Thematic Issue on Horizontal Gene Transfer

Screening of rhizosphere and soil bacteria for transformability

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Natural transformation is assumed to be the most likely mechanism by which DNA from transgenic plants could be horizontally transferred to bacteria. In order to determine the occurrence of naturally transformable bacteria amongst bulk and rhizosphere soil bacteria, different transformation strategies were employed using either plasmid DNA (IncQ plasmids pSM1890 and pSM1885, conferring GFP, Sm^r, Gm^r and GFP, Sm^r, Tc^r, respectively) or genomic DNA from rhizosphere isolates, which were chromosomally tagged with mini-Tn5 (GFP, Tc^r), as transforming DNA. Transformation assays were done in microtiter plates (262 isolates and pSM1890 or pSM1885), on filters (i) with rhizosphere bacterial community mixed with pSM1890 or pSM1885, (ii) with 24 rhizosphere or soil bacterial isolates mixed with genomic DNA of the corresponding mini-Tn5-tagged strains, and in the rhizosphere of tobacco plants inoculated with rifampicin-resistant bacterial isolates and genomic DNA of the corresponding mini-Tn5-tagged strains added. One transformant colony was obtained when *Brevundimonas vesicularis* was transformed with genomic DNA of the corresponding mini-Tn5-tagged strain. Attempts to reproduce this result were unsuccessful. With this single exception, transformants were neither detected in the collection of isolates nor in the rhizosphere bacterial community. *Acinetobacter baylyi* BD413 used as a positive control showed drastically reduced transformation frequencies with plasmid pSM1890 as transforming DNA when mixed with the rhizosphere pellet. All transformants were characterized by BOX-PCR fingerprints, and three different BOX patterns were revealed. Sequencing the 16S rRNA gene showed that all transformants could be assigned to *Acinetobacter* sp. Since transformants were only observed in the positive control, the introduced BD413 either underwent genomic rearrangements, or competence of the *Acinetobacter* population present in the rhizosphere was stimulated by the introduction of BD413. The various transformation assays performed indicate that the proportion of rhizosphere or bulk soil bacteria which are naturally transformable is negligibly low.

Keywords: Horizontal gene transfer / transformability screening / soil bacteria / rhizosphere bacteria / *Acinetobacter baylyi* BD413

INTRODUCTION

Horizontal gene transfer by natural transformation relies on the uptake of naked DNA by competent bacteria from their surroundings, and its subsequent integration into the bacterial genome of the transformed bacterium (Lorenz and Wackernagel, 1994). The uptake of DNA from lysed cells or decaying material, a situation in which the DNA might still be protected, is usually also considered as natural transformation (Nielsen et al., 2000a). Furthermore, spontaneous transformation or transformation by lightning (Cérémonie et al., 2004; 2006) was

also described for some bacteria. Although natural competence is believed to be widespread among bacterial species (Dubnau, 1999; Lorenz and Wackernagel, 1994), the proportion and kind of bacteria in natural settings that can become competent, and the environmental conditions stimulating competence development, are largely unknown.

The molecular biology of natural transformation systems has been studied in great detail only for a rather limited number of bacterial species (reviewed by Dubnau, 1999). Natural competence was shown to be a genetically programmed physiological state permitting the efficient uptake of macromolecular DNA. Natural transformation

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is a tightly regulated process that requires an elaborate machinery with more than a dozen proteins involved. Transformability seems to be a property that is not shared by all isolates belonging to the same species, and transformation frequencies can vary up to four orders of magnitude among transformable isolates of a species (Maamar and Dubnau, 2005; Sikorski et al., 2002). It is important to note that only a certain proportion of isolates belonging to *Bacillus subtilis* or *Pseudomonas stutzeri* were shown to have the ability to become naturally competent (Maamar and Dubnau, 2005; Sikorski et al., 2002). Stable integration of the DNA entering competent cells is most frequently achieved by homologous recombination, by homology-facilitated illegitimate recombination, or as autonomously replicating extra-chromosomal DNA (de Vries and Wackernagel, 2002; Dubnau, 1999). In addition to differences in the DNA uptake processes, bacteria do not exhibit the same efficiency to integrate the incoming DNA by homologous recombination (Sikorski et al., 2002). There have been only a few reports on the development of competence state under environmental conditions (Kay et al., 2002; Nielsen et al., 2000b).

