL) to investigate if the hemin binding of *P. endodontalis* was dependent on the concentration of hemin added.

The Congo red binding assay of *P. endodontalis* proceeded in a similar way. In the Congo red binding experiment, the OD values were measured at 488 nm [18]. To evaluate whether the Congo red binding of *P. endodontalis* is dependent on the concentration of bacteria, Congo red binding was analyzed for various concentrations ($[0-30] \times 10^9$ cells/mL) of bacteria. In order to examine whether the Congo red binding of bacteria was dependent on the amount of Congo red added, several Congo red concentrations (final concentration 0–80 µg/mL) were tested.

Competition analysis of hemin and Congo red

To examine the relationship between hemin binding and Congo red binding, Congo red binding of *P. endodontalis* bound to hemin was analyzed [17]. 1 mL of the bacterial

suspension (1×10^{10} cells/mL) and 0.5 mL of hemin (final concentration 0–40 µg/mL) (Sigma-Aldrich) were incubated in a 37°C water bath for 30 minutes and then centrifuged (12,000 ×g, 4°C, 10 minutes). The bacterial pellet was washed twice with PBS and then resuspended in 1 mL of PBS. Bacterial suspension was incubated with 0.5 mL of Congo red (final concentration 40 µg/mL) (Sigma-Aldrich) in a 37°C water bath for 30 minutes. The OD value of the supernatant was measured by a spectrophotometer at 488 nm after centrifugation (12,000 ×g, 4°C, 10 minutes) [17].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

In order to identify the hemin binding proteins involved in the hemin binding of *P. endodontalis*, SDS–PAGE analysis was carried out by binding hemin–agarose beads to bacterial proteins according to the procedure described by Tai et al. [19]. 50 mL of *P. endodontalis* cultured for 48 hours was centrifuged (12,000 ×g, 4°C, 10 minutes) and then washed twice with PBS to obtain bacterial pellet. The bacterial pellet was resuspended in 10 mL of Tris–NaCl buffer (TN buffer) (50 mM Tris+1 M NaCl, pH 8.0) and kept on ice for 10 minutes. The cells were then lysed with an ultrasonicator (Sonics & Materials, Newtown, CT, USA) (kept on ice intermittently for 30 seconds). After centrifugation (12,000 ×g, 4°C, 10 minutes), 1 mL of the supernatant was incubated with 50 µL of hemin–agarose beads (Sigma-Aldrich) in a 37°C water bath for 1 hour. After centrifugation (12,000 ×g, 4°C, 10 minutes), the supernatant was removed. After resuspension in 1 mL of Tris–NaCl buffer (pH 8.0) containing 10 mM EDTA and 0.75% Sarcosyl, the cells were centrifuged (7,000 ×g, 4°C, 3 minutes). After removing the supernatant, the beads were resuspended in 1 mL of 10 mM Tris–HCl (pH 8.0) supplemented with 1 M NaCl, 10 mM EDTA, and 0.5% Sarcosyl, followed by centrifugation (7,000 ×g, 4°C, 3 minutes) (repeated thrice). The supernatant was removed and the beads were resuspended in 1 mL of 50 mM Tris–HCl (pH 8.0) containing 1 M NaCl, followed by centrifugation (7,000 ×g, 4°C, 3 minutes) (repeated twice). The supernatant was removed and the beads were resuspended by adding 1 mL of TN buffer (pH 8.0), followed by centrifugation (7,000 ×g, 4°C, 3 minutes). The supernatant was removed and the

remaining beads were dissolved in 2X sample buffer (half of the beads volume) and heated at 100°C for 5 minutes to prepare a hemin binding protein sample. 20 μ L of the prepared sample was electrophoresed in 10% polyacrylamide gel (KOMABIOTECH, Seoul, Korea) and stained with Coomassie brilliant blue (Sigma-Aldrich) [19].

Statistical analysis

Statistical significance was determined using a two-sample Student t-test. Student t-test was conducted through the Software Package for Social Sciences (SPSS version 23, IBM Corp., Armonk, NY, USA) program.

Results

Growth test

In order to investigate whether *P. endodontalis* could actually obtain iron through hemin and use it for growth, growth experiments were carried out using NTA, an iron-chelator (Fig. 1). Growth analysis showed that the growth of *P. endodontalis* was observed with a doubling time of about 6 hours in the basal medium without any treatment.

