Associative Nitrogen Fixation by *Klebsiella* spp.: Adhesion Sites and Inoculation Effects on Grass Roots

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Adhesion sites on grass roots for *Klebsiella* strains carrying type 3 or type 1 fimbriae or both were determined. Adhesion of the strains to the roots of *Poa pratensis* and *Festuca rubra* was highly localized; the bacteria adhered strongly to root hairs and with a markedly lower efficiency to the surface of the zone of elongation and to the root cap mucilage. No adhesion to the epidermal cells between root hairs was observed. The adhesion sites were identical for the type 3- and 1-fimbriated bacteria and for *P. pratensis*, *F. rubra*, and *Trifolium pratense*. Inoculation of *P. pratensis* seedlings with *Klebsiella pneumoniae* strain AS resulted in morphological changes in plant roots. The roots of infected plants were heavily covered with root hairs, which often were deformed and branched.

The molecular processes in recognition between plants and bacteria are receiving increasing attention now that bacterial effects on plant morphogenesis, nutrition, and protection against infectious diseases have become apparent (26). Bacterial adhesion to plant surfaces is an important phenomenon involved, e.g., in the induction of nodulation in legumes by rhizobia (2, 12) and of tumors in host plants by agrobacteria (18). In the case of rhizobium-legume interaction, where the mechanisms of the adhesion have been characterized on a molecular level, the adhesion specificity relies on plant lectins that bind to capsular or lipopolysaccharide antigens of the bacterium (2, 12).

Associative nitrogen fixation is carried out by a number of bacterial species living on the roots of nonleguminous plants (25, 28). Nitrogen-fixing enteric bacteria of the genera *Klebsiella* and *Enterobacter* have frequently been isolated from the roots of various plants (10, 25, 28) and are known to be able to adhere to the roots of grasses and cereals in vitro (8). We have recently shown that fimbriae are involved in enterobacterial adhesion to grass roots (8, 9, 17). Two types of fimbria occur on klebsiellas associated with plants: the so-called type 3 and type 1 fimbriae (3, 4, 8). The two fimbrial types differ morphologically and serologically and in their binding specificities: the type 1 fimbriae bind to mannoses (23), but the receptor structure for the type 3 fimbria is not known. Roots of *Poa pratensis* appear to contain receptors for both fimbrial types: purified type 3 (17) as well as type 1 (9) fimbriae bind to grass roots in vitro and fimbria-specific Fab fragments inhibit bacterial adhesion to grass roots (8, 9, 17). In the case of type 1-fimbriated *Klebsiella* or *Enterobacter* spp., bacterial adhesion can be inhibited by the receptor analog methyl-α-D-mannoside (9). Comparative studies, however, suggested that the type 3 fimbriae are more efficient than the type 1 fimbriae in promoting enterobacterial adherence to the roots of various grasses and cereals (8).

Although the basic mechanisms of enterobacterial adhesion to grass roots are known in some detail, nothing is known about the nature of the plant macromolecules active as bacterial receptors or about the distribution of adhesion sites on the root surface. We now report the localization of adhesion sites for fimbriated *Klebsiella* spp. on the roots of *P. pratensis*, *Festuca rubra*, and *Trifolium pratense*. We used immunofluorescence methods that have been applied successfully to the study of enterobacterial adhesion to mammalian tissues (19, 27). We also show that the bacteria have effects on the morphology of grass roots.

**MATERIALS AND METHODS**

**Bacteria.** *Klebsiella pneumoniae* strain AS had been isolated from the roots of *Agrostis stolonifera* and carries both type 1 and 3 fimbriae (8). *K. terrigena* 69/1 carrying type 3 fimbriae (17), *K. pneumoniae* 55/1 carrying type 1 fimbriae, and the nonfimbriated *K. pneumoniae* 5/B149 (8) were originally obtained from J. P. Duguid (Dundee, U.K.). The adhesion of the three strains to *P. pratensis* roots and their fimbriae have been described previously (8, 9, 17). *K. oxytoca* IHK12145 was isolated from the urine of an adult patient with urinary tract infection and carries type 3 fimbriae (M. Kauppi, K. Haahtela, C. Old, I. Ørskov, F. Ørskov, and T. K. Korhonen, manuscript in preparation); the strain is not able to fix nitrogen. The bacteria were grown in static malate broth for 48 h at 28°C (8).

