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Effect on bovine lactoferrin on the activation of the enteroinvasive bacterial type III secretion system

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Abstract

Shigella and enteroinvasive *Escherichia coli* (EIEC) strains secrete virulence proteins by a complex machinery called the type III secretion (TTS) apparatus. Secretion of virulence proteins is a tightly-regulated phenomenon such that the TTS system is weakly active when bacteria are grown in common laboratory media. Activation of the TTS system is triggered by contact with eukaryotic cells, or can be artificially stimulated by the addition of Congo red dye to the growth medium. Exploiting the ability of bovine lactoferrin (bLf) to bind iron we have found that the TTS of EIEC strain HN280 seems to be activated in conditions of low-iron availability, obtained by incubation of bacteria with bLf enclosed within a dialysis bag. Activation of secretion was assessed by measuring the release of IpaB and C, chosen as reporters of secreted virulence proteins. The contribution of small bLf-derived components, diffusing across the dialysis membrane, in the release of Ipa proteins has also been determined. Activation of secretion was not due to bLf-induced damage of the HN280 outer membrane and was not associated with increased transcription of the *mxi* operon. Thus, low-iron availability might be an environmental signal perceived by enteroinvasive micro-organisms in order to modulate secretion of virulence proteins.

Introduction

Shigella flexneri and enteroinvasive *Escherichia coli* (EIEC) are pathogenic micro-organisms that are known to cause disease in humans by a very similar, if not identical, mechanism of pathogenicity. Coordinated expression of a variety of virulence determinants allows these micro-organisms to invade and to multiply within various host cells, including M cells, macrophages and intestinal epithelial cells (Sansonetti 2001). In the expression of these virulence determinants, a key role is played by a virulence plasmid (pINV)-encoded Sec-independent secretion apparatus, called the type III secretion (TTS) apparatus, a structure which spans both the inner and outer membrane and extends into the external milieu (Tran Van Nhieu & Sansonetti 1999). A conserved TTS system is used by a variety of Gram-negative bacteria, which are pathogens for humans, animals or plants, to interact with host cells (Hueck 1998). The TTS system of *Shigella* and EIEC is encoded by the *mxi* and *spa* loci which are located on the pINV within a 31 kb region (the entry region) together with the *ipa* locus. The function of the TTS apparatus is to deliver virulence proteins through the membrane of host cells (translocators) and to inject proteins (effectors) into the cytoplasm to subvert the physiology of the host cell. The activity of the TTS apparatus is controlled by external signals and the system is weakly active when bacteria are grown in vitro (only 5% of proteins are secreted). Contact of bacteria with eukaryotic cells is a signal recognized by enteroinvasive bacteria to activate the TTS system and thereby secretion of

virulence proteins. Secretion can be activated also by addition of the Congo red dye to the growth medium or by inactivating the *ipaB* or *ipaD* genes (Demers *et al.* 1998).

In this report we exploited the iron-binding ability of native bovine lactoferrin (bLf) to evaluate the role of this metal in the activation of the TTS system of enteroinvasive *E. coli*. The results obtained seem to indicate that a low-iron availability induces activation of the TTS system of EIEC strain HN280, and that secretion is not related to increased transcription of the *mxi* operon, nor to bLF-induced outer membrane damages.

Materials and methods

Bacterial strains, culture conditions and reagents

HN280 is a virulent, wild-type EIEC strain of serotype O135:K-:H- (Berlutti *et al.* 1998). HN580 is a derivative of HN280 in which the *mxiC-lacZ* transcriptional operon fusion of *S. flexneri* strain BS184 (Maurelli & Sansonetti 1988) has been transferred to virulence plasmid pHN280 (Colonna *et al.* 1995). Growth media used were Trypticase soy broth and Luria-Bertani (LB) broth. Congo red (Sigma Chemical Co.) was added to Trypticase soy agar to a final concentration of 0.01%.

