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Characterization and Identification of a *Streptomyces* Strain with Biocontrol Activity against *Aeromonas Hydrophila* Causing Haemorrhage Disease in Fish

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Abstract

In this study, experiments were performed to identify and screen *Streptomyces* strains that are antagonistic to *Aeromonas hydrophila*, which causes hemorrhagic disease in fishes. Among 80 isolates, 3 strains capable of antagonizing *Aeromonas hydrophila* by the agar diffusion plate method were obtained. The strain numbered 1083 had a strong activity with a 25 mm diameter clear zone of bacteria. This strain showed grey colonies after 7 days of incubation. After 7 days of incubation,

the grey colonies had white borders, produced soluble pigments on the medium, grew well at 30°C and neutral pH, and adapted well to medium containing a high salt concentration. Strain 1083 was able to utilize different sources of carbon and nitrogen. Sequence analysis of 16S rRNA showed that strain 1083 had a similarity of 99% compared to *Streptomyces antibioticus*. Based on morphological, physiological, and biochemical characteristics, as well as molecular biological analysis, strain 1083 was identified as *Streptomyces antibioticus*.

Keywords

Fish, Sequence analysis, 16S rRNA, *Aeromonas hydrophila*, *Streptomyces* sp

Introduction

Aquaculture industries around the world have been decimated by epidemics of a hypervirulent pathotype of *Aeromonas hydrophila* (Al-Fatlawy and Al-Hadrawy, 2014; Julia and Phillip, 2012). *A. hydrophila* is ubiquitous in warm-water environments and has a diverse host range (i.e., amphibians, birds, fishes, reptiles, and mammals) with an equally diverse number of diseases that includes

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hemorrhagic disease in fishes (FAO, 2014). When environmental conditions are favorable, the pathogen undergoes rapid multiplication causing disease manifestations, and subsequently causing fish to constantly suffer from stress due to adverse conditions in the pond ecosystem like higher temperatures, higher stocking densities, less oxygen, and a heavy organic load, etc. In fact, bacterial infection is one of the leading causes of disease problems in aquaculture (Rasmussen-Ivey *et al*., 2016; Selvakumar *et al*., 2010; Vivekanandhan *et al*., 2004). *A. hydrophila* is the most common disease-causing pathogen, and it can easily spread through accidental abrasions and cause hemorrhagic septicaemia, ulcers, exoph-thalmia, and abdominal distension (Samira *et al*., 2012).

Until now, preventing or controlling aquatic disease has mainly depended on antibiotics and disinfectants. However, the massive use of these chemicals has led to antibiotic resistance in some instances. Also, the rapid expansion of intensive aquaculture industries are often accompanied by rotten uneaten feed, sedimentation of feces, and organic residue. The water quality rapidly deteriorates as a result. In particular, nitrogenous compounds such as ammonia and nitrite build up quickly, both of which are harmful to fish even at low concentrations. Water exchange can be applied to maintain good water quality; however, frequent exchange is not only laborious and costly, but also may incur disease causing agents and pollute nearby water bodies (Dharmaraj *et al*., 2009; Selvakumar *et al*., 2010). Therefore, there is an urgent demand for cost-effective and environment-friendly approaches for remediation of aquaculture water.

Streptomyces consists of widely-distributed groups of microorganisms in nature, which primarily inhabit the soil. Many of them are known to have the capacity to synthesize bioactive secondary metabolites, which include enzymes, herbicides, pesticides, and antibiotics. Around 80% of the world's antibiotics come from *Streptomyces*, mostly from the genera *Streptomyces* and *Micromonospora* (Dharmaraj *et al*., 2009; Mohana and Radhakrishnan, 2014). This study was carried out to screen and identify *Streptomyces* with antagonistic activity against *A. hydrophila* in fish.

Materials and Methods

Materials

The *Aeromonas hydrophila* strains were isolated from diseased catfish (*Ictalurus punctatus*). Several characteristics of this strain were previously identified (Trang *et al*., 2017). *Streptomyces* strains were isolated from various soil samples in Vietnam and stored at the Faculty of Biotechnology, Vietnam National University of Agriculture, Hanoi, Vietnam.

