ELSEVIER

Institut Pasteur

Research in Microbiology xx (2017) 1–11

www.elsevier.com/locate/resmic

Original Article

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

Eva Maria Sternkopf Lillebæk, Stine Lambert Nielsen, Rikke Scheel Thomasen, Nils J. Færgeman, Birgitte H. Kallipolitis*

Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark

Received 19 December 2016; accepted 13 March 2017
Available online ■ ■ ■

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

* Corresponding author.
E-mail addresses: emsl@bmb.sdu.dk (E.M. Sternkopf Lillebæk), sln1990@hotmail.com (S. Lambert Nielsen), rthom13@student.sdu.dk (R. Scheel Thomasen), nils.f@bmb.sdu.dk (N.J. Færgeman), bhk@bmb.sdu.dk (B.H. Kallipolitis).

http://dx.doi.org/10.1016/j.resmic.2017.03.002
0923-2508/

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

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002

(LA; C12:0; Nu-chek prep); palmitic acid (PAL; C16:0; Nu-chek prep); and stearic acid (SA; C18:0; Nu-chek prep). The FFAs were dissolved in 96% ethanol. Ethanol control samples were included in all experiments where cultures were supplemented with FFAs. The ethanol concentration in the control sample was equal to that in the sample stressed with the highest concentration of FFA. After 20 h, growth was measured by determining the colony forming units (CFU) and/or OD_{600} values. Furthermore, the CFU of the diluted ON cultures was determined.

2.3. Purification of RNA and northern blot analysis

L. monocytogenes strains were grown in BHI medium until $OD_{600} = 0.35$. Then, the cultures were split and subinhibitory concentrations of FFAs were added to the following final concentrations: 4 μg/mL EPA, 4 μg/mL GLA and 2 μg/mL PA. As controls, cultures were left untreated or ethanol was added corresponding to the final concentration present in the FFA-treated culture containing the highest amount of ethanol. After 1 h of incubation, 10 mL samples were drawn, snap-cooled in liquid nitrogen and centrifuged at 11,000 relative centrifugal force (rcf) for 3 min at 4 °C. Total RNA was purified as described previously [31]. Northern blot analysis was performed as previously described with few modifications [32]. Briefly, 20 μg of total RNA were loaded on a formaldehyde agarose gel next to 7.5 μL of RNA marker (Dyna-Marker Prestain Marker for RNA High, BioDynamics). Samples were separated for 3 h prior to capillary blotting on a nylon Zeta-Probe GT membrane (Bio-Rad). The membrane was hybridized with ^{32}P-labeled DNA probes (Amersham Megaprime DNA labeling system, GE Healthcare). The forward and reverse primers used for PCR amplification of the DNA probes are listed in Table S1.

2.4. Preparation of protein samples and western blot analysis

L. monocytogenes ON cultures grown in BHI were diluted to $OD_{600} = 0.02$ and grown until $OD_{600} = 0.3$ where the cultures were split. FFAs were added to the following final concentrations: 4 μg/mL EPA; 4 μg/mL GLA; 2 μg/mL PA; 10 μg/mL LA; 3 μg/mL or 100 μg/mL SA; 2 μg/mL or 150 μg/mL PAL. As controls, cultures were left untreated or ethanol was added corresponding to the final concentrations present in two of the FFA-treated cultures. After 1 and 3 h of FFA exposure, 4 mL samples were centrifuged at 3000 rcf for 7 min. Supernatant was removed and pellets stored at −80 °C until further use. Cell pellets were thawed on ice and re-suspended in lysis buffer (0.1 M NaCl, 50 mM Tris−HCl pH 7.5, 10 mM EDTA, 0.5% SDS). Cells were lysed in a FastPrep FPI20 instrument (Thermo Electron Corporation) at speed 6.0 for 2 cycles of 40 s with intermediate cooling. Lysed samples were heated at 95 °C for 5 min and centrifuged at 1200 rcf for 5 min. Supernatants were transferred to new tubes and centrifuged for 10 min at 20,000 rcf. The resulting protein samples were stored at −20 °C until use. Western blot analysis, antibody detection and amido black staining was performed as described earlier [33] with the following modification: blotting was performed at 120 mA for 45 min. For detection of FLAG-tagged PrfA, monoclonal α-FLAG antibody M2 (Sigma) was diluted 1:20,000. Monoclonal primary antibodies α-Act and α-LLO (Abnova) were diluted 1:3000. Mouse HRP-conjugated secondary antibody (DakoCytomation) was diluted 1:3000. All blots were developed using Luminata Forte Western HRP substrate (Millipore) and detected with the ChemiDoc XRS Imaging system (Biorad).

