Stable Transformation of the Actinobacteria *Frankia* spp.

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ABSTRACT A stable and efficient plasmid transfer system was developed for nitrogen-fixing symbiotic actinobacteria of the genus *Frankia*, a key first step in developing a genetic system. Four derivatives of the broad-host-range cloning vector pBBR1MCS were successfully introduced into different *Frankia* strains by a filter mating with *Escherichia coli* strain BW29427. Initially, plasmid pHKT1 that expresses green fluorescent protein (GFP) was introduced into *Frankia casuarinae* strain Ccl3 at a frequency of 4.0×10^{-3} , resulting in transformants that were tetracycline resistant and exhibited GFP fluorescence. The presence of the plasmid was confirmed by molecular approaches, including visualization on agarose gel and PCR. Several other pBBR1MCS plasmids were also introduced into *F. casuarinae* strain Ccl3 and other *Frankia* strains at frequencies ranging from 10^{-2} to 10^{-4} , and the presence of the plasmids was confirmed by PCR. The plasmids were stably maintained for over 2 years and through passage in a plant host. As a proof of concept, a salt tolerance candidate gene from the highly salt-tolerant *Frankia* sp. strain Ccl6 was cloned into pBBR1MCS-3. The resulting construct was introduced into the salt-sensitive *F. casuarinae* strain Ccl3. Endpoint reverse transcriptase PCR (RT-PCR) showed that the gene was expressed in *F. casuarinae* strain Ccl3. The expression provided an increased level of salt tolerance for the transformant. These results represent stable plasmid transfer and exogenous gene expression in *Frankia* spp., overcoming a major hurdle in the field. This step in the development of genetic tools in *Frankia* spp. will open up new avenues for research on actinorhizal symbiosis.

IMPORTANCE The absence of genetic tools for *Frankia* research has been a major hindrance to the associated field of actinorhizal symbiosis and the use of the nitrogen-fixing actinobacteria. This study reports on the introduction of plasmids into *Frankia* spp. and their functional expression of green fluorescent protein and a cloned gene. As the first step in developing genetic tools, this technique opens up the field to a wide array of approaches in an organism with great importance to and potential in the environment.

KEYWORDS actinorhizal symbiosis, cloning vector, gene transfer, genetics, nitrogen fixation, plant-microbe interactions

The actinorhizal symbiosis is an endosymbiotic nitrogen-fixing association between members of *Frankia*, a genus of actinobacteria, and a variety of angiosperms (1, 2). These plants, termed actinorhizal plants, represent over 200 diverse species of dicotyledonous plants classified into 8 distinctive plant families. Symbiosis with *Frankia* spp. allows actinorhizal plants to colonize harsh environments. Actinorhizal plants have a positive impact on the environment, including restoration of disrupted environmental sites, soil stabilization, reforestation, and reclaiming of saline soils (3–5). Besides ecological and economic reasons, the capacity of an individual *Frankia* sp. strain to nodulate and fix nitrogen in association with plants in as many as eight different families makes this system attractive to study.

However, one of the major obstacles in the field of actinorhizal symbiosis is the lack

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of genetic tools for the bacterial partner *Frankia* sp. In fact, the genetics of *Frankia* is still in its infancy. The reasons for the slow development of genetic tools are complex and include the low growth rate of *Frankia* spp. and the difficulty of obtaining single genomic units. Because of these impediments and to provide vital information on its symbiosis, the genomes for many *Frankia* strains have been sequenced (6–8), and these databases have opened up the use of “omics” approaches (9).

At present, there are no known systems for stable gene transfer in *Frankia* spp. Analysis of the *Frankia* genomes revealed the presence of integrated phages (7) and integrating conjugative elements (ICE) in several *Frankia* strains (7, 10), but the use of these elements has not been pursued as a genetic tool. In general, genetic analysis of *Frankia* spp. has been restricted to gene cloning, phylogenetic analyses of selected gene sequences, and some limited studies on plasmid isolation and mutagenesis. Initially, a few studies on spontaneous mutants in *Frankia* spp. have been reported (11–13), and chemical (*N*-methyl-*N*-nitro-*N*-nitrosoguanidine and ethyl methanesulfonate treatments) and physical (UV) mutagenesis approaches have also been investigated with some success but limited potential as tools (14–17).

A number of *Frankia* strains contain plasmids (18). Several of the *Frankia* plasmids have been cloned and sequenced (19–23) to determine their use as a vector. A putative *ori* region common to both pFQ12 and pFQ11 was identified (20, 22), while another one was predicted in pFQ31 (23). The absence of common origins for these indigenous *Frankia* plasmids and their size (>8.5 kb) make their development as cloning vectors difficult. None of these indigenous *Frankia* plasmids have been transferred to another *Frankia* strain. Moreover, there is an absence of efficient and stable transformation methods for *Frankia* spp.

There have been three published attempts at DNA transfer in *Frankia* cells that have resulted in low efficiency of transfer or unstable transformed cells (24–26). The effect of electroporation conditions on cell viability was determined (25). Chromosomal DNA of *Frankia* strain EAN1pec, a lincomycin- and kasugamycin-resistant strain, was electroporated into *Frankia inefficax* strain Eul1c, which is sensitive to lincomycin and kasugamycin (25). Several stable transformants that were resistant to either lincomycin or kasugamycin were identified, stably maintained, and characterized physiologically. No molecular analyses were performed to confirm the presence of the inserted genomic DNA in the transformants. In another study, electroporation of fusion marker genes into *Frankia* spp. resulted in transient transformants (26). The phenotype of these unstable transformants declined over several generations, indicating that the constructs were not stable.

In this study, the introduction of plasmids into *Frankia* spp. via conjugation is reported. Four derivatives of the cloning vector pBBR1MCS (with different antibiotic resistance) were successfully introduced in different *Frankia* strains. The efficiency of transformation, molecular confirmation of the transfer, and copy number of plasmids per *Frankia* genome were evaluated for the different combinations. The plasmid pHTK1 (27), a derivative of the pBBR1 plasmid, which expresses the green fluorescent protein (GFP), was used initially in our study. The stable transfer of pHTK1 into different *Frankia* strains was demonstrated, and it was confirmed by molecular evidence and by observed GFP expression inside *Frankia* strains. As a proof of concept, the pBBR1MCS-3 vector was used for cloning of salt tolerance candidate genes which were introduced into *Frankia* strains, subsequently providing elevated salt tolerance.

