Detection of diketopiperazine and pyrrolnitrin, compounds with anti-*Pythium insidiosum* activity, in a *Pseudomonas stutzeri* environmental strain

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Aims. Screening of bacterial flora for strains producing metabolites with inhibitory effects on the human pathogenic oomycete *Pythium insidiosum*. Separation and characterization of extracts from *Pseudomonas stutzeri* with anti-*Pythium* inhibitory activity. Search for genes with anti-*Pythium* effect within the genome of *P. stutzeri*.

Methods. A total of 88 bacterial strains were isolated from water resources in northeastern Thailand. Two screening methods were used to establish their inhibitory effects on *P. insidiosum*. One strain, *P. stutzeri* ST1302 was randomly chosen, and the extract with anti-*P. insidiosum* activity was fractionated and subfractionated using liquid column chromatography and purified by thin layer chromatography. The chemical structure of purified fractions was determined by Fourier transform infrared spectroscopy, nuclear magnetic resonance and mass spectrometry. Further, search for genes involved in the anti-*Pythium* activity (phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin) was undertaken in this *P. stutzeri* strain using primers described in the literature.

Results. Anti-*P. insidiosum* activity was detected in 16 isolates (18.2%). In *P. stutzeri* ST1302, a subfraction labeled PYK7 exhibited strong activity against this oomycete. It was assigned to the diketopiperazines as cyclo(D-Pro-L-Val). In the search for genes, one gene region was successfully amplified. This corresponded to pyrrolnitrin.

The results suggest the possibility of using the related metabolites against *P. insidiosum*. This is the first report on the inhibitory effects of *P. stutzeri* against this oomycete. The results may contribute to the development of antimicrobial drugs/probiotics against pythiosis.

Key words: *Pythium insidiosum*, *Pseudomonas stutzeri*, antimicrobial, diketopiperazine, gene, pyrrolnitrin

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INTRODUCTION

Pythiosis is a life-threatening disease caused by the oomycete *Pythium insidiosum*, a fungus-like organism belonging to the kingdom *Chromista* and based on DNA sequences, closely related to diatoms and golden-brown algae^{1,2}. The genus *Pythium* comprises over 150 described species but only *P. insidiosum* is capable of infecting mammalian hosts such as cats, dogs, horses, cattle and also humans³⁻⁷. The first report of human pythiosis was that of a patient in Thailand and, since then, other cases have been reported in tropical and subtropical regions worldwide^{3,4}. Clinical forms are usually classified as cutaneous/subcutaneous, ophthalmic, systemic and disseminated and are associated with high morbidity and mortality rates because of lack of specific symptoms and limited diagnostic tools^{3,8}.

In terms of epidemiology, pythiosis is considered an endemic disease restricted mostly to Thailand. This territory consists of many aquatic regions with environments suitable for the life cycle of *P. insidiosum*^{8,9}. Its natural habitat is stagnant, swampy areas, muddy water and wet fields. The oomycete was isolated from 21.6% of aquatic cultivated resources in northern Thailand¹⁰. It has now been demonstrated that watered areas, e.g. rice paddies, irrigation channels and reservoirs, are all potential resources of this emerging pathogen¹⁰.

Currently, there are neither effective antimicrobials nor vaccines against pythiosis^{8,11-14}. Unlike fungi, *P. insidiosum* as an oomycete lacks ergosterol in the cell membrane which is the target of most antifungal drugs^{8,15}. Therefore, attempts to treat animals or humans with antimycotics either in monotherapy or in combination have yielded variable and contradictory results^{11,16}. The effectiveness of

a vaccine from crude protein is ~50% for human, ~30% for canine and ~60% for equine pythiosis^{8,17}. This has led to research on therapeutic alternatives, particularly compounds from medicinal plants and microorganisms. To date, a large number of microbes have been investigated, including the genera *Pseudomonas*, *Bacillus* and *Streptomyces* known to produce a heterogeneous group of potent antimicrobial metabolites^{18,19}. Examples of these are 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, hydrogen cyanide, hydrolytic enzymes, iron-chelating siderophores, and several cyclic lipodepsipeptides^{20,21}. Further, several *Streptomyces* strains have been found to produce phosphomycin, albopeptin B, tautomycin, tautomycetin, and ezomycin S (ref.²²).

The purpose of this study was to screen bacterial strains from aquatic environments in the northeastern area of Thailand for their ability to inhibit the growth of *P. insidiosum*. Further, metabolites of one *Pseudomonas stutzeri* strain chosen for its anti-*Pythium* activity were studied and the genome of this bacterial strain was screened for genes encoding compounds with anti-*Pythium* effect.

