

Production and Characterization of Monoclonal Antibody to *Pseudomonas fluorescens*

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ABSTRACT

Monoclonal antibody (MAb) against a *P. fluorescens* was produced by immunization of Balb/c mice with whole cell of the bacteria. Isotyping test revealed that the MAb was IgG2b with κ light chain. Western blot showed that MAb reacted with whole cell protein of 41 kDa and outer membrane protein (OMP) of 37 kDa. The MAb was considered to be a useful probe for strains identification and rapid diagnosis kit for *P. fluorescens*.

Key words: *Pseudomonas fluorescens*, monoclonal antibody, characterization

INTRODUCTION

Pseudomonas fluorescens is one of the major bacterial pathogens causing a problem in aquaculture. The aquatic animals subjected to some type of stress seem to be more susceptible to the disease. The infected animals show external clinical signs including septicemia, lesion, whirling movement and loss of balance. Disease diagnosis is mainly based on a conventional biochemical test, which is time-consuming and requires a culturing procedure. Therefore, a rapid and more specific diagnosis method would be useful in terms of disease control and farm monitoring.

Monoclonal antibody (MAb) has become an extraordinarily important resource for medical research, diagnosis, therapy, and basic science. MAb probe has made a significant impact on rapid

diagnosis of numerous aquatic animal diseases (Adam *et al.*, 1996). Many MABs have been developed against microbial pathogens such as *Aeromonas hydrophila* (Cartwright *et al.*, 1994), *Mycobacterium* species (Adam *et al.*, 1996; Chen *et al.*, 1997) yellow-head virus (Sithigorngul *et al.*, 2002) and iridovirus (Shi *et al.*, 2003). This study is aimed to develop MAb to *Pseudomonas fluorescens* to be used as a probe for strain identification and a rapid diagnosis kit.

MATERIALS AND METHODS

Culture and preparation of bacterial isolates

P. fluorescens (AAHRI 01419) isolated from infected flame gourami (*Colisa laria*) and other bacteria (Table 1) were grown on tryptic soy broth (TSB) at 30°C for 18 hours (h). The suspension was centrifuged at 3,500 x g for 30

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min and pellet was washed once with 50 ml sterile saline (0.85 % NaCl). It was then resuspended and adjusted to 1.0×10^7 CFU ml⁻¹ with sterile saline and stored at 4°C. The *P. fluorescens* (AAHRI 01419) suspension was subjected to UV at program sterile (GS Gene Linker, BioRad) for 9 min. The viability was confirmed on tryptic soy agar (TSA) and cultured at 30°C for 18 h.

Immunization of mice

Two 8-week-old Balb/c female mice were intraperitoneally (ip) injected with 100 µl of *P. fluorescens* (AAHRI 01419) suspension (1.0×10^7 CFU ml⁻¹) diluted 1:1 with TiterMax Gold adjuvant (CytRx Corporation). Twenty-eight days after the first injection, the second injection was given. Ten days later, blood was collected from tail for antibody titer. Briefly, the serum was serially diluted to test against *P. fluorescens* at 1.0×10^7 CFU ml⁻¹ by indirect enzyme-linked immunosorbent assay (ELISA). Four weeks after the second injection, the third injection was given. The antibody titer test was done 10 days after the injection. Four weeks after the third injection, a mouse was intravenously (iv) injected with 100 µl of bacterial suspension (0.5×10^6 CFU ml⁻¹) without adjuvant. The spleen was collected at day 3 after the injection.

Hybridoma production

Monoclonal antibodies (MAbs) were produced by the method of Campbell (1984) with modifications described by Adam *et al.* (1996). Supernatant of hybridoma cells producing antibodies were screened by indirect ELISA with *P. fluorescens* (1.0×10^7 CFU ml⁻¹). The positive clones were then cloned by the limiting dilution method. Selected hybridoma cells were expanded and maintained in medium (Dulbecco's modified eagle's medium, 2 mM glutamine, 0.5 mM sodium pyruvate, 100 i.u. ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 20% (v:v) fetal bovine serum).

Characterization of MAb

Specificity of the MAb was tested by indirect ELISA. The MAb was further characterized by Western blot. Cross-reaction was tested by indirect ELISA to different bacteria listed in Table 1. The MAb isotyping was tested with a Mouse-Typer[®] Isotyping Panel kit (BIO-RAD) as described by its manufacturer.

