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Plasmid Transfer from *Pseudomonas putida* to the Indigenous Bacteria on Alfalfa Sprouts: Characterization, Direct Quantification, and In Situ Location of Transconjugant Cells

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The transfer of the plasmids pJKJ5 and TOL (pWWO) from *Pseudomonas putida* to the indigenous bacterial community on alfalfa sprouts was studied. Tagging with fluorescent protein markers allowed direct quantification of the introduced donor bacteria and of indigenous bacteria that had received the plasmids. The sprouts were observed for 9 days; during this time alfalfa seeds, inoculated with donor bacteria, developed to edible and subsequently decaying sprouts. The first transconjugants were detected on day 6 after donor inoculation and occurred at frequencies of 3.4×10^{-4} and 2.0×10^{-6} transconjugant cells per donor cell for pKJK5::*gfp* and TOL::*gfp*, respectively. Confocal laser scanning microscopy revealed that the sprouts were heavily colonized with donors and that most transconjugants were located around the hypocotyl and root areas. Randomly selected members of the indigenous bacterial community from both inoculated and uninoculated sprouts, as well as a representative part of the community that had received the plasmids, were characterized by polymorphisms of PCR-amplified ribosomal DNA (rDNA) spacer regions between the 16S and 23S genes, followed by partial 16S rDNA sequencing. This showed that the initially dominating genera *Erwinia* and *Paenibacillus* were gradually replaced by *Pseudomonas* on the fully developed sprouts. Transconjugants carrying either of the investigated plasmids mainly belonged to the genera *Pseudomonas* and *Erwinia*. The numbers of transconjugant cells did not reach detectable levels until 6 days after the onset of germination, at which point these species constituted the majority of the indigenous bacteria. In conclusion, the alfalfa sprouts provided an environment that allowed noteworthy frequencies of plasmid transfer from *P. putida* in the absence of selective pressure that could favor the presence of the investigated plasmids.

Studies of horizontal gene transfer among bacteria colonizing natural or seminatural environments are important for the understanding of the mechanisms leading to bacterial adaptation to a given set of conditions (9, 23, 32), for assessing risks associated with spread of resistance genes to bacteria that infect humans and animals, and for the identification of bacterial populations in the environment that might play a role as reservoirs for resistance genes (for reviews, see references 4 and 35). Investigations contributing to the understanding of the putative transfer of mobile genetic elements through the human food chain have mainly been focused on gene transfer in the digestive tract, whereas only very few reports on gene transfer taking place directly on human food exist (19).

Conjugal gene transfer requires cell-to-cell contact and is thus most likely to occur in environments with a high bacterial density. Alfalfa sprouts sold for consumption constitute such an environment, since production of the sprouts takes place at temperatures and humidity conditions that are favorable for bacterial growth, and it is known that raw sprout products contain a high number of bacteria (10, 24). The same is true for sprouts that are cultivated at home by the consumer from seeds sold for this purpose (15). The bacteria present on sprout products originate from those colonizing the seeds and are thus predominantly rod-shaped soil microorganisms (10). Sprouts are usually consumed raw, and mobile genetic elements present on the sprouts might be transferred to bacteria in the human gut after consumption. To our knowledge, there have been no studies of gene transfer on sprouts, and we felt that such an investigation would be an important contribution to the understanding of the putative spread of mobile genetic elements through the human food chain. In addition, sprouts constitute a good model environment for investigations of plasmid spread on plant surfaces in general, since a single sprout represents an entire plant, including root, hypocotyl, stem, and cotyledons.

We studied the transfer and establishment of two different conjugative plasmids from *Pseudomonas putida* KT2442 (2) to the indigenous bacterial community of alfalfa sprouts grown from seeds sold for use in home sprouting kits. The first plasmid chosen was pKJK5, which is isolated from the barley rhizosphere and confers resistance to tetracycline (28). Recent results from our laboratories have shown that this plasmid is a broad-host-range plasmid belonging to the IncP1 group (L. Mølbak, unpublished data). The second plasmid was TOL (also designated pWW0), which has a narrower host range (26) and carries genes that allow the bacterial host to degrade certain organic compounds, including benzyl alcohol (33, 34).

Fluorescent protein markers have previously proved to be useful in studies addressing spatial distribution of plasmid-carrying bacteria in environmental or seminatural settings (7, 11, 12, 22, 31). In the present study, the plasmids were tagged with the green fluorescent protein (GFP), whereas the donor

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strain was labeled with the *Discosoma* sp. red fluorescent protein (DsRed), thereby allowing in situ visualization of donors and transconjugants directly on the sprouts, as well as quantification by direct counting of single donor and transconjugant cells in homogenized samples. After isolation, green fluorescent transconjugants were characterized by polymorphisms of PCR-amplified ribosomal DNA (rDNA) spacer regions between the 16S and 23S genes (17). Partial 16S rDNA sequencing was used to further identify selected strains representing the different rDNA spacer groups. The indigenous nontransconjugant population present on the sprouts was characterized in the same way. This approach, including direct counting of donor and transconjugant population and characterization of indigenous transconjugant bacteria, has not previously been used in studies of gene transfer on sprouting plants.

