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Identification of *Pseudomonas* sp. 46 NW-04 which Produces Antiviral Agent against Fish Viruses

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Abstract

Identification of *Pseudomonas* sp. 46NW-04 isolated from raceway water of salmonid hatchery which produces an antiviral substance, against fish viruses was carried out in species level.

The antiviral agent was produced when the bacterium was grown at 25°C for 2 days. Molecular weight of this substance was found to be around 1,000 when it was estimated by ultrafiltration of the culture supernatant. This substance in the culture supernatant was found to be thermostable up to 100°C.

The bacterium was identified as *P. fluorescens* biovar I according to Bergey's Manual of Systematic Bacteriology. Mol% G+C of DNA of the bacterium was 59.9%. The bacterium possessed three polar flagella. However, this strain was slightly distinguished from reference strain of *P. fluorescens* biovar I in respect of utilization of trehalose. In addition, antiviral agent appeared not to be produced in culture fluid of the reference strains. Therefore, strain 46NW-04 would be a variant of *P. fluorescens* biovar I.

Introduction

We previously reported the detection of several bacterial isolates with antiviral activity, which were relatively in high incidence in aquatic environments and the rate of detection was higher in estuarine or marine environment than in freshwater environment (Kamei et al., 1987; 1988a). Furthermore, in the case of freshwater-isolates, it was observed that most of the bacteria with antiviral activity belonged to genus *Pseudomonas* (Kamei et al., 1988a).

Pseudomonas sp. 46NW-04 isolated from the raceway water of salmonid hatchery showed potent antiviral activity against infectious hematopoietic necrosis virus (IHNV) (Kamei et al., 1988b). The antiviral substance producing bacteria such as this strain may exert inhibitory effect against pathogenic fish viruses in aquatic environment and it is very important to know the relationship between the bacteria and fish viruses in microbial ecosystem. Therefore, a systematic study involving this bacterium would be required in order to seek the distribution and behavior of the bacteria with antiviral activity in aquatic environment.

In the present study, antiviral substance produced by strain 46NW-04 in the culture filtrate was partially characterized for its biological properties. In addition, identification of the strain was made upto species level by using seven reference strains of *Pseudomonas*.

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Materials and Methods

Plaque assay

Productivity of the antiviral agent by *Pseudomonas* sp. 46NW-04 was ascertained by plaque reduction assay of IHNV (Kamei et al., 1988a). In the case of the antiviral activity of crude extract, it was first dissolved in dimethylsulfoxide (DMSO) and diluted with Hanks' balanced salt solution (Hanks' BSS) to make a stock solution of 1 mg/ml. The stock solution was diluted to 50 μg/ml by Hanks' BSS containing 1% DMSO. A 0.2 ml aliquot of diluted substance was mixed with an equal volume of IHNV suspension (approximately 150 PFU/0.1 ml) and left at 15°C for 3 hours. One % DMSO in Hanks' BSS was used as a control. Plaque assay was then conducted.

Estimation of molecular weight of antiviral agent

Strain 46NW-04 was grown in 100 ml CYG broth (Kamei et al., 1988a) in 500 ml-Sakaguchi flask at 25°C for 2 days. After centrifugation at 6,600×g at 4°C for 20 min, the cultural supernatant was subjected to ultrafiltration using Por Stirred Cell (Spectrum) with filters of 500 and 1,000 molecular weight cut-off (molecularporous membrane disc, Spectrum), and filters of 10,000 (molecut II GC, Millipore) and 30,000 molecular weight cut-off (Immersible CX-30, Millipore). The fractions of the culture fluids obtained from ultrafiltration were sterilized by membrane filtration (Millex-HA, Millipore) and assayed for the comparative antiviral activities. The molecular weight of the antiviral substance was estimated indirectly by selecting the ultrafiltration fraction showing strongest antiviral activity.

Thermostability of antiviral agents in culture fluid

Thermostability of the culture fluid fraction was determined as follows: the culture fluid was filtered with Millex-HA filter and dispensed in 1 ml aliquots in test tubes. Each test tube was kept in a water-bath at 40, 50, 60, 70, 80, 100, and 121°C (autoclave) for 10 min. The heat-treated samples were cooled immediately in ice and assayed for antiviral activity by plaque reduction.