Bacterial preparation

Whole cell bacteria were adjusted to 1.0×10^7 CFU ml⁻¹ with sterile saline and were sonicated for 5 min (Sonopuls HD70, Bandelin).

Outer membrane protein (OMP) was extracted by the method of Bakopoulos *et al.* (1997) with modification. Briefly, bacterial cells grown in TSB were harvested by centrifugation at 3,500 x g for 30 min at 4°C and washed twice in 10 mM tris-HCl, pH 7.8. Washed cells were then resuspended in 3 ml of 10 mM EDTA, pH 7.8, incubated at 45 °C for 30 min, and then cooled to 4°C. The bacterial cells were disrupted with the aid of glass beads (150-212 µm; Sigma Chemical) and vigorous mixing for 5 min. Then DNase and RNase were added at 0.1 µg ml⁻¹ of bacterial suspension and the lysates were incubated at room temperature (RT) for 20 min. Unbroken cells and cell debris were collected by centrifugation at 3,500 x g for 30 min at 4°C. The supernatant containing cell fragments was then sedimented at 72,000 x g (XL-80 Ultracentrifuge; BECKMAN) for 1 h at 4°C, and pellet containing the outer and inner (cytoplasmic) membrane of the bacterial cells was collected. Cytoplasmic membrane was selectively solubilized with 1.5% N-lauroylsarcosine sodium salt (Sigma Chemical) at 22°C for 30 min. Outer membrane and solubilized inner membranes were centrifuged at 72,000 x g for 45 min at 4°C, and the resulting pellet containing the OMPs was resuspended in 10 mM tris-HCl, pH 7.8 and stored at -70°C for the next analysis.

ELISA analysis: 96 well plate ELISA

Table 1 Cross-reactivity (absorbance, 450 nm) of MAb 2E7 against *P. fluorescens* (AAHRI 01419) determined by an indirect antibody capture enzyme-linked immunosorbent assay (ELISA) with the cutting off point at three times of negative control.

Bacteria	Source ^a	Host	Optical density 450	Response (%) ^b
<i>Pseudomonas aeruginosa</i>	ATCC 27853	Blood culture	0.068	5.4
<i>P. putida</i>	DMST 10603	Blood culture	0.072	5.7
<i>P. fluorescens</i>	TISTR 358	Unknown	0.076	6.0
<i>P. fluorescens</i>	AAHRI 01419	Flame gourami	1.264	100.0
<i>P. fluorescens</i>	AAHRI 03418	Gold fish	1.239	98.0
<i>P. fluorescens</i>	AAHRI 03575	Zebra danio	0.553	43.8
<i>P. putida</i>	AAHRI 00127	Frog	0.073	5.8
<i>Pseudomonas</i> sp.	AAHRI 01031	Guppy	0.075	5.9
<i>P. aeruginosa</i>	AAHRI 02007	Striped catfish	0.094	7.4
<i>Staphylococcus aureus</i>	ATCC 25923	Clinical isolate	0.076	6.0
<i>Escherichia coli</i>	ATCC 25922	Clinical isolate	0.067	5.3
<i>Proteus morgani</i>	AAHRI 98095	Soft shell turtle	0.073	5.8
<i>Staphylococcus</i> sp.	AAHRI 00126	Catfish	0.068	5.4
<i>Citrobacter freundii</i>	AAHRI 01013	Giant gouramy	0.067	5.3
<i>Edwardsiella tarda</i>	AAHRI 01041	Tilapia	0.075	5.9
<i>Vibrio cholerae</i>	AAHRI 01260	Tilapia	0.070	5.5
<i>Aeromonas sorbia</i>	AAHRI 03570	Gold fish	0.074	5.8
<i>Streptococcus</i> sp.	AAHRI 04005	Siamese tiger fish	0.074	5.8
<i>A. hydrophila</i>	AAHRI 04031	Siamese fighting fish	0.075	5.9
<i>Plesiomonas shigelloides</i>	AAHRI 04040	Carp	0.073	5.8

