Isolation and identification of a novel ginger tissue pathogenic bacteria *Citrobacter* sp. SJ7

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Abstract. The pathogenic bacteria of ginger plantlets were isolated and identified. Pathogenic strains were isolated, purified, and then identify by morphology observation, physiological and biochemical experiments, and the 16s rDNA sequence was analyzed to verify the classification of the pathogen. This study shows the novel pathogenic bacteria is kind of Gram-negative ones, which can be well root on the ginger tissue as well as cultivated plantlets. The histopathology examination illustrates that the pathogen prefer infect the host plant through ginger tuber. Moreover, the16S rDNA sequence analysis exhibits that it is kind homology species closely relating to *Citrobacter amalonaticus*. In a word, we first isolated a novel pathogenic bacteria of ginger named as *Citrobacter sp* SJ strain, which could successfully recognize the host ginger via xylems.

1. Introduction
Ginger (*Zingiber officinale* Roscoe), as a medicine food homology herb of Zingiberaceae, is used as food, medicine and seasoner. It’s also known as ginger root, spicy cloud, hook finger, Yindixin, fickleness boy, etc. The ginger rhizome (dry ginger), emboli (ginger skin), leaves (ginger leaves) all can be used as medicine. Studies have shown that its unique gingerol has the efficacy in treating digestive diseases, gastrointestinal congestion, esophageal cancer, colon cancer and so on [1-3]. So the research and development of ginger medicinal ingredients has become a hot spot in emerging research field. However, according to the latest investigation data, the ginger planting cost per mu of land was between 7000 yuan and 15000 yuan in 2019, of which the risk cost input was mainly due to the high incidence of soil-borne diseases during the cultivation of ginger. The pathogenic bacteria of ginger blast had the characteristics of complex differentiation, rapid variation, wide host range, difficult prevention and treatment, and long latent period of soil. Once the disease spreads, the production will be reduced by 20-30% or even more than 80%, which seriously restricts the development of ginger industry [4]. In recent years, with the research on ginger cultivation at home and abroad, the quality of ginger has been improved effectively by using tissue culture. However, tissue culture propagation needs to be carried out under aseptic conditions, and pollution events caused by improper human operation were unavoidable. Besides, after planting into the field, bacterium can be spread through vectors like surface water and underground insect pests, leading to the production loss. At present, the annual output value of the main ginger growing areas in China is reduced by 2-3% due to ginger blast,
and the loss of the seriously ill fields is more than 70% [5, 6]. Moreover, up to now, there is still a lack of effective control methods in production practice.

At present, the research on the pathogenic bacteria of ginger blast at home and abroad is mainly focused on the identification of pathogenic bacteria. As a large exporter of ginger, Chinese researchers have pioneered a series of studies. In 1981, Ren et al. discovered that the main pathogenic bacteria was \textit{Pseudomonas solanacearum} [7]. Since then, it has been found that \textit{Pseudomonas solanacearum} is the most common pathogen of ginger blast in different regions. In 1993, Yao et al. discovered that \textit{Erwinia spp.} also can cause ginger blast as a secondary pathogen. \textit{Fusarium spp} and \textit{Rhizoctonia spp.} were proven to have the similar effect in 1998 by Sun [8-10]. Recently, a highly pathogenic strain of \textit{Citrobacter farmeri} was isolated and purified from rotting ginger blocks in southwest China. However, regional differences and large variation of pathogenic bacteria put forward more serious scientific problems for the prevention and treatment of ginger blast.

The process by which plant pathogens cause disease to the host can be divided into the following extremely complex six stages: contact, identification, infection, colonization, expansion and development. Besides, genes participate in the metabolic process of nutrition capture, signal transduction, protein secretion and so on in each step. In the past two decades, the development of genomics has provided a new opportunity to analyze the pathogenicity mechanism of pathogens in detail and design targeted drugs. The complete genome sequencing of \textit{Haemophilus influenzae} has been performed, after that, it has also been completed in many plant pathogens, including \textit{Escherichia coli}, \textit{Bacillus subtilis}, soil bacilli C58, \textit{Caustic xylobacter} strain 9A5C, \textit{Solancorraria} strain GM1000, -\textit{Xanthomonas} strain 306, \textit{Pseudomonas solanacearum} strain DC3000, \textit{Erwinia carotovora subsp. SCR1} [8-12]. A large number of genomic data published earlier [13, 14] provided support for the identification of pathogenic genes of plant pathogens and species-specific genes of these strains by comparative genomics analysis. In 2007, Liu Bin [15] identified three species-specific pathogenic genes of \textit{Pseudomonas solanacearum} by sequencing the genome of 16 plant pathogenic bacteria with whole genome sequence, and further confirmed that one of them was highly toxic to \textit{Arabidopsis} by prokaryotic expression technique. It laid a solid foundation for understanding the mechanism of plant diseases from the perspective of molecular biology and controlling the occurrence of plant diseases more efficiently in the future. However, the research on the pathogenic genes of ginger blast is a blank area all over the world. At the level of genomics, this study focuses on a kind of ginger blast pathogen which has been isolated and identified in our laboratory, and then identify its pathogenic genes by comparing genomes to analyze the biological mechanism of ginger blast caused by different bacterial types. It is of a significant production and application value for analyzing the biological mechanism of ginger blast caused by different bacterial types and effectively prevention and control. Therefore, through the development of this project, we hope to provide theoretical basis for preventing the occurrence and epidemic of ginger blast.

