

Erwinia chrysanthemi strains cause death of human gastrointestinal cells in culture and express an intimin-like protein

X. Duarté a, C.T. Anderson a, M. Grimson a, R.D. Barabote a, R.E. Strauss a, L.S. Gollahon a, M.J.D. San Francisco a,b,*

a Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409, USA
b Institute for Biotechnology, Texas Tech University, Lubbock, TX 79409, USA

Received 27 March 2000; received in revised form 28 June 2000; accepted 3 July 2000

Abstract

The bacterium Erwinia chrysanthemi is a model plant pathogen, responsible for causing cell death in plant tissue. Cell-wall depolymerizing enzymes and avirulence proteins essential for parasitism by this bacterium utilize dedicated type II and type III secretion systems, respectively. Although E. chrysanthemi is not recognized as a mammalian pathogen, we have observed that the bacterium can adhere to, cause an oxidative stress response in and kill cultured human adenocarcinoma cells. These bacteria express a surface protein that bears immunological identity to intimin, a protein required for full virulence of enterohemorrhagic and enteropathogenic Escherichia coli. A type III secretion mutant of E. chrysanthemi was observed to have a significantly lower capability of causing death than the wild-type strain in parallel cultures of human colon adenocarcinoma cells. These observations suggest that E. chrysanthemi has the potential to parasitize mammalian hosts as well as plants. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Erwinia; Mammalian cell adhesion; Type III secretion

1. Introduction

Many members of the genus Erwinia are universally recognized in causing plant disease, infecting a variety of vegetable, fruit and grain crops [1,2]. Important components of the offensive arsenal of these bacteria include the production of secreted plant cell wall-depolymerizing enzymes and Avr (avirulence) proteins essential for parasitism [3]. Secretion of the pectate-degrading enzymes requires a dedicated protein export machinery, designated the Out system, that belongs to the type II protein secretion pathway [4]. Secretion of the Avr proteins requires a type III secretion system to deliver these proteins across plant cell walls and membranes.

Over the years, we have become increasingly aware of common mechanistic processes utilized by bacterial pathogens to infect or cause disease in either their animal or plant hosts [5–10]. Indeed, the opportunistic human pathogen Pseudomonas aeruginosa is also capable of causing disease in plants [7]. The prototype type III protein secretion system first described in the animal pathogenic genus Yersinia [10] has since been observed to function in a variety of bacterial pathogens of animals (Salmonella sp., Shigella sp., Escherichia sp.), and plants (Pseudomonas sp., Xanthomonas sp., Ralstonia sp., Erwinia sp.) [4,9]. Enterohemorrhagic Escherichia coli (EHEC) and enteropathogenic E. coli (EPEC) utilize the type III secretion system to deliver a protein, TIR, into the host that serves as the receptor for the bacterial protein, intimin [11]. Intimate attachment and full virulence of EHEC and EPEC to intestinal mucosa involves the 94 kDa protein, intimin (EaeA) [12,13]. Analysis of intimins from several strains of EHEC and EPEC reveals significant homology at the amino termini but only about 50% identity at their carboxy termini [12]. On the basis of these observations and the potential for E. chrysanthemi to interact with mammalian cells following ingestion of contaminated foods, we investigated whether this bacterium could attach and induce stress in mammalian cells in culture.

* Corresponding author. Tel.: +1 (806) 742-2706; Fax: +1 (806) 742-2963; E-mail: michael.sanfrancisco@ttu.edu