Bacterial strains

Pseudomonas sp. 46NW-04 showed producing low molecular antiviral substance in this study was subjected to identification to species level. Three *Pseudomonas* strains stocked in our laboratory (*P. fluorescens* NCMB129, *P. putida* NCMB406, *P. saccharophila* IAM1504) and also four strains obtained from laboratory of Microbiology, Gifu University School of Medicine, Gifu, Japan (*P. stutzeri* ATCC17588, *P. mendocina* ATCC25411, *P. pseudoalcaligenes* ATCC17440, and *P. acidovorans* ATCC15668) were used as reference strains.

Determination of bacterial characteristics

Characterization of test strains was conducted according to the methods described in Bergey's Manual of Systematic Bacteriology Vol. 1 (1984) and by Stanier et al. (1966). DNA was prepared from bacterial cells by the method of Marmur (1961). Mol% G+C of DNA was measured by the method of Marmur and Doty (1962) and the value was expressed as the average of three trials.

Electron microscopy

Pseudomonas sp. 46NW-04 was grown in Trypticase Soy Broth medium (BBL) at 25°C for 24 hours and the culture was dropped on collodion-membraned mesh. After drying, the specimen was negatively stained with uranyl acetate and observed under a transmission electron microscope (H-300, Hitachi) operating at 75 Kv.

Crude extract

One loopful of stock culture of test strains of *Pseudomonas* was inoculated to 1 L CYG broth in Sakaguchi-flask and incubated with agitation at 25°C for 2 days. The supernatant was extracted twice with an equal volume of ethyl acetate. The ethyl acetate layer was dehydrated with Na_2SO_4 (anhydrous) and concentrated to dryness. Crude extracts obtained were subjected to assay of antiviral activity.

Results and Discussion

Molecular weight of antiviral agent produced by Pseudomonas *sp. 46NW-04*

Molecular weight of the antiviral agent was estimated by assaying antiviral activity of culture supernatant fractionated by ultrafiltration with a gradient of porous membarne filters. The culture filtrates having the components of molecular weight less than 10,000, showed antiviral activity of 99% plaque reduction (Table 1). However, the antiviral activity was slightly reduced when the culture was filtered with the membrane of 1,000 molecular weight-cut off. No antiviral activity was detected in the culture fraction with substances of molecular weight of less than

Table 1. Anti-IHNV activity of cultural filtrate of strain 46NW-04, which was treated with ultrafiltration

Sample	Plaque reduction
500MW-cut*	0%
1,000MW-cut*	92%
10,000MW-cut*	99%
30,000MW-cut*	99%
untreated	100%

* : Indicates cut off molecular weight more than 500, 1,000, 10,000, and 30,000.

Table 2. Thermostability of an anti-IHNV substance in cultural filtrate of strain 46NW-04

Untreated	Heated (C°)						
	40	50	60	70	80	100	121
100%*	100%	100%	100%	100%	100%	100%	96%

* : Plaque reduction of IHNV.

Table 3. Characteristics differentiating strain 46NW-04 from reference strains and species 1–12 (section I) of *Psedudomonas* listed in Bergey's Manual of Systematic Bacteriology

