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Brief Communication

Involvement of soil bacteria in ABO blood mistyping

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ABSTRACT

The current study investigated whether ABO blood mistyping of human biological samples is induced by soil bacteria. A total of 380 bacterial strains were isolated from 50 discrete soil samples using human blood agar, and glycosidase activity evaluated for all strains using 4-nitropheny glycosides (4-nitrophenyl *n*-acetyl-α-D-galactosaminide, 4-nitrophenyl-α-D-galactopyranoside, 4-nitrophenyl-α-L-fucopyranoside) as substrates. Thirteen strains possessed α-galactosidase activity, and 16S rRNA sequence analysis revealed a close relatedness to the genus *Bacillus*. An indirect competitive enzyme-linked immunosorbent assay confirmed seven strains exhibited type B antigen degradation activity. These results demonstrated that 1.8% of the bacteria isolated from soil, were *Bacillus* sp., possessed galactosidase activity, and had the potential to cause ABO blood mistyping.

1. Introduction

The ABO blood group system is one of the most recognized genetic traits in humans, and its antigens are of significant clinical importance, particularly in regard to organ transplantation and blood transfusions. The ABO blood group system has been used in large-scale disasters, to narrow down the search for a missing person [1,2].

Unfortunately, when family DNA is unobtainable, as in the loss of entire families, victim identification by indirect kinship analyses is made difficult. Currently, DNA typing is most commonly used in forensic identification and paternity tests. Blood donation is widespread throughout Japan, and it is legally possible to obtain personal information such as ABO blood groups from samples deposited in medical institutions, and to use it as a reference for identification. ABO blood typing is a very cheap. Therefore paternity test and personal identification using the ABO blood group is a still useful technique in candidate selection from large number of missing person list. Of course the final identification was determined by DNA typing.

ABO blood antigens are present on the surface of red blood cells, in bodily fluids such as saliva [3], and in bones [4], nails [5], and hair [6]. The ABO blood antigens reside on glycoproteins or glycolipids and can be detected in a variety of samples derived from human tissue.

The immunodominant monosaccharide structures of the A, B and H antigens are α-1, 3 linked *n*-acetyl galactosamine (GalNAc), α-1, 3 linked galactose (Gal) and α-1, 2 linked fucose (Fuc), respectively. The genetic method determining the ABO blood group [7,8] is also known. However, discrepancies between phenotypic and genotypic ABO blood groups have been reported [9]. Generally, the serological detection of ABO blood antigens is used to establish ABO blood typing. However, discordance in typing has been reported, whereby the serological and ABO gene analysis of blood stains that had been buried in the ground were determined to be of type AB and B, respectively [9]. The authors found that the specimen was contaminated with *Acinetobacter* sp., which possess components, such as glycoproteins or polysaccharides, similar to type A antigen [9]. Further to this, Yuasa et al. reported that some species of *Aspergillus* and *Penicillium* have a type A antigen [10]. Microorganisms possessing cellular components similar to ABO blood antigens are known to exist in natural environments [11]. Hence, the serologic determination of ABO blood typing would be affected by ABO blood group antigen degradation, caused by glycosidases derived from microorganisms in the environment. It was reported that *Clostridium tertium* produces type A antigen decomposing enzyme (Iseki and Okada) [12], *Bacillus cereus* produces type B antigen decomposing enzyme (Iseki and Ikeda) [13] and *Bacillus fulminans* produces type H antigen decomposing enzyme (Iseki and tsunoda) [14].

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Endo-β-galactosidase(s) produced by *Clostridium perfringens* ATCC10543 were shown to catalyze the hydrolysis of the immunodominant trisaccharide structure of type A and B antigens [15]. One could speculate that if these glycosidases were to degrade either GalNAc in type A or Gal in type B antigen, type O antigen (H antigen) would become exposed. In fact, an enzyme of *Ruminococcus torques* strain IX-70 converted type A to type O [16].

These findings indicated that microorganisms naturally found in the environment could adversely affect ABO blood typing, however, the underlying mechanisms (particularly in soil) is yet to be elucidated. The involvement of microbes (themselves and/or their activities) on ABO blood typing should be evaluated since accurate personal identifications indeed necessary in large-scale disasters.

For this purpose, we surveyed soil microorganisms capable of degrading ABO blood group antigen, and found that 1.8% of the isolated bacteria exhibited glycosidase activity capable of modifying ABO blood group antigen structure.

