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Root-Associated *Enterobacter* and *Klebsiella* in *Poa pratensis*: Characterization of an Iron-Scavenging System and a Substance Stimulating Root Hair Production

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Forty strains of *Enterobacter agglomerans, E. aerogenes, E. cloacae, Klebsiella pneumoniae,* and *K. terrigena* isolated from plants or humans were analyzed for iron-scavenging systems, plant growth-promoting effects on grasses, and production of auxins and related indole compounds. Enterochelin was produced by all isolates of *Klebsiella*, none of which produced aerobactin. None of the isolates of *Enterobacter* from plants produced enterochelin or aerobactin, whereas isolates from humans produced both siderophore types. Inoculation with each enterobacterial isolate significantly increased the number of root hairs of *Poa pratensis*, with no significant difference between bacteria from plants or humans. Cellfree ethyl acetate extracts were tested on newly germinated roots of *P. pratensis*. Extracts obtained at pH 7.0 significantly increased the number of root hairs, whereas extracts obtained at pH 2.8 increased production of root hairs only in few plants. A bioactive compound causing increased production of root hairs was isolated and characterized from the culture supernatant of a strain of *E. agglomerans*. Gas chromatography-mass spectrometry (GC-MS) analysis of this compound proved that the bioactive substance was an auxin, indole-3-acetic acid. Thin-layer chromatographic analysis of the neutral extracts showed that the enterobacterial isolates produced at least 10 indole compounds from which eight were identified by GC-MS. Slight differences in spectra of indole compounds were observed between bacterial isolates from plants and humans, but indole-3-acetic acid was detected in 88% of the enterobacterial isolates.

Additional keywords: associative N_2-fixers, root morphology.

A number of physiological interactions between host plants and bacteria have been identified in associative nitrogen fixation (Okon and Kapulnik 1986; Haahtela *et al.* 1986, 1988a; Hadas and Okon 1987; Kucey 1988). The bacteria probably benefit from nutrients excreted by the host plant, and improved growth of the host plant has been evident in many associations. The bacterial factors responsible for the latter phenomenon remain in part unclear. In many associations, atmospheric nitrogen is fixed and transferred to the host plant by the associative bacteria, but still no definite correlation has been established between the amount of nitrogen transferred to the plant and the observed increases in plant growth or yields (Okon *et al.* 1983; Smith *et al.* 1984; Haahtela *et al.* 1988a; Bashan *et al.* 1989). This has aroused an interest to characterize in more detail the other observed effects of nitrogen-fixers on plants.

Typical effects of *Azospirillum* in cereals include increased growth of roots, an increase in the number of lateral roots and root hairs, and deformation of root hairs (Tien *et al.* 1979; Umali-Garcia *et al.* 1980; Okon and Kapulnik 1986; Jain and Patriquin 1985; Hadas and Okon 1987; Kucey 1988). Similar morphological changes occur in *Poa pratensis* L. infected with root-colonizing enteric bacteria (Haahtela *et al.* 1986, 1988a). Changes in plant roots have also been shown with cellfree extracts from these bacteria, suggesting that they produce phytohormones or other plant growth stimulators (Tien *et al.* 1979; Okon and Kapulnik 1986; Jain and Patriquin 1984, 1985; Horemans *et al.* 1986; Haahtela *et al.* 1988b; Harari *et al.* 1988; Zimmer and Bothe 1988).

Compounds from all groups of plant hormones, auxins, cytokinins, gibberellins, and ethylene, have been isolated and identified as bacterial products (Pegg 1985). The most frequently detected plant hormone among nitrogen-fixing *Azospirillum, Rhizobium*, and *Frankia* is an auxin, indole-3-acetic acid (IAA) (Tien *et al.* 1979; Badenoch-Jones *et al.* 1982; Hartmann *et al.* 1983; Wheeler *et al.* 1984; Jain and Patriquin 1985; Horemans *et al.* 1986; Berry *et al.* 1989; Crozier *et al.* 1988; Harari *et al.* 1988; Zimmer and Bothe 1988; Fallik *et al.* 1989). Also cytokinins (Phillips and Torrey 1972; Tien *et al.* 1979; Horemans *et al.* 1986; Berry *et al.* 1989; Stevens and Berry 1988) and gibberellins (Tien *et al.* 1979; Ernstsen *et al.* 1987) have been detected in these bacteria. All these phytohormones may have a role in changes in root morphology.

Another type of plant-growth-promoting system has been analyzed in root-associated *Pseudomonas*. These bacteria produce efficient iron-scavenging systems, hydroxamate-type siderophores, that apparently are involved in stimulation of plant growth (Kloepper *et al.* 1980; De Weger *et al.* 1986). In this system the effect is most likely not on the plant itself, but siderophores prevent growth of

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deleterious bacteria and fungi (Kloepper *et al.* 1980; De Weger *et al.* 1986) and thus create a favorable environment for plant growth.

We have recently shown that nitrogen-fixing *Klebsiella* and *Enterobacter* adhere to and colonize the roots of *P. pratensis* and that this colonization induces alteration in root morphology (Haahtela *et al.* 1986, 1988a). This study was undertaken to analyze isolates of *Klebsiella* and *Enterobacter* for iron-scavenging systems and to characterize and identify indole compounds responsible for increased growth of root hairs.

MATERIALS AND METHODS

Bacteria. The 11 strains of *E. agglomerans* (Beijerinck) Ewing and Fife, four strains of *K. pneumoniae* (Schroeter) Trevisan, and seven strains of *K. terrigena* Izard *et al.* of plant origin (Table 1) have been described earlier (Haahtela *et al.* 1981; Haahtela and Korhonen 1985). *K. pneumoniae* 55/1 and *K. terrigena* 69/1 were originally obtained from J. P. Duguid (Dundee, U.K.), and *K. pneumoniae* C3 was from J. Thomas (Barcelona, Spain). For comparison, one strain of *E. aerogenes* Hormaeche and Edwards, nine strains of *E. cloacae* (Jordan) Hormaeche and Edwards, and eight strains of *K. pneumoniae* from urine or blood of adult patients were included in the study (A.-M. Tarkkanen, M. Kauppi, K. Haahtela, A. Siitonen, I. Ørskov, F. Ørskov, B. A. Allen, S. Clegg, and T. K. Korhonen, unpublished data) (Table 1). These strains were identified and biotyped with API 20E and API 20CHE test kits (API Systems SA, Montalieu Vercieu, France). The bacteria were grown for 48 hr at 28° C in malate broth (Haahtela *et al.* 1983; Korhonen *et al.* 1983). For analysis of indole compounds, the bacteria were grown with shaking for 4 days in malate broth supplemented with tryptophan (100 μg/ml).

