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Isolation of Endophytic Bacteria Strains in *Mimosa pudica L.*, which grows wild in Hau Giang province, Vietnam.

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ABSTRACT

Recently, many studies have shown that some medicinal plants do not have this disadvantage. *Mimosa pudica* has good antibacterial properties, often used to treat a number of diseases such as rheumatism, back pain, swelling, and ulcer. Therefore, the isolation of endophytic bacteria strains in *Mimosa pudica* was carried out in order to find antibacterial strains that contribute to replacing synthetic antibiotics. Objectives: Isolation of endophytic bacteria strains in *Mimosa pudica* growing wild in Hau Giang province (*Mimosa pudica* L.) with good properties such as antibacterial ability, synthesis of NH_4^+, IAA and ability to dissolve phosphorus which is hard to be soluble. As a result, twenty-two endophytic bacteria strains were isolated from the stems, leaves, roots and root nodules of *Mimosa pudica* . Most of these strains are rod-shaped, gram-negative, and motile. All isolated endophytic bacteria strains were capable of synthesizing ammonium and IAA. Strain L5CTA is the strain that synthesizes the highest amount of ammonium. Besides, the highest amount of IAA was synthesized by the N5CTA strain. Ten strain are capable of solubilizing phosphorus, in which L3CTA strain is the most effective strain. The results of the investigation of antibacterial ability on 3 strains of pathogenic bacteria showed that 15 strains were resistant to Escherichia coli, of which the T1PH strain gave the highest antibacterial results; 12 strains are resistant to *Aeromonas hydrophila*, the L1CT strain is considered the most promising for resistance to *Aeromonas hydrophila*; and 11 strains were resistant to both *Escherichia coli* and *Aeromonas hydrophila* strains. Strain T1PH is resistant to all three pathogenic bacteria *Escherichia coli, Staphylococcus aureus* and *Aeromonas hydrophila*.

Keywords: *Bacillus pumilus*, *Enterobacter ludwigii*, antibacterial, *Mimosa pudica* , endophytic bacteria.

1. Introduction

For many generations, our forefathers have known to use plants and find folk remedies from plants available in nature as medicine. With the great progress of science and technology, many drugs have been created and produced. Thanks to the development of pharmaceutical chemistry, a number of biologically active compounds have been isolated, purified, determined with their chemical structure and pharmacological effects, thereby widely produced and used. However, these chemical-based drugs, in addition to the advantages of being fast-acting, easy to manufacture, store and use, there is a concern that is side effects of the drug, especially for diseases that require long-term treatment. Therefore, today people tend to use natural herbs in the treatment of diseases.

Vietnam is located in the tropical monsoon region. Its climate conditions are favorable for the growth and development of many types of plants, including many species with valuable medicinal properties, creating a rich source of medicinal herbs to serve human health. Many types are popularly used in folk medicine to cure diseases, especially some types have been scientifically proven to have the same effect as today's modern drugs, but are safe for people's health.

A number of medicinal plants with antibacterial properties such as *Wedelia chinensis*, *Houttuynia cordata* and *Phyllanthus urinaria* have been studied to show that they have antibacterial activity because they contain essential oils which are aldehyde groups and ceton derivatives such as methyl n-nonyl ketone, L-decanal, L-dodecanal. The terpen group includes substances a-pinene, camphene,... that have the effect of killing *Streptococcus pneumonia*, *Staphylococcus aureus*, *Shigella*, *Salmonella*, *E. coli* (Do Tat Loi, 2006; Shu-Chen et al., 2008)

Many studies show that in non-legume plants, there are also groups of beneficial microorganisms living in the plant or in the root zone that stimulate plant growth thanks to their ability to fix nitrogen, decompose phosphorus, synthesize growth hormones and compounds that have the ability to directly inhibit some plant diseases or stimulate plants to produce secondary metabolic compounds that help plants resist plant pathogens. In particular, there are endogenous strains of microorganisms in medicinal plants that can produce antibacterial compounds when they live inside medicinal plants. The groups of microorganisms capable of this include species of the genera Azosprillum, Herbapirillum, Gluconacetobacter, Klebsiella...

2. Materials and research methods

2.1. Material

-The whole root, stem, leaves and root nodules of *Mimosa pudica* tree in Hau Giang Province

-*Escherichia coli* and *Staphylococcus aureus* bacteria were provided from the Molecular Biology laboratory, Biotechnology Research and Development Institute, Can Tho University.

