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Development and Potential Use of a Monoclonal Antibody to the Lipopolysaccharide of *Pantoea agglomerans* (IP-PA1)

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Abstract. *Background: The lipopolysaccharide of Pantoea agglomerans (named IP-PA1 by us) has been shown to be effective and safe in the prevention of various diseases such as bacterial or viral infection, lifestyle-related diseases when administered transdermally or orally. To clarify the mechanisms of the preventive or therapeutic effect induced by IP-PA1, a monoclonal antibody to detect IP-PA1 would be useful. For this reason we tried to establish a monoclonal antibody to IP-PA1 and used enzyme-linked immunosorbent assay (ELISA) to measure the amount of IP-PA1. Materials and Methods: Antibodies were raised by immunization using heat killed Pantoea agglomerans, screening was conducted to isolate monoclonal antibodies specific to IP-PA1. Results: Six kinds of IP-PA1 specific monoclonal antibodies with different epitopes were established. An ELISA using the monoclonal antibodies was successfully established which could specifically detect IP-PA1. Conclusion: By use of this ELISA, the staple food content and pharmacodynamic analysis of IP-PA1 could be conveniently estimated.*

Innate immunity is a universal prophylactic system which all multi-cellular animals possess. Macrophages are the cells which play the central role in the innate immune system (1). In 1991, we found a substance derived from a water extract of flour which activated macrophages through oral or intradermal administration (2, 3). The active substance was lipopolysaccharide (LPS) derived from *Pantoea agglomerans*, a Gram-negative bacterium, which grows symbiotically with wheat (4). We named it IP-PA1 (immune potentiator obtained from *P. agglomerans*) (3) and considered it to be applicable to various fields such as health food to prevent and improve metabolic syndromes, skincare products to maintain a healthy state of the skin and feedstuffs for stockbreeding and aquatic culture in which defense against infection is a pressing issue. Accordingly, we demonstrated that IP-PA1 *via* the oral route was useful in preventing and/or restoring various health disorders (3, 5-11).

However the molecular and/or immunological mechanisms involved in the beneficial effect of IP-PA1 were still largely unknown. One of the major difficulties of such analyses was that no method of measuring and detecting IP-PA1 specifically, was yet available. The Limulus test has often been used to measure the amount of LPS, however this reaction is not specific for each LPS. A monoclonal antibody to a specific LPS could fulfill the requirements described above, depending on its biological characteristics. Many monoclonal antibodies recognizing different epitopes of *Escherichia coli* (*E. coli*) LPS have already been established, and these have been quite useful for identifying *E. coli* serotypes such as O157~~:H7~~. Thus, if we could raise monoclonal antibodies to IP-PA1, it would be very helpful not only for discriminating subspecies of *P. agglomerans* but also for analyzing the mode of action of IP-PA1. Also it might be possible to obtain structurally different LPS from subspecies of *P. agglomerans*.

It would be essential, in raising monoclonal antibodies that they bind mainly to the O-antigen which was known to

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Key Words: *Pantoea agglomerans*, low molecular weight LPS, IP-PA1, monoclonal antibody, enzyme linked ~~immunesolvent~~ immunosorbent assay.

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be quite heterogeneous even within each bacterial species (13). The structure of lipid A of IP-PA1 has been analyzed revealing that it quite resembles that of *E. coli* (14), thus we tried to establish monoclonal antibodies with the characteristic that they could bind mainly to the sugar moiety and less to lipid A and the core structure.

Finally, in addition to establishing monoclonal antibodies to meet the criteria described above we also attempted to establish ELISA systems using two different species of antibodies.