Our results suggested that alfalfa sprouts cultivated from commercially available seeds constitute an environment allowing efficient conjugal gene transfer between the colonizing bacteria and that the formation of transconjugants occurred later than reported in other gene transfer studies (18, 20, 22, 27).

MATERIALS AND METHODS

Bacterial strains and plasmids. In all studies, the strain serving as donor was a derivative of *P. putida* KT2442 (2), named LM50 (L. Mølbak, J. E. Johansen, S. J. Sørensen, N. Kroer, and S. J. Binnerup, unpublished data), carrying two independent chromosomal insertions. One is constructed by insertion of the *lacI*^{q} into the chromosome of *P. putida* KT2442 by triparental mating (22). The other is a mini-Tn*5* cassette containing the *dsRed* gene (21) fused to the *Escherichia coli* ribosomal promoter, *rrnB*P1 (31), and was transferred to the chromosome of *P. putida* KT2442::*lacI*^{q} as described by Herrero et al. (16). LM50 is resistant to rifampin (100 μg/ml) and kanamycin (50 μg/ml).

Plasmid pKJK10 is a *gfp*-tagged derivative of the barley rhizosphere conjugative plasmid pKJK5 encoding resistance to tetracycline. For clarity, pKJK10 is referred to as pKJK5::*gfp* here. This plasmid, as well as the *gfp*-labeled TOL plasmid, have been described by Sengeløv et al. (28) and Normander et al. (22), respectively. Both of the plasmids carry mini-Tn*5* insertions (16) of the *gfpmut3b* (8) genes under the control of the LacI-repressible $P_{A1/O4/O3}$ promoter, as well as resistance to kanamycin.

Growth media. Donors harboring pKJK5::*gfp* were grown overnight in brain heart infusion (BHI; Oxoid) supplemented with tetracycline (Sigma) prior to inoculation onto alfalfa seeds and were selectively isolated from sprout samples on 10 mM sodium benzoate supplemented FAB minimal medium (25) containing tetracycline. Indigenous transconjugants were recovered from the sprouts on BHI agar supplemented with tetracycline and identified by the green fluorescence of the colonies.

Donors harboring the *gfp*-labeled TOL plasmid were grown prior to inoculation in BHI supplemented with kanamycin (Sigma) and then selectively isolated from sprout samples on 6 mM benzyl alcohol-supplemented FAB minimal medium containing kanamycin. Indigenous transconjugants were recovered from sprouts on BHI agar supplemented with kanamycin and identified by the green fluorescence of the colonies. In all cases, the concentration of tetracycline was 10 μg/ml, while the concentration of kanamycin was 50 μg/ml.

Isolation and detection of coliform bacteria, pseudomonads, and enterococci from sprout samples was performed on MacConkey agar no. 3, *Pseudomonas* CFC agar, and Slanetz and Bartley agar, respectively. Total counts of bacteria were enumerated on BHI agar. All media were purchased from Oxoid.

Growth of alfalfa sprouts and inoculation of donor bacteria. Four grams of organic alfalfa seeds (*Medicago sativa* L.) imported from the United States (Urtekram, 9550 Mariager, Denmark) was soaked for 1 h at 37°C in 25 ml of (i) tap water, (ii) donor strain *P. putida* LM50/pKJK5::*gfp*, or (iii) donor strain *P. putida* LM50/TOL::*gfp*. Donor bacteria were grown overnight, washed twice, and subsequently diluted 100-fold in tap water before use. Seeds were placed in the sprouting kit (Bergs Biosalat; Dako), from which the liquid was drained off.

The sprouting process was designed to imitate household conditions (consumer-scale). Three perforated horizontal trays stacked on top of an unperforated bottom tray composed the household sprouting kit. The upper tray worked as a lid; the seeds were located in the second tray. During growth some of the sprouts produced long roots moving downwards, and the third tray ensured an adequate distance to the bottom tray, where the surplus of water was collected. For watering, ca. 50 ml of unchlorinated tap water was poured twice a day into the upper tray and recollected as wastewater in the bottom tray. This amount of water, which contained fewer than 10 bacteria per ml, as verified by plating on BHI agar, was sufficient to ensure that the sprouts were kept humid. Temperature was kept constant at 20°C. All experiments were performed at least twice.