Siderophore bioassays. Cross-feeding bioassays for siderophore production were performed on iron-restricted agar medium (containing 200 μM 2,2′-dipyridyl) inoculated with either of two K-12 indicator strains of *Escherichia coli*, as previously described (Carbonetti *et al.* 1986). Strain AN1937 was used to detect enterochelin production (Williams 1979), and strain LG1522 (which carries a ColV-K30*iuc* mutant plasmid) was the indicator for aerobactin secretion (Carbonetti *et al.* 1986). Isolates of *Klebsiella* and *Enterobacter* to be tested were spotted onto the agar surface; siderophore synthesis was indicated by a halo of growth of lawn bacteria around the point of inoculation.

Plant material, inoculation, and conditions of growth. Seeds of *P. pratensis* were surface-sterilized by treatment with 94% ethanol for 1 min and with 5% (w/v) hypochloric acid for 10 min, washed six times with sterile water, and germinated on water agar plates as previously described (Korhonen *et al.* 1983).

For analyzing bacterial effects on root morphology, the surface-sterilized, germinated seedlings 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 solution (Hoagland and Arnon 1938) (one-fourth concentration). Each seedling was inoculated with 10^8 colony forming units of bacterial cells (in 0.2 ml of the malate broth, six seedlings with each inoculant). The seedlings were grown with 300 μg of KNO_3-nitrogen, of which 150 μg was given at planting, followed by 30 μg in 1.5 ml of Hoagland's solution per week. Uninoculated roots were used as controls. The plants

Table 1. Bacterial strains used in this study[a]

Designation	Biogroup[b] /serotype[c]	Fimbria[d]		N_2-fix[e]	Siderophores	
		T1	T3		Aerobactin[f]	Enterochelin[g]
Plant isolates						
E. agglomerans						
Ea Am	G3	+	−	+	−	−
Ea Ca	G3	+	−	+	−	−
Ea Dg1	G3	+	−	+	−	−
Ea Fr1	G3	+	−	+	−	−
Ea Pha	G4	+	−	+	−	−
Ea Php	G3	+	−	+	−	−
Ea Php1	G3	+	−	+	−	−
Ea Php3	G1	+	−	+	−	+
Ea Php5	G1	+	−	+	−	+
Ea Pp1	G3	+	−	+	−	−
Ea Pp2	G3	+	−	+	−	−
K. pneumoniae						
Kp As	K54	+	+	+	−	+
Kp Pp	K54	+	+	+	−	+
Kp C3	ND[h]	+	+	+	−	+
Kp 55/1	K55	+	−	−	−	+
K. terrigena						
Kt Cp	K80	−	+	+	−	+
Kt Pha	K36	+	+	+	−	+
Kt Php1	K80	+	+	+	−	+
Kt Php2	K8,26,74	−	+	+	−	+
Kt Pp1	K32	+	+	+	−	+
Kt Pp2	K8,26,74	−	+	+	−	+
Kt 69/1	K69	−	+	−	−	+
Clinical isolates						
E. aerogenes						
IHK 12151		+	−	−	+	+
E. cloacae						
IHK 12152		+	−	−	+	+
IHK 12153		+	−	−	+	+
IHK 12154		+	−	−	+	+
IHK 12156		+	−	−	+	+
IH 16105		+	−	−	+	+
IH 16138		+	−	−	+	+
IH 16195		+	−	−	+	+
IHK 16218		+	−	−	+	+
IHK 16297		+	−	−	+	+
K. pneumoniae						
IHK 12110	K14	+	+	−	−	+
IHK 12112	K9	+	+	+	−	+
IHK 12114	K30	+	+	−	−	+
IHK 12116	K28	+	+	−	−	+
IHK 12117	K24	+	+	−	−	+
IHK 12120	K54	+	+	+	−	+
IHK 12121	K80	+	−	−	−	+
IHK 12131	K54	+	+	+	−	+

[a] Results concerning biogroup, serotype, and fimbria of plant isolates have been published earlier (Haahtela and Korhonen 1985).

[b] Biogroups for strains of *Enterobacter* are according to Ewing and Fife (1972).

[c] Reaction with anti-*Klebsiella* capsular serum.

[d] Agglutination and immunofluorescence assay with specific antibodies.

[e] Acetylene reduction assay.

[f] Aerobactin bioassay.

[g] Enterochelin bioassay.

[h] ND, not done.

were grown for 5 wk under greenhouse conditions with a photoperiod of 18 hr (fluorescent tubes). After harvesting, the roots were washed thoroughly with sterile phosphate-buffered saline (pH 7.2). For counting the number of bacteria colonizing the roots, two of the roots were homogenized, and samples of dilutions were plated on malate agar as described previously (Haahtela *et al.* 1988a). To evaluate bacterial effects on root morphology, the remaining four roots were examined by light microscopy (Haahtela *et al.* 1986, 1988a).

Extraction of indole compounds. Bacterial cultures (200 ml) were centrifuged at 8,000 × *g* for 30 min. The supernatants were concentrated to 50 ml by evaporation under vacuum, and the pH was adjusted to 7.0 with 1.0 M NaOH. The supernatants were extracted twice with 15 ml of ethyl acetate (extract I). The pH of the residual water phase was adjusted to 2.8 with 1.0 M HCl, and it was extracted twice with 15 ml of ethyl acetate (extract II) (Jain and Patriquin 1985). Ethyl acetate extracts I and II were evaporated to dryness under vacuum and redissolved in 1 ml of methanol. The concentrated extracts were stored at −20° C.

Reference compounds. Authentic indole compounds used as standards (Table 2) for thin-layer chromatography (TLC), gas chromatography-mass spectrometry (GC-MS), and the plant root bioassay were from Sigma (St. Louis, MO). Standards were dissolved in methanol and stored at −20° C.

