Antiprotease inactivation by Salmonella enterica released from infected macrophages

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Summary

The mammalian serine protease plasmin, which has an important role in extracellular matrix degradation during cell migration, is regulated by the plasma antiprotease α₂-antiplasmin (α₂AP). The surface protease PgtE of Salmonella enterica serovar Typhimurium proteolytically inactivated α₂AP. PgtE also activates the plasma zymogen plasminogen to plasmin, and bacteria expressing PgtE promoted degradation of extracellular matrix laminin in the presence of plasminogen and α₂AP. α₂AP inactivation was detected with the rough derivative of S. enterica 14028, but not with the smooth wild-type strain, suggesting that the O-antigen of lipopolysaccharide prevented contact of PgtE with the substrate molecule. After growth of S. enterica 14028 in murine J774A.1 macrophage-like cells, the infected cell lysate as well as bacteria from isolated Salmonella-containing vacuoles (SCVs) cleaved α₂AP. Bacteria from SCVs produced an elevated level of PgtE and had a reduced O-antigen chain length. The lysate from S. enterica 14028-infected macrophages promoted formation of plasmin in the presence of α₂AP, whereas plasmin formation by lysates from uninfected macrophages, or from macrophages infected with the pgtE-negative derivative of 14028, was inhibited by α₂AP. Salmonella disseminates in the host within macrophages, which utilize plasmin for migration through tissue barriers. The results suggest that intracellular enhancement of PgtE activity in Salmonella may promote macrophage-associated proteolysis and cellular migration by altering the balance between host plasmin and α₂AP.

Introduction

Salmonella enterica serovar Typhimurium is a common food-borne pathogen that causes gastroenteritis in the human host, with symptoms of diarrhoea, fever and cramping. In most cases, enteritis remains localized to the intestine, but approximately 5% of infected humans develop bacteraemia (Hohmann, 2001). Serovar Typhimurium infection in mice, on the other hand, resembles the pathogenesis of human typhoid fever that is caused by Salmonella enterica serovar Typhi. Typhoid fever is a severe systemic infection that without treatment has a 10–15% mortality (Ohl and Miller, 2001). Both serovar Typhimurium and serovar Typhi are ingested in contaminated food and enter the host through the epithelial barrier of the small intestine, preferentially via the antigen-sampling M cells but also via invasion of enterocytes (Ohl and Miller, 2001). An important characteristic of Salmonella is the ability to multiply inside macrophages and dendritic cells in submucosa. This is supported by formation of a specific Salmonella-containing vacuole (SCV), a spacious phagosome which avoids fusion with lysosomes (Holden, 2002; Brumell and Grinstein, 2004). In invasive salmonellosis, the infection proceeds to bacteraemia and bacterial dissemination into the reticuloendothelial organs. Salmonella can take advantage of phagocytic host cells for migration: dissemination of Salmonella from the gastrointestinal tract to the liver and the spleen has been shown to take place within macrophages and dendritic cells (Vazquez-Torres et al., 1999). However, one-third of the bacteria found in the blood of patients with serovar Typhi are not associated with phagocytes (Wain et al., 1998), indicating that extracellular dissemination of Salmonella also is possible.

Proteolysis is important in migration of all cell types, both eukaryotic and prokaryotic, across tissue barriers, and dissemination of Salmonella, either inside or outside phagocytic cells, is also likely to require proteolytic activity. Many bacterial pathogens interfere with host proteolytic systems that are involved in extracellular matrix (ECM) breakdown, coagulation, fibrinolysis or complement activation (reviewed in Travis et al., 1995; Lähteenmäki et al., 2001; 2005). The host protease systems are carefully controlled by protease inhibitors, which represent nearly 10% of the total protein mass in mammalian plasma (Travis and Salvesen, 1983). A few invasive bacterial species, which include oral pathogens as well as Yersinia pestis, Staphylococcus aureus and Pseudomonas aeruginosa, have been found to inactivate host antiproteases (Carlsson et al., 1984; Potempa et al., 1986; Maeda and...
Molla, 1989; Grenier, 1996; Kukkonen et al., 2001). The inactivation is thought to enhance uncontrolled proteolysis and promote survival and dissemination of bacteria in the host during infection.

