

# Phenotypic variation in *Acidovorax radialis* N35 influences plant growth promotion

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## Abstract

*Acidovorax radialis* N35, isolated from surface-sterilized wheat roots (*Triticum aestivum*), showed irreversible phenotypic variation in nutrient broth, resulting in a differing colony morphology. In addition to the wild-type form (rough colony type), a phenotypic variant form (smooth colony type) appeared at a frequency of  $3.2 \times 10^{-3}$  per cell per generation on NB agar plates. In contrast to the N35 wild type, the variant N35v showed almost no cell aggregation and had lost its flagella and swarming ability. After inoculation, only the wild-type N35 significantly promoted the growth of soil-grown barley plants. After co-inoculation of axenically grown barley seedlings with differentially fluorescently labeled N35 and N35v cells, decreased competitive endophytic root colonization in the phenotypic variant N35v was observed using confocal laser scanning microscopy. In addition, 454 pyrosequencing of both phenotypes revealed almost identical genomic sequences. The only stable difference noted in the sequence of the phenotypic variant N35v was a 16-nucleotide deletion identified in a gene encoding the mismatch repair protein MutL. The deletion resulted in a frameshift that revealed a new stop codon resulting in a truncated MutL protein missing a functional MutL C-terminal domain. The mutation was consistent in all investigated phenotypic variant cultures and might be responsible for the observed phenotypic variation in *A. radialis* N35.

## Introduction

Phenotypic variation (synonym phase variation) is an adaptive process used by several bacterial species to increase population diversity and fitness during fluctuating environmental conditions (van den Broek *et al.*, 2005; Wisniewski-Dyé & Vial, 2008). Phenotypic variation arises at rates of  $10^{-5}$  per cell per generation or higher, which exceeds the normal spontaneous mutation rate (Henderson *et al.*, 1999). In phenotypic variation, the expression of a given gene is either ON or OFF. These events are usually reversible (ON $\leftrightarrow$ OFF), but in some cases, they are irreversible (ON $\rightarrow$ OFF or OFF $\rightarrow$ ON) (Willems & Gillis, 2005). Various DNA regulatory mechanisms are

involved in phenotypic variation: site specific inversion, recombinational deletion, transposition, spontaneous duplication and mutation, slipped-strand mispairing, genomic rearrangement, and differential methylation. DNA sequence variation resulting from the influence of these mechanisms leads to phenotypically heterogeneous populations (Henderson *et al.*, 1999; van den Broek *et al.*, 2005). In addition, according to van der Woude & Baumber (2004), changes in global regulatory protein expression can lead to an alteration in the expression of several operons, thus resulting in phenotypic variation. In rhizosphere bacteria, different forms of phenotypic variation generate a certain amount of diversity in bacterial colony morphology and physiology. The morphological and

physiological diversity leads to the formation of specific subpopulations that are capable of adaptation to the unpredictable environment in the rhizosphere. Phenotypic variation affects root colonization, biocontrol activity, aggregation, exopolysaccharide production, lipopolysaccharide production, exoenzyme production, and secondary metabolite production (Katupitiya *et al.*, 1995; van den Broek *et al.*, 2005; Vial *et al.*, 2006; Wisniewski-Dyé & Vial, 2008; Lerner *et al.*, 2010).

The genus *Acidovorax* currently contains 14 described species. The species can be separated in two groups: environmental species (including soil and water habitats) and the phytopathogenic species infecting corn, watermelon, and many other plants (Willems & Gillis, 2005; Schaad *et al.*, 2008; Choi *et al.*, 2010; Li *et al.*, 2011). *Acidovorax* strains that grow in soil and water are able to biodegrade some commercial polyesters. For example, *Acidovorax facilis* and *Acidovorax delafieldii* are able to degrade poly-3-hydroxybutyrate (PHB) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) *in vivo* (Mergaert & Swings, 1996). Some phytopathogenic *Acidovorax* species possess antibacterial and antifungal activities, such as *Acidovorax avenae* and *Acidovorax cattleyae*, which demonstrate biocontrol activity against *Rhodotorula mucilaginosa* (Hu & Young, 1998). Recently, we described *Acidovorax radialis* sp. nov., which has plant growth-promoting potential (Li *et al.*, 2011).

*Acidovorax radialis* N35<sup>T</sup> was isolated from surface-sterilized wheat roots and belongs to the environmental *Acidovorax* group (Willems & Gillis, 2005). In a previous study by Li *et al.* (2011), *A. radialis* N35 was shown to undergo irreversible phenotypic variation when grown on NB medium, resulting in differing colony morphologies. One type of morphology observed was a characteristic rough colony shape (wild-type N35) on agar plates and flocculation in liquid medium, whereas the other morphology observed was a smooth colony shape (phenotype variant N35v) without flocculation in liquid medium. However, the 16S rRNA gene coding gene sequence data and DNA–DNA hybridization values of both phenotypes were found to be 100% identical. Nevertheless, the variant N35v lost its motility and swarming ability because of missing flagella. The only observed difference in the ability between the wild type and phenotype variant to metabolize different substrates was a loss of the ability in N35v to utilize L-fucose and formic acid.

The aims of this study were to investigate the consequences of phenotypic variation in *A. radialis* N35 populations for microbe–plant interactions and to elucidate the genetic changes involved in the observed phenotypic variation. In this study, barley (*Hordeum vulgare*) was used as the model system because barley is an important crop

plant worldwide and is easily cultivated in monoxenic and soil systems. We investigated the phenotypic differences and plant growth stimulatory effects of both phenotypes on barley in a soil system. We also compared the root colonization behavior of both phenotypes in both single and co-inoculated monoxenic or dixenic systems using differentially fluorescent-labeled N35 and N35v cells by confocal laser scanning microscopy. To gain insight into the possible differences of both phenotypes at a genetic level, we compared the genome sequence data of *A. radialis* N35 and N35v by 454 pyrosequencing.

