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A novel exopolysaccharide from a clinical isolate of *Prevotella nigrescens*: purification, chemical characterization and possible role in modifying human leukocyte phagocytosis

K. Yamane[1], T. Yamanaka[1], N. Yamamoto[1], T. Furukawa[1], H. Fukushima[1], C. B. Walker[2], K.-P. Leung[3]

[1]Department of Bacteriology, Osaka Dental University, Osaka, Japan, [2]Periodontal Disease Research Clinic, College of Dentistry, University of Florida, Gainesville, FL, [3]Microbiology Branch, U.S. Army Dental Research Detachment, Walter Reed Army Institute of Research, Great Lakes, IL, USA

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Prevotella nigrescens, a gram-negative black-pigmented anaerobic rod, has frequently been isolated from periodontitis and periapical periodontitis lesions. We have isolated an exopolysaccharide-producing *P. nigrescens,* strain 22, from a chronic periodontitis lesion. The purpose of this study was to determine the chemical composition and function of the exopolysaccharide associated with this clinical isolate. The chemical composition and structure of the purified exopolysaccharide from strain 22 were determined by high performance liquid chromatography and methylation analysis. To define the biological function of this exopolysaccharide, a chemically induced exopolysaccharide nonproducing mutant, strain 328, which was derived from strain 22, was established. The biological effects of exopolysaccharide were determined by comparing the ability of strain 22, strain 328 or heat-killed strain 22 to form abscesses in mice and to interfere with the phagocytic activity of peripheral blood polymorphonuclear leukocytes. Chemical analysis showed that isolated exopolysaccharide consisted of mannose (521.6 μg/mg), glucose (25.6 μg/mg), fructose (65.8 μg/mg), galactose (12.5 μg/mg), arabinose (6.2 μg/mg), xylose (3.2 μg/mg), rhamnose (6.1 μg/mg), and ribose (0.6 μg/mg). Methylation analysis of exopolysaccharide indicated that the linkages of mannose were primarily (1 → 2, 1 → 6) (1 → 2) (1 → 6), and (1 → 3). Strain 22 and, to a lesser extent, its heat-killed counterpart induced greater abscess formation in mice than strain 328, even though the enzymatic profile of strain 22 was similar to that of strain 328. The ability of strain 328 to induce abscess formation was restored by adding the purified exopolysaccharide isolated from strain 22 to the cell suspension of strain 328. Exopolysaccharide alone failed to induce abscess formation in mice. Further, strain 328 but not the untreated or heat-killed strain 22, was phagocytosed by polymorphonuclear leukocytes both in the presence and in the absence of opsonic factors. The results suggest that these polysaccharides isolated from strain 22, which primarily consisted of mannose, may play a key role in the development of the chronic inflammatory lesion from which this strain was isolated.

Key words: biofilm; exopolysaccharide; *Prevotella nigrescens*

Hisanori Fukushima, Department of Bacteriology, Osaka Dental University, 8–1 Kuzuhahanazono-cho, Hirakata-shi, Osaka, Japan, 573–1121
Tel.: +81 72 864 3051;
fax: +81 72 864 3151;
e-mail: hisanori@cc.osaka-dent.ac.jp
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Prevotella nigrescens are gram-negative black-pigmented anaerobic pleomorphic rods. These organisms, together with the related *Prevotella intermedia*, are frequently isolated from periodontal pockets of patients with destructive periodontitis (18, 34, 37, 39), acute necrotizing ulcerative gingivitis (28), pregnancy gingivitis (24), and endodontic lesions (2, 38). The frequency with which these related organisms can be isolated and the ratio of *P. nigrescens* to *P. intermedia* varied depending in part on the disease sites from which the clinical samples were obtained (2, 3, 27).

We have previously reported that some strains of *P. intermedia* produce extracellular viscous material which appears as fine fibrous-like structures as revealed by electron microscopy (25). Though the function of these fibrous-like structures has not been determined, morphologically, these structures are similar to the meshwork produced by the mucoid type of *Pseudomonas aeruginosa*. This meshwork, consisting of exopolysaccharides, is considered an important virulent factor that could contribute to the persistent infections associated with *Pseudomonas* (15).

Recently, we isolated a viscous material-producing *P. nigrescens*, designated as strain 22, from a periodontitis lesion. In this report, we describe the chemical composition of this viscous material, determined by means of high performance liquid chromatography (HPLC), gas chromatography (GC), and GC/MS (mass spectrometry) analyses. We also test the pathogenic potential of exopolysaccharide by comparing the exopolysaccharide-producing strain, strain 22, to the exopolysaccharide nonproducing mutant of strain 22, strain 328, with regard to their abilities to induce abscess formation in mice and to interfere with the phagocytic activity of human polymorphonuclear leukocytes.

Material and methods

Bacteria and culture

The exopolysaccharide-producing *P. nigrescens* strain 22, a clinical isolate from a chronic periodontitis lesion, was grown in the enriched-trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, MD) or on trypticase soy blood agar plates supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, MI), hemin (5 mg/l), L-cystine (400 mg/l) and vitamin K_1 (10 mg/l). All bacterial cultures were grown anaerobically in an anaerobic chamber (ANX-3, Hirasawa, Tokyo, Japan) at 37°C in a 5% CO_2−10% H_2−85% N_2 atmosphere. Exopolysaccharide-nonproducing *P. nigrescens*, strain 328, was established from the *P. nigrescens* strain 22 by ethidium bromide treatment as described elsewhere (29) and was grown as described above.

Enzymatic profiles and viscosity of culture medium

The enzymatic activities of strain 22 and its mutant strain 328 were examined for their production of acid phosphatase, alkaline phosphatase, phosphoamidase, and α-glucosidase using the API ZYM system (bioMerieux, Marcy-Etoile, France). Hyaluronidase, chondroitin sulfatase, and DNase activities were examined by inoculating bacterial cells into each appropriate test agar (23, 35). Lecithinase and lipase activities were examined with the LD-egg yolk agar method as described elsewhere (13). The β-lactamase activity was tested using nitrocefin according to the manufacturer's directions (Oxoid, Hampshire, England). The viscosity of the culture medium was measured using a rotary viscometer (Tokisangyo, Tokyo, Japan). For these determinations, strains 22 and 328 were grown in enriched-TSB for 48 h, 550 μl of culture medium was put into a rotor, and the viscosity was measured as shearing stress between a rotor and a rotor shaft at 50 r.p.m. at 20°C.