2.5. β-galactosidase assays

On cultures of *L. monocytogenes* EGD wild type, Δ*prfA* or *prfA** strains containing plasmids p*hly-lac*, p*lhrA36-lacZ* or the empty vector pTCV-lac [34] were diluted to $OD_{600} = 0.02$. At $OD_{600} = 0.3$, the cultures were split and FFAs were added to the following final concentrations: 3 μg/mL EPA; 3 μg/mL GLA; 2 μg/mL PA; 10 μg/mL LA; 3 μg/mL or 100 μg/mL SA; 2 μg/mL or 150 μg/mL PAL. As controls, cultures were left untreated or ethanol was added corresponding to two of the final concentrations present in the FFA-treated cultures. β-galactosidase assays were performed as previously described [28].

3. Results

3.1. Role of PrfA and SigB in the response of L. monocytogenes to unsaturated long-chain FFAs

A possible involvement of the stress sigma factor SigB and the key virulence regulator PrfA in the response to unsaturated long-chain fatty acids was assessed by subjecting *L. monocytogenes* EGD wild type, Δ*sigB* and Δ*prfA* strains to growth in BHI medium containing various concentrations of palmitoleic acid (PA; C16:1) and ɣ-linolenic acid (GLA; C18:3). As the FFAs were diluted in 96% ethanol, control samples were included containing the highest concentration of ethanol added to the cultures. After 20 h of growth, the optical density and CFU values of the cultures were recorded. In line with previous observations, GLA and PA exert an antimicrobial effect on *L. monocytogenes* in BHI medium (Fig. 1) [18,22]. At 20 μg/mL of GLA and 10 μg/mL of PA, growth of the wild type and Δ*sigB* strains was restricted. In contrast to this, the Δ*prfA* mutant was capable of growing in the presence of GLA and PA (Fig. 1). This observation suggests that PrfA acts to increase the sensitivity of *L. monocytogenes* to the unsaturated long-chain FFAs GLA and PA in BHI medium.

To further explore the role of PrfA in the response to various FFAs, growth of *L. monocytogenes* wild type, Δ*prfA* and *prfA** strains was recorded in the presence of increasing concentrations of GLA, PA, the unsaturated long-chain FFA eicosapentaenoic acid (EPA; C20:5), the saturated medium-chain FFA lauric acid (LA; C12:0), and the saturated long-chain FFAs stearic acid (SA; C18:0) and palmitic acid (SAL; C16:0) (Fig. 2). For the saturated long-chain fatty acids SA and PAL, no growth inhibition was observed for any of the