-*Aeromonas hydrophila* was provided from the Department of Fish Diseases, College of Aquaculture and Fisheries, Can Tho University.

2.2. Chemistry

- Chemicals used to process samples: sterile distilled water, 70% alcohol, 3% H_2O_2.

- Chemicals used to stain Gram bacteria: Iodine, Fushin, Crystal violet, 70% alcohol, sterile distilled water.

- Chemicals to measure the amount of ammonium synthesized by bacteria: KCl 2M, $(NH_4)_2SO_4$, phenol nitroprusside, sodium hypochloride.

- Chemicals for measuring IAA: $FeCl_3$, H_2SO_4, K_2HPO_4, KH_2PO_4, commercial IAA.

- Chemicals used to extract isolated bacterial DNA: TE pH (8), SDS 10%, isopropanol, ethanol (alcohol) 70%, CTAB 10%/NaCl 0.7M, Proteinase K (20 mg/ml), sterile double-

distilled water, Chloroform: isoamyl alcohol (24:1 ratio). TE solution includes the following ingredients: 1% Triton X - 100, 1M Tris HCl - 8.5, 0.5 M EDTA - 8.0, sterile distilled water.

- Chemicals used to perform PCR reactions to identify isolated bacteria: PCR buffer, Taq polymerase, isolated bacterial DNA, sterile double-distilled water, dNTPs (dATP, dTTP, dGTP, dCTP).

- Chemicals used to perform gel electrophoresis containing PCR reaction products: Agarose 1.2%, TBE buffer 1X, loading buffer, Pstly standard scale, ethidium bromide (EtBr).

2.3 Research Methods

2.3.1 Method of isolating endogenous bacteria

2.3.1.1 Sample collection

Use a knife to dig the soil around the *Mimosa pudica* tree so that you can get the entire tree, including the roots and root nodules. Then put it in plastic bag and bring it to the Microbiology laboratory, Biotechnology Research and Development Institute, Can Tho University to process samples and isolate endogenous bacteria.

2.3.1.2 Measure the pH of the soil in the rhizosphere

Weigh 20g of soil in the place where the *Mimosa pudica* sample was taken and add 200g of water. Use a glass rod to stir until it is completely dissolved. Measure pH soil with a pH meter.

Result of pH measurement:

- Soil at the rhizosphere of the *Mimosa pudica* tree sampled in Chau Thanh district: pH = 5.8

- Soil at the rhizosphere of *Mimosa pudica* tree sampled in Chau Thanh A district: pH = 5.6

- Soil at the rhizosphere of *Mimosa pudica* tree sampled in Vi Thanh city: pH = 5.7

- Soil at the rhizosphere of *Mimosa pudica* tree sampled in Vi Thuy and Phung Hiep district: pH = 6.1

2.3.2. Sample treatment

According to Cao Ngoc Diep and Phan Thi Nha (2011), in order to eliminate microorganisms that may still cling to the surface, the collected samples are treated as follows:

- Samples were washed under running water to remove soil and dust and then separated stem parts, leaves, roots and root nodules. Then, wash with sterile distilled water and cut into short pieces of 2 - 3 cm and put in fancol tubes.

- Pour 70% alcohol until the sample is submerged, and leave for 30 seconds for leaves, 1 minute for stems and 3 minutes for roots and root nodules. Then discard the alcohol and replace it with H2O2 with the same time as above, then rinse with sterile distilled water 4 times to remove residual chemicals. Then pour once more sterile distilled water until the sample is submerged.

To check the possibility of microorganisms remaining on the surface of the sample after sterilization, aspirate 50 pl of distilled water for the final washing of the sample, spread it in

concentrated PDA medium and incubate at 30ºC. After 24 - 48 hours check for any microbial growth. If no microorganisms grow, it means that the sample has been disinfected on a clean surface and that the bacteria isolated are endogenous in the roots, stems, leaves, and root nodules of the *Mimosa pudica* tree.

- After satisfactory surface sterilization, samples are put into sterilized porcelain mortars and use a sterile ceramic pestle to finely grind the samples, add 1-2ml of sterile distilled water. Use a micropipette to suck the extract into the eppendorf, centrifuge at 8ºC at 5000 rpm for 5 minutes to settle the residue.

