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Adhesion of fimbriated nitrogen-fixing enteric bacteria to roots of grasses and cereals

TIMO K. KORHONEN, EEVA-LIISA NURMIAHO-LASSILA, TUULA LAAKSO and KIELO HAAHTELA
University of Helsinki, Department of General Microbiology, Mannerheimintie 172, SF-00280, Helsinki 28, Finland

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Summary The role of fimbriae in enterobacterial adhesion to roots of grasses and cereals is discussed. All nitrogen-fixing enteric bacteria isolated in Finland had fimbriae. All *Enterobacter* isolates had mannose-binding type-1 fimbriae, whereas most of the *Klebsiella* isolates had both type-1 and type-3 fimbriae. The strains were isolated from a total of ten different grass species, and no specific association was found between grass species and bacterial fimbriation, biogroup or serogroup. Purified, radiolabeled fimbriae bound to roots of *Poa pratensis in vitro*, and bacterial adhesion was inhibited by Fab fragments specific for fimbriae. *Klebsiella* strains carrying type-3 fimbriae adhered to roots of various grasses and cereals more efficiently than type-1- or nonfimbriated strains, and it was concluded that type-3 fimbriae are the major adhesions of *Klebsiella*. Immunofluorescence studies revealed that the bacteria preferentially adhered to root hairs, and to a lesser extent, to the zone of elongation and the root cap mucilage. No strict host specificity in enterobacterial adhesion was observed.

Introduction

Bacterial adherence to plant surfaces and its importance in plant-bacterium interactions is receiving increasing attention. Adherence to plant roots is thought to benefit bacteria by giving them access to nutrients excreted by the roots and by protecting them from predators. Bacterial attachment to specific plant surfaces initiates changes in plant metabolism, *e.g.* rhizobia induce nodulation in legumes[2] and agrobacteria induce tumours in host plants[15]. In *Rhizobium*-legume interaction, adhesion is specified by plant lectins recognizing carbohydrate structures on bacterial lipopolysaccharides and/or capsular antigens[2,9].

It is probable that bacterial adhesion to plant roots also precedes associative nitrogen fixation. *Azospirillum brasilense* strains have been reported to adhere to roots of plants[20,24]; see also Okon and Kapulnik in this volume. Plant-*Azospirillum* interaction is probably initiated also by bacterial chemotaxis towards root mucigel[1] (see also Mandimba *et al.* in this volume).

We have studied the adhesion to roots of grasses and cereals of nitrogen-fixing *Klebsiella* and *Enterobacter* strains, and more specifically, the role of bacterial fimbriae in this process. Fimbriae (pili) are filamentous protein appendages on bacterial cell surfaces; their only