0378-1097/00/$20.00 © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

PII: S0378-1097(00)00325-6
2. Materials and methods

2.1. Bacterial strains, growth conditions and mammalian cell culture

*Erwinia chrysanthemi* strain EC16 (which causes localized necrosis in plants), was obtained from Arun Chatterjee (University of Missouri-Columbia). *E. chrysanthemi* strain CUCPB0873 (which causes systemic infections in African violet), and a type III secretion mutant CUCPB5040 (non-invasive derivative of EC16 with a Tn10 mini-kan insertion [14]), were obtained from Alan Collmer (Cornell University). *E. coli* HB101 was obtained from Gibco-BRL and EPEC strain 2348/69 was obtained from Jim Kaper (University of Maryland). Prior to tissue culture infections, bacterial cultures were grown at 37°C overnight in Luria–Bertani (LB) medium containing an equal volume of Dulbecco’s modified Eagle’s medium (DMEM)+10% fetal bovine serum (FBS). HT29 cells (ATCC number HTB 38) were used as the target host cell. These cells have been derived from an adenocarcinoma of the human colon and were maintained in DMEM+10% FBS under 5% CO₂ at 37°C.

2.2. SDS–PAGE and immunoblotting

Bacterial cultures were grown in LB or DMEM+10% FBS for 2 or 4 h to study expression of intimin. Cells were harvested, washed in 10 mM Tris–HCl (pH 8) and resuspended in the same buffer to an optical density of 0.1 at 600 nm. Total cell proteins were separated on denaturing 8% SDS–polyacrylamide gels. Proteins were electroblotted onto polyvinylidine difluoride membranes (PVDF) (Millipore). Membranes were blocked with 5% non-fat dry milk (NFDM) in Tris-buffered saline, (0.05M Tris–HCl (pH 7.5), 0.2 M NaCl, 0.003 M KCl), (TBS), for 90 min at room temperature and washed with TBS containing 0.05% Tween 20. Polyclonal anti-intimin antiserum was kindly provided by Gad Frankel (Imperial College of Science Technology and Medicine, London). This antiserum is directed against the carboxy terminal domain of the protein [12]. The antiserum was preadsorbed against a cytoplasmic extract of *E. coli* DH5α and diluted 1:2000 in 1% non-fat dry milk in TBS. Alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Bio-Rad) was diluted 1:5000 in 1% NFDM in TBS was used as the secondary antibody. The presence of alkaline phosphatase was detected using p-Nitro Blue Tetrazolium (5'-3' Inc.) and 5-bromo-4-chloro-3-indolyl phosphate (5'-3' Inc.).

2.3. Cell infection, reactive oxygen species and viability measurements

HT29 cells were seeded at 2×10⁴ cells in 24-well plates and grown to a density of approximately 8×10⁴ cells, infected with approximately 1×10⁷ cells of *E. chrysanthemi* EC16, CUCPB0873, CUCPB5040, *E. coli* HB101 or EPEC strain 2348/69 (multiplicity of infection, approximately 125:1), and incubated for 4.5 h under 5% CO₂ at 37°C. Following incubation, excess bacterial cells were washed out with phosphate-buffered saline (PBS), pH 7.2. Dichlorofluorescin diacetate (DCFH-DA) was used to monitor the production of reactive oxygen species (ROS). DCFH-DA is converted to the immobile DCFH by intracellular esterase activity. Oxidative agents, such as hydrogen peroxide, produced in response to bacterial infection convert minimally fluorescent DCFH to the highly fluorescent dichlorofluorescein (DCF) [15]. DCFH-DA (5.0 μM) in PBS was applied to washed cells 4.5 h after inoculation with bacteria and incubated for 15 min at 37°C. Fluorescence of the oxidation product, DCF, was monitored at 515 nm, using an Olympus inverted microscope. Detection of cell viability was studied using the Live/Dead<sup>®</sup> cell viability assay based on plasma membrane integrity and intracellular esterase activity (Molecular Probes). Calcein AM, a cell permeant is enzymatically converted to intensely fluorescent calcein at an excitation wavelength of 530 nm in viable cells. EthD-1, an ethidium homodimer, intercalates into the DNA of cells with plasma membrane disruption, and fluoresces intense red at 600 nm. Calcein AM (0.5 μM) and 5.0 μM EthD-1 were added to the washed infected cells and incubated for 30 min at 37°C. Random fields of vision were photographed using an Olympus inverted microscope with Kodak elite print film. Cell numbers were then counted from the prints for statistical analysis.

