

## Effects of Plant Genotype and Growth Stage on the Betaproteobacterial Communities Associated with Different Potato Cultivars in Two Fields<sup>∇†</sup>

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**Bacterial communities in the rhizosphere are dynamic and susceptible to changes in plant conditions. Among the bacteria, the betaproteobacteria play key roles in nutrient cycling and plant growth promotion, and hence the dynamics of their community structures in the rhizosphere should be investigated. Here, the effects of plant cultivar, growth stage, and soil type on the communities associated with potato cultivars Aveka, Aventura, Karnico, Modena, Premiere, and Désirée were assessed for two different fields containing sandy soil with either a high or low organic compound content. Thus, bacterial and betaproteobacterial PCR-denaturing gradient gel electrophoresis analyses were performed to analyze the effects of plant cultivar and growth on the rhizosphere community structure. The analyses showed that in both fields all cultivars had a rhizosphere effect on the total bacterial and betaproteobacterial communities. In addition, the plant growth stage strongly affected the betaproteobacterial communities in both fields. Moreover, the community structures were affected by cultivar, and cultivars differed in physiology, as reflected in their growth rates, root development, and estimated tuber starch contents. Analyses of betaproteobacterial clone libraries constructed for two selected cultivars (one cultivar that produced low-starch-content tubers and one cultivar that produced high-starch-content tubers), as well as bulk soil, revealed that the rhizospheres of the two cultivars selected for specific bacteria, including plant-growth-promoting bacteria, such as *Variovorax* and *Achromobacter* spp. In addition, quantitative PCR-based quantification of the *Variovorax paradoxus*-specific functional gene *asfA* (involved in desulfonation) indicated that there were clear potato rhizosphere effects on the abundance of this gene. Interestingly, both cultivar type and plant growth stage affected the community under some circumstances.**

Soilborne microbial communities are influenced by plant roots due to, among other factors, the organic compounds in root exudates. Plants thus selectively attract microorganisms to their rhizospheres, and these microorganisms consume particular excreted compounds (9, 13). It clearly follows that microbial communities in the rhizosphere may be differentially influenced by the plant genotype, as well as by the developmental stage, if these factors result in different patterns of root exudation (12, 14, 53). However, there is a paucity of information about the extent to which such shifts in the community occur, about the dynamics of the changes, and about the putative effects on the functioning of the system.

Several initiatives have resulted in novel crop plants (genetically modified plants or plants obtained via traditional breeding) that have genetic systems which enable them to control bacterial and/or fungal pathogens (19, 27, 41). Other crop plants have been developed for diverse industrial purposes (e.g., potato plants that produce tubers with a low amylose content for the paper industry) (21). Given the fact that in

most cases it is not known whether and to what extent novel crops affect the microbial communities in soil, it is important to assess the effects of such crops in relation to the effects of cultivars currently used. A comparative assessment would enable description of the effects of novel cultivars in the context of the effects of existing cultivars and yield a data set that establishes a baseline describing the effects of crop plants on the soil. In particular, establishing the impact on the soil's life support functions (LSF), such as biogeochemical cycles and support of plant health, is crucial.

A sensible strategy to examine the effects of plants on the soil LSF is to focus on a limited set of key organisms involved in several processes. One key group is represented by the betaproteobacteria, as members of this group are important mediators in the cycling of nitrogen, sulfur, and carbon through the soil ecosystem (25, 46). For instance, the *Nitrosomonadaceae* form an important cluster of betaproteobacterial ammonia oxidizers, and *Burkholderia* strains play important roles in the mycorrhization of plants, as well as in symbiotic nitrogen fixation (3, 42). Other members of the betaproteobacteria promote plant growth by synthesizing phytohormones and vitamins (8). For instance, *Burkholderia phytofirmans* typically produces aminocyclopropane-1-carboxylate (ACC) deaminase, which assists in decreasing the level of the stress hormone ethylene in plants. Thus, the growth of plant roots may increase when *B. phytofirmans* is present because of a reduction in the level of this inhibitor of root elongation (47). Furthermore, other *Burk-*

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*holderia* species are important producers of antibiotics that antagonize bacterial and fungal phytopathogens (28). Recently, the betaproteobacterial genera *Variovorax* and *Polaromonas* were found to be capable of desulfonating aromatic sulfonates in the wheat rhizosphere (45). Since the betaproteobacteria mentioned above are key drivers in the LSF of soil, they can be good indicators for assessing the effects of novel crops on soil processes. In particular, the dynamics of desulfonating bacterial communities is important, as freely available sulfur (e.g., sulfate) can be limiting in plant nutrition (23, 46). We thus hypothesized that the betaproteobacteria which perform key tasks like desulfonation may be differentially selected by the rhizospheres of different potato cultivars. Therefore, in this study we assessed the dynamics of betaproteobacterial communities, compared to total bacteria, with different potato cultivars over a growing season in two soils. We focused on the extent to which these cultivars differentially "sampled" the soil microbiota and how the resulting communities changed during a growing season.

## MATERIALS AND METHODS

**Soils and soil sampling.** Two experimental fields, Buinen (soil B) (52°55'N, 6°49'E) and Valthermond (soil V) (52°50'N, 6°55'E), in Drenthe, Netherlands, were used for the experiments. These two fields contained different types of soil; soil B was loamy sand containing 5% organic matter (OM) (pH 5.0), and soil V was sandy peat containing 25% OM (pH 5.0). The fields were under an agricultural rotation regimen. In the previous season, spring barley had been grown in both fields. Six different potato cultivars, cultivars Aveka (A), Aventura (Av), Karnico (K), Modena (M) (modified from cultivar Karnico for low amylose content [7]), Premiere (P), and Désirée (D), were used. Cultivars A, Av, K, and M produced tubers with high starch contents and had a low and/or medium growth rate, whereas cultivars P and D produced tubers with relatively low starch contents and had a high growth rate. The different cultivars had different parental cultivars in the first generation, so their overall pedigrees were complex. For instance, cultivar A was related to cultivar D in the fifth generation and to cultivar K in the third generation (52).

Four replicate plots randomly distributed in the fields were used for each potato cultivar. At the start of the growing season, 20 plants (tubers) were planted in each plot. Standard agricultural practices were used. Samples were taken at the young-plant stage (EC30), flowering stage (EC60), and senescence stage (EC99) (18, 34). The bulk soil sampling times are referred to below as June, July, and September.

At each sampling time, both plants and bulk soil were sampled. For each plot, four replicate plants were removed and taken to the laboratory. The soil loosely adhering to the roots was shaken off, the resulting roots (containing rhizosphere soil) were pooled for each plot, and then the rhizosphere samples were collected by brushing off the soil that was tightly adhering to the root surface. In addition, six composite bulk soil samples, each consisting of four cores for each cultivation area outside the reach of plant roots, were collected. Thus, 24 composite samples for each treatment were obtained for each sampling time and each field. A total of 180 composite samples, including bulk soil samples, were taken during the growing season. In addition, bulk soil samples were taken before planting and after harvesting. All samples were stored in closed plastic bags (containing 1 volume of headspace) at room temperature for <2 days prior to extraction and analysis of soil DNA.