Preparation of exopolysaccharide

The exopolysaccharide was prepared from culture supernatants by the method of Campbell et al. (7). Briefly, *P. nigrescens* strain 22 was grown at 37°C in enriched-TSB for 24 h. Supernatants were separated by centrifuging the liquid culture at 12,000 × ***g*** for 30 min, and sodium acetate was added to a final concentration of 5%. The mixture was stirred for 30 min at 22°C and the exopolysaccharide was isolated by ethanol precipitation from the reaction mixture. The ethanol-precipitated material was collected by centrifugation (18,200 × ***g*** for 15 min at 22°C), resolved in 5% sodium acetate, and then treated with chloroform 1 : butanol (1 : 5, by volume). Water-soluble and chloroform–butanol layers were separated by centrifugation, an equal amount of ethanol was added to the water-soluble layer (this procedure was repeated twice), and the ethanol-precipitated material was freeze-dried. The material, dissolved in distilled water at a final concentration of 10% (w/v) was centrifuged at 217,800 × ***g*** for 2 h to remove the endotoxin. The supernatant was freeze-dried and stored at − 80°C until use. The contaminated endotoxin in this preparation was measured by a limulus test kit (Endospecy, Seikagaku, Tokyo, Japan).

Electron microscopy

For scanning electron microscopy, bacteria grown on trypticase soy blood agar plates for 48 h were collected on a piece of filter paper (Glass fiber GA55, Toyo Roshi, Tochigi, Japan), fixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 2 h and 1% OsO_4 in 0.1 M phosphate buffer for 1 h at 4°C, and dehydrated through an ethanol series and 2-methyl-2-propanol followed by platinum ion coating (E-1030, Hitachi, Tokyo, Japan). Specimens were examined with a scanning electron microscope (S-4000, Hitachi) at an accelerating voltage of 3 kV. For negative staining, bacterial cells grown in enriched-TSB for 24 h were immediately applied to carbon-coated copper grids for 1 min, and stained for 30 s with 2% phosphotungstic acid. Samples were examined in a transmission electron microscope (H7100, Hitachi) at 100 kV.

HPLC and methylation analysis of exopolysaccharide

Neutral monosaccharides were released from purified exopolysaccharide (5 mg) by hydrolysis in a sealed tube with 2 N trifluoroacetic acid (200 ml) at 100°C for 6 h. The hydrolysate was concentrated *in vacuo* and dissolved in 500 ml of distilled water. The sugars were identified by HPLC (LC-9 A, Shimazu, Kyoto, Japan) with a TSK-gel sugar AXG column (15 cm × 4.6 mm) (Tosoh, Tokyo, Japan) using 0.5 M potassium tetraborate buffer (pH 8.7) as a carrier at a flow rate of 0.4 ml/min and a column temperature of 70°C.

Methylation analysis was carried out according to the Hakomori method (19) with a slight modification as described by Hisamatsu et al. (21). The methylated product was treated with 2 ml of 90% (v/v) formic acid for 4 h at 100°C in a sealed tube and hydrolyzed in 2 M trifluoroacetic acid for 6 h under a stream of N_2 at 100°C. Following hydrolysis, sugars were dried *in vacuo*, washed three times with deionized water, reduced overnight at room temperature by $NaBH_4$ (10 mg/ml in EtOH/H_2O, 1 : 1 by volume), and treated with acetic acid to destroy excessive $NaBH_4$. The dried sample was rinsed five times with 2 ml methanol and

acetylated with acetic anhydride in pyridine (1 : 1 by volume, 4 h, 100°C). The resulting partially methylated alditol acetates were analyzed by GC and GC/MS. GC was performed on a Hewlett-Packard 5890 A System equipped with a 25 m × 0.25 mm fused silica capillary column of SPB-5 (Supelco Japan, Tokyo, Japan) using He as carrier gas (flow rate, 2.5 ml/min). The column temperature was programmed at 60°C for 1 min and raised to 280°C by 8°C/min. GC/MS analyses were performed on a JEOL mass spectrometer (JMS-DX-303, JEOL, Tokyo, Japan) interfaced with a Hewlett-Packard 5890 A System. The column temperature was programmed as described above and the ionization potential was set at 70 V. The range was set at 10–500 atomic mass units and scanned at a rate of 1 s/scan. Tested samples were identified by comparison with computer-based library spectra and authentic chemical standards.

Animal studies

The virulence of exopolysaccharide-producing *P. nigrescens* strain 22 was compared with that of exopolysaccharide-nonproducing strain 328 as regards abscess formation in mice. The strains were cultured in enriched-TSB for 48 h. A heat-killed counterpart of strain 22 (at 60°C for 20 min) was also prepared to evaluate how much influence bacterial secreted products other than exopolysaccharide have on abscess formation. Five hundred μl of bacterial suspensions (3×10^7 CFU/ml) was injected subcutaneously into the inguen of each BALB/c mouse (male, 4 weeks). Changes in abscess lesions were recorded photographically using a Nikon FIII camera (Nikon, Tokyo, Japan) set at a fixed magnification for five consecutive days. The abscess area was calculated from a photograph by Image Master (Amersham Pharmacia Biotech, Tokyo, Japan). For characterization of bacterial samples recovered from mouse abscesses, samples were swabbed from the lesions, and the recovered bacteria were examined by gram staining, black pigmentation on blood agar plates, enzymatic profiles, and scanning electron microscopy as described above. To determine whether production of exopolysaccharide is crucial for *P. nigrescens* to induce abscess formation in mice, purified exopolysaccharide from strain 22 was added to cell suspensions of strain 328 at concentrations of 0.5 mg/ml, 2.5 mg/ml, and 5 mg/ml, and 500 μl of each preparation then injected into a mouse inguen. Abscess lesions were recorded photographically as described above.

