Binding of extracellular matrix laminin to *Escherichia coli* expressing the *Salmonella* outer membrane proteins Rck and PagC

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Abstract

*Salmonella* Rck and PagC are closely related virulence-associated proteins. When expressed in non-adherent, non-invasive laboratory *Escherichia coli*, both proteins localised to the outer membrane. Only Rck conferred adhesion to culture cells, but both proteins induced bacterial binding to the cell monolayer background, to extracellular matrix (ECM) preparations, and to the ECM component laminin. Laminin binding was saturable and competitive, and was reduced by removal of carbohydrate side chains. Pre-incubation with laminin targeted recombinant Rck\(^+\) and PagC\(^+\) bacteria directly to the eukaryotic cell surface, and eliminated background binding. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Salmonella*; Cell adhesion; Laminin; Rck; PagC

1. Introduction

Although the virulence-related *Salmonella* outer membrane proteins Rck and PagC have very similar amino acid sequences (53% identical), there is no evidence to confirm a common function. Rck is associated with complement resistance and epithelial adhesion and invasion [1,2], while PagC is required for intramacrophage survival and establishment of systemic infection [3]. This apparent divergence in function has been attributed to the fact that the regions of highest sequence similarity lie in putative transmembrane regions, while higher levels of sequence divergence are located in the extracellular domains of the proteins, domains most likely to determine cell interactions [4]. There are, however, a greater number of conserved residues in the predicted extracellular loops of Rck and PagC than in those of Rck and the Rck and PagC orthologue *Yersinia* Ail, which, like Rck, has been implicated in cell adhesion and invasion [5,6]. This study sought to reconcile this apparent discrepancy, and identified a conserved role for Rck and PagC in laminin-mediated interaction with host tissue.
2. Materials and methods

2.1. Bacterial strains and cultured cell lines

The rck and pagC genes were cloned from Salmonella typhimurium strain SJW1103 [7]. Bacteria were maintained in 2YES broth or on Luria broth (LB) agar at 37°C. E. coli BL21(DE3) derivatives C41(DE3) and C43(DE3) were used for expression of PagC and Rck, respectively, under control of the T7 bacteriophage promoter [8]. Plasmids were maintained by addition of 20 μg ml⁻¹ chloramphenicol. HeLa cells were maintained at 37°C in Dulbecco’s modified Eagle’s media (DME) supplemented with 10% foetal bovine serum, 2 mM l-glutamine, 100 μg ml⁻¹ streptomycin, 100 U ml⁻¹ penicillin (Sigma) under 5% CO₂.

2.2. Plasmid construction

Synthetic oligonucleotides were used to amplify rck (5’-GTTCAGGGAGTTTTCATATGAAAAAATTCG-3’ and 5’-GCCTGCGGATCCGCTCCCTTCGTCTCCCG-3’) and pagC (5’-GGAGTT CATATGAAAAATATTATTATCCAC-3’ and 5’-GCGGAAGGGGATCCTTCCGCATAGCTTA TGCC-3’) from the S. typhimurium SJW1103 chromosome using PCR (Perkin Elmer GeneAmp apparatus). Amplified fragments were digested with NdeI and BamHI and ligated into NdeI/BamHI-digested pACT7 expression vector [9]. This produced plasmids pAMCR1(rck) and pAMCP1(pagC).

2.3. [35S]Methionine labelling, protease treatment and SDS-PAGE analysis

For selective labelling of Rck and PagC, recombinant bacteria were induced with 10 mM IPTG and treated with 300 μg ml⁻¹ rifampicin before addition of [35S]methionine. Protein samples were suspended in SDS loading buffer with 8 M urea and analysed by SDS-15% PAGE [10]. Proteins were visualised by Coomassie staining or autoradiography. For outer membrane localisation, whole cells expressing [35S]Rck or [35S]PagC were suspended in 20 mM Tris, pH 7.4 with proteinase K or trypsin (50 or 200 μg ml⁻¹) and incubated at room temperature for 30 min before addition of 1 mM Pefabloc (Pentafarm) and 10% trichloroacetic acid (TCA) and SDS-PAGE analysis.