Selection of *Streptomyces* strains antagonistic to *Aeromonas hydrophila*

The *Streptomyces* were plated on Gause-1 medium (soluble starch 20 g L^{-1}; K_2HPO_4 0.5 g L^{-1}; $MgSO_4.7H_2O$ 0.5 g L^{-1}, NaCl 0.5 g L^{-1}; KNO_3 0.5 g L^{-1}; $FeSO_4$ 0.01 g L^{-1}; Agar 20 g L^{-1}; pH = 7.0 to 7.4) at 30°C for 5 days. Cultured lumps of agar containing *Streptomyces* 7 mm in diameter were placed into LB medium (10 g tryptone, 10 g NaCl, 5 g yeast extract, 20 g agar, 1 L water, pH 7.0) containing *A. hydrophila*, and then incubated at 4°C for 3 h to diffuse the active ingredient to the medium. The sample was transferred to a 30°C incubator and observed after 12 h, when the diameter of the clear zone (if any) was measured.

Identification of biological characteristics of the selected *Streptomyces*

The selected *Streptomyces* strain numbered 1083 was cultured on Gause-1 medium at 30°C for 7 days and the morphology, color, and size of the colonies were recorded. After 3 days of incubation, the spore chain morphology was observed under the light microscope. The surface morphology of spores was observed under a scanning electron microscope (SEM). Specimen handling, observation, and analysis of the images were carried out in the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam.

Strain 1083 was cultured on ISP-6 medium (Peptone 10 g L^{-1}; yeast extract 1g L^{-1}; iron citrate

0.5 g L^{-1}; Agar 20 g L^{-1}; pH = 7.0 to 7.2) at 30°C. According to the International *Streptomyces* Project (ISP), melanin formation can be determined using the ISP6 medium at 30°C for at least 21 days. Melanin production changes the color of the medium from pale yellow to brown and black (Shirling and Gottlieb, 1966). Thus, the color of the medium used to culture strain 1083 was observed for 21 days.

Checking the ability to assimilate carbon sources: the strain 1083 was cultured on ISP - 9 medium ($K_2HPO_4.3H_2O$ 5.65 g L^{-1}; $(NH4)_2SO_4$ 2, 64 g L^{-1}; KH_2PO_4 2.38 g L^{-1}; $MgSO_4.7H_2O$ 1 g L^{-1}; 1.0 mL of solution B; agar 20 g L^{-1}; pH = 6.8 to 7.0) supplemented with 1% by weight of different sugar sources including D-glucose, D-fructose, D-manotol, sucrose, rhamnose, inositol, L-arabinose, cellulose, D-xylose, and raffinose. The ability to assimilate carbon sources was assessed by the viability and growth of *Streptomyces* on the medium (Shirling and Gottlieb, 1966).

Checking the possibility of using nitrogen sources: strain 1083 was cultured on a nitrate starch medium (Starch 20 g L^{-1}; $NaNO_3$ 2 g L^{-1}; K_2HPO_4 1 g L^{-1}; $MgSO_4.7H_2O$ 0.5 g L^{-1}; KCl 0.5 g L^{-1}; $FeSO_4.5H_2O$ 0.1 g L^{-1}; pH 6.8 - 7.0) as a control. The nitrogen sources including beef extract, KNO_3, NH_4Cl, peptone, $(NH_4)_2SO_4$, and NH_4NO_3 can be replaced with $NaNO_3$.

The effects of temperature, pH, and NaCl concentration on strain 1083 were determined by culturing the strain on Gause-1 medium at different temperatures (4, 20, 30, 40, 45, and 50°C), pH levels (4.0 - 12.0) and NaCl concentrations (1 - 9%) and then looking at the development of colonies on the media.

Identification of the *Streptomyces* strain 1083

Based on the morphological characteristics and culture, including the morphology of colonies, substrate mycelium, aerial mycelium, conidiophore, and surface of spores, the features identified were compared with known *Streptomyces* strains in the international classification system (ISP) (Shirling and Gottlieb, 1966).