Materials and Methods

Antigens. P. agglomerans was grown for 12 to 18 h at 37˚C in a Luria culture broth medium (pH 7.0, 10 g/l bacto tryptone, 5 g/l yeast extract and 10 g/l NaCl). The cultures were harvested by centrifugation at 3000 g at 4˚C and frozen and stored at –20˚C until use. IP-PA1 was purified to over 99% according to the methods described previously (2). In brief, LPS was extracted by the hot phenol method of Westphal and Jann (15). Cells were extracted twice with a 45% (w/w) aqueous phenol at 65 to 68˚C for 20 min. The collected aqueous phase was dialyzed against distilled water several times at 4˚C and applied to a Q-sepharose anion-exchange column. After stepwise elution, a 400 mM NaCl fraction was collected and ultrafiltrated through a membrane (molecular weight cut off 200 kDa, Advantec Toyo, Tokyo, Japan OHP-150). The final samples were frozen and dried. The purity of LPS was >99% (w/w) (protein contamination was 0.5%, nucleic acid contamination was less than 0.35% (w/w)). Low-molecular-mass-IP-PA1 (LMM-IP-PA1) was subfractionated from IP-PA1 by gel filtration in the presence of sodium deoxycholate (16). The LMM-IP-PA1 showed a stained band only at 4 to 6 kDa by analysis using tricine-SDS-PAGE with silver staining. Defatted rice bran extract and rice bran extract were prepared as described below. Two grams of rice bran were suspended and vigorously mixed with LPS-free water at a final concentration of 100 mg/ml and then extracted by heating at 90˚C for 20 min. The extract was then centrifuged at 3000 g for 5 min at room temperature, the supernatant was collected as rice bran extract. Defatted rice bran extract was prepared by the same procedure.

Immunization of BALB/c mice and preparation of the hybridoma. Ten BALB/c mice (6-week-old male) were injected intraperitoneally (*i.p.*) with $1x10^8$ cells of heat killed *P. agglomerans*. A second immunization was given at a 2-week interval with $1x10^8$ cells of heat killed *P. agglomerans*. The mice which showed a high antibody titer to the purified IP-PA1 antigen were given a final *i.p.* injection. The fusion was performed 3 days following the last injection. Isolated spleen cells and P3U1 mouse myeloma cells (5:1) were fused in the presence of 50% polyethylene glycol 1000 (Wako, Osaka, Japan)(17). The fused cells were cultured with HAT selection medium which consisted of hypoxanthine (13.6 μg/ml, Wako), thymidine (3.8 μg/ml, Wako) and aminopterin (0.176 μg/ml, Wako) in RPMI1640 (SIGMA, MO, USA) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), kanamycin (50 μg/ml, Banyu Pharmaceutical, Tokyo, Japan) and ampicillin (60 μg/ml, Wako) at 37˚C in a 5% CO_2 incubator.

ELISA. Purified IP-PA1 was used in a solid-phase indirect ELISA to determine the binding specificities of the monoclonal antibodies. The 96 multi-well ELISA plates (Maxisorp EIA plates, Nunc, Roskiled, Denmark) were coated with 10.0 μg/ml of purified IP-PA1 in 0.05 M carbonate buffer (pH 9.8) at 37˚C for 1 h or at 4˚C over night. The wells were then blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature and then washed with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T), the culture supernatants were added, and the plates were incubated for 1 h at room temperature. Following washing with TBS-T, alkaline phosphatase-labeled goat anti-mouse immunoglobulins IgG, IgA and IgM (Sigma) diluted 1:1000 in 1% BSA-PBS were added for 1 h at room temperature. One hundred microliters of p-nitrophenyl phosphate (p-NPP) (1 mg/ml) (Wako) dissolved in 0.05 M Na_2CO_3, 0.001 M $MgCl_2$ (Wako) was then added. The solution was incubated at room temperature for 1 h before 50 μl of 2 molt NaOH was added and mixed. The optical densities (415 nm) were read with a microplate reader (BIO-RAD, CA, USA, Model 550).

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Cloning of the monoclonal antibody producing hybridomas. The hybridoma supernatants after HAT selection were collected and tested for antibody reactivity against LPS from *P. agglomerans* by the ELISA with alkaline phosphatase-conjugated goat anti-mouse IgG, IgA and IgM. The hybridomas producing antibodies were selected and cloned twice by limiting dilution to ensure stability and clonality following the conventional methods. The clones were expanded as ascites by intraperitoneal injection of $5x10^6$ hybridoma cells in BALB/c mice 10 to 14 days following *i.p.* priming with 0.5 ml of 2, 6, 10, 14-tetramethyl-pentadecane (pristine). Ascitic fluid was collected 7 to 14 days postinoculation of the hybridomas.