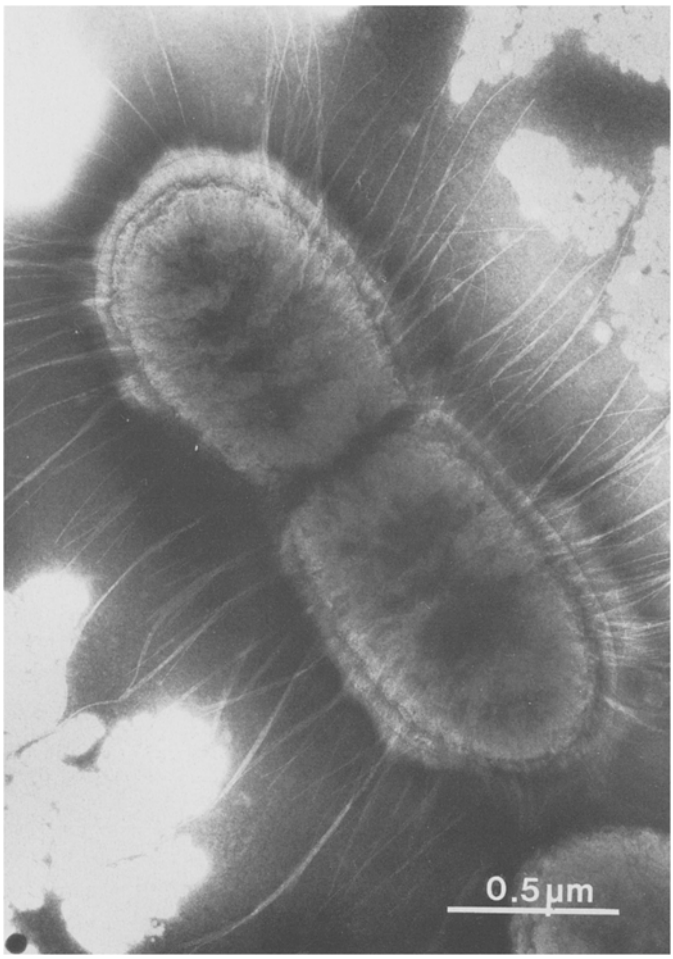

Fig. 1. *K.pneumoniae* strains As after growth for 48 h in static malate broth. The cell is surrounded by fimbriae of 7 nm in diameter and 0.5 to 2.0 µm in length.

known function is in adhesion[3,4]. Figure 1 shows a cell of a nitrogen-fixing *Klebsiella pneumoniae* strain As (isolated from the roots of *Agrostis stolonifera*): the cell is surrounded by fimbriae, which number 200–300 per cell.

Fimbrial functions and binding properties have been studied mainly with pathogenic bacteria and mammalian cells. These studies have revealed several important principles. Bacterial adhesion is specific, i.e. fimbriae recognize a specific receptor structure, mostly a carbohydrate[3,4,13,14,18], on mammalian epithelial cells. A single bacterial species, *e.g. Escherichia coli,* has many different fimbrial types, each of which is usually associated with a given clinical or ecological situation[4,6]. Studies

of fimbrial functions are often complicated by the fact that a bacterial strain can have up to four different fimbrial antigens, which show rapid alternate synthesis, *i.e.* fimbrial phase variation[16,23]. Immunofluorescence studies of cell populations have revealed that the different fimbrial types of an *E. coli* strain mostly occur on separate cells and that the cells can rapidly switch their fimbrial antigens[16]. This means that a bacterial cell population is heterogeneous in respect to fimbriation. In *E. coli,* growth conditions are known to affect fimbriation: some fimbriae are not produced at low temperatures or in rich media[6] and shaking or growth on solid media decreases the formation of so-called type-1 or common fimbriae[4]. The fimbrial filament is composed of one type of protein subunit (fimbrillin) with a molecular weight mostly of 17,000 to 22,000[6,21]. Fimbriae differ genetically too; some are coded by plasmid genes, some by chromosomal genes[6,22].

In this communication we summarize our recent studies on the characteristics and role of fimbriae in enterobacterial adhesion to roots of grasses and cereals. We also discuss the lack of host specificity in this kind of adhesion.

Types of fimbriae on *Klebsiella* and *Enterobacter* strains of plant origin

At least two types of fimbriae occur on *Klebsiella* strains isolated from plants: type-1 fimbriae, which are characterized by their binding to mannosides[3,18], and type-3 fimbriae, whose receptor structures are unknown[3]. Type-1 fimbriae mediate mannose-sensitive attachment of enteric bacteria to many kinds of surface, including mammalian epithelial cells, erythrocytes and yeast cells[4,11,17]. The latter results in agglutination, which can be used in screening for the presence of type-1 fimbriae[11]. Type-3 fimbriae agglutinate human O erythrocytes treated with tannin[3,12] and this property has been used in screening for the presence of these fimbriae[3,4,7]. It is not known whether the agglutination of tannin-treated erythrocytes by type-3 fimbriae results from fimbrial binding to tannin or to structures exposed on erythrocyte surface by the tannin treatment. The two fimbriae also differ morphologically: type-1 fimbriae are 5–7 nm, type-3 fimbriae 3–4 nm, in diameter. Amino acid compositions of the two *Klebsiella* fimbrillins are different and the filaments show weak immunological cross reactivity[12]. The molecular weight of type-3 fimbrillin is 23,500, that of type-1 fimbrillin 18,000[12].