Fig. 1. Growth curve of *P. endodontalis* ATCC 35406. *P. endodontalis* grown in Brain Heart Infusion (BHI) medium ($\diamond$) and in nitrilotriacetic acid trisodium salt (NTA)-treated (iron-depleted) Brain Heart Infusion medium without addition of hemin ($\times$); with hemin (10 μ g/mL) ($\triangle$); or with hemin (25 μ g/mL) ($\square$). Values indicate means of duplicates and the vertical bars denote the standard deviation.

Growth of *P. endodontalis* in NTA-treated media was drastically reduced. However, when hemin was added to NTA-treated medium, growth of bacteria was restored. Bacterial growth in this medium was slower than that in the basal medium. However, the absorbance at the time of maximum growth (hemin 25 μ g/mL) was similar to that of the basal medium. In addition, as the concentration of hemin added to the medium increased, the absorbance of the supplemented medium increased proportionately (Fig. 1).

Hemin binding and Congo red binding analysis

To examine whether the hemin binding ability of *P. endodontalis* is dependent on the bacterial concentration, hemin binding was analyzed at several bacterial concentrations. As a result, the amount of hemin bound to *P. endodontalis* increased in proportion to the concentration of bacteria (Fig. 2). Moreover, the hemin binding ability of bacteria was dependent on the amount of hemin added. However, when hemin was added at a specific concentration (40 μ g/mL) or more, there was no significant difference in the amount of hemin bound to bacteria (Fig. 3). We also

Fig. 2. Effects of bacterial concentration on Hemin binding of *P. endodontalis* ATCC 35406. *P. endodontalis* cells ($0\text{--}20 \times 10^9$ cells/mL) were incubated with hemin at 37°C. After incubation of the mixture for 30 min, the binding reactions were terminated by centrifugation at $12,000 \times g$ for 10 min. The quantity of unbound hemin remaining in the supernatant was measured at 400 nm with a spectrophotometer. The amount of hemin bound to *P. endodontalis* was calculated as the difference between the amount of hemin added and the amount of hemin remaining in the supernatant after reaction. Values indicate means of duplicates and the vertical bars denote the standard deviation.

Fig. 3. Effects of Hemin concentration on Hemin binding of *P. endodontalis* ATCC 35406. The reaction mixture contained 1 mL of bacterial suspension ($OD_{660}=1.5$) and 0.5 mL hemin (final concentration 0–80 $\mu\text{g/mL}$). After incubation of the mixture at 37°C for 30 min, the binding reactions were terminated by centrifugation at 12,000 $\times g$ for 10 min. The quantity of unbound hemin remaining in the supernatant was measured at 400 nm with a spectrophotometer. The amount of hemin bound to *P. endodontalis* was calculated as the difference between the amount of hemin added and the amount of hemin remaining in the supernatant after reaction. Values indicate means of duplicates and the vertical bars denote the standard deviation. If the standard deviation value is too small, the error bar is obscured by the point.

analyzed the hemin binding capacity of *P. endodontalis* at several incubation temperatures to check whether the hemin binding capacity of the bacteria differs depending on the incubation temperature. Hemin binding of bacteria did not show any significant difference with change in the incubation temperature (Fig. 4). Although the amount of hemin bound to bacteria increased with incubation time, no significant difference was observed between the values obtained for 10 minutes incubation and 30 minutes incubation (Fig. 5).

Congo red binding was analyzed at several bacterial concentrations to check whether the Congo red binding of *P. endodontalis* depends on the concentration of bacteria. As a result, Congo red binding increased in proportion to the concentration of bacteria (Fig. 6). In addition, experiments were carried out at various Congo red concentrations to determine whether the Congo red binding of *P. endodontalis* is dependent on the amount of Congo red added. The Congo red binding of the bacteria was shown to be increased upon increase in the concentration of added Congo red (Fig. 7).

Fig. 4. Effects of incubation temperature on hemin binding of *P. endodontalis* ATCC 35406. *P. endodontalis* cells (1×10^{10} cells/mL) were bound to hemin for 30 min at 4°C, 25°C, and 37°C, respectively. Values indicate means of duplicates and the vertical bars denote the standard deviation.

Fig. 5. Effects of incubation time on hemin binding of *P. endodontalis* ATCC 35406. *P. endodontalis* ATCC 35406 cells (1×10^{10} cells/mL) were bound to hemin at 37°C for 1 min, 10 min, and 30 min, respectively. Values indicate means of duplicates and the vertical bars denote the standard deviation. Statistically significances were considered as p -values of ≤ 0.05 and were indicated by *.