- Aspirate 0.5ml of sample solution after centrifugation into a test tube containing 5ml of prepared sterile semi-concentrated Nfb medium. Then close the test tubes for the bacterial fluid to move down.

- After incubation at 30°C for 2 - 3 days to 1 week, bacterial strains extracted from the roots, stems, leaves and root nodules of *Mimosa pudica* . The field is about 2 - 5 mm, this membrane is typical for the growth of endophytic bacteria.

3.2.2 Investigate the characteristics of bacteria

3.2.2.1 Observe morphology, measure colony size

When inoculating bacteria on isolation media, we simultaneously measure the size and observe the morphology of the colonies, including the criteria: color, shape, buoyancy and colony cover with the naked eye.

3.2.2.2 Observing the shape and motility of bacteria

According to Cao Ngoc Diep and Nguyen Huu Hiep (2000), after isolating and separating bacteria, observe the shape and movement of bacteria by pressing drop method under optical microscope at magnification 400 times.

The endophytic bacteria were photographed under the JBS 5500 scanning electron microscope (Japan) at 1000x magnification to better see the cell shape, the tip and side cilia of the bacteria.

Data processing

The obtained data were processed by using Microsoft Office Excel 2003 software and Statgraphics 16.1 statistical software.

3. Results and discussion

3.1. Isolation results and colony characteristics of bacterial strains

Bacterial isolation results

From the roots, stems, leaves and root nodules of the *Mimosa pudica* tree in Hau Giang province, twenty-two endophytic bacteria strains were isolated on PDA medium. Most of these bacterial strains were distributed in all 4 parts of the plant. Isolated bacteria were concentrated in leaves and root nodules, with 7 strains each, followed by roots 5 strains and stems with at least 3 strains.

These endogenous bacteria share the common characteristic of growth and development under microaerobic conditions. When inoculated into semi-solid Nfb medium, the bacteria grew into a pellicle ring white light ring on the medium, 2-5 mm from the surface of the medium. Most of the strains change the color of the original Nfb environment.

Table 7: Isolation results of endophytic bacteria strains in *Mimosa pudica*

No.	Bacterial strain	Isolated position	Place of sample collection
1	L2CT	Leaves	Chau Thanh District - Hau Giang
2	RVT	Root	Vi Thuy District - Hau Giang
3	L1CT	Leaves	Chau Thanh District - Hau Giang
4	R32CT	Root	Chau Thanh District - Hau Giang
5	R34CT	Root	Chau Thanh District - Hau Giang
6	N3PH	Root nodules	Phung Hiep District - Hau Giang
7	T1PH	Stem	Phung Hiep District - Hau Giang
8	N4CT	Root nodules	Chau Thanh District - Hau Giang
9	L22VT	Leaves	Vi Thuy District - Hau Giang
10	L221VT	Leaves	Vi Thuy District - Hau Giang
11	L5CTA	Leaves	Chau Thanh A District - Hau Giang
12	R4CTA	Root	Chau Thanh A District - Hau Giang
13	T3CTA	Stem	Chau Thanh A District - Hau Giang
14	R1CTA	Root	Chau Thanh A District - Hau Giang
15	N61CTA	Root nodules	Chau Thanh A District - Hau Giang
16	N5CTA	Root nodules	Chau Thanh A District - Hau Giang
17	T2CTA	Stem	Chau Thanh A District - Hau Giang
18	N4CTA	Root nodules	Chau Thanh A District - Hau Giang
19	L2CTA	Leaves	Chau Thanh A District - Hau Giang
20	N22CTA	Root nodules	Chau Thanh A District - Hau Giang

21	L3CTA	Leaves	Chau Thanh A District - Hau Giang
22	N21CTA	Root nodules	Chau Thanh A District - Hau Giang

4.1.2 Colony characteristics

After culturing on PDA medium and isolating pure strains, it was found that most of the bacterial strains grow very quickly, the time for the strains to develop into colonies is 12 hours, and 24 hours at the latest. Regarding the morphological characteristics of the colonies:

- Colony color: Most of the colonies are milky white 13/22, ivory white 6/22 and yellow 3/22.

- Colony shape: Most of the colonies are circular in shape 21/22, the rest have irregular shapes 1/24.

