

Review article

Interactions Between N₂-Fixing Enteric Bacteria and Grasses

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Abstract

Adhesion, colonization, and inoculation effects of N₂-fixing *Klebsiella* and *Enterobacter* on roots of grasses are described. Fimbriae-mediated adhesion of the bacteria is mainly localized on root hairs. Receptor-active structures appear to occur on the roots of various grasses, and identical adhesion sites were identified for type-1- or type-3-fimbriated bacteria. Inoculation of *Poa pratensis* and *Triticum aestivum* with enteric bacteria resulted in bacterial colonization of root hairs and expression of fimbriae by the bacteria on root hairs. Variable effects of bacterial inoculation/colonization on the dry matter and N yields of the test plants were observed, and only in half of the established *in vitro* associations atmospheric nitrogen was transferred to the plant. Enterobacterial colonization altered the root morphology; the number of root hairs and lateral roots were increased in infected plants. Similar effects on newly germinated roots of *P. pratensis* were observed with cell-free extracts from culture supernatants of *Klebsiella* and *Enterobacter*.

Keywords: adhesion, colonization, cereals, *Enterobacter*, fimbriae, *Klebsiella*, root morphology

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1. Introduction

Associative N_2 -fixing bacteria have been found on many plants. These bacteria live attached to plant root surfaces, where they are thought to be protected from predators and to benefit from nutrients exudated by the plant roots. Grasses and cereals probably benefit from associative N_2 -fixing bacteria by several mechanisms, such as phytohormones, increased mineral uptake, and improved water status (Okon and Kapulnik, 1986; Patriquin et al., 1983). Associative N_2 -fixers do not induce nodulation in host plants, but colonization on root surfaces has profound effects on root morphology (Patriquin et al., 1983; Jain and Patriquin, 1984; Okon and Kapulnik, 1986; Hadas and Okon, 1987; Haahtela et al., 1988).

In an attempt to reveal the molecular basis of associative N_2 fixation, we have studied interactions between N_2 -fixing enteric bacteria and grasses. A property of N_2 -fixing *Klebsiella* and *Enterobacter* strains isolated from roots of several Finnish grasses is their efficient *in vitro* adhesion to roots of grasses and cereals (Dazzo et al., 1986; Korhonen et al., 1986). This adhesion involves bacterial fimbriae. Fimbriae occur on species of the three enterobacterial genera that are known to include N_2 -fixers, i.e. *Enterobacter*, *Erwinia*, and *Klebsiella* (Duguid, 1959; Duguid and Old, 1980; Korhonen et al., 1987). In Scandinavia, *Enterobacter* and *Klebsiella* are the predominant bacterial species involved in associative N_2 fixation (Haahtela, 1985; Haahtela and Korhonen, 1985; Lindberg and Granhall, 1984), their frequencies obviously overcoming that of *Azospirillum* which is predominant in tropical areas (Döbereiner et al., 1976). In this communication, we summarize our recent studies on adhesion, colonization, and inoculation effects of N_2 -fixing *Klebsiella* and *Enterobacter* on roots of grass species common in the Scandinavian area.

2. Fimbriae-Mediated Bacterial Adhesion to Grass Roots

The root-associated, N_2 -fixing *Klebsiella pneumoniae*, *K. terrigena* and *Enterobacter agglomerans* strains studied in our laboratory express fimbriae (Haahtela and Korhonen, 1985). The *Klebsiella* strains have type-3 and type-1 fimbriae but the *Enterobacter* strains type-1 fimbriae only. The type-3 and type-1 fimbriae differ in the apparent molecular weight of the subunits; type-3 fimbriin molecular weight of *Klebsiella* is 23,500 and that of type-1 is 18,000 for *Klebsiella* and 18,500 for *Enterobacter* (Haahtela and Korhonen, 1985; Korhonen et al., 1983). The type-1 fimbriae bind to α -D-mannosides and occur widely in all enterobacterial species and strains (Duguid, 1959;

Old, 1972). Neither the receptor structure nor the binding specificity of the type-3 fimbriae is known (Duguid, 1959; Korhonen et al., 1983), they are, however, routinely screened by haemagglutination of tannin-treated human erythrocytes (Duguid, 1959). The type-3 and type-1 fimbriae of *Klebsiella* also differ in their amino acid composition and immunological properties (Korhonen et al., 1983). It is noteworthy that the fimbrial types found on *Erwinia* differ from those of *Klebsiella* and *Enterobacter* in chemical and immunological properties (Korhonen et al., 1987).