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002

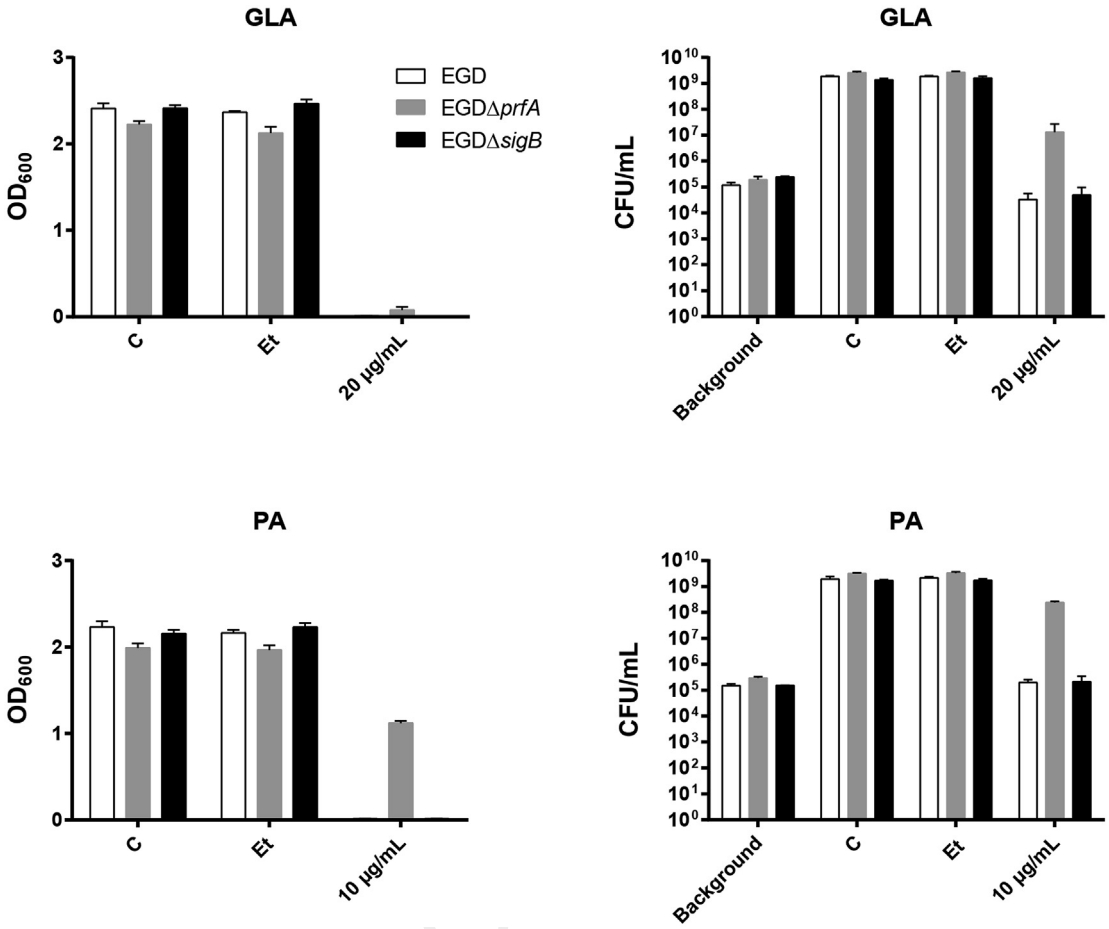

Fig. 1. Growth of *L. monocytogenes* EGD wild type (white bars), EGDΔ*prfA* (gray bars) and EGDΔ*sigB* (black bars) in the presence of 20 µg/mL GLA or 10 µg/mL PA. After 20 h of growth, OD_{600} (left) and CFU values (right) were determined. As controls, growth was measured for untreated cultures (C) and cultures treated with ethanol (Et) corresponding to the final concentrations in samples exposed to GLA (0.4% ethanol) or PA (0.1% ethanol). Furthermore, the CFU/mL at time 0 of the growth experiment was determined (Background). The average of three independent biological replicates is shown.

strains tested, supporting a previous report stating that these FFAs are not active against *L. monocytogenes* in BHI broth [22]. Like GLA and PA, the FFAs EPA and LA exerted an antimicrobial effect against the wild type strain (Fig. 2). With respect to PrfA, growth was recorded for the Δ*prfA* strain in the presence of GLA, PA, EPA and LA, at concentrations that were inhibitory to both the wild type strain and the mutant strain expressing the constitutively active PrfA* protein. Apparently, the presence of wild type PrfA or PrfA* renders *L. monocytogenes* more sensitive towards the medium- and long-chain FFAs exerting an antimicrobial activity against this pathogen. These results further substantiate a role for PrfA in the response of *L. monocytogenes* to antimicrobial FFA.