The serine protease plasmin degrades various ECM components, such as laminin and fibronectin, and has an important role in migration of monocytes and macrophages through tissue barriers (Vassalli et al., 1992; Plow et al., 1999). The zymogen plasminogen, which circulates in plasma in high concentration (180 μg ml⁻¹), is converted to the active form plasmin by specific plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase (uPA). Monocytes and macrophages secrete uPA, and also express receptors for uPA and plasminogen, which serve to localize the forming plasmin activity on migrating cells. Unbound plasmin is efficiently inhibited by the serine protease inhibitor (serpin) α₂-antiplasmin (α₂AP), which circulates in mammalian plasma in 60–70 μg ml⁻¹ concentration. α₂AP also inhibits other serine proteases such as trypsin and chymotrypsin (Travis and Salvesen, 1983; Potempa et al., 1988).

The pgtE gene of Salmonella is present in all sequenced serovar Typhimurium and serovar Typhi genomes, with a conserved sequence and chromosomal location, and codes for the outer membrane protein PgtE that activates plasminogen (Sodeinde and Goguen, 1989; Kukkonen et al., 2004). PgtE belongs to the ompT family of enterobacterial aspartate proteases and has 72% amino acid sequence identity with the omptin of Y. enterocolitica (Kramer et al., 2004). PgtE must be proteolytically cleaved from its precursor to become active (Groisman, 2001; Ohl and Miller, 2001). Our hypothesis was that upregulation of pgtE and LPS modifications inside murine macrophages lead to enhanced proteolytic activity in Salmonella. Here, we present evidence showing that PgtE of macrophage-derived Salmonella proteolytically inactivates the antiprotease α₂AP. Our results also show that PgtE expression is elevated and, further, the LPS O-chain length is altered in Salmonella inside the intracellular compartment.

Results

In vitro inactivation of α₂AP by S. enterica 14028 and its derivatives

Our initial observation was that a c. 50 kDa cleavage product was formed when α₂AP was incubated with the rough S. enterica 14028R, but, instead, α₂AP remained intact when incubated with the virulent parent strain 14028 (Fig. 1A). For the assay, the bacteria were cultivated in PhoP/Q-inducing conditions (N-minimal medium supplemented with 8 μM MgCl₂) (Garcia Véscovi et al., 1996). To determine the role of PgtE in the modification of α₂AP, we constructed a pgtE-negative derivative of 14028R. The resulting 14028R-1 had no detectable effects on α₂AP and cleavage of α₂AP was restored in 14028R-1 complemented with the PgtE-encoding plasmid pMRK3, indicating that it resulted from expression of PgtE. α₂AP remained intact when incubated with 14028R-1 complemented with the vector pSE380 or the plasmid pMRK3, which encodes a proteolytically inactive PgtE (Kukkonen et al., 2004). The corresponding deletion of pgtE (14028-1) and complementations had no significant effect on α₂AP degradation by the wild-type 14028 that expresses smooth LPS (Fig. 1A). Western blotting analysis of bacterial outer membrane preparations showed that 14028 and 14028R produce a similar amount of PgtE (data not shown), indicating that poor cleavage of α₂AP with the wild-type S. enterica 14028 did not result from reduced expression of PgtE. These results suggest that PgtE proteolytically cleaves α₂AP and that O-antigen inhibits this function.

To confirm that the PgtE-mediated cleavage of α₂AP also leads to inactivation of the serpin, α₂AP was first
incubated with bacteria and then with plasmin, and the residual serpin activity was estimated by measuring plasmin activity with a chromogenic substrate. α2AP antibodies. The α2AP and its 50 kDa cleavage product are indicated by arrowheads. α2AP incubated with the smooth S. enterica 14028, the pgtE-negative derivative 14028-1, as well as 14028-1 complemented with PgtE-expressing pMRK3, the vector plasmid pSE380, or pMRK31 which encodes for protease-negative PgtE, are shown in the left panel. The isogenic rough S. enterica 14028R and its corresponding derivatives are shown in the middle panel, and α2AP incubated in buffer is shown in the right panel. The figure shows a representative example of three independent experiments.