Sequence analysis of 16S rRNA: DNA from strain 1083 was extracted according to methods described by Marmur (1961). PCR reaction amplified conservative regions of 16S rRNA with primers named 27F (5' - AGAGTTTGATCCTGGCTCAG - 3') and 1492R (5' - ACGGCTACCTTGTTACGACTT - 3') (Julia and Christina, 2008). PCR products were checked on 1.0% agarose gel, and sent to the company 1tsBASE (Malaysia) for sequencing. The sequenced gene from strain 1083 was compared with strains published in GenBank using the Blast search tool (Altschul *et al.*, 1990). MEGA6 software was used to determine genetic relationships using the maximum parsimony selection method, and the reliability was calculated by the bootstrap algorithm with 1000 repetitions.

Results and Discussion

Screening of *Streptomyces* strains antagonistic against *Aeromonas hydrophila*

Streptomyces is supposed to be antagonistic against other microorganisms because of the presence of compounds with biological activities, especially many types of antibiotics. These substances are secreted to the medium by *Streptomyces* during cultivation, so we used the diffusion method on agar plates for selection and evaluation of *Streptomyces* for antibacterial activity. Gause-1 medium was used to grow the *Streptomyces*, and Lysogeny broth medium was used to grow *A. hydrophila*. Through the screening, three of the 80 studied *Streptomyces* strains were identified to be resistant to *A. hydrophila*. Among them, the strain numbered 1083, which was isolated from mud in Phuc Tho - Ha Noi, was strongly antagonistic with a 25 mm diameter inhibited zone (Figure 1). In recent years, there have been several publications with findings of *Streptomyces* being capable of antagonistic to fish and shellfish pathogens including *A.hydrophila* in *in vitro* conditions (Dharmaraj *et al.*, 2009; Selvakumar *et al.*, 2010). Compared to the previous results, strain 1018 had relatively high activity based on the same methods. This result showed that the *Streptomyces* strain 1083 had high antibacterial activities and the potential for future applications.

Biological characteristics of the *Streptomyces* strain 1083

Morphological characteristics

One of the first criteria to study the biological characteristics and classification of *Streptomyces* is based on morphological characteristics (Miyadoh *et al.*, 2016). The *Streptomyces* strains were grown on Gause-1 medium at 30°C for 7 days to observe the color, size, and shape of the colonies. After 3 days of culture, the colony of strain 1083 showed a round shape, 0.2 - 0.4 mm in size with off-grey color. The color of the colony changed after several days of incubation and the colonies were almost dark brown at day 5. After determining the characteristics of the colonies, we determined the characteristics of the formation of conidiophores, spore chains, and surface of spores from strain 1083. The results observed under an optical microscope at a magnification of 1000 showed that after 48 h of incubation, *Streptomyces* strain 1083 started sporulation. The spores were arranged in long, branched, and clustered chains. After 60 h of culture, the spores began to leave the series and were

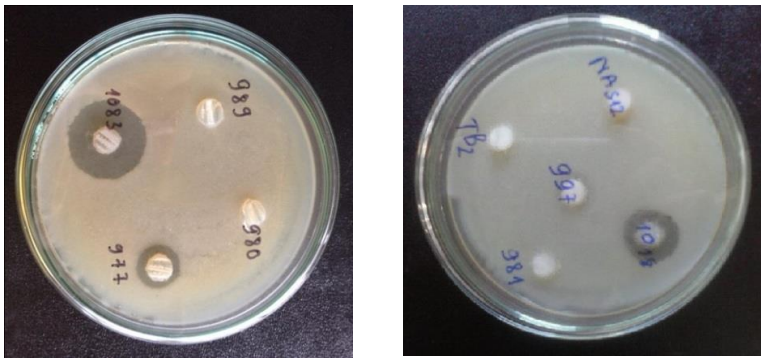

Figure 1. Antagonistic activity of *Streptomyces* samples against *Aeromonas hydrophila* by the diffusion method on agar plates