MATERIALS AND METHODS

Microorganisms for testing

Eighty-eight bacterial isolates, defined morphologically, were obtained from water resources in the region of Khon Kaen, a city located in the northeastern part of Thailand. All isolates were stored in Difco Skim Milk (BD Diagnostic Systems) 100 mg/mL with 33% of glycerol at -20 °C until tested. One strain of *P. insidiosum* SIMI6666, isolated from a corneal ulcer and identified by Angkana Chaiprasert, Department of Microbiology, Mahidol University, Thailand, was used for demonstration of growth inhibition.

Testing of bacteria for anti-*Pythium* activity

Two different techniques were used for testing the inhibitory activity against *P. insidiosum*. Bacterial cultures were grown in 100 mL sterile bottles containing 25 mL of the Brain Heart Infusion (BHI) Broth (BD Diagnostic Systems) and incubated at 37 °C in a shaking incubator (VWR 1575 Signature Benchtop, Shel Lab) at 200 rpm for 3 days. Cells were removed by centrifugation at 8000 × g for 15 min. Supernatants were filtered aseptically through 0.45-μm-pore-sized membrane filters (Millipore). Filtrates were stored at 4 °C.

For the first technique, 100 μL of the filtrate were spread on the surface of a test plate with the Nutrient Agar (Oxoid). A block sized 1×1 cm was cut off from the Sabouraud Dextrose Agar (SDA; Oxoid) with *P. insidiosum* culture grown at 25 °C for 5 days and transferred to the center of the test plate. The plate was incubated at 25 °C and checked after 3, 6 and 9 days for growth inhibition of the culture.

For the second technique, 5 mL of the filtrate were mixed with 15 mL of molten SDA. After solidification, the block from *P. insidiosum* culture was placed on the agar surface as described in the first technique.

Incubation and evaluation of growth inhibition were as described above.

In both techniques, SDA without the filtrate was added as a growth control.

Identification of *P. stutzeri* strain ST1302

One strain identified biochemically using the Vitek 2 Test Card (bioMérieux) as *P. stutzeri* was randomly chosen from isolates showing inhibitory activity. It was named ST1302 and under this designation, it is available in the culture collection of the Centre for Research and Development, Medical Diagnosis Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand.

Identification of this strain was then verified by sequencing analysis. Genomic DNA was prepared using a standard method described by Marmur et al.²³.

The 16S rDNA region of the strain was amplified using PCR with universal primers, fD1 (5'-AGTTTGATCCTGGCTCA-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (ref.²⁴). PCR amplification was carried out in 100 μL of reaction mixture which contained 5 ng of genomic DNA, 1× reaction buffer (Promega), 200 μM each dATP, dTTP, dGTP, and dCTP (Promega), 2 mM of MgCl₂, 50 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Promega). Amplification was performed in the Robocycler Gradient 96 Temperature Cycler (Stratagene) with the cycling conditions as follows: initial denaturation at 95 °C for 2 min followed by 30 cycles (denaturation: 95 °C, 2 min; annealing: 42 °C, 30 s; extension: 72 °C, 4 min) and final extension at 72 °C for 20 min. The PCR product was detected by electrophoresis in 1% agarose gel (Serva Electrophoresis) with ethidium bromide (0.5 μg/mL) running in 1× TBE buffer (90 mM Tris-Borate, 2 mM EDTA, pH 8.0) at 100 V for 1 h. The amplicon was then submitted to the GeneProof a.s., Czech Republic for sequence determination. The sequence was compared and analyzed for the greatest similarity with the genomic databases which are published in the Entrez Nucleotide Database of the National Center for Biotechnology information (NCBI), using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/>).

Extraction, purification and characterization of anti-*Pythium* metabolites from *P. stutzeri*

For preparation of crude extract of metabolites, a single colony of *P. stutzeri* ST1302 from the Nutrient Agar plate was inoculated into each of four 1000 mL bottles containing 500 mL of the BHI Broth and incubated on a rotary shaker at 200 rpm at 37 °C for 3 days. Then, 20 liters of cultured broth were centrifuged at 8000 × g for 15 min and sterilized by filtration through a 0.45-μm-pore-size membrane filter (Millipore). The cell-free filtrate was mixed three times with an equal volume of dichloromethane (Merck). The dichloromethane layer was then dried with anhydrous Na₂SO₄ (Merck) and concentrated by evaporation under reduced pressure in a rotary evaporator (Rotavapor R-210, Buchi Laboratory Equipment).