## Materials and methods

### Bacterial strains, plasmids and growth conditions

All strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, strains were grown in nutrient broth (NB; Fluka, Steinheim, Germany) at 30 °C. *Escherichia coli* strains were grown in Luria–Bertani (LB, modified) medium containing 10 g peptone, 5 g yeast extract, and 5 g NaCl L<sup>-1</sup> at 37 °C (Bertani, 1951). In selective media, 50 µg mL<sup>-1</sup> of kanamycin (Km) was used.

### Phenotypic characterizations

Swarming tests for both types of *A. radialis* N35 were performed on NB semisolid agar plates at 30 °C for 48 h (Daniels *et al.*, 2006). Protease activity, lipase activity, and siderophore production were examined as described earlier (Li *et al.*, 2011).

For calculation of the switch frequency of wild-type N35 to N35v, the wild-type cells were first grown in liquid NB medium. The total colony numbers and the numbers of colonies with switched phenotype were counted on NB solid agar plates at 0 and 16 h of growth in liquid NB medium. The switch frequency was calculated using formulas derived from Stephenson (Stephenson, 2005) and adapted to our study conditions.

$$P_0 = a + (1 - a) \cdot p$$

$$P_{16} = 1 - (1 - a)(1 - p)^{x_{16}} + (1 - a)(1 - p)^{x_{16}} \cdot p$$

(‘*p*’ denotes the switch probability per cell per generation, ‘*a*’ denotes the true proportion of switched cells in the total population, ‘*P*<sub>0</sub>’ is the observed proportion of switched colonies in the total population at 0 h, ‘*P*<sub>16</sub>’ is the observed proportion of switched colonies in the total population at 16 h, and ‘*x*<sub>16</sub>’ denotes the number of bacterial generations in the 16 h interval.)

**Table 1.** Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>Acidovorax radidis</i> N35 <sup>T</sup>	Type strain, wild-type isolate from surface-sterilized wheat roots	Li <i>et al.</i> (2011)
<i>Acidovorax radidis</i> N35v	Phenotype variant of N35	Li <i>et al.</i> (2011)
<i>Escherichia coli</i> TOP10	F <sup>-</sup> <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\Phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74 recA1 araD139 <math>\Delta</math>(<i>ara-leu</i>) 7697 <i>galU galK rpsL</i> (Str<sup>R</sup>) <i>endA1nupG</i></i>	Invitrogen, Carlsbad
<i>Acidovorax radidis</i> N35 GFP & N35v GFP	Km <sup>R</sup> , chromosomally labeled with GFP	This study
<i>Acidovorax radidis</i> N35 YFP & N35v YFP	Km <sup>R</sup> , labeled with YFP	This study
pCR2.1-TOPO	Amp <sup>R</sup> , Km <sup>R</sup> ; <i>lacZ</i> $\alpha$ , cloning vector	Invitrogen
pEYFP	Amp <sup>R</sup> , <i>lacZ</i> , eYFP carrier vector	Clontech, CA
pJBA28	Amp <sup>R</sup> , Km <sup>R</sup> ; carrier plasmid for mini-Tn5-Km-P <sub>A1/04/03</sub> -RBSII- <i>gfpmut3*</i> -T <sub>0</sub> -T <sub>1</sub>	Andersen <i>et al.</i> (1998)
pBBR1MCS-2	Km <sup>R</sup> ; <i>lacZ</i> $\alpha$ , cloning vector	Kovach <i>et al.</i> (1995)
pBBR1MCS-2-eYFP	Km <sup>R</sup> , YFP expression vector	This study

### Plasmid construction

pEYFP with enhanced yellow fluorescent protein was the initial expression vector used (Clontech, CA). The EYFP fragment was PCR amplified with the primer pair eYFP-for (5'-CGCCCAATACGCAAACC-3') and eYFP-rev (5'-GTTGGAATTCTAGAGTCG-3') using an annealing temperature of 50 °C. The PCR products were cloned into a pCR2.1-TOPO vector (Invitrogen, Carlsbad). The resulting plasmid pTOPO-EYFP was cut with EcoRI. To obtain pBBR1MCS-2-EYFP, pBBR1MCS-2 was digested with EcoRI, and the EYFP fragment was ligated to the EcoRI fragment of the pBBR1MCS-2 vector. Colonies containing recombinant plasmids were selected on and isolated from LB medium containing kanamycin at 50  $\mu$ g mL<sup>-1</sup> (LB-Km).

### Green fluorescent protein (GFP) and yellow fluorescent protein (YFP) labeling of N35 and N35v cells

The plasmid pJBA28, a carrier plasmid for the mini-Tn5 transposon cassette that carries a *gfpmut3\** gene and a selective marker for kanamycin (Andersen *et al.*, 1998), was used for chromosomal GFP labeling of *A. radidis* N35 and N35v cells. pJBA28 was isolated using a NucleoSpin plasmid kit (Macherey-Nagel, Düren, Germany) and transferred via electroporation into electrocompetent cells of N35 and N35v as described by Dower *et al.* (1988). A Gene Pulser (Bio-Rad, München, Germany) with a voltage of 2.5 kV for 4.5–5.5 ms was used for the electroporation. Chromosomally GFP-labeled bacteria were selected on Km selective medium, and their GFP fluorescence was verified with a fluorescent microscope with a specific wavelength excitation of 488 nm (Lumar V12; Zeiss, Oberkochen, Germany).

Because satisfactory fluorescence could not be obtained for YFP with chromosomal integration using a similar

construct, a plasmid-based labeling method had to be applied. We used the broad-host-range vector pBBR1MCS-2 to construct a YFP expressing vector, because it is known to be stable in a large number of hosts, even without antibiotic pressure (Kovach *et al.*, 1995). The construct pBBR1MCS-2-EYFP was transferred into electrocompetent cells of *A. radidis* N35 and N35v via electroporation as described earlier and colonies containing the plasmid were selected after growth on LB-Km. We used 1-week-old pure plate cultures for analyzing the expression of YFP and also the fluorescence intensity.

