Escherichia coli Common Pilus (ECP) Targets Arabinosyl Residues in Plant Cell Walls to Mediate Adhesion to Fresh Produce Plants*‡§

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Background: Bacterial fimbriae mediate binding to host tissue through specific interactions.
Results: ECP interacts with arabinosyl residues in pectin and other plant cell wall components.
Conclusion: ECP-arabinan interactions facilitate binding of E. coli to plant hosts.
Significance: The prevalence of arabinan targets in produce plants together with ECP expression may explain the association of pathogenic bacteria in edible plants.

Outbreaks of verotoxigenic Escherichia coli are often associated with fresh produce. However, the molecular basis to adherence is unknown beyond ionic lipid-flagellum interactions in plant cell membranes. We demonstrate that arabinitol is present in different constituents of plant cell walls that are targeted for adherence by E. coli common pilus (ECP; or meningitis-associated and temperature-regulated (Mat) fimbiae) for E. coli serotypes O157:H7 and O18:K1:7. α-Arabinose is a common constituent of plant cell wall that is rarely found in other organisms, whereas ECP is widespread in E. coli and other environmental enteric species. ECP bound to oligosaccharides of at least arabino- or longer in a glycan array, plant cell wall pectic polysaccharides, and plant glycoproteins. Recognition overlapped with the antibody LM13, which binds arabinanase-sensitive pectic epitopes, and showed a preferential affinity for (1–5)-α-linked l-arabinosyl residues and longer chains of arabinitol as demonstrated with the use of arabino-degrading enzymes. Functional adherence in planta was mediated by the adhesin EcpD in combination with the structural subunit, EcpA, and expression was demonstrated with an ecpR–GFP fusion and ECP antibodies. Spinach was found to be enriched for ECP/LM13 targets compared with lettuce. Specific recognition of arabinosyl residues may help explain the persistence of E. coli in the wider environment and association of verotoxigenic E. coli with some fresh produce plants by exploitation of a glycan found only in plant, not animal, cells.

E. coli O157:H7, one of the most important causative agents of fresh produce-associated outbreaks, is often linked with contaminated lettuce and spinach (11, 12).

In mammalian hosts, bacterial adherence is often mediated by lectins present at the fimbrial tip that bind to complementary carbohydrates on the surface of the host tissues. Type 1 fimbriae and P fimbriae are the best characterized in the chaperone-usher family, encoding tip adhesins FimH and PapD, respectively. They recognize α-D-mannosylated proteins and α-D-galactopyranosyl-(1→4)-β-D-galactopyranoside receptor epitope in the globoseries of glycolipids, respectively (13, 14). Conversely, no specific adhesion targets in plant tissue have been elucidated either for phytopathogenic bacteria that encode chaperone-usher fimbriae or for human pathogenic bacteria that can colonize plants as secondary hosts.

The E. coli common pilus (ECP), originated termed meningitis-associated and temperature-regulated (Mat) fimbria, was first identified in newborn meningitis and septicemia E. coli isolate IH3034 (O18:K1:H7) when it was grown at 20 °C (15). The ecp operon is ubiquitous across E. coli and even conserved for some other enteric species (15–19). ECP belongs to the chaperone-usher family encoded by the ecpRABCDE operon where EcpA encodes the pilin domain and EcpD encodes the polymerized tip adhesin. Unusually for classical tip adhesins, EcpD can be polymerized independently, which requires an N-terminal extension in EcpD, or with the major pilin domain (18). Several roles have been described for ECP, including binding to cultured human epithelial cells (16, 17, 20), colonization of infant mice (21), and biofilm development through interorganelle binding of EcpA (22). The regulator EcpR represses the flagellar master operon flhDC, supporting the role for EcpA in biofilms (23). Expression of ECP is strain-dependent in E. coli; some isolates belonging to the B2 phylogenetic group are able to produce these fimbiae in conditions

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The abbreviations used are: ECP, E. coli common pilus; Mat, meningitis-associated and temperature-regulated; HG, homogalacturonan; RG, rhamnogalacturonan; HRGP, hydroxyproline-rich glycoprotein; AGP, arabino-lactotransferase; CDTA, diaminocyclohexanetetraacetic acid; Ara7, arabinohexitolase; AF, arabinofuranosidase; PL, pectin lyase.
mimicking those in the intestine or at 20 °C, whereas others do not retain this capability (24).

Plant cell walls are complex configurations of structures composed mostly of carbohydrates present in polysaccharides and highly glycosylated proteins. Polysaccharides are represented by three different families: cellulose, hemicellulose, and pectin. Cellulose is present as long chains of β-1,4-glucose, and hemicelluloses are branched polysaccharides containing backbones of neutral sugars. Pectins are defined by the presence of uronic acids divided in four polysaccharide “domains”: homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), and xylogalacturonan (25, 26). The polysaccharides are linked together in varying abundances with HG as the most abundant followed by RG-I (27). Cell walls also contain abundant hydroxyproline-rich glycoproteins (HRGPs), a superfamily that encompasses extensins, proline-rich proteins, and arabinogalactan proteins (AGPs). HRGPs are mainly O-glycosylated with arabinose and galactose (28, 29).