**Anti-fimbria antiserum.** Antisera against type 3 fimbria of *K. terrigena* 69/1 and type 1 fimbria of *K. pneumoniae* 55/1 were available from previous work (8, 9, 17). Isolation of the immunoglobulin G fraction from anti-type 1 fimbria serum and its conjugation with fluorescein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, Mo.) were performed as described before (13). The immunoglobulin G was further purified by ion-exchange chromatography on a DEAE-cellulose column (Pharmacia Fine Chemicals, Uppsala, Sweden) before conjugation. A 13-ml portion of the antiserum was used for purification. Unbound stain was removed from the conjugate by gel filtration in a Sephadex G25 column (Pharmacia) with phosphate-buffered saline (PBS; pH 7.1) as eluant. The conjugate was then adjusted to 5 ml and stored at −20°C in aliquots of 100 µl. The fluorochrome/protein ratio of the conjugate was 2.8.

**Plant roots.** Seeds of *P. pratensis*, *F. rubra*, and *T. pratense* were surface sterilized by treatment with 94% ethanol for 1 min and with 5% (wt/vol) hypochloric acid for 1 min.
10 min, washed six times with sterile water, and germinated for 2 to 7 days depending on the plant. Only excised roots 1 cm long were used in the adhesion assays.

**Adhesion assays.** For *K. pneumonia* strain As, adhesion to plant roots was visualized by a direct (with anti-type 1-FITC conjugate) and an indirect (with anti-type 3 fimbria serum) immunofluorescence assay (13). Bacterial adhesion was tested essentially as described recently (8). Briefly, bacteria (2.5 × 10^{10} to 2.5 × 10^{10}) were incubated with the roots in 250 μl of PBS at room temperature for 1 h with occasional shaking. The roots were then washed twice for 15 min with 10 ml of PBS, fixed for 10 min with 3.5% (wt/vol) paraformaldehyde (E. Merck AG, Darmstadt, Federal Republic of Germany), and washed twice with PBS. Adherent bacteria were then stained by incubating the roots with the anti-type 1–FITC conjugate diluted 1:10 in PBS containing 1% (wt/vol) bovine serum albumin (Sigma) at room temperature for 30 min. The roots were then washed with PBS as above, mounted with a drop of 50% (vol/vol) glycerol on glass slides, and examined in a Zeiss standard microscope equipped with an epi-illuminator and filter systems for FITC and tetramethylrhodamine B isothiocyanate (TRITC; Sigma) fluorescence as described previously (20, 21). For the indirect-immunofluorescence assay, the roots with the adherent bacteria were incubated with the anti-type 3 fimbria serum (diluted 1:20 in PBS-bovine serum albumin) for 15 min, washed twice with PBS, and incubated with FITC-labeled swine anti-rabbit immunoglobulin G (Dakopatts a/s, Glostrup, Denmark) for 15 min. The roots were then washed twice with PBS and examined as described above.

Adhesion of strains 69/1, 55/1, and IHK12145 was tested by using FITC- or TRITC-labeled bacteria (19, 33). Labeling with TRITC was used with strain 55/1. Labeling was done by incubating 5 × 10^{10} bacteria for 30 min at room temperature with occasional mixing in 2 ml of 0.1 M Na_{2}CO_{3}, pH 9.0, containing 0.9% (wt/vol) NaCl and 100 μg of the fluorochrome per ml. The bacterial suspension was diluted to 20 ml with PBS containing 0.05% (vol/vol) Tween 20 (BDH Chemicals Ltd., Poole, U.K.), and the bacteria were concentrated by centrifugation (15 min, 2,000 × g, 4°C) and washed once with 20 ml of PBS-Tween. The cells were then suspended in 1 ml of PBS and stored at −20°C in 100-μl aliquots. Agglutination assays with erythrocytes or yeast cells before and after the labeling procedure (19) showed no significant quantitative differences. For the adherence assays, the fluorochrome-labeled suspensions were diluted in PBS-Tween to give 10^4 or 10^5 bacteria per ml, and the adhesion assays were performed as described above (8). In inhibition assays, TRITC-labeled strain 55/1 cells were incubated for 15 min in 5% (wt/vol) methyl-α-D-mannoside or D-glucose (for control) before the adhesion test. The carbohydrates were included in the incubation and washing buffer also.