- Colony cover shape: Most of the colonies have a whole cover 21/22.

- Colony buoyancy: most of the colonies have tissue buoyancy 18/22, the rest are colonies with jagged buoyancy 4/22.

- Colony size: Colony diameter of isolated strains fluctuated about 1 – 6 mm after inoculation on solid PDA medium, incubated at 30ºC for 24 hours.

Comment

After surveying and comparing the ammonium synthesis ability of endophytic bacteria strains in each root organ, stem, leaf and root nodule of *Mimosa pudica* , 22 endophytic bacteria strains were isolated from *Mimosa pudica* with the ability to synthesize ammonium.

In general, the nitrogen fixation capacity of the investigated strains was very variable, showing the different adaptations of these strains to the nitrogen-free environment. This trend is consistent with the descriptions of nitrogen-fixing ability of endophytic bacteria in pineapple (Nguyen Thanh Dung, 2009) and endophytic bacteria in sugarcane (Huynh Thi Hong Phuong et al., 2010).

Among the 22 investigated bacterial strains, the L5CTA strain with the highest ammonium content of 0.430 pg/ml was statistically different from the rest at 5% significance level at day 2 after inoculation. Compared with the nitrogen fixation capacity of the most promising endophytic bacteria in sugarcane by Huynh Thi Hong Phuong et al. (2010) was that the A14 strain synthesized 4.46 pg/ml, the LK3 strain of Nguyen Thanh Dung (2009) synthesized 4.77 pg/ml, the bacterial strains isolated in the *Mimosa pudica* tree were able to fix lower nitrogen determination. From the above studies, it is shown that the nitrogen fixation capacity of endophytic bacteria strains in different plants may be different. On the other hand, bacterial strains isolated from *Mimosa pudica* tree have the ability to synthesize nitrogen, although they are not too high, but they also have the ability to help the plant grow and develop well.

For the bacterial strains isolated from the root nodules, the ammonium synthesized was relatively higher than the others, typically on day 6, the N4CTA strain was the one that synthesized the highest amount of ammonium (0. 34 pg/ml), followed by the strain N21CTA (0.30 pg/ml), which was significantly different from the rest.

4.3 Results of investigation on the ability to synthesize indol-3-acetic acid (IAA) of isolated bacterial strains

After determining the nitrogen fixation ability of twenty-two isolated bacterial strains, continue to culture these strains in liquid Nfb medium supplemented with Tryptophan, monitor and measure the amount of IAA produced. . The results showed that all 22 isolates were capable of synthesizing indole-3-acetic acid (IAA). The ability to synthesize IAA of the bacterial strains is shown by the absorption spectral of the measured samples. The darker pink samples, the greater the spectral absorbance and the higher the ability to synthesize IAA, through days 2, 4, and 6 after inoculation.

Because each strain of bacteria has a different characteristic, the ability to synthesize IAA of each strain is also very different, but there are typical cases as follows:

- The amount of IAA synthesized was lowest on day 2 and highest on day 6. In this case, the explanation is very simple, because bacteria proliferate, the biomass of bacteria increases day by day, so the amount of IAA synthesized also increased gradually from day 2 to day 6. The strains: L22VT, L2CT, L3CTA, N21CTA, N4CTA, N5CTA, R32CT, T1PH, T2CTA, T3CTA, R34CT (11 strains) belong to this case.

- The amount of IAA was low on day 2, on day 4 the amount of IAA synthesized was highest and decreased slightly on day 6. Bacterial strains in this case were: L221VT, N3PH, N4CT, R4CTA, RVT (5 strains).

-3 strains of bacteria L2CTA, L5CTA, N61CTA had the highest amount of IAA on day 2 and decreased until day 6.

- The lowest amount of IAA on day 2, on day 4 this concentration was highest and decreased slightly on day 6. There are 2 strains in this case, L1CT, N22CTA.

- Particularly, the R1CTA strain had the highest IAA content on day 2, this concentration was lowest on day 4 and increased slightly on day 6.

In general, the ability to synthesize IAA of the investigated bacterial strains is very variable. However, the N5CTA strain produced an increasing amount of IAA over days 2, 4 and was highest at day 6 after inoculation compared with all strains, proving that this is the strain with the best ability to synthesize IAA of all strains. Endophytic bacteria strains isolated from *Mimosa pudica* tree.