Natural transformation is thought to be the most likely mechanism for horizontal transfer of transgenic plant genomic or plastid DNA to naturally competent bacteria (de Vries and Wackernagel, 1998; de Vries et al., 2004; Gebhard and Smalla, 1998; Kay et al., 2002; Nielsen et al., 1998). Some naturally transformable bacteria such as *Acinetobacter* sp. or *Pseudomonas stutzeri* were shown to also be able to take up plant DNA, and stable integration of stretches of transgenic plant DNA by homologous recombination or homology-facilitated illegitimate recombination could be demonstrated by marker rescue (de Vries and Wackernagel, 1998; 2002; de Vries et al., 2001; Gebhard and Smalla, 1998; Meier and Wackernagel, 2003). Recently, the naturally transformable *Acinetobacter* sp. ADP1 strain and its derivative BD413 were shown to belong to the newly described species *Acinetobacter baylyi* (Vaneechoutte et al., 2006).

Although more than 80 species have been described as naturally transformable (de Vries and Wackernagel, 2004), we were interested to determine in a collection of recently isolated and identified soil or rhizosphere bacteria (Berg et al., 2002; Milling et al., 2004) the proportion of isolates which is naturally transformable. To be able to select transformants, the antibiotic resistance patterns of the isolates had to be determined. Two different strategies were followed to test the strains for natural transformation: (1) the use of plasmid DNA or lysed cells containing the broad-host-range *gfp*-tagged IncQ plasmids pSM1885 or 1890. Bacterial isolates harvested in the late exponential phase or rhizosphere pellets were tested for transformability with these DNA sources. (2) A selection of strains was chromosomally tagged with *gfp* and an antibiotic resistance marker. The genomic DNA extracted from chromosomally tagged isolates was used in transformation assays with the parental strains.
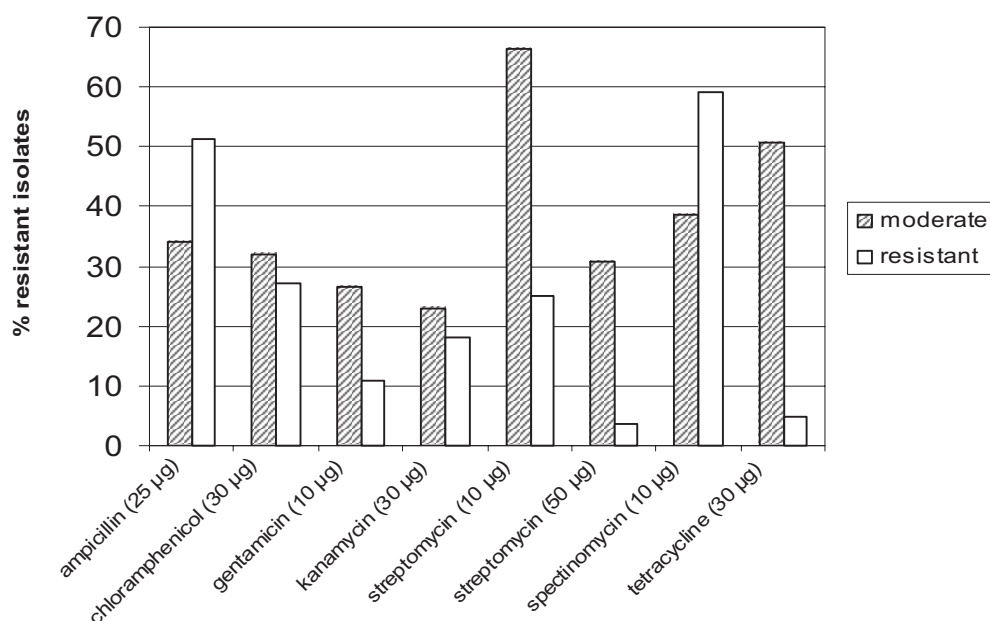
RESULTS AND DISCUSSION