**Agglutination assays.** Hemagglutination of tannin-treated human O erythrocytes by the type 3-fimbriated bacteria and agglutination of yeast cells by the type 1-fimbriated bacteria were tested as described by Duguid (3) and Korhonen (16).

**Composition of *K. pneumonia* As cell population.** The expression of the type 1 and 3 fimbriae on the *K. pneumonia* As cells was assayed by immunostaining of culture samples as recently described (20). A direct staining was done with the FITC–anti-type 1 fimbria conjugate, and an indirect one was done with the anti-type 3 fimbria serum.

**Inoculation experiments.** Surface-sterilized, germinated seedlings of *P. pratensis* were planted in glass tubes (20 cm long, 2.0 cm in diameter; one seedling per tube) containing 20 g of sterile sand and moistened with 7.5 ml of nitrogen-free Hoagland's nutrient solution (11) (one-fourth concentration). Each seedling was inoculated with 10^6 CFU of *K. pneumonia* As (in 0.2 ml of the broth). Ten seedlings were grown without mineral nitrogen and 10 were grown with 300 μg of KNO_{3}·N, of which 150 μg was given at planting, followed by 30 μg in 1.5 ml of Hoagland's solution per week. The concentration of KNO_{3} equaled 1.5 mM. Uninoculated seedlings were used as controls. The plants were grown for 5 weeks under greenhouse conditions with a photoperiod of 18 h (fluorescent tubes). After harvesting, the roots were washed thoroughly with PBS to remove sand. Roots were examined by phase-contrast and interference-contrast microscopy. Some roots were also immunostained with the anti-type 3 fimbria serum as described above.

**RESULTS**

**Adhesion of *Klebsiella* strains to plant roots.** We used two approaches to quantitate adhesion. First, adhesion was performed with fluorochrome-labeled 69/1, 55/1, IHK12145, and 5B149 bacteria. The second approach involved immunostaining of adherent *K. pneumonia* As cells on plant roots. This strain was chosen because it is a typical representative of a nitrogen-fixing klebsiella in that it has both the type 1 and type 3 fimbriae (8). The interpretation of the results with strain As was based on the assumption that the two fimbrial types mostly occur on separate cells, as has been shown for Escherichia coli strains expressing multiple fimbriae (20, 21). To ascertain this, we stained samples of the As culture with the two antisera: 24% of the cells were stained by the anti-type 1–FITC conjugate and 51% were stained by the anti-type 3 fimbriae antiserum, indicating that the cell population was heterogeneous and that a fraction of the cells were nonfimbriated. Control assays showed that strain 69/1 was stained by the anti-type 3 fimbriae serum but not by the anti-type 1–FITC conjugate; reverse staining was observed with strain 55/1. We thus concluded that the two fimbrial types of strain As probably resided on different cells.

We tested bacterial adhesion to the roots of *P. pratensis*, *F. rubra*, and *T. pratense*. In general, the fimbriated bacteria showed a similar binding pattern and no differences were found among the plant species. The examples shown in Fig. 1 were obtained with a bacterial concentration of 5 × 10^6 per ml, which was found optimal for the localization studies. In accordance with our previous adhesion assays with radiolabeled bacteria (8), the nonfimbriated strain 5B149 adhered poorly, showing hardly any adherent cells in assays with 5 × 10^6 bacteria per ml (not shown).