Phagocytosis assay

Peripheral blood polymorphonuclear leukocytes were purified from human volunteers (n = 3; age 20–23 years) by a Polymorphprep™ (Axis-Shield PoC AS, Oslo, Norway) gradient, and cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (MultiSer, Trace Scientific Ltd, Melbourne, Australia), 2 mM L-glutamine, penicillin and streptomycin at 37°C in 5% CO_2. To determine whether strain 22 was resistant to phagocytosis by polymorphonuclear leukocytes, bacterial suspensions of strains 22, 328, and heat-killed strain 22 (1.5×10^6 CFU/ml) were prepared. Polymorphonuclear leukocytes, 1×10^6 cells/ml, were dispensed into the wells of 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) (300 μl/well). To these wells, 100 μl of bacterial suspensions of different tested strains were added and incubated for 60–90 min at 37°C. The cultures were harvested and cytospins were prepared to examine the internalization of bacteria morphologically. The cytospin specimens were fixed with methanol for 1 min, air-dried and stained with Giemsa solution (pH 6.8) for 30 min. Microscopy was performed at ×1000 magnification with a Olympus microscope (BX50, Olympus, Tokyo, Japan) equipped with a CCD video camera system (Cool SNAP, Roper scientific, Chiba, Japan). The proportion of polymorphonuclear leukocyte cells with endocytosed bacteria to the total polymorphonuclear leukocytes was calculated in 50–75 polymorphonuclear leukocytes from five randomly selected fields.

The internalization of bacteria by polymorphonuclear leukocytes was also determined by transmission electron microscopy. Polymorphonuclear leukocytes cocultured with strain 22 or 328 were centrifuged at 8,000 × ***g*** at 4°C for 5 min. Cell pellets were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 2 h, postfixed with 1% OsO_4 in 0.1 M phosphate buffer for 1 h at 4°C, and dehydrated through an ethanol series. Samples were embedded in Epon resin and ultrathin sections were prepared by ultramicrotome (Ultracut, Leica, Tokyo, Japan). Ultrathin sections were placed on a copper grid, stained with uranyl acetate and lead citrate, and observed in a transmission electron microscope (H7100) as described above.

To determine the resistance of strain 22 to phagocytosis by human polymorphonuclear leukocytes under opsonized conditions, a phagocytosis assay was performed in the presence of human AB serum (8% v/v, final concentration; CosmoBio, Tokyo Japan). Briefly, 300 μl of polymorphonuclear leukocytes in RPMI 1640 medium (1×10^6 cells/ml) were dispensed into the wells of 24-well tissue culture plates (Becton Dickinson). To these wells, 100 μl of bacterial suspensions of strain 22 or 328 (1.5×10^6 CFU/ml) together with 35 μl of human AB serum were added. The reaction mixtures were incubated for 60–90 min at 37°C. Cytospins were prepared from the cultures to examine the internalization of bacteria as described above.

Effects of purified exopolysaccharide on phagocytosis

To determine whether the purified exopolysaccharide from strain 22 had any effect on the phagocytosis by polymorphonuclear leukocytes, phagocytosis assays using opsonized fluorolabeled latex beads were performed in the presence of exopolysaccharide. After washing twice with a 10-fold volume of phosphate-buffered saline, fluorolabeled latex beads (Fluoresbrite, Polysciences Inc., Warrington, PA) were incubated (opsonized) with human type AB serum (Cosmo Bio) for 20 min at 37°C and washed twice with phosphate-buffered saline. Polymorphonuclear leukocytes were isolated as described above and dispersed at 1×10^6 cells/well into the wells of 24-well tissue culture plates (1 ml/well). Purified exopolysaccharide from strain 22 was dissolved in RPMI 1640 at a concentration of 1 mg/ml or 5 mg/ml. The serum-coated fluorobeads (approximately 4.55×10^9 particles/ml) were added to the medium containing exopolysaccharide at a concentration of 1 mg/ml or 5 mg/ml, and the components were mixed gently using a rotary mixer overnight at room temperature (RT-5, Taitec, Tokyo, Japan). The mixtures, which contained exopolysaccharide in RPMI 1640 and serum-coated latex beads, were added to culture plates containing polymorphonuclear leukocytes (1 ml for each well). The final concentration of exopolysaccharide in cultures was 0.5 mg/ml and 2.5 mg/ml. Cultures containing opsonized fluorolabeled latex beads and polymorphonuclear leukocytes were used as controls. As a final step, human AB serum was added to each culture (174 μl/well) to give a final serum concentration of 8% (v/v). The cultures

were incubated for 90 min at 37°C in 5% CO_2. The phagocytosing cells were counted by means of flow cytometry (FACScan, Becton Dickinson).

Results

Enzymatic profiles and the viscosity of culture media

As shown in Table 1, the enzymatic activities of strains 22 and 328 were very similar. There was no significant difference regarding the production of enzymes. However, the viscosity of the spent culture medium of the mutant strain 328 was similar to that of the control medium but different than that of strain 22. The viscosity of the control medium and that of the spent culture media obtained from strains 22 and 328 were 1.22±0.01 mPa·s, 1.34±0.01 mPa·s, and 1.25±0.01 mPa·s, respectively.

Morphology and chemical composition of exopolysaccharide

P. nigrescens strain 22, as shown by negative staining, was associated with fibrous structures organized as a fine meshwork. Some of these fibers were in direct contact with the cell wall (Fig. 1). Chemical analyses of these isolated fibrous structures showed that they contained mannose, glucose, arabinose, xylose, rhamnose, and ribose, with the mannose constituting 88% of the polysaccharide (Table 2). Methylation analysis showed that this extracellular polysaccharide was mainly composed of 2,6-substituted (giving 1,2,5,6-tetra-o-acetyl-3,4-di-o-methylmannitol), 2-substituted (giving 1,2,5-tri-o-acetyl-3,4,6-tri-o-methylmannitol), 6-substituted (giving 1,5,6-tri-o-acetyl-2,3,4-tri-o-methylmannitol), and 3-substituted (giving 1,3,5-tri-o-acetyl-2,4,6-tri-o-methylmannitol) mannose (Fig. 2). The relative proportions of partially methylated alditol acetates are summarized in Table 3. The contaminated endotoxin was less than 1.74 EU per mg of tested materials.

