BACTERIAL PATHOGENICITY

Modulation of *actA* gene expression in *Listeria monocytogenes* by iron

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This study analysed the invasiveness of *Listeria monocytogenes* into enterocyte-like Caco-2 cells in which iron depletion was achieved by picolinic acid treatment. Both entry and intracellular multiplication varied depending on the endogenous iron content of bacterial and eukaryotic cells. The behaviour within enterocytes was correlated with a 10-fold increased transcription of the *actA* gene observed in bacterial cells grown under conditions of iron stress.

Introduction

Listeria monocytogenes, a gram-positive bacillus responsible for food-borne infections in animals and man [1], is a facultative intracellular parasite capable of entry, multiplication and movement within target cells [2]. Most of the genes involved in *L. monocytogenes* virulence are clustered on the chromosome [3] and controlled by the transcriptional activator PrfA [4, 5]. Expression is controlled by environmental factors [6]; physicochemical signals, including ferric ions, influence virulence gene expression [7–11].

Iron deficiency significantly increases listeriolysin O production [8–10] and is associated with reduced invasiveness of *L. monocytogenes* cells and with decreased expression of the *inlAB* virulence genes which are required for bacterial entry into host cells [10]. Recent data have shown that transcription of the *prfA* gene is also increased in iron-starved bacteria [12].

However, modulation of the expression of *L. monocytogenes* virulence genes in an intracellular environment has been less extensively studied. It has been suggested that an enhanced transcription of *plcA*, encoding for a phosphatidyl inositol-specific phospholipase C, and several other genes occurs during growth within macrophages [13] and that certain cytosolic signals may stimulate the synthesis of ActA [14, 15], a surface-associated protein of 639 amino acids, which is an essential determinant of *L. monocytogenes* pathogenicity. During intracellular growth, surface-bound ActA supports the formation of tail-like F-actin

structures, allowing the movement of bacteria into the host cytosol and their spread from cell to cell [16–18].

The objective of the present study was to evaluate the influence of iron availability on *actA* gene expression by analysing the invasiveness of *L. monocytogenes* and the transcription of the *actA* gene under different conditions of iron availability.

Materials and methods

Bacterial strain

The clinical isolate of *L. monocytogenes* LM1 used in this study was a haemolytic, wild-type strain capable of invading and multiplying in Caco-2 and HeLa cells, similar to *L. monocytogenes* ATCC 7644 [19]. Iron limitation ($\text{Fe}^{3+} < 1 \mu\text{M}$) was achieved by culturing bacteria in low iron medium (LIM) Brain Heart Infusion (BHI; Merck) plus Chelex-100 [10] and with $100 \mu\text{M}$ ethylenediamine-di-o-hydroxyphenylacetic acid (EDDHA). Iron excess conditions were obtained by culturing bacteria in LIM supplemented with $100 \mu\text{M}$ ferric citrate. Previous reports have shown that the growth rate of *L. monocytogenes* in BHI correlates with the level of assimilable iron [10, 20]. After 18 h, maximal growth rates of *L. monocytogenes* (OD_{600} 1.6) were achieved in iron-rich medium (LIM plus $100 \mu\text{M}$ ferric citrate) as compared with growth rates in LIM (OD_{600} 0.9) [10].

Invasion assays

Caco-2 cells were grown in 24-well plates for 48 h in Eagle's minimum essential medium (MEM). Then the medium was replaced by MEM containing 2 mM picolinic acid and fetal calf serum treated with

Received 8 June 1999; revised version accepted 4 Dec. 1999.

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Chelex-100 10% to remove intracellular iron. After incubation for a further 48 h, iron content was lowered from 125 pM to 18 pM/ 10^6 Caco-2 cells, as determined by measurement in cytosolic fractions with an atomic adsorption spectrophotometer with a minor modification of the procedure described by Mikogami *et al.* [21]. Invasion assays of iron-depleted or iron-loaded Caco-2 cells were performed as described previously [19], at an infective dose of *c.* 100 log-phase bacteria/cell. After incubation for 1 h at 37°C, infected monolayers were washed five times in MEM, and 1 ml of fresh medium containing gentamicin 5 µg/ml was added to each well. After incubation for a further 1 h at 37°C, the cells were lysed by the addition of cold Triton X-100 0.1% and plated to determine the number of viable intracellular bacteria. The intracellular replication of *L. monocytogenes* in iron-depleted or iron-loaded Caco-2 cells was determined according to the method of Conte *et al.* [19].