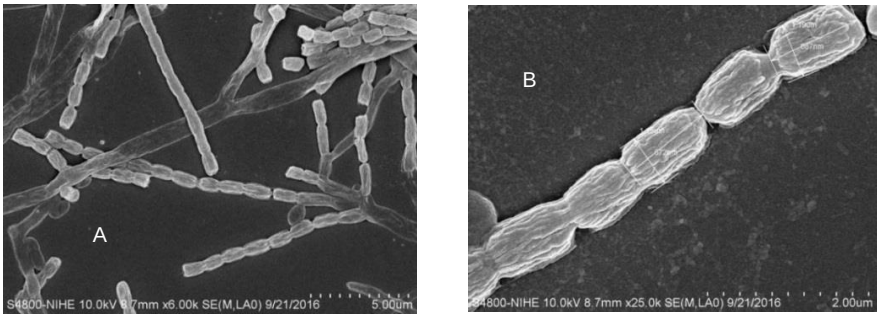

Figure 2. Conidiospores and spore surfaces from strain 1083 under scanning electron microscope at magnification of 6000X (A) and 25000X (B)

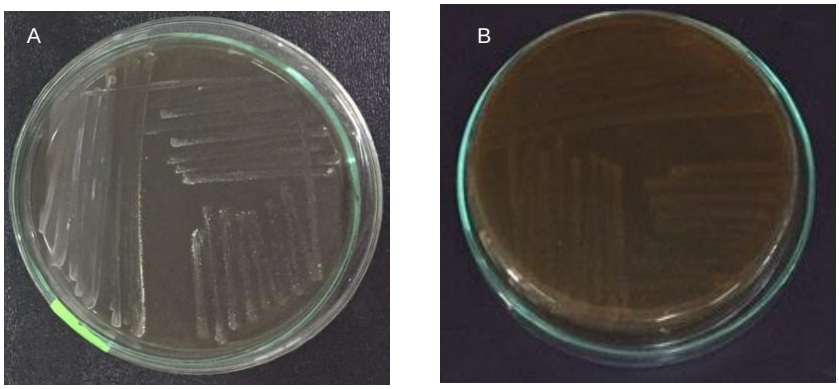

Figure 3. Melanin formation of strain 1083 when cultured on ISP - 6 medium after 21 days, the cultured plate from up-side (A) and bottom-side (B)

released to the culture medium. To more accurately determine the morphology of strain 1083, we observed the morphology of the spore chains and the surface of spores under a scanning electron microscope (SEM). SEM with a 6000X magnification showed that the spore chains of strain 1083 were typical with a white cluster form, each chain bearing 10 - 20 spores (Figure 2A). Spores of strain 1083 had a short oval shape with the size of 0.7 x 1.2 µm and a smooth surface (Figure 2B).

Ability of melanin pigmentation

Strain 1083 was cultured on the ISP6 medium and observed after 21 days of culture. The result showed that the areas around the colonies appeared to be dark brown, indicating that strain 1083 was capable of generating melanin (Figure 3).

The ability of strain 1083 to use carbon and nitrogen sources

The possibility of strain 1083 using carbon and nitrogen sources was investigated. These results served as one of the bases to classify *Streptomyces* according to the ISP system, and at the same time, to provide information on nutrition of strain 1083 for the future fermentation process. Strain 1083 was cultured on ISP-9 medium with different added sugar sources and on the starch nitrate medium where $NaNO_3$ sources were replaced by different nitrogen sources as described in the materials and methods section. The results revealed that strain 1083 could utilize carbon from different sources such as fructose, L-arabinose, raffinose, D-xylose, inositol, D-mannitol, cellulose, and sucrose, etc., in which raffinose, mannitol, dextrose, and galactose showed the highest efficiency (Table 1). These results were consistent with previously published studies (Mohana and Radhakrishnayn, 2014; Miyadoh *et al.*, 2016). Strain 1083 was capable of using nitrogen from various sources such as beef extract, peptone, yeast extract, and KNO_3 (Table 1). In particular, strain 1083 grew persistently in a medium with added nitrogen from beef extract.

