The Diversity of Endophytic Bacteria from the Traditional Medicinal Plants Leaves that Have Anti-phytopathogens Activity

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ABSTRACT

Endophytic bacteria live in plant tissues which utilized in plant protection against phytopathogens. This study aims to investigate the diversity of endophytic bacteria from the leaves of traditional medicinal plants that has anti-phytopathogens properties. Isolation of endophytic bacteria was done by spread plate method. The bacteria were characterised by Gram staining and the 16S rRNA gene analysis. Further screening of anti-phytopathogen activity used disc diffusion method for Ralstonia solanacearum, Xanthomonas campestris, Fusarium oxysporum, and Sclerotium rolfsii. All togethers, sixteen isolates of endophytic bacteria from the leaves of eight medicinal plants species were obtained. Fourteen isolates had an anti-phytopathogen (with eight isolates against R. solanacearum, seven isolates against X. campestris, nine isolates against F. oxysporum, and five isolates against S. rolfsii). From the 14 isolates identified, phylum Firmicutes were dominant (64.3%), followed by Proteobacteria (28.6%), and Actinobacteria (7.1%). Phylum Firmicutes consists of Bacillus indicus (BJF1, TCF1, and MCF2), Bacillus pumilus (CAF4), Bacillus sp. (CAF1), Bacillus subtilis (AAF2, MCF1, CAF3, and MCF3); phylum Proteobacteria consists of Pantoea agglomerans (CAF2), Pantoea stewartii (AAF4), Pseudomonas oryzihabitans (AAF3), and Pseudomonas psychrotolerans (AAF1); and phylum Actinobacteria consists of Kocuria kristinae (CSF1).

Keywords: Diversity, endophytic bacteria, traditional medicinal plants, antimicrobial activity, anti-phytopathogens activity

Introduction

Endophytic bacteria which live in plant tissues is a very interesting subject to study. This organism significantly involves in the protection of plants, increasing growth, and overcoming environmental stresses [1, 2].

Traditional medicinal plants [such as Annona muricata L., Artocarpus altilis (Parkinson) Fosberg, Brucea javanica (L.) Merr., Citrus auranti-folia Swingle, Cheilocostus speciosus (J. Konig) C. Specht, Datura metel L., Manilkara zapota (L.) P. Royen, Morinda citrifolia L., Syzygium cuminii (L.) Skeels., and Tinospora crispa (L.) Miers] have been widely reported to have antimicrobial activity against human pathogen and phytopathogen.

Endophytic bacteria isolated from traditional medicinal plants are reported to produce antibacterial, antifungal and antiseptic compounds [3, 4]. These compounds have the potential to be utilized as biopesticides in controlling pests and diseases in plants.

The use of endophytic bacteria as a biopesticide producer is very beneficial for the environ-
ment. Biopesticide compounds are more environmentally friendly than synthetic pesticides. This is because biopesticides are easily decomposed in nature, specific work targets, have unique properties and work methods, and are not toxic to humans [5].

_Ralstonia solanacearum_ (causes of plant wilt) [6], _Xanthomonas campestris_ (causes of plant blight) [7], _Fusarium oxysporum_ (cause of plant wilt) [8], and _Sclerotium rolfsii_ (cause of root rot) [9] are important phytopathogens for plants. These phytopathogens are soil-borne which quite difficult to control. As phytopathogen resistance increases to existing pesticides, it is necessary to continue to explore in finding new strains of endophytic bacteria that have the potential to be developed as biopesticide producers. This study aims to investigate the diversity of endophytic bacteria from the leaves of medicinal plants that can inhibit the growth of phytopathogens.