2.4. Statistical analysis

Differences in proportional cell mortality among treatments were evaluated by analysis of covariance (ANCOVA) using the General Linear Models procedure of SAS v6.12 (Statistical Analysis System). Counts of dead cells per photograph were used as the response variable, with corresponding total cell counts as the covariate. Although there is a slight positive relationship between the means and variances of cell counts among treatments, the null hypothesis of homogenous variances was not rejected by Levene’s test [16], either for dead-cell counts (*F* = 1.62 with 3 and 45 df, *P* = 0.20) or total-cell counts (*F* = 0.91 with 3 and 45 df, *P* = 0.44); therefore raw rather than square-root transformed counts were used in the ANCOVA. Adjusted mean counts of dead cells per photograph and their standard errors were estimated from the ANCOVA as population marginal means [17]. These are the treatment means expected for a balanced design with the covariate at its mean value. Pairwise *t*-tests were used to determine the statistical significance of differences among treatment means. To maximize power, one-tailed probabilities were estimated, consistent with the prior expectation of an ordered sequence of treatment effects: control < *E. coli* HB101 < *E. chrysanthemi* CUCPB5040 < *E. chrysanthemi*
EC16. Decisions of statistical significance for the set of six pairwise tests were based on a sequential Bonferroni adjustment [18] at an overall ('experimentwise') confidence level of \( \alpha = 0.05 \).

2.5. Immuno-electron microscopy

For pre-embedded immunolabeling studies, a monolayer of HT29 cells was grown on Thermofax® coverslips and inoculated with *E. chrysanthemi* EC16 or *E. coli* HB101 as described above. Following bacterial growth in the presence of HT29 cells for 4.5 h, cells were fixed with 0.1% glutaraldehyde for 10 min, washed three times with PBS and incubated with anti-intimin antibody (1:200) overnight at 4°C. Cells were washed and incubated with gold labeled goat anti-rabbit serum (1:100) for 2 h at room temperature. To retain the bacteria/cell relationship, the coverslips were washed and then fixed in 2% glutaraldehyde, dehydrated and embedded in Spurr’s resin using standard procedures [19]. The coverslips were removed by ‘popping’ them off after immersion in liquid nitrogen and the resulting monolayer sectioned. Samples were examined in a Hitachi HU-11E transmission electron microscope at 75 kV.

3. Results

3.1. *E. chrysanthemi* strains can induce an oxidative stress response in HT29 cells

As a first step, we determined if the presence of *E. chrysanthemi* was able to induce a stress response in cultured mammalian cells. The host cell response was compared using inoculations with *E. chrysanthemi* strains EC16 and CUCPB0873 which are infective on plants and CUCPB5040 which is non-infective on plants. *E. coli* HB101 and EPEC strain 2348/69. The production of ROS was monitored and used as an indicator of the host cell response. All bacterial strains tested grew rapidly in the presence of the cultured mammalian cells. *E. coli* HB101 randomly attached to host cell surfaces (Fig. 1A) and caused production of ROS in approximately 5% of the host cells (Fig. 1B). The two infective strains of *E. chrysanthemi*, EC16 (Fig. 1C) and CUCPB0873 formed aggregates and caused host ROS production in approximately 17% of the cells (EC16 Fig. 1D). The type III secretion mutant CUCPB5040 was less effective in stimulating ROS production (approximately 7%) while the EPEC strain induced ROS production in approximately 25% of the host cells. These data, therefore, represented the first indication that plant infective *E. chrysanthemi* was capable of causing a physiological stress response to mammalian cells in tissue culture.