RESULTS

Efficient mating and transfer of pHTK1 into *Frankia* cells. A reproducible and efficient method of *Frankia* transformation was developed based on mating on a solid-surface medium using *Escherichia coli* as the donor. To counterselect against the donor, *E. coli* strain BW29427, a diaminopimelic acid auxotroph (DAP[−]), was used in this study. This strain will not grow on defined medium, like MOPS-phosphate-nitrogen (MPN) medium, unless supplemented with diaminopimelic acid. Plasmid pHTK1 was initially used in these transformation experiments. Since this plasmid was introduced

TABLE 1 Frequencies of plasmid transformation and spontaneous mutation rates for antibiotic resistances for different *Frankia* strains^a

Strain (clade)	Transformation frequency (no. of transformants/total CFU)			Spontaneous frequency (no. of mutants/total CFU)	
	pHKT1 with 20 μ g/ml Tc	pBBR1MCS with 25 μ g/ml Chlo	pBBR1MCS-3 with 20 μ g/ml Tc	20 μ g/ml Tc	25 μ g/ml Chlo
<i>F. casuarinae</i> Ccl3 (Ic)	4.0×10^{-3}	1.4×10^{-4}	4.3×10^{-3}	1.0×10^{-7}	5.8×10^{-7}
<i>Frankia</i> sp. strain Thr (Ic)	ND	ND	3.0×10^{-4}	8.0×10^{-6}	5.6×10^{-6}
<i>Frankia</i> sp. strain EUN1f (III)	ND	3.2×10^{-3}	1.7×10^{-2}	7.6×10^{-6}	1.0×10^{-6}
<i>F. inefficax</i> Eul1c (IV)	1.0×10^{-4}	ND	8.4×10^{-4}	1.6×10^{-6}	ND

^aTc, tetracycline; Chlo, chloramphenicol; ND, not determined.

into the Gram-positive actinobacteria *Rhodococcus* spp. (28) and another high-G+C-content bacterium, *Burkholderia cepacia* (27), we reasoned that it could potentially work in the high-G+C-content *Frankia* actinobacteria.

To test this idea, *Frankia casuarinae* strain Ccl3, whose plant host range is restricted to Casuarinaceae, was chosen because it has a sequenced genome (6) and is the subject of many studies in the discipline. After mating *F. casuarinae* strain Ccl3 with *E. coli* BW29427(pHKT1), the mixture was plated on MPN medium, as described in Materials and Methods, and incubated at 30°C. After 3 to 4 weeks, isolated small colonies that showed an appearance characteristic of a *Frankia* colony appeared on the selective medium (see Fig. S1 in the supplemental material). The colonies were incubated for another 2 weeks prior to harvesting (Fig. S1). Single colonies with a minimum 3-mm diameter were harvested and transferred to liquid culture containing tetracycline. The transformants were stable and have been maintained in culture for over 3 years in some cases. The frequency of transformation for *F. casuarinae* strain Ccl3 with the vector pHKT1 was 4.0×10^{-3} (Table 1), which is much greater than the frequency of spontaneous tetracycline resistance (1.0×10^{-7}). The controls showed appropriate expected results. Matings lacking a recipient did not grow on MPN medium with kanamycin with or without DAP, while matings lacking a donor generated colonies at a frequency similar to the spontaneous mutation frequency (data not shown).

Confirmation of the presence of the plasmid in *Frankia* cells. All of the transformants were confirmed by several molecular approaches. First, the plasmids were extracted from *F. casuarinae* strain Ccl3(pHKT1) transformants and linearized by enzymatic digestion with NsiI. A single band was observed only for the *F. casuarinae* strain Ccl3(pHKT1) transformants (lanes 2 and 5) and *E. coli* DH5 α (pHKT1) (lane 4) (Fig. 1A). Those bands migrated to the same position in the gel, indicating that they were the same size. The band was absent for the wild-type *F. casuarinae* strain Ccl3 (lane 1), and

FIG 1 Molecular confirmation of the plasmid transfer in different *Frankia* strains. (A) Gel electrophoresis of the extracted plasmid pHKT1. The plasmid was extracted as described in Materials and Methods and run on an agarose gel. Lane 1 represents the negative-control *F. casuarinae* Ccl3 wild-type strain. Lanes 2, 3, and 5 represent *F. casuarinae* strain Ccl3(pHKT1) transformants. Lanes 2 and 5 show the linearized plasmid pHKT1 after treatment with the restriction enzyme NsiI, while lane 3 shows the uncut plasmid pHKT1. Lane 4 shows the linearized plasmid pHKT1 from *E. coli* DH5 α (pHKT1). The pHKT1 plasmid is 6,897 bp in size. (B) Presence of tetracycline amplicon on an agarose gel. The tetracycline PCR fragment amplified from total DNA, as described in Materials and Methods. Lane 1, *F. casuarinae* strain Ccl3(pHKT1) transformant; lane 2, *F. casuarina* strain Ccl3(pBBR1MCS-3) transformant; lane 3, *F. inefficax* strain Eul1c(pBBR1MCS-3) transformant; lane 4, *E. coli* DH5 α (pBBR1MCS-3) (positive control); lane 5, *F. casuarinae* Ccl3 wild-type strain (negative control); lane 6, *F. inefficax* Eul1c wild-type strain (negative control). The expected size of the tetracycline fragment was 488 bp. A 2-log DNA ladder (New England BioLabs, MA) was used as a molecular marker (lane L).

FIG 2 *Frankia casuarinae* strain Ccl3 transformed with pHKT1 fluoresces. Cultures were observed by fluorescence microscopy at $\times 400$ magnification. (A) GFP-labeled *E. coli* DH5 α (pHKT1) after overnight culture in liquid LB medium. (B) GFP-labeled *F. casuarinae* strain Ccl3(pHKT1) transformant after 7 days of growth on MPN liquid medium. (C and D) Control *F. casuarinae* Ccl3 wild-type strain after 7 days of growth in MPN medium but under phase-contrast microscopy (C) and fluorescence microscopy (D). Panels A and B also show fluorescence microscopy.

two bands were observed for the undigested plasmid extracted from *F. casuarinae* strain Ccl3(pHKT1) corresponding to the different plasmid conformations visible on gel electrophoresis (lane 3). Second, a portion of the tetracycline resistance gene found on the plasmid was amplified from DNA extracted from transformants grown in liquid culture (Fig. 1B). Amplicons were detected for *F. casuarinae* strain Ccl3 transformed with pHKT1 plasmid (lane 1), while the wild-type *F. casuarinae* strain Ccl3 did not generate an amplicon (lane 5).

Frankia casuarinae strain Ccl3(pHKT1) transformants were also observed under fluorescence microscopy. The results are presented in Fig. 2. Green fluorescence was observed for the *E. coli* DH5 α (pHKT1) (Fig. 2A) and for a 7-day-old *F. casuarinae* strain Ccl3(pHKT1) transformant (Fig. 2B). The wild-type strain *F. casuarinae* strain Ccl3 did not fluoresce (Fig. 2C and D). All of the *F. casuarinae* strain Ccl3(pHKT1) transformants maintaining the plasmid pHKT1 fluoresced under these conditions. The fluorescence was observed in the hyphae of the *F. casuarinae* strain Ccl3(pHKT1) transformants. These results indicate that exogenous gene expression can occur in *Frankia* strains.