TLC. Aliquots (50 to 200 μl of concentrated extract per centimeter of TLC plate) of extracts I and II and the reference compounds (5 to 40 μg/cm) were analyzed on 0.25-mm-thick silica gel plates (E. Merck AG, Darmstadt, Federal Republic of Germany). Three different solvent systems were used. They are as follows: chloroform-ethyl acetate-formic acid (5:4:1) (solvent system 1), chloroform-ethyl acetate-water (5:4:1) (solvent system 2), and methyl acetate-isopropanol-25% NH_3 (45:35:20) (solvent system 3). After chromatography, the separated compounds were visualized by fluorescence under ultraviolet light (254 nm) and by staining with Ehrlich reagent (Bentley 1962). For purification of different compounds, seven preparative TLC plates were run with solvent system 1, dried, and transversely fractionated on the basis of compounds detected under ultraviolet light. The individual TLC fractions were scraped from the plate, homogenized, eluted with methanol, and finally concentrated by evaporation under vacuum.

Table 2. Indole standards

Indole compound	Abbr.	Mol. wt.	Method used[a] TLC	MS	BT[b]
Anthranilic acid[c]	ANT	137.1	x	x	−
Indole	IND	117.1	x	x	
Indole-2-carboxylic acid	ICA2	161.2	x		−
Indole-3-aldehyde	IAL	145.2	x	x	−
Indole-3-acetic acid	IAA	175.2	x	x	+++
Indole-3-acetaldehyde	IAAL	159.2	x	x	+++
Indole-3-acetamide	IAAM	174.2	x	x	−
Indole-3-acetone	IASE	173.2	x	x	+
Indole-3-acetonitrile	IAN	156.2	x	x	+
Indole-3-butyric acid	IBU	203.2	x	x	+++
Indole-3-ethanol (Tryptophol)	TOL	161.2	x	x	−
Indole-3-carboxylic acid	ICA3	161.2	x	x	−
Indole-3-lactic acid	ILA	205.2	x	x	−
Indole-3-methanol	IMOH	147.2	x	x	
Indole-3-propionic acid	IPR	189.2	x	x	+
Indole-3-pyruvic acid	IPY	203.2	x	x	−
Indole-5-carboxylic acid	ICA5	161.2	x		−
3-Methylindole	MIND	131.2	x	x	
Tryptamine	TRA	160.2	x	x	−
Tryptophan	TPP	204.2	x	x	−
3-β-Indoleacrylic acid	IACR	187.2	x		
3-Hydroxyanthranilic acid[c]	HANT	153.1	x		
5-Hydroxyindole	HIND	133.2	x		−
5-Hydroxyindole-2-carboxylic acid	HICA2	177.2	x		
5-Hydroxyindole-3-acetic acid	HIAA	191.2	x		
5-Hydroxyindole-3-acetamide	HIAAM	190.2	x		
5-Hydroxyindole-3-acetonitrile	HIAN	172.2	x		

[a] TLC = thin-layer chromatography; MS = mass spectrometry; BT = biotest; and x = tested.

[b] The increase in the number of root hairs was quantitated as − (no effect), + (weak), ++ (moderate), and +++ (strong) (Fig. 1); the concentration of each compound was 1 μg/ml (also tested at 1, 10, and 100 ng/ml, data not shown).

[c] Derivative of benzene (not an indole compound).

Table 3. Effect of *Klebsiella* and *Enterobacter* and their cellfree extracts on root hairs of *Poa pratensis*

Strain	Effect on root hairs[a] by Bacteria	Extract I	Extract II
Plant isolates			
E. agglomerans			
Ea Am	++	+++	−
Ea Dg1	++	++	−
Ea Fr1	+	+++	−
Ea Pha	+++	+++	+
Ea Php1	++	+	+
Ea Php5	−	−	−
Ea Pp1	++	+	−
K. pneumoniae			
Kp As	+++	+	−
Kp C3	+	++	−
K. terrigena			
Kt Cp	++	++	−
Kt Pha	+	++	+
Kt Php1	++	++	+
Kt Php2	++	+	−
Kt Pp1	+	+++	+
Clinical isolates			
E. aerogenes			
IHK 12151	++	−	−
E. cloacae			
IH 16105	+++	−	−
IH 16138	+++	−	−
IH 16195	++	+++	−
IHK 16297	+++	+++	−
K. pneumoniae			
IHK 12110	+	+++	−
IHK 12112	+	+	−
IHK 12114	+	+++	++
IHK 12116	+	+++	+
IHK 12117	+++	++	++
IHK 12131	+	+	−
Control[b]	−	−	−

[a] The increase in the number of root hairs was quantitated as − (no effect), + (weak), ++ (moderate), and +++ (strong) (Fig. 1).

[b] Uninoculated plants or roots treated with extract from uninoculated medium.

The fractions obtained were tested by the plant root bioassay (see below), and bioactive fractions were further fractionated by TLC with solvent system 2. After chromatography, the plates were again fractionated, and the identified fractions were eluted and tested by the bioassay and identified by GC-MS (see below).

Plant root bioassay. The effects of the ethyl acetate extracts, or their fractions, and of the reference compounds on root morphology were tested by a modification of the method of Van de Geijn and Van Maaren (1986). Surface-sterilized seeds were germinated in water agar plates with a slope of approximately 60°. Five to 7 days after germination, a small square of cellophane (1×1 cm) was placed underneath the root tips. The samples (40 μl of concentrated extracts) to be analyzed were evaporated to dryness with air, dissolved in 1 ml of distilled water, and adjusted to pH 7.0. The samples were passed through a 0.2-μm membrane filter, heated to 50° C, and mixed with 2 ml of molten sterile agar (1.4%, pH 7.0 in water). The mixture was solidified in a petri dish (3.5 cm in diameter). Small disks (3 mm in diameter) of the agar were taken with a sterile cork borer and placed onto water agar plates on the cellophane in front of the root tips. The roots were incubated for 2 to 4 days and examined by light microscopy. To find a suitable concentration for the bioassay, the ethyl acetate fractions were tested in several concentrations differing from onefold to 125-fold. The reference compounds were tested in four concentrations: 1, 10, 100, and 1,000 ng/ml.