Analysis of sprout samples on solid media. At days 0, 1, 3, 6, and 9 after the seeds were placed in the sprouting kit, three subsamples of 10 seeds or sprouts were collected (by random selection) with sterile tweezers and placed in a stomacher bag. The samples were weighed, diluted 10-fold (wt/vol) in sterile water, and homogenized (Stomacher model 80 laboratory blender; Seward) for 2 min at maximum speed. Dilutions of the homogenized sprouts were spread on solid media for determination of the total numbers of bacteria, pseudomonads, coliform bacteria, and enterococci, respectively. In addition, the dilutions of sprout samples that had been inoculated with the donor strains were spread on solid media (described above) for identification of donor and transconjugant bacteria.

Platemating controls. In order to estimate the amount of gene transfer potentially taking place on the selective plates, 500-μl volumes of overnight culture of the donor strain and 500 μl of homogenized sprouts (without donor strain) containing potential recipient bacteria were mixed in different selected proportions and immediately spotted onto transconjugant selective plates. This assay was performed on each sampling day.

Direct quantification of donor and transconjugant bacteria by microscopy. Samples of homogenized sprouts were diluted and filtered through 25-mm-diameter black polycarbonate membrane filters (pore size, 0.2 μm; Osmonics, Vista, Calif.). The volumes filtered depended on the desired detection level and were chosen so that counting by eye was facilitated.

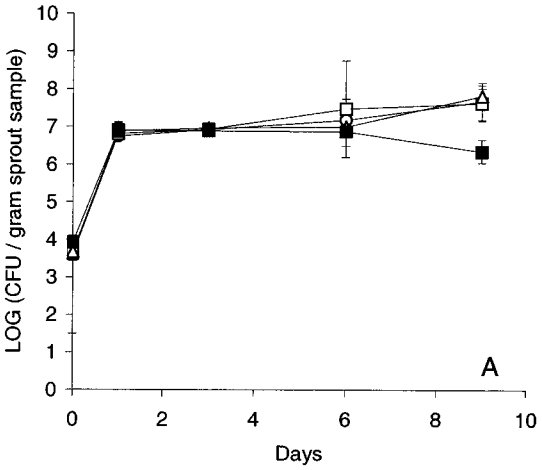
Bacteria were stained by placing the filters on a drop of 10 μg of DAPI (4′,6′-diamidino-2-phenylindole; Sigma)/ml and then washed twice by placing them on drops of distilled water.

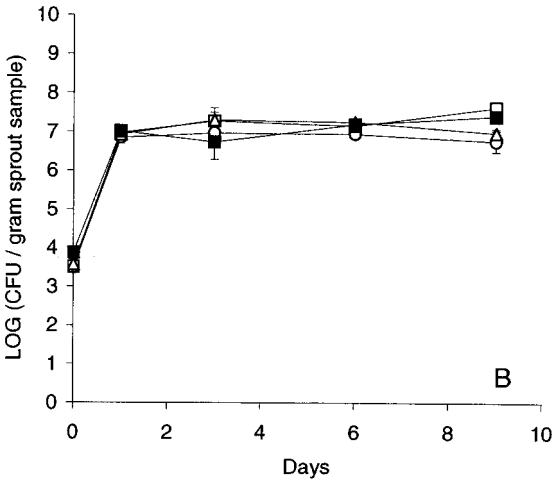
The numbers of DAPI-stained GFP- and DsRed-expressing cells were subsequently counted by using an Axioplan epifluorescence microscope (Carl Zeiss) equipped with a 50-W mercury lamp and the appropriate filter sets, allowing selective visualization of bacteria fluorescing at a given wavelength as desired (25).

Visualization of donor and transconjugant distribution on sprouts. On each day of sampling, three seeds or sprouts were picked from the home sprouting kits for microscopy. Each of the compartments—roots, seeds, stems, and leaves—was systematically investigated, and representative images of chosen areas were obtained. Visualization of GFP- and DsRed-tagged bacteria on sprouts was done by using a model TCS SP1 3 channel confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) equipped with an argon laser (458, 476, 488, and 514 nm) and two HeNe lasers (543 and 633 nm) and a variable spectrometric detection system that simultaneously monitored GFP and DsRed fluorescence. Images were processed for display by using Adobe Photoshop version 6.0.

