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In Vitro Adhesion of N_2-Fixing Enteric Bacteria to Roots of Grasses and Cereals

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Nitrogen-fixing *Klebsiella* and *Enterobacter* strains isolated from several plants were assayed for fimbriae and for adhesion to plant roots in vitro. All eight *Klebsiella* strains formed type 3 fimbriae, and five strains also formed type 1 fimbriae; all 21 *Enterobacter* strains had type 1 fimbriae. Three strains of *Klebsiella* carrying either type 1, type 3, or no fimbriae were used as model organisms in developing an in vitro adhesion test. Adhesion was assayed with bacterial cells labeled with [^{3}H]leucine. Fifteen N_2-fixing strains and the three model strains were compared for adhesion to the roots of seven grasses and five cereals. Type 3-fimbriated *Klebsiella* strains adhered better than the other strains, and type 3 fimbriae appeared to be major adhesins for the *Klebsiella* strains. Although variations between plants were observed, no host specificity for bacterial adhesion was found.

Among the associative nitrogen-fixing bacteria, species of *Klebsiella* and *Enterobacter* have been isolated from the roots of grasses and cereals growing in temperate regions (3, 12, 22) and in tropical areas (1, 18, 25). Associative nitrogen fixers live attached to the plant root surfaces, where they benefit from the organic material exuding from the roots.

Adhesion mechanisms are dependent on the association between plant and bacteria (19). Although the adhesion mechanism of *Rhizobium* spp. has been characterized in detail (13), very little is known about the adhesins and receptors involved in associative nitrogen fixation.

Umali-Garcia et al. (27) showed that cells of *Azospirillum brasilense* adhering to grass roots were associated with granular material on root hairs and fibrillar material on undifferentiated epidermal cells. Root exudates of pearl millet contained substances that bound to azospirilla and promoted bacterial attachment to root hairs. Chemotactic response to root exudates could also be involved in specific recognition (2).

Duguid (5) was the first to propose that saprophytic *Klebsiella* strains adhere, apparently by their fimbriae, to the root hairs of red clover and cress seedlings. We have shown that purified and radiolabeled type 1 and type 3 fimbriae bind to the roots of *Poa pratensis* in vitro and that bacterial adhesion to roots can be inhibited by anti-fimbria Fab fragments (11, 16). This indicates that both fimbrial types function in adhesion to root surfaces. The type 1 fimbriae bind to mannosides (6), whereas the receptor structure for the type 3 fimbriae is not known.

In this communication we describe an in vitro test for assaying the adhesion of associative nitrogen-fixing bacteria to plant roots and compare *Klebsiella* and *Enterobacter* strains for adhesion to the roots of various grasses and cereals. Type 3 and type 1 fimbriae were also compared for their efficiency in promoting adherence.

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MATERIALS AND METHODS

Bacteria. *Klebsiella terrigena* 69/1 (carrying type 3 fimbriae), *K. pneumoniae* 55/1 (carrying type 1 fimbriae), and *K. pneumoniae* 5/B149 (carrying no fimbriae) were gifts from J. P. Duguid (Dundee, United Kingdom); the three strains have previously been typed as *K. aerogenes* (5, 6, 16). Two nitrogen-fixing *K. pneumoniae* strains, six *K. terrigena* strains, and 21 *Enterobacter agglomerans* strains were isolated in Finland between 1978 and 1982 from the roots of various grasses (Table 1) as described previously (10, 12). These strains were identified and biotyped with API 20E and API 50CHE test kits (API Systems SA, France). *Klebsiella* strains were serotyped and classified by Ida and Frits Ørskov (International *Escherichia* and *Klebsiella* Centre, Copenhagen, Denmark). Unless otherwise stated, the bacteria were grown in static malate broth for 48 h at 28°C (9, 16).

Protein estimation. Protein was estimated by the modified Lowry procedure described by Markwell et al. (20), with bovine serum albumin (BSA) as a standard.

Immunological methods. Antisera against the type 3 fimbriae of *K. terrigena* 69/1 and the type 1 fimbriae of *K. pneumoniae* 55/1 were available from previous work (16). Purified immunoglobulin G against type 1 fimbriae of *K. pneumoniae* 55/1 was treated with papain (Sigma Chemical Co., St. Louis, Mo.), and Fab fragments were prepared essentially as described by Porter (23). The Fab fragments were assessed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; no heavy chain of the immunoglobulin G molecule was visible in the gels. Fab fragments against purified type 3 fimbriae from *K. terrigena* 69/1 were available from previous work (16). Antibody titers of antisera and Fab fragments were determined in an enzyme-linked immunosorbent assay (ELISA) (7) as previously described (17).