**Extraction of soil DNA.** Pooled samples of bulk soil were used directly for extraction of DNA, whereas rhizosphere soil was pooled for each plot to obtain one of four replicates. For extraction of soil DNA, a Powersoil DNA extraction kit (Mo Bio Laboratories Inc., NY) was used with 0.5 g of soil according to the manufacturer's instructions, slightly modified as follows. Glass beads (diameter, 0.1 mm; 0.25g) were added to the soil slurries, and the cells were disrupted by bead beating (mini-bead beater; BioSpec Products, United States) three times for 60 s. To assess the quantity and purity, the crude DNA extracts were run on 1.5% agarose gels at 90 V for 1 h in 0.5× Tris-acetate-EDTA (TAE) buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA; pH 8.0) using a fixed amount (5 µl) of a 1-kb DNA ladder (Promega, Leiden, Netherlands) as the molecular size and quantity marker. Gels were stained with ethidium bromide for 20 min (1.2 mg/liter ethidium bromide in 0.5×TAE buffer). The quantity of extracted DNA

was estimated by comparison to the ladder. DNA quality (average molecular size and purity) and quantity were estimated from gel, using the degree of DNA shearing (average molecular size) as well as the amounts of coextracted compounds (quality) and comparison to known amounts of DNA (quantity) in the marker.

**Quantitative PCR (qPCR).** Quantification of 16S bacterial rRNA genes was performed with primers 341F and 518R as described previously (37), using an annealing temperature of 55°C and no added betaine. Standards ( $10^4$  to  $10^8$  molecules per reaction mixture) were prepared using PCR products from *Variovorax paradoxus* type strain DSM30034. Statistical analyses (*t* tests) were performed to assess the significance of the differences between the numbers of target genes in the different cultivars and at different growth stages.

Quantitative PCR of *Variovorax* sp. *asfA* genes was performed using primers *asfA\_Varx\_F1* (CTGTCGGGCATGGAGTCT) and *asfA\_Varx\_R1* (AGCGT CACCGAAAGTGCT) to obtain 302-bp *asfA* gene products, as described by Schmalenberger et al. (45). The reaction mixture (total volume, 10 µl) contained 5 ml of DyNamo capillary SYBR green qPCR master mixture (Finnzymes, Helsinki, Finland), 1 M betaine, 0.3 pmol of forward and reverse primers (*asfA\_Varx\_F1* and *asfA\_Varx\_R1*, respectively) and 5 ng of template DNA. The PCR conditions were 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 20 s at 60°C, and 20 s at 72°C (45).

**PCR amplification for DGGE community fingerprinting.** Using DNA extracted from the rhizosphere and bulk soils, PCR amplification targeting the 16S rRNA genes of total bacteria and betaproteobacteria was performed. Total bacterial communities were assessed by PCR directly using DNA extracted from soil. Briefly, each 25-µl PCR mixture used for bacterial PCR-denaturing gradient gel electrophoresis (DGGE) analysis contained 5 µl PCR buffer [60 mM Tris-HCl, 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5.5 mM MgCl<sub>2</sub>; pH 9.0], 0.5 µl formamide, 0.5 µg T4 gene 32 protein (Roche, Almere, Netherlands), 10 nmol of each deoxyribonucleoside triphosphate, 10 pmol of each primer (GC-341F and 518R) (37), and 5 U of AmpliTaq DNA polymerase (Stoffel fragment; Applied Biosystems, Foster City, CA) in pure water. After addition of about 5 ng of template DNA, the mixtures were placed in a GeneAmp PCR system 9700 cyclor (Applied Biosystems, Foster City, CA), and thermal cycling was performed as follows: initial denaturation for 5 min at 94°C, followed by 35 cycles consisting of 1 min at 94°C, 1 min at 57°C, and 3 min at 72°C and then extension for 30 min at 72°C. A nested PCR approach was used for amplification of the betaproteobacterial community DNA. The initial amplification was done with primers 27F and 865R using a touchdown protocol (30 cycles) as described by Cunliffe and Kertesz (5). This PCR was followed by a second (fresh) PCR, in which 1 µl (5 ng) of the primary PCR product was used as the template DNA with DGGE primers GC-341 and 518R (25 cycles).

**DGGE community fingerprinting.** All DGGE profiles were generated using the Ingeny Phor-U system (Ingeny International, Goes, Netherlands). The PCR products obtained with the soil DNA, at estimated concentrations of 200 ng, were loaded onto polyacrylamide gels containing 6% (wt/vol) acrylamide in 0.5× Tris-acetate-EDTA (TAE) buffer (2.42 g Tris base, 0.82 g sodium acetate, and 0.185 g EDTA in 1 liter of H<sub>2</sub>O). The bacterial and betaproteobacterial amplicons were run on 35 to 65% denaturant gradient gels at 100 V for 16 h at 60°C. All gels were silver stained (20) and air dried, after which they were digitized for further analysis.

**Computer-assisted analysis of DGGE fingerprints.** The profiles of the different DGGE gels were stored as TIFF files. Images were normalized using the markers, and the patterns were subsequently compared by using clustering methods. Similarity matrices consisting of defined numbers in each gel (e.g., nine different samples in triplicate) were generated using Pearson's correlation coefficient (*r*). Subsequently, the patterns were clustered using the unweighted pair group method with arithmetic averages (UPGMA) with GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). In addition, data derived on the basis of Jaccard correlation (a band-based analysis) were used for redundancy analysis (RDA) using CANOCO (Microcomputer Power, Ithaca, NY). Community similarities based on relative band intensities and positions were analyzed by performing canonical correspondence analyses with Monte Carlo permutation tests (CANOCO 4.0; Microcomputer Power). The Monte Carlo tests were based on 199 random permutations of the data to establish statistical significance. Moving window analyses (MWA) were used to calculate the rate-of-change parameter ( $\Delta t$ ) for bulk soil during the season. First, the similarities of the densitometric curves of DGGE patterns were calculated based on the Pearson correlation coefficient. The percent change (100% - % similarity) was recalculated as described by Marzorati et al. (30).

**Cloning and sequencing of betaproteobacterial 16S rRNA gene amplicons generated from selected samples.** Three clone libraries of betaproteobacterial gene fragments were generated (using primers 27F and 865R) to compare the

bulk soil and cultivar A and P rhizosphere betaproteobacterial communities. The amplicons were ligated into pGEM-T Easy vectors (pGEM242-T vector system II; Promega, Madison, WI), which was followed by introduction into competent *Escherichia coli* JM109 cells by transformation according to the manufacturer's instructions. White colonies were picked and replated on LB agar plates for a second check. The samples were sequenced by AGOWA (Berlin, Germany).

