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Original Article

Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*

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Abstract

The foodborne pathogen *Listeria monocytogenes* is the causative agent of the invasive disease listeriosis. Infection by *L. monocytogenes* involves bacterial crossing of the intestinal barrier and intracellular replication in a variety of host cells. The PrfA protein is the master regulator of virulence factors required for bacterial entry, intracellular replication and cell-to-cell spread. PrfA-dependent activation of virulence genes occurs primarily in the blood and during intracellular infection. In contrast, PrfA does not play a significant role in regulation of virulence gene expression in the intestinal environment. In the gastrointestinal phase of infection, the bacterium encounters a variety of antimicrobial agents, including medium- and long-chain free fatty acids that are commonly found in our diet and as active components of bile. Here we show that subinhibitory concentrations of specific antimicrobial free fatty acids act to downregulate transcription of PrfA-activated virulence genes. Interestingly, the inhibitory effect is also evident in cells encoding a constitutively active variant of PrfA. Collectively, our data suggest that antimicrobial medium- and long-chain free fatty acids may act as signals to prevent PrfA-mediated activation of virulence genes in environments where PrfA activation is not required, such as in food and the gastrointestinal tract.

Keywords: *Listeria monocytogenes*; PrfA; Transcription regulation; Antimicrobial free fatty acids

1. Introduction

Listeria monocytogenes is a Gram-positive bacterial pathogen that thrives in various environments, ranging from soil and foods to different types of hosts [1]. The bacterium enters the gastrointestinal tract of the host via ingestion of contaminated foods. In susceptible individuals, *L. monocytogenes* may cross the intestinal barrier, the placental barrier and the blood–brain barrier, causing life-threatening diseases such as bacteremia and meningitis [1]. During infection, the bacterium gains access to the cytoplasm of host cells, multiplies within the intracellular niche and spreads from cell to cell through host actin polymerization [1,2]. The transcription regulator PrfA controls the expression of key virulence factors that are necessary for the intercellular lifestyle of *L. monocytogenes* [2,3]. To activate transcription, the PrfA protein binds to a specific DNA sequence, the PrfA box, located in the promoter regions of virulence genes belonging to the core PrfA regulon [3]. PrfA-regulated virulence genes encode proteins essential for bacterial entry into non-professional phagocytic cells (internalins InlA and InlB), escape from host cell vacuoles (hemolysin LLO, phospholipases PlcA and PlcB, metalloprotease Mpl), replication in the host cytosol (sugar phosphate transporter Hpt) and actin polymerization and cell-to-cell movement (surface protein ActA, internalin InlC) [2–4]. Furthermore, transcription of *prfA* is positively autoregulated by the PrfA protein itself [3,4]. At the post-transcriptional

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level, PrfA is regulated by an RNA thermosensor leading to increased production of PrfA at body temperature [5]. The activity of the PrfA protein is usually very low in the external environment but, upon infection of mammalian cells, PrfA undergoes a conformational switch from an "inactive" to its "active" form leading to a strong induction of PrfA-regulated virulence genes [2–4]. Bacterial and host-derived glutathione was recently described as a signaling molecule facilitating PrfA activation in the intracellular environment [6–8]. Furthermore, a small peptide pheromone (PplA) produced by *L. monocytogenes* may also facilitate PrfA activation [9]. Several mutant variants of PrfA locked in the "active" conformation have been identified [2–4,10]. *L. monocytogenes* carrying such a *prfA** mutation constitutively expresses PrfA-regulated virulence genes in vitro and bypasses the need for bacterial and host-derived signaling molecules for PrfA activation [2–4,6,9–11].

The alternative stress sigma factor, Sigma B (SigB), controls expression of genes that are important for growth and survival of *L. monocytogenes* under general stress conditions, such as low pH and osmotic stress [12]. Furthermore, substantial evidence suggests a role for SigB in *L. monocytogenes* virulence. SigB is known to modulate PrfA-mediated virulence factor expression; firstly, one of the promoters upstream from *prfA* depends on SigB [13,14]; secondly, the PrfA-regulated internalin coding genes are preceded not only by a PrfA box, but also a SigB promoter [15]. SigB and PrfA most likely act to promote growth and survival of *L. monocytogenes* at different stages of infection. Whereas PrfA is important for transcription activation of virulence genes during replication in the blood and the intracellular environment, SigB primarily plays a role in controlling gene expression during adaptation to the intestinal environment [16,17].