B. Loss of serpin activity in α2AP. α2AP was first incubated with bacteria for 2 h and then incubated with plasmin and a chromogenic substrate for 90 min to measure the residual plasmin activity. Samples with the smooth S. enterica 14028 and its derivatives are shown in the left panel, and the rough 14028R and its derivatives in the middle panel. For comparison, plasmin activity in samples with α2AP incubated in buffer instead of bacteria, as well as plasmin in the absence of α2AP (no α2AP), are shown in the right panel. The bars represent means and the error bars ranges of absorbance values of duplicate samples from a representative experiment performed in three independent repetitions.

α2AP inactivation by recombinant E. coli expressing PgtE

To confirm the role of PgtE in antiprotease inactivation in a well-defined laboratory host background, we studied the efficiency of α2AP degradation with the PgtE-encoding recombinant E. coli XL1(pMRK3). Similarly to S. enterica 14028R, E. coli XL1(pMRK3) rapidly produced a c. 50 kDa cleavage product of the 70 kDa α2AP molecule (data not shown), and nearly completely degraded α2AP during a prolonged incubation (Fig. 2A). Corresponding loss of serpin activity towards plasmin was seen with α2AP incubated with E. coli XL1(pMRK3), whereas α2AP incubated with E. coli XL1 harbouring the vector plasmid pSE380 was not cleaved and retained protease inhibitor activity (Fig. 2B). Loss of serpin activity was also studied in relation to the other reported proteolytic activity of PgtE, i.e. conversion of plasminogen to plasmin, and with a natural macromolecular substrate. Laminin is an important component of ECMs and a well-characterized substrate of plasmin, and incubation of E. coli XL1(pMRK3) on immobilized, radioactively labelled laminin together with plasminogen promoted release of radioactivity from the wells (Fig. 2C), whereas only a small release of radioactivity was seen with E. coli XL1(pSE380). This indicates that PgtE-mediated conversion of plasminogen to plasmin promoted laminin degradation. When α2AP was added in the wells together with E. coli XL1(pMRK3) and plasminogen, laminin degradation was only slightly reduced, whereas laminin degradation by purified plasmin was effi-
ciency inhibited by α2AP (Fig. 2C). Loss of plasmin inhibition was not as complete as when α2AP was pre-incubated with bacteria before plasmin was added (Figs 1B and 2B); however, the results indicate that PgtE-generated plasmin degrades laminin also in the presence of α2AP. Efficient PgtE-mediated degradation of laminin in the presence of plasminogen and α2AP was seen also in rough Salmonella background, whereas the smooth Salmonella 14028, which did not inactivate α2AP (Fig. 1), and did not activate plasminogen because of the presence of O-antigen repeats (Kukkonen et al., 2004), did not promote laminin degradation (data not shown). These results indicate that PgtE can generate uncontrolled plasmin by concomitant plasminogen activation and α2AP inactivation, and this way enhances degradation of a major component of tissue barriers.

α2AP cleavage after growth of S. enterica 14028 inside murine macrophages

As the PgtE-mediated inactivation of α2AP described above was detected with the rough S. enterica 14028R but not with the virulent, smooth 14028, the relevance of the antiprotease inactivation by Salmonella remained questionable. Significant changes in Salmonella surface architecture are induced inside macrophages (Ernst et al., 2001; Eriksson et al., 2003), and we reasoned that Salmonella may express fully functional PgtE while growing inside macrophages. To study this, we infected murine J774A.1 macrophage-like cells with S. enterica 14028 and 14028-1. Infected cells were lysed after 20 h, and we first analysed the lysates for α2AP degradation. The lysate from macrophages infected with S. enterica 14028 promoted α2AP cleavage far more efficiently than did bacteria cultivated in vitro in Luria broth (Fig. 3), suggesting that macrophage infection promotes PgtE activity in Salmonella. The lysate from macrophages infected with the pgtE-negative S. enterica 14028-1, similarly to uninfected cell lysate, did not enhance α2AP cleavage. We normally used a 45 min initial infection period for J774A.1 cells, but to exclude possible cytotoxic effects we also tested lysates from macrophages infected with salmonellae for...
only 15 min; again, lyse from 14028-infected, but not from 14028-1-infected, cells promoted \( \alpha_2 \)AP cleavage (data not shown). S. enterica 14028 grown in PhoP/Q-inducing medium only very weakly enhanced formation of the 50 kDa cleavage product of \( \alpha_2 \)AP (Fig. 3). As PhoP activity is promoted by macrophage acidification in vivo, and, to a lower extent, by low-pH growth medium in vitro (Alpuche Aranda et al., 1992), we analysed Salmonella 14028 cultivated also in N-minimal medium at pH 5, but detected no significant enhancement of \( \alpha_2 \)AP cleavage (data not shown).