Plants. The following grasses were used: *Dactylis glomerata* cv. Hera Daehnfelt, *Festuca pratensis* cv. Tammisto, *Festuca rubra* cv. Echo Daehnfelt, *Lolium perenne* cv. Valinge, *Phalaris arundinacea* cv. LH404, *Phleum pratense* cv. Tammisto, and *Poa pratensis* cv. Arina Dasas. The following cereals were used: *Avena sativa* cv. Nasta, *Hordeum vulgare* cv. Etu, *Secale cereale* cv. Pekka, *Triticum*

TABLE 1. Nitrogen-fixing bacterial strains

Strain	Plant of origin	Serotype[a] or biogroup[b]	Fimbria	
			Type 3	Type 1
K. pneumoniae				
As	*Agrostis stolonifera*	K54	+	+
Pp	*Poa pratensis*	K54	+	+
K. terrigena				
Cp	*Carex pallescens*	K80	+	−
Pha	*Phalaris arundinacea*	K36	+	+
Php1	*Phleum pratense*	K80	+	+
Php2	*Phleum pratense*	K8, 26, 74	+	−
Pp1	*Poa pratensis*	K32	+	+
Pp2	*Poa pratensis*	K8, 26, 74	+	−
E. agglomerans				
Am	*Achillea millefolium*	G3	−	+
Ca	*Calamagrostis arundinacea*	G3	−	+
Dg1	*Dactylis glomerata*	G3	−	+
Dg2	*Dactylis glomerata*	G3	−	+
Dg3	*Dactylis glomerata*	G3	−	+
Er	*Elytrigia repens*	G3	−	+
Fr1	*Festuca rubra*	G3	−	+
Fr2	*Festuca rubra*	G3	−	+
Fr3	*Festuca rubra*	G3	−	+
Fr4	*Festuca rubra*	G3	−	+
Pha	*Phalaris arundinacea*	G4	−	+
Php	*Phleum pratense*	G3	−	+
Php1	*Phleum pratense*	G3	−	+
Php2	*Phleum pratense*	G3	−	+
Php3	*Phleum pratense*	G1	−	+
Php4	*Phleum pratense*	G3	−	+
Php5	*Phleum pratense*	G1	−	+
Php6	*Phleum pratense*	G3	−	+
Pp1	*Poa pratensis*	G3	−	+
Pp2	*Poa pratensis*	G3	−	+
Pp3	*Poa pratensis*	G3	−	+

[a] Reaction with anti-*Klebsiella* capsular serum.
[b] Biogroups are according to Ewing and Fife (8).

aestivum cv. Ruso, and *Triticum sativum* cv. Aura. The seeds were surface sterilized by treatment with 94% ethanol for 1 min and with 5% (wt/vol) hypochloric acid for 6 to 10 min, washed six times with sterile water, and germinated on water-agar plates for 3 to 8 days depending on the plant. Only excised roots 1 cm long were used.

In vitro adhesion test. The bacteria were labeled during growth by supplementing the culture medium with L-[4,5-^{3}H]leucine (5 μCi/ml; Amersham Corp.), washed gently with 17 mM potassium phosphate buffer (pH 6.9), and suspended in it to give 10^9 to 10^{10} bacteria per ml. Bacteria (10^8 or 10^9; we always used two concentrations) were incubated with roots in 1 ml of buffer at room temperature for 1 h with occasional shaking, and then the roots were washed by soaking in 40 ml of buffer. This was repeated four times (until the radioactivity level of the washing buffer remained constant). After being washed, the roots were incubated with 200 μl of solubilizer (Lumasolve; Lumac Co., Basel, Switzerland) at 37°C for 1 h, suspended in 2.5 ml of scintillation liquid (Lipoluma; Lumac), and allowed to stabilize at 4°C for 5 h. Radioactivity was measured in a 1215 Rackbeta scintillation counter (LKB Wallac, Bromma, Sweden). In inhibition tests, bacteria were incubated with increasing concentrations of Fab fragments or BSA for 30 min at room temperature before adhesion tests. The numbers given represent means of 10 determinations.