Antibiotic resistance patterns

The sensitivity of a total of 81 gram-negative soil and rhizosphere isolates towards a range of antibiotics was screened by disc diffusion test, in order to determine which strains were sensitive to the antibiotics tested, and thus to choose suitable resistance markers to select potential transformants (Fig. 1).

The proportion of isolates that were resistant (inhibition zone < 8 mm) or moderately resistant (inhibition zone: 8–15 mm) to the antibiotics tested is shown in Figure 1. The majority of the isolates were sensitive towards high-level streptomycin (64.2%), followed by gentamicin (61.4%), kanamycin (57.8%) and tetracycline (43.4%). The two plasmids pSM1890 (conferring streptomycin and gentamicin resistance) and pSM1885 (coding for streptomycin and tetracycline resistance), both belonging to the IncQ group, were selected as transforming DNA, depending on the sensitivity of the soil or rhizosphere isolates towards the antibiotics to which pSM1890 or pSM1885 confer resistance.

Transformation of rhizosphere and soil bacteria by plasmids pSM1890 or pSM1885

Plasmids pSM1890 and pSM1885 (derived from the IncQ plasmid pIE723), both hosted in *E. coli,* were used either as plasmid DNA or as *E. coli* cell lysates in transformation assays performed in microtiter plates. Plasmids belonging to the IncQ group have a wide host range. The additional antibiotic resistance markers and the *gfp* marker gene should facilitate the detection of transformants. Furthermore, the presence of the IncQ plasmid in putative transformants can be confirmed by PCR with primers targeting IncQ replicon-specific sequences. A total of 262 potato rhizosphere or soil isolates harvested in the late exponential phase were tested in microtiter plate assays for transformability using cell lysates of *E. coli* pSM1890 or *E. coli* pSM1885. Cell lysates were used because Nielsen et al. (2000a) reported that the lack of DNA purity did not affect the uptake by the competent *Acinetobacter baylyi* BD413 strain. Putative transformants observed for different strains, which all contained the IncQ plasmid and the *gfp* gene, turned out to have the BOX fingerprints of *E. coli* pSM1890. Despite boiling overnight-grown *E. coli* cells, obviously some cells must have escaped this process.
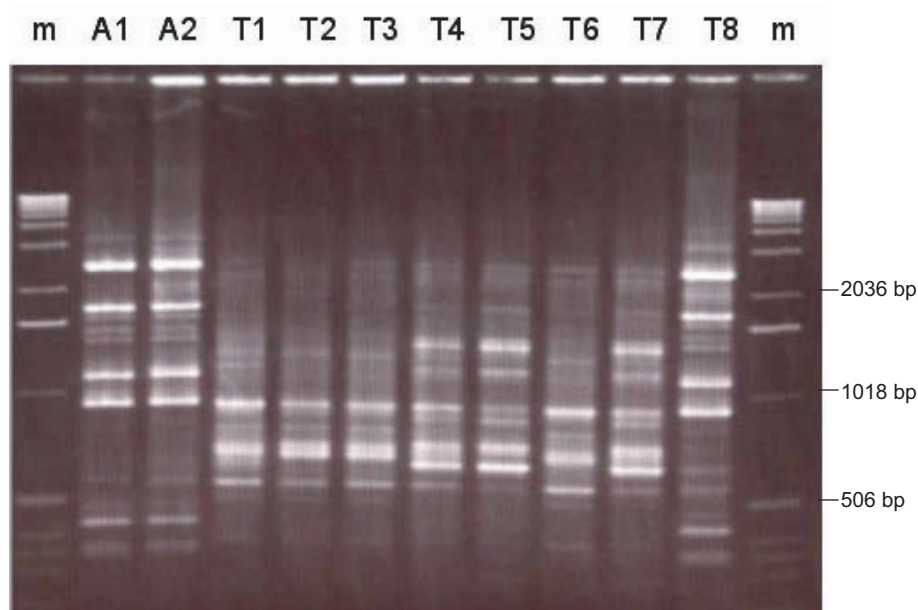
Figure 1. Proportion of potato rhizosphere and soil isolates (%) resistant (inhibition zone < 8 mm) or medium resistant (inhibition zone 8–14 mm) to the antibiotics tested in the disc diffusion test: inhibition zones measured after 24 h incubation at 28 °C.

