Site-directed mutagenesis to deactivate two nitrogenase isozymes of *Kosakonia radicincitans* DSM16656^T

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Site-directed mutagenesis to deactivate two nitrogenase isozymes of *Kosakonia radicincitans* DSM16656^T

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Abstract

Biological nitrogen fixation (BNF) is considered one of the key plant growth-promoting (PGP) factors for diazotrophic organisms. Whether the Fe and the FeMo nitrogenases of *Kosakonia radicincitans* contribute to its PGP effect is yet to be proven. Hence, for the first time we conducted site-directed mutagenesis in *K. radicincitans* to knock out *anfH* and/or *nifH* as means to deactivate BNF in this strain. We used $^{15}\text{N}_2$ labeled air to trace BNF activities in ΔanfH , ΔnifH and $\Delta\text{anfH}\Delta\text{nifH}$ mutants. Assessing bacterial growth, nitrogen content and ^{15}N incorporation revealed that BNF is impaired in *K. radicincitans* DSM16656^T ΔnifH and $\Delta\text{anfH}\Delta\text{nifH}$. However, we detected no significant contribution of the Fe nitrogenase to biological dinitrogen assimilation under our pure bacterial culture experimental conditions. Such non-diazotrophic *K. radicincitans* DSM16656^T mutants represent excellent tools for investigating nitrogen nutrition in *K. radicincitans*-inoculated plants.

Keywords: BNF, nitrogenase, $^{15}\text{N}_2$ labeling, site-directed mutagenesis, PGPB

Introduction

Nitrogenase enzymes are essential for converting atmospheric nitrogen (N_2) into biologically available ammonia (NH_3); however, they are only present in some prokaryotes (Pereira e Silva et al. 2011). This process is termed biological nitrogen fixation (BNF) and is carried out by a group of prokaryotic organisms collectively called diazotrophs, which can be symbiotic, plant-associated, or free-living (Hartmann and Barnum 2010; Pereira e Silva et al. 2011; Seefeldt et al. 2009).

Kosakonia radicincitans DSM16656^T is a plant-associated Gram-negative facultative anaerobe, originally extracted from winter wheat of temperate regions (Brady et al. 2013; Kämpfer et al. 2005). It is associated with plant leaves, roots and even endophytic tissues (Remus et al. 2000). *K. radicincitans* is an emerging plant growth-promoting bacterium (PGPB) that boosts production yields in various cereal crops and non-leguminous vegetable crops upon inoculation in both greenhouses and fields (Berger et al. 2013; Berger et al. 2015; Krey et al. 2011; Remus et al. 2000; Ruppel et al. 2006). These positive growth improvements across different crops elevate its potential as a bio-inoculant in modern agriculture.

It is not yet known which of *K. radicincitans* genes contribute to the growth improvements observed in inoculated plants; however biochemical tests confirmed that *K. radicincitans* is diazotrophic (Ruppel and Merbach 1997; Scholz-Seidel and Ruppel 1992). This is also strengthened by genome publications revealing a nitrogenase-encoding *nifHDK* operon in *K. radicincitans* strains (Bergottini et al. 2015; Mohd Suhaimi et al. 2014; Witzel et al. 2012) (<https://www.ncbi.nlm.nih.gov/nuccore/CP015113.1>). Through sequence analysis of the published genomes, we discovered that *K. radicincitans* DSM16656^T, YD4, UMEnt01/12 and

GXGL-4A also have an *anfHDGK* operon encoding an additional nitrogenase. Whereas, *nifHDK* encodes a component of the iron-molybdenum (FeMo) nitrogenase, which is found in all diazotrophs, *anfHDGK* encodes for a component of the iron-only (Fe) nitrogenase found secondarily in only some diazotrophs (Dixon and Kahn 2004; Dos Santos et al. 2004; Oda et al. 2005; Raymond et al. 2004; Rubio and Ludden 2005; Teixeira et al. 2008). BNF has long been connected with plant growth improvements associated with *K. radicincitans*, particularly in non-leguminous crops (Bergottini et al. 2015; Brock et al. 2013; Kämpfer et al. 2005; Ruppel and Merbach 1995; Scholz-Seidel and Ruppel 1992; Witzel et al. 2012). However, a direct contribution of *K. radicincitans* nitrogenases to growth boosts observed in *K. radicincitans* inoculated plants is yet to be practically proven. Genetic manipulation tools that are crucial for investigating gene functions still have not been applied to the genus *Kosakonia*.

As an initial step towards investigating *K. radicincitans* nitrogenases, we set out to generate *K. radicincitans* nitrogenase mutants and assess their contribution to BNF activities in this strain. We applied “hit and run” site-directed mutagenesis (Clerico et al. 2007; Sharan et al. 2009) to knock out the dinitrogen reductase subunit encoding genes *anfH* and *nifH*, and deactivate the Fe and FeMo nitrogenases in *K. radicincitans* DSM16656^T. We used natural ¹⁵N abundance and ¹⁵N₂ labeled air experiments under N₂ fixing conditions to functionally prove the lack of BNF two of our knock-out mutant strains.

Materials and Methods

nifH and *anfH* DNA sequence analyses

Genome comparison was performed with sequences from the NCBI database and the BLAST® Command Line Applications BLAST+ (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/>). The

nitrogenase gene clusters were displayed according to the genomic location of the genes on the chromosome using BLAST ring image generator (BRIG) (<http://brig.sourceforge.net/>), followed by an in-house script for drawing linear clusters as scalable vector graphics (SVG). Operons within the clusters were predicted with the FGENESB bacterial operon and gene prediction tool offered by Softberry (www.softberry.com). AnfH and NifH protein sequence alignments were executed with Clustal Omega of the MegAlign Pro software version 11.2.1 (DNASTAR Inc, Madison, USA). To obtain central conserved protein domains, reserved position specific-BLAST (RPS-BLAST) was performed by uploading the AnfH/NifH protein sequences as query sequences into the NCBI's CD-Search tool (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) for conserved domains and protein classification and also in the MOTIF search tool on GenomeNet (<http://www.genome.jp/tools/motif/>).