James Duguid suggested in 1959 that the adhesion of klebsiellas to plant roots is mediated by fimbriae. We have confirmed that purified ^{125}I -labeled type-1 and type-3 fimbriae as well as fimbriated cells of *Klebsiella* and *Enterobacter* bind specifically to the roots of blue grass, *Poa pratensis* (Korhonen et al., 1983; Haahtela et al., 1985). Although both fimbrial types are involved in the adhesion to root surfaces, the type-3 fimbriae appear to be more efficient in promoting adherence (Haahtela and Korhonen, 1985). The *Klebsiella* and *Enterobacter* strains adhere to the roots of their natural host plants but also to those of *Trifolium pratense* not known to host associative N_2 -fixers. Moreover, roots of different grasses and cereals do not differ markedly in taking up bacteria (Haahtela and Korhonen, 1985). We thus concluded that there is no strict host specificity in enterobacterial adhesion to plant roots.

We used two methods to localize the adhesion sites on root surfaces: electron microscopy and indirect immunofluorescence by staining adherent bacteria on the roots with fimbriae-specific antibodies. Both methods gave closely similar results. Whereas the non-fimbriated model strain adhered only poorly, the fimbriated bacteria showed an efficient and highly localized adhesion to root hairs of *P. pratensis* (Fig. 1). Similar results were obtained with roots of fescue grass *Festuca rubra* (Fig. 2). No differences in the binding sites were observed between the type-1 and the type-3 fimbriae or between the grass species studied (Haahtela et al., 1986).

3. Colonization of Roots

We compared 8 strains of *Klebsiella* and 11 strains of *Enterobacter* with different fimbriation, serotypes or biogroups (Haahtela and Korhonen, 1985) for colonization efficiency on roots of *P. pratensis* and *T. aestivum*. With an inoculum of $ca\ 10^8$ colony forming units (cfu), each strain colonized both plants in numbers ranging from 10^4 to 10^7 bacteria per root. To demonstrate expression of fimbriae during colonization and to locate bacterial colonization on roots we stained roots by indirect immunofluorescence using

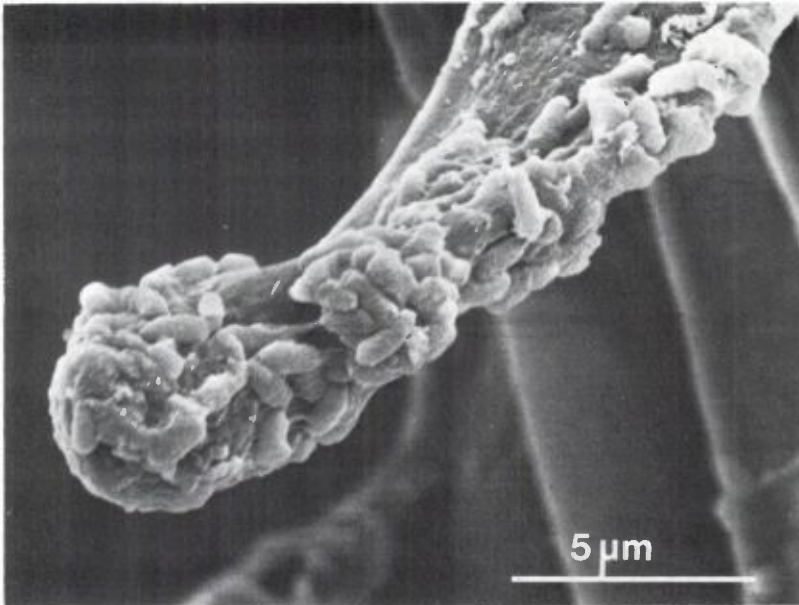


Figure 1. Scanning electron micrograph of *E. agglomerans* Php1 attached *in vitro* to a root hair of *P. pratensis*.

fimbriae-specific antibodies. Immunostained bacteria were found exclusively on surfaces of root hairs, and both the type-1 and the type-3 fimbriae were expressed during colonization (Haahtela et al., 1986; 1988).