Characteristics	test strains								reference species								
	46NW-04	*P. fluorescens* (NCMB 129)	*P. putida* (NCMB 406)	*P. stutzeri* (ATCC 17588)	*P. mendocina* (ATCC 25411)	*P. pseudoalcaligenes* (ATCC 17440)	*P. acidovorans* (ATCC 15668)	*P. saccharophila* (IAM 1504)	1. *P. aeruginosa*	Species 2, 3 and 4 : see Table 3-5-9	5. *P. putida*	6. *P. syringae* pvs.	7. *P. viridiflava*	8. *P. cichorii*	9. *P. stutzeri*	10. *P. mendocina*	11. *P. alcaligenes*	12. *P. pseudoalcaligenes*
No. of flagella	>1								1	>1	>1	>1	1-2	>1	1[a]	1[a]	1	1
Fluorescent pigments	+	+	+	−	−	−	−	−	d	+	+	+	+	+	−	−	−	−
Pyocianine	−	−	−	−	−	−	−	−	d	−	−	−	−	−	−	−	−	−
Carotenoids	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	d	−
Growth at 41°C	−	−	−	+	+	+	+	+	+	−	−	−	−	−	d	+	+	+
Levan formation from sucrose	+	+	−	−	−	−	−	−	−	d	−	d		−	−	−	−	−
Arginine dihydrolase	+	+	+	−	+	+	−	−	+	+	+	−	−	−	−	+	+	d
Oxidase reaction	+	+	+	+	+	+	−	+	+	+	+	−	−	+	+	+	+	+
Denitrification	−	−	−	+	−	−	−	−	+	d	−	−	−	−	+	+	+	+
Gelatin hydrolysis	+	+	−	−	−	−	−	−	+	+	−	d	+	−	−	−	d	d
Starch hydrolysis	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−
Utilization of:																		
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Trehalose	−	+	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−
2-Ketogluconate	+	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	−	−
Meso-Inositol	+	+	+	−	−	+	−	−	−	+	−	d	+	d	−	−	−	−
Geraniol	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	+	−	−
L-Valine	+	+	+	+	+	+	+	+	d	+	+	−	−	−	+	+	−	−
β-Alanine	+	+	−	−	+	+	−	+	+	+	+	−	−	−	−	+	d	d
DL-Arginine	+	+	+	+	−	+	+	+	+	+	+	d	+	+	−	+	+	+

[a]: Lateral flagella of short wavelength may be also be produced under certain conditions. d: Indicates that depends on strains.

500. From these results, the molecular weight of the antiviral substance was estimated to be around 1,000.

Thermostability of the antiviral agent

The antiviral activity of the culture fluid fraction remained unchanged after heat-treatment at 100°C, but slightly decreased on following autoclaving at 121°C (Table 2). This result suggested that the antiviral agent was thermostable. Therefore, low molecular weight and thermostable properties of the antiviral agent suggests that it is probably different from the substances such as antiviral proteolytic bacterial enzymes reported to date (Knowlton and Ward, 1987; Toranzo et al., 1982; Ward et al., 1986).

Identification of a Pseudomonas *sp. 46NW-04*

Identification of *Pseudomonas* sp. 46NW-04 was carried out on the basis of taxonomical procedures of *Pseudomonas* as described in Materials and Methods, comparing with 7 reference strains (*P. fluorescens* NCMB129; *P. putida* NCMB406; *P. stutzeri* ATCC17588; *P. mendocina* ATCC25411; *P. pseudoalcaligenes* ATCC17440; *P. acidovorans* ATCC15668; *P. saccharophila* IAM1504). Results of these tests and characteristics of *Pseudomonas* described in Bergey's Manual were compared and listed in Table 3.

PHB accumulation, the most significant taxonomical property of *Pseudomonas* sp., was observed in reference strains of *P. pseudoalcaligenes*, *P. acidovorans*, and *P. saccharophila*, but not in strain 46NW-04. This result indicated that strain 46NW-04 could be assigned to section I of *Pseudomonas* listed in Bergey's Manual, which includes *P. aeruginosa*, *P. fluorescens* biovars, *P. chlororaphis*, *P. aureofaciens*, *P. putida* biovars, *P. syringae* pathovars, *P. viridiflava*, *P. cichorii*, *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, and *P. pseudoalcaligenes*. Furthermore, since production of fluorescent pigment and arginine dihydrolase activity were positive in strain 46NW-04, the bacterium was classified in species 1-5 of section I, namely *P. aeruginosa*, *P. fluorescens* biovar I-V, *P. chlororaphis*, *P. aureofaciens*, or *P. putida* biovars.

