I. Introduction

Iron is essential for the growth, reproduction, and survival of all prokaryotic and eukaryotic species. Iron has also been shown to be essential for the initiation and progression of several infectious diseases. In order to survive therefore, prokaryotes and eukaryotes have devised unique mechanisms to compete for, and sequester the small amounts of iron which are available to them.

Eukaryotic cells compete for iron by synthesizing large number of high-affinity iron-binding proteins (i.e., transferrin, lactoferrin), hemoglobin-binding protein (haptoglobin), heme-binding proteins (hemopexin, albumin), and iron-storage proteins (ferritin, hemosiderin) which are capable of competing for, and storing iron. Therefore, in order for host-associated bacteria to survive, they must be capable of competing with the host's large number of high-affinity iron-binding proteins. Prokaryotic cells compete with eukaryotes for this iron by employing two mechanisms: synthesizing their own high-affinity binding and transport proteins, siderophores, and outer membrane associated iron-binding protein.

The siderophores are low molecular weight proteins, which bind iron by chelating it from the host, and transferring it to the specific outer membrane receptors which then transport it directly into the cell. Several Gram-negative bacteria contain, in addition to, or in place of siderophores, iron (hemin) binding proteins in their outer membrane. These outer membrane-associated iron-binding proteins are capable of binding iron directly, and transporting it across the outer membrane into the cell. For the most part, these outer membrane expressed iron-binding proteins are expressed during iron stress, and are formed in response to very low levels of iron in the environment.

* This study was supported by Medical Research Institute Grant from Pusan National University Hospital.
While there are several studies which have dealt with the hemin-binding and hemin-regulated proteins in the oral pathogen, Porphyromonas gingivalis\textsuperscript{20,22-25}, there is no such studies which has studied hemin-binding in Prevotella intermedia. In the study presented here, we have identified, purified, and characterized a putative hemin-binding protein in Prevotella intermedia.

II. Materials and Methods

1. Bacterial Strains and Growth Conditions

Prevotella intermedia (P. intermedia) ATCC 25611 was used in this study. Cells were grown anaerobically on the surface of enriched Trypticase soy agar, or in 2.1% (w/v) Mycoplasma broth base (BBL, Becton Dickinson, Cockeysville, MD) supplemented with 1µ/ml menadione and 5µ/ml hemin\textsuperscript{23}. Plate grown cultures were routinely incubated for 4 days and used as the inoculum for liquid growth. Liquid grown cells were incubated for approximately 24h, equivalent to late exponential growth phase. For hemin restriction (i.e., hemin starvation), late exponential or early stationary phase cultures were grown with excess hemin (i.e., 7.7µ M hemin), and serially passaged at least 5 times as a 10% inoculum into hemin-free medium. Liquid grown cells were incubated for approximately 24h, equivalent to late exponential growth phase. For hemin restriction (i.e., hemin starvation), late exponential or early stationary phase cultures were grown with excess hemin (i.e., 7.7µ M hemin), and serially passaged at least 5 times as a 10% inoculum into hemin-free medium. All glassware was washed in chromic acid and rinsed in deionized water to remove contaminating iron and hemin. The cultures were grown in a Coy anaerobic chamber (Coy Laboratory Product Inc.) in a N$_2$-H$_2$-CO$_2$ (85%-5%-10%) atmosphere at 37°C. Growth was monitored over a 60h period in duplicate by measuring the absorbance at 660 nm. Culture purity was assessed by Gram staining and plating to solid medium.

2. Cell Envelope Preparation

P. intermedia ATCC 25611 was grown into late exponential-early stationary phase. The cells were harvested by centrifugation at 12,000 X g, for 20 min at 4°C, washed 3 times in cold phosphate-buffered saline (PBS, pH 7.2), and resuspended in PBS containing a protease inhibitor cocktail consisting of 2mM each of phenylmethylsulfonyl fluoride (PMSF), Na- P- tosyl- L-lysine chloromethyl ketone (TLCK), and benzamidine. Cell envelopes were prepared by sonication of whole cells in PBS by four disruption cycles. The cell envelopes were recovered after low-speed (10,000 X g, 30 min) and high-speed (200,000 X g, 2h) centrifugation. The envelopes were suspended in PBS and stored at 20°C until used.

3. Protein Determination

Protein concentration was determined using the bicinchoninic acid (BCA) assay of Pierce (Pierce, Rockford, IL) modified by Kennel and Holt\textsuperscript{26}. The assay was modified to a microassay for protein determination in flat-bottomed microtiter plates. Briefly, 20 µl cells or various cell fractions were serially diluted in the microtiter wells with distilled water. 200 µl of protein assay reagent was added to each well, the plates were incubated at room temperature for 30 min,