**Material and Methods**

**Isolation of endophytic bacteria**

Leaves of plants _A. muricata_ L., _Art. altilis_ (Parkinson) Fosberg, _B. javanica_ (L.) Merr., _C. aurantiifolia_ Swingle, _Che. speciosus_ (J. König) C. Specht, _D. metel_ L., _M. zapota_ (L.) P Royen, _Mor. citrifolia_ L., _S. cumini_ (L.) Skeels., and _T. crispa_ (L.) Miers were collected from the garden in Lakuk, Simpang Haru Village, Padang, West Sumatra Province, Indonesia. The collection of plant leaves was carried out according to de Melo et al. (2009) [10]. Leaves were sterilized by following the method developed by Araujo et al. (2001) [11].

Isolation was carried out using the method of de Melo et al. (2009) [10] with modification. Sterile plant leaf segments (1 gram) were mashed with a mortar and sterile pestle, then suspended into 9 mL physiological NaCl (Merck®) 0.85%. The suspension was homogenized and serially diluted. Then 0.1 mL of the suspension from each dilution was inoculated into a Petri dish containing medium Tryptic Soy Agar (TSA) (Merck®) using a spread plate method, then incubated at 27°C for 1-3x24 hours. Growing isolates were observed for the colony morphology.

Bacterial colonies that grow are purified on TSA plates using a quadrant streak plate method. Purified endophytic bacterial isolates were then stained through Gram staining. Gram staining is done by referring to Cappucino & Sherman (2014) [12], using the Gram (Merck®) staining kit.

**Screening of endophytic anti-phytopathogens activity**

All isolated endophytic bacterial isolates were screened to determine its anti-phytopathogens activity against _R. solanacearum_, _X. campestris_, _F. oxysporum_, and _S. rolfsii_. _R. solanacearum_ and _X. campestris_ were from culture collection of Microbiology Laboratory, Faculty of Agriculture, Andalas University, Padang, Indonesia. _F. oxysporum_ and _S. rolfsii_ were from culture collection of Phytopathology Laboratory, Faculty of Agriculture, Andalas University, Padang, Indonesia. Screening of anti-phytopathogen activity was carried out disk diffusion method which refers to Melliaawati et al. (2006) [13]. Positive control used 15 ppm of chloramphenicol for bacteria, while positive control of fungus used 15 ppm of ketoconazole.

**Identification of endophytic bacteria through 16S rRNA Analysis**

The assay was done by planting Pure culture of endophytic bacteria was inoculated into a test tube containing 4 mL of Tryptic Soy Broth medium (Merck®) and incubated using a shaker for 24 hours. The results of the culture were taken as much as 3 mL, then centrifuged at a speed of 13,000 rpm, for 5 minutes. Pellets are taken to extract the genome DNA using the Wizard® Genomic DNA purification kit (Promega Corp.) following the manufacturer's instructions. DNA amplification was carried out by preparing the mix reagent mixture for PCR as follows (KAPA Taq ReadyMix Kit - KAPA): 20 µL dH₂O, 25 µL master mix PCR, 2 µL primer 9F (20 pmol) (5’-GAG TTT GAT CCT GGC TCA G 3’), 2 µl Primer 1541R (20 pmol) (5’-AAG GAG GTG ATC CAG CC-3’), and 1 µL DNA Template with a total volume of 50 µL. PCR amplification was carried out as many as 30 cycles with the following programs: preheat at 96°C for 5 minutes, denaturation at 96°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute, extension at 72°C for 7 minutes. PCR results were electrophoresed in 1% agarose gel using 1x TAE buffer then visualized using gel illuminator. Agarose gel is photographed as documentation. The PCR results are cleaned using the SV Gel and PCR Clean-Up System (Promega) Wizard.

The purified DNA extract was sent to First
Bacteria have been found in plants, including obligate endophytic bacteria, which cannot be cultured. Hardoim et al. (2008) [16] explained that many endophytic bacteria were obligate endophytes. Gayathri et al. (2010) [17] and Anjum & Chandra (2015) [18] also obtained Gram-positive bacteria as endophytic bacteria in their study.