Thus, characteristics of strain 46NW-04 and *P. fluorescens* NCMB129 used as reference strain were compared with main properties of species 1-5 of section I and shown in Table 4. Strain 46NW-04 was clearly distinguished from *P. aeruginosa*, especially in the point of negative production of pyocyanine and also distinct from *P. putida* biovars based on positive gelatin-liquefaction and lecithinase. These results indicated that strain 46NW-04 could be assigned to any of *P. fluorescens* biovar I-V, *P. chlororaphis*, or *P. aureofaciens*.

As the final step of identification, characteristics of strain 46NW-04 and *P. fluorescens* NCMB129 were also compared with properties of *P. fluorescens* biovar I-V, *P. chlororaphis*, and *P. aureofaciens* (Table 5). Strain 46NW-04 did not produce chlororaphis and phenazine monocarboxylate, suggesting that it was different from *P. chlororaphis* and *P. aureofaciens*. Therefore, this strain should belong to biovar I-V of *Pseudomonas fluorescens*. However, further characterization revealed that it was not only different from biovar II, III, and IV in respect of denitrification, but also from biovar V in respect of levan formation from sucrose.

From these results, strain 46NW-04 could be identified as *P. fluorescens* biovar

Table 4. General characteristics of strains 46NW-04 and NCMB 129 of *Pseudomonas fluorescens* and species 1-5 of *Pseudomonas* (section I) listed in Bergey's Manual of Systematic Bacteriology.

Caracteristics	test strains		reference species									
	46NW-04	*P. fluorescens* (NCMB 129)	1. *P. aeruginosa*	2. *P. fluorescens* biovar I	2. *P. fluorescens* biovar II	2. *P. fluorescens* biovar III	2. *P. fluorescens* biovar IV	2. *P. fluorescens* biovar V	3. *P. chlororaphis*	4. *P. aureofaciens*	5. *P. putida* biovar A	5. *P. putida* biovar B
Cell diameter, μm	0.9		0.5-0.7	0.7-0.8	0.7-0.8	0.8	0.7	0.8	0.7-0.8	0.7-0.8	0.7-1.1	0.7-1.1
Cell length, μm	2.2		1.5-3.0	2.3-2.8	2.0-2.8	2.0-2.8	2.0-2.5	2.0-3.0	1.5-3.0	1.9-2.8	2.0-4.0	2.0-4.0
Flagella number	>1		1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Pyocyanin production	−	−	+	−	−	−	−	−	−	−	−	−
Pyoverdin production	+	+	+	+	d	+	+	d	d	+	+	d
Chlororaphin production	−	−	−	−	−	−	−	−	+	−	−	−
Phenazine monocarboxylate production	−	−	−	−	−	−	−	−	−	+	−	−
Other pigments (not carotenoids)	−	−	+	−	−	−	d	−	−	−	−	−
Yellow-orange cellular pigments	−	−	−	−	−	−	−	−	−	−	−	−
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
PHB accumulation	−	−	−	−	−	−	−	−	−	−	−	−
Levan formation from sucrose	+	+	−	+	+	−	+	−	+	+	−	−
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	−	−
Starch hydrolysis	−	−	−	−	−	−	−	−	−	−	−	−
Autotrophic growth with H_2			−	−	−	−	−	−	−	−	−	−
Lecithinase (egg yolk)	+	+	−	+	±	+	+	d	+	d	−	−
Lipase (Tween 80 hydrolysis)	−	−	±	d	−	d	d	d	+	d	d	d
Extracellular PHB hydrolysis	−	−	−	−	−	−	−	−	−	−	−	−
Growth at 4°C	+	+	−	+	+	+	+	d	+	+	d	+
Growth at 41°C	−	−	+	−	−	−	−	−	−	−	−	−
Denitrification	−	−	+	−	+	+	+	−	+	−	−	−
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+
Catechol, ortho cleavage			+	+	+	+	+	+	+	+	+	+
Protocatechuate, ortho cleavage	+	+	+	+	+	+	+	+	+	+	+	+
Mol % G+C of DNA	59.9	60.7	67.2	60.5	61.3	60.6	59.4	60.5	63.5	63.6	62.5	60.7

d: Indicates that depends on strains.