In the present study, we tested the hypothesis that bacteria interact with plant tissue by targeting specific glycans. We identified targets for two different ECP variants in plants by using high throughput plant glycan arrays and enzyme-linked immunosorbent assay (ELISA). We were able to capitalize on the extensive body of work for ECP/Mat from newborn meningitis and septicemia E. coli strain IHE3034 (O18:K1:H7) but expanded this to investigate the role of ECP from the human pathogen E. coli O157:H7 (strain Sakai). The interactions were characterized with plant polysaccharides and from functional in planta adhesion assays.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Media—**E. coli O157:H7 strain Sakai (Shiga-toxin negative) (30) and E. coli K-12 strain JT1 (31) were grown in either Luria-Bertani (LB) broth or rich defined MOPS supplemented with 0.2% glucose, thiamine, and essential and non-essential amino acids (32). Antibiotics were included where necessary to maintain transformed plasmids or for selection with adherence assays at the following final concentrations: 50 µg ml⁻¹ kanamycin, 12.5 µg ml⁻¹ chloramphenicol, 50 µg ml⁻¹ ampicillin, 10 µg ml⁻¹ tetracycline. Induction of ecp in recombinant E. coli strains was carried out with 5 µM isopropyl β-D-thiogalactopyranoside. All media, antibiotics, and inducers were purchased from Sigma-Aldrich. E. coli strain JT1 was selected to overexpress ECP fimbriae. This strain lacks flagella and type 1 fimbriae, and it encodes a copy of the ecp cluster even though it belongs to serogroup K-12, which does not contain strong, active promoters for the expression of native ecp (24).

**Cloning and Mutagenesis—ecpA (ECs0323) and ecpA–D (ECs0323–0320) deletions were constructed using allelic exchange as described previously (33). Primers used for crossover PCR are listed in Table 1. A PstI site in the ecpA upstream sequence required that the PCR products were blunt end-cloned via T4 polynucleotide kinase into the pTOF24 vector. A Flp recombinase target-flanked tetracycline cassette was sub-cloned into the NotI site introduced into pTOF24, creating allelic exchange vectors pAH002 for ecpA and pAH003 for ecpA–D. The promoter region of ecpR (440 bp of the 5’-UTR) was PCR-amplified from E. coli O157:H7 Sakai genomic DNA with primers 0324.5.XbaI and 0324.3.XbaI (Table 1) and cloned into pKC026 using XbaI, creating the transcriptional fusion pAH001. Two overexpression constructs for E. coli, ecpA–D and ecpA–E, were generated by cloning the regions into pSE380 (Table 2), generating pYR006 and pYR007, using HindIII-HindIII and XbaI-HindIII, respectively. Enzymes were purchased from New England Biolabs (Ipswich, MA) or Roche Applied Science. Plasmid clones were confirmed by appropriate restriction digests and Sanger sequencing. Mutants were confirmed by phenotype and diagnostic PCR.

**ECP Preparation and Antibodies—**ECF from pYR006 (EcpA–E from E. coli O157:H7 Sakai) and pMat3 (EcpA–E from E. coli O18:K1:H7 IHE3034) transformed in E. coli JT1 were isolated as described with modifications (34). In short, bacteria were cultured in LB medium supplemented with isopropyl β-D-thiogalactopyranoside and ampicillin at 37 °C for 16 h in static conditions, harvested by centrifugation, resuspended in cold Tris-buffered saline (TBS), detached from the bacterial cell by using a blender (three times for 30 s), and centrifuged twice at 4,000 x g for 30 min. Ammonium sulfate was added slowly into the supernatant containing the fimbriae with vigorous stirring to achieve two-thirds saturation. After an overnight incubation at 4 °C, the fimbriae were harvested by centrifugation at 15,000 x g for 20 min at 4 °C and then suspended in cold TBS + 0.5% deoxycholate. The preparation was subjected to a sucrose gra-

**TABLE 1**

| Product                        | Primer name          | Primer sequence                      |
|-------------------------------|----------------------|--------------------------------------|
| ecpA–D                        | ecpAHindIII          | CCGAACCTTTGCTCCATTAATCACTGG          |
|                              | ecpDHindIII          | CCGAACCTCCTGGAGCAATGCCTGG           |
|                              | EcpAXbaI             | GCACATACGGCTCTTTGAAAGTCTTGG          |
|                              | EcEpEHindIII         | TCCTGATTTGAAGAATCTCCCTTBGGG         |
| 5′-flanking region of ecpA for deletions | 0324.5.XbaI         | GCTGCTGATTTTAATTTGACGCC             |
|                              | 0324.3.XbaI          | GCCTGCACACTTTCAAAACCTGC             |
| 3′-flanking region of ecpA deletions | 0323.NotI.3F        | CCGTCTTCGGCGCTTTGAAAATCCCTTGG       |
|                              | 0323.PstI.3R         | CGCTTCGGCGCTTTGAAAATCCCTTGG         |
| 3′-flanking region of ecpD for deletions | 0320.NotI.3F        | CGAGTTTCTGCTCCTTTGAAAGTCTTGG       |
|                              | 0320.PstI.3R         | TTATGTGCTTACTTGAACCGCCCTGC          |
| ΔecpA.NotI linker             | 0323.Sall.5F         | CTGGTCTTGCTGCTTTGAAAATCCCTTGG       |
|                              | 0323.PstI.3R         | CGCTTCGGCGCTTTGAAAATCCCTTGG         |
| ΔecpA–D.NotI linker           | 0323.Sall.5F         | GCACATACGGCTCTTTGAAAGTCTTGG         |
|                              | 0320.PstI.3R         | TCCTGATTTGAAGAATCTCCCTTBGGG         |