Confirmation of the stability of the transformant *in planta*. The *F. casuarinae* strain Ccl3(pHKT1) transformant was inoculated into the host plant *Casuarina glauca* in order to observe the stability of the transformant *in planta*. The resulting nodules were collected 2 months after inoculation and surface sterilized. The nodules were fragmented and plated on growth medium. After a 2-month incubation, single colonies were harvested and subcultured into liquid growth medium. The cultures were incubated for 2.5 months, and genomic DNA (gDNA) was extracted from liquid culture. A PCR approach was used to identify these isolates as *Frankia* strains maintaining the pHKT1 plasmid (Fig. 3). The chromosomal gene *Franci3_3203* (UTRase gene) was used to identify *F. casuarinae* strain Ccl3 (Fig. 3A). As expected, amplicons were detected for both the *F. casuarinae* strain Ccl3(pHKT1) transformant isolated from *C. glauca* nodules and the wild-type *F. casuarinae* strain Ccl3. No amplicon was observed for the negative

FIG 3 Molecular confirmation of the stability of the *F. casuarinae* strain Ccl3(pHKT1) *in planta*. The *F. casuarinae* strain Ccl3 UTRase gene (*Francci3_3203*) was amplified as described in Materials and Methods, and the amplicons were visualized on an agarose gel. (A) Gel electrophoresis of the UTRase gene amplicons. Lane 1 shows *F. casuarinae* strain Ccl3(pHKT1) isolated from *C. glauca* nodules; lane 2, *F. casuarinae* Ccl3 wild-type strain (positive control); lane 3, *E. coli* DH5 α (pBBR1MCS-3) (negative control). The expected size of the amplicon was 539 bp. (B) Gel electrophoresis profile of plasmid-specific amplicon (the tetracycline resistance gene). Lane 1 shows *F. casuarinae* strain Ccl3(pHKT1) isolated from *C. glauca* nodules; lane 2, *F. casuarinae* Ccl3 wild-type strain (negative control); lane 3, *E. coli* DH5 α (pBBR1MCS-3) (positive control). The expected size of the tetracycline fragment was 854 bp. Gels were spliced to remove duplicate lanes.

control corresponding to the plasmid extracted from *E. coli* DH5 α (pHKT1). The same samples were used to detect the presence of the plasmid pHKT1 by PCR amplification of the tetracycline resistance gene (Fig. 3B). Amplicons were detected for the *F. casuarinae* strain Ccl3(pHKT1) transformant isolated from *C. glauca* nodules and the plasmid extracted from *E. coli* DH5 α (pHKT1). The wild-type *F. casuarinae* strain Ccl3 did not generate an amplicon. The amplicons obtained in Fig. 3A were sequenced (Fig. S2). The sequences for amplicons of the *F. casuarinae* strain Ccl3(pHKT1) transformant (nodule isolate) and *E. coli* strain DH5 α (pHKT1) are identical. These results confirmed the presence and the stability of the *F. casuarinae* strain Ccl3(pHKT1) transformant within *C. glauca* nodules and after reisolation. Under these conditions, the plasmid was maintained for over 6 months.

Use of the pBBR1MCS plasmid in the transformation of *Frankia* spp. The plasmid pHKT1 is mobilized via the RP4/RK2 mating system and possesses an origin of replication (*OriV*) and the replication gene (*rep*) from plasmid pBBR1 (27). Thus, pHKT1 is a derivative plasmid of the pBBR1MCS plasmid family (27) that expresses the green fluorescent protein (GFP). We reasoned that other pBBR1MCS plasmids should be compatible with *Frankia* strains, and three derivative plasmids of the broad-host-range cloning plasmid pBBR1MCS (pBBR1MCS, pBBR1MCS-3, and pBBR1MCS-5) were used to transform different *Frankia* strains. Four *Frankia* strains (Ccl3, Thr, Eul1c, and EUN1f) belonging to three different clades were transformed. The frequencies of transformation are presented in Table 1. Transformants with pBBR1MCS-5, carrying a gentamicin resistance gene, were unable to be transferred into liquid culture with gentamicin (data not shown). The transformation frequencies obtained for pHKT1, pBBR1MCS, and pBBR1MCS-3 were between 3.2×10^{-2} and 8.4×10^{-4} . These frequencies are higher than the frequencies of spontaneous mutations (5.6×10^{-6} to 5.8×10^{-7}) to tetracycline or chloramphenicol (Table 1). The transformants were transferred into liquid medium with the appropriate antibiotic and exhibited the same rate of growth as the wild-type strain.

Similar to studies on *F. casuarinae* strain Ccl3(pHKT1) transformants, the presence of pBBR1MCS in *Frankia* spp. was confirmed by PCR (Fig. 1B). Figure 1B presents the molecular confirmation of *F. casuarinae* strain Ccl3(pBBR1MCS-3) and *Frankia inefficax* Eul1c(pBBR1MCS-3) transformants. The tetracycline resistance gene amplicons were detected for the two transformants and the control *E. coli* strain. The two *Frankia* wild-type strains, *F. casuarinae* strain Ccl3 and *F. inefficax* Eul1c, did not produce a band. These data confirm the presence of the plasmid in the transformants.

Determination of the plasmid copy number per *Frankia* genome. A quantitative PCR (qPCR) approach was used to determine the average plasmid copy number per

TABLE 2 Plasmid copy number per *Frankia* genome

Strain (clade)	Copy no. ^a		
	pHKT1 with 20 µg/ml Tc	pBBR1MCS with 25 µg/ml Chlo	pBBR1MCS-3 with 20 µg/ml Tc
<i>F. casuarinae</i> Ccl3 (Ic)	3–4	9–10	12–15
<i>F. inefficax</i> Eul1c (IV)	1	ND	1
<i>Frankia</i> sp. EUN1f (III)	ND	ND	19

^aTc, tetracycline; Chlo, chloramphenicol; ND, not determined.