GC-MS. For GC-MS analysis, the bioactive thin-layer fractions and extract I from the Am strain of *E. agglomerans* were derivatized by methylation with diazomethane (Schlenk and Gellerman 1960) or by silylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA, E. Merck AG) (Badenoch-Jones *et al.* 1982). For methylation, the samples were evaporated to dryness and 100 μl of diazomethane in chloroform was added. After incubation for 24 hr at room temperature, the samples were evaporated to dryness and redissolved in heptane. For silylation, the dry samples were incubated with 100 to 400 μl of BSTFA-acetonitrile (1:1) for 2 hr at 70° C, evaporated, and redissolved in heptane. Underivatized samples were redissolved directly in heptane. The samples were analyzed in a Hewlett-Packard HP 5880 (Hewlett-Packard, Palo Alto, CA) gas chromatograph equipped with an HP 5970 A mass selective detector, HP 9000 computer system, and an HP-1 capillary column. Methylated samples were introduced in the splitless mode (0.5 min splitless time) at 270° C, and a temperature program of 1 min at 50° C, 30° C per minute to 150° C and 10° C per minute to 280° C was used; the detected mass area was 50.0 to 300.0 (5 to 17 min). Silylated samples were introduced in the splitless mode (2 min splitless time) at 225° C, and a temperature program of 3 min at 60° C, 30° C per minute to 130° C and 7° C per minute to 235° C was used; the detected mass area was 50.0 to 450.0 (5 to 23 min) (Ernstsen *et al.* 1987).

RESULTS

We have previously reported characteristics of root-associated enteric bacteria (Haahtela and Korhonen 1985). For comparison, fimbriation, K-types, and nitrogenase activity of the human isolates of *Enterobacter* and *Klebsiella* are shown in Table 1. The fimbriation in the plant and human isolates was similar, that is the strains of *Klebsiella* had type 3 and type 1 fimbriae, and the strains of *Enterobacter* had only type 1 fimbriae. Only three of the human isolates of *Klebsiella* possessed nitrogenase activity; interestingly two of those strains were of the K-type 54 that was found among the plant isolates as well.

Siderophores in strains of *Klebsiella* and *Enterobacter*. None of the plant-associated strains of *Klebsiella* was able to synthesize aerobactin (Table 1), but all were positive in the bioassay for enterochelin. Similar results were obtained for the human isolates of *Klebsiella*. In contrast, only two of the plant-associated strains of *Enterobacter* produced either siderophore, whereas the human isolates were positive for both aerobactin and enterochelin.

Effects of bacterial inoculation on root hairs. At an inoculum of 10^8 bacterial cells per root, each test strain colonized the plants in numbers ranging from 8.1×10^4 to 6.6×10^6 bacteria per root after growth for 5 wk (details not shown). Microscopic examination revealed that the inoculated roots contained significantly more root hairs than did the uninoculated roots (Table 3, Fig. 1). Only one strain of *E. agglomerans*, Php5, failed to give a response, and there was no significant difference between the human and the plant isolates. The differences in the effect on root hair formation seen between individual strains did not correlate with the number of bacteria colonizing the roots (not shown), or with the type of fimbriation, N_2 fixation, or siderophore production.

Plant response to bacterial culture extracts and reference compounds. The bioactivities of extracts I (pH 7.0) and II (pH 2.8) were tested with newly germinated roots (Table 3). Compared to untreated roots, the roots of plants grown with extract I showed significantly increased numbers of root hairs, whereas extract II increased production of root hairs only in few plants. Of the indole reference compounds (Table 2), indole-3-acetaldehyde and indole-3-butyric acid (IBU) occasionally had a strong effect on root hair production, and only at a high concentration (1 μg/ml), whereas IAA was the only one which repeatedly had a significant and concentration-dependent effect on root hair production (Fig. 1).

Preliminary TLC identification of indole compounds produced by *Enterobacter* and *Klebsiella*. Several indole compounds in extract I were preliminarily identified by TLC (Table 4). We realized that the identification of indole compounds was not possible by TLC alone, but the preliminary analysis was performed to detect the possible common compounds in extract I from different strains. Most strains of *Klebsiella* and *Enterobacter* produced compounds with the same R_f values as indole-3-acetamide (IAAM); indole-3-aldehyde (IAL)/tryptophol (TOL) (IAL and TOL migrated similarly during TLC); IAA/indole-3-carboxylic acid (ICA3); and indole-3-acetone (IASE)/IBU/indole-3-propionic acid. Klebsiellas of plant origin did not produce 5-hydroxyindole-3-acetamide, and only a few of the plant isolates produced indole-3-lactic acid (ILA); otherwise, the spectrum of indole compounds detected in plant and human isolates was similar, although the intensity of the compounds varied. The profile of indole compounds of *E. agglomerans* Php3 and Php5 was quite different from the other strains; these two strains produced at least three so far unidentified compounds (possibly other than indoles), which were not produced by other strains (details not shown). Compounds in extract II were so colored that their analysis by TLC was not possible.

Fractionation of extract I from *E. agglomerans* Am. Extract I from *E. agglomerans* Am was chosen for purification and identification of the substances that affected *P. pratensis* root hair formation. This strain expressed high bioactivity on roots (Table 3) and, with the exception of indole-3-methanol (IMOH), produced all the indole compounds detected by TLC (Table 4). After TLC with solvent system 1, only one fraction (with an R_f value of 0.80) of the 11 observed fractions actively promoted root hair formation. TLC with solvent system 2 separated this fraction further to 17 fractions of which only one (R_f value of 0.20) was bioactive. In both solvent systems, these bioactive fractions migrated identically to the IAA reference. After fractionation with solvent system 2, the bioactive compound was also analyzed by two-dimensional TLC first developed with solvent system 3 and then with solvent system 2. TLC was performed with authentic IAA as the reference and also by adding IAA to the active fraction; the two compounds also comigrated in this system (not shown).

Identification of the bioactive compound with GC-MS. The bioactive compound was methylated and analyzed by GC-MS and compared to the mass spectra of methylated standards listed in Table 2. The GC-MS analysis verified that the bioactive compound was IAA (Fig. 2).

GC-MS of indole compounds in extract I from *E. agglomerans* Am. Extract I was directly analyzed by GC-MS after methylation and silylation. Reference compounds were silylated both separately (IAL, IMOH, IAA, and IAAM) and as a mixture of the compounds indicated in Table 2 (except for indole-3-pyruvic acid and tryptophan). The indole compounds identified in extract I from *E. agglomerans* Am by methylation were IAA, IASE, ICA3, and TOL, and by silylation IAA, IAAM, IAL, ICA3, ILA, IMOH, and TOL. A total of eight indole compounds were identified, while some compounds characterized as indoles according to their ion peaks remained unidentified.