Transcriptional analysis of *actA* gene

Total cellular RNA was extracted [10, 22] from log-phase cells of *L. monocytogenes* in LIM and LIM supplemented with ferric ions. Samples of 5 µg of total RNA were mixed with three volumes of denaturing solution (0.4 M 3-(N-morpholino)-propanesulphonic acid, 10 mM sodium acetate, 1 mM EDTA; formamide 50%; 2.2 M formaldehyde), incubated at 65°C for 5 min, and placed on ice. After addition of 600 µl of 10 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) samples were vacuum-blotted with a BioRad slot-blot apparatus on to HybondTM-C extra nitrocellulose membranes (Amersham) previously soaked in the same solution. RNA was cross-linked to the membrane by baking at 80°C for 2 h. The total

amount of RNA present in each slot was checked by probing the membranes with a 16S rDNA probe from *L. monocytogenes* LM1. Membrane hybridisation and washings were performed as described previously [10]. Transcription of *actA* was monitored by an intragenic probe made by PCR in which two oligonucleotides annealing to bp 907–925 of the non-coding strand and to bp 1285–1306 of the coding strand of the published sequence were used as primers [23]. Hybridisation was monitored by a blot analyser (Betascope model 603, Betagen, MA, USA).

Results and discussion

The invasion of Caco-2 cells by *L. monocytogenes* varied depending on the endogenous iron in both bacteria and cells (Fig. 1a and b). At 3 h after the addition of bacteria to untreated Caco-2, the number of viable intracellular bacteria grown in iron-limited medium was significantly less than for bacteria grown in iron excess conditions ($p < 0.05$) (Fig. 1a). Iron availability in Caco-2 cells also affected bacterial invasion during the first 3 h of incubation; viable intracellular bacterial cells decreased from 5.25 to 4.6 log₁₀cfu/ml for iron-limited bacteria and from 4.6 to 4.3 log₁₀cfu/ml for iron-rich bacteria (Fig. 1a and b). Further incubation in iron-loaded Caco-2 cells resulted in a rapid increase in the number of intracellular iron-limited bacteria to numbers similar to those seen with iron-rich bacteria (Fig. 1a). Similarly, further incubation in iron-depleted Caco-2 cells resulted in increased numbers of intracellular bacteria whether grown under iron-limited or iron-rich conditions (Fig. 1b). After incubation for 7 h, the numbers of intracellular bacteria were very similar whatever the iron availability conditions.

Fig. 1. Growth curves of iron-rich (●-●) and iron-starved (■-■) *L. monocytogenes* in (a) untreated Caco-2 cell monolayers and (b) picolinic acid-treated cell monolayers. Results are presented as the mean (SD) log₁₀ cfu of four independent experiments.

Microscopy of intracellular bacteria stained with tetramethyl rhodamine isothiocyanate-conjugated phalloidin showed that the ability to form tail-like structures (dependent upon the polymerisation of host actin by ActA) was more efficient when bacteria and host cells were iron-depleted (data not shown).

To investigate whether iron affects the invasive phenotype of *L. monocytogenes* LM1 by regulating the expression of the *actA* gene at the transcriptional level, slot-blot analyses were performed with total cell RNA extracted from *L. monocytogenes* cells grown in LIM or LIM supplemented with ferric ions. Hybridisation with the *actA* probe of RNA extracted from iron-deficient bacteria resulted in a 10.6-fold increase in hybridisation signal as compared with RNA from iron-rich bacteria (Fig. 2). However, a growth condition of iron excess was not sufficient to repress totally the transcription of the *actA* gene.

The role of iron as a regulator of virulence gene expression in *L. monocytogenes* is well established [8, 10, 12]. Within host cells, two genes, *hly* and *actA*, which encode for listeriolysin and ActA, respectively, are expressed preferentially during bacterial intracellular growth and are required to establish intracellular infection [15]. The present study has provided further insight into the role of iron as a regulator of virulence gene expression. It demonstrated that the transcription of the *actA* gene in iron-stressed bacteria is 10-fold higher than in iron-rich bacteria, indicating that the *actA* gene is up-regulated under iron-stress conditions, similarly to the *hly* and *prfA* genes located in the same virulence cluster [10, 12]. Consequently, in iron-depleted cell monolayers the increased bacterial growth rate observed may be related not only to enhanced listeriolysin O synthesis which occurs in iron-stressed bacteria [8, 10], but also to increased *actA* expression, although other effects of iron depletion on mammalian cell physiology cannot be ruled out.

This work was supported by Cofin 9806260379-004 and MURST grants.

Fig. 2. Transcriptional analysis of the *actA* gene of *L. monocytogenes* LM1 grown under iron-poor ($\text{Fe}^{3+} < 1 \mu\text{M}$) and iron-rich conditions ($100 \mu\text{M Fe}^{3+}$).

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