Native bLf (approximately 20% iron-saturated) was provided by Dicofarm, Roma, Italy. Iron-saturated bLf was prepared as described by Rossi *et al.* 2002. Iron-free phosphate buffer solutions (PBS) were prepared by means of bLf, enclosed in a dialysis membrane, and overnight dialysis at 4 °C using a bLf concentration of 1.0 mg per ml of PBS.

Assays for bLf-induced activation of the type III secretion apparatus

Exponentially growing EIEC strain HN280 (OD_{600} = 0.8) was washed, concentrated ten times (6 ml) in iron-free PBS and incubated for 1 h at 37 °C in the absence and in the presence of native bLf or in the presence of iron-saturated bLf (12 mg of protein dissolved in 1 ml of PBS and separated from bacterial cells by a dialysis membrane). As positive control, bacterial cells were incubated in iron-free PBS in the presence of Congo red (7 μg/ml). At the end of the incubation period, bacteria were centrifuged and any proteins released in the supernatant fractions were filtered, precipitated by the addition of 1/10th volume of trichloroacetic acid (TCA) and suspended in a volume of Laemmli sample buffer (1/300th of the initial volume). Secreted proteins were separated by SDS-PAGE and either stained with Comassie brilliant blue or transferred to a PVDF membrane and subjected to Western blot analysis using anti-IpaB and -IpaC monoclonal antibodies (provided by A. Phalipon, Unité de Pathogenie Microbienne Moleculaire, INSERM, Institut Pasteur, France) and peroxidase-labelled anti-mouse secondary antibodies. Induction of secretion was assessed by measuring the release in the supernatant of IpaB and C proteins, chosen as reporters of the TTS apparatus activation. Quantification of secreted IpaB and C proteins was done by densitometry by the aid of Kodak 1D software.

Alteration of the HN280 outer membrane was assessed by Western blot analysis by determining the presence of apyrase in the secreted proteins preparations. Apyrase is a 27.5 kDa periplasmic protein produced by HN280 (Berlutti *et al.* 1998) chosen as reporter of the integrity of outer membranes. Secreted proteins were analyzed using mouse anti-apyrase specific antibodies.

β-galactosidase assays

β-galactosidase assays were performed on SDS-chloroform-permeabilized exponentially growing bacterial cells (Miller 1972) after incubation for 1 h at 37° in the presence and in the absence of bLf, as described above.

Results and discussion

Low-iron availability activates the TTS system

Activation of the TTS system of *Shigella* and enteroinvasive *E. coli* (EIEC) is a tightly-regulated phenomenon. Virulence proteins are stored in the bacterial cytoplasm, some associated with a specific chaperone, and transcription/translation of some effector genes is regulated in response to secretion. Under secreting conditions, in addition to Ipa, at least 15 other effector proteins are secreted by the TTS system of enteroinvasive micro-organisms (Mavris *et al.* 2002). Differential secretion of virulence proteins is probably occurring in response to different environments and to the different phases of the virulence pathway. To cause disease in humans, enteroinvasive micro-organisms have to face various hostile environments and to interact and multiply within different host cells (M cells,