The results of surveying the ability to synthesize IAA of endophytic bacteria strains in *Mimosa pudica* showed that the N5CTA strain was the most promising strain, capable of synthesizing the highest amount of IAA. According to the research results of Chen T et al. (2014), endophytic bacteria strains in ginger are capable of synthesizing the amount of IAA from 7.45 to 162.9 pg/ml. Meanwhile, the strain Bacillus isolated from maize produced an IAA of 105.11 pg/ml (Szilagyi-Zecchin VJ et al., 2014). However, the *Bacillus cereus* strain isolated from *Sophora alopecuroides* only synthesized 0.368 mg/ml IAA (Longfei Zhao et al., 2011). From the above studies, it shows that bacterial strains isolated from *Mimosa pudica* have the ability to synthesize IAA, although not too high, but also have the ability to help plants grow and develop well.

Survey results on the ability to dissolve insoluble phosphorus of isolated bacterial strains

After investigating the ability to fix nitrogen and synthesize IAA, 22 bacterial strains continued to be investigated for their ability to dissolve insoluble phosphorus on concentrated NBRIP medium. The strains that grow on this medium have strains that turn the medium green, but there are also strains that turn the medium yellow. These strains that turn the medium yellow are the strains that may have the ability for phosphorus solubility.

The results showed that 10/22 bacterial strains were able to dissolve phosphorus from insoluble form to easily absorbable form (accounting for 45.5% of total isolates). These 10 bacterial strains grow well on solid NBRIP medium. Bacterial strains with the ability to dissolve insoluble phosphorus are easily identified by creating halo light rings.

After incubation for 2 days at 30ºC, all strains showed colonies, however, only 6 strains appeared halo rings, the remaining 16 strains did not appear halo rings. The strain with the highest phosphorus dissolution efficiency was the strain N2CTA with 28.967%, a statistically significant difference compared with other strains. Most of the remaining strains had nearly equal phosphorus dissolution efficiency.

In general, 10/22 endophytic bacteria strains in *Mimosa pudica* have the ability to dissolve insoluble phosphorus, as shown by the efficiency of phosphate E, in which L3CTA is the most effective strain (121.43%) at day time. Fourth, this shows that this is a strain that adapts to the environment quite quickly and has good ability to dissolve insoluble phosphorus. According to research by Nguyen Khanh Ngoc (2013), two strains LA8 and LB12 endogenously living in rice are the two strains with the highest ability to dissolve phosphorus with phosphorus dissolution efficiency of 165% and 175%, respectively. The NDH15 strain of Nguyen Thanh Binh (2013) that lives endogenously in watermelon plants has the highest phosphorus solubility with a phosphorus dissolution efficiency of 246% at day 5 after inoculation. Thereby, it shows that endophytic bacteria strains in different plants have different phosphate solubilization efficiency, but in general, the insoluble phosphate dissolving efficiency of endophytic bacteria strains in *Mimosa pudica* tree is still quite high.

Antibacterial ability of the isolated bacterial strains

All bacterial strains were tested for their antibacterial ability against 3 types of pathogenic bacteria: *E. coli*, *Staphylococcus aureus* and *Aeromonas hydrophila*. Resistance to *E. coli*, *Staphylococcus aureus* and *Aeromonas hydrophila* of the isolates was demonstrated through the formation of a light ring around the filter paper impregnated with bacterial inoculum on the PDA medium that was spread with pathogenic bacteria. Observe the formation of light rings for 3 consecutive days.

When tested for antibacterial ability with 3 species of pathogenic bacteria such as *E. coli*, *Staphylococcus aureus* and *A. Hydrophila*, 15 out of 22 bacterial strains were found to be resistant to enteric pathogens (*E. coli*), 12 of 22 strains were resistant to *Aeromonas hydrophila* and 1 strain was resistant to human pathogens *Staphylococcus aureus*. Of which, 11 strains are resistant to both *E. coli* and *Aeromonas hydrophila*, and 1 strain is resistant to all 3 pathogenic bacteria.

Resistance to *Staphylococcus aureus* bacteria

When investigating the antibacterial ability of endophytic bacteria strains isolated from human pathogenic bacteria *Staphylococcus aureus*, only 1 strain isolated, the T1PH strain, it was resistant to *Staphylococcus aureus*. The T1PH strain was also the only strain that is resistant to all 3 pathogenic bacteria used in the study.