2.4. Adhesion assays

Culture slides (Nunc, 16-well Labtek glass chamber slide) were layered with 1 × 10⁸ HeLa cells (incubated for 24 h), 25 μl Matrigel (Sigma, polymerised as instructed), or soluble extracellular matrix (ECM) components (Sigma, 50 μl 50 μg ml⁻¹ unless otherwise stated, incubated overnight at 4°C before removal and blocking of slides with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) for 1 h at 4°C). E. coli carrying pAMCP1 or pAMCR1 were grown to mid-exponential phase and induced with IPTG (1 mM) for 2–3 h. Cells were washed in RPMI (Hepes modification) and resuspended in the same buffer (for competition experiments 50 μg ml⁻¹ laminin was added to suspensions unless otherwise stated and cells were pre-incubated at 37°C for 30 min). Monolayers and slides coated with ECM proteins were incubated with 1 × 10⁷ bacterial cells for 1 h before washing 10 times in PBS, fixing in 4% formaldehyde and staining with Meyer’s haematoxylin (Sigma, as instructed). ECM preparations were inoculated with 2.5 × 10⁶ bacterial cells, incubated for 10 min, washed twice in PBS, fixed with 4% formaldehyde, and stained with Giemsa. In cell adhesion assays, the average number of bacteria bound per eukaryotic cell was calculated from evaluation of 40 individual cells.

2.5. Adhesion of radiolabelled bacteria to ECM proteins immobilised on nitrocellulose

Laminin, collagen type IV, fibronectin, fetuin, and bovine serum albumin (10 μl 1 mg ml⁻¹) were dried on nitrocellulose and the blot was blocked with PBS, 5% BSA for 1 h. Bacteria (5 × 10⁸) expressing [35S]Rck or [35S]PagC protein were resuspended in 10 ml PBS, 5% BSA, and incubated with the blots for 16 h at room temperature. Blots were washed 10 times in PBS, dried and results were visualised by exposure to Kodak X-Omat LS film.
3. Results and discussion

3.1. Expression of Salmonella Rck and PagC in the outer membrane of laboratory E. coli

The rck and pagC genes were amplified by PCR from S. typhimurium SJW1103 and cloned downstream of the T7 promoter in the expression vector pACT7. IPTG induction of mid-exponential phase cells of E. coli BL21(DE3) derivatives carrying the resulting pAMCR1 or pAMCP1 recombinant plasmids produced high levels of, respectively, the expected 17-kDa Rck and 18-kDa PagC proteins, as determined by SDS-PAGE of whole cell lysates (Fig. 1A). Intact E. coli cells expressing [35S]Rck or [35S]PagC were treated with proteinase K and trypsin. While neither protein possessed exposed trypsin cleavage sites, both proteins were degraded by proteinase K (Fig. 1B), confirming outer membrane localisation for both.

3.2. While only Rck mediates cell adhesion and entry, both Rck and PagC mediate binding to the ECM

Use of recombinant E. coli allowed the activity of Rck and PagC to be assayed without the influence of other Salmonella virulence proteins encoded. After
1 h incubation with HeLa cells, E. coli carrying the plasmid vector was unable to bind to the epithelial cell monolayers. Induction of Rck, but not PagC, in recombinant E. coli carrying pAMCR1 or pAMCP1 conferred the ability to bind to the HeLa cell monolayers (approximately 8–10 bacteria bound each eukaryotic cell). Additionally, we noted that following repeated washes, E. coli expressing either Rck or PagC remained bound to monolayer backgrounds whereas the control strain did not (not shown). This background binding suggested that E. coli expressing Rck and PagC could interact with secreted eukaryote protein(s).

To determine whether ECM proteins were capable of mediating such background adherence, binding of both PagC- and Rck-expressing cells to the Matrigel preparation of mouse basal lamina was examined. Both Rck and PagC conferred upon the non-adherent E. coli the ability to bind to the ECM (Fig. 2). Direct interaction with the ECM is a mechanism utilised by many pathogens and may serve to promote bacterial colonisation of open wounds, to provide an indirect mechanism for binding to the mammalian host cell, or to mediate penetration of subepithelial layers following cellular invasion [11–13]. The in vivo significance of this result remains to be

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**Fig. 3.** E. coli expressing Rck or PagC visualised by light microscopy.

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**Fig. 4.** Attachment to ECM proteins by E. coli expressing PagC or Rck. A: Binding of E. coli expressing Rck (R), PagC (P) or neither protein (−) to the ECM components indicated, immobilised on glass slides. Values indicate the mean of five randomly chosen microscope fields; bars show S.E.M. B: Binding of E. coli expressing Rck or PagC to glass slides coated with increasing laminin concentrations. Values are mean percentages of bacteria bound per microscope field, calculated from three separate experiments, with the value at 100 μg ml⁻¹ taken as 100%. S.E.M. was 5–10%. C: E. coli cells expressing [35S]PagC incubated with the ECM proteins indicated, immobilised on nitrocellulose and visualised by autoradiography. The lower panel shows the effect of preincubation of immobilised laminin with (+) or without (−) equivalent numbers of unlabelled E. coli expressing PagC.
Fig. 5. Adhesion to HeLa cell layers by *E. coli* (control), and *E. coli* expressing Rck or PagC following pre-incubation in RPMI, Hepes modification, with or without laminin. Arrows indicate the location of recombinant bacteria pre-incubated in the presence of laminin and bound to eukaryotic cells.
determined, but, if conserved in Salmonella, ECM binding may play a role in basal lamina penetration.