2. Materials and methods

2.1. Experimental materials

Seven strains of pathogenic bacteria were isolated from the ginger in Yongchuan Cucumber Mountain in Chongqing. Ginger tissue culture seedlings (3-4 leaves, 4-5 roots) cultured in aseptic condition and normal ginger tubers were selected. The materials were provided by Chongqing characteristic plant seedling science and technology city.

2.2. Isolation and purification of strain

Through plate screening and marking purification, seven strains of ginger pathogenic bacteria were screened and purified. Then, the colonies were selected and named SJ1-7, respectively, preserving at 4°C. SJ7 was finally identified as a new type of ginger pathogenic bacteria by Koch's rule for follow-up experiments.
2.3. **Morphological observation**
The morphology of individual pathogenic bacteria SJ7 was observed under optical microscope and the cell size was measured. The morphological structure was observed by Gram stain method according to the previous reference [2].

2.4. **Pathogenicity identification of isolated bacteria**
The bacteria and four known bacteria that were previously isolated and placed in the inclined surface tube were taken, and the original solution was diluted into bacterial solution with the concentration of $10^9$, $10^7$, $10^5$, $10^3$ and $10^1$ by red blood cell counting board [3]. All operations were performed in a sterile environment with the aseptic water treatment as the control group.

2.4.1. **Pathogenicity identification of ginger tissue culture seedlings.** 2 mL bacterial solution was injected into the culture medium for the normal growing ginger tissue culture seedling roots. Three parallel groups of each solution were set up, with aseptic water treatment group as the control. After 5-7 d culture in a 28°C constant temperature incubator, the rooting status of ginger tissue culture seedlings was observed, and the roots were stained red [4].

2.4.2. **Pathogenicity identification of ginger tuber.** Ginger was treated in two ways: 1. Cutting the ginger tuber into the same size using sterilized aseptic knife, and then gently cutting a few evenly shallow wounds on the ginger block with knife tip. 2. After soaking in bacteria solution for 2 hours, gently covering the treated ginger and incubating them in the sterile operating table to prevent the bacteria. A processing ginger is placed in each petri dish and sealed with fresh-keeping film. Three parallel repeats were set for each bacterial solution, and a control group of aseptic water was set for each. The pathogenicity of ginger surface was observed after incubation in 28°C constant temperature incubator for 3-5 days.

2.5. **Genome extraction and 16S rDNA amplification**
The bacterial genomic DNA rapid extraction kit (OMEGA) was used to extract the strain DNA. After that, 16s rDNA was amplified by PCR.

Positive primers: 5’-AGAGTTGATCCTGGCTCAGAACGAACGCT-3’; reverse primers: 5’-TACGGCTACCTTGTTACTTCACCCC-3’. PCR products were detected with 1% agarose gel electrophoresis. Sequence analysis was performed by Shanghai Meiji Biomedical Technology Co., Ltd.

2.6. **Genetic evolutionary analysis**
Firstly, sequences of homologous species were retrieved and downloaded from the Nr/Nt library. Secondly, multiple sequences were compared using Clustal X 1.81, then MEGA 5.0 was used to construct the phylogenetic tree by adjacency method, maximum likelihood method and minimum evolution method respectively after the sequence alignment. At last, the image was integrated by Adobe IllustratorCS4.

3. **Results and discussion**

3.1. **Dyeing observation and pathogenicity identification of pathogenic bacteria**
The pathogenic bacteria in 20 bottles of tissue culture samples were isolated and purified by plate separation. Finally, an unknown pathogenic bacteria SJ7 was identified according to the growth pattern. Morphological observation under optical microscope showed that the unknown strain SJ7 was rod-shaped, about 1.0 μm in diameter and 2.0–6.0 μm in length. It was a gram-negative bacterium, mainly distributed in single and in pairs with motility (Fig. 1).
In order to verify its pathogenicity, the bacteria were inoculated with ginger by 5 concentration gradients and incubated for 5 days. The results showed that, compared with the aseptic water treatment group, the colonization ability of ginger cultured with SJ7 was significantly enhanced with the increasing concentration of bacteria solution. And the inoculation site showed dark color, watery patches, and wilting of ginger. The results showed that SJ7 had certain pathogenicity to ginger, and the pathogenicity was enhanced with the concentration of inoculated bacteria (Fig. 2).