Table 1. Enzymatic activities of strain 22 and strain 328

Enzymatic activities	Strain 22	Strain 328
Hyaluronidase	+	+
DNase	+	+
Lecithinase	+	+
Lipase	+	+
Chondroitin sulfatase	–	–
Collagenase	–	–
β-lactamase	–	–
Acid phosphatase	+	+
Alkaline phosphatase	+	+
Phosphoamidase	+	+
α-Glucosidase	+	+

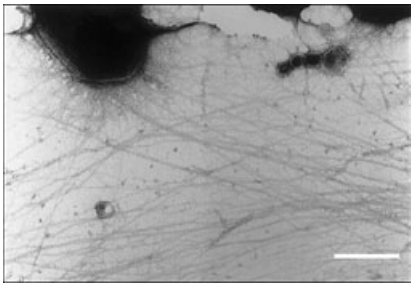

Fig. 1. Transmission electron microscopy of negatively stained exopolysaccharide-producing *P. nigrescens* strain 22. Note the fine net-like structures of exopolysaccharide around the cells and on the substrate. Bar = 0.5 μm.

Table 2. Neutral sugar components of exopolysaccharide isolated from strain 22

Neutral sugars	Content (μg/mg)
Mannose	521.6
Glucose	25.6
Fructose	15.8
Galactose	12.5
Arabinose	6.2
Xylose	3.2
Rhamnose	6.1
Ribose	0.6
Total	591.6

Animal studies

The abscesses in mice induced by subcutaneous injections of strain 22 and heat-killed strain 22 were significantly larger than those in mice injected with strain 328 (Fig. 3). The ability of heat-killed strain 22 to induce abscesses was comparable to that of the untreated strain 22, although the abscesses in mice injected with heat-killed strain were absorbed faster than those of mice injected with strain 22. On day 4, the abscesses were still present in mice injected with untreated or heat-killed strain 22-injected mice, but not in mice injected with strain 328, the exopolysaccharide nonproducing strain (Fig. 3). The morphology of each strain used to induce the formation of abscess was examined by scanning electron microscope before and after the animal passage. While substantial amounts of the meshwork-like structures were observed around the bacterial cells of strain 22 before the animal passage, the structures associated with the cells that had recovered from the abscess appeared to be significantly denser (Fig. 3A, G). In regard to the heat-killed strain 22 cells, while the cell integrity appeared to be damaged by the heat treatment, the meshwork-like structures were well preserved (Fig. 3C). Further, no morphological changes were observed in strain 328, which lacked the fibrous structures, before and after the passage (Fig. 3B, H). When the purified exopolysaccharide from strain 22 was added to the cell suspension of strain 328, meshwork-like structures formed around strain 328 cells (Fig. 4A). These cells were capable of inducing abscess formation when injected into mice (Fig. 4B). In contrast, the injection of exopolysaccharide alone did not induce abscess formation (Fig. 4C).

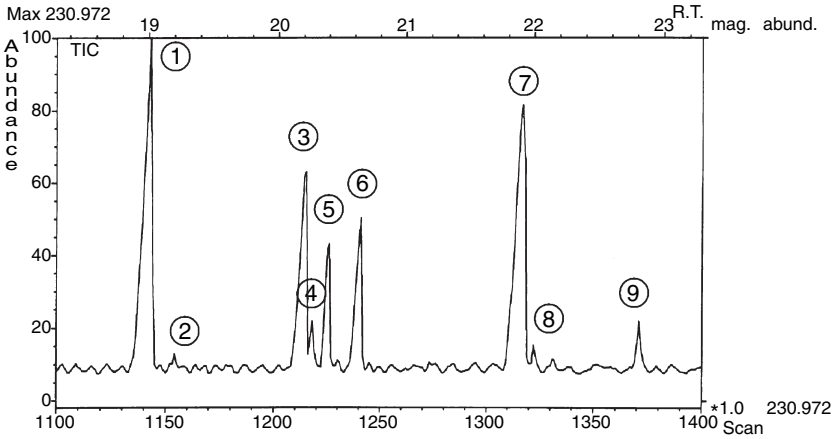

Fig. 2. Total ion current chromatogram of the alditol acetates of methylated sugars from exopolysaccharide produced by *P. nigrescens* strain 22. Exopolysaccharide purified from *P. nigrescens* strain 22 were methylated, hydrolysed, reduced, acetylated, and analyzed by GC/MS. **1**: 1,5-di-o-acetyl-2,3,4,6-tetra-o-methylmannitol. **2**: 1,5-di-o-acetyl-2,3,4,6-tetra-o-methylgalactitol. **3**: 1,2,5-tri-o-acetyl-3,4,6-tri-o-methylmannitol. **4**: 1,4,5-tri-o-acetyl-2,3,6-tri-o-methylmannitol. **5**: 1,3,5-tri-o-acetyl-2,4,6-tri-o-methylmannitol. **6**: 1,5,6-tri-o-acetyl-2,3,4-tri-o-methylmannitol. **7**: 1,2,5,6-tetra-o-acetyl-3,4-di-o-methylmannitol. **8**: 1,3,5,6-tetra-o-acetyl-2,4-di-o-methylmannitol. **9**: 1,2,4,5,6-pent-o-acetyl-3-o-methylmannitol.

Table 3. Methylation analysis data of exopolysaccharide

Methylated sugars	Relative proportions[a]
2, 3, 4, 6-Man	1.00
2, 3, 4, 6-Gal	0.03
3, 4, 6-Man	0.47
2, 3, 6-Man	0.07
2, 4, 6-Man	0.22
2, 3, 4-Man	0.32
3, 4-Man	0.74
2, 4-Man	0.02
3-Man	0.07

[a]Proportions were obtained as a ratio to 1,5-di-o-acetyl-2,3,4,6-tetra-o-methylmannitol.