3.2. Exposure to subinhibitory concentrations of antimicrobial FFAs reduces the mRNA levels of PrfA-activated virulence genes

To assess the effects of antimicrobial FFAs on the expression of PrfA-regulated genes, we performed a northern blotting experiment. First, *L. monocytogenes* wild type, Δ*prfA* and *prfA** cultures were grown to early exponential phase in BHI medium. Then, the cultures were split and exposed to subinhibitory concentrations of EPA, PA or GLA. Samples corresponding to cells grown under non-stress conditions or exposed to ethanol were included as controls. Growth was recorded for all strains tested throughout the experiment (Fig. S1). One h after FFA exposure, cells were harvested for RNA extraction. The mRNA level of PrfA-dependent virulence genes was assessed by northern blot analysis using radiolabeled probes specific for virulence genes *prfA*, *plcA*, *plcB*, *hly*, *actA* and *inlA*. An overview of the promoters and expected transcript lengths of the PrfA-regulated virulence genes is shown in Fig. 3, and the result of the northern blot analysis is shown in Fig. 4. In BHI broth, PrfA-dependent activation of the virulence genes in the wild type strain is known to be relatively low. In line with this, faint bands corresponding to the expected transcripts were observed for the control samples of the wild type strain (EGD; lanes C and Et). Except for *inlA*, these bands were not observed in the control samples of the mutant strain lacking PrfA (EGDΔ*prfA*; lanes C and Et), supporting that PrfA is essential for the expression of most virulence genes. In the *prfA** mutant strain, all virulence genes tested were highly expressed in a PrfA-dependent