To study \( \alpha_2 \)AP cleavage with macrophage-derived bacteria in conditions containing as little material from the eukaryotic cells as possible, we next isolated SCVs from S. enterica 14028- and 14028R-infected macrophages, lysed them, and measured \( \alpha_2 \)AP cleavage with bacteria from the vacuoles. Formation of the 50 kDa cleavage product of \( \alpha_2 \)AP was seen with both 14028 and 14028R from isolated SCVs; however, nearly complete degradation of \( \alpha_2 \)AP was seen only with 14028R grown in PhoP/Q-inducing conditions (Fig. 4, top). Similarly to infected macrophage lysates, the \( pgtE \)-negative 14028-1 from SCVs promoted no cleavage of \( \alpha_2 \)AP (data not shown). The SCV preparations, the macrophage lysates or the in vitro grown bacteria showed no reaction with \( \alpha_2 \)AP antibodies in the absence of \( \alpha_2 \)AP (data not shown). Altogether, our results suggest that the activity of PgtE is enhanced during growth of Salmonella inside macrophages and that this promotes degradation of host \( \alpha_2 \)AP by bacteria released from macrophages.
The results of the present work give evidence that PgtE-bound basement membranes promote migration of lagen breakdown by activating latent procollagenases involved in cell migration; it directly degrades ECM and et al specific adhesin (Sodeinde and Goguen, 1989; Kukkonen also converts plasminogen to plasmin and is an ECM-responsible for the inactivation of the antiprotease. PgtE from uninfected cells or from cells infected with plasminogen antibodies. Macrophages convert plasminogen to plasmin in an uPA-dependent manner (Vassalli et al., 1992), and we observed formation of plasmin with all J774A.1 macrophage lysates (Fig. 5). The lysate from Salmonella 14028-infected cells, however, converted plasminogen to plasmin more rapidly than the lysates of uninfected cells or from cells infected with Salmonella 14028-1 (Fig. 5 and data not shown). Furthermore, plasmin formation by lysates from uninfected macrophages or from Salmonella 14028-1-infected cells was inhibited by α2AP, whereas the lysate from Salmonella 14028-infected cells converted plasminogen to plasmin also in the presence of α2AP (Fig. 5). These results suggest that PgtE-mediated proteolysis promotes macrophage-associated plasminogen activation in the presence of α2AP.

Discussion

The serpin α2AP is a highly efficient inhibitor of plasmin and also inhibits other serine proteases. Our results show that the commonly used virulent strain 14028 of S. enterica serovar Typhimurium cleaves α2AP after release from murine macrophages, and modifies α2AP into an inactive form that no longer inhibits plasmin. We identified the surface protease PgtE of Salmonella as the component responsible for the inactivation of the antiprotease. PgtE also converts plasminogen to plasmin and is an ECM-specific adhesin (Sodeinde and Goguen, 1989; Kukkonen et al., 2004). Plasmin is a broad-spectrum protease involved in cell migration; it directly degrades ECM and basement membrane glycoproteins and mediates collagen breakdown by activating latent procollagenases (Plow et al., 1999), and we have earlier found that surface-bound plasmin promotes migration of Salmonella through basement membranes in vitro (Lähteenmäki et al., 1995). The results of the present work give evidence that PgtE-generated α2AP degradation is enhanced in bacteria from macrophages and in vitro leads to uncontrolled plasmin activity that can be targeted onto ECM laminin. This is likely to enhance cellular migration associated with Salmonella infection.