* Restriction enzyme sequences are underlined.
TABLE 2
Plasmids and strains
FRT, Flip recombinase target; Tet, tetracycline.

| Plasmid | Relevant features | Source/Ref. |
|---------|-------------------|-------------|
| pSE380  | Expression vector, tetr primer | Invitrogen |
| pYR006  | pSE380; Amp<sup>R</sup>, Xhol-HindIII insertion of EcpA–E (Sakai) | This study |
| pYR007  | pSE380; Amp<sup>R</sup>, HindIII–HindIII insertion of EcpA–D (Sakai) | This study |
| pMat3   | pSE380; Amp<sup>R</sup>, EcpA–E (IHE3034) | 15 |
| pTOF24  | Temperature-sensitive allelic exchange vector | 33 |
| pTOF1-Tet<sup>R</sup> | FRT Tet cassette | 27 |
| pKC26   | pAI145 with P <sup>R</sup> rpmM replaced by PgyrA, Cam<sup>R</sup> | This study |
| pAH001  | pKC26: PgyrA replaced with PcepR, Cam<sup>R</sup> | This study |
| pAH002  | pTOF24: PstI–Sall blunted end insertion of ecpa. NotI linker + NotI insertion of Tet from pTOF1-Tet<sup>R</sup> | This study |
| pAH003  | pTOF24: PstI–Sall blunted end insertion of ecpa–D. NotI linker PCR + NotI insertion of Tet from pTOF-Tet<sup>R</sup>. | This study |

TABLE 3
Monoclonal antibodies

| Antibody | Specificities | Ref. |
|----------|--------------|-----|
| LM1      | Extensin (plant glycoprotein) | 78  |
| LM2      | AGPs | 79  |
| LM5      | (1→4)-β-Galactan | 80  |
| LM6      | (1→5)-α-Arabinin and arabinoglactan (plant glycoprotein) | 39, 43 |
| LM13     | (1→5)-α-Arabinin | 38, 44, 45 |
| LM15     | Non-fucosylated xylglucan (XXXG) | 56  |
| LM16     | Uncharacterized RG-I epitope | 38, 44 |
| LM21     | Heteromannan | 50  |
| LM25     | XXXG/galactosylated xylglucan | 38  |
| JIM7     | Partially methyl-esterified homogalacturonan | 55  |
| JIM8     | AGPs | 45  |
| JIM19    | Extensin (plant glycoprotein) | 45  |

Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and 1× plant protease inhibitor mixture (Sigma-Aldrich). The samples were incubated on ice for 30 min and then centrifuged at 400 × g at 4 °C for 3 min. Protein concentrations were determined using the BCA protein assay. The proteins were kept at −80 °C for no longer than 1 week.

Cell Wall Polymer Extraction—Lettuce and spinach leaves or roots were ground by mortar and pestle to a fine powder in liquid nitrogen. Six volumes of ethanol 70% were added to 1 volume of powder and incubated at room temperature for 10 min with rotation and then centrifuged at 3,000 × g 10 min. This step was repeated five times. The pellet was rinsed twice with acetone and dried. This alcohol-insoluble residue preparation was then followed by sequential extraction using two solvents: 50 mM CTDA, pH 7.5 and 4 M NaOH. The two extraction solvents used are known to solubilize pectins and noncellulosic polysaccharides, respectively.

Enzymatic Treatment—Highly purified enzymes (Megazyme, Bray, Ireland) included endo-β-mannanase (Bacillus sp.) used in 0.1 M glycine, pH 8.8 for 2 h at 40 °C; arabinofuranosidase (Aspergillus niger) used in 200 mM acetate buffer, pH 4 for 2 h at 40 °C; arabinanase (recombinant from Cellvibrio japonicus) used in 100 mM potassium phosphate buffer, pH 7 for 2 h at 40 °C; and pectate lyase (C. japonicus) used in 50 mM CAPS buffer, pH 10 for 2 h at 40 °C. All enzymes were used at 10 units/ml.

Glycan Array Printing and Screening—Glycan array printing was performed as described (38), and arrays were screened as described previously (4). For detailed information on all oligosaccharide samples, see Table 4. The results shown are based on six individual experiments. A competition assay with BSA-conjugated arabinohexaptasose (Ara7) was performed by co-incuba-
tion with purified ECP (pYR006) at 20 μg/ml and was based on two independent experiments.

**ELISA and mAb Screening for Extract Characterization and Bacterial Interaction**—ELISAs were performed in 96-well microtiter plates (Nunc, Maxisorb) coated with spinach proteins or arabinan at 50 μg/ml and at a 1:10 dilution of plant polysaccharide extract (in 0.1 M NaHCO₃ buffer, pH 9.6) in a volume of 100 μl incubated at 4°C overnight, similar to the concentrations used by others (39). The plates were washed three times with TBS and blocked with protein-free blocking
buffer (Thermo Fisher Scientific Inc.) for 1 h at room temperature. Plates were again washed three times with TBS and then incubated for 2 h with 100 μl of bacteria at a concentration of ~2 × 10^7 cfu/ml (A₆₀₀ adjusted to 0.02 in TBS) or primary antibodies (Table 3) (at 1:20 for all plant polysaccharides/glycoproteins). After 2 h, the wells were washed three times with TBS and incubated with a rabbit anti-E. coli antibody (Abcam) diluted at 1:500 in TBS or incubated directly with a horseradish peroxidase anti-rabbit IgG conjugate (diluted at 1:1000). For detection of bacteria, after 1 h, the wells were washed three times with TBS and incubated with 100 μl of horseradish peroxidase anti-rabbit IgG conjugate (diluted at 1:1000). After 1 h, all secondary antibodies were washed three times with TBS. The color reaction was developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) solution: 22 mg of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) diluted in 100 ml of citrate buffer (50 mM sodium citrate, 0.05% H₂O₂, pH 4.0). The absorbance at A₆₀₅ was measured on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Extracts enriched in glycoproteins were pipetted onto nitrocellulose membrane as immunodot blots at 100 μg each, allowed to dry, and probed with the monoclonal antibodies (Table 3) essentially as described for the ELISA with the following modifications: nonspecific interactions were blocked with 3% skimmed milk, primary antibodies were used at 1:100 dilution, the secondary anti-rabbit HRP conjugate was used at 1:5000 dilution, and the blots were washed with PBS containing 0.08% Tween 20. Gum arabic was used at 20 μg as a positive control. ECL substrate was used to detect binding (Millipore, Billerica, MA) and was exposed to ECL Hyperfilm for 10 min.

