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Evaluation of the 23S rRNA gene as target for *q*PCR based quantification of *Frankia* in soils

Suvidha Samant[a], Rudolf I. Amann[b], Dittmar Hahn[a,*]

[a] Texas State University, Department of Biology, 601 University Drive, San Marcos, TX 78666, USA
[b] Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany

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ABSTRACT

The 23S rRNA gene was evaluated as target for the development of Sybr Green-based quantitative PCR (*q*PCR) for the analysis of nitrogen-fixing members of the genus *Frankia* or subgroups of these in soil. A *q*PCR with a primer combination targeting all nitrogen-fixing frankiae (clusters 1, 2 and 3) resulted in numbers similar to those obtained with a previously developed *q*PCR using *nif*H gene sequences, both with respect to introduced and indigenous *Frankia* populations. Primer combinations more specifically targeting three subgroups of the *Alnus* host infection group (cluster 1) or members of the *Elaeagnus* host infection group (cluster 3) were specific for introduced strains of the target group, with numbers corresponding to those obtained by quantification of nitrogen-fixing frankiae with both the 23S rRNA and *nif*H genes as target. Method verification on indigenous *Frankia* populations in soils, i.e. in depth profiles from four sites at an *Alnus glutinosa* stand, revealed declining numbers in the depth profiles, with similar abundance of all nitrogen-fixing frankiae independent of 23S rRNA or *nif*H gene targets, and corresponding numbers of one group of frankiae of the *Alnus* host infection only, with no detections of frankiae representing the *Elaeagnus*, *Casuarina*, or a second subgroup of the *Alnus* host infection groups.

Introduction

The genus *Frankia* represents nitrogen- and non-nitrogen-fixing actinomycetes that form root nodules on some non-leguminous woody plants [4,10]. Root nodule formation is host plant-specific, with clusters 1, 2, and 3 representing nitrogen-fixing frankiae of the *Alnus*, *Dryas* and *Elaeagnus* host infection groups [2], and cluster 4 representing non-nitrogen-fixing strains [39]. Root nodules resemble highly homogeneous environments occupied by enrichments of generally one major *Frankia* population only which allowed for extensive studies on nodule-forming *Frankia* populations by both growth-dependent and -independent methods (e.g. [4,10,14]). Frankiae inhabit a second ecological niche, i.e. soils, with vastly contrasting conditions compared to root nodules. Soils are highly heterogeneous environments that can support a large microbial community with more than 10^9 cells g^{-1} soil [7,40] at a tremendous diversity [8,9,33,34], and frankiae generally present in small numbers only (approx. 10^4–10^6 cells g^{-1} soil) [11,22,26,31]. The complexity of soils and the low abundance of frankiae negatively affects population analyses by both growth-dependent and -independent methods, and consequently information on populations of *Frankia* in soil is much more limited than that on *Frankia* in root nodules (see [6] for review).

We recently reported on the development of a SYBR Green based *q*PCR method for the quantification of the genus *Frankia* in soils using *nif*H gene sequences as target [31]. This *q*PCR method allowed us to quantify frankiae in different mineral soils, with cell density estimates for frankiae of up to 10^6 cells [g soil {dry wt.}]$^{-1}$ depending on the soil [31]. The study, however, also revealed some problems using *nif*H genes as target for the quantification of frankiae. First, primers developed only detected frankiae of the *Alnus* and *Elaeagnus* host infection groups (clusters 1 and 3, respectively), but not frankiae of the *Dryas* host infection group (cluster 2) or non-nitrogen-fixing strains (cluster 4). Second, indications for *nif*H gene transfer were observed with up to 99.3% sequence similarity of *nif*H gene fragments of confirmed members of the genus *Frankia* to those of *Micromonospora lupini* that was isolated from root nodules of *Lupinus angustifolius* [35]. These issues prompted us to look for other genes that could be used as target in *q*PCR applications to quantify all members of the genus *Frankia* but also to distinguish clusters or specific subgroups within the genus. A promising target was found in an actinomycetes-specific insertion in Domain III of the 23S rRNA and its gene [28] that had been used as target for oligonucleotide probing before [29]. A small database of about 60 sequences of this insertion from different *Frankia* strains was available from previous taxonomic studies

* Corresponding author. Tel.: +1 512 245 3372; fax: +1 512 245 8713.
E-mail address: dh49@txstate.edu (D. Hahn).

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Table 1
Primer combinations targeting *nif*H or 23S rRNA gene sequences representing nitrogen-fixing members of the genus *Frankia* or subgroups within the genus.