Agglutination tests. Hemagglutination and yeast cell agglutination tests with whole bacterial cells were performed as previously described (15). In inhibition tests, bacteria were incubated with Fab fragments for 15 min at room temperature. Human blood group type O erythrocytes were treated with tannin as described by Duguid (5). Bacterial agglutination with antisera prepared against the various fimbriae were performed in 1:10 and 1:100 dilutions of antisera.

RESULTS

Characterization of nitrogen-fixing *Klebsiella* and *Enterobacter* strains. The characteristics of the strains used are listed in Table 1. In API 20E tests, all of the nitrogen-fixing *Klebsiella* strains were identified as *K. pneumoniae*. A more detailed classification (API 50CHE and other tests) and serotyping differentiated the *Klebsiella* strains into several serotypes of *K. pneumoniae* and *K. terrigena* (Table 1). The *Enterobacter* strains were identified as *E. agglomerans*. Physiologically, all of the *E. agglomerans* strains belonged to the aerogenic group described by Ewing and Fife (8). Positive Voges-Proskauer tests (VP^+) and negative indole tests (IND^-) placed 18 strains in biogroup G3 within the aerogenic group (Table 1). Strain Pha was assigned to G4 (VP^+, IND^+), and strains Php3 and Php5 were assigned to G1 (VP^-, IND^+). The nitrogen-fixing strains were isolated from 10 plant species. It should be noted that the same plant species could host either *Klebsiella* or *Enterobacter* strains, including different serotypes and biogroups.

All eight *Klebsiella* strains were able to form type 3 fimbriae (Table 1) as tested by agglutination with tannin-treated human blood group type O erythrocytes and with antisera prepared against purified type 3 fimbriae from *K. terrigena* 69/1. Five strains also had type 1 fimbriae (Table 1) as tested by agglutination with yeast cells and antisera prepared against type 1 fimbriae from *K. pneumoniae* 55/1. All of the *E. agglomerans* strains had type 1 but lacked type 3 fimbriae, i.e., they agglutinated yeast cells in a mannose-sensitive manner.

Adhesion assay. The adherence of *K. terrigena* 69/1 and *K. pneumoniae* 55/1 and 5/B149 to the roots of *P. pratensis* is shown in Fig. 1. Since adhesion was significantly dependent on the concentration of bacteria in the incubation buffer, we decided to screen strains routinely at two concentrations (10^8 and 10^9 bacteria per ml) found within the linear phase of the adhesion curve (results are given for a concentration of 5×10^8 cells per ml). *K. terrigena* 69/1, carrying type 3

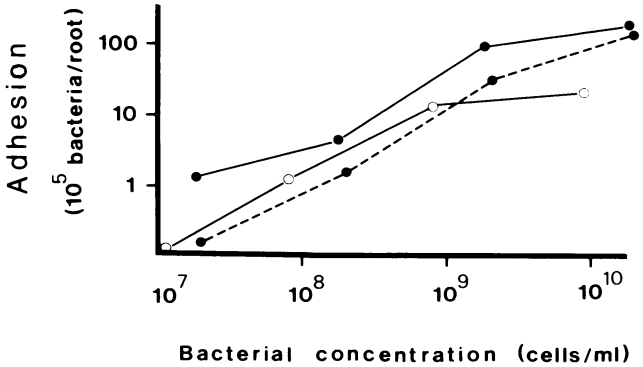

FIG. 1. Adhesion of *Klebsiella* model strains to roots of *P. pratensis*: type 3-fimbriated *K. terrigena* 69/1 (●———●); type 1-fimbriated *K. pneumoniae* 55/1 (○———○); nonfimbriated *K. pneumoniae* 5/B149 (●– – –●).

TABLE 2. Adhesion of *Klebsiella* and *Enterobacter* strains to the roots of grasses