a = ATCC: American Type Culture Collection

DMST: Department of Medical Sciences Thailand

TISTR: Thailand Institute of Scientific and Technological Research

AAHRI: Aquatic Animal Health Research Institute

b = Response was the mean of triplicate wells, expressed as a percentage of the optical density obtained by strain AAHRI 01419 (OD 1.264); OD450 of negative control (PBS) was 0.050

plates (NUNC™, Apogent) were coated with 50 µl of 0.01% (w/v) poly-L-lysine in 0.125 M carbonate-bicarbonate buffer pH 9.5. Plates were incubated for 1 h at RT before washing twice with low salt wash buffer (LSW; 0.02 M phosphate, 0.38 M NaCl, 0.05% Tween-20, pH 7.3). *P. fluorescens* and other bacteria were added to each well at 1.0×10^6 CFU ml⁻¹ final concentration and incubated at 4°C overnight. A solution of 0.05% glutaraldehyde in PBS was added into each well with a 20-minute-further incubation. The plates were then washed three times with LSW. Non-specific binding sites were blocked with 1%

bovine serum albumin (BSA) for 2 h at RT. After washing the plates with LSW, supernatants or antisera (100 µl well⁻¹) were added and incubated at RT for 2 h. The plates were then washed three times with high salt wash buffer (HSW; 0.02M phosphate, 0.5M NaCl, 0.1% Tween-20, pH 7.7) with a 5-min soaking on the last wash. Goat antimouse immunoglobulin-G (IgG) labeled with horseradish peroxidase (Sigma), diluted 1:5,000 in PBS was added at 100 µl well⁻¹ and incubated at RT for 1 h. Plates were then washed again as described above. The assay was developed with chromogen in substrate buffer (0.1M citric acid,

0.1M sodium acetate (pH 5.4), and 0.33% (v:v) H₂O₂) at 100 µl well⁻¹. Finally, the reaction was stopped with 2M H₂SO₄ and measured at 450 nm (BIO-TEK instruments, CERES UV900 HDi).

SDS-PAGE and Western blot (WB) analysis: Bacterial whole cell and the OMP were diluted with sample buffer (0.5M Tris-HCl pH 6.8, glycerol, 10% SDS, 0.05% bromophenol blue, β-mercaptoethanol) based on protein concentration (50 µg protein well⁻¹) and boiled for 4 min. They were then centrifuged at 3,500 x g for 5 min before applying into the 12% gels. The gels were then subjected to an electric current at 120V for 60 min. A half of the gel was cut and stained with Coomassie brilliant blue R-250, while another was blotted onto nitrocellulose membrane using semi-dry Transblot apparatus (BioRad). Then the membrane was removed and incubated with blocking solution [1% BSA in TBS (0.02M TrisHCl pH 7.5, 154 mM NaCl)] for 1 h at 37°C. Subsequently, it was washed three times, each 5 min with TTBS (0.5% Tween-20 in TBS) and incubated with neat hybridoma supernatants overnight at 4°C. After incubation, the blot was washed as previously described and incubated with goat-anti-mouse IgG horseradish peroxidase conjugate for 1 h at RT. The membrane was washed again and incubated with substrate solution (4-chloro-1-naphthol; BioRad) until bands were visible. The reaction was finally stopped with distilled water.

RESULTS

Production of MAb

Out of 13 clones, 1 clone (MAb 2E7) was positive to *P. fluorescens*. Its isotype of this MAb was IgG2b with Kappa light chain. The MAb recognized *P. fluorescens* (AAHRI 01419, 03418 and 03575) but it did not react to *P. fluorescens* (TISTR 358) as shown in Table 1.

Cross-reactivity of the MAb

The result of testing to different bacteria showed that MAb 2E7 had no cross reactivity to other bacteria (Table 1).

Western blot analysis

Prior to WB analysis, the gels of both whole cell and OMP were stained with Coomassie blue. The result showed major bands of protein profile of the whole cell at 105, 41 and 20 kDa, while those of the OMP were 46 and 21 kDa. (Fig. 1A & B). With WB analysis, MAb 2E7 reacted to one band of the whole cell *P. fluorescens* (AAHRI 01419) at 41 kDa (Figure 1C) whereas reacted to the OMP at 37 kDa (Figure 1D).