Bacterial growth conditions and screening for antibiotic sensitivity

Under non-BNF growth conditions, the wild type strain *K. radicincitans* DSM16656^T and *Escherichia coli* S17-1 λ *pir* were cultured in Luria-Bertani (LB) agar or LB broth (Life technology, Darmstadt, Germany) at 30°C. Where antibiotic selection was required, 100 $\mu\text{g ml}^{-1}$ ampicillin, and 30 $\mu\text{g ml}^{-1}$ kanamycin were added to the media. For blue/white colony screening 119.5 mg l^{-1} IPTG and 0.08 $\mu\text{g ml}^{-1}$ X-gal were added to the medium, while 10% sucrose LB agar was used to cure the vector backbone after conjugation. Under BNF growth conditions, the wild type strain *K. radicincitans* DSM16656^T and mutants were cultured in a nitrogen-free medium (Rennie 1981). The nitrogen-free medium (MR) has the following composition per liter: 15 g microbiological agar, 5 g mannitol, 5 g sucrose, 0.8 g K_2HPO_4 , 0.2 g KH_2PO_4 , 0.1 NaCl, 0.2

g MgSO₄·2H₂O, 0.06 g CaCl₂·2H₂O, 100 mg yeast extract, 25 mg Na₂MoO₄·2H₂O, 10 mg FeSO₄·7H₂O, 28 mg Na₂Fe EDTA, 5 µg biotin, 10 µg p-aminobenzoic acid and 1 ml 50% sodium lactate solution.

To screen for antibiotic resistance, *K. radicincitans* DSM16656^T wild type strain was cultured overnight in LB liquid medium at 30°C on a rotary shaker at 120 rpm. Cultures were harvested under sterile conditions, suspended in 0.05 M NaCl (as a physiological buffer solution) and the optical density (OD₆₂₀) was adjusted to 0.2. LB agar plates were prepared with different antibiotics at concentrations stated in Supplementary Table S1¹, and 100 µl of *K. radicincitans* DSM16656^T inoculum was spread on antibiotic-containing LB agar plates or antibiotic-free LB agar plates as a positive control. Each treatment was replicated 6 times, and the experiment was repeated once. All plates were incubated overnight at 30°C. Antibiotic resistance was assessed by counting only plates with no bacterial colony as sensitive or no resistance, while any plate with at least a single colony was noted as resistant to the tested antibiotic.

Nucleic acid extraction

The genomic DNA of *K. radicincitans* DSM16656^T was extracted using Gene aid Presto™ Mini gDNA Bacteria Kit (Biozentrum, Hamburg, Germany), while vector DNA isolation was performed using Zyppy™ Plasmid Miniprep Kit (Zymo Research, Freiburg, Germany). PCR product gel bands were extracted and purified with a HiYield Gel/PCR DNA purification kit (Sued-laborbedarf GmbH, Gauting, Germany). RNA was extracted from pure bacteria cultures using InnusPEED bacteria/fungi RNA kit (Analytik Jena, Jena, Germany).

PCR and sequencing

115 To amplify the regions flanking *nifH* or *anfH*, a 50 µl PCR reaction comprising 38 µl water, 5 µl
116 Thermopol buffer (10x), 2 µl primer for (10 pM), 2 µl primer rev (10 pM), 1.5 µl dNTPs, 0.5 µl
117 (1 unit) Vent DNA polymerase (New England Biolabs, Hitchin, United Kingdom) and 1 µl DNA
118 template (60 ng) was prepared. PCR of the flanking regions was performed using the following
119 program: 94°C for 5 min, 25x (94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min), 72°C for 7 min.
120 To fuse the in-frame DNA-inserts, a crossover PCR was performed using 0.25 µl (1.25 units)
121 Taq DNA polymerase (Thermo scientific, Darmstadt, Germany) and 1 µl (70 ng µl⁻¹) *anfH* and
122 *nifH* flanking regions PCR products as DNA templates. The following program was used: 94°C
123 for 5 min, 30x (94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min), and 72°C for 10 min. All PCRs
124 were performed in a Peqstar 96 universal thermocycler (PEQLAB, Erlangen, Germany). PCR
125 products were visualized on a 1% agarose gel calibrated with a FastRuler middle range DNA
126 ladder (Thermo Scientific, Darmstadt, Germany). DNA samples were sequenced at Eurofins
127 Genomics (Ebersberg, Germany) and sequence data were analyzed with DNASTar SeqMan pro
128 software version 12.1.1 (DNASTar Inc, Madison, USA). Primers used in this study are shown in
129 Supplementary Table S2¹.

130 **Cloning of deletion vectors**

131 To delete *anfH*, 540 bp (F1) upstream *anfH* was PCR-amplified using primer pair F1 rvs fusion
132 and F1 fwd BamHI, while the 513 bp (F2) downstream *anfH* was PCR amplified with primer
133 pair F2 fwd fusion and F2 rvs BamHI. The PCR-amplified homology regions F1 and F2 were
134 linked to a 1053 bp in-frame DNA-insert (F12) in a crossover PCR with primer F1 fwd BamHI
135 and F2 rvs BamHI (Fig. 1A). The resulting F12 was first PCR purified and cloned into the
136 pGEM-T easy vector system I (Promega, Mannheim, Germany) and transformed into chemically

competent *E. coli* DH5 α cells. White ampicillin resistant colonies were screened for the in-frame DNA-insert with the Invitrogen CloneChecker (Life Technologies, Darmstadt, Germany) using BamHI restriction. F12 in-frame DNA-insert were excised by BamHI restriction digest and cloned into the BamHI linearized suicide vector pNPTS138-R6KT (Lassak et al. 2010) to form a hybrid pNPTS138-R6KT-F12. The hybrid vector pNPTS138-R6KT-F12 was transformed into EC100D *pir* cells for easy screening of the correct recombinants through blue and white screening. Similarly, to delete *nifH*, 467 bp upstream (F3) *nifH* was amplified with F3 fwd BamHI and F3 rvs fusion, while 501 bp downstream (F4) was amplified with F4 fwd fusion and F4 rvs BamHI. F3 and F4 were fused to a 968 bp F34 in-frame DNA-insert in a crossover PCR with primer pairs F3 fwd BamHI and F4 rvse BamHI. Correct integration of F12 and F34 into pNPTS138-R6KT-F12 and -F34, was verified by sequencing at Eurofins Genomics (Ebersberg, Germany). The *anfH* deletion vector pNPTS138-R6KT-F12 and the *nifH* deletion vector pNPTS138-R6KT-F34 were used to transform chemically competent conjugation donor *E. coli* 17-1 λ *pir* cells. Bacterial strains and vectors used in this study are shown in Table 1. A detailed protocol on conducting site-directed mutagenesis in *K. radicincitans* is provided in Supplementary Files S2¹.