Duguid and coworkers[3,4,19] have surveyed the occurrence of fimbriae on *Klebsiella* and *Enterobacter* strains. They found that most of the *Klebsiella* strains isolated from plants had both type-3 and type-1 fimbriae. Most *Enterobacter* species, on the other hand, had only type-1

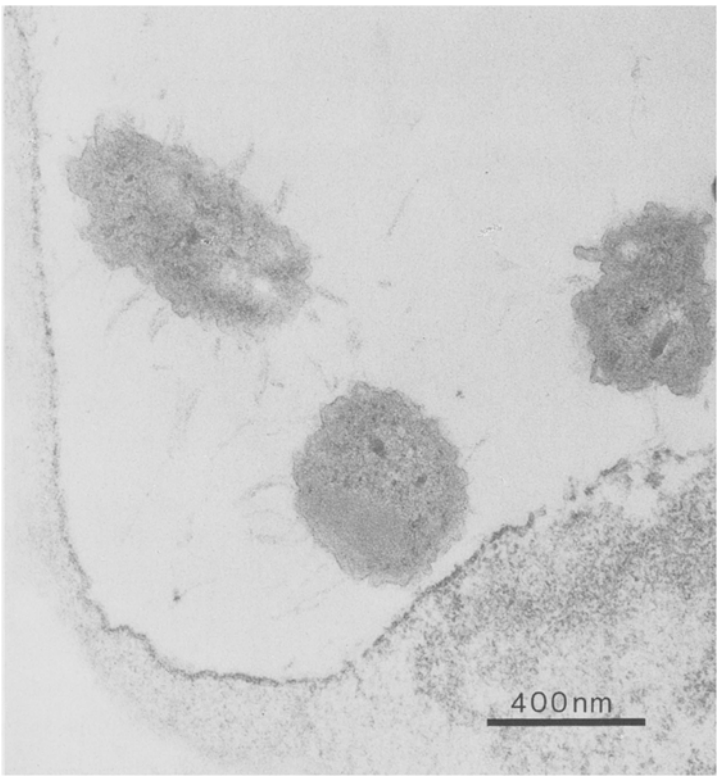

Fig. 2. Electron micrograph of *K. terrigena* strain 69/1 adhering to *P. pratensis* root surface *in vitro*. Bundles of fimbrial filaments can be seen between bacteria and root surface.

fimbriae. Recently, nitrogen-fixing *Klebsiella* and *Enterobacter* strains isolated from roots of ten grass species were screened for fimbrial antigens[7]. We found that all eight *Klebsiella* strains had type-3 fimbriae and five of them also had type-1 fimbriae, and all 21 of the *Enterobacter* strains had type-1 fimbriae. The *Klebsiella* strains were typed either as *K. pneumoniae* or as *K. terrigena,* and the *Enterbacter* strains as *E. agglomerans* belonging mostly to the biogroup G3 of Ewing and Fife[5]. No specific association was found between grass species and bacterial serotype, biogroup or fimbriation[7]. It thus seems that all nitrogen-fixing enteric bacteria are fimbriated and that *Klebsiella* and *Enterobacter* differ in that most of the former have two fimbrial antigens, type-1 and type-3.

Role of fimbriae in adhesion to plant roots

Electron microscopic studies on the adhesion (Fig. 2) showed numerous filaments, probably bundles of fimbriae, between bacterial

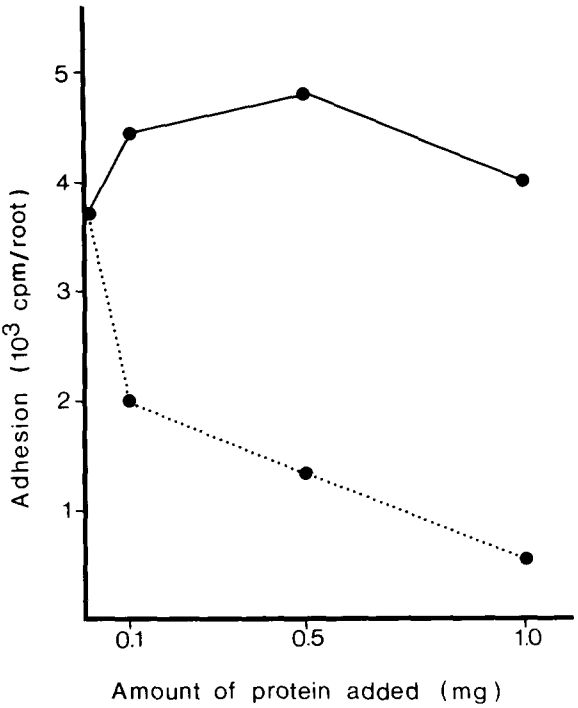

Fig. 3. Effect of anti-type-3-fimbriae Fab fragments on the adhesion of *K. terrigena* strain Php2 to roots of *P. pratensis*. Adhesion in the presence of bovine serum albumin (•——•), of Fab fragments (•·····•).

cells and root surface. This indicated that fimbriae might be active in adhesion.