Monosaccharide Analysis—Monosaccharide analysis of cell walls was carried out as described (40). Acid hydrolysis (2 M trifluoroacetic acid for 1 h at 120 °C) was carried out, and the samples were loaded on a high performance anion exchange chromatography column and run on a Dionex ICS3000 system with a Dionex AS autosampler fitted with a 25-μl loop. The monosaccharides fucose, galactose, glucose, xylose, mannose, galacturonic acid, and glucuronic acid were separated on 3 × 150-mm PA-20 column with a 3 × 30-mm guard column. Eluents were ultrapure water (18.2 megaohms), 200 mM NaOH, and 1 M sodium acetate as described. The monosaccharides arabinose and rhamnose were separated on 4 × 250-mm PA-100 column with a 4 × 50-mm guard column eluted isocratically at 1 ml min⁻¹ with 200 mM NaOH. The flow rate was 0.4 ml min⁻¹ (40). The data are presented as a percentage of the total (mg/mg dry weight) to allow direct comparison.

Bacterial Adherence Assays—Adherence assays were performed as described (4). Briefly, plant tissue was aseptically separated, washed, and incubated with a bacterial suspension at a concentration of ~2 × 10^7 cfu/ml (A₆₀₀ adjusted to 0.02 in PBS) for 2 h at 18 °C. The tissue was then washed three times and macerated, and the bacteria were enumerated on selective medium.

Confocal Microscopy—Plant tissues were analyzed as described (4). Briefly, plant tissues were fixed, deparaffinized, treated with enzymes, and incubated successively with protein-free blocking buffer and antibodies. Primary antibodies were used at 1:50 and secondary (anti-rat conjugated to Alexa Fluor 568) at 1:5000 (Sigma–Aldrich). The sample was incubated for 10 min with Calcofluor white and then mounted with Dako fluorescent mounting medium (Dako, Carpentaria, CA). Fresh leaves were stained with polyclonal antiserum against ECP for 1 h (1:500) or propidium iodide. After three washes in TBS, the leaf samples were incubated for 45 min with an Alexa Fluor 568-conjugated anti-rat antibody (1:5000). Imaging was performed on a Leica TCS-SP2 AOBS microscope (Leica Microsystems, Germany). Photoshop CS software (Adobe Systems) was used for postacquisition image processing.

Sequence and Phylogenetic Analysis—The ecpD ORF derived from E. coli isolate Sakai was used to carry out a BLASTn analysis against a range of E. coli sequences (supplemental Table 1). The ORFs were identified, and amino acid sequences were derived using a bioinformatics program (CLC Bio, Aarhus, Denmark). These sequences were aligned using ClustalW (41). Sequences that did not encode a full-length ecpD ORF were excluded from subsequent alignment.

Statistical Analyses—The means ± S.E. were calculated for each experimental group, and the statistical significance was evaluated with Student’s t test or one-way analysis of variance. The results were considered as significant for a p value ≤0.05.

RESULTS

ECP Binds to Arabinose Oligosaccharides—Recombinant ECP was purified from E. coli JT1 overexpressing ECP from high copy number plasmid pMat3 (ECP derived from newborn
meningitis and septicemia *E. coli* strain IHE3034 (O18:K1:H7)) (15) to generate a polyclonal antibody. The antibody reacted with a protein of the predicted size of the main structural subunit, EcpA (Fig. 1, A–C). Induction of pMAT3 resulted in fimbrial elicitation with fine, hairlike structures (Fig. 1, D and E).

The antibody reacted to a similar level to ECP derived from *E. coli* O157:H7 isolate Sakai, which encodes an identical copy of the mature peptide of the main structural subunit, EcpA, but not to the mock fimbrial preparation from *E. coli* JT1 (pSE380). Although the host strain encodes a native ecp cluster, it belongs to serogroup K-12, which expresses minimal or non-detectable levels of ECP (24). ECP derived from *E. coli* O157:H7 strain Sakai was cloned and purified. We used this strain because it was isolated from a massive foodborne outbreak in Japan in 1996 associated with white radish sprouts (42). Purified ECP fimbriae were used to screen a plant glycan array printed with oligosaccharides coupled to BSA including galacturonate, galactose, arabinose, mannose, xylose, xyloglucan, and glucose and probed with the ECP antibody (detailed in Table 4).

