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Properties of hemagglutination by *Prevotella melaninogenica*

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Abstract

Although *Prevotella melaninogenica* belongs to the commensal oral microbiota, some strains possess putative virulence factors. For example, we have previously described fimbriated, hemagglutinating strains of *P. melaninogenica*, isolated from patients with periodontal disease. The aim of this investigation was to compare some chemical and physical properties of hemagglutination (HA) of *P. melaninogenica* with those of other pigmented gram-negative anaerobes. HA of 13 *P. melaninogenica* strains proved to be considerably weaker than that of the major periodontal pathogen, *Porphyromonas gingivalis*. Vigorous shaking reduced HA of shaken cells but the shaken supernatant had the same hemagglutinating activity as non-shaken cells. The hemagglutinating agent on *P. melaninogenica* seemed to be a protein, which can be separated from the cell and binds to lactose-, galactose-, and raffinose-containing carbohydrates on the erythrocytes. Adherence to epithelial cells did not differ significantly between the hemagglutinating and non-hemagglutinating strains of *P. melaninogenica*. Although *P. melaninogenica* is able to agglutinate erythrocytes, this potential virulence factor is of a considerably lower magnitude than that of major periodontal pathogens.

Keywords: *Prevotella melaninogenica*; Hemagglutination; Adherence; Fimbria; Erythrocytes

1. Introduction

Adherence of the bacterium to host cells is the initial step in colonization and pathogenicity. Hemagglutination (HA) is the aggregation of erythrocytes caused by bacterial molecules adhering to two or more erythrocytes, and has been widely used as a model for testing bacterial adhesins and their lectin specificity. HA seems to be linked with fimbriae [1,2] and these fimbriae are claimed to play an important role in the virulence of a number of oral and non-oral pathogens [3].

Prevotella melaninogenica is a gram-negative, anaerobic, rod-shaped, pigment-producing bacterium that colonizes the oral cavity during early infancy [4,5] and stays as a ubiquitous member of the oral commensal microbiota [6]. Many strains among the genus *Prevotella* present phenotypic properties usually regarded as virulence factors, such as hemolysis [7–10] and HA [11–13]. We have previously described a hemagglutinating variant of *P. melaninogenica*, isolated from patients with periodontitis [13]. It does not seem to be a separate species of the *Prevotella* genus, but is genotypically similar to *P. melaninogenica*, *Prevotella veroralis*, and *Prevotella loescheii*. When examined with an electron microscope, these isolates appeared fimbriated, but the non-hemagglutinating isolates did not [13].

The aim of this study was to examine various physical and chemical properties of HA among clinical *P. melaninogenica* isolates and to compare their hemagglutinating activity with that of hemagglutinating and non-hemagglutinating reference strains, and further to investigate whether hemagglutinating strains exhibited enhanced adhesion to oral epithelial cells.

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Table 1
Strains used in this investigation (HA on microscopic slides [13,22])

HA+ *P. melaninogenica* isolates	G 1, G 9, G 14, G 68, G 107, G 109, G 144, G 153, G 156f, G 156k, G 161, G 197, MCS 105	$n = 13$
HA−*P. melaninogenica* isolates	G 3, G 11, G 150	$n = 3$
HA−*P. melaninogenica* reference strains	WPH 150, VPI 4196	$n = 2$
HA−*P. intermedia* reference strain	ATCC 25611	$n = 1$
HA+*P. gingivalis* reference strains	ATCC 33277, CCUG 14449, CCUG 14450, OMGS 720	$n = 4$

G isolates = clinical isolates from this laboratory, MCS 105 = clinical isolate kindly provided by Dr. George Bowden, WPH 150, VPI 4196, CCUG 14449, CCUG 14450, and OMGS 720 = reference strains kindly provided by Dr. Ellen Frandsen, ATCC 25611 and ATCC 33277 = reference strains from the American Type Culture Collection.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The investigation included 16 clinical *P. melaninogenica* isolates and seven reference strains representing *P. melaninogenica*, *Prevotella intermedia*, and *Porphyromonas gingivalis* (Table 1). Bacterial strains were grown on Fastidious Anaerobe Agar (FAA; LabM, Bury Lancs, UK) with 5% defibrinated horse blood, incubated at 37 °C for 3 days in an anaerobic cabinet (Don Whitley Scientific, Shipley, West Yorkshire, UK) containing 7% H_2, 8% CO_2, and 85% N_2.

2.2. Erythrocytes

Human erythrocytes collected from a volunteer (blood group O, Rh+) in the laboratory, and erythrocytes from sheep, rabbit, rat, guinea-pig, and horse were included. Each selection of erythrocytes was washed three times in phosphate buffered saline (PBS, pH 7.2) and suspended in the same buffer to a 2% (vol/vol) concentration.

2.3. Microtiter plate assay of hemagglutination

HA was assessed using a modification of the method of Okamoto et al. [14]. Bacterial cells were harvested from FAA plates with sterile cotton swabs, suspended in PBS, and their concentration was adjusted to OD 1.000 (±0.050) at 550 nm. Two-fold dilutions of bacterial strains were made in microtiter plates with V-shaped bottoms. An equal volume of 2% erythrocyte suspension was added to each well and incubated at 4 °C for 4 h. Erythrocytes incubated with an equal volume of PBS served as a negative control. Full HA was registered as an erythrocyte precipitate equally distributed over the well bottom, whereas an erythrocyte sediment in the bottom of the V-shaped well indicated no HA. The relative hemagglutinating strength was calculated as the mean of the dilution numbers giving a full and visible agglutination against OD.

For studying adhesion and inhibition and the effect of various physical and chemical treatments on HA, two clinical hemagglutinating *P. melaninogenica* isolates (G 9 and G 107) were selected as the representatives of the two clusters found with 16S rDNA PCR-RFLP [13] and one clinical *P. melaninogenica* isolate (G 11) as the representative of the non-hemagglutinating isolates.

2.4. Inhibition of hemagglutination

Inhibition by *N*-acetyl-D-glucosamine (Sigma-Aldrich, St. Louis, MO, USA), D-galactose, D-glucosamine, D-lactose, D-mannose (ICN Pharmaceuticals, Costa Mesa, CA, USA), and D-glucose (Merck KgaA, Darmstadt, Germany) was tested by adding the sugars to the bacterial/erythrocyte suspension to 1% and 2% (wt/vol) final concentration. The inhibitory effect of L-arginin (1% wt/vol; ICN) and antipain (1 mM; ICN) was tested in the same manner.

2.5. Effect of various physical and chemical treatments on hemagglutination

2.5.1. Heat treatment of bacteria

The effect of heat on HA was tested by incubating the selected strains at 50 and 80 °C for 30 min. Non-heated bacteria incubated at room temperature for 30 min were used as a negative control.

2.5.2. Shaking of bacteria

A suspension of the selected bacterial strains was vigorously shaken on a vortex mixer for 15 min. After shaking, the suspension was spun down in a microcentrifuge and the bacterial pellet re-suspended in equal volume of PBS. Both re-suspended pellet and the supernatant were tested for HA. Non-shaken cells were used as a negative control.

2.5.3. Enzyme treatment of bacteria

Each bacterial suspension was incubated with 2 mg/mL of trypsin and chymotrypsin (Sigma-Aldrich) in separate digestions at 37 °C for 1 h. Bacteria were then