Characterization of transconjugants and indigenous sprout bacteria. On days 1, 3, 5, and 9 of the experiment, ca. 30 colonies representing the indigenous sprout flora (including 10 from the uninoculated sprouts and 10 from the sprouts inoculated with each of the donor strains) were randomly picked, while the number of transconjugant colonies picked depended on the numbers of green colonies available. Several subcultivations (i.e., the transfer of green colonies to a new agar plate by using a sterile loop) were often required to obtain clean transconjugant colonies. A total of 394 colonies were isolated, including 120 representing the indigenous flora of the uninoculated sprouts, 118 representing the indigenous flora of spouts inoculated with a donor strain, 100 representing transconjugants carrying pKJK5::*gfp*, and 56 representing transconjugants carrying TOL::*gfp*. The 394 isolates were subjected to PCR amplification of the highly variable rDNA spacer regions between the 16S and 23S genes as described by Jensen et al. (17) by using the primers ITS-16S-1392-S-15 and ITS-23S-206-A-21 (P. Willumsen and B. M. Hansen, unpublished data). These isolates represented 20 different gel patterns and thus belonged to 20 different DNA spacer polymorphism groups. Of the 394 isolates, 59 were selected for partial 16S rDNA sequencing, ensuring that all DNA spacer groups were represented at least once.

PCR amplification of the 16S rDNA gene (first 500 bp) was performed with the primers SDBact0008aS20 and S*UNIV518Aa18 (1). PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Partial sequencing of the PCR products was carried out by MWG-Biotech AG by using an NEN Global IR^2 DNA Sequencer Li-COR and the primer SDBact0008aS20. The sequences obtained were compared to sequences in nucleotide databases by using the NCBI/BLASTN program (http://www.ncbi.nlm.nih.gov). The closest match was accepted as the closest relative.





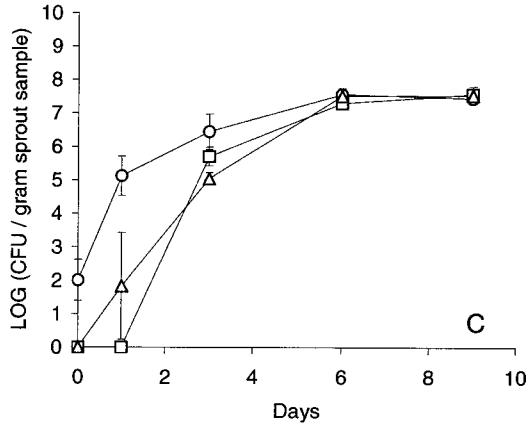


FIG. 1. Numbers of CFU of the total bacterial population (○), non-lactose-fermenting gram-negative bacteria (△), *Pseudomonas* including the donor strain (□), and the donor strain (■) measured on sprouts inoculated with donor bacteria containing either pKJK5::*gfp* (A), TOL::*gfp* (B), or without donor inoculation (C). The error bars represent the standard errors.

In vitro transfer of pKJK5::*gfp* and TOL::*gfp* to selected recipients. From the group of randomly isolated indigenous sprout bacteria, six representatives were chosen. The six isolates were collected on different sampling days and represented a number of different taxa on the uninoculated sprouts. Among the indigenous bacteria present at day 1, *Paenibacillus* sp. and *Erwinia* sp. were selected, while from day 3, another *Erwinia* sp. and a *Pseudomonas* sp. were selected. From days 5 and 9, strains of *Pseudomonas gessardii* and *Stenotrophomonas maltophilia*, respectively, were chosen. The six isolates were used as recipients of pKJK5::*gfp* and TOL::*gfp* in filter-mating experiments. From overnight cultures grown in BHI, 100 μl of donor and recipient strains (12 combinations) were mixed, placed on 25MM Whatman filters on nonselective agar plates, and incubated overnight at 30°C. Quantification of donor and transconjugant bacteria was done by fluorescence microscopy as described for sprout samples. The experiment was performed in triplicates.

RESULTS

Characterization of bacterial flora on alfalfa sprouts. Three days after introduction into home sprouting kits, most of the alfalfa seeds began to develop tiny sprouts. After 6 days, the sprouts appeared ready to eat, while advanced decay was observed after 9 days.

At the time of introduction into the home sprouting kit, the uninoculated seeds carried ca. 10^2 CFU/g, while the seeds inoculated with either of the two *P. putida* donor strains carried ca. 10^4 CFU/g. After 1 day of germination, the inoculated sprouts carried 10^7 CFU/g, and this number remained constant for the rest of the sampling period (Fig. 1A and B). On the uninoculated seeds, 10^5 CFU/g was detected after 1 day of germination. This number gradually increased to 10^7 CFU/g on day 6 and remained constant thereafter (Fig. 1C).

The donor bacteria constituted the major proportion of the bacteria present on the inoculated seeds (Fig. 1A and B), while bacteria belonging to the genus *Pseudomonas* were not detected on the uninoculated seeds until day 3 (Fig. 1C). A proportion of the bacteria present on the uninoculated seeds at day 1 represented non-lactose-fermenting gram-negative bacteria appearing as colorless colonies on MacConkey agar plates.