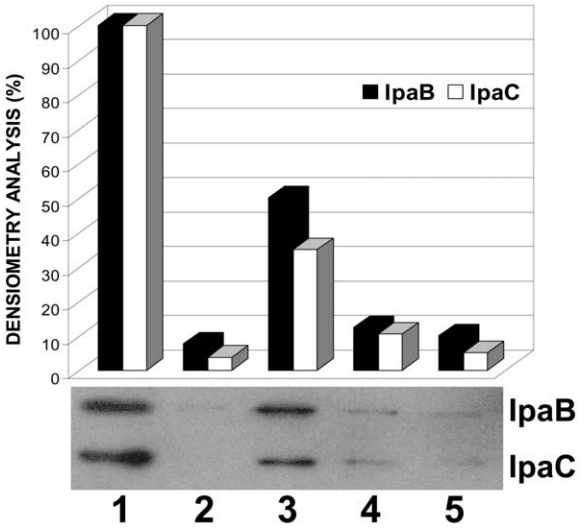

Fig. 1. Effect of bLf on secretion of IpaB and C by EIEC strain HN280 by Western blot and densitometry analysis. Exponentially-growing HN280 bacterial cells were washed, suspended in 6 ml of iron-free PBS and incubated 1 h at 37 °C in the presence of Congo red (lane 1); iron-free PBS (lane 2); bLf (lane 3); bLf and iron-citrate (lane 4); iron-saturated bLf (lane 5). bLf and iron-saturated bLf were separated from bacterial cells by a dialysis membrane (see Materials and Methods for details). At the end of incubation, secreted proteins were TCA-precipitated, separated by SDS-PAGE, transferred to a PVDF membrane and probed with anti-IpaB and C antibodies. The position of secreted IpaB and C proteins are indicated on the right. Secreted IpaB and C proteins were quantified by densitometry (top).

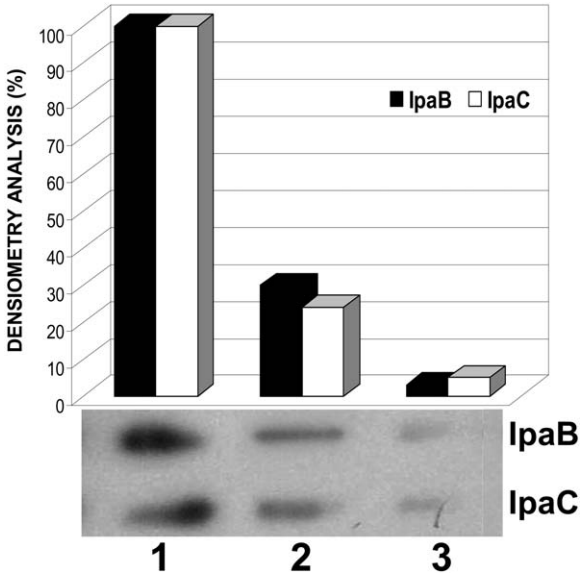

Fig. 2. Effect of bLf-derived dialyzable small products on secretion of IpaB and C by EIEC strain HN280. Twelve mg of bLf were dissolved in 1 ml of iron-free PBS, enclosed within a dialysis bag, and incubated for 1 h at 37 °C in 6 ml of iron-free PBS to induce formation and diffusion of bLf-derived products. After removal of bLf, the PBS solution was used straight away to suspend exponentially growing HN280 and the release of IpaB and C was determined by Western blot analysis (lane 2). Controls were bacteria incubated with bLf enclosed within a dialysis bag (lane 1), or simply with iron-free PBS (lane 3). The position of secreted IpaB and C proteins are indicated on the right. Densitometry of secreted proteins is depicted on the top.

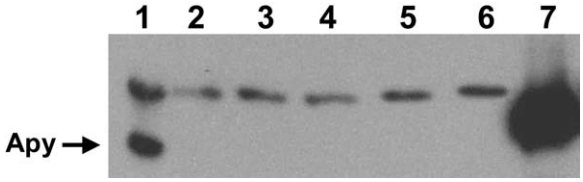

Fig. 3. The release of IpaB and C is not due to bLf-induced alterations of the outer membrane of EIEC strain HN280. Secreted proteins were separated by SDS-PAGE, transferred to a PVDF membrane and probed with anti-apyrase specific antibodies. Preparations of secreted proteins, the same shown in Figure 1 (see Figure 1 legend for details), were obtained from bacteria incubated with Congo red (lane 2); iron-free PBS (lane 3); bLf (lane 4); bLf and iron-citrate (lane 5); iron-saturated bLf (lane 6). A whole-bacterial extract of HN280 is shown in lane 1; purified recombinat apyrase is shown in lane 7. The position of apyrase (Apy) is indicated on the left.

macrophages, intestinal epithelial cells), environments presenting different iron-availability. In this context, differences in the availability of iron (as well as of other important metal ions) might represent signals that are recognized by these facultative intracellular pathogens in order to modulate secretion of virulence proteins through the TTS system.