**Anti-phytopathogens activity of endophytic bacteria**

In general, isolated endophytic bacteria have anti-phytopathogens activity. Eight isolates had anti-phytopathogens activity against *R. solanacearum*; seven isolates had anti-phytopathogens activity against *X. campestris*, nine isolates had anti-phytopathogens activity against *S. rolfsii* (Table 2). The endophytic bacteria that have been isolated have low anti-phytopathogens activity against phytopathogenic bacteria (2 mm) compared to anti-phytopathogens activity against phytopathogenic fungi (8 mm – 17 mm). Based on the criteria by Davis and Stout (1971) [19] anti-phytopathogens activity against phytopathogenic bacteria in this study was weak (inhibitory zone was < 5 mm), whereas anti-phytopathogens activity against phytopathogenic fungi was moderate (inhibitory zone was 5 – 10 mm) and strong (inhibitory zone was 10 – 20 mm).

The results obtained indicate that the endophytic bacteria obtained have the potential to be developed as a biocontrol agent or as a biopesticide producer against phytopathogenic fungi. Endophytic bacteria can produce lysis enzymes against compounds such as chitin, protein [20], cellulose, and hemicellulose [21]. These enzymes can result in direct suppression of the activity of phytopathogenic microbes [21].

The ability of endophytic bacteria to produce anti-phytopathogens compounds was very beneficial for host plants, because host plants can be used against phytopathogen [22]. According to Haas & Defago (2005) [23], this was caused by...
active plants responding to various environmental stimuli and can also respond to various chemical compounds stimulated by microbes. Both by soil microbes, as well as microbes associated with plants.

In general, endophytic bacteria have an excellent ability to inhibit phytopathogenic fungi, when compared to phytopathogenic bacteria. According to Bloemberg & Lugtenberg (2001) [24], this ability was caused by the ability of endophytic bacteria to produce diffuse and volatile antifungal compounds. Endophytic bacteria could mediate de novo antimicrobial synthesis and new antifungal secondary metabolites, which have been accepted as potential fungicides to prevent the spread of phytopathogens [25].

Identification of endophytic bacterial isolates that have anti-phytopathogens activity

Based on the results of 16S rDNA sequence analysis, 14 isolates were identified into nine different species with similarity ranged from 94-99% to the nearest strain (Table 3). The isolates which have similarities > 97% can be identified as the same species, however, the similarity < 97% is identified as the same genus [30]. All identified isolates are new strains, and each is registered in the National Center for Biotechnology Information (NCBI) database with accession numbers KY806221-KY806234.

Anti-phytopathogens activity test results (Table 2) shows that strains from the isolated Bacillus genus have diverse anti-phytopathogens activities. B. indicus BJF1 has anti-phytopathogens activity against R. solanacearum and F. oxysporum; B. indicus TCF1 only has anti-phytopathogens activity against X. campestris; and B. indicus MCF2 has anti-phytopathogens activity against R. solanacearum and X. campestris. B. indicus was first discovered by Suresh et al. (2004) [31] in the waters of West Bengal, India, and these bacteria were resistant to arsenic. Hong et al. (2008) [32] report-

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolate</th>
<th>Gram staining</th>
<th>R. solanacearum</th>
<th>X. campestris</th>
<th>F. oxysporum</th>
<th>S. rolfsii</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>AAF1</td>
<td>Negative</td>
<td>2</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>AAF2</td>
<td>Positive</td>
<td>2</td>
<td>2</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>3.</td>
<td>AAF3</td>
<td>Negative</td>
<td>2</td>
<td>0</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>4.</td>
<td>AAF4</td>
<td>Negative</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.</td>
<td>AMF1</td>
<td>Positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6.</td>
<td>BJF1</td>
<td>Positive</td>
<td>2</td>
<td>0</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>7.</td>
<td>CAF1</td>
<td>Positive</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8.</td>
<td>CAF2</td>
<td>Negative</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.</td>
<td>CAF3</td>
<td>Positive</td>
<td>0</td>
<td>2</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>10.</td>
<td>CAF4</td>
<td>Positive</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>11.</td>
<td>CSF1</td>
<td>Positive</td>
<td>0</td>
<td>2</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>12.</td>
<td>MZF1</td>
<td>Positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13.</td>
<td>MCF1</td>
<td>Positive</td>
<td>0</td>
<td>2</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>14.</td>
<td>MCF2</td>
<td>Positive</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15.</td>
<td>MCF3</td>
<td>Positive</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>16.</td>
<td>TCF1</td>
<td>Positive</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17.</td>
<td>Positive Control</td>
<td>21</td>
<td>20</td>
<td>26</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>
ed *B. indicus* to be safely used as a prebiotic. However, there have been no reports of these bacteria as endophytes and their ability to produce anti-phytopathogens compounds. This study was first reported that the bacterium as endophytic and has anti-phytopathogens activity. Further research was needed to determine the potential of these strains as biopesticide producers.