PCR amplification of the highly variable rDNA spacer regions between the 16S and 23S genes and subsequent partial 16S rDNA sequencing of randomly selected strains isolated from the uninoculated sprouts on BHI revealed that the dominant groups of bacteria at day 1 belonged to the genera *Erwinia* and *Paenibacillus.* With time, these genera were gradually replaced by *Pseudomonas* (Fig. 2). Low numbers of other bacteria (i.e., species of *Bacillus* and *Stenotrophomonas*) occurred occasionally (data not shown). Enterococci as defined by growth on Slanetz and Bartley agar or coliform bacteria as defined by red colonies on MacConkey agar no. 3 were not detected at any time (data not shown).

On alfalfa sprouts inoculated with donor bacteria, *Erwinia* and *Pseudomonas* were the only other genera found. Similar to what was observed for uninoculated sprouts, *Erwinia* spp. dominated the indigenous part of the microflora at day 1 but were gradually replaced by indigenous *Pseudomonas* spp. that dominated 9 days after the onset of the experiment (data not shown).

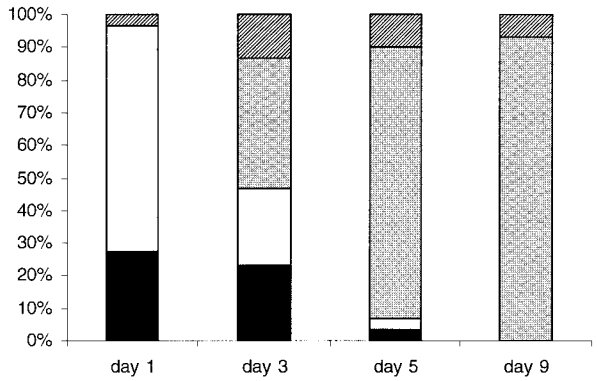


FIG. 2. Relative densities of major bacterial populations present on uninoculated sprouts at selected time points as identified by polymorphisms of PCR-amplified rDNA spacer regions and subsequent partial 16S rDNA sequencing. The results for *Pseudomonas* (▨), *Erwinia* (□), and *Paenibacillus* (■) spp. and for species either not identified or present in proportions of <5% (▨) are presented.

Transfer of pKJK5::*gfp* and TOL::*gfp* on sprouts. Direct counting of green (GFP), red (DsRed), and blue (DAPI) bacteria in sprout samples revealed the average numbers of transconjugants, donors, and total bacterial cells on the sprouts, respectively. In all cases the total numbers of bacteria per gram of sprout sample were ca. 10^8, gradually increasing to just less than 10^9 at the end of the experiments. The numbers of donor cells were generally between 5- and 10-fold lower (Fig. 3).

After 3 days of germination, the numbers of transconjugants were below or close to the detection limit of approximately two transconjugants per gram of sprout sample for both of the investigated plasmids (data not shown). After 6 days, however, ca. 10^4 transconjugants per g of sample were detected on the sprouts inoculated with *P. putida* LM50/pKJK5::*gfp*, and no noteworthy change in this number was seen after 9 days (Fig. 3A). The highest measured frequency of pKJK5::*gfp* transconjugant to donor cells occurred on day 6 and was ca. 3.4×10^{-4}.

The sprouts inoculated with *P. putida* LM50/TOL::*gfp* carried on the average 3.4×10^1 transconjugants per g of sample on day 6; this level increased to 2.7×10^2 per g of sample on day 9 (Fig. 3B). The highest measured frequency of TOL::*gfp* transconjugant cells per donor cell occurred on day 9 and was 2.0×10^{-6} per g of sample.

PCR amplification of the variable rDNA spacer regions between the 16S and 23S genes ($n = 394$), followed by partial 16S rDNA sequencing of a subset ($n = 59$) of strains together representing all of the 20 rDNA spacer groups identified, showed that most bacteria that had received plasmid pKJK5::*gfp* ($n = 100$) belonged to the genera *Erwinia* (64%) and *Pseudomonas* (28%), whereas 8% were not identified. Also, most indigenous sprout bacteria that had received TOL::*gfp* ($n = 52$) were identified as *Erwinia* (41%) and *Pseudomonas* (34%) spp., whereas 25% remained unidentified.

Control experiments performed to verify that the transconjugants picked for PCR amplification were not the result of conjugation events that took place after sampling showed that no transfer occurred on the selective plates used for transconjugant isolation.

Spatial distribution of donors and transconjugants on sprouts. Systematic confocal laser scanning microscopy of the sprouts revealed that the *P. putida* LM50 donor cells heavily colonized the alfalfa sprouts from day 1.