DISCUSSION

The fimbrial types that mediate enterobacterial adhesion to root hairs (Haahtela *et al.* 1986) are found in plant and clinical isolates of *Klebsiella* and *Enterobacter* (Table 1) and in many other members of the Enterobacteriaceae as well (Duguid and Old 1980). This indicates that enteric bacteria from diverse ecosystems possess the capacity to adhere to plant roots. Our results also show that the capacity to colonize roots of *P. pratensis* is not restricted to enterobacterial isolates from plants. It was particularly striking that we found no systematic difference in the efficiency of colonization of *P. pratensis* roots between

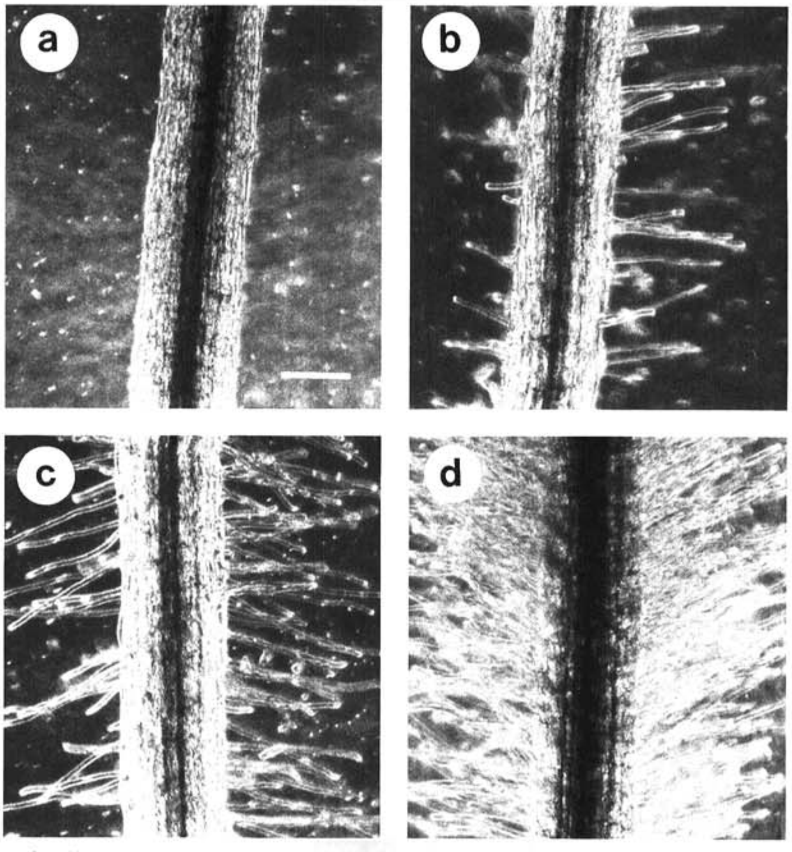

Fig. 1. Quantitation of the root hair effect: -, no effect (**a**); +, weak (**b**); ++, moderate (**c**); and +++, strong (**d**). The plants were tested with 1 (**b**), 10 (**c**), or 100 (**d**) ng/ml of indole-3-acetic acid; **a** shows roots without indole-3-acetic acid. The bar represents 100 μm.

plant and clinical isolates. *P. pratensis* is a common host for N_2-fixing *Klebsiella* and *Enterobacter* in Scandinavia (Haahtela *et al.* 1981; Haahtela and Korhonen 1985; Lindberg and Granhall 1984). It could be that the selection of the particular strains of *Klebsiella* and *Enterobacter* as associative nitrogen-fixers in this plant is dependent on factors other than adhesive and colonization capacities. One such factor could be the ability of the bacteria to survive in soil during nongrowth periods.

All the strains isolated from plants fixed nitrogen. Interestingly, three of the clinical klebsiellas (Table 1) were also able to fix nitrogen. Two of these strains had the capsular antigen K54, which was also found in some of the *K. pneumoniae* plant isolates. All the other clinical strains were nitrogenase-negative. In view of the conflicting results on the efficiency of nitrogen transfer to plants by the associative enteric bacteria (Haahtela and Kari 1986; Haahtela *et al.* 1988a), it is somewhat surprising that nitrogenase activity is the characteristic which most clearly separates plant from clinical isolates. It could be that the main biological role of nitrogenase activity in associative enteric bacteria is to increase their potential to survive outside the rhizosphere.

Production of siderophores improves competition for iron and also root colonization sites (Kloepper *et al.* 1980; Kloepper and Schroth 1981) and prevents action of soilborne pathogens on plants. This may be benefical for the bacteria during colonization of roots and also relevant for protection of plant roots from invading pathogens (De Weger *et al.* 1986). Such a mechanism might be possible for associative klebsiellas, which produced enterochelin as frequently as did the klebsiellas of human origin (Table 1). In contrast, most of the plant-associated strains of *Enterobacter* did not produce enterochelin or aerobactin, and therefore they may have other systems with which to compete in the root environment. It cannot, of course, be ruled out that other siderophores active in the two bioassays were produced by some of the strains; only by isolation and structural analysis can definitive indications be made. However, it is generally the case that bacterial ferrisiderophore receptors are specific, and as far as we are aware, there have been no reports that the aerobactin and enterochelin receptors act as receptors for other siderophores.