Strain As adhered strongly to root hairs but not to the epidermal cells between root hairs, as shown by the direct immunostaining with the anti-type 1 fimbriae serum (Fig. 1A) and by the indirect staining with the anti-type 3 fimbriae serum (Fig. 1D to F). Control stainings (not shown) without adherent bacteria gave only weak fluorescence, against which background the adherent bacteria could easily be distinguished. In Fig. 1F, a typical bacterial staining with anti-fimbriae antibodies (20) can be seen: the bacteria stained strongly at the cell edges. The type 1-fimbriated strain 55/1 (Fig. 1B) and also the type 3-fimbriated strains 69/1 and IHK12145 (not shown) adhered selectively to root hairs. The molecular specificity of adhesion was shown by the inhibition of the adhesion of strain 55/1 by methyl-α-D-mannoside (Fig. 1B and C). Adherent bacteria were found (but in...
significantly lower numbers than in root hairs) also in the mucilage surrounding sloughed root cap cells and on the surface of the elongation zone (Fig. 1G and H).

**Effect of bacteria on root morphology.** Inoculation of *P. pratensis* seedlings with *K. pneumoniae* As had a profound effect on root morphology. Inoculated seedlings were incubated for 5 weeks, and colonization of the roots by the bacteria was confirmed by immunostaining with the anti-type 3 fimbriae serum, which revealed bacteria predominantly on root hairs (not shown). Roots of the inoculated plants were larger than those of the control plants, the difference being clearer with plants grown under nitrogen-free conditions (Fig. 2A and B). Roots of the inoculated plants contained significantly more numerous root hairs than did roots of the control plants (Fig. 2C to F). Also, the zone of elongation was shorter in the inoculated roots (Fig. 2C and D). Numerous curled and branched root hairs could be seen in the inoculated but not in the control roots (Fig. 2G to I).

**DISCUSSION**

In this communication we demonstrate that fimbricated *Klebsiella* strains show a highly localized adhesion to the roots of grasses and red clover. It is generally assumed that adhesion to plant roots is beneficial for associative nitrogen fixers by giving them access to plant exudates abundant at root surfaces, and our results show that colonization of *P. pratensis* roots by the bacteria has profound effects on root morphology.

Most nitrogen-fixing *Klebsiella* strains isolated in Finland possess both type 3 and type 1 fimbiae (8), and we have previously demonstrated that both fimbral types are involved in enterobacterial adhesion to grass roots (8, 9, 17). Since the type 1 and 3 fimbiae have differing binding specificities, it was somewhat unexpected that the bacteria carrying these fimbiae adhered to the same areas on the root surfaces. Immunofluorescence staining of strain As, which expressed both fimbral types, by anti-type 3 or anti-type 1 fimbria sera revealed bacteria attached mostly to root hairs (Fig. 1A to F). The type 3-fimbriated strains 69/1 and H1K12145 and the type 1-fimbriated strain 55/1 (Fig. 1B) showed localization in their adherence similar to that of strain As, which confirms that the two fimbral types really bind to approximately the same areas on the roots of *P. pratensis*. Bacteria appeared to adhere, albeit in lower numbers, also to the root cap mucilage (Fig. 1G and H) and to the epidermal cells at the elongation zone. No bacteria were found on the sloughed cells at the root cap.

Fimbriae are bacterium-binding proteins that recognize specific carbohydrate receptors on eucaryotic cells (4, 23). The localized binding of fimbricated enteric bacteria to grass roots demonstrated here thus indicates that access to the receptor-active molecules is unevenly distributed on the root surface. Information on the distribution of carbohydrates of plant surfaces is scarce, but some evidence of a localized expression of carbohydrates exists. Vermeir and McCully (34) used a fucose-specific lectin to localize fucose residues on the roots of *Zea mays* L.. The lectin bound only to root caps and to the epidermis of mature root hairs, which indicates that fucose has a localized distribution on maize roots. Similarly, Werker and Kislev (35) observed two types of mucilaginous material in *Sorghum* roots. One type occurred near the tip of root hairs and the other type occurred on the root hairs and on the outer wall of ordinary root epidermal cells. Ridge and Rolfe (29) described recently the localized binding of several lectins to the roots of the tropical legume *Macropitum atropurpureum*.