YFP-labeled single cells of both types showed a maximum emission wavelength of 530 nm, whereas the GFP emission maximum is at 510 nm. Therefore, the YFP-and/or GFP-labeled *A. radidis* N35 and N35v cells were detectable and distinguishable using the  $\lambda$ -mode of the confocal laser scanning microscopy (CLSM, 510 META; Zeiss).

### Bacterial colonization in a monoxenic and a dixenic system

The barley (*H. vulgare* L.) variety 'Barke' used in this study was purchased from the National Breed Josef Breun GdbR (Herzogenaurach, Germany).

For sterilization, barley seeds were shaken in 70% ethanol for 2 min and incubated in 2% NaOCl for 15 min. After that, the seeds were washed with demineralized water (H<sub>2</sub>O<sub>dem.</sub>) and incubated in a 600 mg L<sup>-1</sup> penicillin and 250 mg L<sup>-1</sup> streptomycin solution for 30 min. Finally, the seeds were incubated on NB plates at 30 °C and germinated for 3 days. Following this, they were inspected for contaminations. Only uncontaminated seedlings were selected for inoculation.

Uncontaminated barley seedlings were inoculated by dipping the roots of axenically germinated seeds into GFP-labeled N35 or N35v cell suspensions (10<sup>8</sup> cells mL<sup>-1</sup>) for 1 h. For cocolonization experiments, the roots of seedlings

were inoculated in the same way with a 1 : 1 mixture of GFP-labeled N35 and YFP-labeled N35v or YFP-labeled N35 and GFP-labeled N35v cell suspensions. Inoculated seedlings were transferred to glass tubes ( $\varnothing$  30 mm, Schott AG, Mainz, Germany) filled 5–6 cm in height with sterilized quartz sand ( $\varnothing$  1.0–2.5 mm, Sakret, Ottobrunn) containing 10 mL Murashige and Skoog mineral salt medium (Sigma, Steinheim, Germany). All plants were grown under a 16 °C/12 °C day/night cycle, 50% relative humidity, and a photo period of 12 h (metal Halide lamps of 400 W) for 3 weeks until two-leaf stage.

### Plant growth-promoting effect on barley in a soil system

For the cultivation of barley in soil, topsoil (0–20 cm) was taken from a site in Neumarkt, Germany (latitude 48.150, longitude 11.733), which has been used for agriculture in the past. The soil was classified as Haplic Arenosol, with a high content of sand (88% sand) and a low content of total organic carbon (1%) and nitrogen (0.1%), and pH 5.8. The pots were filled with 2 L sieved soil (2 mm), and 50 mL  $H_2O_{dem.}$  was added to obtain a water content similar to 60% of the maximum water-holding capacity. The barley seeds were germinated on moist filter paper by incubation for 3 days at room temperature in the dark (nonsterile conditions). The seedlings were then inoculated with GFP-labeled *A. radialis* N35 or N35v cell suspensions ( $10^8$  cells  $mL^{-1}$ ) for 1 h as described earlier. For each treatment (noninoculated, inoculated with N35 GFP, and inoculated with N35v GFP), eight pots with three plants per pot were cultivated for 4 months. The plants were watered two times a week with 50 mL  $H_2O_{dem.}$  in the first month and three times per week in the second and third months. Throughout the experiment, the plants were fertilized two times with 150 mL of 0.7% Wuxal Top N (12% N, 6%  $P_2O_5$ , 6%  $K_2O$ , Aglukon Spezialdünger, Düsseldorf, Germany) and 150 mL of 0.02 M  $MgSO_4$  per pot. Plants were grown for 16 weeks in a greenhouse at the same conditions as described for the monoxenic or the dixenic system until spike appearance stage.

### Microscopic analysis

Root samples from the monoxenic, the dixenic, and the soil systems were harvested and washed with  $1\times$  PBS. The intact sections of the roots were placed on a slide and overlaid with Citifluor-AF1 (Citifluor, London, UK). The root samples were analyzed with a CLSM 510 META with an argon laser (laser line 488 nm) and a helium-neon laser (laser line 543). A water immersion objective C-Apochromat 63x1.2W Korr (aperture number 1.2)

with 63 times magnification was used for all analyses and image acquisitions.  $\lambda$ -mode (META module) was applied for the identification of characteristic emission spectra of GFP with a maximum emission wavelength of 510 and 530 nm for YFP. The image analysis was performed using the LSM IMAGE BROWSER software, Version 3.5 (Zeiss).

Scanning electron microscopy (SEM) images were taken as previously described in Li *et al.* (2011).

### 454 pyrosequencing

Genomic DNA of *A. radialis* N35 and N35v, grown independently in liquid NB medium, was isolated, and shotgun libraries were prepared for sequencing. Emulsion PCR (emPCR), emulsion breaking of DNA-enriched beads and sequencing of the shotgun libraries was performed on a second-generation pyrosequencer (454 GS FLX Titanium; Roche, Mannheim, Germany) using Titanium reagents and Titanium procedures as recommended by the developer following protocols for shotgun sequencing.

Quality filtering of the pyrosequencing reads was performed using the automatic standard signal processing pipeline of the GS Run Processor (Roche) to remove failed and low-quality reads from raw data and to remove adaptor sequences.

The data resulting from 454 pyrosequencing were assembled using the GS FLX NEWBLER software 2.0.01 (Roche) with a minimum overlap length of 40 bp and a minimum overlap identity of 90% (submitted to NCBI with the IDs AFBG01000001-AFBG01000030 for N35 and AFBH01000001-AFBH01000033 for N35v). For selected regions, an annotation was performed according to homologous sequences identified by 'BLAST' (<http://blast.ncbi.nlm.nih.gov/>) (Altschul *et al.*, 1997), and protein sequences from translated DNA were obtained using ExPASy (Swiss Institute of Bioinformatics, <http://www.expasy.ch/tools/dna.html>).