In this report we exploited the extremely high affinity of native bLf for iron to study the role of this element in the activation of the TTS apparatus of enteroinvasive *E. coli*. EIEC strain HN280 was incubated in the presence of bLf which was separated from the bacteria by a dialysis membrane, as described in Materials and Methods. At the end of the incubation (1 h at 37 °C), secreted TCA-precipitated proteins were analyzed by Western blot. Controls were bacteria incubated with iron-free PBS alone (negative control), or supplemented with Congo red (7 μg/ml) which is known to induce secretion of the TTS apparatus (positive control) (Demers *et al.* 1998). Western blot and densitometry analysis of secreted IpaB and C proteins (Figure 1) showed that exposure of HN280 to Congo red activates the TTS machinery (lane 1), while iron-free PBS alone did not (lane 2). Exposure of bacteria to bLf (lane 3) activates secretion albeit at a lower extent than that induced by Congo red. Activation of secretion was abolished when iron-citrate (50 μM final concentration) was added to the bacterial suspension (lane 4) or when bacteria were incubated

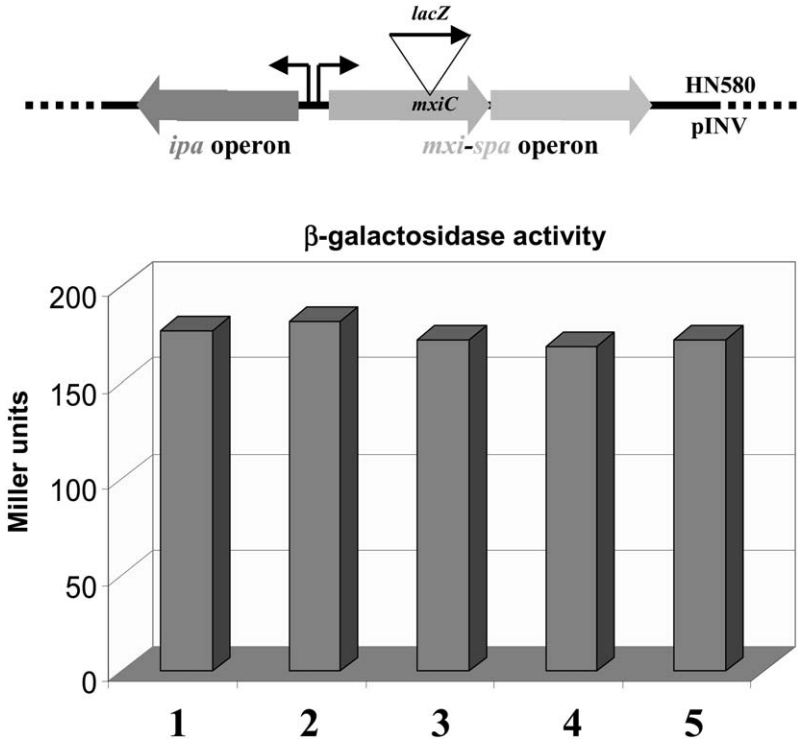

Fig. 4. Expression of β-galactosidase by HN580, a mxiC-lacZ fusion mutant derivative of EIEC strain HN280. EIEC strain HN580 was incubated with bLF under the same experimental conditions as those described for HN280 in the legend of Figure 1. Lane 1, bacteria incubated 1 h at 37 °C in the presence of Congo red (lane 1); in iron-free-PBS (lane 2); in the presence of bLf (lane 3); in the presence of bLF and iron-citrate (lane 4); or iron-saturated bLf (lane 5). At the end of the incubation period, β-galactosidase activity was measured as described in Materials and Methods. Units of β-galactosidase are as defined by Miller 1972. The top shows a graphical representation of the *mxiC-lacZ* fusion in HN580; bent arrows indicate the trancriptional start sites of the *ipa* and *mxi-spa* operons and the direction of transcription; pINV, virulence plasmid.

with iron-saturated bLf (lane 5). These results suggest that activation of the TTS apparatus of enteroinvasive bacteria might be regulated in response to low-iron availability.