Table 5. Characteristics of strains 46NW-04 and NCMB 129 of *Pseudomonas fluorescens* and species 2 (biovars I-V), 3 and 4 of *Pseudomonas* (section I) listed in Bergey's Manual of Systematic Bacteriology.

Characteristics	46NW-04	*P. fluorescens* (NCMB 129)	2. *P. fluorescens* Biovar I	Biovar II	Biovar III	Biovar IV	Biovar V	3. *P. chlororaphis*	4. *P. aureofaciens*
P. fluorescens biovars as designed by Stanier et al. (1966)			A	B	C	F	G	D	E
Nonfluorescens pigments :									
Green (chlororaphin)	−	−	−	−	−	−	−	+	−
Orange (phenazine-1-carboxylate)	−	−	−	−	−	−	−	−	+
Blue, nondiffusible	−	−	−	−	−	+	−	−	−
Levan formation from sucrose	+	+	+	+	−	+	−	+	+
Denitrification	−	−	−	+	+	+	−	+	d
Carbon sources used for growth :									
L-Arabinose	+	+	+	+	d	+	d	−	+
Sucrose	+	+	+	+	−	+	d	+	d
Saccharate	−	−	+	+	d	+	d	+	+
Propionate	+	+	+	+	d	+	+	+	+
Butyrate	+	+	−	d	d	+	d	+	+
Sorbitol	+	+	+	+	d	+	d	−	−
Adonitol	+	+	+	−	d	−	d	−	−
Propylene glycol	+	+	−	+	d	−	d	−	−
Ethanol	−	−	−	+	d	−	d	d	−

d : Indicates that depends on strains.

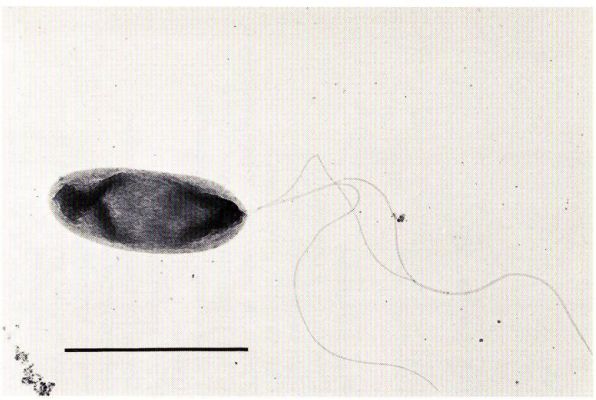

Fig. 1. Electron micrograph of *Pseudomonas* sp. 46NW-04 by negative stain with uranyl acetate.
Bar in micrograph indicates 2 μm.

Table 6. Comparative antiviral activities of *Pseudomonas fluorescens* strains in the cultural filtrates and crude extract

Strain	Plaque reduction of IHNV	
	Filtrate	Crude extract (25 μg/ml)
46NW-04	98%	100%
NCMB 129	67%	0%
ATCC 13525	54%	ND

ND: Not determined.

I. In addition, this identification was strongly supported by the measurement of mole% G+C of DNA, which was 59.9% and similar to that of *P. fluorescens* NCMB129 and all other characteristics were also same as that of *P. fluorescens* NCMB129 except for utilization of trehalose. Electron micrograph showed that strain 46NW-04 possessed three polar flagella and a cell length of 2.2 μm (Fig. 1).

Thus, since *Pseudomonas* sp. 46NW-04 was identified as *P. fluorescens* biovar I, antiviral activity against IHNV was further investigated with culture fluids of strain NCMB129 used in this study and *P. fluorescens* ATCC13525. However, none of the fluids showed potent antiviral activity. Furthermore, the same antiviral agent was not detected in crude extracts from those fluids by ethyl acetate: antiviral agent could be extracted with ethyl acetate from the culture fluid of strain 46NW-04 and this crude extract showed 100% plaque reduction of IHNV at 25 μg/ml (Table 6). The overall results suggest that strain 46NW-04 probably is an unique bacterium producing antiviral agent among the bacteria belonging to *P. fluorescens* biovar I and may play an important role in inactivating fish viruses in the microbial ecosystem under fish environments.

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