Frankia genome. Specific primer sets targeted to the tetracycline gene for pBBR1MCS-3 and pHKT1 plasmids or the *lacZ* gene for the pBBR1MCS plasmid were used to quantify the amount of plasmid in a sample. A specific primer set targeted to a single-copy gene within the *Frankia* genome was used to determine the number of genomes present in a sample. Dividing these two values determines the plasmid copy number. The data presented in Table 2 represent the average of 4 to 5 measurements for a minimum of 2 independent transformants. *Frankia casuarinae* strain Ccl3 transformants contained 12 to 15 copies of pBBR1MCS-3 and 9 to 10 copies of pBBR1MCS per *F. casuarinae* genome. The copy number for pHKT1 found in *F. casuarinae* strain Ccl3 was lower by 3 to 4 copies per genome and may be explained by the larger size of the plasmid. Plasmid size is negatively correlated with the plasmid copy number per cell (29). *Frankia inefficax* strain Eul1c maintained a low copy number (about 1) for pBBR1MCS-3 and pHKT1. These *F. inefficax* strain Eul1c transformants grew with appropriate antibiotics, and the presence of the plasmid was detected by PCR. These results were repeated with four independent transformants using different primers sets (Table 2). *Frankia* sp. strain EUN1f (clade III) had 18 copies of pBBR1MCS-3 per genome. *Frankia* sp. strain Thr (clade Ic) had an average of 9 to 11 copies for pBBR1MCS-3, similar to the value obtained with *F. casuarinae* strain Ccl3 (clade Ic).

To test the stability of these plasmids, *F. casuarinae* strain Ccl3(pBBR1MCS-3) and *F. casuarinae* strain Ccl3(pHKT1) transformants were subcultured every 2 to 3 weeks for 9 months and divided into MPN liquid medium with or without the selective antibiotic for 2 weeks. The copy numbers for pBBR1MCS-3 or pHKT1 were similar under both conditions. For pBBR1MCS-3, the average copy number was 9 with or without tetracycline, while pHKT1 had an average copy number of 3 to 4.

Many of these transformants have been maintained in the lab for over 3 years. After 9 months of subculture, transformant *F. casuarinae* strain Ccl3(pBBR1MCS-3), which had an initial copy number of 12, maintained a similar copy number of 10. A plasmid copy number of 10 was maintained for transformants cultured for 22 months. The *F. casuarinae* strain Ccl3(pHKT1) transformant also maintained a similar value for the average copy number, as follows: 3 to 4 copies initially, remained unchanged 9 months later, and 6 to 7 copies after 22 months of incubation. These data confirm the stability of these plasmids in different *Frankia* strains over a 22-month period.

Proof of concept of this mating transformation method based on salt tolerance of *Frankia casuarinae* strain Ccl3. Genetic, transcriptomic, and proteomics approaches identified seven candidate genes that are responsive to salt stress (30). These candidates are present in the salt-tolerant *Frankia* sp. strain Ccl6 and absent in the salt-sensitive *F. casuarinae* strain Ccl3. They are upregulated under stress conditions in *Frankia* sp. strain Ccl6. Gene *Ccl6_RS22605* from *Frankia* sp. strain Ccl6 was selected for the study and is predicted to code for a zinc peptidase. The predicted coding region and 350 bp upstream of the predicted start codon were cloned into pBBR1MCS-3 plasmid (pBBR1MCS-3-22605). *F. casuarinae* strain Ccl3, a salt-sensitive strain, was transformed with this construct. The efficiency of transformation was 1.25×10^{-4} .

To confirm the presence of the construct in *F. casuarinae* strain Ccl3 cells, a PCR approach was used on two distinct transformants. Figure 4A shows amplification of a fragment of tetracycline gene and part of a mobility region for the plasmid for the two different transformants and the positive control (purified pBBR1MCS-3-22605 plasmid).

FIG 4 Molecular confirmation of the transformation of *F. casuarinae* strain Ccl3 with pBBR1MCS-3-22605. (A and B) Gel electrophoresis profiles of amplicons of the tetracycline resistance and mobility genes on the vector and *Ccl6_RS22605* genes for the insert, respectively. (A) Results of amplification of tetracycline resistance and mobility gene fragments with an expected size of 1,269 bp. Lane 1, *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformant 1; lane 2, *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformant 2; lane 3, *F. casuarinae* strain Ccl3(pBBR1MCS-3) transformant; lane 4, *F. casuarinae* Ccl3 wild-type strain (negative control). (B) Results of amplification of the *Ccl6_RS22605* gene fragments with an expected size of 451 bp. Lane 1, *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformant 1; lane 2, *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformant 2; lane 3, *F. casuarinae* Ccl6 wild-type strain (positive control); lane 4, *F. casuarinae* Ccl3 wild-type strain (negative control); lane 5, *F. casuarinae* strain Ccl3(pBBR1MCS-3) transformant with the empty vector (negative control). (C) Gel electrophoresis profile of endpoint RT-PCR for *Ccl6_RS22605* gene. Lane 1, *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformant 1; lane 2, *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformant 2; lane 3, *F. casuarinae* strain Ccl3(pBBR1MCS-3) transformant with the empty vector (negative control); lane 4, *F. casuarinae* Ccl3 wild-type strain (negative control); lane 5, *Frankia* sp. Ccl6 wild-type strain (positive control). The size expected for the cDNA amplification was 450 bp. Gels were spliced to remove duplicate lanes.

As expected, no amplification was obtained for the wild-type *F. casuarinae* strain Ccl3. The presence of the gene *Ccl6_RS22605* in these *F. casuarinae* strain Ccl3 transformants was also confirmed by PCR (Fig. 4B). The amplicon of the *Ccl6_RS22605* gene was generated with the two *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformants and the positive control, the wild-type strain *F. casuarinae* Ccl6 DNA. The amplicon was absent in both in the wild-type *F. casuarinae* strain Ccl3 and the vector control, the *F. casuarinae* strain Ccl3(pBBR1MCS-3) transformant containing the empty vector. Colony PCR was also performed to confirm the presence of the plasmid in *F. casuarinae* strain Ccl3 cells (Fig. S3). With this technique, *Frankia* colonies could be screened after 5 to 6 weeks of growth instead of waiting 5 extra weeks of growth in liquid culture for DNA extraction (30). These data confirmed the presence of the constructs in the two *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformants tested.

The expression of the *Ccl6_RS22605* gene in these *Frankia* transformants was determined by endpoint reverse transcriptase PCR (RT-PCR) (Fig. 4C). The *Ccl6_RS22605* gene was only expressed in the two *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformants and the positive-control *Frankia* sp. strain Ccl6 wild type. Both the *F. casuarinae* strain Ccl3 containing the empty vector pBBR1MCS-3 and *F. casuarinae* Ccl3 wild-type strain did not express the gene. These results show that the cloned gene was expressed in the transformants.

The effect of the cloned gene on the salt tolerance by *F. casuarinae* strain Ccl3 was tested by a growth assay (Fig. 5). Wild-type *Frankia* sp. strain Ccl6 was the most resistant to salt stress and had an MIC value of 1,000 mM NaCl, while wild-type *F. casuarinae* strain Ccl3 was more sensitive to salt concentrations, having an MIC value of 475 mM NaCl, similar to previous results (30). The two *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformants, clones 1 and 6, showed an increase in the level of salt tolerance (MIC value, 650 mM NaCl) compared to wild-type *F. casuarinae* strain Ccl3 but less than that of *Frankia* sp. strain Ccl6. *Frankia casuarinae* strain Ccl3(pBBR1MCS-3) containing the empty vector exhibited a salt tolerance pattern (MIC value, 475 mM NaCl) similar to that of the wild-type *F. casuarinae* strain Ccl3. These results demonstrate that the cloned *Ccl6_RS22605* gene, encoding a zinc peptidase, was expressed and provided an increase in salt-tolerant *F. casuarinae* strain Ccl3.