**Analysis of betaproteobacterial gene sequence diversity.** Prior to analyses of the sequences, these were checked for chimera formation using Bellerophon v.3 (<http://greengenes.lbl.gov>). Altogether, 84, 115, and 121 sequences were obtained from cultivar Av, cultivar P, and bulk soil, respectively. The sequences were classified using the Ribosomal Database Project II (RDP) classifier with a confidence threshold of 80% (<http://simo.marsci.uga.edu>). To determine the closest phylogenetic relatives of the sequences, BLAST-N was used with the nonredundant NCBI database. Sequence alignments and tree building were carried out using the Molecular Evolutionary Genetics Analysis (MEGA) software package (51) and the Kimura two-parameter algorithm (24) with bootstrap tests of inferred phylogeny with 1,000 replications. Pairwise sequence similarities were calculated with DNADIST (<http://cmgm.stanford.edu/phylib/dnadist.html>) using the Kimura two-parameter algorithm (24). On the basis of the similarity matrix generated, the sequences were assigned to operational taxonomic units (OTUs) using DOTUR (44). The frequency data assigned to an OTU at the "species" (97% similarity) and "genus" (95% similarity) levels were used to obtain rarefaction curves and Chao1 richness estimates. Sequences were also subjected to library shuffling analysis using LIBSHUFF (49) to determine if the clone libraries were significantly different.

**Nucleotide sequence accession numbers.** The sequences generated in this study have been deposited in the GenBank database under accession numbers GU472842 to GU473161.

## RESULTS

**Plant development during the growing season.** No signs of disease or nutrient limitation were seen in the potato cultivars during the growing season (2008) in all plots of the two soils. For all cultivars and in both soils, the young-plant stage (EC30) occurred around 30 days postplanting (dpp) (i.e., the end of June). However, subsequently, the growth rates of cultivars A, Av, M, and K and the growth rates of cultivars P and D were different in both soils. The flowering stage (EC60) occurred between 50 and 60 dpp for cultivars D and P and between 80 and 85 dpp for cultivars A, Av, K, and M (July). Finally, the senescence stage (EC99) was between 110 and 115 dpp for cultivars P and D, between 135 and 140 dpp for cultivar A, and between 145 and 150 dpp for cultivars Av, K, and M. Interestingly, cultivars P and D produced shorter roots that at the flowering stage were on average about 15 cm long, whereas all other cultivars had root systems that were around 25 cm long.

**Dynamics of bacterial abundance in bulk and rhizosphere soils as assessed by qPCR.** The abundance of each of the bacterial populations in the different samples during the growing season was estimated using the bacterial 16S rRNA gene abundance as determined by qPCR. In the two bulk soils during the growing season, the copy numbers of the target genes were fairly stable (statistically similar) and ranged from  $3 \times 10^7$  to  $8 \times 10^8$  gene copies/g soil in both bulk soils. However, specific trends were observed in both soils. The gene copy numbers first decreased, albeit insignificantly ( $P > 0.05$ ), from the start of the experiment until June in both fields. Following this, the numbers of gene copies increased progressively until the end of the growing season (see Fig. S1A in the supplemental material).

When the bacterial abundance in the rhizosphere was examined, different trends were observed for each cultivar over time for the two fields. In soil B, significant rhizosphere effects on the total bacterial abundance were observed for cultivars A, M,

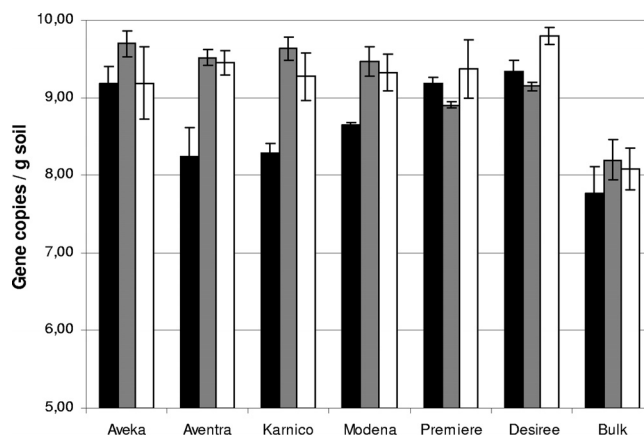


FIG. 1. Abundance of bacterial 16S rRNA genes in the rhizosphere and bulk soil at different stages of growth of cultivars in Buinen soil. The error bars indicate standard errors. Black bars, young-plant stage; gray bars, flowering stage; open bars, senescence stage.

P, and D at the young-plant stage and for all cultivars at the flowering stage. In addition, we also found significant effects of the rhizosphere for cultivars Av, K, M, and D at the senescence stage. When the dynamics over time was considered, the total bacterial abundance increased from the young-plant stage to the flowering stage and then decreased until the senescence stage for cultivars A, Av, K, and M (significantly, except for cultivar A). In contrast, the bacterial abundance for cultivar D increased significantly from the flowering stage to the senescence stage (Fig. 1).

The bacterial abundance analyses for soil V showed different trends. In most cases, no significant rhizosphere and/or cultivar effects were found at the different growth stages. However, the rhizosphere community abundance increased insignificantly over time for cultivars A and M and decreased for cultivars P and D, whereas the abundance was roughly stable for cultivars Av and K (data not shown). The ranges were  $5 \times 10^8$  to  $4 \times 10^9$  gene copies/g soil for the young-plant stage,  $1.5 \times 10^9$  to  $4.2 \times 10^9$  gene copies/g soil for the flowering stage, and  $3 \times 10^9$  to  $6.6 \times 10^9$  gene copies/g soil for the senescence stage.

**Dynamics of bacterial diversity and community composition in bulk and rhizosphere soils as assessed by PCR-DGGE analysis.** For both bulk soil and rhizosphere samples, the bacterial PCR-DGGE patterns generated for all four replicate plots revealed high within-treatment similarities for each cultivar and sampling time (data not shown). This suggested that the variability resulting from plot, sampling, DNA extraction, PCR amplification, and DGGE was generally low.

The bacterial PCR-DGGE patterns generated using bulk soil revealed that there was a ~40% change during the growing season in both soil B and soil V. For soil B, the patterns obtained in June were different from those obtained before planting, as well as from those obtained in July and September (Fig. 2A). In addition, the patterns obtained for bulk soil collected 1 year later (unplanted soil) were different from the patterns from September (Fig. 2A). For soil V, there was only a ~30% (gradual) change in the bulk soil patterns during the growing season; however, an ~80% change was observed