For preparation of fractions from the crude extract, separation by liquid column chromatography (LCC) was used. The extract was applied on the top of a glass column filled with a slurry of silica gel 60 (0.015-0.040 mm; Merck), which was preconditioned with dichloromethane. Gradient mixtures of dichloromethane and finally with methanol added were used as mobile phases. The eluent was collected. Fractions with similar patterns were combined and tested for anti-*Pythium* activity by the disc diffusion method as described below. The fractions with inhibitory activity were separated again using LCC with gradient mixtures of dichloromethane and hexane (Merck) as mobile phases. Thin layer chromatography was then used for differentiation of single fractions according to retention factors (R_f) and for purity checking. Detection was done under exposure to UV light at 256 and 364 nm.

The chemical structure of one purified fraction was determined by Fourier transform infrared spectroscopy (FTIR) followed by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Infrared spectra were measured in potassium bromide discs at the wavelengths 450-4000 cm^{-1} and detected using the PE 1600 FTIR spectrometer (Perkin Elmer). NMR spectroscopy was carried out at the Faculty of Sciences, Chiang Mai University, Chiang Mai, Thailand. The following analyses were performed: proton and carbon NMR, double quantum filtered - correlation spectroscopy, heteronuclear multiple quantum coherence and heteronuclear multiple bond coherence. MS evaluating molecular weight of fractions was carried out at the Faculty of Sciences, Mahidol University, Bangkok, Thailand. The data were analyzed and chemical structures of the fractions were established.

Disc diffusion method for determination of anti-*Pythium* activity of *P. stutzeri* metabolites

Using this method, both crude extract and purified fractions were tested for anti-*Pythium* activity. The test solution was prepared by dilution of 1 mg of the dried filtrate in 1 mL of dichloromethane. Paper discs (6.0 mm

in diameter; Gibthai, Thailand) filled with 20 μL of test solutions were placed on the SDA plate which was then inoculated by the SDA block with *P. insidiosum* as described above. A disc containing 20 μL of dichloromethane only was included as a negative control. The plates were kept at room temperature for 2 h in a laminar flow box to allow diffusion of the test solution. They were then incubated at 25 °C for 3, 6 and 9 days. If inhibition of *P. insidiosum* growth around the disc was observed, the effect of the compound was evaluated as positive.

Detection of anti-*Pythium* genes in *P. stutzeri*

Search for genes encoding compounds with antimicrobial inhibitory activity in *P. stutzeri* strain ST1302 was performed with five sets of primers: PCA2a (5'-TGCCAAGCCTCGCTCCAAC-3') and PCA3b (5'-CGCGTTGTTCTCGTTCAT-3'); 30-84/1 (5'-CAGTTCATCCGGCGGGCTGCAG-3') and 30-84/2 (5'-CCCGTTTCAGTAAGTCTTCCATGATGCG-3') for phenazine-1-carboxylic acid; Phl2a (5'-GAGGACGTCGAAGACCACA-3') and Phl2b (5'-ACCGCAGCATCGTGTATGAG-3') for 2, 4-diacetylphloroglucinol; PltBf (5'-CGGAGCATGGACCCCCAGC-3') and PltBr (5'-GTGCCCCGATATTGGTCTTGACCGAG-3') for pyoluteorin and PrnCf (5'-CCACAAGCCCGGCCAGGAGC-3') and PrnCr (5'-GAGAAGAGCGGGTCGATGAAGCC-3') for pyrrolnitrin^{20,21,25,26}. All primers were synthesized by Metabion International, Germany. DNA was prepared in the same way as for sequencing. PCR amplification was performed according to Kumar et al.²⁷.

RESULTS AND DISCUSSION

Antimicrobial activity testing and molecular genetic identification of *Pseudomonas stutzeri* ST1302

All 88 bacterial isolates obtained from water resources were tested for activity against *P. insidiosum*. Using both

Table 1. Searching for genes with anti-*Pythium insidiosum* activity in the strain *Pseudomonas stutzeri* ST1302.

Gene-specific primer pair	Antimicrobial product (DNA size in bp)	Presence (+) or absence (-) of the product
PCA2a-PCA3b	Phenazine-1-carboxylic acid (1150)	-
30-84/1-30-84/2	Phenazine-1-carboxylic acid (1200)	-
Phl2a-Phl2b	2,4-diacetylphloroglucinol (745)	-
PltBf-PltBr	Pyoluteorin (773)	-
PrnCf-PrnCr	Pyrrolnitrin (719)	+

screening techniques, 16 isolates (18.2%) were found to inhibit its growth. The correlation between techniques was 100%.

Phenotypic identification of the strain *P. stutzeri* ST1302 was verified by sequencing. The 783-bp PCR product derived from the 16S rDNA region revealed 98% similarity to *P. stutzeri* strain SN1 (accession No. JF 461537.1) included in the NCBI database.