Our present results agree with the previous observation that PgtE is cryptic in Salmonella cultivated in rich laboratory culture media. Plasminogen activation (Kukkonen et al., 2004) and α2AP inactivation are not seen with the smooth S. enterica 14028, whereas the isogenic rough derivative 14028R inactivated α2AP after cultivation in PhoP/Q-inducing conditions. Biological importance of antiprotease degradation was suggested by the observation that the lysate of murine macrophage-like J774A.1 cells infected with Salmonella 14028, as well as 14028 from isolated macrophage SCVs, promoted cleavage of α2AP. In contrast, the lysate of cells infected with the pgtE-negative 14028-1 and the lysate of non-infected macrophages did not cleave α2AP. It is noticeable that degradation of α2AP by 14028 from SCVs was less efficient than by the lysate from 14028-infected cells containing the same amount of bacteria (Figs 3 and 4). Moreover, the rough 14028R degraded α2AP more efficiently when cultivated in vitro under PhoP/Q-inducing conditions than when isolated from SCVs, although increased expression of PgtE was seen in bacteria from both conditions. The reason for this phenomenon remains speculative at the moment, but a likely explanation is that both upregulation of pgtE and reduction in the O-antigen chain length take place when Salmonella grows inside the macrophage.
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Monocytes and macrophages utilize plasmin in their migration (Ploplis et al., 1998), and inactivation of α2AP may promote macrophage migration and hence enhance dissemination of Salmonella within the phagocytes. On the other hand, plasmin acts as a chemoattractant for monocytes (Syrovets et al., 1997), and uncontrolled plasmin may promote interaction of Salmonella with monocytes and macrophages by this mechanism also. In our study, lysates of J774A.1 macrophages converted plasminogen to plasmin, probably via the uPA-mediated activation cascade that is well characterized on macrophage surface and requires plasmin-catalysed activation of macrophage-produced pro-uPA (Vassali et al., 1992). Because of inhibition of plasmin, α2AP restrains plasminogen activation by human mononuclear cells (Duval-Jobe and Parmely, 1994), and we observed that α2AP completely inhibited plasmin formation by uninfected J774A.1 macrophages and macrophages infected with the pgtE-negative 14028-1. Remarkably, the lysate from macrophages infected with S. enterica 14028 converted plasminogen to plasmin also in the presence of α2AP. Furthermore, plasmin formation in the absence of α2AP was more rapid with the lysate from macrophages infected with S. enterica 14028 than with lysates from uninfected or 14028-1-infected cells. This suggests that the plasminogen activator activity of PgtE also becomes functional in Salmonella inside macrophages. The rate of plasmin formation by lysates from both uninfected and Salmonella-infected cells varied considerably in different infection experiments, probably reflecting the amount of cellular uPA, but only the lysates from the cells infected with the wild-type Salmonella 14028 promoted generation of plasmin regardless of the presence of α2AP. α2AP is present in tissue locations where plasmin is formed, and the capacity to inactivate α2AP and activate plasminogen that Salmonella gains during intracellular growth is likely to increase plasmin activity in host tissues. Our ongoing work suggests that S. enterica 14028 isolated from SCVs has PgtE-mediated activity also towards other macromolecular substrates (our unpublished data). Thus, the increased proteolysis described here probably holds for other polypeptide substrates of PgtE as well.