The rhizoplane was generally covered by a thick matrix of donor bacteria (Fig. 4A), many of which were motile. On the stem, the donor cells formed a monolayer, and the highest densities were observed in the curvatures of the epidermis cells. Transfer of pKJK5::*gfp* and TOL plasmids preferentially took place on the roots (Fig. 4A) and particularly at the hypocotyl (Fig. 4B) of the alfalfa seedlings. However, transconjugant cells were occasionally observed on the stem. Most of the bacterial cells that had received either of the plasmids were rod shaped and were observed as single cells, as well as clusters or chains of bacteria (Fig. 4B and C).

In vitro transfer of pKJK5::*gfp* and TOL::*gfp* to selected recipients. Filter-mating experiments showed that in vitro transfer of pKJK5::*gfp* occurred readily from the donor strain to three of the selected bacterial isolates naturally present on

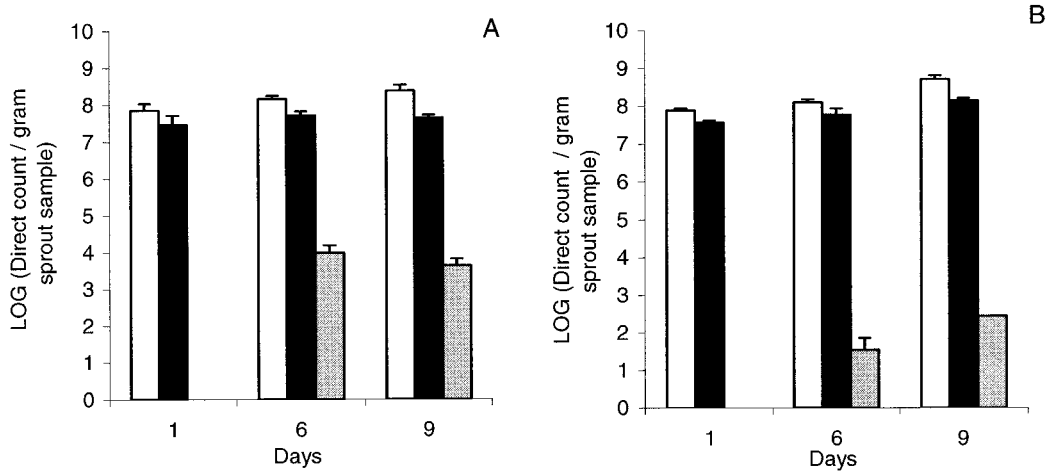


FIG. 3. Direct counts of total bacteria (□), donor cells (■), and transconjugant cells (▨) counted in sprout samples at days 1, 6, and 9 after inoculation with donor cells containing either pKJK5::*gfp* (A) or TOL::*gfp* (B). The error bars represent the standard errors.

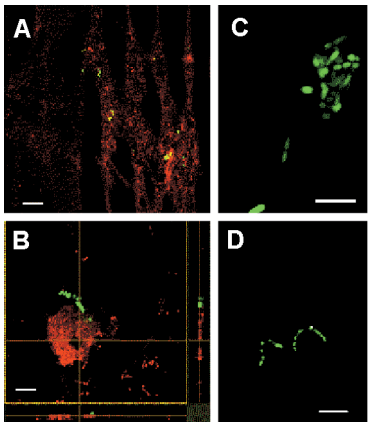


FIG. 4. Confocal laser scanning micrographs of alfalfa sprouts 6 days after the onset of germination and inoculation with red fluorescent donor bacteria *P. putida* LM50/pKJK5::*gfp*. Bacteria belonging to the indigenous microbial population on the sprouts, which have received the pKJK5::*gfp* plasmid, appear green. (A) Root area heavily colonized by donor cells. Several transconjugant bacteria are visible. Scale bar, 20 μm. (B) Xyz scan of hypocotyl area with microcolony of donor and transconjugant bacteria. Scale bar, 5 μm. (C and D) Micrographs of transconjugant bacteria with different cell morphologies. Scale bar, 5 μm. Observations of sprouts inoculated with *P. putida* LM50/TOL::*gfp* were similar to those of sprouts colonized with *P. putida* LM50/pKJK5::*gfp* (data not shown).