**FIGURE 2.** Plant glycan interactions with ECP. A, plant glycan arrays comprising 36 defined oligosaccharides probed with purified ECP<sub>Sakai</sub> (pYR006) or ECP<sub>IHE3034</sub> (pMat3) detected with a specific anti-ECP antibody on ECL film. The negative control, no ECP, is also shown. The glycans are arranged on a grid pattern, each spotted four times, twice at 1 mg ml<sup>−1</sup> (upper) and twice at 0.2 mg ml<sup>−1</sup> (lower); refer to Table 4 for detail and a pictorial map. B, glycan arrays probed with anti-arabinan antibodies LM6 and LM13 or co-incubated with purified ECP<sub>Sakai</sub> (pYR006) and Ara7. Numbers 1–7 refer to the number of arabinosyl residues in each oligosaccharide.

Arabinofuranosidase (AF) is a glycoside hydrolase that specifically targets α-L-arabinofuranosides containing (1→3) and/or (1→5) linkages found in RG-I as well as arabinoxylans and arabinogalactans. Pretreatment of the glycan array with AF prevented binding of purified ECP<sub>Sakai</sub> (pYR006) to any residues and severely affected LM13 binding to Ara7 so that a signal was only just visible after a long exposure time (Fig. 3A). In
Commercial purified branched and debranched long arabinan polymers (>110 residues/molecule), which were recognized by LM6 and LM13 (data not shown), were included to better characterize the interaction. Both ECP variants (Sakai and IHE3034) bound to spinach pectin and not to hemicellulose or branched arabinan, demonstrating a tropism for pectin. Adherence of ECP to isolated pectin from roots was >2-fold higher than on leaves presumably because this tissue type is richer in arabinan as shown with LM6 (Fig. 4B). Interestingly, ECP\textsubscript{Sakai}, expressed from \textit{E. coli} JT1 (pYR006) was significantly more adherent to debranched arabinan ECP\textsubscript{IHE3034} (pMat3) (p < 0.05) as well as pSE380. The ability of bacteria expressing ECP to inhibit antibody adhesion (LM1, LM6, and LM13) to the CDTA extract of spinach leaves was tested. The presence of bacteria expressing ECP\textsubscript{Sakai} or ECP\textsubscript{IHE3034} reduced LM6 and LM13 binding by ~35% (Fig. 4C).

**ECP Interaction with Spinach Arabinosyl Residues in Glycoproteins—**An ELISA was performed with spinach leaf or root extracts enriched in glycoproteins and \textit{E. coli} JT1 overexpressing ECP\textsubscript{IHE3034} (from pMat3) or the empty plasmid (pSE380) as a control (Fig. 5A). Expression of ECP\textsubscript{IHE3034} resulted in a ~2-fold increase in adherence to the extracts from both leaves and roots compared with the control. Enzymatic treatment with AF abolished the increase in binding of ECP-expressing bacteria to levels similar to those seen with the pSE380 control, whereas treatment for mannanases (mannanase) or pectins (pectin lyase (PL)) did not affect the interaction. The presence of arabinans in the spinach extracts was detected with antibodies LM6 and LM13 and was reduced by AF enzymatic treatment (Fig. 5B). There was no recognition of extensin using the LM1 antibody. Characterization of the extracts with the cell wall antibodies confirmed the presence of AGPs (with LM2 and JIM8) and showed the presence of the major pectin polysaccharide domain, homogalacturonan, partially methyl-esterified, using JIM7 (Fig. 5C). This is not unexpected as it is not possible to generate completely pure glycoprotein preparations. LM13 was detected albeit in relatively low amounts. A positive control for AGPs, gum arabic, was strongly bound by LM2 (not recognized by LM13 or LM6). The binding data together with the presence of antibody epitopes and response to enzymatic treatment indicate an interaction of ECP with AGPs.

**Functional Binding of ECP in Planta—**ECP knock-out mutants were constructed to determine whether ECP plays a functional role for \textit{E. coli} O157:H7 in planta on roots and leaves. The main structural subunit, EcpA, is involved in biofilm formation and cell-cell binding; indeed, imaging of purified ECP fimbriae indicated organelle interactions and bundling (Fig. 1, D and E). Therefore, to distinguish the biofilm effect from a specific interaction, adherence to plants for an ecpA mutant was compared with that for a whole cluster ecpA–D mutant where structural and adhesin genes were removed. Deletion of ecpA in \textit{E. coli} O157:H7 Sakai resulted in an ~10-fold reduction in the number of bacteria recovered from spinach leaves that was further reduced with the removal of the whole cluster ecpA–D (p < 0.05) (Fig. 6A). A significant reduction in adherence for the ecpA and ecpA–D mutants was also observed in roots, although the magnitude of the decrease was not as great. Complementation of the ecpA–D mutant (with
pYR007) restored the WT phenotype in leaves (p < 0.001) and roots (p < 0.005). Oddly, the mutant complemented with the control plasmid pSE380 became more adherent on spinach roots compared with Sakai WT, which we postulated was due to the effect of isopropyl β-D-thiogalactopyranoside (the inducing agent) elsewhere on the genome. Addition of isopropyl β-D-thiogalactopyranoside to E. coli O157:H7 Sakai WT and both ecp mutants resulted in an increase in bacterial adherence (data not shown), confirming this idea.

Pretreatment of spinach roots with AF, arabinanase, PL, or mannanase reduced the ability of E. coli O157:H7 Sakai to adhere, although the greatest difference was observed with PL treatment (p < 0.005), indicating the possibility of multiple interactions, including arabinose-dependent ECP (Fig. 6B). The PL buffer (pH 10) was included because the high pH has been demonstrated previously to degrade pectin backbones that break down under basic conditions (50). Moreover, arabinan can contain various esters (e.g. acetyl and feroyl) that are also base-sensitive (51, 52).