DISCUSSION

For many years, one of the major obstacles hindering the study of *Frankia* actinobacteria was the lack of genetic tools, including a stable transformation method. In this study, a mating transformation protocol was designed using filter mating methods. This

FIG 5 The expression of the *Ccl6_RS22605* gene, a salt tolerance gene, in *F. casuarinae* strain Ccl3 transformants increases its salt tolerance level. *Frankia* cultures were grown for 14 days in growth medium containing different NaCl concentrations, as described in Materials and Methods. The growth yield was determined by measuring the total protein content and corrected for the inoculum content. Growth curves were standardized to 300 $\mu\text{g/ml}$ for the control conditions (0 mM NaCl). Symbols represent *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformant clone 1 (open triangles), *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformant clone 6 (closed squares), *F. casuarinae* strain Ccl3(pBBR1MCS-3) transformant containing the empty vector (closed triangles), *F. casuarinae* Ccl3 wild-type strain (open circles), and *Frankia* sp. Ccl6 wild-type strain (closed circles).

is a successful method developed for stable plasmid transfer in *Frankia* spp. that allows the expression of cloned genes. Several lines of evidence have been presented to demonstrate that the approach is successful. First, antibiotic resistance transformants were generated at a higher frequency (at least 2 log) than the spontaneous mutation rate. Second, the presence of the plasmid was detected by molecular approaches, including detection in an agarose gel and PCR amplification of plasmid genes. Third, transformants expressed the genes encoded on the plasmid, including GFP and antibiotic resistance or genes cloned into the vector. These plasmids were stably maintained in the transformants over 3 years. PCR approaches confirmed the presence of the plasmid in 22-month-old transformants, and the copy number of these plasmids did not change after 22 months of culturing. The plasmid was also maintained after passage through a host plant and sequential recovery of the strain from the host plant, providing further evidence of the plasmid stability. Thus, the plasmid was stably maintained by *Frankia* spp. and was not either degraded or expelled.

The four *Frankia* strains used in this study represent three of the four *Frankia* clades, which are based on molecular phylogenetic analysis and host plant range (1). Clade I consists of *Frankia* strains that associate with host plants in the Casuarinaceae, Betulaceae, and Myricaceae families, while members of clade II are infective on Rosaceae, Coriariaceae, Datisceae, and the genus *Ceanothus* (Rhamnaceae). Members of clade III are the most promiscuous and are infective on Elaeagnaceae, Rhamnaceae, Myricaceae, Gymnostoma, and occasionally the genus *Alnus*. Clade IV consists of "atypical" *Frankia* strains that are unable to reinfect actinorhizal host plants or form ineffective root nodule structures that are unable to fix nitrogen. Clade I contains subclades, and subclade Ic contains strains limited to *Casuarina* and *Allocasuarina* host plants.

The mating method of plasmid transfer in *Frankia* spp. also had a higher efficiency (10^{-2} to 10^{-4} , Table 1) than in our previous study using electroporation (10^{-5}) (25). Other studies using electroporation generated unstable transformants (24, 26). Mating transformation produced a larger amount of viable *Frankia* cells than under electroporation conditions. Thus, the mating method allows screening a larger population of cells (10^6 to 10^7 CFU) than electroporation (10^4 to 10^5 CFU) (25). It seems that the viability of *Frankia* cells decreases as the capacitance of the electrical pulse increased (15). The filter mating method with the use of a vacuum increased the physical contact

between the donor and recipient cells, which elevated the transfer efficiency. For *Frankia* spp., transformation protocols using mating methods appear to allow for a more successful outcome than does electroporation.

The mating method used in this study generates a single CFU on agar, which should produce a homogenous population of transformants. Liquid transformation methods can result in a mixture of cells that does not homogeneously contain plasmids. The major drawback for the mating method is that it requires a minimum 13 to 14 weeks from the plating to the molecular confirmations of the transformants (Fig. S1). This long chronology is mainly due to the low growth rate of *Frankia* cells. *Frankia* cells will grow faster in liquid culture, but plating is the only method to select and isolate transformants for further study. However, time could be saved at the molecular confirmation step (Fig. S1) by using an efficient colony PCR method that will cut out the 8- to 9-week time span to confirm the presence of the plasmid in bacterial colony (31).

The plasmids used in this study are derivative plasmids from the broad-host-range cloning plasmid pBBR1 isolated from Gram-negative *Bordetella bronchiseptica* (28). These derivative plasmids of pBBR1 have been successfully transferred into the Gram-positive actinobacteria of *Rhodococcus* (32) and in numerous Gram-negative bacteria (33). Moreover, the high stability of these plasmids has been demonstrated in *Brucella* spp. pBBR1MCS was still detected in different *Brucella* strains after over 50 days in the absence of antibiotic selection (34). These plasmids are mobilized via the RP4/RK2 mating system and possess an OriV, origin of replication, and replication gene (*rep*) (33). They combine a Gram-positive mobilization mechanism with a Gram-negative replication mechanism. This replication mechanism does not use the rolling-circle mechanism that is commonly found in Gram-positive plasmids. pBBR1 uses the theta mechanism, which consists of the melting of parental strands, synthesis of primer RNA, and initiation of DNA synthesis by extension of the RNA primer (35). Thus, the key for successful plasmid transfer and stability in *Frankia* spp. appears to be the use of a plasmid containing a Gram-positive mobilization mechanism and an uncommon Gram-negative replication, the theta mechanism. This conclusion is supported by previous attempts at introducing other plasmids that have different origins without success (our unpublished data). We propose that other plasmids containing similar characteristics could be introduced into *Frankia* spp. by this transformation method to test this hypothesis.

Introduction of pHKT1 in *F. casuarinae* strain Ccl3 provided evidence of exogenous gene expression within *Frankia* cells. These transformed cells fluoresced due to the presence of the *gfp* gene on the plasmid (Fig. 2). Older cultures (1 month old or older) lost fluorescence, suggesting that other factors might be involved, including bacterial growth state. Subculturing these transformants into fresh growth medium caused the recovery of fluorescence. Bacterial gene expression is regulated by growth rate and state (36). Different growth states cause a change in the abundance of repressor or activator proteins involved in gene expression regulation. A proof-of-concept experiment was developed to test if a cloned gene could be expressed in *Frankia* spp. Our results demonstrate that the *Ccl6_RS22605* gene from *Frankia* sp. strain Ccl6 was successfully introduced into *F. casuarinae* strain Ccl3. Several lines of evidence show that the gene was present and expressed in its *Frankia* host (Fig. 4 and 5). The expression of that gene resulted in an elevated level of salt tolerance (Fig. 5) but not to the level of *Frankia* sp. strain Ccl6. This gene is upregulated under salt stress conditions for salt-tolerant *Frankia* sp. strain Ccl6 and absent in the salt-sensitive *F. casuarinae* strain Ccl3 (30).