For a more detailed study of the role of fimbriae in the adhesion system under discussion three approaches were made. Thus if fimbriae are involved in adhesion, then (1) Fab fragments prepared from immunoglobulin G specific for the fimbriae should inhibit bacterial adhesion; (2) purified, radiolabeled fimbriae should bind to plant roots *in vitro;* and (3) fimbriated bacteria should adhere more efficiently than nonfimbriated ones.

We immunized rabbits with purified type-3 fimbriae of *K. terrigena* 69/1 and type-1 fimbriae of *K. pneumoniae* 55/1, purified immunoglobulin G from the hyperimmune sera and prepared Fab fragments against both fimbrial antigens[8,12]. Both strains are plant isolates[3,4,19]. It should be noted that antisera or immunoglobulins as such should not be used in inhibition studies, since they aggregate bacteria and reduce the number of free cells; this will lead to decreased adhesion in an unspecific manner. Figure 3 shows the effect of anti-type-3-fimbriae

Fab fragments on the adhesion of *K. terrigena* strain Php2 to roots of *P. pratensis*. For adhesion tests, bacteria were grown in static malate broth containing ^{3}H-leucine, and adhesion was quantitated as radioactivity remaining on the roots after thorough washing. Roots, each 1 cm long, from axenically germinated seeds were used in each assay. A control experiment was performed in the presence of bovine serum albumin. A dose-dependent inhibition by Fab fragments was observed, which indicated that type-3 fimbriae were involved in the binding. Similar inhibition was observed with anti-type-1-fimbriae Fab fragments using strain 55/1, which has only type-1 fimbriae[8]. Moreover, adhesion of type-1-fimbriated *Enterobacter* and *Klebsiella* strains was inhibited by mannoside in a dose-dependent manner[8].

Type-3 and type-1 fimbriae retained their lectin-like binding properties after purification, *i.e.* type-3 fimbriae agglutinated tannin-treated human O erythrocytes and type-1 fimbriae showed a mannose-sensitive agglutination of yeast cells[8,12]. We then labeled both fimbriae with ^{125}I and tested their binding to roots of *P. pratensis in vitro*. The binding of both fimbriae was inhibited by Fab fragments specific for the fimbriae but not by bovine serum albumin[8,12]. Moreover, the binding of type-1 fimbriae was also inhibited by mannoside[8]. It was concluded that both type-3 and type-1 fimbriae can mediate enterobacterial attachment to roots of *P. pratensis*.

We tested various nitrogen-fixing *Klebsiella* and *Enterobacter* strains for adhesion to roots of grasses (*Dactylis glomerata, Festuca pratensis, F. rubra, Lolium perenne, Phalaris arundinacea, Phleum pratense,* and *Poa pratensis*) and to roots of cereals (*Avena sativa, Hordeum vulgare, Secale cereale, Triticum aestivum, T. sativum*)[7] (Fig. 4). The adhesion tests were made with bacterial concentrations of 5×10^8 bacteria per ml. We found that the *Klebsiella* strains adhered to all roots in significantly higher numbers than did the *Enterobacter* strains. For *Klebsiella* the mean number of bacteria per root was 10^6 with grasses and 1.5×10^6 with cereals; for *Enterobacter* it was 4×10^5 and 5×10^5, respectively. A root, 1 cm long, of *Poa pratensis* could accept up to about 10^7 bacteria[7].

Adhesion with three *Klebsiella* model strains was also tested: a type-3-fimbriated strain 69/1, a type-1-fimbriated strain 55/1 and a nonfimbriated strain 5/B149. The strains are among those described by Duguid and coworkers[3,4,19]. The strain 69/1 adhered to most test roots in numbers about ten times higher than those of other strains. At low bacterial concentrations the strain 55/1 adhered slightly better than the strain 5/B149[7].