3.2. *E. chrysanthemi* EC16 and CUCPB0873 can kill HT29 cells

To study if continued incubation of HT29 cells with *E. chrysanthemi* resulted in further host cell perturbation, a viability assay was performed. Fig. 2A shows that HT29 cells grown in the absence of bacteria maintained their healthy appearance. Under fluorescence microscopy, (Fig. 2B), approximately 94% of the cells under control conditions appeared viable (green). *E. coli* HB101 did not cause significant host cell perturbation and resulted in approximately 14% cell death (Fig. 2D). Adherence of infective *E. chrysanthemi* strains EC16 or CUCPB0873 to
HT29 cells resulted in morphological changes and membrane collapse in the host. Fig. 2E and F depict HT29 cells incubated with strain CUCPB0873 under bright field and fluorescence microscopy, respectively. Approximately 30% of the host cells were observed to be dead.

3.3. The type III secretion system appears to be important for \textit{E. chrysanthemi} virulence on HT29 cells

Host cell killing was monitored with \textit{E. chrysanthemi} EC16, \textit{E. chrysanthemi} CUCPB5040 and \textit{E. coli} HB101 to quantitatively assess the virulence of the bacterial species being studied and to compare differences among them. The ANCOVA indicated a significant relationship between dead-cell and total-cell counts ($F = 18.7$, $df = 1,44$, $P < 0.001$), and a significant treatment effect after adjustment for the covariate ($F = 36.7$, $df = 3,44$, $P < 0.001$). The increasing sequence of adjusted mean dead-cell counts (Table 1), control HT29 < \textit{E. coli} HB101 < \textit{E. chrysanthemi} CUCPB5040 < \textit{E. chrysanthemi} EC16, strongly supports the hypothesis that \textit{E. chrysanthemi} is capable of causing significant host cell death. The type III secretion mutant was found to be defective in its ability to kill HT29 cells and was comparable to \textit{E. coli} HB101 with the adjusted mean dead cell numbers being 53.3 and 42.8, respectively. All pairwise comparisons among treatments were significant at a level of $P = 0.042$ or less, except for the control HT29-HB101 and HB101-CUCPB5040 differences ($P = 0.155$). The control HT29-HB101 comparison is significant by itself, but not when adjusted for the set of six simultaneous hypothesis tests.

3.4. \textit{E. chrysanthemi} expresses a surface protein similar to intimin

The intimin protein is expressed as an outer membrane protein in EHEC and EPEC and binds to a host cell receptor of bacterial origin that is secreted by the type III secretion pathway. When total cell proteins from \textit{E. chrysanthemi} strains EC16 and CUCPB0873, grown under varying environmental conditions were probed in immunoblots with anti-intimin antiserum, an immunoreactive polypeptide of approximately 86 kDa was observed (Fig. 3). This protein was expressed following growth for 2 h (early exponential phase) in either LB or DMEM+FBS, but its expression was down-regulated after 4 h (mid to late exponential) of growth in both media (Fig. 3, lanes 4–7). Extended bacterial growth (4 h) in the presence of HT29 cells, however, resulted in continued production of the protein (Fig. 3, lanes 1 and 2). Immunogold labeling to detect the localization of \textit{E. chrysanthemi} proteins that reacted with the intimin antibody clearly showed the presence of immunologically reactive proteins on the bacterial cell surface (Fig. 4A). Gold particles were also observed at the regions of contact between the bacterium and host cell surfaces (Fig. 4A, arrow). Approximately 30–40% of the

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of pictures</th>
<th>Mean total cells (per photograph)</th>
<th>Mean dead cells</th>
<th>Percent mortality</th>
<th>Adjusted mean dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>264.5 ± 21.3</td>
<td>21.3 ± 4.1</td>
<td>8.05</td>
<td>24.7 ± 7.1</td>
</tr>
<tr>
<td>HB101</td>
<td>13</td>
<td>311.0 ± 28.5</td>
<td>44.5 ± 9.4</td>
<td>14.3</td>
<td>42.8 ± 7.0</td>
</tr>
<tr>
<td>CUCPB5040</td>
<td>12</td>
<td>288.0 ± 20.1</td>
<td>50.5 ± 8.7</td>
<td>17.5</td>
<td>53.3 ± 7.3</td>
</tr>
<tr>
<td>EC16</td>
<td>13</td>
<td>339.1 ± 22.0</td>
<td>121.8 ± 7.2</td>
<td>35.7</td>
<td>114.5 ± 7.2</td>
</tr>
</tbody>
</table>

Adjusted means are from the analysis of covariance.
bacteria were observed to be labeled with the gold particles. There was no indication of an immunologically similar protein on the E. coli HB101 cell surface (Fig. 4B).