DISCUSSION

The MAb 2E7 produced in this study against the whole cell of *P. fluorescens* (AAHRI 01419) was evaluated by ELISA and WB. Its isotype was IgG2b class. As the affinity to the antigen of IgG is higher than that of the IgM antibody, this may suggest that the MAb could be used widely for serological tests (Shi *et al.*, 2003). With ELISA analysis, MAb 2E7 reacted to most of *P. fluorescens*, except isolate TISTR 358 which was isolated from Japan. As Swain *et al.* (2003) reported an existing of a number of serotypes and strains found in *P. fluorescens*, it may suggest that MAb 2E7 recognized some serotypes. However, the MAb 2E7 did not cross-react to the other tested bacteria.

Outer membrane protein is also one of this work interests. It has been reported that the OMP of gram-negative pathogenic bacteria has an important role in their pathogenicity to the host including adherence, uptaking of nutrients, and eliminating host-defence mechanisms (Seltman and Holst, 2002). Protein OprF (37 kDa) is a common OMP reported in *Pseudomonas aeruginosa* and its gene (*oprF*) seemed to be conserved within pseudomonas groupI (Ullstrom

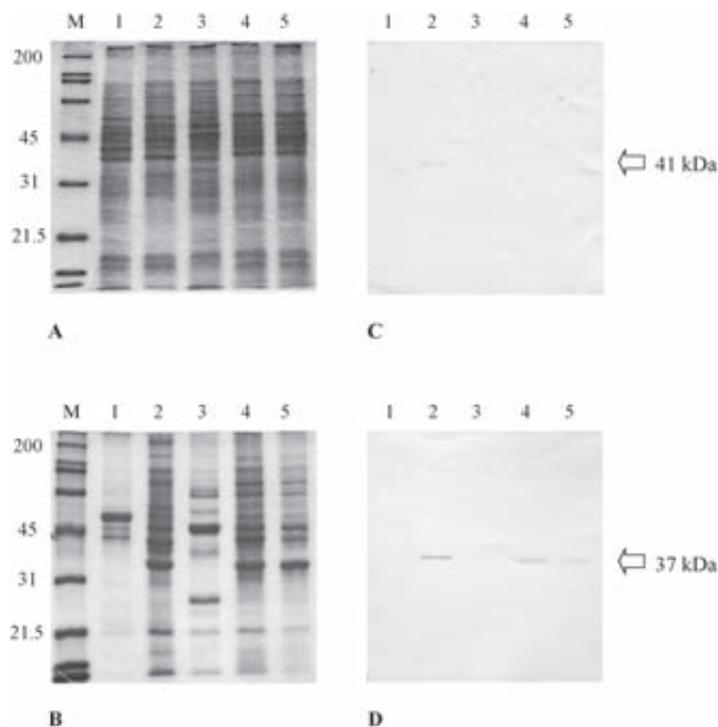


Figure 1 Whole cell and OMP were analysed by SDS-PAGE at 12% gel showing a wide range of protein profile (A, B). WB analysis was tested to whole cell and OMP with MAb 2E7(C, D). Lane M was standard molecular weight marker (kDa); Lane 1 was *Pseudomonas* sp. (AAHRI 01031); Lane 2 was *P. fluorescens* (AAHRI 01419); Lane 3 was *P. aeruginosa* (AAHRI 02007); Lane 4 was *P. fluorescens* (AAHRI 03418); Lane 5 was *P. fluorescens* (AAHRI 03575).

et al., 1991). The 37-kDa OprF-like protein has also been reported in *P. fluorescens* and was suggested to involve with adhesion between a *P. fluorescens* strain and plant roots (De Mot *et al.*, 1991; Kragelund *et al.*, 1996). Here, the result showed that the MAb 2E7 recognized an epitope present on the 37 kDa OMP of *P. fluorescens*. This epitope could be a probe moiety intact on both whole cell particle (41 kDa) and the OMP fragment (37 kDa). It is premature to conclude that the OMP fragment (37 kDa) is a distinct virulent factor among the pathogenic strains. More work is needed to characterize this OMP fragment, until then the present MAb will be most useful for the strain identification. Considering the bacteria detection, the MAb 2E7 is having a good character for a further development of a rapid diagnosis kit

to screen *P. fluorescens* infection in aquatic animals. This would envisage the disease situation of the fish ponds as well as provide relevant information for farm management.

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