Taken together, our results show that both type-3 and type-1 fimbriae

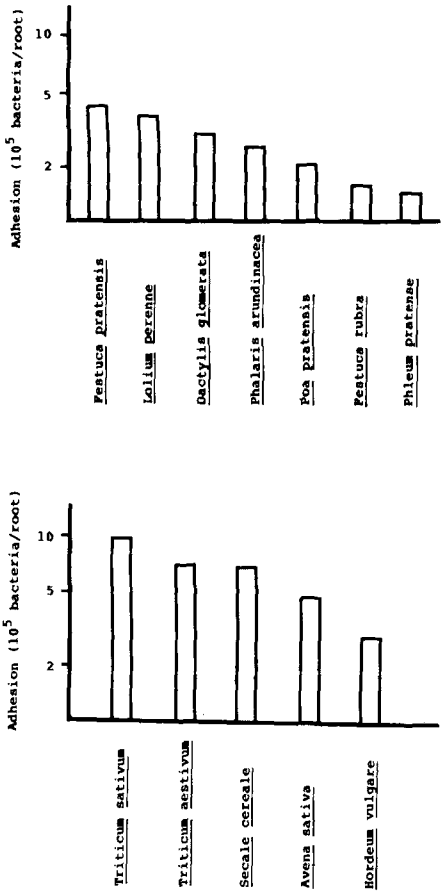

Fig. 4. Bar diagrams of adhesion of *K. terrigena* strain Cp to the roots of grasses and cereals.

can mediate enterobacterial adhesion to roots of grasses and cereals and that type-3 fimbriae are more efficient in promoting adherence. Type-3 fimbriae occur mostly on klebsiellas[3,4,7], which are nonmotile and may have to compensate their lack of mobility (and hence of possible chemotaxis towards root exudates) by a more efficient capacity of attachment.

Lack of host specificity in enterobacterial adhesion

To establish possible host specificity we assayed six *Klebsiella* and nine *Enterobacter* strains for adhesion to roots of the 7 grasses and the 5 cereals listed above[7].

Figure 4 shows results of adhesion assays with strain *K. terrigena* Cp. The strain, isolated from the roots of *Carex pallescens*, had only type-3

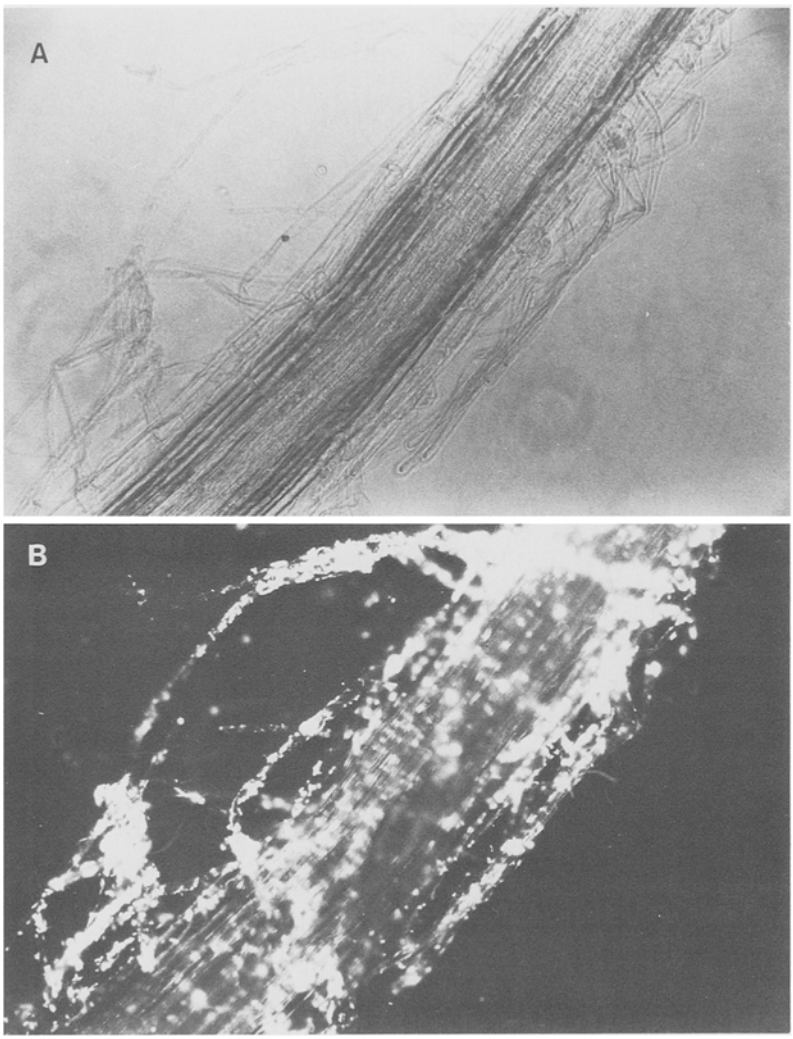

Fig. 5. An immunofluorescence study of adhesion of *Klebsiella* strain As to a root of *F. rubra*. A, micrograph of a root that is shown in B for immunostaining of adherent bacteria. The staining was done with fluorochrome-conjugated anti-type-3-fimbriae antibodies. An efficient bacterial adhesion to root hairs can be seen.