**Figure 1.** Gram Staining and Bacterial Morphology of Unknown Pathogenic Bacteria SJ7 Isolated from Ginger (×40)

![Figure 1. Gram Staining and Bacterial Morphology of Unknown Pathogenic Bacteria SJ7 Isolated from Ginger (×40)](image)

In order to further confirm its pathogenicity, the strain SJ7 was inoculated into the culture medium for 3-year-old ginger tissue culture seedlings in a light incubator with constant temperature for 5 days. As shown in Fig. 3, compared with the control group, growth retardation, leaf chlorosis, leaf diameter shortening, leaf vein shallowness, root rot and so on were observed in ginger tissue culture seedlings after inoculating the strain SJ7. And with the increasing infection concentration, the whitening degree of leaves became more obvious. Therefore, it was concluded that the parasitism of the pathogen can interfere with the growth of the host ginger seedlings and has strong pathogenicity to the ginger seedlings.

**Figure 2.** Pathogenicity Identification of Unknown Bacteria SJ on Ginger Tuber.

![Figure 2. Pathogenicity Identification of Unknown Bacteria SJ on Ginger Tuber.](image)
3.2. Histopathological observation
According to the tissue biopsies of ginger tissue culture seedling after infection (Fig. 4), it was observed that the infection of the pathogen led to the increased cell spaces in xylem fiber of the plant and deepen color of the catheter, suggesting that the bacterium may be a xylem decomposing enzyme-type pathogen.

3.3. Biochemical properties analysis of pathogenic bacteria
16S rDNA, which encodes a subunit of ribosomal RNA, has the characteristics of conservatism and universality, as well as the stability of the sequence itself. It can be used widely as phylogenetic marker for the analysis of bacterial community structure (or to classify and identify different strains and genera by the difference of variable region sequence). Therefore, the genome of unknown strain SJ7 was extracted using genomic extraction kit and amplified by PCR detection by agarose gel electrophoresis with 16S rDNA primer (Fig. 5). Then, the positive bacteria were sequenced.
Figure 5. 16S rDNA amplification of unknown ginger pathogenic bacteria SJ7. Note: M means DL5000 DNA Marker; 1-3 are stands for 16s rDNA PCR amplification from respective Ginger root after infected by unknown bacteria SJ7 for 48h at 30°C; 4-6 are respective labelled 16s rDNA PCR amplification from Ginger tissue culture seedlings after infected by unknown bacteria SJ7 for 48h at 30°C at 2-3 leaves-drawing stage; And target gene band was noticed by red arrow.

Then, the phylogenetic tree was constructed with the 16s rDNA sequence and threshold of 1000 repeats using Mega 5.0 software with adjacency method, maximum likelihood method and minimum evolution method. *E. coli* was recruited as the out-group, the phylogenetic evolution analysis results showed that the pathogen was *Citrobacter* sp (Fig. 6), which was consistent with the observation of bacterial morphology.

Figure 6. Systematic evolutionary analysis of ginger pathogen SJ7. Note: Numbers represent the confidence value of maximum likelihood method, minimum evolution method and adjacency method, respectively.

4. Discussion

*Citrobacter* is a gram negative facultative anaerobe and a common intestinal habitant bacteria, belonging to Enterobacteriaceae [16]. It was widely distributed in soil, water, food and other natural environment. And it acts as a conditional pathogen to cause aquatic organisms and human inflammation, necrosis, pus, bacteriaemia and even death, showing clinical multi-drug resistance to most antimicrobial agents [17]. Thus, it was indicated that the pathogen was an opportunistic infection pathogen for animal hosts with broad-spectrum drug resistance. Wang et al. [18] found that *Citrobacter* can be co-produced on a Mexican leguminous plant as an endophyte with Erwinia,
Klebsiella, Salmonella, Enterobacter, etc. to enhance the plant’s adaptability to the environment. Botanists confirmed that the transition from endophytic bacteria to pathogenic bacteria or saprophytic bacteria existed in liberobacter asiaticum [19, 20], gum disease of peach tree [21], root rot of watermelon [22]. Therefore, *Citrobacter sp SJ7* may convert into a pathogen of ginger by the influence of environment, showing strong pathogenicity to tissue culture seedlings. Following research will focus on the screening of specific genes of *Citrobacter sp SJ7* and bioantagonistic bacteria in order to provide more effective solutions for the production detection and control of ginger pathogenic bacteria.

5. Conclusion

Based on morphological, pathological biochemical, and physiological features, and sequence analysis of 16S rDNA, a new pathogenicity of *Citrobacter sp SJ7* was identified, which could lead to the rot of ginger, water-stained patches and softening. It also could cause the yellow wilting of ginger seedlings, shortening of leaf diameter, whitening of seedlings and root rot of tissue culture seedlings. There have been no reports on the disease caused by *Citrobacter* in ginger. In this study, a new kind of ginger pathogenic bacteria was isolated, and histopathological results showed that it could cause tissue pathological changes and increase cell space in the xylem of ginger, leading to the growth retardation and even death of ginger seedlings.

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