4. Discussion

Members of the bacterial genus Erwinia, that cause disease on a wide variety of fruit, vegetable and grain crops, belong to the same family as Escherichia, Salmonella, Shigella and Yersinia, many species of which are recognized agents of gastrointestinal disorders in humans. With the current explosion of knowledge on the identification and characterization of bacterial virulence factors, we have become increasingly aware of common cellular processes shared by pathogens of animals and plants. Remarkably, mechanisms for exploitation of host surface molecules for adherence, systems for the delivery of bacterial virulence proteins and mechanisms to subvert host defenses, have been found to be highly conserved among bacterial pathogens of plants and animals [1,5,7–9,20–23].

Our research findings demonstrate that two strains of E. chrysanthemi EC16, and CUCPB8073, can adhere to mammalian cultured cells, induce an oxidative stress response and cause death of these cells. The adhesion phenotype of E. chrysanthemi to host cells appeared to be a combination of both localized and aggregative [24]. The laboratory strain of E. coli HB101, although able to grow in the presence of HT29 cells, did not cause much cell death as EC16. Thus, this stress response of the cultured mammalian cells infected with E. chrysanthemi was probably not directly related to nutrient depletion in the medium. E. chrysanthemi strains, EC16 and CUCPB8073 express an envelope protein of approximately 86 kDa that bears strong immunological identity to the intimin protein expressed by EPEC and EHEC. In EPEC 2348/69, intimin is recognized to be a 94-kDa surface protein. Expression of an immunologically related protein in E. chrysanthemi was observed to be similar to the expression pattern of intimin observed with EPEC 2348/69 cultures where the protein was observed to be at a higher concentration in tissue culture medium than in L broth. Higher levels of the immunoreactive protein were observed in EC16 cultures grown in the presence of HT29 cells than in their absence. This points to the involvement of a host factor for continued expression of the protein. Our immunogold labeling studies with strain EC16, show that the intimin immunological homologue is a cell surface protein. Distribution of the immunogold label over the bacterial cell surface was, however, much lower than that observed with enteropathogenic E. coli strain E2348/69 [12]. Type III secretion systems have been shown to exist in bacterial pathogens of plants and bacterial pathogens of animals. These secretion systems serve to deliver virulence associated proteins into their hosts. In E. chrysanthemi, avirulence (Avr) proteins which are required for pathogenesis and survival of bacteria in plant cells are secreted by a type III protein secretion system [3]. As a model plant pathogen, E. chrysanthemi is not recognized as a mammalian pathogen. The mechanism(s) utilized by these bacteria to kill cultured mammalian cells are currently unknown. Our observation of the greatly reduced capability of type III protein secretion mutant of E. chrysanthemi (CUCPB5040), to cause death in cultured mammalian cells compared to wild-type strains, has important implications. Although intimin is not absolutely essential for pathogenesis by EHEC and EPEC, it is required for full manifestation of virulence. Are intimin-like proteins important in plant pathogenesis? Studies in our laboratory are also focusing on the pathogenic potential of the Erwinia on mammalian cells.

Acknowledgements

This work was supported by the office of the Provost and the Department of Biological Sciences, Texas Tech University. This research was supported in part by a Howard Hughes Medical Institute grant through the Undergraduate Biological Sciences Education Program to Texas Tech University (X.D.). We thank Jim Kaper for helpful discussions.

References

transfers its receptor for intimate adherence into mammalian cells. Cell 91, 511–520.