E. coli expressing Rck bound to eukaryotic cell monolayers and extracellular matrix preparations in clumps (Fig. 2), but this was due to autoagglutination, also seen in the absence of monolayers and ECM (Fig. 3). In contrast, expression of PagC did not induce autoagglutination.

3.3. E. coli expressing either Rck or PagC bind laminin

To elucidate the mechanism by which Rck or PagC expression induced ECM binding, we examined the ability of E. coli expressing Rck or PagC to bind to Matrigel components laminin and collagen type IV, which are common ligands used for pathogenic interaction. For comparison, binding was assessed in parallel to soluble fibronectin, the glycoprotein fetuin, and bovine serum albumin. The ECM protein components and control proteins were immobilised on glass slides and incubated with E. coli expressing Rck or PagC (Fig. 4A). Both bound to laminin-coated slides. Cells expressing Rck, but not PagC, also bound to soluble fibronectin. Bacterial binding to both proteins is unsurprising as other bacterial adhesins, e.g., Yersinia YadA, mediate binding to multiple ECM components [12]. No significant binding was seen in either case to collagen type IV, the carbohydrate-rich glycoprotein fetuin or to the BSA control. E. coli carrying the vector alone did not bind to any of these proteins.

To demonstrate that laminin binding was specific, inhibition and saturation of laminin binding was examined. Increasing concentration of laminin immobilised on glass slides resulted in increased binding of recombinant bacteria (Fig. 4B) at concentrations ranging from 0 to 100 µg ml\(^{-1}\) laminin solution. No significant difference was seen in the number of bacteria bound to slides coated with 100–200 µg ml\(^{-1}\), suggesting saturation. Binding of E. coli expressing PagC to slides coated using 50 µg ml\(^{-1}\) laminin was reduced to 44% (±7%) by pre-incubation of these recombinant bacteria with laminin at a concentration of 25 µg ml\(^{-1}\). The same could not be shown for E. coli expressing Rck due to autoagglutination.

E. coli expressing \[^{35}S\]Rck (not shown) or \[^{35}S\]PagC (Fig. 4C) also bound to laminin immobilised on nitrocellulose. Pre-incubation of laminin-coated nitrocellulose with E. coli expressing unlabelled PagC significantly reduced subsequent binding of cells expressing the labelled protein (Fig. 4C), while pre-incubation with the E. coli C43(DE3) did not (not shown). Similar assay of E. coli expressing Rck was precluded by the autoagglutination of these cells.

3.4. Binding of soluble laminin targets E. coli expressing Rck and PagC directly to the eukaryotic cell surface

To determine whether soluble laminin was able to inhibit eukaryotic cell binding by Rck, bacteria expressing the protein were preincubated with laminin (Fig. 5, Table 1). No inhibition of binding was observed, instead bacterial binding to eukaryotic cells was enhanced, and binding to the background appeared to decrease. E. coli cells expressing PagC were similarly pre-incubated with laminin. Incubation of PagC-expressing cells with laminin agglutinated the bacteria (not shown). Such treatment allowed PagC-expressing E. coli to bind directly to the cell surface, and prevented their binding to the eukaryotic cell monolayer background. This pattern of binding was not confined to epithelial cell monolayers, but was identical when adherence to murine monocyte-derived cell layers was studied. Direction of bacteria to the eukaryotic cell by pre-incubation with laminin could indicate that laminin acts as a bridging molecule, efficiently targeting cells to the eukaryotic cell surface.

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<tr>
<th>Strain</th>
<th>Bacteria bound per eukaryotic cella</th>
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<tr>
<td></td>
<td>−laminin</td>
</tr>
<tr>
<td>Rck</td>
<td>9.0±1.5</td>
</tr>
<tr>
<td>PagC</td>
<td>0.4±0.3</td>
</tr>
<tr>
<td>Control</td>
<td>0.0±0.0</td>
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\(^{a}\)Results represent the average number of bacteria bound per eukaryotic cell and the S.E.M. of this value, calculated from 40 individual eukaryotic cells in a representative experiment. Average values may prove to be artificially low due to agglutination phenotypes.
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