Inhibitory effects of exopolysaccharide on phagocytic activity of human leukocytes

One possible mechanism that might contribute to the ability of exopolysaccharide-producing strain 22 and its heat-killed counterpart to induce abscess formation in mice was their resistance to uptake by the host phagocytic cells. In the phagocytosis experiments, strain 328 cells were readily internalized by polymorphonuclear leukocytes after 90 min of incubation. Many of these bacteria were found in the cytoplasm of the polymorphonuclear leukocytes (Fig. 5B). In contrast, strain 22 and its heat-killed cells were rarely internalized, though many of these cells were bound to the cell surface of polymorphonuclear leukocytes (Fig. 5A, C). Phagocytosis profiles similar to the ones described above were obtained for strains 22 and 328 under opsonized conditions (Fig. 5D, E).

Examination of samples by transmission electron microscopy confirmed that strain 328 cells (Fig. 6C, D), but not strain 22 cells (Fig. 6A, B), were internalized by polymorphonuclear leukocytes, and the ingested bacteria appeared to be surrounded by membranes (Fig. 6C, D).

Anti-phagocytic effect of purified exopolysaccharide from strain 22

As compared to controls (Fig. 7A), addition of exopolysaccharide to the culture medium significantly inhibited the internalization of human type AB serum-coated latex beads by polymorphonuclear leukocytes, as indicated by the significant reductions in the number of polymorphonuclear leukocytes containing fluorolabeled latex beads (Fig. 7). This inhibition appeared to be dose dependent (Fig. 7B, C).

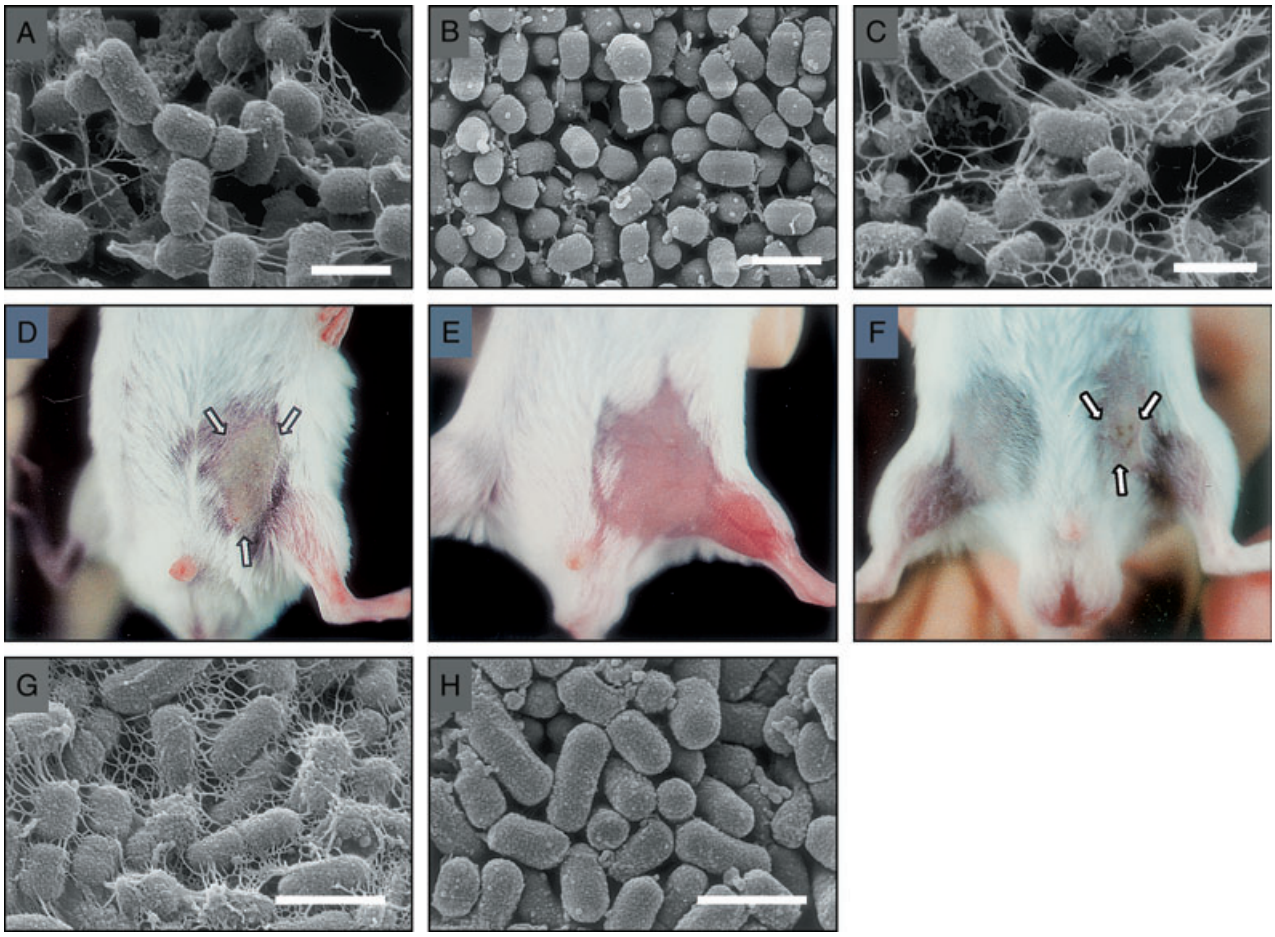

Fig. 3. Abscess induction in mice and the morphology of exopolysaccharide-producing and nonproducing strains before and after passage. Scanning electron micrographs showing surface structures of exopolysaccharide-producing strain 22 (A), nonproducing mutant 328 (B) and heat-killed strain 22 (C) before passaged into mice. A net-like structure was present around strain 22 and the heat-killed strain 22 cells (A and C). Abscesses were induced when 500 μl of bacterial cell suspension (3×10^7 CFU/ml) of strain 22 (D) or heat-killed strain 22 (F) was injected into an inguinal area of the mouse. No abscess was observed when mice were injected with strain 328 (E). The abscess lesion induced by strain 22 (D, arrows) was larger than that induced by heat-killed strain 22 (F, arrows). Both lesions persisted for 4 days. In contrast, the swelling induced by the subcutaneous injection of strain 328 resolved by day 2. After the animal passage, the net-like structure became more abundant (G) around cells of strain 22, but not around cells of exopolysaccharide-nonproducing mutant strain 328 (H). Bar = 1.0 μm.