Conjugation and mutant screening

For conjugation, the donor strain *E. coli* 171 *pir*-pNPTS138-R6KT-F12 or -F34 and the recipient strain *K. radicincitans* DSM16656^T were grown overnight at 30°C in a rotary shaker at 120 rpm. The cultures were harvested under sterile conditions by centrifuging at 3000 rpm for 15 min, rinsed three times with NaCl (0.05 M) and suspended in 200 μ l NaCl (0.05 M). 100 μ l of *E. coli* 171 *pir* carrying the hybrid vector was added to a 100 μ l of the recipient strain *K. radicincitans*

DSM16656^T in a centrifuge tube and mixed by pipetting. The mixture was incubated at room temperature for 20 min before it was pipetted onto LB agar plates and incubated overnight at 30°C in a stationary incubator. Colonies were suspended in 2 ml LB broth, plated on LB/ampicillin/kanamycin plates in serial dilutions 10¹, 10², 10³ and incubated overnight. Ampicillin was used to repress *E. coli* 171 *pir*-pNPTS138-R6KT-F12/F34, whereas resistance against kanamycin was used to identify the vector transfer and the first recombination events into *K. radicincitans* DSM16656^T.

Colony PCR of the post conjugation kanamycin resistant colonies was performed with primer pair *anfH* fwd/F2 rvs BamHI targeting a 1070 bp in-frame DNA-insert (F1 integration 5') or a 1898 bp wild type fragment (F2 integration 3'). Colonies that gave the wild type band from the first screening were further screened with primer pair F1 fwd BamHI/*anfH* rev for a 1070 bp in-frame DNA-insert (Fig. 1B). Integration of pNPTS138-R6KT- F34 (*nifH*) was performed and analyzed in the same manner. To create $\Delta anfH \Delta nifH$, the vector pNPTS138-R6KT- F34 (*nifH*) was used to delete *nifH* in the $\Delta anfH$ strain.

Correct post conjugation recombinants were plated on 10% sucrose LB agar plates and incubated overnight at 30°C to cure the vector. 10% sucrose colonies were again streaked on new 10% sucrose LB agar plates and in parallel also on kanamycin LB agar plates to identify colonies that had lost kanamycin resistance. At this stage loss of kanamycin resistance identified the second recombination event and the positive loss of the pNPTS138-R6KT (Fig. 1C). Therefore, only colonies that grew on sucrose but failed to grow on kanamycin were further screened for the desired mutants by colony PCR. Gene knockout was verified by sequencing PCR products and by lack of gene expression analyzed by qPCR (data not shown).

The growth of the resultant nitrogenase mutants was investigated in LB broth to determine whether the absence of *anfH* and *nifH* affected the mutants' growth under non-BNF conditions. *K. radicincitans* DSM16656^T wild type, $\Delta anfH$, $\Delta nifH$ and $\Delta anfH\Delta nifH$ OD₍₆₂₀₎ was measured by an automated Anthos HTIII spectrophotometer (Anthos Mikrosysteme GmbH, Germany) every hour for 48 hours and the growth rates were calculated per hour during the mid-exponential growth phase.

¹⁵N₂ labeling

¹⁵N₂ was produced by the thermal disintegration of ¹⁵N labeled NO₄NO₂. For this, 69 g of NaNO₂ were dissolved in 100 ml H₂O and heated to 70°C in a round-bottom flask with an inlet for salt solutions and an outlet for resulting N₂. 33 g of non-labeled (NH₄)₂SO₄ and 33 g of ¹⁵N labeled (NH₄)₂SO₄ (95 atom %) were dissolved in 100 ml H₂O and added to a NaNO₂ solution. Both salts dissociated in water and reacted to NH₄NO₂, which disintegrates to N₂ and 2H₂O at 70°C. This produced about 20 L of ¹⁵N labeled ¹⁵N₂, which was collected in an air-tight bag until the gas production stopped.

K. radicincitans DSM16656^T wild-type, $\Delta anfH$, $\Delta nifH$, and $\Delta nifH\Delta anfH$ were cultured in a 35 ml semi-solid nitrogen-free medium (Rennie 1981) in 100 ml Erlenmeyer flasks (EMFs). In addition, medium inoculated with 0.05 M NaCl solution was used as negative control. The experiment comprised 60 EMFs, 12 EMFs per treatment. The EMFs were placed randomly in 6 air-tight plastic bags (10 EMFs per bag). Half of these bags were filled with 8.23 l room air (non-labeled air) and the other 3 were filled with 6.5 l of ¹⁵N labeled ¹⁵N₂, plus 1.73 l O₂ to create an atmosphere with a composition similar air to room air. All EMFs were incubated at room temperature for seven days.

¹⁵N abundance measurements in cell cultures

During sampling, the cultures were mixed thoroughly for even cell distribution, transferred into centrifuge tubes and stored at -28°C. They were then moved to -70°C for 24 hours, before they were lyophilized with an ALPHA 2-4 LSC freeze dryer (Martin Christ GmbH, Germany). For nitrogen analysis, 30 to 40 mg of lyophilized sample was weighed into a tin capsule and combusted in a VARIO elemental analyzer (Elementar Analysensysteme GmbH, Germany) at 950°C under O₂ addition and continuous flow of helium (He). Nitrous gases released by combustion of samples were reduced to N₂ at 500°C using elemental copper. The resultant N₂ was transferred into a NOI-6 PC emission spectrometer (Fischer Analysen Instrumente GmbH, Germany) to measure ¹⁵N abundance.