In plant leaves, stomatal guard cell walls are rich in pectin and contain arabinan epitopes (53, 54). Expression of functional ECP fimbriae significantly increased the number of bacteria associated with stomata for both E. coli O157:H7 isolate Sakai and E. coli JT1 overexpressing ECPSakai (from pYR006) (Fig. 7, A and B). To identify unspecific molecular binding mediated by ECP, natural wool was used as an inert, biological substrate to mimic plant root morphology in the absence of any plant glycans (Fig. 7C). There was no significant difference in the number of E. coli JT1 ± ECP (pMat3 or pSE380) recovered from wool (p = 0.721) in contrast to a significant increase exhibited by ECP ΔE3034 (pMat3) on spinach roots (p < 0.05).

Identification of LM13 Epitopes in Spinach—The distributions of LM13 (long arabinans) and LM6 (pectin) epitopes in spinach roots were identified using antibody probes together with enzymatic treatment (Fig. 8). Cortical cells appeared to be enriched in pectin as indicated by LM6 and
**FIGURE 5. ECP interactions with spinach glycoproteins.** Shown are the results of ELISA of spinach enriched with glycoproteins from roots or leaves with *E. coli* JT1 expressing ECP<sub>HE3034</sub> (pMat3) or the negative control (pSE380) (A) or with antibody LM6 (all arabinose), LM13 (arabinan), or LM1 (HRGPs) (B). Detection was carried out with or without (native) enzymatic treatments: AF, mannanase (Mase), and PL. Spinach root extracts were characterized from dot blots with the monoclonal antibodies (C). The labels refer to each antibody (Table 3), and “LM2 (gum)” refers to the interaction with gum arabic as a positive control. Significance levels are as follows: ***, p < 0.001; **, p < 0.005; *, p < 0.05; NS, non-significant. Error bars represent S.E.

**FIGURE 6. Functional adherence of ECP to plant tissue.** A, bacteria recovered from spinach leaves and roots following a 2-h adherence assay for *E. coli* O157:H7 Sakai WT, mutants ΔecpA and ΔecpA–D, and the complemented ΔecpA–D mutant with plasmid pYR007 or the control pSE380. The average number of bacteria is expressed as log<sub>10</sub> cfu/g of fresh tissue. B, bacteria recovered from spinach roots following a 2-h adherence assay with AF, arabinanase (Arase), PL, and PL buffer treatments for *E. coli* O157:H7 Sakai WT. Significance levels are as follows: ***, p < 0.001; **, p < 0.005; *, p < 0.05; NS, non-significant. Error bars represent S.E.
LM13 detection and AF and PL treatment effects (Fig. 8, D, E, G, and H). Although AF treatment reduced signals from all of the antibody probes, PL treatment had a greater effect on LM13 detection. JIM7 (homogalacturonan) (55) and PL buffer treatment alone were included as controls. Epitopes for LM13 and LM6 were also identified in spinach guard cells (Fig. 8, M and N). It was not possible to assess enzymatic treatment on guard cells because of their destructive effects on stomata (53, 54).

Comparative adherence assays for *E. coli* O157:H7 WT and ΔecpA–D showed a more pronounced role for ECP to spinach compared with lettuce (*L. sativa*) (Fig. 9) (the data are expressed as a ratio to compare plant species). To determine whether increased ECP-dependent adherence was a result of greater availability and/or abundance of the ECP target in spinach, cell wall polysaccharides from the roots and leaves of both plant species were analyzed using a range of antibodies to detect epitopes for pectin (LM5, LM6, and LM13), hemicellulose (LM21 and LM25), and extensin (LM1) (Table 3). As expected, the pectin probes reacted most strongly with CDTA-derived extracts, whereas the hemicellulose probes reacted with NaOH-derived extracts (Fig. 9, C and D). LM13 and LM6 gave signals in both plant species, but higher levels were detected in spinach. The trend was the same for both leaves and roots albeit with differences in the complement of the specific residues. Monosaccharide quantification of plant extracts, including arabinose (Table 5), determined from HPLC analysis correlated with the epitope presence (Fig. 9E).

**Functional Expression of ECP in Planta**—*E. coli* O157:H7 has been shown to encode an active promoter site for the *ecp* operon (24). To observe ECP transcription and production in...
planta, we examined spinach leaves inoculated with E. coli O157:H7 Sakai transformed with a plasmid-encoded ecpR-GFP transcriptional fusion after anti-ECP detection. Expression of ecpR-GFP (green) was evident in most cells, and elicitation of ECP fimbriae (red) was also observed. Differences in the green fluorescence levels suggested that ecpR transcription may be heterogeneous in planta (Fig. 10). A different plant species was evaluated (a) to confirm that ECP expression is not restricted to spinach plants and (b) to observe any co-localization with particular plant tissues. N. benthamiana line mGFP5-ER expresses GFP fused to an endoplasmic reticulum protein (36). Inspection of a guard cell shows the green network of the endoplasmic reticulum together with bacteria expressing ecpR (green) and ECP fimbriae (red) adjacent to a chloroplast (purple) and on the leaf epidermis (Fig. 10B).