Purification and characterization of metabolites with anti-*Pythium* activity

Crude extract of the *P. stutzeri* strain ST1302 which was active against *P. insidiosum* using the disc diffusion method, was separated into 20 fractions with different R_f but in only three was such activity detected. A fraction labeled F10, composed of eight subfractions, was chosen for testing inhibitory effects. Using the disc diffusion method, only a subfraction labeled PYK7 with $R_f = 0.7$ exhibited anti-*Pythium* activity. This was selected, purified and analyzed in the light of its chemical structure. PYK7 was a white, crystalline powder. From the results of the FTIR analysis, the peak at $\nu > 3000 \text{ cm}^{-1}$ indicated a typical secondary amine. The peak at $\nu = 1671 \text{ cm}^{-1}$ corresponded to a carbonyl group. However, the peaks at $\nu = 1429\text{--}1295 \text{ cm}^{-1}$ showed a difference in C-O- bonds. The MS data showed the molecular ion peak of the PYK7 substance at m/z 197 (M+1) with a molecular formula $\text{C}_{10}\text{H}_{17}\text{N}_2\text{O}_2$ (Fig. 1). Based on the analysis of NMR spectra, this was assigned to diketopiperazines (DKPs) as cyclo(D-Pro-L-Val). DKPs are the smallest possible cyclic peptides commonly biosynthesized from amino acids by different organisms²⁸. Due to their rigid structure, chiral nature and varied side chains, they are an attractive scaffold for drug design. Both natural and synthetic DKPs have a wide variety of biological activities including antitumor, antiviral, antifungal and antibacterial activities.

The data from all spectroscopic methods used were in agreement with those described in the literature. The chemical structure of PYK7 was determined by interpretation of the MS and NMR data and compared with appropriate data from the literature including physicochemical features²⁹. It was found that the main anti-*Pythium* compound is DKP. Jayatilake et al. reported the production of DKP from a *Pseudomonas aeruginosa* strain associated with an Antarctic sponge³⁰. However, to the best of our knowledge, the production of DKP by *P. stutzeri* found

in our study is the first report of its occurrence in this species.

Detection of antimicrobial genes

The results of screening for genes with antimicrobial inhibitory activity in our *P. stutzeri* strain using PCR are summarized in Table 1. Only one gene region was successfully amplified. This corresponded to pyrrolnitrin. It is an antifungal antibiotic synthesized by some *Pseudomonas* spp. from tryptophan and initiated by a chloroperoxidase enzyme system³¹. Pyrrolnitrin inhibits fungal growth by inhibiting the respiratory electron transport system. It is most active against dermatophytic fungi, especially from the genus *Trichophyton*.

In accord with the amplification products described by Raaijmakers et al., no genes encoding phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol and pyoluteorin

Fig. 2. Demonstration of searching results for genes with anti-*Pythium insidiosum* activity in the strain *Pseudomonas stutzeri* ST1302 by PCR. (M) 200–1500 bp ladder; (N) negative control; amplification by (1) phenazine-1-carboxylic acid primer pair, PCA2a-PCA3b; (2) phenazine-1-carboxylic acid primer pair, 30-84/1-30-84/2; (3) 2,4-diacetylphloroglucinol primer pair, Phl2a-Phl2b; (4) pyoluteorin primer pair, PltBf-PltBr and (5) pyrrolnitrin primer pair, PrnCf-PrnCr. Only amplification of the pyrrolnitrin gene was positive.

Fig. 1. Structure of cyclo(D-Pro-Val) (ref.³⁵).

were found in this study^{20,25,26}. However, a product sized approximately 700 bp corresponding to the pyrrolnitrin gene was amplified in our *P. stutzeri* strain and compared to strains of *P. aeruginosa* and *P. fluorescens* as reported earlier (Fig. 2) (ref.²⁰). Several strains belonging to the genus *Pseudomonas* derived from clinical materials and agricultural soil have been reported to promote the growth of plants and act as effective biocontrol agents^{18,32,33}. Some metabolites of *Pseudomonas* spp. such as hydrogen cyanide and pyoluteorin were found to be active against plant pathogens like *Pythium ultimum*, *Rhizoctonia solani*, *Phytophthora capsici* and *Fusarium oxysporum*³². However, pyrrolnitrin was the only compound with known antimicrobial inhibitory activity (including anti-*Pythium* effects) we found in our *P. stutzeri* strain.

At present, there is no effective drug for treating pythiosis. Data on the antimicrobial activity of some aquatic bacteria suggest their metabolites could be used against *P. insidiosum*. This study is the first report on the inhibitory activity of *P. stutzeri* against this pathogenic oomycete and on its metabolite PYK7, identified as DKP cyclo(D-Pro-Val). However, understanding the contribution of this compound to the pathophysiology of *P. insidiosum* needs to be further explored. Our results may contribute to the development of novel antimicrobial drugs/probiotics.

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