Other functions of PgtE have also been connected to macrophage environment: PgtE has been proposed to promote survival of Salmonella inside macrophages via cleavage of cationic anti-microbial peptides (Guina et al., 2000). Our results support this hypothesis, as during the 20 h incubation within the macrophages the number of the wild-type 14028 cells increased to c. 170% of internalized bacteria, whereas the pgtE-negative 14028-1 was reduced to c. 50% (data not shown). Salmonella induces both necrotic and apoptotic cell death in infected macrophages, and various bacterial and host cell components have been identified in host cell death (Knodler and Finlay, 2001; Monack et al., 2001). In Salmonella-infected human macrophages, cell death involves PhoP activity (Detweiler et al., 2001), and thus high-level expression of functional PgtE may correlate with host cell death. A recent mouse infection model demonstrated that the growth of Salmonella...
in tissues relies on a continuous distribution of bacteria to new phagocytes (Sheppard et al., 2003; Mastroeni and Sheppard, 2004), suggesting that bacterial release from the macrophages may be a common event during infection. Our results suggest that the bacterial population that is released from macrophages is proteolytically active, and our hypothesis is that a main function of PgtE is to enhance survival and spread of Salmonella after bacterial release from dying phagocytes.

The homologue of PgtE in Y. pestis, Pla, also cleaves αAP (Kukkonen et al., 2001). Comparison of αAP inactivation by recombinant E. coli expressing the two omptins showed that PgtE inactivates the inhibitor as efficiently as Pla (data not shown), whereas PgtE is significantly less efficient in plasminogen activation than Pla (Kukkonen et al., 2004). The substrate preference of the Salmonella omptin for αAP may reflect differences in the pathogenetic mechanisms of the infections. Y. pestis is a predominantly extracellular pathogen, and formation of uncontrolled plasmin in tissue fluids may be of primary importance for bacterial dissemination in plague. Bacterial release from dying phagocytes in tissues relies on a continuous distribution of bacteria to new phagocytes (Sheppard et al., 2003; Mastroeni and Sheppard, 2004), suggesting that bacterial release from the macrophages may be a common event during infection. Our results suggest that the bacterial population that is released from macrophages is proteolytically active, and our hypothesis is that a main function of PgtE is to enhance survival and spread of Salmonella after bacterial release from dying phagocytes.

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**Experimental procedures**

**Bacterial strains and plasmids**

Bacterial strains and plasmids used in this work are listed in Table 1. In frame deletion of pgtE of S. enterica 14028R was performed by site-specific mutagenesis with the suicide vector pCVD442 (Donnenberg and Kaper, 1991) as described earlier for 14028 (Kukkonen et al., 2004).

**Cleavage and inactivation of αAP**

For optimal in vitro expression of PgtE, wild-type S. enterica and their pgtE-negative derivatives were grown overnight in N-minimal medium, pH 7.4, containing 38 mM glycerol, 0.1% casaminoacids, 2 μg ml⁻¹ thiamine and 8 μM MgCl₂. When indicated, bacteria were grown for comparison also in Luria broth. Complemented pgtE-negative derivatives of Salmonella as well as recombinant E. coli were grown in the presence of 5 μM IPTG (Promega) for induction of the trc promoter of the pSE380 plasmid as detailed by Kukkonen et al. (2001). Bacteria were collected, washed, adjusted to the indicated cell densities and incubated with human plasma αAP (50 μg ml⁻¹; Calbiochem) in a 40 μl total volume in PBS-Dulbecco at 37°C. Samples were resolved in 12% reducing SDS-PAGE and transferred onto nitrocellulose membrane. The peptide bands were probed with rabbit anti-human αAP IgG (Calbiochem) and detected with alkaline phosphatase-conjugated anti-rabbit IgG (Dako) and the phosphatase substrate. To measure the level of αAP inactivation, αAP (5.75 μg ml⁻¹) was first incubated with bacteria for 2 h in a 200 μl total volume. Human plasmin (2.5 μg ml⁻¹; Fluka) and a chromogenic substrate of plasmin, Val-Leu-Lys-dihydrochloride (S-2251; 0.45 mM; Chromogenix), were added, and the residual plasmin activity was measured as breakdown of the substrate at 405 nm after 90 min incubation.