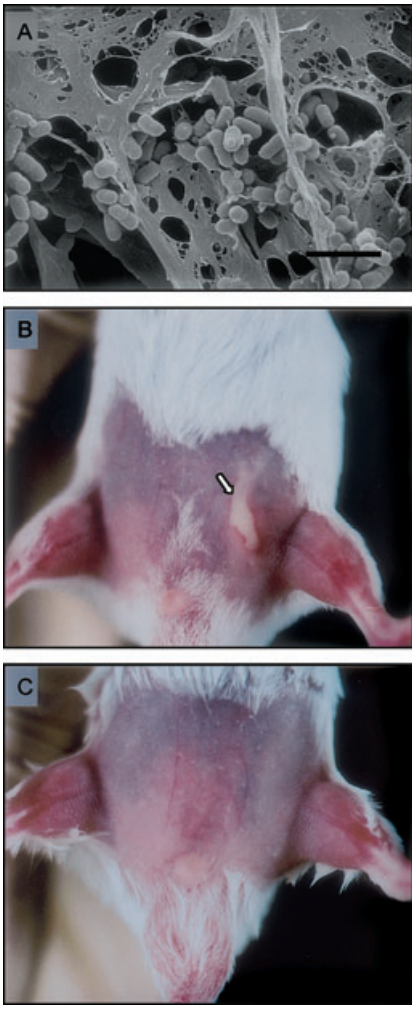

Fig. 4. Abscess formation by strain 328 supplemented with purified exopolysaccharide isolated from strain 22. Purified exopolysaccharide from strain 22 was added to cell suspensions of strain 328 at a concentration of 0.5 mg/ml. The added exopolysaccharide formed a net-like structure around strain 328 cells (A, bar = 3 μm). 500 μl of cell suspension (3×10^7 CFU/ml) with purified exopolysaccharide was injected into an inguinal area of a mouse. The abscess lesion was photographed 3 days after injection (B). Abscesses did not form in mice injected with 500 μl of exopolysaccharide (0.5 mg/ml) each (C).

Discussion

The pathogenic potential of *P. nigrescens* and the related *P. intermedia* varies among strains. For example, different strains of *P. nigrescens* and *P. intermedia* vary in their ability to adhere to buccal epithelial cells (25), invade human oral epithelial cells (11), agglutinate mammalian erythrocytes, produce enzymes which include lecithinase (14), elastolytic serine protease (33) or acid phosphatases (9), and degrade IgG (22). In this communication, we report the identification of an exopolysaccharide that was unique to a clinical strain of *P. nigrescens* isolated from an oral lesion. Ideally, genetically targeted mutants that are deficient in exopolysaccharide biosynthesis should be used for studying the biological effects of exopolysaccharide since we hypothesized that the exopolysaccharide served as a virulence factor promoting the infection associated with this clinical isolate. However, a genetic transfer system for introducing DNA into *P. intermedia/nigrescens* remains to be developed (26). This is further complicated by our observation that these bacteria may possess potent restriction systems for excluding foreign DNA (unpublished data). Therefore, to circumvent the lack of adequate molecular tools for constructing gene knockouts in these organisms, we elected to establish a chemically induced exopolysaccharide nonproducing mutant, strain 328, from exopolysaccharide-producing strain 22, for studying the function of exopolysaccharide. While there are concerns about the stability of the chemically induced mutants and the possibilities of multiple mutations induced by the process, both strains 328 and 22 exhibited similar enzymatic profiles (Table 1) and growth patterns (data not shown). During this study, we did not observe the emergence of revertants from these mutants.

In this study, we found that the exopolysaccharide-producing isolate strain 22 was far more potent in causing abscess formation in mice than was strain 328, the chemically induced mutant that lacked exopolysaccharide. This was the case despite both strains 22 and 328 being