sprouts: one *Pseudomonas* sp. and two different isolates of *Erwinia* (isolates 1 and 2). However, filter-mating experiments with the donor strain containing TOL::*gfp* resulted in detectable transfer only to one of the six tested isolates: *Erwinia* isolate 2. Transfer of pKJK5::*gfp* to *Erwinia* isolate 1 and *Pseudomonas* sp. occurred at relatively high frequencies of ca. 5.6×10^{-2} (standard error of the mean [SEM] = 3.2×10^{-3}) and 1.6×10^{-2} (SEM = 5.5×10^{-3}) transconjugant per donor, respectively. Transfer to the third recipient, *Erwinia* isolate 2, occurred with significantly lower frequencies of ca. 1.5×10^{-4} (SEM = 1.0×10^{-4}) for pKJK5::*gfp*, and 7.2×10^{-4} (SEM = 1.7×10^{-4}) for TOL::gfp. Similar in vitro mating experiments with indigenous isolates of *Paenibacillus* sp., *S. maltophilia*, and *P. gessardii* as recipients did not result in detectable transfer of either of the investigated plasmids (data not shown). Consistently, these isolates represented rDNA spacer groups, which did not include transconjugants formed on sprouts.

DISCUSSION

Within the field of food microbiology, several studies addressing gene transfer in the intestinal tract of animals or humans have been performed. However, studies of gene transfer occurring directly on food products also contribute significantly to an understanding of the putative spread of genetic elements among bacteria throughout the human food chain.

Vegetables sold as ready-to-eat products are often colonized with a large amount of bacteria (10, 24). The conditions on the surface of such products are likely to be conducive to conjugational gene transfer, since it requires cell-to-cell contact, and for most plasmids present in gram-negative bacteria this takes place preferentially between surface-associated bacteria (5, 6). Alfalfa sprouts, cultivated in a home sprouting kit, provided a very useful nonsterile model system that allowed us to follow the development and decay of entire plants. The absence of soil in the system reduced the number of parameters to consider when interpreting the results, made the microscopy much easier, and reduced the necessary manipulations to a minimum. Also, a low content of chlorophyll, which is highly autofluorescent, facilitated the microscopy.

Our study included a rough mapping of the dominating groups of indigenous aerobic bacteria present on organically produced alfalfa seedlings during their development from seeds to edible and subsequently decaying sprouts. To the best of our knowledge, this has not previously been done. Bacteria belonging to the *Erwinia* and *Paenibacillus* genera dominated the microbial flora originally present on the seed but, as time progressed, these were outnumbered by pseudomonads (Fig. 1 and 2). These observations are consistent with another study showing that mainly rod-shaped bacteria are present on the surface of alfalfa sprouts (10).

Comparison of numbers from direct counts (Fig. 3) with data obtained by plating (Fig. 1) indicated that the donor cells were generally fully culturable, whereas a fraction of the total indigenous bacteria were not capable of growth on the media used in the present study. This should be kept in mind when the characterization of the indigenous sprout flora (Fig. 2) is evaluated, since only cultured bacteria were sequenced and identified. Also, it must be taken into account that the chosen agar medium (i.e., BHI) inevitably influenced which genera were found. The choice of this medium was based on the assumption that sprouts, which are known to be heavily colonized with bacteria (10, 24), represent an environment rich in nutrients, and that a rich medium would therefore support growth of the major fraction of sprout-associated indigenous bacteria.

Our data showed that the surface of alfalfa sprouts is an environment allowing plasmid transfer and that, in this environment, *Erwinia* and *Pseudomonas* spp. acted as recipients for both investigated plasmids. The DsRed-labeled *P. putida* donor strains readily colonized the surface of the sprouts (Fig. 1), and transfer of the GFP-labeled TOL and pKJK5 plasmids occurred (Fig. 3), resulting in transfer ratios comparable to (or lower than) the ratios obtained in other reports on *Pseudomonas* plasmid transfer in natural or seminatural systems (3, 22, 30).

Ratios of transconjugants per donor obtained in the filter-mating experiments by using selected indigenous isolates as recipients and designed to give a maximal yield of transconjugants were higher than the ratios observed on the sprouts. Isolates of *Paenibacillus* sp., *S. maltophilia*, and *P. gessardii*, which were present in significant numbers on the sprouts but not identified among the transconjugants, did not receive any of the two investigated plasmids in filter-mating experiments. *Erwinia* and *Pseudomonas* spp. were both identified among the transconjugants carrying either of the investigated plasmids, but the TOL::*gfp* plasmid was only transferred to one of the three selected representatives of these strains in filter-mating experiments. This indicates that the phenotype defined as the "putative TOL recipient" is not necessarily affiliated with a given species but more likely is affiliated with a given isolate. The isolates picked from the background microbial flora of uninoculated sprouts and subsequently used as recipients in filter-mating experiments may not have been identical to those identified as transconjugants from the sprout samples, and it is

known that conjugation ratios can vary considerably within different isolates of the same species (14).