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002

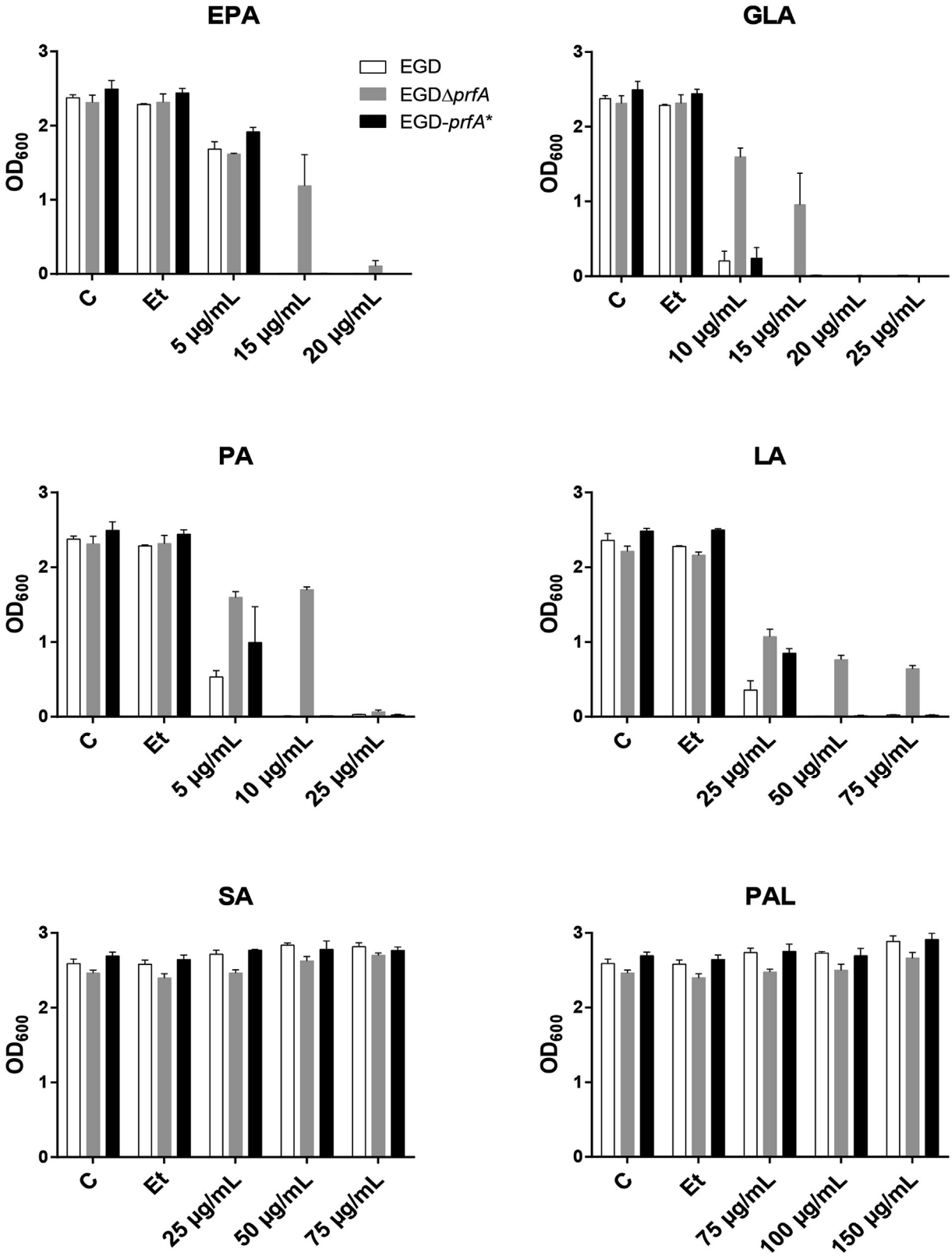

Fig. 2. Growth of *L. monocytogenes* EGD wild type (white bars), EGDΔ*prfA* (gray bars) and EGD-*prfA** (black bars) in the presence of increasing concentrations of EPA, GLA, PA, LA, SA and PAL. OD_{600} values were determined after 20 h of growth. As controls, OD_{600} was measured for untreated cultures (C) and cultures treated with 0.07–0.36% ethanol (Et) corresponding to the final concentrations in cultures exposed to FFAs. The average of three independent biological replicates is shown.