### SNP and InDel analysis

The N35 Newbler assembled contigs (IDs see above) were used as reference. For the SNP and InDel analysis of the variant genome N35v data, we started by mapping the N35v raw reads to the reference using the MOSAIK aligner (1.1.0021 linux 64bit, <http://bioinformatics.bc.edu/marthlab/Mosaik>). The raw reads were obtained in the trimmed format from the Roche sff file using 'sff2fastq' (version 0.8.0, <https://github.com/indrael/sff2fastq>). The used parameters were hashsize 14 and alignment candidate threshold 17 as proposed by the MOSAIK authors. The number of allowed mismatches was set to 20 suited

for longer 454 reads, and the reads were mapped uniquely (compare Misra *et al.*, 2011).

The final BAM-format mapping file was then obtained using MOSAIK's conversion tool. Postprocessing and SNP calling were performed with SAM Tools [version 0.1.7-18 (r605)] (Li *et al.*, 2009). For variant calling, we explicitly stated a monoploid SNP calling model. This ensures calling only high-quality SNPs that are present throughout the sequenced sample.

The obtained variants were filtered in multiple steps as carried out by Hedges *et al.* (2009) and Ng *et al.* (2010): First, we subjected the data to the SAM Tools varFilter to remove all low-quality and suspicious variants (minimum and maximum read coverage 5 and 9999, respectively) as recommended by the SAM Tools authors (Li *et al.*, 2009). Second, SNPs were filtered for a SNP quality  $\geq 20$  and coverage of  $\geq 8$ . In a third filtering, we discarded all InDels that did not have evidence from  $\geq 50\%$  of all reads covering the respective position(s). In addition to the evidence criteria, we also filter for InDel score (as obtained from samtools)  $\geq 50$ . Essentially, the InDel filtering is mainly carried out because of the known issue of homonucleotide calling of 454 technology (Alderborn *et al.*, 2000; Ronaghi, 2001) and effectively removes 99.6% of all InDels, leaving only 97 high-quality InDels. Annotation of genes with detected variants was retrieved from the PEDANT (Walter *et al.*, 2009) database. The PEDANT gene model was predicted with the tool Prodigal (Hyatt *et al.*, 2010).

### Verification of the stability of the *mutL* deletion

Six colonies of wild-type N35 and ten colonies of phenotype variant N35v were independently picked from NB agar plates. Genomic DNA was isolated with a FastDNA SPIN Kit for soil (MP, Illkirch, France), and the *mutL* gene region was PCR amplified using a newly designed primer pair MutL-s (5'-AACTGTTCTTCTCCACCC-3') and MutL-as (5'-TCACGAAGGGTGCGGC-3') with an annealing temperature of 55 °C. The PCR products were purified using a NucleoSpin Extract II kit (Macherey-Nagel) and sequenced with the BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, CA) using an ABI 3730 sequencer (Applied Biosystems).

### Statistical analyses

A standard *t*-test ( $n = 24$ ,  $P < 0.05$ ) was used to compare the differences between the harvested root and shoot samples of each treatment.

The Genbank/EMBL/DDBJ accession number for the *mutL* gene sequence of *A. radicans* N35 is HQ718776 and for *A. radicans* N35v is HQ718777.

## Results

### Phenotypic characterization of *A. radicans* N35 wild type in comparison with its variant N35v

Phenotypic variation was regularly observed in *A. radicans* N35 when grown in NB medium; rough (wild-type N35) and smooth (phenotype variant N35v) colony morphologies could be distinguished. The wild-type N35 switched to the phenotype variant N35v at a rate of  $3.2 \times 10^{-3}$  per cell per generation on NB medium. This was calculated from  $2 \times 10^6$  switched colonies of  $3.8 \times 10^7$  total colonies at 0 h and  $7.2 \times 10^7$  switched colonies of  $1.1 \times 10^9$  total colonies at 16 h using the formulas as described in the Material and methods section. There was no detectable reversion from the phenotype variant to the wild type or other phenotype, even at different growth conditions, such as in mineral medium or after the colonization of barley roots (data not shown).

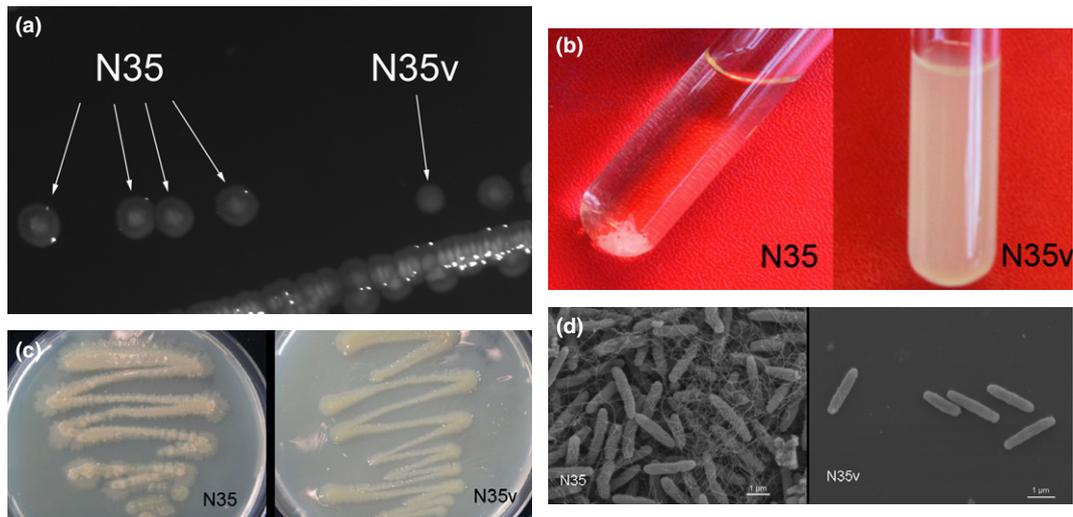
The colony shapes, growth in liquid medium, physiological, and biochemical characteristics of both phenotypes are summarized in Fig. 1 and Table 2. Wild-type N35 demonstrated swarming on soft-agar plates, whereas phenotype variant N35v could not swarm (Fig. 1c). SEM images revealed that wild-type cells were often arranged in clusters with filaments (extracellular microfibrils), whereas variant cells were found to be separate from each other (Fig. 1d).