Statistical analyses

All data were tested for normal distribution using the Shapiro-Wilk test, and homogeneity of the variances using the Levene test. None of the data sets fulfilled parametric test assumptions. Hence, the non-parametric Mann Whitney U test was used to test for significant differences between the wild type strain and each of the mutant strains in each experiment, using Mathematica, version 9 (Wolfram Research, Harborough, United Kingdom) and SPSS 22 (IBM, Ehningen, Germany) software.

Results

The *anf* and *nif* gene clusters of *K. radicincitans* DSM16656^T

Analyses of the *K. radicincitans* DSM16656^T genome revealed that this strain has two nitrogenase gene clusters at two different chromosome loci. The *anf* nitrogenase gene cluster comprises 7 genes *anfHDGKnimAOA* divided into two putative transcriptional units defined as

operons. The *nif* gene cluster comprises 19 genes *nifJHDKTYENXUSVWMFLABQ*, which are divided into 7 putative transcriptional units defined as operons. On both clusters, the genes are divided into four functional groups: synthesis of the nitrogenase, synthesis of the nitrogenase cofactor, electron transport to the nitrogenase and regulation of the nitrogenase functions. Although the *anf* cluster has fewer genes than the *nif* cluster, the *anf* genes represent all the functional groups encoded by the genes on the *nif* cluster. On the *anf* cluster the synthesis of the nitrogenase enzyme complex is encoded by *anfHDGK*, while on the *nif* cluster the synthesis of the nitrogenase enzyme complex is encoded by *nifHDK* (Fig. 2A).

BLAST analysis of AnfH and NifH from *K. radicincitans* DSM16656^T was performed against AnfH and NifH of various strains to investigate protein conservation. Protein sequence comparison showed that both AnfH and NifH are 100% identical within investigated strains of the genus *Kosakonia* (*K. radicincitans* DSM16656^T, GXGL-4A, YD4, UMEnt01/12 and also in *K. oryzae* Ola51). Inter-genera assessments revealed protein sequence variations in AnfH and NifH between *Kosakonia* and the investigated non-*Kosakonia* diazotrophs. *Kosakonia* AnfH sequences vary by 11% from *A. vinelandii*, 13 % from *R. capsulatus* and *R. palustris* and by 21% from *P. durus*. *Kosakonia* NifH protein sequences vary by 12% from *Azotobacter vinelandii*, 29% from *Rhodobacteria capsulatus*, 28% from *Paenibacillus durus* and by 31% from *Rhodopseudomonas palustris*.

To identify conserved protein domains, AnfH and NifH were analyzed using the NCBI central conserved protein domain database. Both AnfH and NifH proteins contain the three conserved protein domains documented in the central protein domain for nitrogenases. Both AnfH and NifH have the Walker A motif, Switch region I and II. The Walker A motif and Switch region I

are involved in nucleotide binding, while the Switch region II holds the Fe₄S binding site. All three conserved domains are located towards the N-terminus of both proteins. The MOTIF blast search revealed two conserved subdomains of the NACHT (NTPases) and major histocompatibility complex (MHC) domains. AnfH analyses show that the AnfH C-terminus overlaps an MHC subdomain, while the NifH N-terminal end overlaps a NACHT NTPases subdomain.

Antibiotic resistance and mutant construction

To find a suitable gene deletion system, screening for antibiotic resistance revealed that *K. radicincitans* DSM16656^T is resistant to a wide range of antibiotics but sensitive to spectinomycin, tetracycline, and kanamycin (Supplementary Table S1¹). The Fe nitrogenase was deactivated by knocking-out the dinitrogenase reductase-encoding gene *anfH* as described in methods and depicted in Fig. 1A-C, Fig. 2B and Fig. 3. FeMo nitrogenase was deactivated by knocking out *nifH*.

¹⁵N₂ fixation

To check whether the absence of the nitrogenase genes affected non-BNF related cellular functions, we investigated the growth of the wild type and mutants under non-BNF growth conditions with adequate mineral nitrogen sources (LB broth). Under these growth conditions, all strains showed the same growth behavior: wild type (0.057±0.002), Δ *anfH* (0.057±0.002), Δ *nifH* (0.057±0.002) and Δ *anfH* Δ *nifH* (0.056±0.006).

To study BNF activities, nitrogen incorporation into cell cultures was quantified in cultures grown in the MR medium after 7 days of incubation under both non-labeled and ¹⁵N₂ labeled air. Nitrogen concentrations in the wild type were significantly higher (p< 0.05) than in the Δ *nifH*

and $\Delta anfH\Delta nifH$, but remained unaffected in the $\Delta anfH$ cultures (Fig. 4). Additionally, nitrogen incorporation was also quantified in cultures cultivated in MR medium with molybdenum content reduced to $17 \mu\text{g l}^{-1}$ compared to the original MR medium containing $9460 \mu\text{g l}^{-1}$. The results obtained from the reduced molybdenum medium did not differ from those observed in the MR medium (data not shown) suggesting that low levels of molybdenum did not affect Fe nitrogenase or FeMo nitrogenase activities. Unfortunately, attempts to grow the bacteria in a molybdenum-free medium were unsuccessful, making it impossible to measure ^{15}N changes. To trace N_2 assimilation into the bacterial cultures due to BNF, we measured ^{15}N abundance in cultures grown under $^{15}\text{N}_2$ labeled air after 7 days of incubation. These experiments verified the lack of active BNF in *K. radicincitans* DSM16656^T $\Delta nifH$ and $\Delta anfH\Delta nifH$ strains; where *nifH* was knocked out, ^{15}N abundance in these cultures did not differ from the negative control without bacteria inoculation (Fig. 5A). In addition, natural ^{15}N abundance was quantified in the experiment conducted with non-labeled air and incubated for 7 days, which further supported the lack of active BNF in the two cultures without *nifH*. Here, due to active nitrogenase enzyme discrimination against the heavier isotope (^{15}N), wild type and $\Delta anfH$ cultures had significantly lower ^{15}N abundance compared to the $\Delta nifH$, $\Delta anfH\Delta nifH$ and negative control, which cannot fix N_2 (Fig. 5B). Thus BNF activity was lost in *K. radicincitans* DSM16656^T $\Delta nifH$ and $\Delta anfH\Delta nifH$ where the FeMo nitrogenase (*nif*) was deactivated, whereas BNF in the $\Delta anfH$ remained equivalent to the wild type.