*B. subtilis* belongs to *Bacillus* genus which has the most strains in this study (four strains). Three strains had anti-phytopathogens activity against phytopathogenic bacteria and fungi, namely *B. subtilis* CAF3, *B. subtilis* MCF1, and *B. subtilis* AAF2, whereas one strain only had antibacterial activity, namely *B. subtilis* MCF3 (Table 2). *B. subtilis* was generally found in soil, water, and associated with plants [33]. Several strains of *B. subtilis* have been reported as endophytes and have the ability to inhibit soil-borne phytopathogens [34], such as *X. campestris* [35], *R. solanacearum* [36], *F. oxysporum* [37, 38] and *S. rolfsii* [39]. *B. subtilis* was reported to have the ability to produce antimicrobials, such as antimicrobial lipopeptides [40], so that these bacteria have considerable antimicrobial activity.

Strains from other *Bacillus* genera also have anti-phytopathogens activity, such as *Bacillus* sp. CAF1 against *R. solanacearum*; and *B. pumilus* CAF4 against *F. oxysporum* and *S. rolfsii*. Sturz *et al.* (2005) [41] reported that *B. pumilus* isolated from endorhiza and potato exorhiza could have anti-phytopathogens activity against *F. oxysporum*. de Melo *et al.* (2009) [10] also reported that these bacteria also had anti-phytopathogens activity against *S. rolfsii*. Therefore, it can be concluded that *Bacillus* was a genus of endophytic bacteria that has the potential to be developed as a biocontrol agent because it can inhibit the growth of bacteria and fungi. Forchetti *et al.* (2007) [42] mentions that strains of the *Bacillus* have the advantage of being developed as biopesticides compared to other bacteria. Strains from this genus are easily to be cultured and stored; can be applied as spores, or inoculants to seeds, or bioactive compounds produced; shows a protective effect on various pathogenic microbes and improve plant growth.

*K. christinae* CSF1 has anti-phytopathogens activity against *X. campestris* and *F. oxysporum*. This bacterium is also known as *Micrococcus kristinae*. This bacterium was first isolated by Kovacs *et al.* (1999) [43] from the roots of *Typha angustifolia*. These bacteria are found as endophytes in some plants, such as *Solanum tuberosum* [41], *Carica papaya* [44], *Panicum virgatum* [45], and *Durio* sp. [46]. *K. kristinae* can inhibit the growth of *F. oxysporum* [41], dissolve phosphate [46], and have pectinase enzyme activity [44].