Ten wild-type colonies and ten phenotypically variant colonies were screened for lipase and siderophore activities. Equivalent lipase and siderophore activity was observed in both phenotypes (Table 2).

### Plant growth promotion of barley

To assess the effect of *A. radicans* N35 on barley, the dry weight of roots and shoots was determined after inoculation with wild-type N35 and compared with the dry weight of the roots and shoots of phenotype variant N35v and the noninoculated controls.

After 4 months of growth in a greenhouse, the root dry weight of wild-type N35 inoculated barley plants was significantly ( $P < 0.05$ ) increased by 40% relative to noninoculated control plants. In the plants inoculated with the phenotype variant N35v, an increase of 13% in comparison with control could be detected, but the difference was not statistically significant (Fig. 2). The shoot dry weight also increased significantly ( $P < 0.05$ ) when barley was inoculated with wild-type N35, with 20% more biomass being observed in comparison with noninoculated controls. Following inoculation with the phenotype variant N35v, the shoot dry weight showed only a slight non-significant increase when compared with noninoculated controls (Fig. 2).



**Fig. 1.** Phenotypic comparison between wild-type N35 and variant N35v. (a) Wild-type N35 (rough colonies) could switch to phenotype variant N35v (smooth colony) on NB agar plate; small white spots: light reflections. (b) Growth distinction between wild-type N35 and variant N35v in NB liquid medium. (c) Swarming motility of both types of N35 and N35v on NB semisolid agar plate. (d) Scanning electron micrographs showed that N35 cells occurred in cluster, whereas N35v cells separated from each other. Bars, 1  $\mu$ m.

**Table 2.** Phenotypic characteristics of *Acidovorax radicus* N35 wild type in comparison with its phenotype variant

Characteristics	Wild-type N35	Phenotype variant N35v
Colony shapes*	Rough	Smooth
Growth in liquid medium*	Flocculation	Turbid
Swim*	+	–
Swarming ability	+	–
Flagellum*	One polar flagellum	No flagellum
Lipase activity	+	+
Siderophore activity	+	+
Utilization of L-fucose*	+	–
Utilization of formic acid*	+	–

Activations are indicated as follows: +, activation was detected; –, no detectable activation.

\*Results were described by Li *et al.* (2011).

### Root colonization ability of *A. radicus* N35 and N35v cells

The colonization of barley roots by GFP-labeled *A. radicus* N35 and N35v cells was examined in monoxenic or dixenic quartz sand and a soil system using CLSM. Root samples were taken after 3 weeks in the monoxenic or the dixenic system and after 12 weeks in a separate experiment in the soil system.

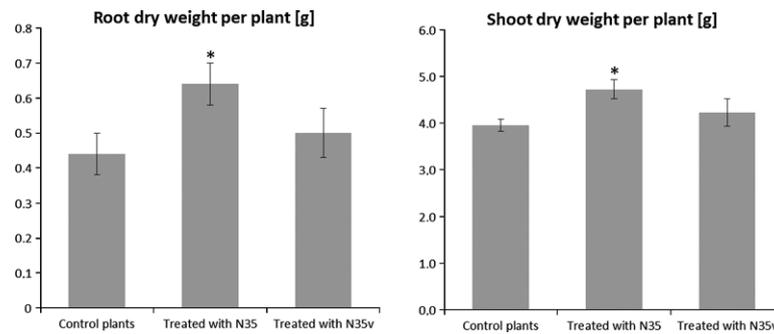
Three weeks after separate inoculations in the monoxenic system, both the wild type and phenotype variant N35v had colonized the surface of roots and root hairs in large numbers forming biofilm-like aggregates. Twelve weeks after inoculation, wild-type N35 cells (Fig. 3a) and phenotype variant N35v cells had endophytically

colonized the roots of soil-grown barley plants. Some cells even appeared to be located within root cells. Thus, when inoculated individually in either monoxenic or soil systems, both types of N35 could endophytically colonize barley roots 12 weeks after inoculation, and no apparent differences in colonization behavior were found between wild-type N35 and the phenotype variant N35v.

To determine whether the colonization behavior of *A. radicus* N35 was associated with phenotypic variation, cocolonization experiments of both N35 and N35v cells on barley roots were performed. Barley seedlings were inoculated with a 1 : 1 mixture of GFP-labeled N35 and YFP-labeled N35v cells or YFP-labeled N35 and GFP-labeled N35v cells and grown in the dixenic system. After 3 weeks, the plants were harvested and microscopically inspected using CLSM. Bacterial cells colonized mostly the basal part of the roots, but there was a clear difference in colonization behavior between the wild type and the phenotype variant. GFP-labeled wild-type N35 cells colonized root surfaces in high numbers, forming aggregates, whereas only a few YFP-labeled N35v cells could be observed (Fig. 3b). These results were independent of the fluorescent protein used. When the bacteria were labeled with different fluorescent proteins, the differential colonization patterns of N35 vs. N35v were still observed (Fig. 3c).