Discussion

We generated site-directed mutagenesis to specifically knock out the dinitrogenase reductase-encoding genes *anfH* or *nifH* to deactivate the Fe and FeMo nitrogenases in *K. radicincitans*

291 DSM16656^T. Significant reduction in nitrogen concentration and ¹⁵N abundance (in the ¹⁵N₂
292 labeled air experiment) in bacterial cultures under N₂ fixing conditions showed the loss of active
293 BNF in the $\Delta nifH$ and $\Delta anfH\Delta nifH$. This is also verified by the natural ¹⁵N abundance in the non-
294 labeled air experiment.

295 Since this is the first time that site-directed mutagenesis has been conducted in *K. radicincitans*
296 DSM16656^T, antibiotic resistance data provide insights into appropriate antibiotic vector
297 markers. Initially, we adopted the helper vector pSIM 19 harboring kanamycin resistance genes
298 for lambda Red recombineering (Datta et al. 2006), a technique often used in *E. coli* and
299 *Salmonella* (Datsenko and Wanner 2000; Datta et al. 2006; Hu et al. 2013; Lesic and Rahme
300 2008; Sharan et al. 2009); however this did not work for *K. radicincitans* DSM16656^T. In our
301 study, expressing the helper vector in *K. radicincitans* DSM16656^T resulted in tiny single
302 colonies after an overnight incubation that could not grow any further into bigger colonies,
303 probably due to the toxicity of the lambda proteins. Lambda proteins toxicity has been reported
304 before in *Pantoea ananatis* (Katashkina et al. 2009). An in-frame deletion approach using the
305 suicide vector pNPTS138-R6KT (Lassak et al. 2010) was used to delete *anfH* and *nifH* in *K.*
306 *radicincitans* DSM16656^T.

307 Genome data of *K. radicincitans* DSM16656^T enabled graphical illustration of the nitrogenase
308 clusters as well as domain identification. The presence of conserved nitrogenase domains
309 suggests that both *anfH* and *nifH* of *K. radicincitans* DSM16656^T encode dinitrogenase
310 reductases that functionally resemble the previously studied nitrogenases of *Azotobacter*
311 *vinelandii*, *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *Clostridium pasteurianum* (Dos
312 Santos et al. 2004; Oldroyd and Dixon 2014). The two nitrogenases function in the same way,

except that the molybdenum element on the *nifHDK* protein is replaced by iron in *anfHDK* (Dos Santos et al. 2004). The *nif* operon in *K. radicincitans* DSM16656^T harbors many regulatory genes that are absent on the *anf* operon (Fig. 2), as reported in other studied diazotrophs such as *Rhodopseudomonas palustris* (Oda et al. 2005).

Natural ¹⁵N abundance measurements are often variable, not only due to natural ¹⁵N abundance variation in different environments (Hauck 1973) but also because biological systems and processes discriminate against heavier isotopes in the presence of lighter ones (Carlsson et al. 2006; Unkovich 2013). Consequently, in experiments conducted in natural air, active BNF results in higher ¹⁴N and lower ¹⁵N abundance in cell cultures. Therefore, both ¹⁵N₂ labeled and non-labeled air experiments were conducted in this study to assess active BNF in *K. radicincitans* DSM16656^T and draw reliable conclusions.

Both the nitrogen concentration and ¹⁵N quantification findings show that the FeMo nitrogenase plays an important role in BNF in *K. radicincitans* under our experimental conditions: its deactivation in the $\Delta nifH$ and $\Delta anfH\Delta nifH$ lead to a complete loss of active BNF in these strains. Similar findings are reported in *Azotobacter vinelandii*, and *Rhodobacter capsulatus*, where the FeMo nitrogenase encoded by the *nif* operon is recorded as the main nitrogenase dominating BNF over the Fe nitrogenase (Dixon and Kahn 2004; Oda et al. 2005). It is worth mentioning the insignificant contribution of the Fe nitrogenase evident in the $\Delta anfH$ cultures in our experiments. Fe nitrogenase is sensitive to molybdenum and functional only in environments free of molybdenum traces (Dixon and Kahn 2004; Teixeira et al. 2008; Thiel and Pratte 2013; Wang et al. 1993). Perhaps the reduced molybdenum level used in this study was still adequate to inhibit

334 Fe nitrogenase functions. Future research should thus inspect Fe and FeMo nitrogenases in *K.*
 335 *radicincitans* inoculated plants.

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 357 nov., respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into *Kosakonia* gen.
 358 nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae*
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459 **Fig. 1** A scheme of the series of events leading to *anfH* deletion in *K. radicincitans* DSM16656^T.
460 **(A)** Construction of the in-frame DNA-insert F12 and the insertion of F12 into pNPTS138-
461 R6KT. **(B)** Post conjugation and integration of the vector pNPTS138-R6KT-F12 into the *K.*
462 *radicincitans* DSM16656^T chromosome. **(C)** The final recombination event that cured the vector
463 backbone and resulted in the desired mutants.

464 **Fig. 2** A scheme of the nitrogenase-encoding *nifHDK* and *anfHDKGK* operons and deletions
465 generated. **(A)** The location of the atmospheric nitrogen fixing gene clusters *anf* and *nif* on the *K.*
466 *radicincitans* DSM16656^T genome map. The genetic arrangement of the genes within each
467 cluster is accompanied by pattern codes indicating the functions of each gene. **(B)** Mutant strains
468 created in this study.

469 **Fig. 3** Colony PCR screening and visualization of the PCR products amplified using Δ *anfH* and
470 Δ *nifH* primer pairs anfHrev/anfHfwd and nifHfwd/nifHrev on a 1% Agarose gel, next to the
471 FastRuler ladder DNA ladder (L). Wild type *K. radicincitans* DSM16656^T wild type is included
472 as a positive control.