The ability of strain 1083 to adapt to culture medium conditions

Investigation of medium culture factors on the growth and development of the *Streptomyces* strain 1083 provides useful information about culture conditions for further research. *Streptomyces* strain 1083 was cultured on Gause medium at different temperatures, pH levels, and salt concentrations. Observation of the growth and development of strain 1083 after 5 days of culture are summarized in Table 2. The results showed that strain 1083 was capable of adapting to the test conditions relatively well. However, strain 1083 developed the best at 30 - 45°C, in neutral or slightly alkaline media with a pH in the range of 7.0 - 9.0, and had the ability to withstand concentrations of NaCl up to 5.0% (Table 2). Since strain 1083 could grow in medium up to 5.0% of salt concentration, it could be classified as belonging to the moderate salt endurance group, but it thrived the best at a salt concentration of 1.0 - 2.0%. The results found in this study were similar to those published by Mohana and Radhakrishnan (2014).

Table 1. Ability of strain 1083 in using various carbon and nitrogen sources

Carbon source	Development of the strain 1083 after 5 days of culture	Nitrogen source	Development of the strain 1083 after 5 days of culture
Sucrose	+	KNO_3	+++
R - Hamnose	++	Beef Extract	++
α - Lactose	++	NH_4Cl	-
D - glusose	+	Peptone	+++
Maltose	+++	$(NH_4)_2SO_4$	-
D - xylose	+	NH_4NO_3	+
D - sobitol	+		
Dextrin	+		

Note: (+) strain 1083 can grow; (+++) strain 1083 can grow well; (-) strain 1083 cannot grow.

Table 2. The influence of environmental conditions on the development of strain 1083

Factors	Optimum value	Endurance value
Temperature (°C)	30 - 45	20 - 45
NaCl (%)	< 3	< 5
pH	7.0 - 9.0	5.0 - 12.0

Identification of *Streptomyces* strain 1083

The identification of *Streptomyces* strain 1083 was based on the similarity of the 16S rRNA gene fragment with gene sequences from other *Streptomyces* strains uploaded to GenBank. The primers 27F and 1492R were used to amplify the 16S rRNA gene fragment of strain 1083. Electrophoresis of PCR product showed a single band with the size of about 1500 bp (Figure 4).

PCR products were purified and sequenced at the 1stBASE company (Malaysia). The obtained sequence was compared with other sequences in GenBank by the blast tool. The phylogenetic tree for strain 1083 was established (Figure 5).

The resulting phylogenetic tree based on sequencing the 16S rRNA gene showed that *Streptomyces* 1083 was located in the same branch with *Streptomyces antibioticus* with a 99% bootstrap value. Combined with information of the nucleotide sequence, the compatibility of the 16S rRNA sequence of *Streptomyces* 1083 with *Streptomyces antibioticus* was 99%, and comparison of the similarity in the characteristics studied further strengthened the reliability of the species relationship (Miyadoh *et al*., 2016). Based on the combination of biological characteristics and molecular biological methods, we concluded that the strain 1083 belongs to *Streptomyces antibioticus.*

Conclusions

Three *Streptomyces* strains which expressed antagonistic activity against *Aeromonas hydrophila*, which causes hemorrhagic disease in fishes, were screened from 80 isolated strains. Strain 1083 showed stronger antagonistic activity with a clear zone of 25 mm in diameter. The morphology, characteristics of culture physiology, and biochemistry, as well as molecular biology of *Streptomyces* strain 1083 were identified. Combination of characteristics suggested that *Streptomyces* strain 1083 was *Streptomyces antibioticus.*