The results of this study provide an important genetic tool for this Gram-positive bacterium. The availability of many sequenced *Frankia* genomes and other “omics” databases provides vital information on potential genes of interest involved in the actinorhizal symbiosis. The development of the cloning vector and subsequent exogenous gene expression in *Frankia* spp. provide a useful tool that will allow the potential of a unique and environmentally important organism to be more fully realized.

TABLE 3 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description ^a	Reference(s) or source
Strains		
<i>Escherichia coli</i>		
DH5 α	F ⁻ Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (<i>r_K⁻ m_K⁺</i>) <i>phoA supE44 λ^- thi-1 gyrA96 relA1</i>	43
BW29427	<i>hsdS</i> <i>lacZ</i> Δ M15 RP4-1360 Δ (<i>araBAD</i>)567 Δ <i>dapA1341::</i> (<i>erm pir</i>) <i>tra</i>	Datsenko and Wanner, unpublished data
DH5 α (pHKT1)	<i>E. coli</i> DH5 α containing pHKT1	This study
DH5 α (pBBR1MCS)	<i>E. coli</i> DH5 α containing pBBR1MCS	33
DH5 α (pBBR1MCS-3)	<i>E. coli</i> DH5 α containing pBBR1MCS-3	33
DH5 α (pBBR1MCS-3-22605)	<i>E. coli</i> DH5 α containing pBBR1MCS-3-22605	This study
BW29427(pHKT1)	<i>E. coli</i> BW29427 containing pHKT1	This study
BW29427(pBBR1MCS)	<i>E. coli</i> BW29427 containing pBBR1MCS	This study
BW29427(pBBR1MCS-3)	<i>E. coli</i> BW29427 containing pBBR1MCS-3	This study
BW29427(pBBR1MCS-3-22605)	<i>E. coli</i> BW29427 containing pBBR1MCS-3-22605	This study
<i>Frankia</i> spp.		
<i>F. casuarinae</i> strain Ccl3	Wild type, Gm ^r Kan ^r Kas ^r	38, 44
<i>Frankia</i> sp. strain Ccl6	Wild type	45
<i>F. inefficax</i> strain Eul1c	Wild type, Nov ^r	38, 46
<i>Frankia</i> sp. strain EUN1f	Wild type, Str ^r Nov ^r	38, 47
<i>Frankia</i> sp. strain Thr	Wild type	48
Ccl3(pHKT1)	<i>F. casuarinae</i> strain Ccl3 containing pHKT1	This study
Ccl3(pBBR1MCS-3)	<i>F. casuarinae</i> strain Ccl3 containing pBBR1MCS-3	This study
Ccl3(pBBR1MCS)	<i>F. casuarinae</i> strain Ccl3 containing pBBR1MCS	This study
Eul1c(pHKT1)	<i>F. inefficax</i> strain Eul1c containing pHKT1	This study
Eul1c(pBBR1MCS)	<i>F. inefficax</i> strain Eul1c containing pBBR1MCS	This study
EUN1f(pBBR1MCS-3)	<i>Frankia</i> strain EUN1f containing pBBR1MCS-3	This study
EUN1f(pBBR1MCS)	<i>Frankia</i> strain EUN1f containing pBBR1MCS	This study
Ccl3(pBBR1MCS-3-22605)	<i>F. casuarinae</i> strain Ccl3 containing pBBR1MCS-3-22605	This study
Plasmids		
pHKT1	<i>gfp</i> fragment, Tc ^r	27
pBBR1MCS-3	Broad-host-range vector, Tc ^r	33
pBBR1MCS	Broad-host-range vector, Chlo ^r	33
pBBR1MCS-3-22605	pBBR1MCS-3 containing gene 22605 from strain Ccl6	This study

^aGm^r, gentamicin resistant; Kan^r, kanamycin resistant; Kas^r, kasugamycin resistant; Nov^r, novobiocin resistant; Str^r, streptomycin resistant; *gfp*, green fluorescent protein; Tc^r, tetracycline resistant; Chlo^r, chloramphenicol resistant.

MATERIALS AND METHODS

Growth and maintenance of bacterial strains. Table 3 lists all of the plasmids and bacterial strains used in this study. *Frankia* stock cultures were grown and maintained in basal MP growth medium (37, 38) supplemented with 5.0 mM NH₄Cl and the appropriate carbon source. Basal MP growth medium consisted of morpholinepropanesulfonic acid (MOPS)-phosphate buffer (50 mM MOPS, 10 mM K₂HPO₄ [pH 6.8]) supplemented with 1 mM Na₂MoO₄, 2 mM MgSO₄, 20 μ M FeCl₃ with 100 μ M nitrilotriacetic acid (NTA), and modified trace salts solution (37). For *F. casuarinae* strain Ccl3 and *Frankia* sp. strains Ccl6 and Thr, 5 mM sodium propionate was used as a carbon and energy source, while 20 mM glucose or fructose was used with *F. inefficax* strain Eul1c and *Frankia* sp. strain EUN1f, respectively. Standing cultures were incubated at 30°C. Liquid cultures of *Frankia* strains or transformants were subcultured every 2 to 3 weeks. *Escherichia coli* strains were grown at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with appropriate antibiotics when required.

Transformation of *Frankia* spp. by mating. Prior to conjugation with *Frankia* strains, the plasmids used in this study were introduced into the donor *E. coli* BW29427, a diaminopimelic acid (DAP) auxotroph, by electrotransformation. Plasmids were introduced into recipient *Frankia* strains through filter mating with the donor *E. coli* BW29427. Briefly, a *Frankia* culture (100 ml) was grown to logarithmic phase (7 days old) and harvested by centrifugation (20 min at 4,300 $\times$ g). The cells were resuspended in 1 ml of MP medium. The *Frankia* cells were mixed with 500 μ l culture of *E. coli* BW29427 grown to an optical density of 0.6. The *Frankia*-*E. coli* mixture was collected by filtration on a sterile 0.22- μ m polycarbonate filter under vacuum pressure. The polycarbonate filter was aseptically transferred onto the surface of an LB agar plate supplemented with 300 μ M DAP and incubated at 28°C for 24 h. After incubation, the filter was removed and suspended in 3 ml MPN medium. The suspension was vortexed to separate the cell pellet from the filter. The *Frankia*-*E. coli* cell pellet was fragmented by gently grinding in a sterile glass tissue homogenizer. This step was necessary to fragment the *Frankia* mycelia. The homogenized mixture was used directly or was diluted 10⁻¹- to 10⁻⁶-fold in MP medium. Five hundred microliters of the 10⁰, 10⁻¹, or 10⁻² dilution was mixed with 3 ml of 0.8% agar MP and the appropriate antibiotics. For example, for the transformation of *F. casuarinae* strain Ccl3 with pHKT1, 20 μ g/ml tetracycline and 25 μ g/ml kanamycin were used. Tetracycline was used to stress the plasmid and kanamycin to select for *Frankia* spp. and counterselect against the donor. The antibiotic concentrations