473 **Fig. 4** Nitrogen assimilation into lyophilized MR semi-solid media (control) and cell cultures
474 (MR semi-solid media with bacterial cells) through BNF under ¹⁵N₂ labeled and non-labeled air.
475 Error bars represent standard deviations of the means. Significant differences between the wild
476 type and either of the treatments are shown by asterisks in ¹⁵N₂ labeled air and plus signs in non-
477 labeled air at 0.01 significance level (Mann-Whitney U test, n=6).

478 **Fig. 5** ¹⁵N assimilation into lyophilized MR semi-solid media (control) and cell cultures (MR
479 semi-solid media with bacterial cells) through BNF in **(A)** cultures incubated under ¹⁵N₂ labeled
480 air and **(B)** cultures incubated under non-labeled air. Error bars represent standard deviations of

481 the means. Asterisks show significant differences between the wild type and either of the
482 treatments at 0.01 significance level (Mann-Whitney U test, n=6).

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Table 1 Strains and vectors used in this study

Strains/Vectors	Genotypes	Source
Bacterial strains		
<i>K. radicincitans</i> DSM16656 ^T (D _{5/23})	<i>K. radicincitans</i> DSM16656 ^T wild type	Kaempfer et al, 2005
<i>E.coli</i> EC100D <i>pir</i>	<i>F</i> <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ - <i>rpsL</i> (<i>Str</i> ^R) <i>nupG</i> <i>pir</i> ⁺ (<i>DHFR</i>)	Epicente
<i>E. coli</i> S17-1 λ <i>pir</i>	<i>fhuA2</i> <i>lac</i> (<i>del</i>)U169 <i>phoA</i> <i>glnV44</i> Φ 80' <i>lacZ</i> (<i>del</i>)M15 <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i>	Hamburg University, Biozentrum
<i>E. coli</i> DH5 α	<i>fhuA2</i> <i>lac</i> (<i>del</i>)U169 <i>phoA</i> <i>glnV44</i> Φ 80' <i>lacZ</i> (<i>del</i>)M15 <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i>	Bioline
Kr1	<i>K. radicincitans</i> DSM16656 ^T Δ <i>anfH</i>	This study
Kr2	<i>K. radicincitans</i> DSM16656 ^T Δ <i>nifH</i>	This study
Kr3	<i>K. radicincitans</i> DSM16656 ^T Δ <i>anfH</i> Δ <i>nifH</i>	This study
Vectors		
pNPTS138-R6KT	<i>mobRP4</i> ⁺ <i>ori</i> -R6K <i>sacB</i> ; suicide plasmid for in-frame deletions; Km ^r	Lassak et al, 2010
pGEM-T easy system I	ori-F1, <i>lacZ</i> Amp ^r ; TA cloning vector	Promega
pGEM-T easy system I-F12	<i>anfH</i> in-frame deletion construct in pGEM-T easy vector system I	This study
pGEM-T easy system I-F34	<i>nifH</i> in-frame deletion construct in pGEM-T easy vector system I	This study
pNPTS138-R6KT- F12	<i>anfH</i> in-frame deletion construct in pNPTS138-R6KT	This study
pNPTS138-R6KT- F34	<i>nifH</i> in-frame deletion construct in pNPTS138-R6KT	This study

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Table S1. Antibiotic resistance of *K. radicincitans* DSM16656^T. “Yes” stands for resistance while “no” stands for no resistance against the tested antibiotic.

Antibiotic	Concentration µg ml ⁻¹	Bacteria growth
Ampicillin	100	yes
Rif-Ampicillin	50	yes
Lincomycin	25	yes
Erythromycin	50	yes
Chloramphenicol	50	yes
Blasticidin	100	yes
Hygromycin	100	yes
Kanamycin	30	no
Spectinomycin	50	no
Tetracycline	15	no
Gentamycin	15	no

Table S2. Primers used in this study.

Primer names	Sequences (5 to 3)	Base pairs	Functions
F1 fwd BamHI ^{ad}	<u>ggatcc</u> GCTGGCGGATCTGCCGGTA	25	F1 amplification
F1 rvs fusion	GTAAAATAACCGGACGAAGATATTTCCTTGATG	35	
F2 fwd fusion	CATCAAGAGGAAATATCTTCGTCCGGTTATTTTAC	35	F2 amplification
F2 rvs BamHI ^{ac}	<u>ggatcc</u> CATCCATCACTTCCTGCGCAATG	29	
F3 fwd BamHI ^{bf}	<u>ggatcc</u> GGCGACATCAGTAAAGGCATACG	29	F3 amplification
F3 rvs fusion	CCTGTTGGCGGGCGTGCAGGTCATGGTGTTTC TCC	35	
F4 fwd fusion	GGAGAAACACCATGACCTGCACGCCCGCCAA CAGG	35	F4 amplification
F4 rvs BamHI ^{be}	<u>ggatcc</u> CAGGCCGACCGGGGCATTC	24	
AnfH fwd ^c	GCACAGCGATTTGCTGGCACC	21	<i>anfH</i> verification mutant
AnfH rev ^d	CGAAAATATCCACTTCCGGACGC	23	
NifH fwd ^e	GGGGGTAATGGGGTAAATCGC	21	<i>nifH</i> verification mutant
NifH rev ^f	CGGCTTCAATGTCGTCGCC	19	
Erad 2680vor	GTGGACGGTGAAAAAGAGTTTCTCG	25	qPCR amplification <i>anfH</i>
Erad 2680rev	GGTGACGGTTTTCTTGTTGAATTC	24	
Erad 6038vor	GCCAACGCCCATCACCATGGACG	23	qPCR amplification <i>nifH</i>
Erad 6038rev	CGTTTTCTTCTGCGGCGGTTTTTACC	25	
Erad 4235vor	CGCAGGGCTTTTCCGTGGTG	20	qPCR <i>K. radicincitans</i> reference gene 1
Erad 4235rev	GCCGCCGTCGTCTGCATCATAGT	23	
Erad 4483vor	ATACCCCGTCGCCAGATAAAGT	22	qPCR <i>K. radicincitans</i> reference gene 2
Erad 4483rev	TCACCGCGCCGAAGACGATA	20	

Note: The underlined sequences represent the inserted restriction sites. Superscript letters on some primer names shows different primer combination: ^a primer pairs for the crossover PCR to fuse F1 and F2; ^b crossover PCR that fused F3 and F4; ^{cd} *anfH* post conjugation colony screening, ^{ef} *nifH* post conjugation colony screening.