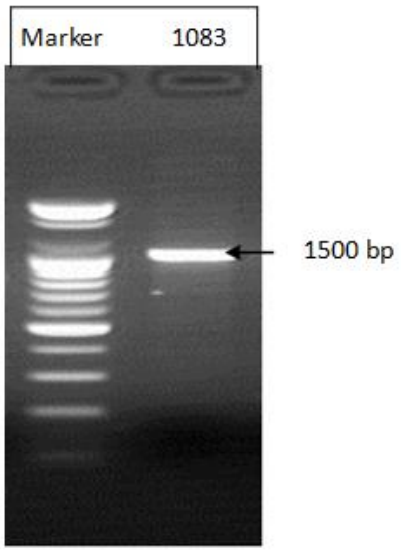

Figure 4. Electrophoresis of PCR product on 1.2% agarose gel

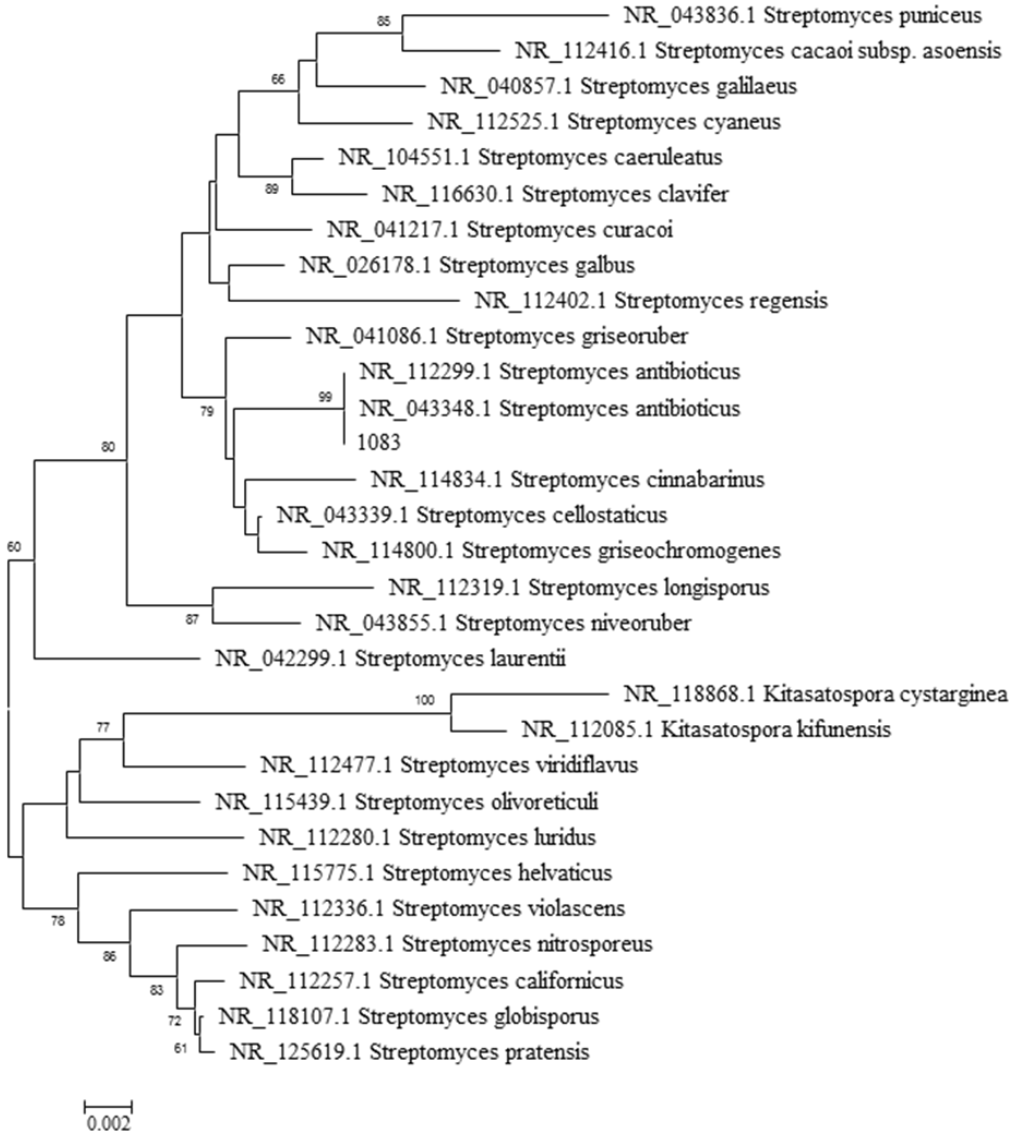

Figure 5. Phylogenetic tree of the *Streptomyces* strain 1083 based on 16S rRNA sequence

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