Competition analysis of hemin and Congo red

Congo red binding was analyzed after binding of *P. endodontalis* with hemin in order to examine the association between hemin binding and Congo red binding in *P. endodontalis*. As a result, Congo red binding of *P. endodontalis* was inhibited by about 21.4% when 2.5 $\mu\text{g/mL}$ hemin was pre-bound and about 39.5% when 10 $\mu\text{g/mL}$ hemin was prebound. When hemin was pre-bound at a concen-

Fig. 6. Effects of bacterial concentration on Congo red binding of *P. endodontalis* ATCC 35406. *P. endodontalis* cells ($0\text{--}30 \times 10^9$ cells/mL) were incubated with Congo red at 37°C . After incubation of the mixture for 30 min, the binding reactions were terminated by centrifugation at $12,000 \times g$ for 10 min. The quantity of unbound Congo red remaining in the supernatant was measured at 488 nm with a spectrophotometer. The amount of Congo red bound was determined by measuring the amount of dye remaining in the supernatant. Values indicate means of duplicates and the vertical bars denote the standard deviation. If the standard deviation value is too small, the error bar is obscured by the point.

Fig. 7. Effects of Congo red concentration on Congo red binding of *P. endodontalis* ATCC 35406. The reaction mixture contained 1 mL of bacterial suspension ($\text{OD}_{660}=1.5$) and 0.5 mL Congo red (final concentration $0\text{--}80 \mu\text{g/mL}$). After incubation of the mixture at 37°C for 30 min, bacteria were removed by centrifugation, and the amount of Congo red bound was determined by measuring the amount of dye remaining in the supernatant. Values indicate means of duplicate experiments and the error bars indicate standard deviations of the mean.

Fig. 8. Inhibition of Congo red binding of *P. endodontalis* ATCC 35406 by hemin prebinding. Hemin was allowed to prebind to *P. endodontalis*, and the excess hemin was removed by washing with PBS, followed by the addition of Congo red (final concentration $40 \mu\text{g/mL}$). Amount of Congo red bound to bacteria was measured with a spectrophotometer. Values indicate means of duplicate experiments and the error bars indicate standard deviations of the mean. Statistically significances were considered as p -values of ≤ 0.05 and were indicated by *.

Fig. 9. SDS-PAGE analysis of hemin binding protein of *P. endodontalis* ATCC 35406. Bacterial components bound to hemin-agarose beads were extracted from *P. endodontalis*. The samples were analyzed by SDS-PAGE with Coomassie Brilliant blue staining. Lane 1 (molecular weight markers) represents the mass (kDa) of a protein molecule. Lane 2 represents the hemin binding protein of *P. endodontalis*.

tration of 40 µg/mL, it was confirmed that the inhibition was about 76% (Fig. 8).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

In order to identify proteins involved in hemin binding of *P. endodontalis*, hemin-agarose beads were bound to bacterial proteins and SDS–PAGE analysis was performed. The band of protein presumed to be involved in the hemin binding of *P. endodontalis* was observed at 40 kDa (Fig. 9).

Discussion

Porphyromonas genus requires iron for growth [13], and bacteria have a mechanism for obtaining iron from the environment [3]. Heme compounds can supply enough iron to grow [8], and it has been shown that iron can be obtained from hemin in *P. gingivalis* [8,18,20]. In this study, the growth of *P. endodontalis* in NTA-treated media was drastically reduced. However, when hemin was added to NTA-treated medium, growth of *P. endodontalis* was restored. This observation supports the possibility that *P. endodontalis* can obtain iron required for growth using hemin. The ability to use heme compounds such as hemin or hemoglobin has been implicated in the binding of these molecules to bacteria, as revealed by studies on bacteria that require hemin for growth [21]. The hemin binding of *P. endodontalis* was dependent on the bacterial concentration and the concentration of hemin added. However, when the concentration of hemin above a certain value was used, the change in the hemin binding ability of the bacteria was insignificant. This suggests that bacteria can bind to a certain amount of hemin and that a certain number of hemin binding sites exist in a single bacterium.