### Identification of the influence of the *mutL* gene on phenotypic variation in *A. radicus* N35

To investigate the mechanism and genetic basis behind the observed phenotypic variation, the entire genome of the wild-type N35 and phenotype variant N35v was



**Fig. 2.** Plant growth-promoting effect of *Acidovorax radialis* N35 and N35v on barley. Barley plants were grown in eight different pots containing an agricultural soil with three plants per pot in a greenhouse for 16 weeks. The setup allowed 24 plants for each treatment to be analyzed. Values were means of 24 plants from eight different pots. Error bars: standard errors. \*Indicated statistically significant differences between roots and shoots of inoculated and noninoculated plants (*t*-test,  $P < 0.05$ ).

sequenced using 454 pyrosequencing. After sequencing, 30 contigs for N35 and 35 contigs for N35v were obtained from the assembly, and the average assembly coverage for both types was 40 $\times$ . The average contig size and the largest contig size for N35 were 190.123 and 887.230 kb and for N35v 148.988 and 840.687 kb, respectively. The genome size of both types predicted by the software was the same, nearly 5.5 Mb, estimated using the NEWBLER software (Roche).

Mapping of the N35v reads yields an average mapping coverage of nearly 45 $\times$  for all 30 contigs of the N35 reference. The SNP calling on the mapping delivers an unfiltered total of 90 867 SNVs and larger InDels. Applying quality filtering steps (as described in Material and methods), we identified 20 high-quality homozygous SNPs and 97 high-quality InDels.

Among these InDels, the largest and highest scoring (InDel score of 2087) is a deletion of 16 nt, at position 531111 of contig00020. In the wild-type genome of N35, this region contains the DNA sequence 'CGCCAGCCCCTGCTGC'. Of a total of 30 reads covering that position, 28 show evidence for the deletion, suggesting an almost homogenous sample population. The detailed results are accessible via the PEDANT website ([pedant.gsf.de](http://pedant.gsf.de)), and a summary thereof is provided in two tables as supplementary material.

Using the annotation of the reference contigs, this position is identified as 'DNA mismatch repair protein MutL' with the UniProt (TheUniProtConsortium, 2011) accession number 'A1WQM3' (Fig. 3a). The next closest neighboring InDels to the MutL deletion are both beyond the MutL gene (positions 470'957 and 542'919 on contig00020). When looking at close-by SNPs, only two SNPs are reported for the contig00020 in total. None is residing in the MutL region (positions 140'408 and 687'912 respectively). This suggests that the identified deletion within the MutL gene is not influenced by close-by SNPs.

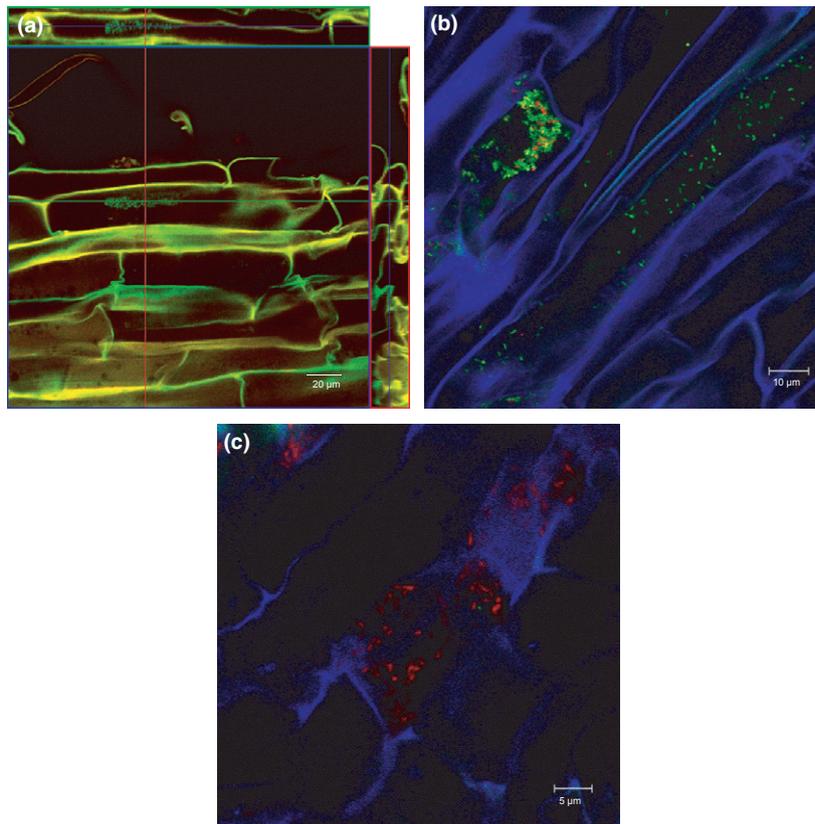
The PFAM active site method (Mistry *et al.*, 2007; Finn *et al.*, 2010) was used to identify highly significant matches of the MutL proteins of N35 and N35v. We were able to identify three highly significant matches for N35 and two highly significant matches for N35v. One of the domains is identical to HATase\_c, which is an ATPase-like histidine kinase, DNA gyrase B, and HSP90. The other domain matched DNA\_mis\_repair. These two domains were detected in both phenotypes of N35 and N35v; however, the third domain, the MutL\_C-terminal dimerization domain, is only detected in wild-type N35 (Fig. 3b). Thus, in N35v, the MutL protein is truncated because of a deletion-caused frameshift creating a stop codon in the coding region, while in N35, the complete MutL protein can be expressed.

To verify that the impaired MutL protein was not an individual event randomly occurring in one studied isolate, six colonies of wild-type N35 and ten colonies of the phenotype variant N35v were independently picked from NB agar plates, and the *mutL* gene region was amplified using primer pair MutL-s and MutL-as. The 16-nucleotide deletion of the *mutL* gene was observed in all N35v colonies, whereas the *mutL* gene in all N35 wild-type colonies was unaltered. These results indicate that the *mutL* gene deletion in N35v is a stable event coupled to phenotypic variation that always occurs at the same position within the *mutL* gene in the N35v cells.