However, an alternative explanation is that small dialyzable peptides were being released from bLf, which is known to be more susceptible to proteolytic cleavage than the iron-saturated form (Kuwata *et al.* 1998), and that these peptides are responsible for the activation of the TTS system. Among these dialyzable small peptides, lactoferricin is known to retain the ability of Lf to bind bacterial lipopolysacarides (LPS), and thus to induce changes in bacterial cell surfaces (Kuwata *et al.* 1998). In order to ascertain if the release of Ipa proteins was due to bLf-related small peptides, which might have been generated and diffused across the dialysis membrane during the 1 h incubation step at 37 °C, bLf was dissolved in iron-free PBS (12 mg/ml), enclosed in a dialysis bag, and incubated 1 h at 37 °C (i.e. for the same time-period used to incubate bacteria and bLf) in 6 ml of iron-free PBS. After removal of the dialysis bag, the PBS solution was directly used to suspend exponentially growing HN280; thereafter bacteria were incubated for 1 h at 37 °C. Secreted proteins were TCA-precipitated and analyzed by Western blot. Figure 2 shows that bLf-derived dialyzable products are able to induce release of Ipa proteins at an higher degree than upon incubation of HN280 with iron-free PBS (lane 2 vs lane 3, respectively). However, diffusible bLf-derived products did not entirely account for the observed effect, since the amount of IpaB and C released (lane 2) accounted for only 30% and 24%, respectively, of the IpaB and C proteins recovered in the supernatant of HN280 incubated with bLf (lane 1). This finding indicates that, in the presence of dialysis membrane-separated bLf, activation of the TTS system might occur. Even though we do not have data regarding either the stability of these dialyzable components or the ability to bind iron, taken together these results

seem to indicate that bLf might induces release of Ipa proteins in part by the effect of bLf-generated dialyzable products, which most probably bind to bacterial surfaces, and in part by activating the TTS system of HN280, probably by the ability of bLf to withhold iron. On this basis, a low-iron availability might be a signal perceived by enteroinvasive bacteria in order to modulate secretion of virulence proteins.

Secretion of Ipa proteins is not due to alteration of bacterial outer membrane

To assess whether secretion of Ipa proteins is due to damage of the outer membrane of HN280 induced by the release of small peptides or by the ability of bLf to bind Ca^{2+} and/or Mg^{2+} ions (Rossi *et al.* 2002), the preparations of secreted proteins shown in Figure 1 were subjected to Western blot analysis to determine the release of apyrase, a periplasmic protein produced by HN280 (Berlutti *et al.* 1998) chosen as reporter of the outer membrane integrity. Apyrase was not detected in any of the samples assayed (Figure 3). This result clearly rules out that the bLf-induced secretion of IpaB and C proteins was due to severe alterations of the outer membrane of HN280.

Induction of secretion does not induce increased transcription of TTS genes

Congo red is known to induce activation of secretion by the TTS system of enteroinvasive bacteria without enhancing transcription of the *ipa* and of the *mxi* operons (Demers *et al.* 1998). To determine whether the observed induction of secretion enhances transcription of TTS genes, we determined the expression of the mxi operon by measuring the β-galactosidase activity of HN580, a derivative of EIEC strain HN280 carrying a pINV-encoded *mxiC-lacZ* transcriptional fusion. Bacteria were incubated with bLf under the same experimental conditions described above. Controls were bacteria incubated with Congo red, iron-free PBS, iron citrate, and iron-saturated bLf. HN580 produced β-galactosidase at approximately the same extent (in the range of 180 Miller units) under all experimental conditions (Figure 4). These results indicate that, as for Congo red, induction of secretion is not related to increased transcription of the *mxi* operon.

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