pristane

Characterization of the monoclonal antibodies. The Ig isotypes were determined with condensed supernatant of the culture medium by a mouse monoclonal antibody isotyping kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cross-reactivity of the monoclonal antibodies to other kinds of LPS was tested by the ELISA assay using 16 kinds of LPS (*E. coli* 127 B8 (Difco), *Salmonella typhimurium* (*S. typhimurium*) BOag(BioCarb), *S. typhimurium* DOag O:9(BioCarb), *Klebsiella pneumoniae*, *Serratia marcescene*, *Salmonella minnesota* wild-type, *Salmonella minnesota* R595, *E. coli* K12, *E. coli*-J5 (Rc), *Bordettera pertussis* L65, Pseudomonas aeruginosa F-D TYPE 1, *Vibrio cholerae* Inaba 569B-LPS (all List Biological Laboratories Inc, CA, USA), *Yersinia*-LPS and a synthetic lipid A (Synthetic lipid A LA-15-PP) (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan)) as the coated antigen.

Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. Electrophoretic analysis of LPS was performed as described elsewhere (2) on 15% polyacrylamide gels. Prestained protein molecular weight markers, (Broad range, New England BioLabs Inc., Mass., USA) were used to determine molecular size of the LPS bands on gels stained by a silver staining procedure using Silvesteron kit (Nacalai Tesque, Inc, Kyoto, Japan). The LPS separated on gels were electro-blotted onto polyvinylidene difluoride membranes (Sequi-Blot, Bio-RAD), using a semidry blotting apparatus (TAITEC, Saitama, Japan, SB-160) at 80 V for 90 min, transfer buffer was 20 mM Tris-glycine containing 5% methanol (pH 8.3). The blotted sheets were cut longitudinally into sections, and each one was tested by incubation with a different

Table I. *Characteristics of established monoclonal antibodies.*

Monoclonal antibody	Isotype Heavy chain/Light chain	Antigen(s) Putative Recognition site
32-G2	IgG2b/κ	O-antigen
34-G2	IgM/κ	O-antigen
4-E11	IgG3/κ	O-antigen
20-A8	IgG1/κ	O-antigen
49-F2	IgG3/κ	O-antigen
29-D3	IgM/κ	Lipid A
103-E11	IgM/κ	Lipid A
86-F11	IgM/κ	O-antigen

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antibody solution. Then, alkaline phosphatase conjugated anti-mouse IgG, IgA and IgM goat antibodies were used as secondary antibodies. Finally, IP-PA1 was visualized with nitro blue tetrazolium / bromochloroindolyl phosphate (NBT/BCIP).

IP-PA1 quantification by anti-IP-PA1 monoclonal antibodies. Anti-IP-PA1 monoclonal antibody (IgM, 34-G2) which excluded IgG by a protein G column (Pharmacia, Mich., USA) was used in a solid-phase antibody for IP-PA1 sandwich ELISA. ELISA plates (Maxisorp EIA, Nunc) were coated with 50 µl of diluted IgM, 34-G2 in PBS, at 37°C for 1 h or at 4°C over night. The wells were then blocked with 3% BSA-PBS for 1 h at room temperature and washed with TBS (pH 7.5)-0.05%Tween 20 (TBS-T), 50 µl of standard diluted IP-PA1 or the unknown sample solution diluted with 1% BSA-PBS were added, and the plates were incubated for 1 h at room temperature. Following washing with TBS-T, 50 µl of diluted secondary anti-IP-PA1 monoclonal antibody was added, and the plates were incubated for 1 h at room temperature. Following washing with TBS-T, alkaline phosphatase-labeled goat anti-mouse IgG (Sigma) diluted 1:1000 in 1% BSA-PBS was added for 1 h at room temperature. The plates were then washed and 100 µl of p-NPP (1 mg/ml, Wako) was added for 1 ng/ml to 100 ~~g/ml~~ ng/ml concentration of IP-PA1. After 30 to 60 min, the absorbance at 415 nm was measured by a microplate reader (Model 550, BIO-RAD). For the measurement of 0.2 ng/ml to 12.5 ng/ml of IP-PA1, ELISA amplification kit (Invitrogen, Calif., USA) was used instead of the p-NPP according to the instructions provided by kit.