In a second approach, the transformability of tomato rhizosphere bacteria by plasmid pSM1890 was tested without prior isolation of bacteria. The microbial pellet freshly obtained from the rhizosphere of tomato plants grown under greenhouse conditions was mixed with *E. coli* pSM1890 cell lysates. To verify the transformability of *Acinetobacter baylyi* BD413 under these conditions, the tomato rhizosphere pellet, mixed with *Acinetobacter baylyi* BD413 cells harvested at the late exponential stage, and cell lysates of *E. coli* pSM1890 were used as positive controls. Green fluorescent colonies could only be detected for the positive controls. No effect of the nutrient solution added (Nielsen et al., 1997b) was found, because two green fluorescent colonies were detected in the assay with nutrients added and six for the two controls without nutrients added. The presence of pSM1890 could be verified for all transformants of the positive control by PCR and Southern blot analysis (data not shown). Surprisingly, the analysis of BOX-PCR profiles of all eight transformants revealed three distinct profiles (Fig. 2). Partial 16S rRNA gene sequencing of one representative of each BOX type showed that all sequences had the highest similarity with *Acinetobacter* sp. DSM587. The addition of competent cells of *Acinetobacter baylyi* BD413 to the rhizosphere pellet seemed to have stimulated transformation of other *Acinetobacter* strains, and it is tempting to speculate on the presence of a competence stimulating-compound that is recognized also by other members of the *Acinetobacter* complex.

Obviously, transformation frequencies of *Acinetobacter baylyi* BD413 added as positive control were drastically reduced in the presence of the rhizosphere microbial community. This confirms earlier observations of drastically reduced transformation frequencies for *Acinetobacter baylyi* BD413 in non-sterile soil microcosms (Nielsen et al., 1997a; 2000a). No transformants were detected in the experiments without *Acinetobacter baylyi* BD413 added. Few colonies grown on the selective medium used to select transformants were isolated and their genomic DNA was used as template for IncQ *oriV* PCR. No signal was detected, and thus it can be concluded that under the conditions used no bacterial cells from the rhizosphere pellet were transformed with the IncQ plasmid pSM1890.

Transformation of rhizosphere bacteria by chromosomal DNA

Transformation frequencies of *Acinetobacter baylyi* BD413 with chromosomal DNA were reported to be at least two orders of magnitude higher than for plasmid DNA (Gebhard and Smalla, 1998). Furthermore, several studies have shown that the presence of homologous sequences is essential for successful chromosomal integration either by homologous recombination or by homology-facilitated illegitimate recombination (de Vries and Wackernagel, 2002; Gebhard and Smalla, 1998; Meier and Wackernagel, 2003). In order

Figure 2. BOX-PCR of GFP-positive transformants obtained after filter transformation of *Acinetobacter baylyi* with pSM1890 in the presence of the microbial pellet obtained from the tomato rhizosphere. Lane 1 *Acinetobacter baylyi* BD413, lane 2 *Acinetobacter baylyi* BD413 (pSM1890), lane 3 to lane 10 GFP-positive transformants. Partial sequencing of the 16S rRNA gene revealed that T6, T7, T8 were identical and showed 99% sequence identity with *Acinetobacter* sp. ADP1 (CR.543861.1).