2. Materials and methods

2.1. Materials

Soil samples from 58 locations in Gifu Prefecture, Japan, were collected with sterilized spatulas into plastic centrifuge tubes. Glycosidase substrates, 4-nitrophenyl *n*-acetyl-α-D-galactosaminide (*p*NP-αGalNAc), 4-nitrophenyl-α-D-galactopyranoside (*p*NP-αGal), 4-nitrophenyl-α-L-fucopyranoside (*p*NP-αFuc) and 4-nitrophenol were obtained from Sigma (St. Louis, MO, USA), and dissolved in phosphate-buffered saline (PBS; pH 7.5). α-Galactosidase derived from green coffee bean was obtained from Prozyme (Hayward, CA, USA). Polyacrylamide-conjugated blood group antigen type B (tri)-PAA was obtained from GlycoTech (Gaithersburg, MD, USA) and dissolved in PBS. Microtiter-plates (F96 MaxiSorp Immunoplate) were obtained from Nunc (Roskilde, Denmark). Luria–Bertani (LB) medium, bovine serum albumin (BSA) and polyoxyethylene (20) sorbitanmonolaurate (Tween 20) were obtained from Wako (Tokyo, Japan). The washing solution was PBS containing 0.05% Tween 20 (PBST). Blocking solution was PBST containing 1% BSA (PBST-BSA). Anti-B monoclonal antibody (MAb) was obtained from Immucor (Norcross, GA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgM MAb (anti-mouse IgM-HRP) was obtained from American Qualex (San Clemente, CA, USA). Anti-B MAb was diluted with PBST-BSA. Ten milligrams of *o*-phenylenediamine (OPD; Sigma) was dissolved in 20 mL McIlvaine buffer (10.3 mL 0.2 M Na_2HPO_4 and 9.7 mL 0.1 M citrate, pH 5.0). Human blood (type AB) no longer suitable for transfusions because of their expiration date, were kindly provided by the Japanese Red Cross Society. Agar with 5% human blood was prepared as described elsewhere [17]. Briefly, human blood (type AB, whole blood) was added aseptically to molten 1.5% agar and 0.85% NaCl (autoclaved and cooled to 60 °C), and immediately dispensed into sterile Petri dishes. Saliva (type B) stains were collected from filter paper placed in the mouths of healthy adult volunteers for one minute. The saliva stain was then air-dried at room temperature and extracted in 20 mL PBS. The extract was heat-treated for 20 min in boiling water to deactivate salivary enzymes, concentrated by dialysis, and dissolved in 1 mL PBS. The protein concentration in the saliva extract was established as 0.177 μg/mL by the Bradford method. Ex taq HS was obtained from TaKaRa (Shiga, Japan).

2.2. Strain isolation

One gram of soil was suspended in 1 mL sterilized saline (0.85% NaCl), and 100 μL aliquots of serial 10-fold dilutions applied to human blood agar, and incubated at 25 °C for 48 h. Colonies were randomly selected according to colony morphology (color and size), dye production, and hemolysis. Pure isolates were obtained by repeat culturing of selected isolates on 5% human blood agar. A total of 380 bacterial strains were isolated. These strains were expressed that the first number means the location where soil was collected, and the second number is a serial colony number picked up from a plate culture.

2.3. Glycosidase activity

*p*NP-αGalNAc (A antigen GalNAc), *p*NP-αGal (B antigen Gal), and *p*NP-αFuc (O antigen Fuc) were used as substrates for α-N-acetylgalactosaminidase, α-galactosidase and α-fucosidase activity measurements, respectively.

All 380 strains were cultured in 3 mL LB medium at 25 °C and 120 rpm for 48 h. The bacterial cultures were centrifuged at 25,000×*g* for 10 min, the supernatant recovered, and the cell pellets suspended in 1 mL PBS. The pellets were washed three times in PBS, resuspended in 200 μL PBS, and stored in liquid nitrogen. The pellet was transferred to a thermostatic bath at 37 °C immediately for thawing, and the freeze–thaw procedure repeated three times. The resultant cell free extract and culture supernatant were subjected to the following experiments.

A 40 μL aliquot of *p*NP-αGalNAc, *p*NP-αGal or *p*NP-αFuc were added to the wells of a 96-well microtiter plate. A 10 μL aliquot of cell free extract or culture supernatant was added to each well and incubated for 1 h at 37 °C. The reaction was terminated by the addition of 50 μL 0.5 M sodium carbonate, and the optical density (OD) measured at 405 nm on a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). PBS was used as negative control. The calibration curve was generated by 2-fold serial dilutions of 4-nitrophenol (0.125 mM) with PBS. Protein concentrations of crude enzyme were measured using the Bradford method, with BSA as standard. Boiled crude enzymes were included as controls.

2.4. Indirect competitive ELISA for the detection of B antigen degradation

Thirteen strains exhibiting α-galactosidase activity were pre-cultured in 3 mL LB medium at 25 °C and 120 rpm for 48 h, followed by subculturing in 100 mL LB medium under the same conditions. Cells were pelleted by centrifugation at 25,000×*g* for 10 min, and washed three times with 15 mL PBS. The cell pellet (wet weight 1 g) was placed in a mortar with liquid nitrogen, ground with a pestle, and extracted with 1 mL PBS. The cell free extract was recovered by centrifugation at 25,000×*g* for 15 min. Strain 9-1 was cultured same conditions repeatedly and the cell pellet was collected. The cell pellet (wet weight 5 g) was extracted with 5 mL PBS. The cell free extract was concentrated by dialysis, and dissolved in 1 mL PBS. The cell free extract was recovered by centrifugation at 25,000×*g* for 15 min.

Equal volumes (15 μL) of cell free extract and saliva extract (serial 2-fold dilution 1:1600 with PBS) were combined (total 30 μL), and incubated at 37 °C for 24 h. The mixture was heat treated at 95 °C for 15 min, centrifuged at 25,000×*g* for 15 min, and the supernatant recovered. The supernatant was combined with 30 μL of anti-B monoclonal antibody (MAb) diluted 1:800, and incubated at 4 °C for 12 h. The supernatant/anti-B MAb solutions were used in the indirect competitive enzyme-linked immunosorbent assay (ELISA).

The indirect competitive ELISA for the detection of B antigen was performed as previously described [18]. A 50 μL aliquot of diluted type B (tri)-PAA was added to each well of a microtiter plate and incubated at 4 °C for 12 h. Unbound antigen was