In our previous inoculation experiments considering similar associations (Haahtela and Kari 1986; Haahtela *et al.* 1986, 1988a), we have observed great variability in bacterial effects on the dry matter and nitrogen yields of the host plants and in the transfer of atmospheric nitrogen to plants. These parameters varied from significant increases in some associations to decreases in others. A similar variability is obvious in reports on other associative systems (Okon *et al.* 1983; Smith *et al.* 1984). However, in all our experiments the bacterial effects on root morphology, especially root hair production, have been pronounced and repeatable (Haahtela *et al.* 1986, 1988a; Table 3). In this study we found that the enteric bacteria both of plant and human origin increased the production of root hairs (Table 3). Interestingly, the clearest effects were produced by strains of *E. cloacae* of human origin, none of which could fix nitrogen (Tables 1 and 3). A related finding was recently reported for the *A. brasilense* Tarrand *et al.*-tomato association. Bashan *et al.* (1989) showed that the contribution of *A. brasilense* to the improvement of

Table 4. TLC analysis of indole compounds produced by plant and clinical isolates of strains of *Klebsiella* and *Enterobacter*[a]

	Number of strains producing indole compounds			
	Klebsiella		***Enterobacter***	
Indole	**Plant isolates ($n = 11$)**	**Clinical isolates ($n = 8$)**	**Plant isolates ($n = 11$)**	**Clinical isolates ($n = 10$)**
IMOH[b]	5	2	2	4
IND	8	6	5	9
IASE, IBU, IPR[c]	10	6	10	10
IAA, ICA3[c]	10	6	11	8
IAL, TOL[c]	11	6	11	9
IAAM	11	6	11	8
ILA	3	5	3	8
HIAAM	0	7	9	8
TRA	3	6	11	8
TPP	11	7	9	10

[a] Compounds were identified from extract I.
[b] For abbreviations see Table 2.
[c] All compounds in a group migrated with the same R_f value.

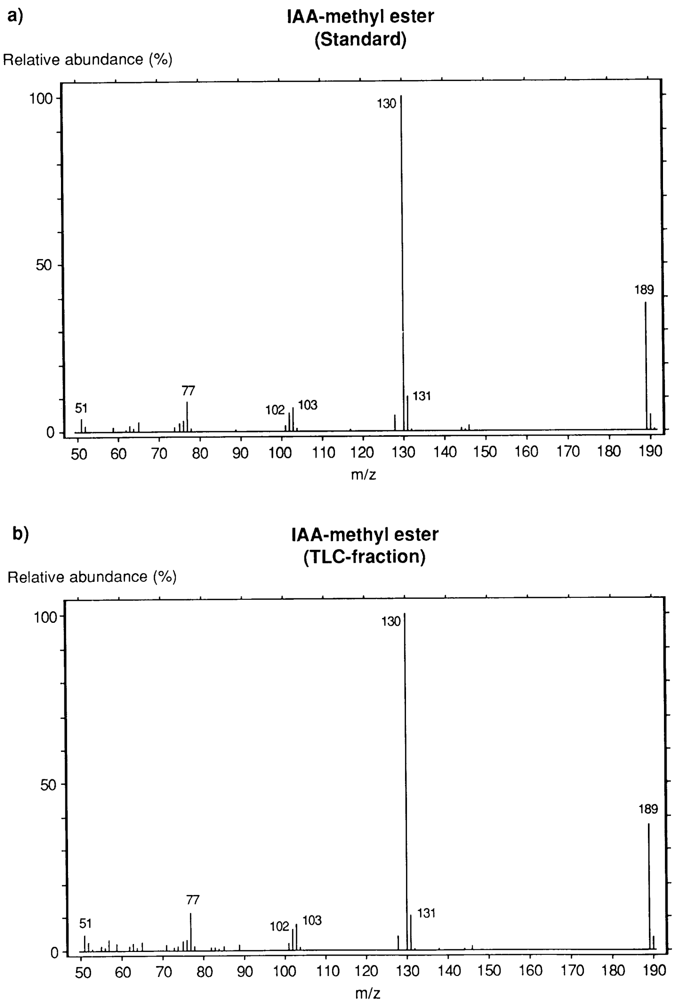

Fig. 2. Mass spectra of (**a**) the reference compound indole-3-acetic acid (IAA) and (**b**) the bioactive compound in extract I from the culture supernatant of *Enterobacter agglomerans* Am.

tomato seedling growth is not through N_2 fixation. The inoculation effects on root morphology and root hair proliferation by the enteric bacteria are very similar to those caused by *Azospirillum* in wheat, maize, and millet (Umali-Garcia *et al.* 1980; Kapulnik *et al.* 1985; Okon and Kapulnik 1986; Harari *et al.* 1988).

Neutral extracts (I) mimicked the effect of the bacterial cells and significantly increased plant root hair production (Table 3). In our studies the extracts from Ea IHK 12151, Ec IH 16105, and Ec IH 16138 did not show root hair effects, although this effect was clear with bacterial inoculations. It is obvious that other phytohormones may also have an effect on root hair proliferation and that small differences in the composition of phytohormonal mixtures can give rise to variable effects in plants (Wightman *et al.* 1980; Wightman and Thimann 1980; Tien *et al.* 1979; Hartmann *et al.* 1983; Van de Geijn and Van Maaren 1986; Harari *et al.* 1988). The effects of similar compounds might also vary in different plant-bacterium interactions. In *Azospirillum*-cereal associations, production of IAA has been reported to cause increased shoot (Tien *et al.* 1979) and root (Zimmer and Bothe 1988) dry weight, increased proliferation of root hairs (Harari *et al.* 1988; Van de Geijn and Van Maaren 1986), and deformation of root hairs (Jain and Patriquin 1985). In *Frankia-Alnus* and *Rhizobium*-legume symbioses, the role of IAA might be in the regulation of nodulation (Wheeler *et al.* 1984; Ernstsen *et al.* 1987). Production of IAA by pathogenic *Agrobacterium* causes and regulates tumor production in plants (Schröder 1987).

The analysis of indole compounds by TLC indicated production of several compounds by the enteric bacteria (Table 4). Most of these compounds could be preliminarily identified, or at least separated into groups of putative compounds, by TLC alone (Table 4). It was evident that the enterobacterial isolates produced similar patterns of indole compounds, and there were no significant differences between the clinical and the plant isolates.

Further identification of indole compounds in extract I was conducted with *E. agglomerans* Am. The separation and purification of indole compounds on TLC plates suggested that the bioactive substance causing root hair proliferation was IAA. This identification was verified by GC-MS analysis (Fig. 2). In associative nitrogen-fixers, production of IAA was reported earlier for *A. brasilense* (Harari *et al.* 1988; Jain and Patriquin 1985; Zimmer and Bothe 1988; Tien *et al.* 1979), but not for associative enteric bacteria. The physiological effects of *A. brasilense* in wheat, maize, and millet (Kapulnik *et al.* 1985; Okon and Kapulnik 1986; Harari *et al.* 1988) are very similar to the effects seen with associative enteric bacteria in *P. pratensis* (Haahtela *et al.* 1986, 1988a; Table 3). Roots of maize inoculated with *Azospirillum* have been found to have higher amounts of both free and bound IAA than those of control plants (Fallik *et al.* 1989), which possibly indicates transfer to plants of IAA excreted by *Azospirillum*. IBU, which is widely used in agriculture as a synthetic hormone (Nickell 1982), was also present in roots inoculated with *Azospirillum* (Fallik *et al.* 1989).