used were 20 µg/ml tetracycline, 25 µg/ml kanamycin, and 25 µg/ml chloramphenicol. The 0.8% agar mixture, containing the appropriate antibiotics, was poured onto the surface of MPN growth medium with 2% agar. Mixing was done by tipping the plates from side to side before the agar hardened. The plates were sealed with Parafilm and incubated at 30°C. To determine the total number of *Frankia* CFU, 500 µl of the 10⁻² to 10⁻⁶ dilutions was mixed with 3 ml of 0.8% agar MP without antibiotics. The diluted 0.8% agar mixtures were poured onto MPN growth medium without antibiotics, mixed, and sealed as described above. After 5 to 6 weeks of incubation, single 3-mm-diameter *Frankia* colonies were picked and grown in liquid culture (Fig. S1).

To determine the frequency of transformation, the number of CFU with antibiotic resistance was divided by the total number of *Frankia* CFU. The frequency of a spontaneous mutation giving an antibiotic resistance was determined as described previously (15). Briefly, homogenized *Frankia* hyphae were diluted and plated as described above with lower dilutions under antibiotic stress and higher dilutions without antibiotics. The frequency of spontaneous mutation was determined by the number of antibiotic-resistant CFU divided by the total *Frankia* CFU.

Molecular detection of the plasmid. (i) Total DNA extraction from *Frankia* spp. Total DNA extraction from *Frankia* strains or transformants were performed by a cetyltrimethylammonium bromide (CTAB) DNA extraction protocol (39). Six milliliters of *Frankia* liquid culture was used to start the extraction.

(ii) PCR. Primers used to confirm the presence of pBBR1MCS-3, pHKT1, and pBBR1MCS-3-22605 plasmids were targeted the tetracycline gene (Tc^r PCR, pHKT1 PCR, or Tc^r-1,269-bp PCR) (Table 4). PCR molecular confirmation of the presence of pBBR1MCS plasmid was targeted to the replication gene (*rep* PCR Fw and Rev) (Table 4). PCRs were carried out using 50 to 100 ng of DNA, 10 µM the forward and reverse primers (Table 4), and 25 µl OneTaq Hot Start 2× mastermix (New England BioLabs, MA) in a final volume of 50 µl. The thermocycling parameters were as follows: (i) an initial denaturation of 94°C for 5 min; (ii) 40 cycles of denaturation at 94°C for 30 s, primer annealing for 30 s at 66°C, 64°C, 60°C, 55°C, or 54°C, depending of the primer sets (Table 4), and (iii) extension 72°C for 1 min to 1.5 min, with a final elongation at 72°C for 7 min. PCR amplicons were visualized by agarose gel electrophoresis.

(iii) Plasmid extraction from *Frankia* culture. A 7-day-old *Frankia* culture (150 ml) was harvested and washed twice in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). The pellet was resuspended in the same TE buffer and incubated at 80°C for 30 min. The cells were incubated for 2 h at 37°C with 2% lysozyme following with and incubation at 65°C for 15 min with 2% SDS. NaOH at 0.1 N was added, and the contents were mixed and allowed to stand for 10 min at room temperature. For neutralization, 1 M potassium acetate (pH 5.2) was added and mixed by inversion. The solution was centrifuged for 5 min at room temperature. The clear supernatant was transferred to a new tube and precipitated by adding 2 volumes of 100% ethanol and centrifuging at maximum speed for 15 min. The DNA was finally resuspended in 40 µl of distilled water.

To remove linear chromosomal DNA, 1× NEB buffer 4 (New England BioLabs, MA) was supplemented with 1 mM ATP and 2 µl of exonuclease V (RecBCD) (New England BioLabs). Incubation at 37°C for 1 h was done. The exonuclease was heat inactivated by incubating at 70°C for 30 min.

(iv) Colony PCR of *Frankia* spp. Colony PCR was performed as previously described (31). Primers used to confirm the presence of the pBBR1MCS-3 and pHKT1 plasmids were targeted the tetracycline gene Tc^r (Tc^r PCR colony FW and Rev) (Table 4).

(v) PCR fragment Sanger sequencing. PCR fragments were sequenced after purification of the PCR mixture by using the QIAquick PCR purification kit (Qiagen, CA). Samples were sent to GeneWiz for DNA Sanger sequencing. The samples were prepared according to the guidelines of the GeneWiz company.

Plasmid copy number determination. The plasmid copy number was determined by quantitative PCR (qPCR) (40). A specific primer set targeted to a single-copy gene in the *Frankia* genome was used to quantify the *Frankia* chromosome, as follows: uracil phosphoribosyltransferases (UTRase gene) for *F. casuarinae* strain Ccl3 and *Frankia* sp. strain Thr, *copC-1* for *F. inefficax* strain Eul1c, and *rpoB* for *Frankia* sp. strain EUN1f. To determine the amount of plasmid, specific primer sets were targeted to the following genes on the plasmids: tetracycline gene for pBBR1MCS-3 and pHKT1 and the *lacZ* gene for pBBR1MCS (Table 4). Standard curves were made with various concentrations of gDNA (0 to 10 ng) and plasmid (0 to 10 ng) with the corresponding primer sets (Table 4). qPCR mixtures consisted of 5 µl of 10 ng/µl gDNA, primer mix (0.3 µM), and SYBR green PCR mastermix (Applied Biosystems). The thermocycling parameters for the Agilent Mx3000P qPCR system (Agilent Technologies) were as follows: (i) 95°C for 15 min, (ii) 40 cycles of 95°C for 15 s and 60°C for 30 s, and (iii) a thermal dissociation cycle of 95°C for 60 s, 55°C for 30 s, and incremental increases of the temperature to 95°C for 30 s. The samples were measured in triplicate. Averages and standard deviations of the results from a minimum 2 independent transformants were calculated.