4. Effects of Bacterial Inoculation on Plant Growth

In inoculated plants, variable effects on shoot dry matter yields, total N yields, and N content were observed both in *P. pratensis* and *T. aestivum*. Incorporation of atmospheric N_2 into plants was analyzed by the ^{15}N dilution method (Haahtela and Kari, 1986; Haahtela et al., 1988). The percentage of N derived from atmosphere varied in different associations. Thus in all associations N_2 -fixing bacteria were present on roots, as demonstrated by positive acetylene reduction assay, but atmospheric nitrogen was transferred to host plants only in half of the associations tested (Haahtela et al., 1988). Despite the high % nitrogen derived from atmosphere values in some associations, there was no correlation between nitrogen fixation and yield increases (Table 1).

5. Bacterial Effects on Root Morphology

Because inoculation with *Klebsiella* and *Enterobacter* caused increases in plant yields, which did not, however, correlate with incorporation of atmospheric nitrogen (Table 1), we also studied possible changes in plant root morphology during association. For this purpose plant roots were examined by electron and light microscopy (Haahtela et al., 1988).

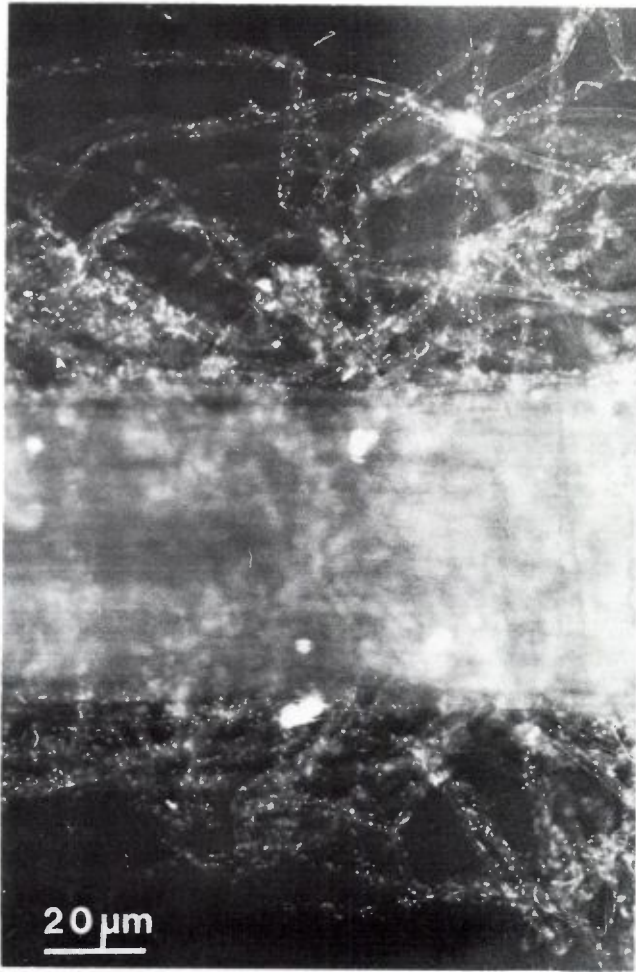


Figure 2. Adhesion of *K. pneumoniae* As to the roots of *F. rubra*, visualized by indirect immunofluorescence with anti-type-1 fimbria antiserum. Note strong adhesion to root hairs.

An altered root morphology in infected plants was evident in electron microscopy (Fig. 3). Inoculated roots contained significantly more root hairs than did uninoculated roots. Also the length of the elongation zone was shortened. We tested 19 of *Klebsiella* and *Enterobacter* strains and 13 of them strongly effected the root hair formation of *P. pratensis*. In *T. aestivum*, increased production of lateral roots was the dominant bacterial effect. Effects on root morphology were clearly detectable even at low inocula of $< 10^7$ cfu per root, which gave $ca 10^8$ colonized bacteria per root.