Direct GC-MS analysis of extract I from *E. agglomerans* Am verified the presence of eight indole compounds also detected by TLC: IAA, IAL, IAAM, IASE, ICA3, ILA, IMOH, and TOL (Table 4). *E. agglomerans* Php3 and Php5 produced at least three unidentified compounds, which were not produced by any other strain. They also produce enterochelin not found in the other strains of *Enterobacter* from plants. Interestingly, strains Php3 and Php5 differ also in their biotype and localization of *nif* genes (Väisänen *et al.* 1985). IAA, TOL, IAL, and IMOH have been found in *R. phaseoli* 8002 (Ernstsen *et al.* 1987), and IAA, TOL, IMOH, ICA3, and ILA have been found in *Frankia* sp. strain HFPArI3 (Berry *et al.* 1989). An interesting finding was the presence of IASE in *E. agglomerans* Am, since it has not been previously reported in plant-associated bacteria.

In summary, the morphological changes in *P. pratensis* roots infected with strains of *Klebsiella* and *Enterobacter* of either plant or human origin were similar, despite the fact that the latter lack nitrogenase activity. Almost all strains stimulated the proliferation of root hairs and produced several indole compounds, including IAA. Purification of the bioactive substance from extract I of *E. agglomerans* Am ascertained that IAA was one, perhaps the main, compound which affected root hair formation. However, the presence of other substances that affected the growth and root hair production of plants, either alone or in combination with IAA, cannot be ruled out.

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LITERATURE CITED

Badenoch-Jones, J., Summons, R. E., Djordjevic, M. A., Shine, J., Letham, D. S., and Rolfe, B. G. 1982. Mass spectrometric quantification of indole-3-acetic acid in *Rhizobium* culture supernatants: Relation to root hair curling and nodule initiation. Appl. Environ. Microbiol. 44:275-280.

Bashan, Y., Singh, M., and Levanony, H. 1989. Contribution of *Azospirillum brasilense* Cd to growth of tomato seedlings is not through nitrogen fixation. Can. J. Microbiol. 67:2429-2434.

Bentley, J. A. 1962. Analysis of plant hormones. Methods Biochem. Anal. 9:75-124.

Berry, A. M., Kahn, R. K. S., and Booth, M. C. 1989. Identification of indole compounds secreted by *Frankia* HFPArI3 in defined culture medium, Plant Soil 118:205-209.

Carbonetti, N. H., Boonchai, S., Parry, S. H., Väisänen-Rhen, V., Korhonen, T. K., and Williams, P. H. 1986. Aerobactin-mediated iron uptake by *Escherichia coli* isolates from human extraintestinal infections. Infect. Immun. 51:966-968.

Crozier, A., Arruda, P., Jasmim, J. M., Montero, A. M., and Sandberg, G. 1988. Analysis of indole-3-acetic acid and related indoles in culture medium from *Azospirillum lipoferum* and *Azospirillum brasilense*. Appl. Environ. Microbiol. 54:2833-2837.

De Weger, L. A., Van Boxel, R., Van der Burg, B., Gruters, R. A., Geels, F. P., Schippers, B., and Lugtenberg, B. 1986. Siderophores and outer membrane proteins of antagonistic, plant-growth-stimulating, root-colonizing *Pseudomonas* spp. J. Bacteriol. 165:585-594.

Duguid, J., and Old, D. C. 1980. Adhesive properties of *Enterobacteriaceae*. Pages 185-217 in: Bacterial Adherence. E. H. Beachey, ed. Receptors and Recognition, Ser. B, Vol. 6. Chapman and Hall, London.

Ernstsen, A., Sandberg, G., Crozier, A., and Wheeler, C. T. 1987. Endogenous indoles and the biosynthesis and metabolism of indole-3-acetic acid in cultures of *Rhizobium phaseoli*. Planta 171:422-428.

Ewing, W. H., and Fife, M. A. 1972. *Enterobacter agglomerans* (Beijerinck) comb. nov. (the Herbicola-Lathyri bacteria). Int. J. Syst. Bacteriol. 22:4-11.

Fallik, E., Okon, Y., Epstein, E., Goldman, A., and Fischer, M. 1989. Identification and quantification of IAA and IBA in *Azospirillum brasilense*-inoculated maize roots. Soil Biol. Biochem. 21:147-153.

Haahtela, K., and Kari, K. 1986. The role of root-associated *Klebsiella pneumoniae* in the nitrogen nutrition of *Poa pratensis* and *Triticum aestivum* as estimated by the method of ^{15}N isotope dilution. Plant Soil 90:245-254.

Haahtela, K., and Korhonen, T. K. 1985. In vitro adhesion of N_2-fixing enteric bacteria to roots of grasses and cereals. Appl. Environ. Microbiol. 49:1186-1190.

Haahtela, K., Wartiovaara, T., Sundman, V., and Skujins, J. 1981. Root-associated N_2 fixation (acetylene reduction) by *Enterobacteriaceae* and *Azospirillum* strains in cold-climate spodosols. Appl. Environ. Microbiol. 41:203-206.

Haahtela, K., Helander, I., Nurmiaho-Lassila, E.-L., and Sundman, V. 1983. Morphological and physiological characteristics and lipopolysaccharide composition of N_2-fixing (C_2H_2-reducing) root-associated *Pseudomonas* sp. Can. J. Microbiol. 29:874-880.

Haahtela, K., Laakso, T., and Korhonen, T. K. 1986. Associative nitrogen fixation by *Klebsiella* spp.: Adhesion sites and inoculation effects on grass roots. Appl. Environ. Microbiol. 52:1074-1079.

Haahtela, K., Laakso, T., Nurmiaho-Lassila, E.-L., and Korhonen, T. K. 1988a. Effects of inoculation of *Poa pratensis* and *Triticum aestivum* with root-associated, N_2-fixing *Klebsiella, Enterobacter* and *Azospirillum*. Plant Soil 106:239-248.