On the sprouts, we observed a higher maximal frequency of transconjugants per donor cell for pKJK5::*gfp* (3.4×10^{-4}) than for the TOL plasmid (2.0×10^{-6}). We suggest that one explanation for this could be the difference in host range for these two plasmids. Although it has been shown that only intraspecies transfer of TOL takes place in soil (26), pKJK5 is an IncP1 plasmid and thus able to transfer to a number of different genera within the group of proteobacteria (Mølbak, unpublished). However, as mentioned above, characterization of transconjugant cells suggested that the indigenous bacteria acting as recipients for either of the plasmids belonged to the same genera (*Pseudomonas* and *Erwinia*). Still, as was the case for the characterization of indigenous sprout bacteria based on rDNA spacer regions, the choice of culture medium might have influenced the detection of these particular genera. An important difference between the two plasmids investigated was that filter-mating experiments to selected indigenous recipients showed generally higher transfer frequencies for pKJK5::*gfp* than for TOL:*gfp*. We therefore speculate that the lower mating efficiency of TOL donors than of pKJK5 donors observed on sprouts might be solely related to the maximal efficiency with which these plasmids are able to transfer.

Many reports on gene transfer in natural ecosystems show that transfer takes place, if at all, immediately after the introduction of a donor strain (13, 18, 20, 22). Still, in the present study, we detected no transconjugants until 6 days after introduction of the donor strain (Fig. 3) even though the concentration of donor bacteria reached its maximum already the first day after inoculation onto the seeds (Fig. 1). We suggest that the most important explanation for this is that the combined fraction of putative *Erwinia* and *Pseudomonas* recipients was initially low (Fig. 2). In some of the listed previous investigations (18, 20), a known recipient strain is added to the ecosystem and is thus present in relatively high numbers at the time of donor introduction. In the other studies (13, 22), which investigated plasmid transfer to indigenous microbial flora, the ecosystems were in a stable condition at the time of donor introduction, and it is therefore unlikely that large changes in the amount of putative recipients occurred as in the present study.

It is important to note that, unlike most other investigations of plasmid transfer, our study took place in an ecosystem that was undergoing a continuous development during germination of the seed and development of seedlings. In addition to the availability of recipients, other factors that were changing during the experiment and that might influence gene transfer could include the physical conditions on the surface of a seed versus that of a mature sprout. Transfer of each of the investigated plasmids took place preferentially on the root (Fig. 4), which was not fully developed until 6 days after the onset of germination, i.e., on the same day that the first transconjugants were detectable. Some studies indicate that plasmid transfer happens most efficiently when the availability of bacterial substrate is relatively high (29), and for the TOL plasmid it has been demonstrated that the physiological state of the recipient cells influence their ability to participate in transfer (7). We suggest that high concentrations of plant exudates around the root and the hypocotyl causes a high density of donors and putative recipients and perhaps also enhanced plasmid transfer and that these factors together explain why the large majority of transconjugants was observed in these zones of the seedlings (Fig. 4). An additional explanation could be that the roots of the sprouts growing in the home sprouting kit were exposed to higher humidity than other parts of the plants and that the presence of a liquid film on the surface of the roots would promote diffusion of nutrients, as well as bacterial motility. The latter would increase the number of encounters between donor and recipient bacteria and thus the frequency of transfer events. However, the decay of the sprouts that had taken place in the period between days 6 and 9 did not result in an increased amount of transfer (Fig. 3), even though it can be assumed that decay results in an increased release of nutrients as well as liquid from decomposed plant parts.

Some of the observed (green) bacterial cells on the sprout surface might originate from proliferation of the initially formed transconjugants, which would be expected to occur in areas with high substrate availability. Based on the observation that no increase in transconjugant numbers occurred from day 6 to day 9 (Fig. 3), we speculate that presence of the investigated plasmids did not result in increased bacterial fitness. On the contrary, transconjugants formed on the sprouts might have a lower potential for growth and colonization of the alfalfa plants than before reception of the plasmids due to the metabolic cost of expressing extra traits, which do not constitute a selective advantage in the given ecosystem. Since, to the best of our knowledge, the environment constituted by alfalfa sprouts did not contain benzyl alcohol, it was expected that the TOL plasmid would not provide the transconjugants with increased fitness. The rhizosphere plasmid pKJK5 has not yet been sequenced and might have provided the bacteria with an advantage by expression of uncharacterized phenotypes but, as explained here, this did not seem to be the case.

The present results can be relevant in the assessment of risks associated with the microbiological safety of ready-to-eat sprout products. We believe that our observations can be extrapolated to many other vegetables that are consumed raw, and we emphasize that mobile genetic elements can enter the human gastric system not only through food deriving from animals but also from ready-to-eat vegetable products. In addition, our observations contribute to the understanding of gene exchange between bacteria in dynamically developing ecosystems and underline the importance of describing the succession of bacterial populations indigenously present in such systems.

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