Conducting site-directed mutagenesis in *K. radicincitans*

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Summary

This protocol describes step-by-step procedures used to construct $\Delta anfH$ and $\Delta nifH$ of *K. radicincitans*. The protocol is adapted from (Clerico et al. 2007) and modifications were added to optimize it for *K. radicincitans*.

MATERIALS

Enzymes

BamHI-HF (New England Biolabs, Hitchin, UK)

Taq DNA polymerase (Thermo Fischer Scientific, Darmstadt, Germany)

Vent DNA polymerase (New England Biolabs, Hitchin, UK)

Strains and vectors

E. coli DH5 α (Bioline, London, UK)

EC100DTM *pir*-116 *E. coli* (Epicenter, Madison, USA)

PGEM-T EASY vector system I (Promega GmbH, Mannheim, Germany)

pNPTS138-R6KT (Lassak et al. 2010)

Transformax EC100DTM *pir*-116 *E. coli* (Epicenter, Madison, USA)

K. radicincitans (IGZ, Großbeeren, Germany)

E. coli S17-1 λ *pir* (Biozentrum, Hamburg, Germany)

Kits

CloneChecker (Thermo Fischer Scientific, Darmstadt, Germany)

Epicentre Fast-link DNA Ligation kit (Biozym, Oldendorf, Germany)

Gene aid Presto™ Mini gDNA Bacteria kit (Biozentrum, Hamburg, Germany)

HiYield Gel/PCR DNA purification kit (Sued-laborbedarf GmbH, Gauting, Germany)

InnuSPEED bacteria/fungi RNA kit (Analytik Jena, Jena, Germany)

Zyppy™ Plasmid Miniprep kit (Zymo Research, Freiburg, Germany)

Media

Luria-Bertani (LB) broth/agar (Life Technology, Darmstadt, Germany)

LB-sucrose (10%) agar

SOC medium:

Add 10 ml of solution B to 965 ml solution A to form solution AB.

Adjust the pH to 7 with NaOH.

In separate bottles, autoclave solution AB and Solution C then cool to room temperature.

Filter-sterilize solution D.

Add 5 ml of Solution C and 20 ml of solution D to solution AB.

Solution A:

20 g tryptone

5 g yeast extract

0.5 g NaCl

Dissolve in 965 ml distilled water.

Solution B: 250 mM KCl

Solution C: 2 M MgCl₂

Solution D: 20 mM glucose

Reagents

Ampicillin, final concentration in the media: 100 µg ml⁻¹.

Kanamycin, final concentration in the media: 30 µg ml⁻¹.

IPTG, final concentration in the media: 119.5 mg l⁻¹.

X gal, final concentration in the media: 0.08 µg ml⁻¹.

PROCEDURES

Part 1 Assembling the in-frame DNA-insert

a) Overnight culture

- i. Use *K. radicincitans* -80 glycerol stocks to streak single colonies on LB agar plates and incubate at 30°C overnight.
- ii. Pick a single colony and inoculate 50ml LB broth in 250 ml Erlenmeyer flask (EMF).
- iii. Incubate at 30°C overnight.

b) PCR of the harboring flanks (recommended size not less than 500 bp)

- i. Harvest the cells from an overnight culture and extract genomic DNA with the Gene aid Presto™ Mini gDNA Bacteria kit.
- ii. In two separate PCR reactions, amplify the region downstream and upstream the target gene using the appropriate primers, with the following PCR reaction mix: 50 µl reaction comprising 38 µl water, 5 µl Thermopol buffer (10x), 2µl primers for (10 pM), 2µl primer rev (10 pM), 1.5 µl dNTPs, 0.5 Vent DNA polymerase and 1 µl (60 ng) DNA template and the following program: step 1: hold at 94°C for 5 min, step 2: (25 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min), and step 3: (72°C for 7 min).
- iii. Visualize the PCR products on an agarose gel.

Note: If the PCR only produce the desired product, proceed to the cross over PCR; however if more products are produced the desired product must be gel-purified HiYield Gel/PCR DNA purification kit before used in the crossover PCR.

The use of proof reading polymerase in this step is important for a crossover PCR.

c) Fusing the flanks via crossover PCR

In a crossover PCR, the primers are designed with complementary 5' ends to enable fusion of the two flanks.

- i. Prepare a 50 µl crossover PCR reaction mix: comprising 0.25 µl (1.25 units) Taq DNA polymerase, 37.75 µl water, 5 µl Thermopol buffer (10x), 2 µl primers for (3 pM dilutions), 2 µl primer rev (3 pM, 2 µl dNTPs, 0.5 µl (70 ng) upstream flank and 0.5µl

(70 ng) downstream flank and run with the following program: step 1: 94°C for 5 min, step 2: 30 cycles (94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min), and step 3: 72°C for 10 min.

- ii. Purify the crossover PCR products (in-frame DNA-insert), with HiYield Gel/PCR DNA purification kit.