to detect the transformation of rhizobacteria by chromosomal DNA, a total of 24 gram-negative strains belonging to different taxonomic groups (Tab. 1) were chromosomally tagged by introducing a mini-Tn5 carrying a *gfp* gene and a tetracycline resistance gene. The genomic DNA of tagged strains was used for transformation of the parental strain. Only one green colony was detectable for a *Brevundimonas vesicularis* isolate (identified by fatty acid methylester analyses with ID 0.78%). The BOX fingerprints of the *gfp*-tagged strain and the original strain were identical, indicating that indeed this strain acquired genomic DNA and stably integrated it into its chromosome. However, this result could not be reproduced.

Transformation of rhizosphere and soil bacteria by DNA of gfp- and tet-tagged genomic DNA in soil microcosms

Demanèche et al. (2001) reported that natural transformation of *Pseudomonas fluorescens* and *Agrobacterium tumefaciens* isolates was observed under soil conditions but not on plates. These striking observations stimulated us to perform microcosm experiments with seven soil and eight rhizosphere isolates for which bright fluorescence was detected after tagging. Overnight cultures of the non-tagged rifampicin-resistant mutants re-suspended in sterile saline were introduced in soil microcosms by

watering the tomato plants. Approx. 1 mg.mL^{-1} of genomic DNA from the corresponding *gfp*- and *tet*-tagged isolate in 5 mL water was added to the plants inoculated with the non-tagged rifampicin resistant mutant. Controls were treated similarly, but without adding genomic DNA.

Plating the cell suspensions recovered from the rhizosphere of tomato plants after 24 h onto selective media containing rifampicin and tetracycline revealed that none of the inoculated strains captured the *gfp* and the *tet* marker genes.

CONCLUSIONS

The results of the transformation assays performed indicate that the number of naturally transformable bacteria colonizing bulk or rhizosphere soils might be low. However, we are fully aware that only a very limited number of bacterial isolates could be tested. In addition, it might well be that the transformation conditions optimized for *Acinetobacter baylyi* BD413 are less appropriate for isolates belonging to other species.

Sikorski et al. (2002) describe that within a population of *Pseudomonas stutzeri* strains from soil, non-transformability and different levels of transformability were associated with distinct genetic subgroups. Furthermore, the detection of naturally transformable bacteria

Table 1. Bacterial isolates which were chromosomally labeled with *gfp* and *tet*.

Strain	Species	FAME ID **or 16S sequence similarity**
RN86*	***Pseudomonas cannabina***	**99%**
KS49*	***Pseudomonas lutea***	**97%**
KN89	***Pseudomonas fluorescens*****	**99%**
KN92	*Pseudomonas putida* (biotype B)	0.49%
KS70	***Pseudomonas fulgida*** (DSM14938)	**98%**
KS63	*Pseudomonas syringae* (glycine)	0.79
KF85	n.d.**	
KS25	*Pseudomonas agarici*	0.66
KN74*	***Pseudomonas fluorescens F113***	**99%**
KS16*	***Pseudomonas fluorescens* (F113)**	**100%**
KU20	***Pseudomons fluorescens***	**99%**
21 (30/99)*	*Pseudomonas putida*	0.74
23 (30/99)*	*Cedacea davisae*	0.67
58 (30/99)*	*Pseudomonas putida*	0.84
64 (30/99)*	*Acidovorax facilis*	0.77
229 (60/99)*	*Pseudomonas mendocina*	0.8
282 (60/99)*	*Pseudomonas chlororaphis*	0.59
226 (99/99)*	*Stenotrophomonas maltophilia*	0.73
76 (60/00)*	*Stenotrophomonas maltophilia*	0.84
96 (60/00)*	*Varivorax paradoxus*	0.56
120 (60/00)	*Comamonas acidovorans*	0.57
137 (60/00)	*Agrobacterium radiobacter*	0.86
190 (60/00)*	*Ochrobactrum anthropi*	0.93
69 (99/00)*	*Brevundimonas vesicularis*	0.78