Results

Establishment of hybridoma clones producing monoclonal antibody which specifically bound to IP-PA1. Two mice spleen which showed high ~~serum titer~~ against IP-PA1 were used for fusion with the myeloma cells. Approximately 1000 wells containing hybridoma cells were obtained and the monoclonal antibody content of the supernatant of the cultured cells assayed. One hundred and twenty five wells showed IP-PA1 antibody positive supernatant. The IP-PA1 monoclonal antibody producing hybridoma cells, were expanded further to establish the clones that were used to produce the monoclonal antibody that bound with IP-PA1. Finally, 8 clones, denoted as 4-E11, 20-A8, 29-D3, 32-G2, 34-G2, 49-F2, 86-F11 and 103-E11 were successfully isolated (Table I). The isotype of monoclonal antibody produced by each clones was determined as shown in Table I.

serum antibody titer

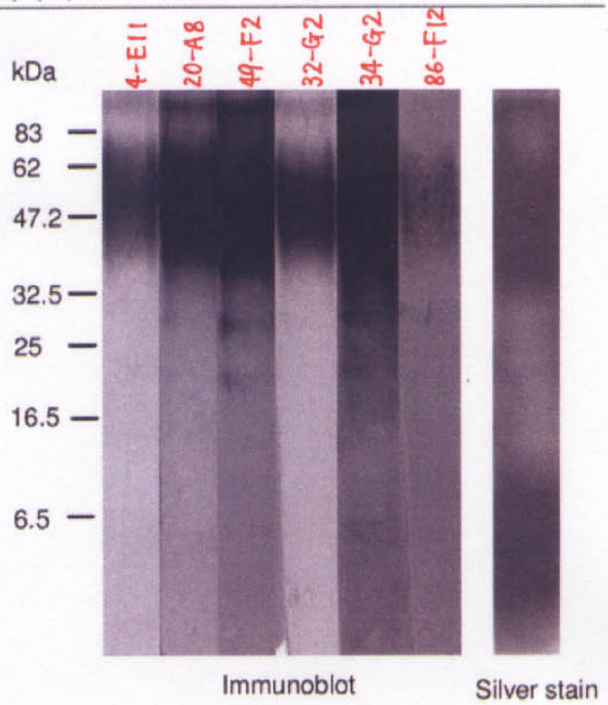

Figure 1. *Immunoblots showing reactivities of monoclonal antibodies with IP-PA1 (LPS antigen bands derived from Pantoea agglomerans), following* ~~*tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis*~~. *IP-PA1 were fractionated on a 15% gel and transferred by electroblotting onto sheets of polyvinylidene difluoride membrane. Subsequently, the sheets were cut into strips, treated with individual monoclonal antibodies followed by alkaline phosphatase conjugated anti-mouse IgG, IgA, and IgM antibody. IP-PA1 were visualized with NBT/BCIP. Silver stain: IP-PA1 visualized by silver staining after tricine-SDS-PAGE. The numbers refer to molecular weight of protein standard.*

tricine-SDS-PAGE

Cross-reactivity for various LPSs. The characteristics, in terms of specificity, each monoclonal antibody epitope produced by the 8 clones were determined using 17 different LPS by ELISA ~~assay~~ (Table II). Five clones (4-E11, 20-A8, 32-G2, 49-F2, and 86-F11) out of the 8 clones specifically reacted with IP-PA1, and they did not react with the other LPS or synthetic Lipid A (Figure 1, Table II). In contrast, 34-G2 reacted with IP-PA1 and LMM-IP-PA1 and 29-D3 and 103-E11 reacted with all the LPS samples examined including synthetic Lipid A (Table II). Immunoblots analysis (Figure 1) and ELISA ~~assay~~ (Table II) results strongly suggested that the 5 clones (4-E11, 20-A8, 32-G2, 49-F2, and 86-F11) specifically reacted with the polysaccharide part of the IP-PA1, and the 2 clones (29-D3 and 103-E11) reacted with the lipid A portion.