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002

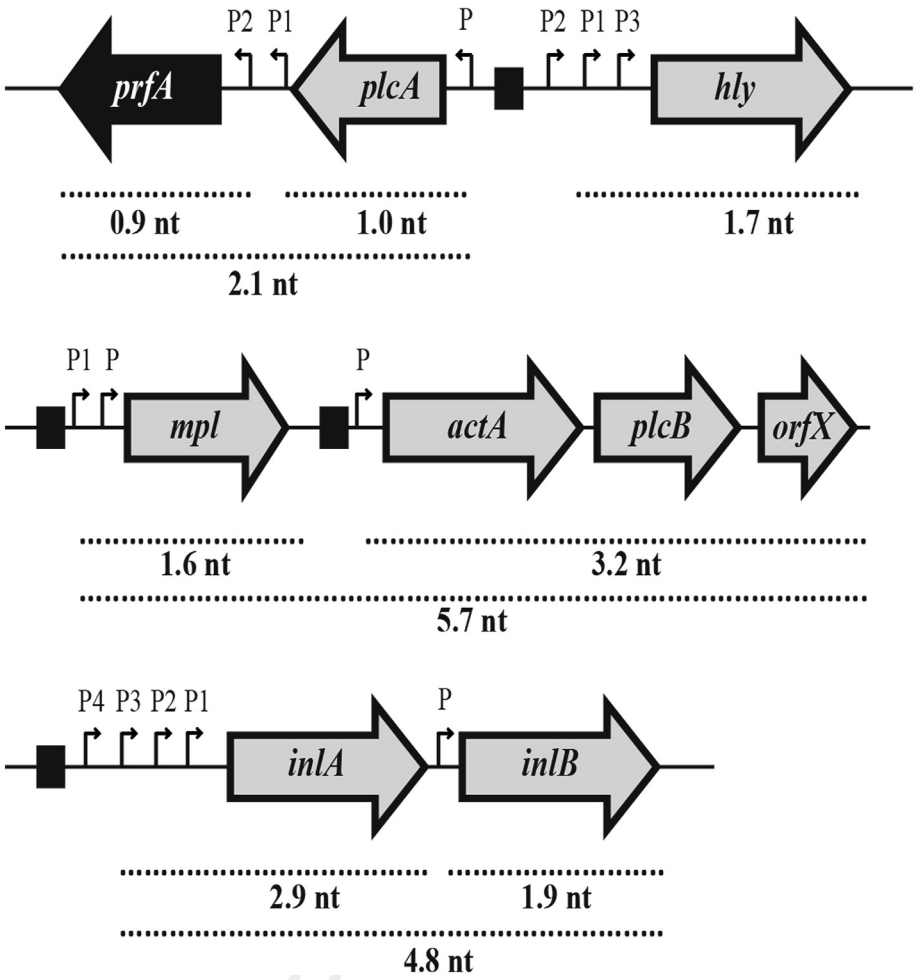

Fig. 3. Schematic overview of the PrfA-regulated genes and transcripts tested in this study. The PrfA binding sites are indicated by black boxes; the known promoters (P) and transcripts (dotted lines) are indicated. Below the dotted lines, the approximate lengths in of the transcripts are shown [3,42–44]. Modified from Ref. [3].

manner under the control conditions, clearly demonstrating the ability of the constitutively active PrfA* protein to activate transcription during growth in BHI medium (EGD-*prfA**; lanes BHI and Et). Intriguingly, upon exposure to subinhibitory concentrations of EPA, PA or GLA, PrfA-dependent virulence gene expression was repressed in the *prfA** mutant strain as well as the wild type strain. These results indicate that at subinhibitory levels, antimicrobial FFAs act as a signal to repress transcription of PrfA-dependent virulence genes in *L. monocytogenes*.

3.3. The cellular levels of virulence factors LLO and ActA are downregulated in response to subinhibitory concentrations of antimicrobial FFAs

To further investigate the effect of FFAs on PrfA-dependent virulence gene expression, the cellular protein levels of PrfA, ActA and LLO were assessed by western blot analysis. For these experiments, a strain encoding a FLAG-tagged PrfA* variant was constructed, which allows detection of PrfA* protein levels using α-FLAG antibodies (Fig. S2A and S2B). The FLAG-tag did not interfere with the ability of PrfA* to activate transcription of *hly*, and at the RNA level, the strains EGD-*prfA**-FLAG and EGD-*prfA** respond equally well to the presence of subinhibitory concentrations of EPA (Fig. S2C). Levels of the virulence factors ActA and LLO were assessed using antibodies raised against these proteins. The strain expressing FLAG-tagged PrfA* was grown in BHI medium to early exponential phase; the culture was split and cells were exposed to subinhibitory concentrations of EPA, GLA, PA or LA. Alternatively, cells were treated with comparable levels of SA or PAL, or the highest concentration of SA or PAL that could be added to the cultures without causing precipitations in the growth medium. As controls, cultures were left untreated or were exposed to two relevant concentrations of ethanol. Growth of the cultures was followed throughout the experiment (Fig. S3C). After 1 and 3 h of FFA exposure, cells were harvested and lysed and the cellular fractions were employed for western blot analysis (Fig. 5A and B; Fig. S3A and S3B). After 1 h of FFA exposure, only minor differences between the FFA treated and control samples were observed for LLO and ActA (Fig. 5A), whereas, after 3 h, their levels were clearly reduced in cells exposed to the antimicrobial FFAs EPA, GLA, PA and LA (Fig. 5B;