fimbriae. Its adhesion values varied from 10^5 to 10^6 bacteria per root and were higher with cereal roots than with grass roots. Roots 1 cm long were used in each assay, but obviously the roots differed in surface area. No attempts were made to adjust adhesion values for this variation. However, it is evident that the roots did not differ markedly in taking up bacteria. The other strains gave similar results[7], *i.e.* adhesion values for a particular strain were within a power of ten for all roots,

and all strains adhered better to roots of cereals than to roots of grasses.

We conclude that there is no strict host specificity in enterobacterial adhesion to roots of grasses or cereals. This is supported by the fact that the strains were isolated from the roots of a number of plant species and that the same plant species could host either *Klebsiella* or *Enterobacter* strains of different fimbriation, serotypes or biogroups[7].

Adhesion sites for *Klebsiella* on grass roots

We then located the adhesion sites for fimbriated *Klebsiella* strains on the roots of *P. pratensis* and *F. rubra*. This was done by performing the adhesion assay in a routine manner and staining the adherent bacteria with fluorchrome-labelled anti-fimbriae antibodies. Figure 5 shows an example of such a staining. A highly localized adhesion can be seen: bacteria bind effectively to root hairs but not to the root surface. The fimbriate bacteria also bind, but apparently with a lower efficiency, to the zone of elongation and the root mucilage, *i.e.* to the areas where plant exudates are available. Similar results were obtained with type-1- and type-3-fimbriated strains, so it appears that both fimbriae bind to the same sites on the root. No difference was observed between the roots of *P. pratensis* or *F. rubra*.

Conclusions

All nitrogen-fixing enteric bacteria isolated in Finland had fimbriae and adhered to the roots of various grasses and cereals. No strict host specificity in the adhesion could be observed, which was to be expected since the strains were isolated from a total of ten different grass species[7]. Thus, interaction between enteric bacteria and grasses seems to differ from *Rhizobium*-legume interaction, where specific bacterial adhesion to plant roots establishes symbiosis[2,9]. *Rhizobium* strains adhere specifically to root hairs; whereas the nitrogen-fixing enteric bacteria also bind to the zone of elongation and the root cap mucilage. In all, *Klebsiella* strains seem to adhere to those areas on the grass root where they have the best access to plant exudates.

Enterobacterial adhesion to roots of *P. pratensis* is mediated by fimbriae[8,12] and it seems that type-3 fimbriae are more efficient in promoting adherence than mannose-binding type-1 fimbriae. In accordance with the results of Duguid and coworkers[3,4,19] we found type-3 fimbriae on nitrogen-fixing klebsiellas but not on *Enterobacter* strains[7]. At present we cannot totally exclude other adhesion mechanisms, such as plant lectins, but their role is probably not significant, in view of the

efficient inhibition by fimbriae-specific Fab fragments[8,12] (Fig. 3) and receptor analogues[8]. Interestingly, it has been proposed that fimbriae are active in rhizobial adhesion to soybean roots[25] and a mannose-binding lectin, possibly a protein similar to the type-1 fimbriae of enteric bacteria, has been identified on a strain of *Rhizobium leguminosarum*[10]. One may speculate that adhesion mediated by fimbriae or other bacterial lectins is not restricted to enteric bacteria but represents a more general recognition mechanism between plants and bacteria.

The biological significance of the fimbriae-mediated adhesion is still partly in doubt. The fact that all nitrogen-fixing enteric bacteria have fimbriae suggests that such structures function in associative nitrogen fixation. *In vitro* adhesion tests showed that *Klebsiella* strains adhered better than *Enterobacter* strains, but the latter were isolated from grass roots more frequently than klebsiellas[7]. This could be due to bacterial chemotaxis towards plant roots[1], a phenomenon which is lacking in nonmotile *Klebsiella* strains. Adhesion to plant roots, which enables enteric bacteria to benefit from the organic materials exuding from the roots, is apparently not the only factor that establishes associative nitrogen fixation. We are now cloning fimbrial genes from *Klebsiella* for a more detailed study of their role and significance in associative nitrogen fixation.

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