Grass	% of bacteria adhering[a]										
	K. pneumoniae			*K. terrigena*				*E. agglomerans*			
	Pp	55/1	5/B149	Pha	Php1	Pp1	69/1	Am	Dg1	Pha	Php1
Dactylis glomerata	0.19	0.01	0.04	0.44	0.12	0.05	0.35	0.02	0.19	0.24	0.10
Festuca pratensis	0.39	0.02	0.02	0.78	0.15	0.08	0.73	0.03	0.11	0.11	0.10
Festuca rubra	0.26	0.02	0.06	0.18	0.15	0.02	0.25	0.02	0.16	0.19	0.04
Lolium perenne	0.31	0.01	0.05	0.38	0.14	0.06	0.98	0.01	0.15	0.07	0.10
Phalaris arundinacea	0.20	0.01	0.06	0.21	0.13	0.05	0.68	0.02	0.08	0.04	0.06
Phleum pratense	0.08	0.01	0.01	0.13	0.10	0.02	0.38	0.01	0.05	0.23	0.04
Poa pratensis	0.10	0.05	0.04	0.60	0.08	0.02	0.34	0.01	0.08	0.24	0.04
$\bar{x}$	0.22	0.02	0.04	0.39	0.12	0.04	0.53	0.02	0.12	0.16	0.07

[a] Percentage of the initial number of bacteria (5×10^8 cells per ml) used in the incubation buffer which adhered to the roots. Values are means of 10 determinations.

fimbriae, adhered better than *K. pneumoniae* 55/1 and 5/B149 to *P. pratensis* roots; the difference was highly significant ($P < 0.001$, Student's t test). Interestingly, nonfimbriated *K. pneumoniae* 5/B149 also adhered well at the highest bacterial concentrations. Electron microscope examinations (not shown) revealed that bacteria adhered to all areas on the root surface, not only to root hairs.

Before doing routine adhesion assays, we tested the assay conditions for some parameters. This was done with the three model strains and *P. pratensis* (details not shown). The incubation time was determined by estimating the number of adherent bacteria after incubation for 15, 30, 60, 120, and 180 min. Adsorption was almost complete (80% of maximum) after 30 min and did not increase after 60 min. Therefore, 60-min incubation times were used in subsequent studies. Adhesion was not significantly dependent on pH between 5.2 and 7.8, on temperature between 4 and 37°C, or on ionic strength (we tested 0.15 to 1.0 M NaCl in the incubation buffer). We therefore decided to perform adhesion tests at room temperature in potassium phosphate buffer (17 mM, pH 6.9), which is also used in the culture medium. The effect of bacterial age on adherence was tested with 1-, 2-, 3-, and 4-day-old cultures. Adhesion was best with 2-day-old cultures.

The sensitivity of the adhesion assay was 10^4 bacteria per root, and we had to use concentrations of at least 10^7 cells per ml of incubation buffer (Fig. 1). Variations between determinations were considerable; standard deviations in single tests ranged from 30 to 60%. Variations were similar for the mean values of tests performed on different days.

Adhesion of *Klebsiella* and *Enterobacter* strains to roots of grasses and cereals. We compared six *Klebsiella* and nine *E. agglomerans* strains for adhesion to the roots of seven grasses and five cereals (Tables 2 and 3). The results for four nitrogen-fixing strains and the three model strains of *Klebsiella* and for four *Enterobacter* strains are given in Table 2 for grasses and in Table 3 for cereals. One bacterial culture was tested with either all grass roots or all cereal roots.

The strains adhered to all the roots tested (Tables 2 and 3). The observed adhesion values ranged from 0.01 to 0.98%, which correspond to 5×10^4 to 5×10^6 bacteria per root. However, for a particular strain the range was much smaller and usually of the same order of magnitude. It is obvious that the adhesion values were affected by differences in root surface area. No attempts were made to account for these differences, and therefore no statistical analysis was used in comparing different roots. However, some trends in adhesion to different roots were observed.

Adhesion values for the N_2-fixing *Klebsiella* strains ranged from 0.02 to 0.78 ($\bar{x} = 0.19$) with grasses and from 0.06 to 0.63 ($\bar{x} = 0.28$) with cereals. Adhesion was better with cereals than with grasses ($P < 0.05$). Adhesion percentages for *Enterobacter* strains ranged from 0.01 to 0.24 ($\bar{x} = 0.09$) with grasses and from 0.01 to 0.39 ($\bar{x} = 0.10$) with cereals. Nitrogen-fixing klebsiellas adhered better than *Enterobacter* strains to both grasses ($P < 0.05$) and cereals ($P < 0.01$). Of the model strains, type 3-fimbriated *K. terrigena* 69/1 adhered to the roots of both grasses ($P < 0.001$) and cereals ($P < 0.02$) much better than the type 1-fimbriated *K. pneumoniae* 55/1 or the nonfimbriated strain 5/B149. Adhesion values for strains 55/1 and 5/B149 were equal.