* Strains with bright GFP-fluorescence which were selected for transformation tests performed in tobacco-soil microcosm; species names given in bold were determined by 16S rRNA gene sequencing. n.d.: Not determined.

might be impaired because not all members of a transformable species can develop natural competence, and also the efficiency of genomic integration is variable. For *Bacillus subtilis* it was recently shown that only 10% of the cells of a population develop natural competence as a result of different expression of genes coding for proteins involved in competence development (Berka et al., 2002; Maamar and Dubnau, 2005).

Although long-term persistence of transgenic plant DNA under field conditions has been shown (de Vries et al., 2003; Gebhard and Smalla, 1999), the small proportion of naturally transformable soil and plant-associated bacteria observed in this study supports the conclusions drawn by van den Eede et al. (2004) that for genetically modified (GM) food and feed there is only very little reason to expect, to assume that their consumption would imply any generalized risk. The finding that only a very small proportion of soil and plant-associated bacteria was able to take up free DNA which was of bac-

terial origin and should have ensured its stable establishment, provides further support for the assumption that the likelihood of gene acquisition from pollen, rhizodeposits or decaying plant material from GM plants by bacteria is extremely low, considering that plant genome complexity was reported to be another factor limiting *in situ* transfer (Bertolla et al., 2000).

MATERIALS AND METHODS

Strains and plasmids

Bacterial strains tested for natural transformability using several approaches were previously isolated from soil or rhizosphere of potato plants in the study of Milling et al. (2004), from the rhizosphere of oilseed rape, potato and strawberry plants (Berg et al., 2002) and from suppressive soils (Adesina et al., 2007). All strains were previously

identified by analysis of fatty acid composition (FAME) or 16S rRNA gene sequencing.

E. coli strains containing non-selftransferable IncQ plasmids pSM1890 (GFP, Gm^r, Sm^r) and pSM1885 (GFP, Tc^r, Sm^r) were provided by Søren Molin (Technical University Denmark, Lyngby) and the construction of the plasmids was described by Haagensen et al. (2002).

The naturally transformable strain *Acinetobacter baylyi* BD413 (Palmen et al., 1993) was used as positive control for transformation with rhizosphere cell pellets obtained from tomato plants.

The *E. coli* (MT102) JB538 pJBA137 (mini-Tn*5* with tetracycline and gfp mut 3* constitutively) strain was used as donor and IncP RK600 in *E. coli* HB101 as helper plasmid in triparental matings to chromosomally tag a rhizosphere isolate.

Monitoring of antibiotic resistance phenotypes

Eighty-one bacterial isolates from potato rhizosphere and soil (Milling et al., 2004) were screened by disc diffusion test for antibiotic resistance towards ampicillin (25 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (10 and 50 µg), spectinomycin (10 µg) and tetracycline (30 µg). The inhibition zone was measured after 24 h incubation at 28 °C.

Plating

All transformation assays were performed on Mueller-Hinton agar (MHA, Merck). To select putative transformants that acquired pSM1890 or pSM1885 in transformations performed in microtiter plates, the resuspended bacterial lawns were serially diluted and plated onto MHA supplemented with streptomycin (50 µg.mL^{-1}) and gentamicin (20 µg.mL^{-1}) or streptomycin (50 µg.mL^{-1}) and tetracycline (20 µg.mL^{-1}), respectively. To determine recipient counts, serial dilutions were dropped in triplicates on MHA without antibiotics added.

Putative transformants in rhizosphere cell pellets of tomato plants were selected by plating on MHA supplemented with streptomycin (50 µg.mL^{-1}), gentamicin (50 µg.mL^{-1}), chloramphenicol (30 µg.mL^{-1}), kanamycin (50 µg.mL^{-1}) and cycloheximide (100 µg.mL^{-1}) to select for transformants, and only MHA supplemented with cycloheximide for recipient counts.