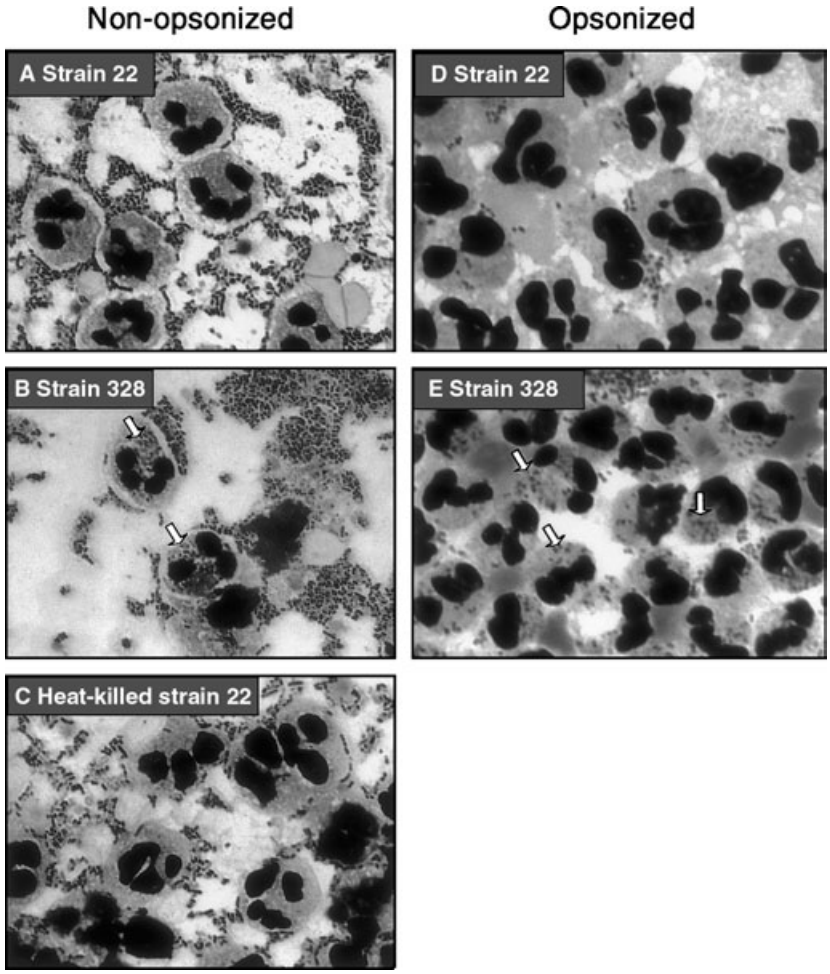

Fig. 5. Anti-phagocytic effect of exopolysaccharide to phagocytosis by polymorphonuclear leukocytes. Exopolysaccharide-producing strain 22 (A) and heat-killed strain 22 (C) were not internalized by polymorphonuclear leukocytes, but exopolysaccharide-nonproducing strain 328 was internalized. Many bacteria are located in the cytoplasmic vacuoles (B, arrows). Under opsonized conditions (8%, v/v, human AB serum in the medium), strain 22 was not internalized by polymorphonuclear leukocytes (D) but strain 328 was (E, arrows). The data are from one of three independent experiments. Magnification: ×1000.

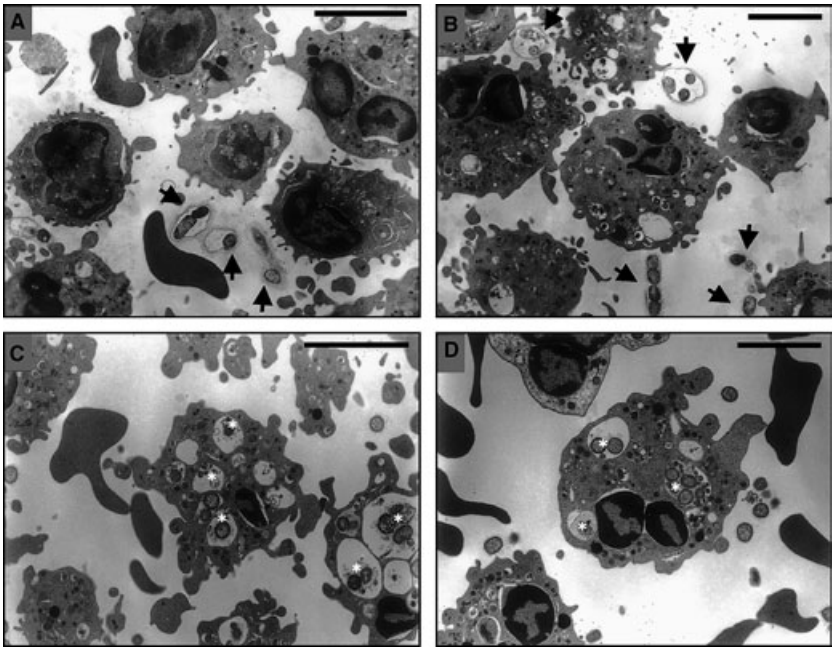

Fig. 6. Resistance of exopolysaccharide-producing strain 22 against the phagocytic activity of human polymorphonuclear leukocytes. Strain 22 cells (A and B, arrows) were not internalized by polymorphonuclear leukocytes. Exopolysaccharide-nonproducing strain 328 cells, on the other hand, were internalized and the ingested bacteria appear to be enclosed within a cytoplasmic vacuole (C and D, asterisks). A & C and B & D represent two independent experiments. Bars = 2.8 μm.

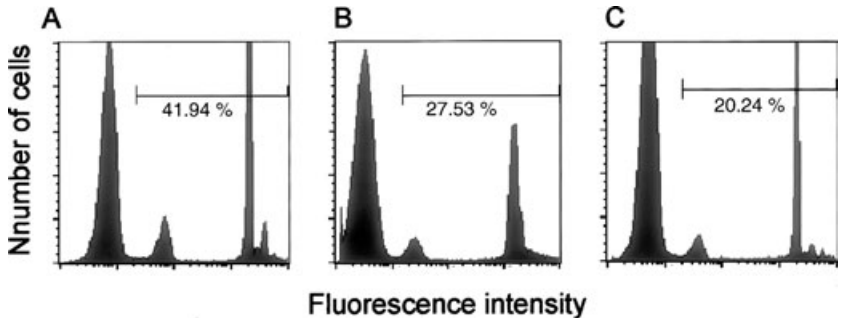

Fig. 7. Anti-phagocytic effect of purified exopolysaccharide from strain 22. Opsonized fluorescent latex beads, mixed with purified exopolysaccharide from strain 22, were incubated with human polymorphonuclear leukocytes for 90 min. The phagocytosing samples were analyzed by FACS. The filled histogram in A indicates the data from human type AB serum only; the histograms in B and C indicate the inhibitory effects of 0.5 mg/ml and 2.5 mg/ml exopolysaccharide on phagocytosis by polymorphonuclear leukocytes, respectively. The data are from one of two independent experiments.