## Discussion

### Phenotypic variation in *A. radialis* N35

According to the definition of phenotypic variation in literature, phenotypic variation occurs at a frequency higher than  $10^{-5}$  switches per cell per generation (Henderson *et al.*, 1999; Saunders *et al.*, 2003; Wisniewski-Dyé & Vial, 2008). In case of *A. radialis* N35, the rough colony type



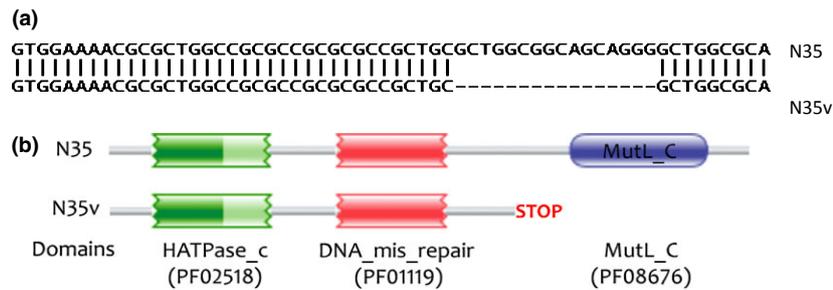
**Fig. 3.** CLSM image showing the localization of GFP-labeled *Acidovorax radialis* N35 on barley roots in a soil system as well as cocolonization of both types of *A. radialis* N35 on barley roots in a dioxenic system. GFP-labeled bacterial cells are shown in green, YFP-labeled bacterial cells in red, and the roots in green (a) and in blue (b and c). (a) Middle part of roots after inoculation of GFP-labeled *A. radialis* N35 after 12 weeks in the soil system. Orthogonal view of a 3D confocal image created from a z-stack of xy-scans. The top view, framed in blue, shows one picture from the middle of this z-stack. The red and green lines represent vertical optical cuts through the stack, which result in the side-view images framed in red and green, respectively. In these side views, the blue line marks the vertical position, where the top view image is located within the z-stack. For wild-type N35, the GFP-labeled bacteria are observed in aggregates in the top view, and the white arrows in the side view show the bacteria colonizing the inside of the root cells. (b) Co-inoculation of GFP-labeled N35 and YFP-labeled N35v on barley roots. GFP-labeled N35 cells colonize the roots in a large number forming aggregates, where YFP-labeled N35v are observed only in a few cells. (c) Co-inoculation of YFP-labeled N35 and GFP-labeled N35v on barley roots. YFP-labeled N35 cells constitute the vast majority, while only one GFP-labeled N35v cell can be observed.

(wild type) switched to the smooth colony type (phenotypic variant) at a frequency of about  $3.2 \times 10^{-3}$  per cell per generation in nutrient broth.

Previously, it has been reported that an increased proportion of phenotype variants of *Pseudomonas fluorescens* F113 (Sánchez-Contreras *et al.*, 2002) and *Pseudomonas brassicacearum* NFM42 (Achouak *et al.*, 2004) was generated during colonization of roots. However, when sterile barley roots were inoculated with wild-type N35, which was then re-isolated after 2 weeks from the root material, the same switch rate was observed as in a planktonic culture after plating on NB agar (data not shown). This indicates that the variant was not formed during the growth period under the given experimental conditions or only at very low rates.

Usually in phenotypic variation, the variants are unstable and able to revert to the original phenotype, but in

some cases, the switch is irreversible (Wisniewski-Dyé & Vial, 2008). Vial *et al.* (2009) described the generation of irreversible phenotype variant cells following phenotypic variation in *Burkholderia ambifaria* HSJ1, where the variants proved to be stable even after re-isolation from their eukaryotic hosts. Similarly, in our study, no reversion of the phenotype variant N35v to the wild-type N35 was observed despite multiple subcultures in both laboratory culture conditions and the barley rhizosphere (data not shown). The phenotypic variation was shown to be based on a 16-nucleotide deletion, which may explain the irreversibility of the phenotypic variation. However, deletions in a gene are not necessarily irreversible because in the human pathogen *Campylobacter coli* UA585, reversible insertions and deletions in a short homopolymeric tract of thymine residues located in the *flhA* gene were observed. The mechanisms involved in the reversible



**Fig. 4.** Comparison of the *mutL* gene in *Acidovorax radicles* N35 and N35v. (a) Sixteen-nucleotide deletion in *A. radicles* phenotype variant N35v in comparison with wild-type N35 in *mutL* gene region. (b) The domains of *A. radicles* N35 and N35v *mutL* gene: HATPase and DNA\_mis\_repair domains are found in both types, whereas the MutL\_C dimerization domain is deleted in N35v because of the insertion of a stop codon.

insertion and deletion of the T residues in the *flhA* gene remain to be determined (Park *et al.*, 2000). Considering that a reversion to the wild-type form might require very specific conditions, the question whether phenotypic variation is reversible in N35 cannot be conclusively answered.

#### Plant growth-promoting effect and competitive colonization of *A. radicles* N35 and its phenotype variant in barley

When compared with the phenotype variant N35v, the wild-type N35 was better in promoting growth of barley (Fig. 2). Phenotypic variation has been described in many PGPR such as *Azospirillum lipoferum* 4B (Vial *et al.*, 2004), *Azospirillum brasilense* Sp7 (Lerner *et al.*, 2010), and *B. ambifaria* HSJ1 (Vial *et al.*, 2009), but only very few studies have discussed the possible influence of phenotypic variation on plant growth promotion. It is possible that the reduced competitive colonization behavior of N35v observed on barley roots could also be involved in its inferior plant growth-promoting effect. A highly competitive colonization of plant roots by introduced bacterial cells is most important for effective bacteria–plant interaction (Chebotar *et al.*, 2001; Kamilova *et al.*, 2005).