Table 1. Effect of inoculation with *Klebsiella* and *Enterobacter* on growth of *P. pratensis* and *T. aestivum*

Inoculum/ plant	% Ndfa ^a	Increase (%) in	
		Dry matter yield	Total N yield
<i>K. pneumoniae</i> Pp / <i>P. pratensis</i>			
Experiment 1 ^b	7	19	48
Experiment 2 ^b	34	0	0
Experiment 3 ^c	17	-13	19
<i>E. agglomerans</i> Am/ <i>P. pratensis</i> ^c			
	1	23	28
<i>K. pneumoniae</i> Pp/ <i>T. aestivum</i>			
Experiment 1 ^b	12	-10	0
Experiment 2 ^c	0	0	18
<i>E. agglomerans</i> Am/ <i>T. aestivum</i> /m ^c			
	2	0	0

^a Nitrogen derived from atmosphere (Haahtela and Kari, 1986; Haahtela et al., 1988).

^b (Haahtela and Kari, 1986)

^c (Haahtela et al., 1988)

We prepared neutral (pH 7.0) and acidic (pH 2.8) ethyl acetate (Jain and Patriquin, 1985) extracts from culture supernatants of 12 strains of *Klebsiella* and 11 strains of *Enterobacter* and analyzed them by thin layer chromatography (TLC) (Tien et al., 1979). The 2 extracts were tested with newly germinated roots of *P. pratensis* (Van de Geijn and Van Maaren, 1986). Compared with control roots (Fig. 4A), roots growing with the neutral fraction significantly increased the number of root hairs and decreased the length of elongation zone (Fig. 4B), whereas roots growing with the acidic fraction caused early initiation of lateral roots (Fig. 4C). Preparative TLC was used to separate compounds in the neutral extract from *E. agglomerans* and a compound increasing root hair formation has been purified. Characterization of this substance is under progress, and gas chromatography-mass spectrometry suggests that it is indole-3-acetic acid. The substances in the acidic extract, causing initiation of lateral roots have not yet been analyzed.

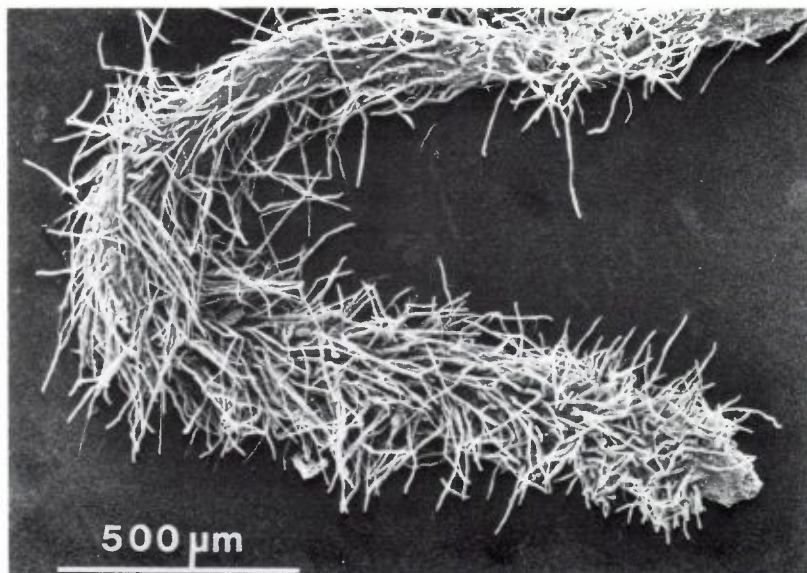


Figure 3. Scanning electron micrograph of a root of *P. pratensis* inoculated with *K. pneumoniae* Pp. Note the numerous root hairs and the short zone of elongation.