Fluorescence microscopy. *F. casuarinae* strain Ccl3 and *E. coli* cells containing the pHKT1 plasmid were observed by fluorescence microscopy. *Frankia* cells with pHKT1 were grown for 7 days in MPN growth medium supplemented with 20 µg/ml tetracycline. As a control, *Frankia* cells without the plasmid were also grown for 7 days in MPN growth medium without antibiotics. The control *E. coli*(pHTK1) strain was grown overnight in LB medium with tetracycline. Cells were observed under an Olympus BH-2 microscope without sample preparation. The GFP was excited at 488 nm and observed under ×400 magnification. Images were treated with the QImaging software.

Plant inoculation and bacterial isolation from plant. Three 2-month-old *Casuarina glauca* plants were inoculated with 2 ml of a concentrated culture of *F. casuarinae* strain Ccl3(pHKT1) transformant. After 2 months incubation, an average of 35 nodules per plant were collected and sterilized (41). After a week of incubation in LB medium, 6 nodules were crushed with a sterile mortar. The mixture was plated

TABLE 4 Oligonucleotides used in this study

Target	Direction	Sequence ^a	T _m (°C) ^b	Purpose	Reference or source
Tc ^r gene PCR	Fw	TAGGAAGCAGCCACAGTAGTA	55	Confirmation of conjugants by PCR on tetracycline gene	This study
	Rev	CTGTAGGCATAGGCTTGTTAT			
pHKT1 PCR	Fw	TAGGAAGCAGCCACAGTAGTA	55	Confirmation of conjugants by PCR on tetracycline gene (used on colonies isolated from nodules)	This study
	Rev	CGTTGAATCGGGATATGC			
Tc ^r gene-1,269bp PCR	Fw	TAGGAAGCAGCCACAGTAGTA	64	Confirmation of conjugant by PCR on the tetracycline gene + <i>mob</i> gene	This study
	Rev	CATCCACCGTGACGAAACC			
UTRase gene PCR	Fw	CGCATCGCGCCGTCCCGCA	66	Amplification on coding region of <i>Francci_3203</i> gene	This study
	Rev	GGTCAGCAGCCGCTCGGCG			
<i>rep</i> PCR	Fw	GGTCAGCAGAGACACTTT	54	Confirmation of conjugants by PCR of pBBR1MCS	This study
	Rev	ATTCTCCGTTTCGGTCAAG			
Tc ^r qPCR	Fw	CATTAGGAAGCAGCCACAGTAG	60	Copy no. per cell quantification for PTHK1 and pBBR1MCS-3	This study
	Rev	TGTTTCGGCGTGGGTATG			
<i>lacZ</i> qPCR	Fw	CAATTCCGCCCTATAGTGAGTCG	60	Copy no. per cell quantification for pBBR1MCS vector family	This study
	Rev	CTGGCGTAATAGCGAAGAGG			
Ccl3 UTRase gene qPCR	Fw	GTCCGCAGACGACATCAC	60	Copy no. of genome of Ccl3	This study
	Rev	GTACTCCGAGCAGGACGA			
Eul1c <i>copC-1</i> qPCR	Fw	TCTACCGCTGTCTACCGGAT	60	Copy no. of genome of Eul1c	42
	Rev	ACGAACCGGACCCCTACGTCA			
EUN1f <i>ropB</i> qPCR	Fw	CTGGAGAAACCTTCTTCAACC	60	Copy no. of genome of EUN1f	This study
	Rev	CTCCAGCTTCTGTGACCTT			
Ccl6-22605	Fw	ATATGGGCCCCAGACGAGTCGGGCGGACCA	60	Cloning of CCl6_22605 gene into pBBR1MCS-3	This study
	Rev	TCATACTAGTAAGGCTCGGCCCTCGGAGA			
Ccl6-22605 RT-PCR	Fw	GGCTCTGTTCGATCGCACCA	55	RT-PCR on 22605 gene	This study
	Rev	GCCACGTGCGACGCCCCAGT			

^aUnderlining indicates restriction sites used for cloning.

^bT_m, melting temperature.

on MPN medium–2% agar with an MPN–0.8% agar top layer. A month later, single *Frankia* colonies appeared on the plates. Liquid cultures were started, and the total gDNA was extracted from these colonies 2.5 months after plating.

Cloning and expression of a potential salt tolerance gene. The *Ccl6_RS22605* gene was amplified by PCR from *Frankia* sp. strain Ccl6 gDNA using a specific primer set (Table 4). The amplicon was purified using the QIAquick PCR purification kit protocol (Qiagen Sciences) and digested by the enzymes *SpeI* and *Apal* (New England BioLabs, MA), as was pBBR1MCS-3. The digestion mixtures were purified using the same QIAquick PCR purification kit and ligated. The construct was introduced by chemical transformation into competent NEB5 α competent *E. coli* cells (New England BioLabs). The construct was confirmed by PCR and DNA sequencing. The confirmed construction was introduced into the donor *E. coli* BW29427 by electroporation and mated into *Frankia* spp., as described above.

Endpoint RT-PCR. An endpoint RT-PCR was used to confirm the expression of the cloned gene (*Ccl6_RS22605*) in *F. casuarinae* strain Ccl3(pBBR1MCS-3-22605) transformants. RNA extraction was done using a Triton X-100 method (38, 39). DNA was removed by DNase I treatment (New England BioLabs, MA).

cDNA synthesis was performed using the GoScript reverse transcription system, according to the manufacturer's instructions (Promega Corporation, Madison, WI). RNA (300 ng) and primers (20 μ M) for the *Ccl6-22605* gene (Table 4) were denatured at 70°C for 5 min before the addition of 1 μ l of GoScript reverse transcriptase, 4 μ l GoScript 5 $\times$ reaction buffer, MgCl₂, 0.5 mM dinucleoside triphosphates (dNTPs), and nuclease-free water to a final volume of 20 μ l. The primers were annealed at 25°C for 5 min and extended at 42°C for 60 min. The cDNA (500 ng) was used as the template for PCR using forward and reverse gene-specific primers (Table 4). The thermal cycling conditions included initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation for 30 s (94°C), primer annealing for 30 s (58°C), and extension for 2.5 min (72°C), with a final elongation at 72°C for 7 min. PCR amplicons were visualized by agarose gel electrophoresis.

Salt sensitivity assay. The salt tolerance levels of *Frankia* strains were determined by measuring the total cellular protein content after growth under salt stress, as described previously (30). Briefly, cells were grown in propionate basal medium with 5 mM NH₄Cl containing different NaCl concentrations (0 mM, 200 mM, 400 mM, 600 mM, 800 mM, and 1,000 mM). The inoculum was adjusted to 40 μ g/ml total protein, and the 24-well plates were incubated at 28°C for 14 days. Growth was measured by total cellular protein content using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher). Relative growths under different salt treatments were evaluated by comparison to the initial growth without salt after 14 days. To compare the different strains, the growth curves were standardized at 0 mM NaCl to 300 μ g/ml protein.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00957-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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