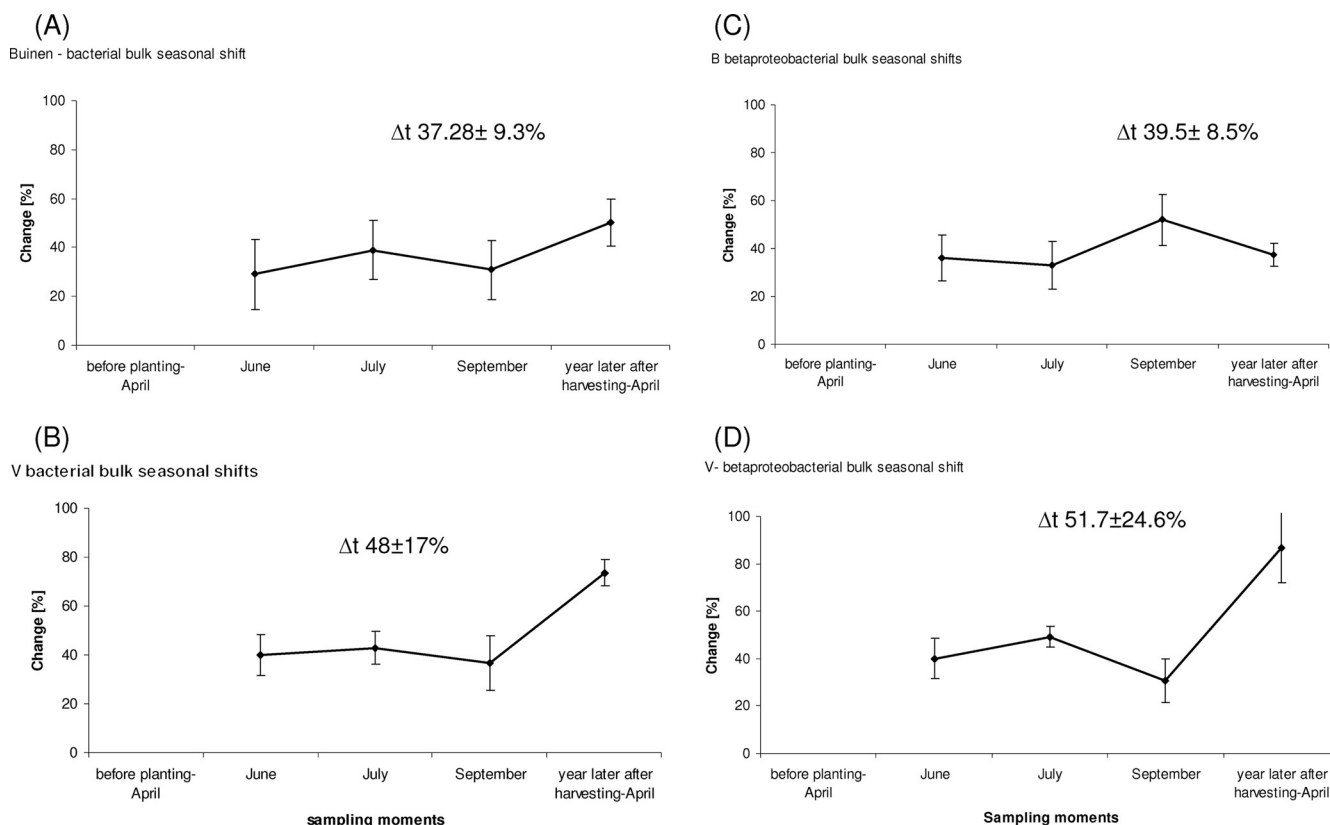


FIG. 2. Results of moving window analyses to evaluate the percent change for bacterial communities in soil B (A) and soil V (B) and for betaproteobacterial communities in soil B (C) and soil V (D) at different sampling times. The rate of change ( $\Delta t$ ) was calculated by determining the average of the corresponding moving window curve data points.

compared with the patterns for samples collected 1 year later (Fig. 2B).

**(i) Rhizosphere effect.** At all growth stages in the two soils, the rhizosphere bacterial PCR-DGGE patterns grouped separately from the corresponding bulk soil patterns (Fig. 3), indicating that there were clear rhizosphere effects on the bacterial community compositions. This was confirmed by the fact that for both soils Monte Carlo permutations showed that the bulk soil patterns were significantly different from the rhizosphere patterns ( $P < 0.05$ ). Cultivar, growth stage, and soil type also had an effect on the clustering of the patterns (Fig. 3A).

**(ii) Cultivar effect.** Five of the six bacterial PCR-DGGE patterns for the potato cultivars in soil B (the exception was the cultivar A pattern) grouped together at the young-plant stage. However, at the flowering stage, the patterns for cultivars K, M, and P grouped together ( $P > 0.05$ ), while the patterns for cultivars A, Av, and D clustered separately, each as a separate unit ( $P < 0.05$ ). At the senescence stage, two main clusters were obtained (cultivars M, P, and D and cultivars A, Av, and K) (Fig. 3B). For soil V, the bacterial patterns for all six potato cultivars grouped closely together at the young-plant stage and were separate from the corresponding bulk soil patterns (data not shown). However, at the flowering stage, the patterns for cultivars A, Av, and K grouped together, whereas those for cultivars M, P, and D formed a separate cluster (Fig. 3C). At the senescence stage, the patterns for all cultivars

again grouped together, except for the cultivar A pattern; these patterns did not cluster with the bulk soil patterns (data not shown).

**(iii) Plant growth effect.** For soil B, plant growth effects were clearly observed for all cultivars; that is, the bacterial patterns in the rhizosphere changed over time. For all cultivars, the patterns at the young-plant and flowering stages clustered closer to each other than those at the senescence stage. For soil V, plant growth stage also affected the bacterial patterns. For all cultivars, the patterns at the senescence stage were significantly different from those at the young-plant and flowering stages ( $P < 0.05$ ).

**Dynamics of betaproteobacterial communities in bulk and rhizosphere soils.** The betaproteobacterial communities in both bulk soils collected in June were different from those in the bulk soils collected in July and September. For soil B, the differences were around 40% during the year. However, for soil V a similar difference occurred during the growing season: the patterns for samples obtained 1 year later in the spring showed strong shifts compared to the patterns for samples obtained before planting (Fig. 2D).

**(i) Rhizosphere and cultivar effects.** At each plant growth stage in both soils, the rhizosphere-generated betaproteobacterial PCR-DGGE patterns grouped separately from the corresponding bulk soil patterns (Fig. 4B). For soil B, the patterns for all six cultivars tended to group together at the young-plant stage, whereas cultivar effects were observed at the flowering