Haahtela, K., Laakso, T., Nurmiaho-Lassila, E.-L., Rönkkö, R., and Korhonen, T. K. 1988b. Interactions between N_2-fixing enteric bacteria and grasses. Symbiosis 6:139-149.

Hadas, R., and Okon, Y. 1987. Effect of *Azospirillum brasilense* inoculation on root morphology and respiration in tomato seedlings. Biol. Fertil. Soils 5:241-247.

Harari, A., Kigel, J., and Okon, Y. 1988. Involvement of IAA in the interactions between *Azospirillum brasilense* and *Panicum miliaceum* roots. Plant Soil 110:275-282.

Hartmann, A., Singh, M., and Klingmuller, W. 1983. Isolation and characterization of *Azospirillum* mutants excreting high amounts of indole acetic acid. Can. J. Microbiol. 29:916-923.

Hoagland, D. R., and Arnon, D. 1938. The water-culture method of growing plants without soil. Calif. Agric. Exp. Stn., Berkeley, Circ. 347.

Horemans, S., De Koninck, K., Neuray, J., Hermans, R., and Vlassak, K. 1986. Production of plant growth substances by *Azospirillum* sp. and other rhizosphere bacteria. Symbiosis 2:341-346.

Jain, D. K., and Patriquin, D. G. 1984. Root hair deformation, bacterial attachment, and plant growth in wheat-*Azospirillum* associations. Appl. Environ. Microbiol. 48:1208-1213.

Jain, D. K., and Patriquin, D. G. 1985. Characterization of a substance produced by *Azospirillum* which causes branching of wheat root hairs. Can. J. Microbiol. 31:206-210.

Kapulnik, Y., Gafny, R., and Okon, Y. 1985. Effect of *Azospirillum* spp. inoculation on root development and NO^-_3 uptake in wheat (*Triticum aestivum* cv. Miriam) in hydroponic systems. Can. J. Bot. 63:627-631.

Kloepper, J. W., and Schroth, M. N. 1981. Plant growth-promoting rhizobacteria and plant growth under gnotobiotic conditions. Phytopathology 71:642-644.

Kloepper, J. W., Leong, J., Teintze, T., and Schroth, M. N. 1980 Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature 286:885-886.

Korhonen, T. K., Tarkka, E., Ranta, H., and Haahtela, K. 1983. Type 3 fimbriae of *Klebsiella* sp.: Molecular characterization and role in bacterial adhesion to plant roots. J. Bacteriol. 155:860-865.

Kucey, R. M. N. 1988. Plant growth-altering effects of *Azospirillum brasilense* and *Bacillus* C-11-25 on two wheat cultivars. J. Appl. Bacteriol. 64:187-196.

Lindberg, T., and Granhall, U. 1984. Isolation and characterization of dinitrogen-fixing bacteria from the rhizosphere of temperate cereals and forage grasses. Appl. Environ. Microbiol. 4:683-689.

Nickell, L. S., ed. 1982. Plant Growth Regulators, Agricultural Uses. Springer, Berlin.

Okon, Y., and Kapulnik, Y. 1986. Development and function of *Azospirillum*-inoculated roots. Plant Soil 90:3-16.

Okon, Y., Heytler, P. G., and Hardy, R. W. F. 1983. N_2 fixation by *Azospirillum brasilense* and its incorporation to host *Setaria italica*. Appl. Environ. Microbiol. 46:694-697.

Pegg, G. F. 1985. Pathogenic and non-pathogenic microorganisms and insects. Pages 599-624 in: Encyclopedia of Plant Physiology, Vol. 11. R. P. Pharis and D. M. Reid, eds. Springer-Verlag, Berlin.

Phillips, D. A., and Torrey, J. G. 1972. Studies on cytokinin production by *Rhizobium*. Plant Physiol. 49:11-15.

Schlenk, H., and Gellerman, I. H. 1960. Esterification of fatty acids with diazomethane on a small scale. Anal. Chem. 32:1412-1414.

Schröder, J. 1987. Plant hormones in plant-microbe interactions. Pages 40-63 in: Plant-Microbe Interactions: Molecular and Genetic Perspectives, Vol. 2. T. Kosuge and E. W. Nester, eds. Macmillan Publishing Co., New York.

Smith, R. L., Schank, S. C., Milam, J. R., and Baltensperger, A. A. 1984. Responses of *Sorghum* and *Pennisetum* species to the N_2-fixing bacterium *Azospirillum brasilense*. Appl. Environ. Microbiol. 41:342-345.

Stevens, G. A., Jr., and Berry, A. M. 1988. Cytokinin secretion by *Frankia* sp. HFPArI3 in defined medium. Plant. Physiol. 87:15-16.

Tien, T. M., Gaskins, M. H., and Hubbell, D. H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl. Environ. Microbiol. 37:1016-1024.

Umali-Garcia, M., Hubbell, D. H., Gaskins, M. H., and Dazzo, F. B. 1980. Association of *Azospirillum* with grass roots. Appl. Environ. Microbiol. 39:219-226.

Väisänen, O., Haahtela, K., Bask, L., Kari, K., Salkinoja-Salonen, M., and Sundman, V. 1985. Diversity of *nif* gene location and nitrogen fixation among root associated *Enterobacter* and *Klebsiella* strains. Arch. Microbiol. 141:123-127.

Van De Geijn, S., and Van Maaren, J. M. M. 1986. A fast screening method for bacterial isolates producing substances affecting root-growth. Symbiosis 2:67-75.

Wheeler, C. T., Crozier, A., and Sandberg, G. 1984. The biosynthesis of indole-3-acetic acid by *Frankia*. Plant Soil 78:99-104.

Wightman, F., and Thimann, K. V. 1980. Hormonal factors controlling the initiation and development of lateral roots. I. Sources of primordia-inducing substances in the primary root of pea seedlings. Physiol. Plant. 49:13-20.

Wightman, F., Schneider, E. A., and Thimann, K. V. 1980. Hormonal factors controlling the initiation and development of lateral roots. II. Effects of exogenous growth factors on lateral root formation in pea roots. Physiol. Plant. 49:304-314.

Williams, P. H. 1979. Novel iron uptake system specified by ColV plasmids: An important component in the virulence of invasive strains of *Escherichia coli*. Infect. Immun. 26:925-932.

Zimmer, W., and Bothe, H. 1988. The phytohormonal interactions between *Azospirillum* and wheat. Plant Soil 110:239-247.