The plant macromolecules acting as fimbral receptors remain to be identified. The obvious candidates are mucilages exuded at root hairs and at the root cap. Maize root cap mucilage consists of a neutral β(1-4)-glucan polymer and of acidic polymers containing fucose, arabinose, galactose, galacturonic acid, and, as minor constituents, xylose, glucose, and mannos (6). The same carbohydrates occur also in the rice root cap slime but in different molar ratios (1). Type 1 fimbiae are characterized by their binding to α-D-mannosides (16, 23) but are also able to bind glucosides substituted with hydrophobic residues (5) so they might bind to plant mucilages. The binding specificity of the type 3 fimbra has not been characterized so far.

The fimbricated *Klebsiella* strains are not as restricted in their adhesion as are strains of *Rhizobium* spp. which adhere first to the tips of root hairs of their leguminous host plants (2, 12). Fimbriated *Klebsiella* strains adhere well to the roots of a number of grasses and *Sinapis* (8), which is compatible with the fact that such bacteria have been isolated from the roots of a number of plant species (8, 25, 28). The *Klebsiella* strains adhered also to the root hairs of *T. pratense*, which to our knowledge has not been reported to support associative nitrogen fixers in its roots. Fimbriae are known to occur on species of the three enterobacterial genera that are known to include nitrogen fixers, i.e., *Erwinia, Enterobacter*, and *Klebsiella* (3, 4, 8), and the adhesion here described probably represents a general mechanism for plant-enteric bacteria interaction. Interestingly, fimbrliae-like proteins are involved also in the specific adhesion of a plant pathogen, *Pseudomonas syringae* pv. *phaseolicola*, to the stomata of bean leaves (30).

We have previously shown that nitrogen is fixed and transferred to the plant in the *Klebsiella* sp.-*P. pratensis* association (7). Of importance is the present finding that inoculation of *P. pratensis* seedlings with *K. pneumoniae* As resulted in increased root mass (Fig. 2A and B) and number of root hairs, as well as in decreased length of the zone of elongation (Fig. 2C and D). Similar findings have been reported in pearl millet (31, 32), wheat (14, 24), and sorghum (22) inoculated with *Azospirillum* sp., an associative nitrogen-fixing bacterium. The effects on pearl millet roots by *Azospirillum* sp. were suppressed in the presence of nitrate (32), which could be seen in the *Klebsiella* sp.-*P. pratensis*
FIG. 2. Effect of *K. pneumoniae* As on morphology of *P. pratensis* roots. (A) An un inoculated (left) and an inoculated (right) root grown in nitrogen-free medium; (B), same, but in nitrogen-containing medium. (C) Control root grown in nitrogen-free medium; (D), an inoculated root grown under similar conditions. Note the numerous root hairs and the short zone of elongation in (D). (E and F) A control and an inoculated root grown in nitrogen-containing medium. (G) Root hairs of a control plant and of (H and I) inoculated plants. Arrows point to branched root hairs resembling "tuning forks"; also note moderately curled tips of root hairs in (H) and (I). Bar, 100 μm in (C to F); 10 μm in (G to I).
association also (Fig. 2C to F). Finally, similarly to Azospirillum sp.-grass associations (14, 22, 24), infection of *P. pratensis* with strain A caused root hair deformation (Fig. 2G to I). These effects are probably due to plant growth substances produced by the bacteria (15, 22, 31). In rhizobia, root hair deformation is strain specific and assumed to be correlated with the ability of different Rhizobium strains to nodulate the host (36). Interestingly, Okon and Kapulnik (22) reported that, in contrast to Azospirillum spp., Klebsiella strains did not increase the root surface area of wheat. Whether this reflects some degree of host specificity in Klebsiella sp.-grass interactions remains to be resolved.

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LITERATURE CITED