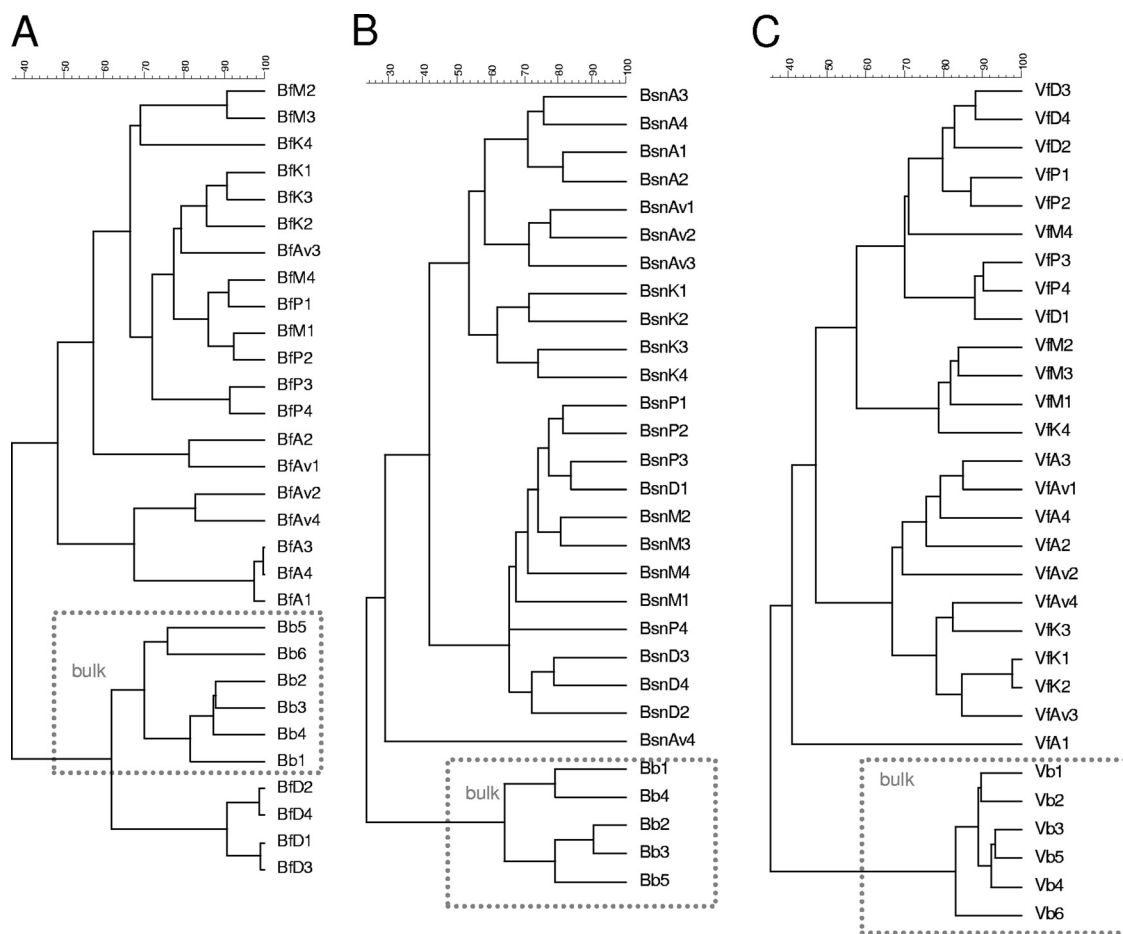


FIG. 3. Dendrograms showing the similarity of PCR-DGGE profiles generated with the bacterial 16S rRNA DGGE system for soil B at the flowering (A) and senescence (B) stages and for soil V at the flowering stage (C). B, Buinen soil; A, cultivar Aveka; Av, cultivar Aventura; K, cultivar Karnico; M, cultivar Modena; P, cultivar Premiere; D, cultivar Désirée; V, Valthermond soil; s, young-plant stage; f, flowering stage; sn, senescence stage.

and senescence stages. Specifically, at the flowering stage, cultivars A, Av, and K grouped together, while cultivars M, P, and D clustered separately (Fig. 4A). Monte Carlo permutation analysis showed that, at this growth stage, the two clusters were significantly different from each other ( $P < 0.05$ ). At the senescence stage, the clustering was comparable to that at the flowering stage, and the patterns for cultivars P and M became more similar (Fig. 4B). In contrast, for soil V the six cultivars showed clustering trends that were different from those for soil B. At the young-plant stage, the patterns for all cultivars except cultivars K and D grouped together. Monte Carlo analysis showed that the cultivar K and D patterns were significantly different from those for the other cultivars ( $P < 0.05$ ). At the flowering stage, the patterns for cultivars Av, M, P, and D clustered together, whereas those for cultivars A and K clustered separately from this group. The patterns for the six cultivars grouped together at the senescence stage ( $P > 0.05$ ).

Finally, these analyses also showed that there was a clear effect of soil type on the plant-associated betaproteobacterial communities, since for the same cultivars the community structures were different for the two fields (data not shown).

**(ii) Plant growth effect.** A plant growth effect on the betaproteobacterial communities was observed for all six cultivars for both soils. However, there were different trends over time for the two soils. For cultivars A and Av in soil B, the patterns at the senescence stage were significantly different from those at the young-plant and flowering stages (which clustered together in RDA). Monte Carlo permutation tests supported this finding, as the young-plant stage patterns were statistically similar to those for the flowering stage ( $P > 0.05$ ). For cultivar K, the patterns for all three growth stages grouped together, whereas for cultivars M, P, and D the patterns were significantly different for the three growth stages for each cultivar ( $P < 0.05$ ).

For soil V, the patterns for cultivars Av, K, and D clustered similarly: the patterns for the young-plant and flowering stages clustered together, whereas the patterns for the senescence stage clustered separately. Monte Carlo permutation tests showed that the differences were significant. While the patterns obtained for the flowering and senescence stages of cultivars A and M grouped together, those for the young-plant stage grouped separately from the combined cluster.