Strains of the isolated *Pantoea* genus in this study only had antibacterial activity. *Pan. agglomerans* CAF2 have anti-phytopathogens activity against *R. solanacearum* and *X. campestris*, while *Pan. stewartii* AAF4 only has anti-phytopathogens activity against *R. solanacearum*. *Pantoea*

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Table 2. Strains of endophytic bacteria based on the results of molecular identification through 16S rDNA analysis

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolate</th>
<th>Accession number assigned</th>
<th>Closest type strain (accession number)</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>AAF1</td>
<td>KY806234</td>
<td><em>Pseudomonas psychrotolerans</em> AP9-27B (KM891562)</td>
<td>99</td>
</tr>
<tr>
<td>2.</td>
<td>AAF2</td>
<td>KY806226</td>
<td><em>Bacillus subtilis</em> SSCT68 (AB210968)</td>
<td>99</td>
</tr>
<tr>
<td>3.</td>
<td>AAF3</td>
<td>KY806233</td>
<td><em>P. oryzihabitans</em> AF31 (LC015573)</td>
<td>99</td>
</tr>
<tr>
<td>4.</td>
<td>AAF4</td>
<td>KY806232</td>
<td><em>Pantoea stewartii</em> M073</td>
<td>99</td>
</tr>
<tr>
<td>5.</td>
<td>BFF1</td>
<td>KY806221</td>
<td><em>B. indicus</em> (KF791344)</td>
<td>99</td>
</tr>
<tr>
<td>6.</td>
<td>CAF1</td>
<td>KY806225</td>
<td><em>B. cereus</em> RNS_01 (KT380683)</td>
<td>94</td>
</tr>
<tr>
<td>7.</td>
<td>CAF2</td>
<td>KY806231</td>
<td><em>Pantoea agglomerans</em> ZFJ-15 (EU931554)</td>
<td>99</td>
</tr>
<tr>
<td>8.</td>
<td>CAF3</td>
<td>KY806228</td>
<td>*B. subtilis 55C1-1 (JN366797)</td>
<td>99</td>
</tr>
<tr>
<td>9.</td>
<td>CAF4</td>
<td>KY806224</td>
<td><em>B. pumilus</em> FI39 (KT318787)</td>
<td>99</td>
</tr>
<tr>
<td>10.</td>
<td>CSF1</td>
<td>KY806230</td>
<td><em>Kocuria kristinae</em> LCT (KR230389)</td>
<td>99</td>
</tr>
<tr>
<td>11.</td>
<td>MCF1</td>
<td>KY806227</td>
<td><em>B. subtilis</em> subsp. <em>inaquosorum</em> MER_77 (KT719652)</td>
<td>99</td>
</tr>
<tr>
<td>12.</td>
<td>MCF2</td>
<td>KY806223</td>
<td><em>B. indicus</em> WJB 131 (KU877665)</td>
<td>99</td>
</tr>
<tr>
<td>13.</td>
<td>MCF3</td>
<td>KY806229</td>
<td><em>B. subtilis</em> SR41 (KY203664)</td>
<td>99</td>
</tr>
<tr>
<td>14.</td>
<td>TCF1</td>
<td>KY806222</td>
<td><em>B. indicus</em> WJB 131 (KU877665)</td>
<td>99</td>
</tr>
</tbody>
</table>
Figure 1. Phylogenetic tree strains of endophytic bacteria that have anti-phytopathogens activity
was found in Citrus spp. [11], Arachis hypogea L. [47], Zea mays L. [48], and in several other plants that have been studied. Pantoea spp. reported to produce IAA [47], siderofor [49], and have the ability to fix nitrogen [48]. This bacterium does not have antifungal activity against phytopathogenic fungi [50]. Both strains of Pantoea identified in this study have opportunistic properties. Pan. agglomerans are opportunistic to cotton plants [51], however, Pan. stewartii was opportunistic towards maize plants [52, 53].

P. psychrotolerans AAF1 and P. oryzihabitans AAF3 have anti-phytopathogens activity against R. solanacearum and F. oxysporum. Lamessa & Zeller (2007) [54] reported that Pseudomonas was a potential genus that can control R. solanacearum. Chaiharn et al. (2009) [55] and Munif et al. (2012) [56] adding this genus can also inhibit the growth of F. oxysporum. Pseudomonas was capable of producing siderophore [57], and these compounds can inhibit the growth of phytopathogen microbes [58].