EcpD Sequence Variation—ECP fimbriae are almost ubiquitous in E. coli and are also encoded by related members of the Enterobacteriaceae, although the EcpA (main structural subunit) and EcpD (adhesin) coding sequences are not completely conserved. Alignment of 66 E. coli EcpD amino acid sequences

FIGURE 8. Epitope detection of spinach root and leaf sections. Shown are confocal microscopy images of fixed spinach root sections either untreated (A–C) or treated with AF (D–F), PL (G–I), or PL buffer (Buf) (J–L) and then probed with antibody LM6 (all arabinose; A, D, G, and J), LM13 (arabinan; B, E, H, and K), or JIM7 (homogalacturonan; C, F, I, and L). Spinach leaves were probed with LM6 (M) or LM13 (N). Root and leaf tissues were stained with Calcofluor white (light blue), and antibodies were detected with the secondary Alexa Fluor 568-conjugated antibody (red). Scale bars, 8 µm.
FIGURE 9. **Plant species-dependent differences for adherence and polysaccharide composition.** A and B, bacteria recovered from lettuce and spinach leaves or roots following a 2-h adherence assay for *E. coli* O157:H7 Sakai WT and ΔecpA–D. The average number of bacteria recovered is expressed as a ratio of WT versus ΔecpA–D mutant. C and D, ELISA of lettuce and spinach polysaccharide extracts (CDTA and NaOH) from roots and leaves with LM16 (uncharacterized RG-I epitope), LM13 (arabinan), LM6 (all arabinose), LM5 (galactan), LM25 (galactosylated xyloglucan), LM21 (mannan), or LM1 (HRGPs). E, HPLC analysis of arabinose monosaccharides present in a total polysaccharide extraction of lettuce and spinach root and leaves. Significance levels are as follows: ***, p < 0.001; **, p < 0.005; *, p < 0.05. Error bars represent S.E.
Sugar beet arabinan consists of an oligomer of arabinose residues that are either single or double substituted (64). Treatment of unbranched arabinan to remove potential intermolecular connections between cell wall components, enzymatic treatments that do not target arabinan specifically may nonetheless impact its availability. ECP interaction with spinach extracts enriched in glycoproteins was only affected by arabinofuranosidase treatment. The data indicate that despite the presence of HG backbone in the extracts, arabinan was not abundant and that ECP also interacts with AGPs. The apparent lack of pectate lyase activity on the HG component can be accounted for by the requirement for Ca^{2+} for catalysis; Ca^{2+} ions are absent in the methylated form of HG (67). In summary, it appears that arabinan from pectin polysaccharides and possibly plant glycoproteins play a role in E. coli ECP-dependent adhesion in planta. In turn, the distribution of arabinan could affect tropism for the bacteria: toward guard cells in leaves and cortical cells within root tissue just as is seen for PapD-dependent tropism to galactans in renal tissue.

Functional binding of ECP to plant tissue appears to be conferred by the adhesin, EcpD, and is supported by the presence of the main structural subunit, EcpA. Because ECP did not significantly contribute to binding to woody substrates, the structural context is correct (45). The overlap in recognition shows that ECP recognized pectic arabinans and potentially O-glycosylated hydroxyproline proteins (AGPs), which contain arabinose and galactose, ranging from a single residue to up to 75 arabinogalactan residues (28, 61).

Stomatal guard cells, which are rich in pectic arabinan (53, 54) and have been shown to be a target for bacteria (4, 62), were targeted by bacteria expressing ECP. ECP fimbiae bound preferentially to pectin fractions in polysaccharides extracted from spinach compared with long oligomers of arabinan purified from beet roots that were either branched or unbranched (63). Sugar beet arabinan consists of an α-(1→5)-linked backbone of L-arabinosyl residues that are either single or double substituted (64). Treatment of unbranched arabinan to remove (1→2)- and (1→3)-α-L-arabinofuranosyl branches revealed a specificity of ECP 

**TABLE 5**

Monosaccharide composition and content (%) of spinach and lettuce tissue

| Monosaccharide  | Lettuce leaves | Spinach leaves | Lettuce roots | Spinach roots |
|-----------------|----------------|----------------|--------------|--------------|
| Fucose          | 0.31           | 1.17           | 0.13         | 0.98         |
| Galactose       | 1.06           | 1.57           | 5.25         | 1.83         |
| Glucose         | 54.54          | 25.94          | 29.90        | 5.77         |
| Xylose          | 1.27           | 4.50           | 2.57         | 2.48         |
| Mannose         | 5.50           | 2.96           | 2.63         | 7.39         |
| Galacturonic acid| 4.78           | 5.18           | 1.30         | 3.62         |
| Glucuronic acid | 3.62           | 1.83           | 0.61         | 12.39        |
| Arabinose       | 24.82          | 47.23          | 47.08        | 63.83        |
| Rhamnose        | 4.10           | 9.61           | 10.54        | 1.71         |

highlighted variable residues (Table 6). The types of substitutions for the most frequent changes included conserved (e.g., L145M); non-polar aliphatic to polar, uncharged (e.g., R232L); and vice versa (e.g., A117T). The mature peptide of EcpD from E. coli from isolates Sakai and IHE3034 (used in this study) contains three amino acid substitutions.

**DISCUSSION**

Identification and characterization of the molecular targets underlying the adhesion of bacteria (phytopathogenic or human pathogenic) to plants is limited compared with that for mammalian and animal hosts (57, 58). So far, only an interaction between E. coli flagella and ionic lipids has been described where flagella can intercalate into plant plasma membranes (4). The interaction between α-(1→5)-l-arabinose in plant cell walls and ECP derived from a well known human pathogen associated with outbreaks from fresh produce is the first example of a target for bacteria (4). The apparent lack of pectate lyase activity on the HG component may in turn reflect the amount of ECP organelle expressed (24).