In competitive colonization experiments, wild-type N35 cells were dominant over N35v (Fig. 3b and c). The observed dominance can be explained by the loss of motility of N35v because of the absence of flagella (Tab. 2) and loss of swarming capacity (Fig. 1c). The presence of flagella is required in rhizobacteria for the most effective colonization of roots, such is seen in the *P. fluorescens* WCS374 colonization of potato roots (de Weger *et al.*, 1987). Previous studies have shown that flagella-mediated chemotaxis toward plant root exudates plays a major role in competitive root colonization of rhizobacteria (Zhulin & Armitage, 1992; de Weert *et al.*, 2002).

Swarming is defined as bacterial group motility across a surface, which is driven by lateral bacterial flagella

(Harshey, 2003; Butler *et al.*, 2009). Alexandre & Bally (1999) suggested that swarming across root surfaces may be responsible for the long-term colonization of *A. lipoferum* 4B. According to this hypothesis, the variant phenotype N35v cells, which lack flagella and swarming ability, are devoid of the ability to move on root surfaces and are therefore less competitive in barley root colonization.

Finally, wild-type N35 was nutritionally also more versatile than the phenotype variant. The wild-type N35 was found to readily metabolize fucose and formic acid as carbon source, whereas the phenotype variant N35v lost the ability to utilize these two substrates (Table 2). Fucose and formic acid provided by plant root exudates (Basic *et al.*, 1986; Sandnes *et al.*, 2005) could be utilized as carbon sources by N35, and consequently, wild-type N35 is probably better adapted to the rhizosphere in comparison with phenotype variant N35v.

Owing to its various deficiencies in rhizosphere colonization, N35v could be considered a planktonic culture type. Colony heterogeneity within the bacterial population may provide flexibility in response to environmental changes (i.e. planktonic vs. attached or nutrient-rich vs. nutrient-poor life styles).

#### Genetic mechanisms behind the phenotypic variation of *A. radicles* N35

The mismatch repair proteins MutS and MutL, which are truncated in N35v, are evolutionarily conserved and are involved in DNA mismatch repair by correcting replication errors (Modrich & Lahue, 1996). In *E. coli*, MutS recognizes mispaired or misinserted bases and binds to MutL in the presence of ATP forming a MutS–MutL–DNA mismatch repair complex (Grilley *et al.*, 1989; Habraken *et al.*, 1998; Schofield *et al.*, 2001; Junop *et al.*, 2003).

MutL contains a highly conserved N-terminal ATPase domain and a less conserved C-terminal domain (Guarné *et al.*, 2004). The MutL C-terminal domain plays a key role in the homodimerization of MutL in prokaryotes,

and the activity of the MutL protein is regulated by the ATP-dependent dimerization of the MutL C-terminal domain (Guarné *et al.*, 2004; Kosinski *et al.*, 2005). In the *A. radialis mutL* gene product, two domains were identified in both wild-type N35 and phenotype variant N35v, namely HATase\_c and DNA\_mis\_repair, whereas the third domain MutL\_C (MutL C-terminal domain) was only detected in N35, not in N35v (Fig. 4b). Because the MutL C-terminal domain mediates the dimerization of the MutL protein, the absence of the MutL C-terminal domain abolishes the activity of the MutL protein, leading to a loss of the protein's DNA repair ability in *A. radialis* N35v. By sequencing the *mutL* region of ten independently picked colonies of N35v, we were able to verify that the impaired MutL protein was not an individual random event in one studied colony but always occurs in exactly the same way in each phenotypic variant. The mechanisms behind the formation of this specific deletion are still unknown.

The mismatch repair system has been reported to influence the phenotypic variation frequency in human pathogenic bacteria, such as *Neisseria meningitidis* (Richardson & Stojiljkovic, 2001) and *Haemophilus influenzae* (Bayliss *et al.*, 2004). In this study, the impaired MutL protein in N35v might abolish the mismatch repair function during DNA replication resulting in a higher frequency of mutations in a given cell at one or more sites. If the mutation is advantageous for the bacteria, a new mutant colony (variant) can arise. Consequently, the impaired MutL protein could induce other mutations that allow the adaptation to the changed nutrient and colonization conditions. Several point mutations were found in N35v in comparison with N35 using a bioinformatic SNP and InDel analysis on the 454 sequencing data, located, for example, in genes for a TonB-dependent receptor protein and diguanylate cyclase-phosphodiesterase protein. However, in contrast to the *mutL* deletion, the stability of these point mutations could not be verified in N35v after resequencing of several randomly selected colonies (data not shown).

In *Streptococcus pyogenes* (Bormann & Cleary, 1997) and *E. coli* (Blyn *et al.*, 1990), changes in the expression of global regulatory proteins leading to phenotypic variation have been reported. The changes in a single regulatory protein can lead to variable expression of multiple cellular proteins through the changes in the regulation of the initiation of transcription at the main promoter of operons (van der Woude & Baumler, 2004). MutL also possesses a nonspecific DNA-binding activity (Guarné *et al.*, 2004). The DNA-binding activity of MutL might lead to an up- or down-regulation of the expression of multiple specific proteins resulting in the observed phenotypic variation in *A. radialis* N35. Because the impaired

repair mechanism of MutL usually led to unspecific and undirected mutations, which did not match the observed phenotype of N35v, the phenotypic variation in *A. radialis* N35 was more likely mediated by the MutL up- or down-regulating mechanism. Further elucidation of this mechanism will require another more in-depth molecular study.

In conclusion, our data provide strong evidence that *A. radialis* N35 undergoes phenotypic variation at laboratory growth conditions to adapt to a planktonic lifestyle. This requires special care, when propagating it for an inoculation as PGPR because this variation decreases its PGPR efficiency.

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