Transformation assays and controls using genomic DNA tagged with *gfp* and *tet* as transforming DNA were selected by plating on Plate-Count-Agar (PCA, Merck) supplemented with cycloheximide (200 µg.mL^{-1}), rifampicin (50 µg.mL^{-1}) and tetracycline (20 µg.mL^{-1}).

Recipient cells were dropped on PCA only supplemented with cycloheximide 200 µg.mL^{-1} and rifampicin 50 µg.mL^{-1}.

All plates were incubated at 28 °C for two to three days, and colonies grown were checked for green fluorescence.

Genomic and plasmid DNA extraction

Preparation of genomic DNA from putative transformants or wild-type strains was done by a first lysis step according to the Qiagen protocol, and followed by genomic DNA preparation using the Ultra clean TM^{15} DNA purification kit (MoBIO).

Plasmid DNA was extracted with Qiagen plasmid extraction kit (maxi), while plasmid DNA from putative transformants was extracted using the MoBio mini plasmid preparation kit or Qiagen plasmid DNA extraction kit (mini).

Cell lysates of the strains *E. coli* pSM1890 or pSM1885 were prepared by harvesting overnight-grown cells by centrifugation, resuspension of the cell pellets in distilled water and freeze-boiling of aliquots.

PCR conditions and sequencing

To check putative transformants for the presence of the IncQ plasmid pSM1890 or pSM1885, PCR with the primers IncQ/oriV1 5' CTC CCG TAC TAA CTG TCA CG 3' and IncQ/oriV2 5' ATC GAC CGA GAC AGG CCC TGC 3' was done according to Götz et al. (1996).

BOX-PCR fingerprints were obtained after PCR amplification of genomic DNA with the primer BOX A1R 5' CTA CGG CAA GGC GAC GCT GAC G 3' as described by Rademaker and de Bruijn (1997).

PCR detection of the *gfp* gene in putative transformants in soil or tobacco rhizosphere was done with the primers gfp F: 5' ATA TAG CAT GCG TAA AGG AGA AGA ACT TTT CA 3' and gfp R: 5' CTC TCA AGC TTA TTT GTA TAG TTC ATC CAT GC 3' according to Andersen et al. (1998). PCR mixes contained Taq polymerase (Stoffel fragment), Stoffel buffer, 0.2 mM deoxynucleoside triphosphates, 3.75 mM MgCl$_2$, 0.2 µmol of primers gfp F and gfp R. After a 7 min step at 94 °C, 30 cycles of amplification followed, consisting of 1 min denaturation at 94 °C, 1 min of primer annealing with annealing temperature of 67 °C, and 1 min of primer extension at 72 °C, followed by a 10 min final extension step at 72 °C.

Putative *Acinetobacter baylyi* BD413 transformants (positive control) obtained after transformation in the presence of tomato rhizosphere cells which displayed different BOX-PCR patterns were chosen for partial 16S

rRNA gene sequencing. For sequence evaluation the computer program "arb" was used.

Southern blot hybridization

Plasmid-DNA or IncQ *oriV* PCR-products amplified from putative transformants obtained after microtiter plate screening were analyzed by Southern blotting (Sambrook et al., 1989) and hybridization of Southern blotted DNA (Boehringer, Mannheim, Germany) using a digoxigenin labeled IncQ *oriV* specific probe (Götz et al., 1996).