**Degradation of immobilized laminin**

Entactin-free laminin (from mouse Engelbreth-Holm-Swarm tumour; BD Biosciences) was labelled with 125I (Amersham); specific activity obtained was 2.5 × 10⁶ cpm per microgram of protein. Laminin (200 ng in 100 μl of PBS) was coated on polystyrene Microstrip (Labsystems) wells by incubating overnight at room temperature, the wells were washed and bacteria (2 × 10⁵ ml⁻¹ in PBS-Dulbecco) were added on the wells together with human Glu-plasminogen (20 μg ml⁻¹; American Diagnostica) and αAP (30 μg ml⁻¹). Control wells contained plasmin in 20 μg ml⁻¹ concentration; total volume in the wells was 200 μl. Microstrips were incubated at 37°C with mild agitation, and samples were taken at indicated time points to measure the radioactivity released from the wells.

**Table 1.** Bacterial strains and plasmids.

| Bacterial strain | Relevant characteristics | References |
|------------------|--------------------------|------------|
| S. enterica 14028 | Smooth LPS               | ATCC       |
| S. enterica 14028-1 | ΔpgtE derivative of 14028 | Kukkonen et al. (2004) |
| S. enterica 14028R | Rough LPS derivative of 14028 | Wick et al. (1994) |
| S. enterica 14028R-1 | ΔpgtE derivative of 14028R | This work |
| E. coli XL1     | K12, rough LPS           | Stratagene |
| Plasmid         | pgtE in pSE380           | Kukkonen et al. (2004) |
| pMRK3           | pgtE D206A in pSE380     | Kukkonen et al. (2004) |
| pMRK31          | Expression vector, trc promoter | Invitrogen |

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Bacterial phagocytosis and analysis of infected macrophage lysates

J774A.1 murine macrophage-like cells (ATCC TIB-67) were grown on six-well cell culture plates (Nunc) in Dulbecco's modified Eagle medium ( Gibco) supplemented with 10% fetal bovine serum (Hyclone). In all subsequent steps, 4 ml of medium per well was used. The cells were washed with PBS and fresh medium was added. S. enterica 14028 and 14028-1 grown to stationary phase in Luria broth were added on J774A.1 cells at multiplicity of infection (moi) of 10, 12 wells per bacterial strain. The plates were gently centrifuged to facilitate contact between the bacteria and the cells and incubated for 45 min for phagocytosis to occur. After washing, extracellular bacteria were killed by 1 h treatment with gentamicin (100 μg ml⁻¹ in PBS), and the cells were incubated for 20 h in fresh medium containing 20 μg ml⁻¹ gentamicin. After washing, the cells were lysed by 0.2% Triton X-100. The lysates were pelleted, washed, suspended in 200 μl of PBS-Dulbecco, and 40 μl samples were analysed for α,-AP cleavage as described for in vitro grown bacteria. The lysates were also incubated with human Glu-plasminogen (56 μg ml⁻¹; American Diagnostica) in the presence and absence of α,-AP (130 μg ml⁻¹) in a 40 μl total volume, resolved in 10% reducing SDS-PAGE and analysed by Western blotting with anti-plasminogen-IgG (Dako).

Isolation and analysis of Salmonella-containing vacuoles

Isolation of SCVs from J774A.1 macrophages was performed essentially as in Desjardins et al. (2002). Briefly, after 20 h infection the macrophages from 36 wells were collected with a cell scraper, washed and resuspended in 4 ml of ice-cold 250 mM sucrose, 3 mM imidazole, pH 7.4. Cells were gently broken by subsequent passages through a Pasteur pipette and a syringe with a 22G needle. Unbroken cells and nuclei were pelleted, and SCVs were isolated from the supernatant in a 10–62% sucrose gradient ultracentrifugation. SCVs were diluted in 10 ml of ice-cold PBS, pelleted and lysed by 500 μl of 0.2% Triton X-100. The lysates were pelleted, washed, suspended in 200 μl of PBS-Dulbecco, and analysed for α,-AP cleavage. Bacteria were also analysed by Western blotting with anti-PgtE anti-serum after electrophoresis in reducing 10% SDS-PAGE (Kukkanen et al., 2004) to detect expression of PgtE. For detection of LPS, whole-cell lysates were treated with proteinase K (Hitchcock and Brown, 1983), and after electrophoresis of the samples in 15% SDS-PAGE LPS was detected by silver staining (Tsai and Frasch, 1982).

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