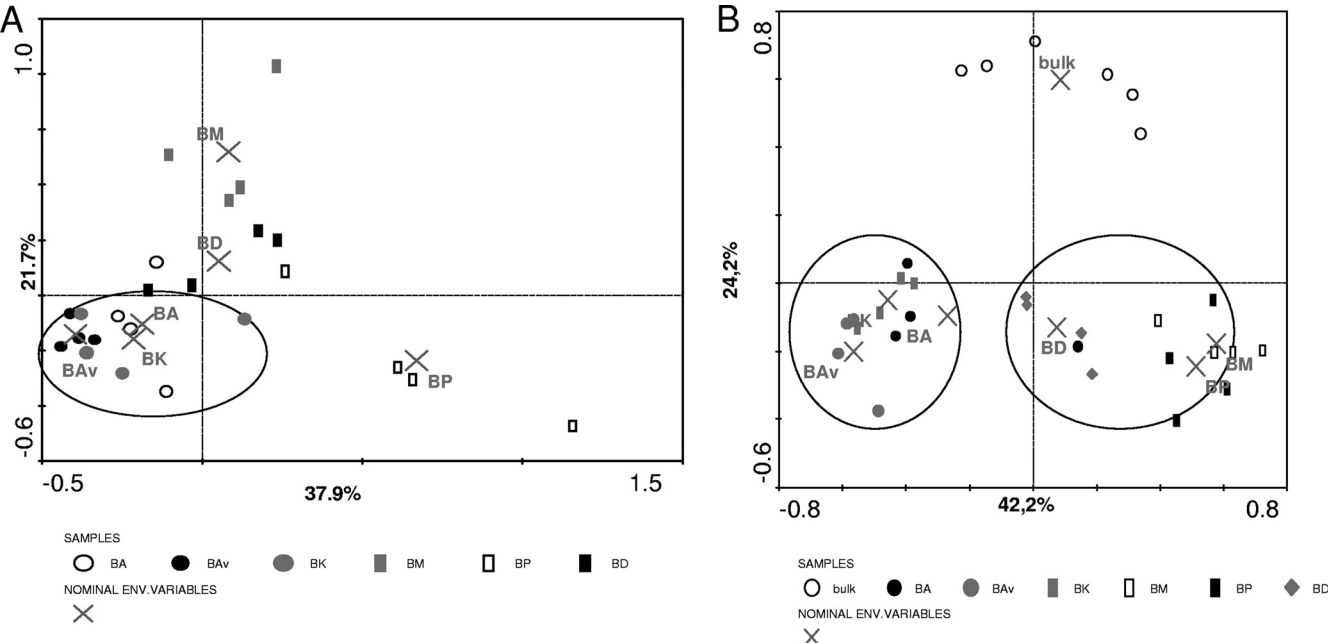


FIG. 4. Ordination biplot diagrams generated by redundancy analysis (RDA) of betaproteobacterial communities in the soil B rhizosphere and bulk soil at the (A) flowering and (B) senescence stages. B, Buinen soil; A, cultivar Aveka; Av, cultivar Aventura; K, cultivar Karnico; M, cultivar Modena; P, cultivar Premiere; D, cultivar Désirée; V, Valthermond soil; s, young-plant stage; f, flowering stage; sn, senescence stage; ENV., environmental. The eigenvalues on the axes indicate the percent variation in PCR-DGGE ribotypes; percent variation is indicated on the axes.

Contrary to the results for soil B, the patterns obtained during growth of cultivar P grouped closely together, which was supported by Monte Carlo analysis ( $P > 0.05$ ).

**Analysis of betaproteobacterial clone libraries.** Since the betaproteobacterial PCR-DGGE analyses revealed that there were clear differences between the cultivars with high-starch-content tubers and the cultivars with low-starch-content tubers at the flowering stage, one high-starch-content cultivar and one low-starch-content cultivar (cultivars A and P, respectively), in addition to corresponding bulk soil, were used for construction of three betaproteobacterial 16S rRNA gene clone libraries. After quality and chimera checks, 121, 115, and 84 sequences were obtained from bulk soil and the cultivar A and P rhizospheres, respectively. Shuffling analysis (49) showed that each library was significantly different from the other libraries. Rarefaction curves were then generated to assess the depth of sampling and the richness of the libraries, using 97 and 95% cutoffs for grouping of OTUs at the “species” and “genus” levels, respectively. None of these curves reached the plateau level. Although we did not sample to saturation, both rarefaction and Chao1 analyses showed that there were significant cultivar effects on the betaproteobacterial communities, and the library obtained from cultivar A was the most diverse library based on rarefaction curves and nonparametric Chao1 richness estimates (Fig. 5B). In addition, these analyses showed that the cultivar P library had the lowest richness, and the bulk soil library was intermediate.

All sequences, even those that were similar to database entries at low levels of similarity (i.e., <97%), were affiliated with betaproteobacterial 16S rRNA gene sequences (see Fig. S2 and S3 in the supplemental material).

Using RDP library comparison at a confidence threshold of

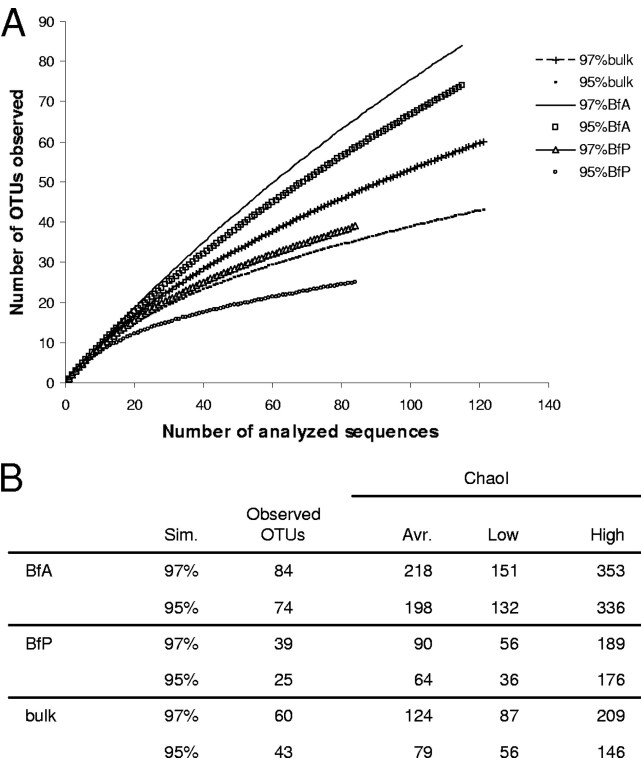


FIG. 5. (A) Rarefaction curves for observed operational taxonomic units (OTUs) at the “species” (97%) and “genus” (95%) levels for partial betaproteobacterial 16S rRNA gene sequences retrieved from soil B for cultivars P and A at the flowering stage and from bulk soil. (B) Chao1 richness estimates with the corresponding confidence limits (95%), as well as the number of OTUs determined with DOTUR for each case evaluated. B, Buinen soil; f, flowering stage; A, cultivar Aveka; P, cultivar Premiere; Sim., similarity.

TABLE 1. Comparison of clone libraries (RDP analysis)

Level	Taxon	% of <i>Betaproteobacteria</i> at flowering stage in soil B		
		Cultivar P	Cultivar A	Bulk soil
Family	<i>Comamonadaceae</i>	20.2	25.2	0.8
Genus	<i>Variovorax</i>	10.7	7	
Genus	Unclassified <i>Comamonadaceae</i>	7.1	13.9	0.8
Family	<i>Oxalobacteriaceae</i>	14.3	11.3	0.3
Genus	<i>Massilia</i>	6	1.7	0.8
Genus	Unclassified <i>Oxalobacteriaceae</i>	7.1	7	
Family	<i>Nitrosomonadales</i>	4.8	2.6	16.5
Genus	<i>Nitrospira</i>	4.8	2.6	16.5
Family	<i>Alcaligenaceae</i>	4.8	14.8	0.8
Genus	<i>Achromobacter</i>	1.2	11.3	0.8
Genus	<i>Dexia</i>	2.4		
Family	<i>Incertae sedis 5</i>	8.3	7	7.4
Family	<i>Burkholderiaceae</i>	28.6	15.7	28.1
Genus	<i>Burkholderia</i>	28.6	14.8	27.2
	Unclassified betaproteobacteria	15.5	3.5	32.2

80%, the majority of the sequences were affiliated with recognized classes of the betaproteobacteria (4), whereas the remainder (15 to 30%) were affiliated with unclassified betaproteobacteria (Table 1). In addition to differences in richness, there were significant differences in community makeup with respect to the prevalence of particular groups between the two rhizospheres and the bulk soil. For instance, sequences assigned to the family *Comamonadaceae* accounted for 25.2 and 20.2% of the clones from the rhizospheres of cultivars A and P, respectively, whereas this group made up only 0.8% of the amplicons generated from the bulk soil. Within the *Comamonadaceae*, the genus *Variovorax* was found to be abundant in both rhizospheres, accounting for 50 and 28% of the *Comamonadaceae* for cultivars P and A, respectively. In contrast, this genus was completely absent from the bulk soil library. The second highly abundant family in the rhizosphere was the *Oxalobacteriaceae*. The family *Alcaligenaceae* was also selected in both rhizospheres, but mostly in cultivar A rhizospheres. In particular, the genus *Achromobacter* ( $P < 0.0003$ )

was significantly dominant in cultivar A rhizospheres. On the other hand, the genus *Nitrospira* was more abundant in the bulk soil than in the rhizosphere. Finally, *Burkholderiaceae* were found to be equally (28%) abundant in the cultivar P and bulk soil libraries, whereas they accounted for 14% of the cultivar A library (Table 1).