Results of identification of promising bacterial strains isolated from *Mimosa pudica* tree.

No.	Bacterial strain	Identification results	Similarity (%)
1	L1CT	*Enterobacter ludwigii* IARI-THD-17	97%
2	T1PH	*Bacillus pumilus* GR21	97%

Sequences of genes encoding 16S-rRNA of the L1CT strain

The DNA fragment of the 1137 bp L1CT has 97% homomorphism rate with the 16S-rRNA gene region sequence of *Enterobacter ludwigii* IARI-THD-17.

According to a study by Aarab et al. (2013), *Enterobacter ludwigii* isolated from the rhizosphere of *Lupinus hirsutus* L. had a high ability to synthesize IAA and solubilize phosphorus (298.66 mg/L), which increased root length (53%) and increase the dry weight of rice plants. On the other hand, *Enterobacter ludwigii* isolated from the rhizosphere of Lolium perenne L., by its ability to synthesize IAA and solubilize phosphorus, also increased shoot height (20%) and increased root weight by 50% (M. Schoebitz et al., 2007)

Through the results of isolation and investigation of biochemical characteristics of endophytic bacteria in *Mimosa pudica* tree, it was shown that endogenous L1CT in *Mimosa pudica* leaves has a high ability to synthesize IAA (39.49 pg/ml). and also has the ability to dissolve phosphorus (16.9%), in addition to the ability to fix nitrogen (0.26 pg/ml on the 4th day after inoculation). In particular, this study also showed that this strain also has high antibacterial ability, resistant to *E. coli* and *Aeromonas hydrophila* bacteria with an antibacterial ring diameter of 6.67 mm and 17.67 mm, respectively, was the strongest *Aeromonas hydrophila* -resistant strain of the twenty-two isolates. Sequencing results showed that the L1CT had 97% homomorphism rate with the DNA sequence of *Enterobacter ludwigii*.

Sequence of genes encoding 16S-rRNA of T1PH strain

The 1218 bp DNA fragment of the T1PH strain has 97% homomorphism with the 16s-rRNA gene region sequence of the *Bacillus pumilus* GR21.

According to Chari (2011), *Bacillus pumilus* is resistant to *E. coli* and *Staphylococcus aureus.* In addition, *Bacillus pumilus* is also capable of synthesizing IAA (36.7 pg/ml) and dissolving insoluble phosphate (37.3 pg/ml). Not only that, the culture of this bacteria is also resistant to fungi even after heat treatment. Besides, they also secrete some extracellular enzymes capable of breaking down cellulose, proteins and chitin (Murugappan, 2013).

After isolating and examining biochemical characteristics, it was found that the T1PH strain had a high ability to synthesize IAA and fix nitrogen (37.35 pg/ml and 2.23 pg/ml,

respectively). In particular, this strain is resistant to all three types of pathogenic bacteria *E. coli*, A. hydrophila and S. aureus with an antibacterial ring diameter of 8.67mm for *E. coli*; 3.67 mm for *Aeromonas hydrophila* and 7.67 mm for *Staphylococcus aureus*. The identification results show that the T1PH strain has a homomorphism rate of 97% with the sequence of *Bacillus pumilus*, which shows that T1PH is a promising bacterial strain that can be used in cultivation, medicine and aquatic applications. products, can be used to produce potential microbial antibiotics, which can replace some synthetic antibiotics in fighting pathogens in aquaculture and cultivation.

4. Conclusion

Twenty-two bacterial strains were isolated from the roots, stems, leaves and root nodules of *Mimosa pudica*. All 22 strains of these bacteria have the ability to fix nitrogen, synthesize IAA. 10/22 strains created a halo ring, showing the efficiency of dissolving insoluble phosphorus. There are 15 strains of bacteria that are resistant to *E. coli*, 12 strains of bacteria that are resistant to *Aeromonas hydrophila*. In particular, there are 11 strains that are resistant to both *E. coli* and *Aeromonas hydrophila* bacteria. Two promising bacterial strains, L1CT and T1PH, were identified, respectively, as *Enterobacter ludwigii* and *Bacillus pumilus*, with a homomorphic rate of 97%.

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