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002

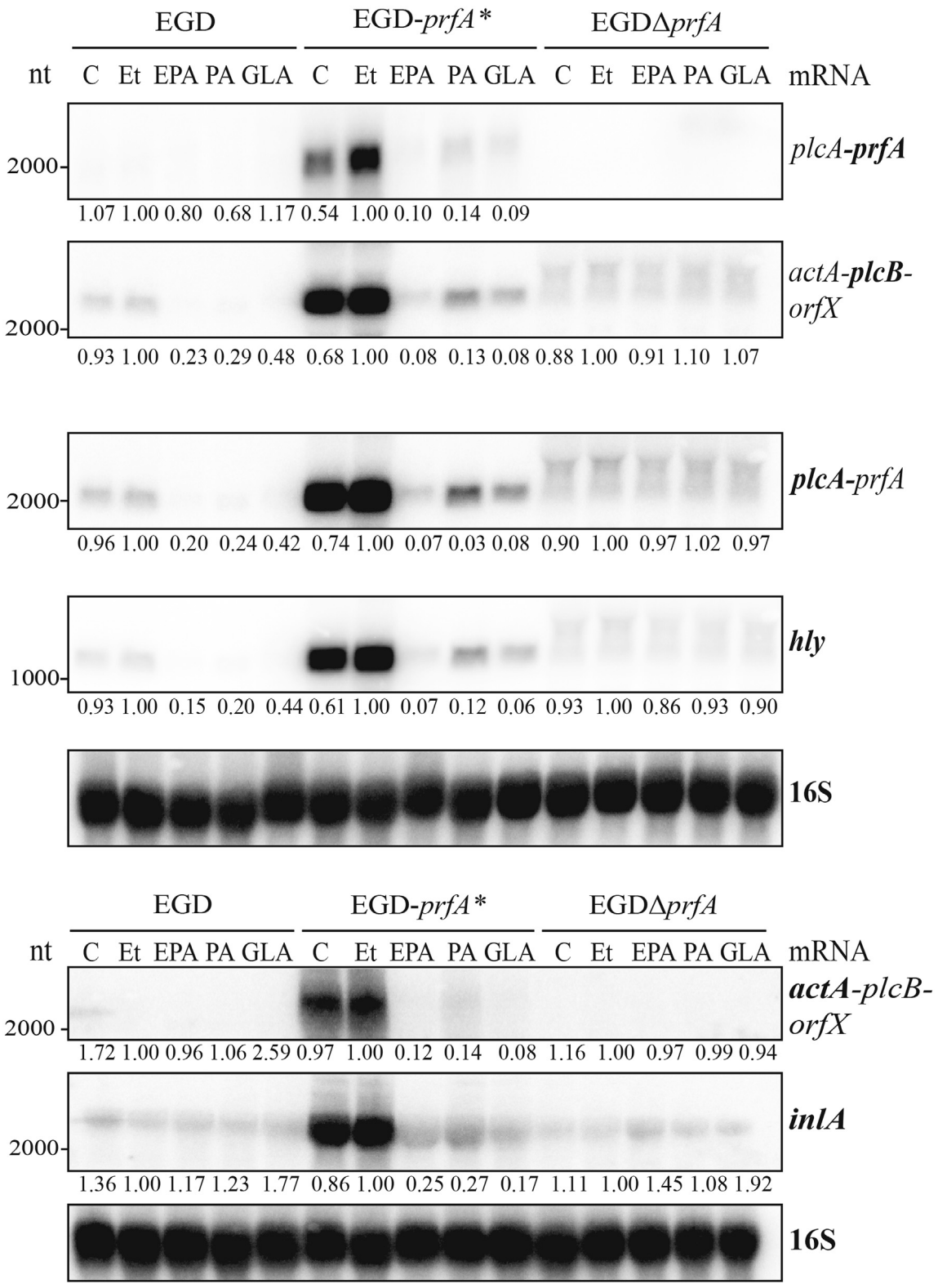

Fig. 4. Northern blot analysis of PrfA-regulated virulence genes in *L. monocytogenes* EGD wild type, EGD-*prfA** and EGDΔ*prfA*. Samples were taken from cultures exposed to subinhibitory concentrations of EPA, PA or GLA for 1 h as well as control samples from untreated cultures (C) or cultures treated with 0.08% ethanol (Et). The northern blots were probed for *prfA*, *plcB*, *plcA*, *hly*, *actA*, *inlA* and 16S (loading control). Relative levels of the transcripts (normalized to 16S) are shown below each lane. For comparison, the transcript lengths of the size marker are shown to the left.