Effect of Fab fragments on bacterial adhesion. We have earlier shown that both type 3 and type 1 fimbriae can mediate enterobacterial adhesion to grass roots (11, 16). Since our results above suggested that type 3-fimbriated *Klebsiella* strains adhered better than the other strains, we decided to compare anti-type 3 and anti-type 1 fimbriae Fab fragments for inhibition of adhesion. This was done with *K.*

TABLE 3. Adhesion of *Klebsiella* and *Enterobacter* strains to the roots of cereals

Cereal	% of bacteria adhering[a]										
	K. pneumoniae			*K. terrigena*				*E. agglomerans*			
	Pp	55/1	5/B149	Pha	Php1	Pp1	69/1	Am	Dg1	Pha	Php1
Avena sativa	0.50	0.01	0.13	0.21	0.06	0.08	0.16	0.04	0.06	0.01	0.33
Hordeum vulgare	0.21	0.01	0.01	0.29	0.24	0.11	0.07	0.07	0.05	0.02	0.17
Secale cereale	0.26	0.01	0.08	0.50	0.23	0.17	0.33	0.04	0.05	0.02	0.27
Triticum aestivum	0.25	0.01	0.05	0.60	0.20	0.15	0.44	0.07	0.05	0.03	0.24
Triticum sativum	0.35	0.01	0.05	0.53	0.63	0.13	0.66	0.07	0.06	0.03	0.39
$\bar{x}$	0.31	0.01	0.05	0.42	0.27	0.13	0.33	0.06	0.05	0.02	0.28

[a] See Table 2, footnote *a*.

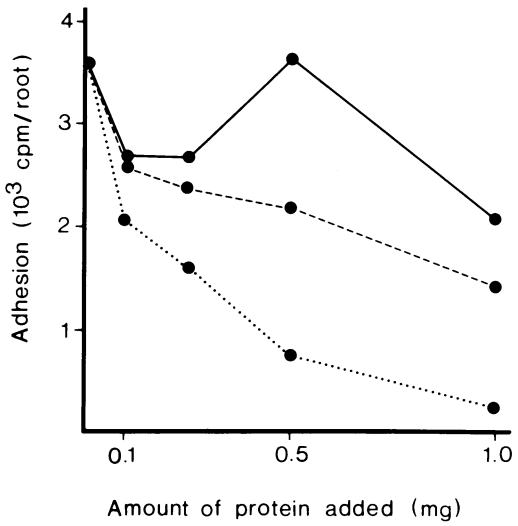

FIG. 2. Adhesion of N_2-fixing *K. pneumoniae* As to the roots of *P. pratensis* in the presence of BSA (———), anti-type 3 fimbriae Fab fragments (· · ·), and anti-type 1 fimbriae Fab fragments (– – –).

pneumoniae As, which carries both type 3 and type 1 fimbriae, and *P. pratensis* roots (Fig. 2). BSA had no effect on adhesion, whereas 500 and 1,000 μg of anti-type 3 fimbriae Fab fragments inhibited adhesion by 79 and 86%, respectively (for both concentrations, $P < 0.001$). Anti-type 1 fimbriae Fab fragments inhibited adhesion by 49 and 30%, respectively ($P < 0.02$) (Fig. 2). There was also a significant difference between inhibition by anti-type 3 and anti-type 1 fimbriae Fab fragments ($P < 0.001$). The Fab fragment concentrations causing 50% inhibition compared with a control without added Fab fragments were 0.20 mg/ml for anti-type 3 fimbriae Fab fragments and 0.92 mg/ml for anti-type 1 fimbriae Fab fragments. The ELISA titers of anti-type 3 and anti-type 1 fimbriae Fab fragments (at concentrations of 5 and 9 mg of protein per ml, respectively) were 9×10^4 and 7.5×10^4, respectively, and the concentration of Fab fragments required to cause 50% inhibition of hemagglutination or yeast cell agglutination was 0.56 mg/ml for anti-type 3 and 0.63 mg/ml for anti-type 1 fimbriae Fab fragments.