Part 2 Cloning the in-frame DNA-insert and strain transformation

a) Ligation of the in-frame DNA-insert and the PGEM-T EASY vector system I

- i. Ligate the PGEM-T EASY vector system I and the in-frame DNA-insert.
- ii. Use the ligation mixture to transform chemically competent *E. coli* DH5 α .
- iii. Inoculate 100 μ l of the transformation mixture onto LB/ampicillin/IPGT/X-gal agar plates.
- iv. Incubate at 30°C overnight.
- v. Screen the white colonies with the CloneChecker kit for the desired recombinants.
- vi. Culture the correct recombinants in LB broth/ampicillin.
- vii. Incubate at 30°C in a rotary shaker at 150 rpm overnight.
- viii. Harvest the cells and extract the vector pGEM-T easy vector system I-in-frame DNA-insert with the Zyppy™ Plasmid Miniprep kit.
- ix. Extract the recombinant DNA from pGEM-T easy vector system I-in-frame DNA-insert using BamHI-HF.
- i. Gel-purify the in-frame DNA-insert with a HiYield Gel/PCR DNA purification kit.

b) Ligation of the in-frame DNA-insert and the suicide vector pNPTS138-R6KT

- i. Linearize the vector pNPTS138-R6KT, with BamHI-HF.
- ii. Gel-purify the linearized vector with a HiYield Gel/PCR DNA purification kit.
- iii. Ligate the linearized pNPTS138-R6KT and the in-frame DNA-insert using the Epicenter Fast-link DNA Ligation kit.
- iv. De-activate the ligation reaction mixture at 65°C for 10 min then cool it down to room temperature.
- v. Use the ligation mixture to transform chemically competent EC100D™ *pir*-116 *E. coli*.

- vi. Inoculate 100 μ l of the transformation mixture onto LB/kanamycin/IPGT/X-gal agar plates.
 - vii. Incubate at 30°C overnight.
 - viii. Screen the white colonies with the CloneChecker kit for the positive recombinants.
 - ix. Culture the correct recombinants in LB broth/kanamycin.
 - x. Incubate at 30°C in a rotary shaker at 150 rpm overnight.
- c) Transformation of the conjugation donor *E. coli* S17-1 λ *pir*
- i. Harvest the cell pellet and extract the vector pNPTS138-R6KT-in-frame DNA-insert with the Zyppy™ Plasmid Miniprep kit.
 - ii. Use the extracted vector pNPTS138-R6KT-in-frame DNA-insert to transform chemically competent *E. coli* S17-1 λ *pir*.
 - iii. Inoculate 100 μ l of the transformation mixture onto LB/kanamycin agar plates.
 - iv. Incubate at 30°C overnight.
 - v. Screen the colonies with the CloneChecker kit.
 - vi. Culture the correct recombinants in LB broth/kanamycin overnight.
 - vii. Make glycerol stocks and store at -80°C.

Part 3 Conjugation

- a) Mixing the donor strain and the recipient strain
- i. Prepare *K. radicinicans* overnight culture in LB broth.
 - ii. Prepare *E. coli* S17-1 λ *pir*-pNPTS138-R6KT-in-frame DNA-insert overnight cultures in LB broth/kanamycin.
 - iii. Harvest the cells and suspended each culture in 200 μ l 0.05 M NaCl.
 - iv. In a centrifuge tube, add 100 μ l of *K. radicinicans* DSM16656^T and 100 μ l of *E. coli* S17-1 λ *pir*-pNPTS138-R6KT then mix by shaking.
 - v. Inoculate the mixture onto LB agar plates in aliquots/drops ($\approx$ 50 μ l).
 - vi. Inoculate the unmixed cultured onto separate LB plates as controls.
 - vii. Incubate at 30°C overnight.
 - viii. Wash off the post-conjugation and the control cultures from the LB agar plates with 1 ml 0.05 M NaCl.

- ix. Inoculate the conjugation mixture onto LB/kanamycin/ampicillin agar plates in dilutions (undiluted, 10^{-1} ; 10^{-2} ; 10^{-3}).
 - x. Inoculate the control cultures undiluted onto LB/Kanamycin/ampicillin agar plates.
 - xi. Incubate at 30°C overnight.
- b) Screening for the 1st homologous recombination event
- i. Screen several colonies with following 50 μl PCR reaction mix comprising 37.75 μl water, 5 μl Thermopol buffer (10x), 2 μl primers for (10 pM), 2 μl primer rev (10 pM), 2 μl dNTPs, 0.25 Taq DNA polymerase, 1 μl colony mix (a single colony dissolved in 10 μl sterile distilled water) and the run the PCR with the following program: step 1: hold at 94°C for 5 min, step 2: (30 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min), and step 3: (72°C for 10 min).
 - ii. Visualize the PCR product on an agarose gel.
 - iii. Prepare overnight cultures of positive colonies in LB broth.
- c) Curing the vector backbone and screening for 2st homologous recombination event
- i. Harvest the cells and inoculate onto 10% sucrose LB agar plates.
 - ii. Incubate at 30°C overnight.
 - iii. Harvest the cells and inoculate (in a grind system) onto 10% sucrose LB agar plates, also on kanamycin plates in parallel.
 - iv. Incubate at 30°C overnight.

Part 4 Mutant screening

a) Colony PCR screening

Note: Screen only the colonies that have lost kanamycin resistance but grow on sucrose plates.

- i. Screen several colonies with following 50 μl PCR reaction mix comprising 37.75 μl water, 5 μl Thermopol buffer (10x), 2 μl primers for (10 pM), 2 μl primer rev (10 pM), 2 μl dNTPs, 0.25 Taq DNA polymerase, 1 μl colony mix (a single colony dissolved in 10 μl sterile distilled water) and run the PCR with the following program: step 1: hold at 94°C for 5 min, Step 2: (30 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min), and step 3: (72°C for 10 min).
- ii. Visualize the PCR product on agarose gel.

iii. Prepare overnight cultures of the positive colonies.

b) Sequence verification

- i. Harvest the cells and extract the genomic DNA with Gene aid Presto™ Mini gDNA Bacteria kit.
- ii. Do a PCR with the mutants screening primers, with the following 50 µl PCR reaction mix comprising 37.75µl water, 5µl Thermopol buffer (10x), 2 µl primers for (10 pM), 2 µl primer rev (10 pM), 2 µl dNTPs, 0.25 Taq DNA polymerase, 1 µl (60 ng) DNA template and run the PCR with the following program: step 1: hold at 94°C for 5 min, step 2: (30 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min), and step 3: (72°C for 10 min).
- iii. PCR product purification with a HiYield Gel/PCR DNA purification kit.
- iv. Sequence the purified PCR products.
- v. Prepare glycerol stocks of the verified mutants and store at -80°C.

References

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