**Quantification of desulfonating *Variovorax* spp. by using *asfA*-based qPCR.** Because of the abundance of *Variovorax* spp. in the rhizosphere libraries and the importance of *V. paradoxus* in the desulfonation process (45), the abundance of *asfA* genes was determined by qPCR to assess the putative effects of rhizosphere and cultivar on the desulfonation process. The *asfA* gene copy number did not change in the B bulk soil during the growing season. In contrast, in the V bulk soil a rapid increase in the *asfA* gene copy number was observed from June to July (see Fig. S1B in the supplemental material), while the copy number did not change between July and September.

In both soils, a rhizosphere effect on the *Variovorax asfA* gene abundance was clearly observed, since the abundance was much greater in the rhizospheres than in the corresponding bulk soils. This rhizosphere effect was significant at all growth stages for all cultivars, except for cultivar A at the young-plant stage in soil B (Fig. 6A). Moreover, an effect of cultivar on the dynamics of the *asfA* gene abundance was also observed. In soil B, the *asfA* gene abundance increased significantly for cultivars A, Av, K, and M from the young-plant stage to the flowering stage, and for cultivars P and D it increased from the flowering stage to the senescence stage. In soil V, the *asfA* gene abundance with cultivars A, Av, and K showed similar trends; i.e., the *asfA* gene abundance increased from the seedling stage to the flowering stage ( $P > 0.05$ ), and there were no significant differences for cultivars M, P, and D.

In soil B, the *asfA* gene abundance with the cultivars that produce high-starch-content tubers (cultivars A, Av, and K) showed trends that were different from the trends observed with the cultivars that produce low-starch-content tubers (cultivars P and D). In addition, the *asfA* gene abundance with cultivar M showed different trends depending on the soil; in

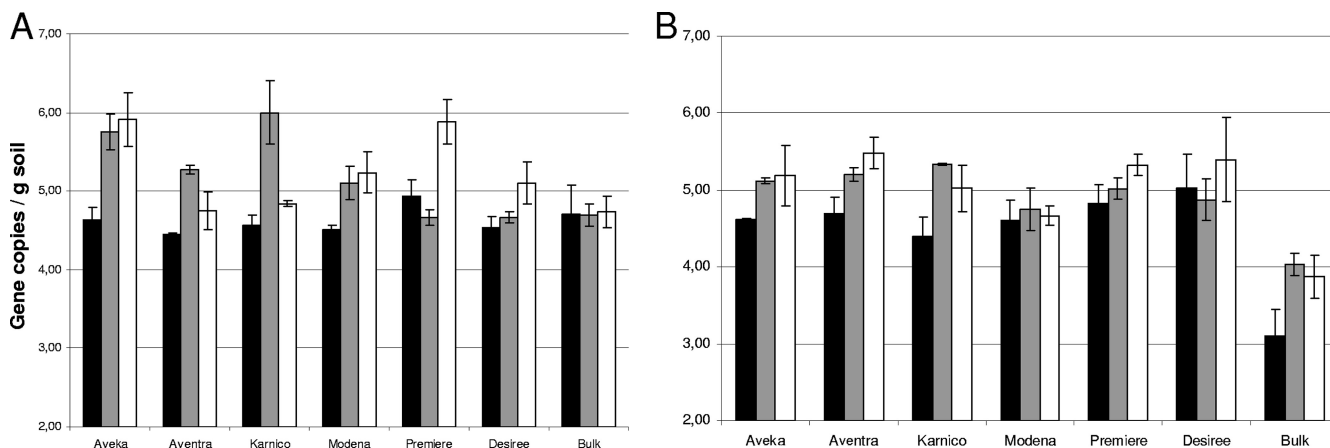


FIG. 6. Abundance of *Variovorax asfA* genes in rhizospheres of different potato cultivars and corresponding bulk soil at different growth stages in soil B (A) and soil V (B). The error bars indicate standard errors. Black bars, young-plant stage; gray bars, flowering stage; open bars, senescence stage.

soil B, the *asfA* gene abundance was between that with parent cultivar K and that with low-starch-content tuber cultivar P (Fig. 6B).

## DISCUSSION

In this study, we assessed the dynamics of the abundance and community structure of selected soil bacterial communities as a function of plant cultivar, growth stage, and soil type, using six potato cultivars grown in two soils with different textures. Clearly, effects of soil type, cultivar, and growth stage on plant-associated communities could be measured by our techniques. Soil microbial communities can be affected by many factors, such as soil characteristics, environmental conditions, and crop management strategies, like rotation and removal of crop residue (15, 33). Accordingly, community changes were observed over time in the bulk soil in both fields. Differences in the microbial community in bulk soil between the preplanting and young-plant stages have been shown previously (26). Interestingly, in our study drastic changes were observed in soil V after 1 year, which could have been caused by the accumulation of water in the field in the year after harvesting, due to the soil type. Thus, the changes in bacterial community structure might be explained by differences in water contents and oxygen limitations in water-logged soil. The changes in community structure were not associated with changes in bacterial abundance, except for the changes at the beginning of the growing season (before planting). This indicates that the agricultural practices used when the soil is prepared for planting (plowing and fertilization) might reduce the bacterial abundance regardless of the soil type.

When the effect of plants on bacterial abundance and community structure was analyzed, significant differences were observed in both fields at all growth stages. The increase in abundance in the rhizosphere compared to the bulk soil was expected, as the presence of substrates released by plant roots by exudation would have a direct effect on bacterial abundance. In soil V, however, the bacterial abundance remained roughly the same during the growing season. It is possible that due to the higher nutritional status of soil V (organic matter content and nutrients), the bacterial abundance in this soil was affected much less by root exudates.

Moreover, the rhizosphere bacterial and betaproteobacterial communities were also significantly different from the corresponding bulk soil communities. Rhizosphere microbial communities are known to be affected by complex interactions among soil type, plant species (genotypes), and growth (29, 43). Previous studies indicated that the rhizosphere population decreases as a plant matures (2, 43), whereas other studies showed that microbial diversity increases with plant age (22, 38). Even though in our study the rhizosphere did not always harbor more bacteria than the corresponding bulk soil, our findings do not imply that there was no rhizosphere effect. In fact, the influence of roots on bacterial populations in the rhizosphere may be small or ephemeral, but it is still present.