Transformation with plasmid DNA

To test a large number of bacterial strains for natural transformability, transformation assays were done in 24-well microtiter plates filled with MHA using freeze-boiled cell lysates of the strains *E. coli* pSM1890 or pSM1885 according to the sensitivity tested as transforming DNA. Overnight-grown strains were harvested by centrifugation, and the cell pellets were resuspended in 0.85% NaCl. In each transformation assay 100 μL of each culture were mixed with 50 μL cell lysates per well (done in duplicate), controls were cultures without transforming DNA. Microtiter plates were incubated at 28 °C for 24 h. After resuspending cells in 1 mL 0.85% NaCl, different dilution steps were dropped in three replicates on selective media. Altogether 262 strains were screened for transformability.

To screen for naturally transformable bacteria in the rhizosphere, the microbial cell pellets obtained from tomato roots were mixed with plasmid DNA of pSM1890. Bacterial cell pellets from rhizosphere of tomato plants were obtained directly by diluting 5 g of rhizosphere with sterile water and shaking out the cells using a stomacher (Stomacher 400, Seward, Westbury, USA). To remove soil particles and root residues, the supernatant was centrifuged twice at $500 \times g$ for 3 min. The cell pellet was harvested from the supernatant by centrifugation at $20\,800 \times g$ for 30 min in Sorvall tubes. The microbial cell pellet was resuspended with 2 mL 0.85% sterile saline. One hundred μL of rhizosphere solution was mixed with 40 μL of plasmid DNA of pSM1890 or with 200 μL cell lysates of *E. coli* pSM1890. Controls were done without DNA and cell lysates. As a positive control, transformation assays with plasmid DNA or cell lysates and 360 μL of competent *Acinetobacter baylyi* BD413 cells were performed (Kay et al., 2002), and as negative control the rhizosphere pellet was mixed with *Acinetobacter baylyi* BD413 without DNA added. One replicate of these assays was done with nutrient solution (5X M9 buffer + 2% lactic acid) to stimulate the competence of bacterial strains as described by Nielsen et al. (1997b). Filter transformation was done according to Nielsen et al. (1997a).

Transformation with genomic DNA of *tet*-labeled strains

To test for the ability of bacterial strains to take up and stably integrate their own DNA, chromosomal DNA tagged with *tet* and *gfp* was used as transforming DNA. Twenty-four strains were tagged, and afterwards the identity of the transformed strains was confirmed by BOX-PCR. The intensity of GFP fluorescence varied depending on the strain. Chromosomal DNA of *gfp*- and *tet*-tagged strains with strong fluorescence was used to transform wild-type strains by filter transformation according to Nielsen et al. (1997a). Seven isolates from soil and eight isolates from rhizosphere were selected for transformation assays in soil or rhizosphere of tobacco plants. Spontaneous rifampicin-resistant mutants were generated by plating an overnight culture on PCA supplemented with 50 μg.mL^{-1} rifampicin. Rifampicin-resistant mutants were grown overnight at 28 °C, harvested by centrifugation and resuspended in 30 mL 0.85% NaCl. Cfu counts of the inoculum were determined by the drop test method. Fifteen mL of this culture was used to water tobacco plants grown in pots under greenhouse conditions for inoculation of rhizosphere or soil of tobacco plants, depending on from where the isolates originated. Controls of rhizosphere and soil were inoculated with 15 mL culture but no DNA was added later. Pots were incubated for 24 h and afterwards 6 mL per pot of the nutrients solution 10 X M9L-25P [10 X M9 buffer + 2% lactic acid + 25 X phosphate salts (169.5 g.L^{-1} Na$_2$HPO$_4$ and 75 g.L^{-1} K$_2$HPO$_4$)] was added according to Nielsen et al. (1997b) to stimulate the competence of bacterial strains. One hour after the addition of the nutrient solution, 1μg.μL^{-1} of *gfp*- and *tet*- labeled DNA in 5 mL distilled water was added to the appropriate wild-type strain. No DNA was added to the control pots. After 24-hour incubation sampling was done. One g of soil or rhizosphere was sampled in five replicates and resuspended with 9 mL sterile saline by inverting for 20 min, afterwards tubes were left for 20 min to let soil particles settle. The supernatant was plated in different dilution steps on selective media.

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