DISCUSSION

The in vitro adhesion test described in the present communication showed that all of the nitrogen-fixing *Klebsiella* and *Enterobacter* strains adhered to all of the grass and cereal roots tested. Adhesion values for all strains and roots, at a concentration of 5×10^8 bacteria per ml, varied between 5×10^4 and 5×10^6 bacteria per root, and adhesion of a particular strain to different roots usually varied by less than 10-fold. Thus, no strict host specificity was observed in enterobacterial adhesion to plant roots; this is in accordance with the fact that the nitrogen-fixing strains were isolated from the roots of a number of grass species (Table 1). Thus, enteric bacterium-plant interaction in associative nitrogen fixation differs from *Rhizobium*-legume interaction, in which specific bacterial adhesion to plant roots determines the establishment of symbiosis (4, 13). *Rhizobium* species also adhere preferentially to root hairs; our preliminary localization studies have not shown such specificity in enterobacterial adhesion to the roots of *P. pratensis*. Besides showing lack of host specificity, our in vitro adhesion test has proven valuable in determining the bacterial cell components involved in adhesion (11, 16).

Variations in the adhesion test were considerable, but this is a general feature of most in vitro adhesion tests, (14, 26). However, the fact that we were able to inhibit bacterial adhesion by receptor analogs (11) or by Fab fragments specific for bacterial adhesins (11, 16) (Fig. 2) indicated that we were measuring specific adhesion. We have shown that fimbriae mediate enterobacterial adhesion to grass roots (11, 16), and recent work on *Escherichia coli* fimbriae (21, 24) has shown that fimbriae are susceptible to phase variation. This means that only a fraction of cells in a population carry fimbriae and that the percentage of fimbriated cells is dependent on the strain, culture conditions, and growth phase. This is reflected as large day-to-day and strain-dependent variations in adhesion tests. We were unable to correct the adhesion values to allow for the actual root surface available for bacterial adhesion. It is obvious that the roots used in the experiments described in Tables 2 and 3 differ in surface area, e.g., due to different numbers of root hairs and variations in root thickness, although we used roots of equal length. This makes comparison of different roots very difficult, but it is obvious that they do not markedly differ in taking up bacteria. Also, the adhesion values obtained were quite low, between 0.01 and 0.98%, but this was to be expected since there was a marked disparity between root surface area and test volume. It appears that a 1-cm-long root of *P. pratensis* can take up maximally about 10^7 bacteria (Fig. 1).

Both type 3 (16) and type 1 (11) fimbriae of enteric bacteria can mediate enterobacterial adhesion to plant roots. Our results indicate that type 3 fimbriae are more efficient than type 1 fimbriae in promoting adherence. This was supported by two lines of evidence. First, of the model strains used, the type 3-fimbriated strain was clearly the most effective in adhesion (Fig. 1, Tables 2 and 3), and the mean adhesion value for the *Klebsiella* strains, all of which had type 3 fimbriae (Table 1), was higher than that for the *Enterobacter* strains, which lacked type 3 fimbriae (Tables 1 through 3). Second, adhesion of a strain carrying both type 1 and type 3 fimbriae was inhibited more efficiently by Fab fragments against type 3 fimbriae than by those against type 1 fimbriae (Fig. 2). It should be noted that the Fab fragments were tested at comparable concentrations, as shown by equal ELISA and agglutination inhibition test results.

Adhesion values for the type 1-fimbriated model strain and *E. agglomerans* strains were only marginally higher than those for the nonfimbriated model strain (Fig. 1, Tables 2 and 3). We cannot, at present, exlude other adhesion mechanisms, such as plant root lectins, although their contribution to the adhesion system under discussion is probably not significant in view of the efficient inhibition by receptor analogs or fimbriae-specific Fab fragments (11, 16) (Fig. 2).

It is interesting that strains of *Enterobacter* were isolated from grass roots more frequently than were klebsiellas (Table 1), although the latter were more efficient in adhesion. One explanation for this could be bacterial chemotaxis towards plant roots, as has been observed in associative nitrogen fixation involving *Azospirillum brasilense* (2). *Klebsiella* strains are nonmotile and may have to offset their lack of chemotaxis with a more efficient adhesive capacity.

All of the nitrogen-fixing enteric bacteria isolated in Finland were fimbriated (Table 1) and thus able to adhere to plant roots and other surfaces. The in vitro adhesion test here described indicated a lack of adhesion-based host specificity for associative nitrogen-fixing enteric bacteria

and is useful in identifying mechanisms of bacterial adhesion to plant roots in general. Bacterial adhesion to plant roots should also be considered in planning field trials or constructing inocula for plants that support associative nitrogen fixation.

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