Target group	Primer combination (5′ → 3′)	Anneal temperature (°C)	Fragment size (bp)	Reference
Target gene: *nif*H				
Nitrogen-fixing *Frankia* strains (clusters 1, and 3)	nifHf1 (5′ GGC AAG TCC ACC ACC CAG C) nifHr158 (5′ GAC GCA CTT GAT GCC CCA)	64	191	[1]
Target gene: 23S rRNA				
Nitrogen-fixing *Frankia* strains (clusters 1, 2, and 3)	23Fra1655f (5′ CTG GTA GTA GGC AAG CGA TGG) 23Fra1769r (5′ GGC TCG GCA TCA GGT CTC AG)	64	133	This study
Alnus host infection group (cluster 1)				
Subgroup I (ArI3)/*Casuarina*	23Ar/Cas1579f (5′ GTT GTG CTA ACC ATC TGA TCG GAT) 23Fra1769r (5′ GGC TCG GCA TCA GGT CTC AG)	66	209	This study
Subgroup II (Ag45/Mut15)	23Mut1555f (5′ TTG ATG CGT CCA TGC TGA GG) 23Fra1769r (5′ GGC TCG GCA TCA GGT CTC AG)	66	233	This study
Casuarina-infective strains	23Cas1610f (5′ TGTCTC TTC GGA GGT GTG TTC G) 23Fra1769r (5′ GGC TCG GCA TCA GGT CTC AG)	66	178	This study
Elaeagnus host infection group (cluster 3)	23EAN1579f (5′ GTT TGT GCT AAC CGT TCT GGT) 23Fra1769r (5′ GGC TCG GCA TCA GGT CTC AG)	64	209	This study

that demonstrated sufficient sequence variation to distinguish several subgroups within the genus *Frankia* [13,16]. This database was amended with published and unpublished sequences obtained from whole genome sequencing projects for *Frankia* [25].

The aim of this work was to evaluate the usefulness of this 23S rRNA insertion as target for *q*PCR applications aimed at the specific detection and quantification of *Frankia* in soils. The evaluation was set up as a comparative study with our previously developed SYBR Green based *q*PCR method using *nif*H gene sequences as target [31]. For this purpose, the 23S rRNA insertion was evaluated as target for the detection of all nitrogen-fixing frankiae only, excluding the non-nitrogen-fixing members of the genus, and within the nitrogen-fixing frankiae on the subgroup level using host plant group assignments of frankiae (i.e. frankiae of the *Elaeagnus* and *Alnus* host infection groups, with *Casuarina*-infective strains separated from the latter).

Materials and methods

Primer design and evaluation

Partial sequences of about 160 bp that represented nitrogen-fixing frankiae of the *Alnus* host infection group including *Casuarina*-infective strains (cluster 1, $n = 35$ and 5, respectively), the *Elaeagnus* host infection group (cluster 3, $n = 12$), and the *Dryas* host infection group (cluster 2, $n = 1$) and non-nitrogen-fixing strains (cluster 4, $n = 12$) [13,16] were aligned in Geneious 5.5.7 (Biomatters Ltd, Auckland, New Zealand). This alignment was further amended with sequences of *Frankia* strains CcI3, ACN14a [both cluster 1], EAN1pec, EUN1f [both cluster 3], EuI1c [non-nitrogen-fixing frankiae] and the uncultured endophyte of *Datisca glomerata* [cluster 2], and other actinomycetes (*Acidothermus*, *Geodermatophilus*, *Nakamurella*, *Streptomyces*, *Streptosporangium*, and *Kitasatospora*) retrieved from the SILVA rRNA database project (www.arb-silva.de, accessed 08/18/2011) [27]. The SILVA rRNA database also provided information on copy numbers of the 23S rRNA gene per genome, with two copies in strains CcI3, ACN14a and the endophyte in *D. glomerata*, and three copies in strains EAN1pec, EUN1f and EuI1c.

The alignment was screened for conserved as well as for group-specific sequences within the genus *Frankia*. Selected primers were checked for low potential of self- and hetero-dimer formation using OligoAnalyzer 3.1 (www.idtdna.com/analyzer/Applications/OligoAnalyzer) and for target specificity using TestPrime 1.0 [15] from the SILVA rRNA database project (accessed 12/06/2012).

qPCR design and evaluation

Annealing temperatures for all primer combinations were tested in *q*PCRs with DNA of representative *Frankia* strains of the *Elaeagnus* and *Alnus* host infection groups and of *Casuarina*-infective strains ($n = 54$) [12]. Non-nitrogen-fixing *Frankia* strain AgB1.9 (cluster 4) and several streptomycetes (*S. albireticuli* NRRL-B5493, *S. flavogriseus* NRRL-B1671, *S. erythrogriseus* NRRL-B3808 and *S. griseus* NRRL-B2682) retrieved from database searches with small mismatches in the forward and reverse primer, respectively, served as non-target organisms. *q*PCR quantification of frankiae was performed for each sample in triplicate in a total volume of 10 μl containing 5 μl of SsoADV SybrGreen Mix (BioRad, Hercules, CA), 0.2 μl of each primer (100 nM each) and 1 μl of template in an Eco Real-time PCR system (Illumina, San Diego, CA) using an initial denaturation at 95 °C for 5 min, and 40 cycles of denaturation at 95 °C, annealing at 64 or 66 °C depending on the primer combination (Table 1), and extension at 72 °C, each for 30 s, as in our previous study [31]. The amplification was followed by a melting curve analysis. Quantification was based on standard curves generated from purified PCR products of strains $EAN1_{pec}$, ArI3, CcI3 or Ag45/Mut15 depending on the primer combination. Amplicons were generated using the genus-specific primers, and concentrations measured with a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Copy numbers were calculated from concentrations (http://www.uri.edu/research/gsc/resources/cndna.html) and normalized after *q*PCR quantification with the primer combination targeting all nitrogen-fixing frankiae. Copy