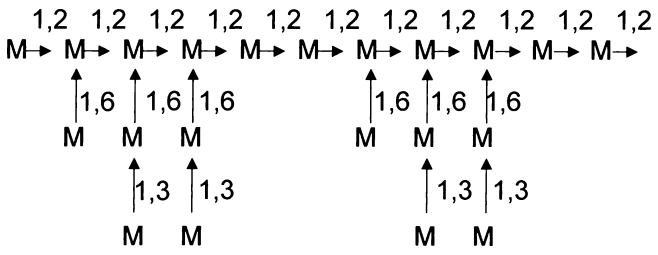

Fig. 8. Putative glycosidic linkages of mannose residues in exopolysaccharide from strain 22.

capable of producing similar hydrolytic enzymes, including lecithinase and lipase, and exhibiting similar enzymatic profiles (see Table 1). In this mutant strain, we did not detect the presence of any mesh-like structures morphologically on the cell wall of strain 328 cells, nor were we able to isolate any substantial amount of exopolysaccharide from the culture medium of this mutant. When the purified exopolysaccharide of strain 22 was added to the cell suspension of strain 328, the ability of strain 328 to induce abscess formation in mice was restored. These findings were consistent with the observation that exopolysaccharide of strain 22, which was seen as meshwork structures around the cells used as the inoculum, became significantly denser around the cells that were recovered later from the lesions (Fig. 3G). The data from the heat-killed counterpart of strain 22 also indicated that this structure, but not other secreted bacterial products, was essential for inducing the abscess formation. To lend more support to our observation, we also determined the viscosity of the culture supernatants isolated from strain 22 as compared to that of the strain 328 and the control medium. While the high viscosity of the culture supernatant is usually indicative of the presence of exopolysaccharide, the viscosity of the culture supernatant of strain 328 was similar to that of the control medium, suggesting that there was a lack of exopolysaccharide production in these cells. Though we were uncertain whether the deficiency in exopolysaccharide production found in these cells was due to the lack of machinery for the synthesis, assembly, or secretion of exopolysaccharide, on the basis of the results obtained we conclude that the exopolysaccharide produced by strain 22 might be associated with the pathogenic properties of this clinical isolate.

Exopolysaccharide, which appears in the forms of capsular polysaccharide, viscous material, or slime, is produced by many microorganisms that infect plants, animals, or humans (10). These materials are considered important virulence factors due to their possible roles in promoting colonization and persistence of infection exhibited by many of the microorganisms (10). For example, an exopolysaccharide produced by *Vibrio cholerae* is required for permanent immobilization (20). Clinical isolates of coagulase-negative staphylococci produce a hexosamine that is involved in mediating the adherence of the organisms to medical devices and is responsible for their resistance to host defenses (1). Further, *Pseudomonas aeruginosa*, a major pathogen of cystic fibrosis, produces large amounts of alginate-like polysaccharides, which are associated with the invasiveness of this organism (16). Alginate is a family of unbranched, non-

repeating copolymers consisting of variable amounts of (1-4)-linked β-D-mannuronic acid and its epimer α-L-guluronic acid (31). *Burkholderia cepacia*, previously known as *Pseudomonas cepacia*, has also been shown to produce PS I and PS II (other forms of exopolysaccharide), which are also considered potential virulence factors (8). The structure of the exopolysaccharide produced by a clinical isolate of *B. cepacia* is a repeated unit of −3)GlcA(1-3)-Man(1-3)-Glc– with branches of a disaccharide Gal(1, 2)-Rha– and a single galactosyl residue (8). In addition, colanic acid consists of repeated trisaccharides −3)Fuc(1-4)-Fuc(1-3)-Glc– with an oligosaccharide branch Pyr-Gal(1-4)-GlcA(1-3)-Gal(1-4)– (36) and is an important constituent of the biofilms formed by most *Escherichia coli* strains and other members of Enterobacteriaceae (17, 36). Colanic acid is crucial for the survival of these organisms in various hostile environments, especially under desiccated conditions (30). In contrast, the main constituent of exopolysaccharide isolated from strain 22 was mannose (Table 2) and this polysaccharide was different from other exopolysaccharides as described above. Results from the methylation analysis indicated the presence of a highly branched chain in this polysaccharide. A putative repeated unit in the exopolysaccharide from strain 22 is described in Fig. 8. To this end, a mannose-rich polysaccharide produced by *Capnocytophaga ochracea* has been reported (12). This exopolysaccharide, which contains 80% mannose (12), suppresses both human and murine lymphocyte responses to the mitogens, lipopolysaccharide and concanavalin A *in vitro* (4, 6) and activates human complement (5).

The capability of strain 22 to induce abscess in mice might be attributable to the cell-associated exopolysaccharide protecting the bacteria from the phagocytic system without evoking host immune responses, thereby ensuring a longer survival of the organisms in the host. Our *in vitro* data indicate that the exopolysaccharide isolated from strain 22 had an antiphagocytic effect on polymorphonuclear leukocytes under opsonized conditions. We also learned that when human peripheral blood mononuclear cells or HT3T (monocytic linear) were cocultured with the exopolysaccharide isolated from strain 22 or the culture supernatant of strain 22, interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor-α were not induced to any significant degree (data not shown). In fact, the stimulatory effects observed after these cells were exposed to lipopolysaccharide were reduced in the presence of exopolysaccharide isolated from strain 22. In contrast, strain 328, which did not possess the fibrous material (exopolysaccharide) and was readily phagocytosed by polymorphonuclear leukocytes in the *in vitro* tests, lacked the ability to induce abscess formation in mice. It is conceivable that exopolysaccharide could play an important role in determining the susceptibilities of strains 22 and 328 to phagocytosis, which could influence the formation of abscess when these bacteria are injected into mice. There are indeed studies in the literature indicating that the exopolysaccharide (alginate) of *P. aeruginosa* plays a key role as an antiphagocytic factor in the establishment of opportunistic infection (32). Although exopolysaccharide could be an antiphagocytic factor in this study, other surface structures (for example lipopolysaccharide) were altered in these chemically induced mutants, rendering them more susceptible to phagocytosis by polymorphonuclear leukocytes. However, the observation that exopolysaccharide isolated from strain 22, which essentially was devoid of lipopolysaccharide as tested, was capable of restoring the ability of strain 328 to induce the abscess formation and of suppressing the phagocytosis of opsonized latex beads by polymorphonuclear leukocytes strongly argues for the pathogenic potential of exopolysaccharide. The definitive determination of the virulence properties associated with the exopolysaccharide isolated from *P. nigrescens* awaits the development of a suitable molecular tool that is capable of introducing specific targeted mutagenesis in these organisms.

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