Free fatty acids (FFAs) have long been known to exhibit potent antimicrobial activity against bacteria [18]. The primary target of FFAs is the bacterial cell membrane, where they disrupt essential biological processes that occur within the membrane, but the exact mechanism underlying the antimicrobial activity of FFAs is not well understood [18]. As a foodborne pathogen, *L. monocytogenes* encounters a variety of both unsaturated and saturated medium- and long-chain FFA in foods and in the gastrointestinal tract of the host. Besides being present in our diet, specific FFAs, such as the unsaturated long-chain FFAs linoleic acid (C18:2), oleic acid (C18:1) and arachidonic acid (C20:4), are also known as active constituents of bile [19]. Interestingly, at subinhibitory concentrations, unsaturated long-chain FFAs have been shown to inhibit the expression of virulence factors in the intestinal pathogens *Vibrio cholerae* [19] and *Salmonella enterica* [20,21]. Studies of FFAs present in milk revealed that the saturated medium-chain FFA lauric acid (C12:0), as well as unsaturated long-chain FFAs linoleic acid (C18:2) and ɣ-linolenic acid (C18:3), exhibit a strong bactericidal effect on *L. monocytogenes* [22,23]. Intriguingly, the invasive efficiency of *L. monocytogenes* in Caco-2 enterocyte-like cells, which is mediated by the PrfA- and SigB-regulated internalin InlA, was strongly decreased in the presence of subinhibitory concentrations of these FFAs [22]. The mechanism underlying the inhibitory effect of medium- and long-chain FFAs on invasion was not elucidated.

In this study, we sought to examine the effect of medium- and long-chain FFAs on virulence gene expression in *L. monocytogenes*. Interestingly, we found that subinhibitory concentrations of antimicrobial FFAs act to downregulate the expression of PrfA-activated genes. Importantly, the inhibitory effect was also seen in the strain EGD-*prfA**, expressing a constitutively active variant of PrfA. Our findings suggest that selected medium- and long-chain FFAs may act as signaling molecules to prevent PrfA-dependent activation of virulence genes in *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

For this study, the wild type *L. monocytogenes* EGD serotype 1/2a and its isogenic mutant derivatives Δ*prfA* [24] and Δ*sigB* [25] were used. The strain EGD-*prfA**, expressing the mid-level constitutively active PrfA mutant derivative PrfA-G155S [10,11], and the strain EGD-*prfA**-FLAG, expressing N-terminal 3 × FLAG-tagged PrfA-G155S, were constructed by using the temperature-sensitive shuttle vector pAUL-A [26]. For construction of strain EGD-*prfA**, primers G155S-A, -B, -C and -D were used for a 2-step PCR amplification of a fragment containing the desired *prfA** substitution (for primers, see Table S1). For N-terminal FLAG-tagging of *prfA**, the primers PrfA-3 × FLAG-1, -2, -3 and -4 (Table S1) were employed for a 2-step PCR amplification of a fragment containing the 3 × FLAG-tag coding sequence inserted between codon 2 and 3 of *prfA**. The DNA fragments were inserted into pAUL-A and the resulting plasmids were introduced into *L. monocytogenes* as described previously [27]. Homologous recombination was achieved as described in [28]. The plasmid p*hly-lacZ*, containing a transcriptional fusion between the *hly* promoter and the *lacZ* gene, was constructed in a previous study [29]. The plasmid p*lhrA36-lacZ*, containing a transcriptional fusion between the core promoter of the *lhrA* gene and *lacZ*, was constructed previously [30]. *L. monocytogenes* was routinely grown at 37 °C with aeration in brain heart infusion broth (BHI, Oxoid). When appropriate, cultures were supplemented with kanamycin (50 μg/mL) or erythromycin (5 μg/mL). For cloning in pAUL-A, *Escherichia coli* TOP10 (Invitrogen) was grown at 37 °C in Luria–Bertani broth supplemented with 150 μg/mL erythromycin.

2.2. Growth experiments in the presence of FFAs

Overnight (ON) cultures in BHI were diluted to optical density at wavelength 600 (OD_{600}) = 0.0002, and 5 mL was transferred to glass tubes and supplemented with increasing concentrations of FFAs. The following FFAs (purity ≥ 99%) were used: eicosapentaenoic acid (EPA; C20:5; Sigma–Aldrich); ɣ-linolenic acid (GLA; C18:3; Sigma–Aldrich); palmitoleic acid (PA; C16:1; Sigma–Aldrich); lauric acid