Based on the results obtained it can be concluded that endophytic bacteria that are identified are quite diverse and have various anti-phytopathogens activities. Host plants can be a limiting ability. This can be seen from the anti-phytopathogens ability of B. subtilis isolated from different plants having different anti-phytopathogens activity against phytopathogens. B. subtilis is a fairly dominant species found in this study. B. subtilis was reported by Jacobsen et al. (2004) [59] can produce antimicrobial peptides and contribute to leaf and root disease. Also, the bacteria also produce lipopeptide, which is an amphiphilic compound with surfactant activity [60]. Bacillus has secondary metabolite products that are attractive with a broad antimicrobial spectrum and very diverse structures [61].

The Bacillus is the most common genus found as endophytes. This is supported by the results of several studies which claim the Bacillus is dominant in some plants, such as Jacaranda decurrens [62], Panax spp. [63, 64], Manihot esculenta [10], Solanum lycopersicum [56], Polygonum cuspidatum [65], and Musa spp. [66]. Bacillus is known as a cosmopolitan genus and has high survival ability. This can be caused by the presence of endospores. Moat et al. (2002) [67] stated that endospores contained in cells have a role in protecting organisms against unfavorable environmental conditions. This leads to organisms that have them will be able to survive and maintain their existence.

**Phylogenetic endophytic bacterial isolates that have anti-phytopathogens activity**

The results of phylogenetic analysis of endophytic bacterial strains that have anti-phytopathogens activity using UPGMA (Figure 1) show strains of endophytic bacteria that have anti-phytopathogens activity grouped into three phyla. Firmicutes are dominant phylum (64.3%), followed by Proteobacteria (28.6%), and Actinobacteria (7.1%). The three phyla are commonly found as endophytes in various plants [68, 69]. Some results of the study report that phylum Firmicutes is the dominant phylum [66, 70, 71], followed by Proteobacteria and Actinobacteria, respectively [66, 71].

Phylogenetic tree show that the strains obtained were grouped into two groups originating from different ancestors. The first group was Pan. stewartii AAF4, P. oryzihabitans AAF3, and P. psychrotolerans AAF1, while the second group was K. kristinae CSF1, Bacillus sp. CAF1, Pan. agglomerans CAF2, B. indicus BJF1, B. indicus TCF1, B. indicus MCF2, B. pumilus CAF4, B. subtilis CAF3, B. subtilis AAF2, B. subtilis MCF1, and B. subtilis MCF3. In general, the strains that were owned have a close relationship with strains that have similarities with them, only B. subtilis AAF2 and B. subtilis MCF1 strains were different. B. subtilis AAF2 has similarities with B. subtilis SSCT68, but has a kinship with B. subtilis subsp. inaquosorum MER_77, B. subtilis MCF1 has similarities with B. subtilis subsp. inaquosorum MER_77, but has a kinship with B. subtilis 55CI-1. Triana (2005) [87] found that different species have close molecular relationships, namely Photorhizobium thomsonianum BTA1 with Blastobacter denitrificans.

**Conclusion**

Sixteen isolates of endophytic bacteria from eight species of medicinal plants were obtained. Fourteen isolates had anti-phytopathogens activity (eight isolates against R. solanacearum, seven isolates against X. campestris, nine isolates against F. oxysporum, and five isolates against S. rolfsii). Identification of the fourteen isolates showed that Firmicutes were dominant phylum (64.3%), followed by Proteobacteria (28.6%) and Actinobac-
bacteria (7.1%). Phylum Firmicutes consist of **B. indicus** (BJF1, TCF1, and MCF2), **B. pumilus** (CAF4), **Bacillus** sp. (CAF1), **B. subtilis** (AAF2, MCF1, CAF3, and MCF3); phylum Proteobacteria consists of **P. agglomerans** (CAF3), **P. stewartii** (AAF4), **P. oryzihabitans** (AAF3), and **P. psychrotolerans** (AAF1); and phylum Actinobacteria consists of **K. kristinae** (CSF1).

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