**E. coli Common Pilus Binds to Arabinose**

Sequences of EcpD, the adhesin, are extremely well conserved in all E. coli genomes and especially between the serotype O157:H7 strains. However, differences in the EcpD mature protein may be sufficient to alter receptor specificity as has been observed for type 1 fimbiae (65). ECP tropism for pectic arabinan was confirmed with pectate lyase treatment, which degrades homogalacturonan chains, the main component of pectin polysaccharides (66), and releases the arabinose-substituted pectin polysaccharides. The high potency of pectate lyase activity means that it may have some arabinase activity even at low concentrations. Furthermore, because of the many intermolecular connections between cell wall components, enzymatic treatments that do not target arabinan specifically may nonetheless impact its availability. ECP interaction with spinach extracts enriched in glycoproteins was only affected by arabinofuranosidase treatment. The data indicate that despite the presence of HG backbone in the extracts, pectic arabinans were not abundant and that ECP also interacts with AGPs. The apparent lack of pectate lyase activity on the HG component can be accounted for by the requirement for Ca^{2+} for catalysis; Ca^{2+} ions are absent in the methylated form of HG (67). In summary, it appears that arabinan from pectin polysaccharides and possibly plant glycoproteins play a role in E. coli ECP-dependent adhesion in planta. In turn, the distribution of arabinan could affect tropism for the bacteria: toward guard cells in leaves and cortical cells within root tissue just as is seen for PapD-dependent tropism to galactans in renal tissue.

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Both ECP variants from E. coli isolates Sakai and IHE3034 demonstrated enhanced adhesion to spinach root polysaccharides compared with leaves, which supports previous findings of increased colonization in the roots (69). Furthermore, higher levels of ECP-mediated adhesion of E. coli O157:H7 occurred in spinach compared with lettuce, which may be a result of the
FIGURE 10. **ECP expression and elicitation in planta.** Shown are confocal images of spinach (A) or *N. benthamiana* expressing mGFP5-ER (B) leaves inoculated with *E. coli* O157:H7 Sakai WT expressing *ecpR*-GFP (green) and ECP fimbriae (red) 4 h postinoculation. Chloroplast autofluorescence is false colored purple, and endoplasmic reticulum is green for *N. benthamiana*. Scale bars, 20 μm.
reduced arabinan in lettuce. It is of note that verotoxigenic E. coli are isolated from spinach plants at higher rates compared with lettuce (70, 71) and that consumption of raw spinach has risen by 180% in the United States from 1992 to 2005 (49). This information together with ECP specificity for arabinose may go some way to explain why verotoxigenic E. coli outbreaks are more frequently associated with spinach.

Demonstration of adherence mediated by flagella together with specific recognition of a plant oligosaccharide by an adhesin (4) suggests a stepwise process of E. coli adherence to plant cells. First, a broad molecular recognition occurs between flagella and plasma membranes without any particular tropism. This is then followed by a specific, adhesin-mediated adherence, e.g. ECP to cell wall pectin polysaccharides. Exploitation of such an abundant plant-specific glycan may aid the spread of E. coli in the environment, and given the ubiquity of ECP, it could be a reasonably common mechanism of binding to plants (and for related enterobacteria). However, differences in ecp cluster expression and EcpD amino acid sequence are likely to affect both the occurrence of binding and the affinity.

The ability of mammalian pathogenic bacteria to target an intermediate host for survival and transmission is not without precedent: the spread of Vibrio cholerae, a well known human pathogen, through freshwater and marine environments (72, 73) is dependent on its interaction with chitin polysaccharide in zooplankton (74, 75). Furthermore, pathogenic strains of V. cholerae show increased fitness in bivalves compared with environmental isolates (76). Similarly, ECP-arabinan interactions may play an important role in allowing pathogenic E. coli to use plants as a secondary host and as a vehicle to spread to other primary hosts such as cattle or humans.

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TABLE 6

| AA position | Consensus | Substitution | Percentage (n = 66) |
|-------------|-----------|--------------|--------------------|
| −15         | Thr       | Ala          | 43.9               |
| −5          | Val       | Ala          | 39.4               |
| −3          | Ala       | Glu          | 19.7               |
| 14          | Arg       | Ser          | 1.5                |
| 51          | Asn       | Thr          | 42.4               |
| 117         | Ala       | Thr          | 18.2               |
| 130         | Ala       | Thr          | 3                  |
| 145         | Leu       | Met          | 15.2               |
| 157         | Thr       | Ile          | 1.5                |
| 170         | Ala       | Asn          | 10.6               |
| 180         | Ser       | Thr          | 1.5                |
| 222         | Ala       | Thr          | 1.5                |
| 229         | Ile       | Met          | 1.5                |
| 232         | Arg       | Leu          | 21.2               |
| 252         | Thr       | Ile          | 1.5                |
| 319         | Leu       | Glu          | 25.8               |
| 354         | Ile       | Leu          | 7.6                |
| 380         | Thr       | Ala          | 1.5                |
| 392         | Thr       | Ile          | 1.5                |
| 441         | Thr       | Ala          | 9.1                |
| 448         | Thr       | Ile          | 1.5                |

* Assumes a signal peptide from position −1 to −23.

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