Previous studies have shown that Congo red binding is inhibited by hemin and protoporphyrin IX [15]. In this study, Congo red binding of *P. endodontalis* was dependent on bacterial concentration and concentration of added Congo red. However, when the amount of added Congo red was above a certain level, the degree of Congo red binding decreased gradually. These results indicate that bacteria can bind only to a certain amount of Congo red.

In addition, when *P. endodontalis* was prebound to hemin before Congo red binding, Congo red binding of bacteria was reduced by about 76%. Studies on the association between hemin and Congo red binding have been conducted in several bacteria. Daskaleros and Payne [16] showed that *S. flexneri* inhibited Congo red binding by more than 30% when bound to hemin before binding to Congo red. In addition, Kay et al. [22] showed that Congo red binding of *Aeromonas salmonicida* was inhibited by more than 40% when hemin was prebound. This Congo red and hemin competition analysis suggests that these two compounds can bind to the same or closely related sites in bacteria. Nevertheless, when hemin was prebound, Congo red was still bound to bacteria, although the Congo red of bacteria binding was inhibited. There are a few possible reasons for this observation. First, the molecular structure of Congo red and hemin is similar, but not identical, and Congo red can additionally bind to the hemin-bound receptors of bacteria [16]. Second, an assumption is made that these two compounds can bind to distinct sites other than the common binding site on bacterial surfaces [16]. Third, hemin is weakly bound to bacteria, and after the hemin has detached from the bacteria, the Congo red may bind to the site.

Several previous studies have shown that proteins are involved in the hemin binding of bacteria [23,24]. A 39.5 kDa protein was identified as a protein involved in the hemin binding of *Haemophilus influenzae* [23]. In addition, in *Vibrio vulnificus*, a 36.5 kDa protein is involved in hemin binding [24]. And a 40 kDa protein located in the outer membrane of *P. gingivalis* was found to be involved in hemin binding [20]. According to this study, the 40 kDa protein of *P. gingivalis* was essential for the growth of bacteria in hemin-depleted environments [20]. In this study, protein predicted to be involved in the hemin binding of *P. endodontalis* was observed at 40 kDa. It is not known whether the 40 kDa protein found in *P. endodontalis* and the 40 kDa protein previously reported in *P. gingivalis* are identical.

Recently, Gao et al. [25] identified an 18 kDa protein from *P. gingivalis*, which is different from the previously found 40 kDa hemin binding protein (HBP35) [20]. This protein has been identified as a Dps (DNA binding protein from

starved cells) protein homolog. According to these studies, Dps of *P. gingivalis* could bind to hemin. In addition, Yamamoto et al. [26] identified a Dpr (20 kDa), Dps family protein, in *Streptococcus mutans*. The Dpr protein is structurally similar to the Dps protein and has been reported to bind iron. According to Mieno et al. [27], Dpr of *S. mutans* and Dps of *P. gingivalis* could be evolved as members of a hemin binding family. Therefore, further studies are needed to confirm the possibility that the hemin-binding protein (40 kDa) of *P. endodontalis* has evolved from Dps family proteins.

The presence of hemin in the growth environment of bacteria is related to bacterial virulence. According to the study by Love [28], hemin influences bacterial penetration into the dentinal tubule. *Peptostreptococcus micros* and *Prevotella intermedia* could invade into the dentinal tubules when incubated in the presence of hemin, but not when hemin was absent [28]. These results suggest that the presence of hemin can increase bacterial invasion.

Hemin is likely to be present in dentinal tubules in the free form or in the form bound to heme compounds, which were released from damaged red blood cells [28]. In addition, heme compounds were found in the gingival crevicular fluid and gingiva [29]. Heme compounds are degraded by acids to form hemin [30]. In addition, hemin may be present in root canal environment due to the influx of blood during endodontic treatment [31]. Thus, the ability of *P. endodontalis* residing in root canal to bind to hemin is likely to play an important role in the growth of this bacterium.

In this study, we analyzed the hemin binding properties of *P. endodontalis* and confirmed the association of Congo red and hemin binding in this bacterium. In addition, SDS-PAGE analysis using hemin agarose beads confirmed the presence of proteins involved in hemin binding. It was confirmed that *P. endodontalis* could grow by obtaining iron through hemin. In order to clearly understand the mechanism of iron uptake in *P. endodontalis*, further studies such as analysis of structure and localization of hemin binding protein and study of mechanism of iron migration in bacteria are needed.

Conflicts of Interest

The authors declare that they have no competing interests.

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