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Table II. *ELISA reactivity of monoclonal antibodies.*

Antibody	*P.a.*		*E. coli*					*S.mi.*		*S.typh.*		*S.ma.*	*P.ae.*	*V.ch.*	*K.pn.*	*Y.*	*B.p.*
	IP-PA1	LMM-IP-PA1	synthetic LipidA	O111: B4	O127	J5 (Re)	K12 mm 294	R595 (Re)	wild-type	BO	DO		F-D type 1				
32-G2	100[a]	2.0	0.4	0.4	0.6	0.5	-0.1	0.3	0.2	0.2	-0.4	0.2	0.3	0.1	-0.7	0.4	0
34-G2	100	17.5	1.0	0.8	3.4	0.8	0.6	1.0	0.6	2.9	2.8	0.7	0.9	0.7	0.4	3.0	3.8
4-E11	100	1.6	-0.3	0.2	0.2	0.4	0.4	0.3	0.5	0.7	-1.2	0.4	0.4	0.4	1.2	-0.4	-1.0
20-A8	100	0.4	0.2	0	0.6	-0.1	-0.2	-0.1	-0.1	0.2	0.1	-0.2	-0.1	-0.2	-0.5	0.1	0.2
49-F2	100	0.4	0.3	0.2	7.0	0.2	-0.2	0.1	0	0.7	-0.6	0.5	0.2	0.1	0	0	0.1
29-D3	100	102.5	121.9	68.2	61.3	99.8	94.3	101.2	54.5	63.2	89.9	101.6	79.5	41.6	117.9	105.9	121.2
103-E11	100	107.3	205.1	73.4	75.4	102.6	103.8	108.3	59.2	32.5	85.5	105.3	101.6	42.3	109.9	153.8	120.5
86-F11	100	0.5	0.6	-0.7	0.3	1.3	0.2	0.3	1.2	0.2	0.3	0	0.8	0.8	-0.4	0.6	0.5

a: indicated numbers in table means relative % for the absorption of IP-PA1 (100%). P.a.: *Pantoea agglomerans*, S.mi.: *Salmonella minnesota*, S.ma.: *Serratia marcescene*, P.ae.: *Pseudmonas aeruginosa*, V. ch.: *Vibrio cholerae*, K.pn.: *Klebsiella pneumoniae*, B.p.: *Bordettera pertussis*, Y.: *Yersinia*-LPS, S.typh.: *Salmonella typhimurium*.

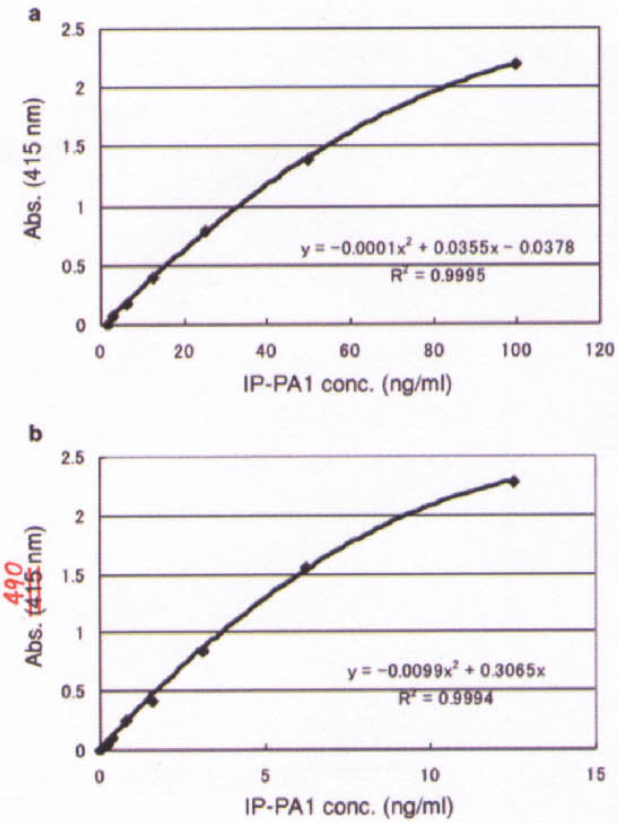

Figure 2. *Standard curve of IP-PA1 by two ELISA methods. a) p-NPP b) ELISA amplification kit. Measurement method is described in the Materials and Methods.*