6. Conclusions

All *Enterobacter* and *Klebsiella* strains selected originally from grass roots for their ability to fix nitrogen produce fimbriae (Haahtela and Korhonen, 1985). Although fimbriation is not obligatory for nitrogen fixation, it may be an advantage for the bacteria by giving them an intimate contact to plant root surfaces. The nature of the plant macromolecules active as bacterial receptors in fimbriae-mediated adhesion is not known. The fimbriae-mediated adhesion of enteric bacteria to root hairs does not seem host-specific (Haahtela and Korhonen, 1985; Haahtela et al., 1986). Bacterial competition in the rhizosphere may influence the outcome of establishment of N_2 -fixing associations on plant roots. High numbers of saprophytic *Pseudomonas* cells diminished the influence of *Azospirillum* on roots of maize surface (Fallik et al., 1988). Therefore the effective, specific adhesion of enteric bacteria to root hairs might significantly increase their competence. Similar evidence for attachment and colonization of *Azospirillum* to grass roots including root hairs, has been described (Umali-Garcia et al., 1980).

Efficiency of N_2 transfer to the plants in associative N_2 fixation seems to vary considerably in different associations (Table 1; Okon et al., 1983; Malik et al., 1987) and our experiments leaves open the biological significance of N_2 fixation. The improved dry matter and N yields in the host plants may

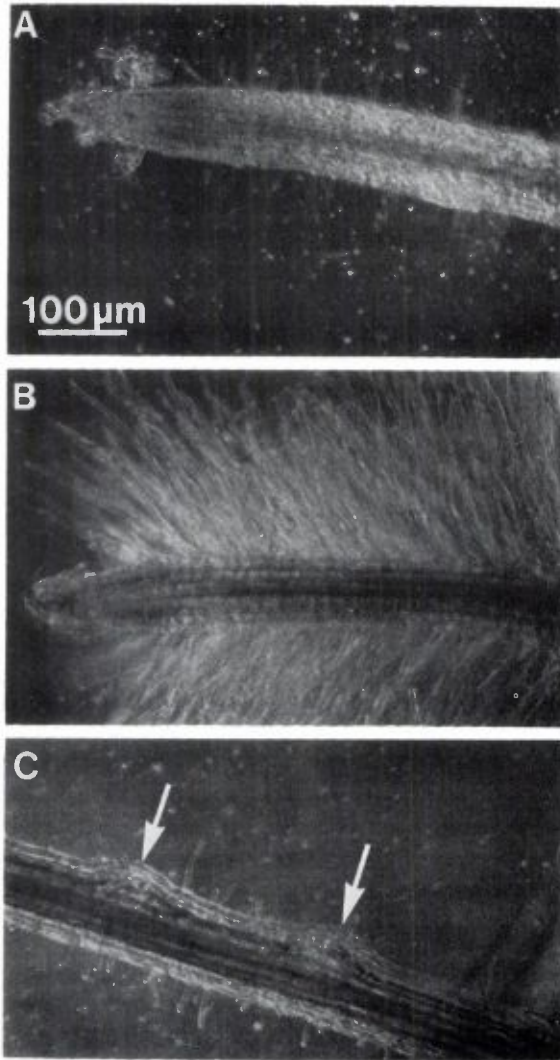


Figure 4. Effects of extracts from culture supernatant of *E. agglomerans* Am on newly germinated roots of *P. pratensis*. A root growing without extracts (A), with neutral (pH 7.0) ethyl acetate extract (B), and with acidic (pH 2.8) ethyl acetate extract (C). Note effective root hair formation in B and early initiation of lateral roots in C (arrows).

be caused by bacterial factors other than the Nif^+ phenotype, such as plant-growth promoting substances. Many rhizosphere bacteria are known to produce phytohormone-like substances (Horemans et al., 1986; van de Geijn and van Maaren, 1986). The ability of *Azospirillum* to increase root hair develop-

ment, root branching and root surface by producing indole-3-acetic acid is evident (Jain and Patriquin, 1984; Jain and Patriquin, 1985; Morgenstern and Okon, 1987). Inoculation with N₂-fixing *Klebsiella* and *Enterobacter* strains, as well as substances in bacterial culture medium, significantly change plant root morphology (Figs. 3 and 4). Interestingly, 2 types of morphological changes were seen (Fig. 4), effective formation of root hairs, which seems to be caused by indole-3-acetic acid, and early initiation of lateral roots, which is caused by unidentified substances.

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