We obtained evidence that there was a cultivar effect, which was especially evident for the structure of the betaproteobacterial community. The usual PCR biases (1) may affect the frequency and/or presence of sequences in DGGE or clone library analyses. Preferential annealing is one such bias, and

selection of the most abundant sequences at the expense of low-abundance sequences is another bias. The less dominant groups might become apparent in PCR-DGGE analysis only if specific primers are used to reduce the complexity, which improves resolution of the rarer types. The analysis of the betaproteobacteria allowed us to examine such a less dominant group, which has previously been shown to account for around 4 to 16% of a soil bacterial community (50). Interestingly, the observed cultivar effect, which correlated with the tuber starch content and root development, also correlated with growth stage; this effect was absent at the young-plant stage and was more evident at the flowering stage. In the case of soil B, the cultivar effect became even stronger at the senescence stage for both bacterial community structure and betaproteobacterial community structure. For soil V, clear separation between cultivars was observed only at the flowering stage for the total bacterial and betaproteobacterial communities, but the correlation between tuber starch content and community structure was observed mainly for bacterial communities. As mentioned above, the difference for each soil can be a result of soil characteristics. Different cultivars with different growth rates and differences in root development are likely to release organic compounds to different extents, and the bacterial populations in the rhizospheres for the two cultivar groups with different tuber starch contents might have consisted of species that utilize different carbon sources. Thus, in response to changing root exudation patterns, the microbial community composition in the rhizosphere also changes with time and varies during the life cycle and with the seasonal response of plants (10). Also, the amount and type of compounds in the root exudate might show genotype-specific variations. In some cases, plant species may have a greater influence on microbial community composition than soil type (16, 55), whereas the effect of soil type on the community may be greater than the effect of plant species in other cases (6, 11, 48). Plant genotype-specific selectivity of plant roots for rhizobacterial community structure has also been observed in previous studies (11, 12, 35, 41, 53).

In order to better understand the effect of the differences in plant physiology (related to tuber starch content and root development) on rhizosphere bacterial diversity, clone libraries for two cultivars growing in soil B during the flowering stage were compared to clone libraries for the corresponding bulk soil. Surprisingly, the three libraries varied remarkably in terms of the estimates of the diversity of the betaproteobacteria. Specifically, the rhizosphere of the cultivar that produces high-starch-content tubers showed the greatest betaproteobacterial diversity, whereas the communities associated with the low-starch-tuber cultivar were the least diverse. It is reasonable to expect that the physiological changes that lead to plants with different tuber starch contents and growth rates incite changes in the quality and/or quantity of the exudates released by the roots. In this context, one could speculate that the roots of slowly growing plants that produce high-starch-content tubers would release more diverse organic compounds, which would sustain greater betaproteobacterial diversity. On the other hand, the low diversity observed in the rhizosphere of the cultivar that produces low-starch-content tubers and has a high growth rate could be due to simpler exudation patterns. For instance, differences in the abundance of *Achromobacter* were



observed. *Achromobacter piechaudii* has been shown to contain ACC deaminase activity, reduce the level of ethylene (32), and increase resistance to salt (31), flooding (17), and pathogen stress (54). Despite the differences in estimated diversity, the rhizosphere communities also showed some general trends, as several genera found in the clone libraries are known to contain plant growth-promoting bacteria. *B. phytofirmans*, which was found in both rhizosphere samples, can also reduce the level of ethylene (47). *Comamonadaceae* and *Oxalobacteriaceae*, which were very dominant in both rhizospheres, were found to be preferentially associated with mycorrhizal roots in *Medicago trunculata* (39, 40). Arbuscular mycorrhizal fungi were also shown to be present in most of the rhizospheres independent of cultivar type (E. Hannula, personal communication). Within the family *Comamonadaceae*, the genus *Variovorax* was dominant, accounting for up to 50% of the *Comamonadaceae* clones obtained from the cultivar with high starch content, whereas it was completely absent from the bulk soil. Moreover, based on our results, *V. paradoxus*, which has been found to be the key desulfonating species in the wheat rhizosphere (45), seems to be an important rhizobacterium with potato as well, indicating the relevance of this group for potato plants. It would be interesting to determine whether these results apply to other crops. Additionally, the role of the unclassified members of the *Comamonadaceae* in the rhizosphere, which accounted for 7 to 14% of the bacteria, is unclear, but they might be involved in desulfonation. Schmalenberger and Kertesz (46) showed that related species, such as *Polaromonas* and *Acidovorax* species, are involved in desulfonation in the wheat rhizosphere.

A remarkable observation was the abundance of the *asfA* gene in the soil V rhizosphere compared with the abundance in bulk soil, whereas there was a clear difference after the young-plant stage between soil B rhizospheres and bulk soils. Regardless of the soil type, the abundance of *asfA* in the cultivars varied during growth; for the high-starch-content tuber cultivars, the number of *asfA* gene copies increased as the plants matured from young plants to the flowering stage, whereas for the low-starch-content tuber cultivars, the *asfA* gene copy number remained rather stable throughout the growing season. The effect of the physiology of potato plants that produce tubers with different starch contents on the rhizosphere *V. paradoxus asfA* gene number may have overcome the soil type effect, even though the community structures of the betaproteobacteria associated with the soils were different. More studies are needed to confirm the role of *V. paradoxus* in desulfonation in the potato rhizosphere, as well as the role of other potential desulfonating bacteria.

In our study, the values for the genetically modified cultivar M fell in the range of values for the other cultivars for all variables measured. Thus, cultivar M did not have any outstanding effect on bacterial community structure and abundance. Previously, Milling et al. investigated the effects of a transgenic potato (which produced tubers with an altered starch composition) on the composition of bacterial and fungal communities in the rhizosphere (36). They compared a parent, a transgenic line, and another nontransgenic cultivar for three growing seasons. They did not observe any significant influence of the modification on the dominant members of the rhizosphere bacterial communities. Based on our results, we con-

cluded that although the cultivar M patterns were different from the cultivar K patterns, they were still similar to the patterns for the other cultivars (the baseline), particularly cultivars P and D. Interestingly, all three cultivars produced high-starch-content tubers, like cultivar M.

Our study showed that there was a strong effect of plant growth stage as well as soil type on the bacterial communities that were associated with potato. The potato cultivars grouped based on the starch content of the tubers, and hence the plant-specific variations in the rhizosphere bacterial communities correlated with effects of the tuber starch content on, e.g., the rhizosphere or root architecture. *V. paradoxus* was found to be abundant in the potato-associated bacterial communities, and the *asfA* gene, which is involved in desulfonation, was abundant in several cases. Moreover, the genetically modified cultivar fit the baseline, which fluctuated between low-starch-content potato cultivars and high-starch-content potato cultivars.

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