Quantification of IP-PA1 by ELISA. To establish the quantification method for IP-PA1 by ELISA, clone 34-G2 was used to capture IP-PA1, and clone ~~4E-11~~ 4-E11 was used as a

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Table III. *Spike test.*

Sample	IP-PA1 conc. (μg/ml)
E. coli #127 LPS (16 μg/ml)	ND
IP-PA1 (16 μg/ml) + *E. coli* #127 LPS (16 μg/ml)	16.0
Defatted rice bran extract	ND
Rice bran extract	ND
Rice bran extract + IP-PA1 (16 μg/ml)	16.0

ND: not detected (15.6< ng/ml). *E. coli* O127 LPS and IP-PA1 samples were diluted from 2.0 mg/ml solution. Rice bran extract containing IP-PA1 (final concentration: 16 μg/ml) was prepared by addition of 1/125 volume of IP-PA1 (2.0 mg/ml) to the rice bran extract. Sixty four to 2,056 times diluted samples were used as samples for ELISA.

specific counterpart of the IP-PA1-34-G2 complex. As shown in Figure 2a, this assay system could detect 1.6 ng/ml to 100 ng/ml of IP-PA1 (R^2>0.99). Moreover, the commercially available ELISA amplification kit enhanced the detection limit to 0.2 ng/ml to 12.5 ng/ml (R^2>0.99) (Figure 2b).

To determine that the IP-PA1 was measured accurately and not the coexistence of other kind of LPS, the IP-PA1 was measured by using the ELISA system with the coexistence of *E. coli* LPS or other materials. As shown in the Table III, the IP-PA1 could be accurately measured with the coexistence of extract of *E. coli* LPS and rice bran which is one of the candidate substances for inclusion in IP-PA1 containing fermented wheat extract. (Rice bran had no detectable IP-PA1.) From these results, the ELISA system would be sufficiently sensitive to measure IP-PA1 in a mixed material such as food, and to monitor localization or absorption or distribution by immuno-histochemical study.

Discussion

Six kinds of monoclonal antibodies to IP-PA1 were obtained which did not react with LPSs prepared from other species of Gram negative bacteria or synthetic lipid A, at least as examined in this investigation (Table II). The O-antigen polysaccharide structure of LPS is thought to be heterogeneous even within the same species of bacteria. The results suggested that the monoclonal antibodies to IP-PA1 obtained in this study may recognize the O-antigen polysaccharide structure of the IP-PA1. The monoclonal antibodies to IP-PA1 may serve as tools for analysis of the sugar chain structure of IP-PA1, which has not yet been determined.

We were able to construct a quantification method for IP-PA1 by an indirect sandwich ELISA system using two of the monoclonal antibodies to IP-PA1. Using this system, the amount of IP-PA1 in a fermented wheat extract and in various kinds of material which included rice bran or lactose were measurable (data not shown). In addition, the IP-PA1 content at around 100 ng/g in feeding stuff for chickens or aqua farming could be detected as an estimated amount (data not shown).

The monoclonal antibodies to IP-PA1 may be of use for the analysis and tracking of IP-PA1 *in vivo* and *in vitro*, for example, absorption, distribution, metabolism and excretion of IP-PA1 in the gastrointestinal tract after oral administration or, absorption from the skin. In addition, monoclonal antibodies to IP-PA1 make it broadly possible to analyze the observed difference of bioactivity between *E. coli* LPS and IP-PA1 at a cellular and molecular level (2, 6).

Recently *P. agglomerans* has been focused upon as a useful Gram-negative bacterium of edible plant origin. *P. agglomerans* which could fix nitrogen and phosphorus has been isolated as a symbiotic bacterium not only from wheat, but also, from rice (18) and sweet potato (19), and has been investigated for growth promotion activity in these plants (18, 19). Moreover, the potential of *P. agglomerans* to control the major post-harvest diseases of Golden Delicious apples has been reported (20). Therefore the monoclonal antibodies to IP-PA1 obtained in this study are thought to be a powerful tool for identifying strains belonging to the *P. agglomerans* species.

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