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002

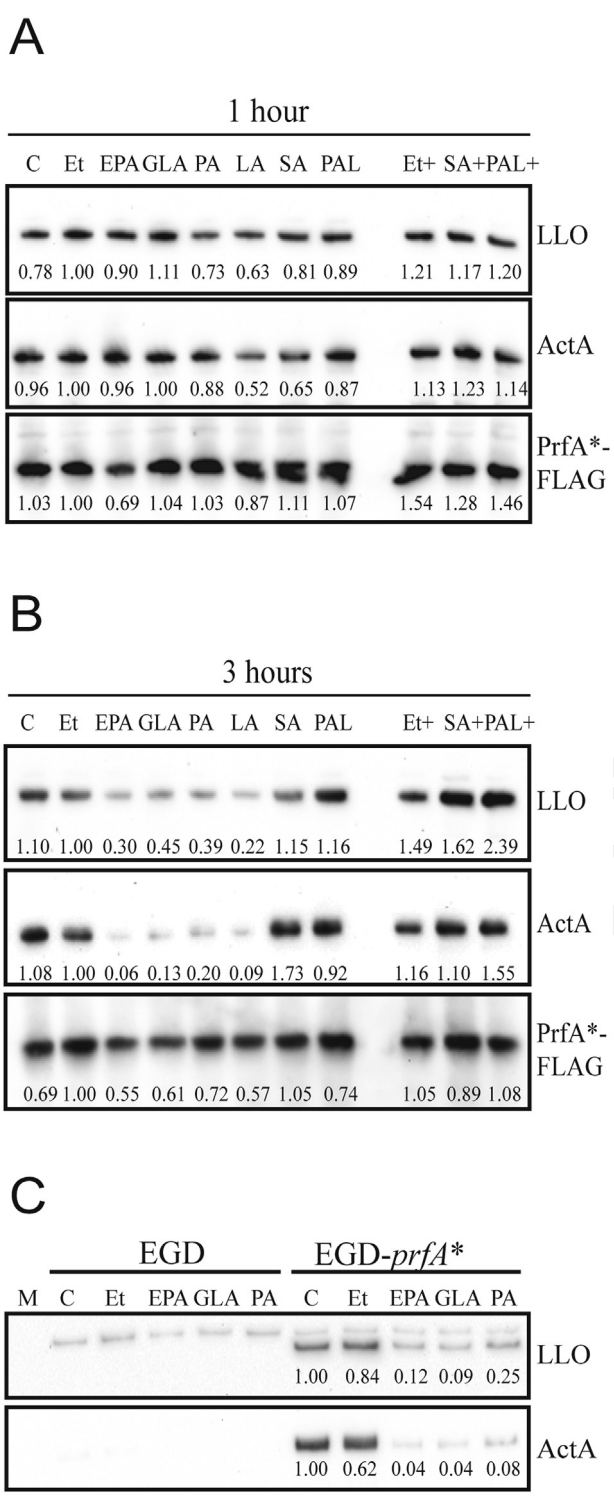

Fig. 5. Western blot analysis of LLO, ActA and PrfA*-FLAG. For the experiment presented in (**A**) and (**B**), samples were taken from *L. monocytogenes* EGD-*prfA**-FLAG exposed to subinhibitory concentrations of EPA, GLA, PA, LA or comparable levels of SA or PAL for 1 h (**A**) or 3 h (**B**), respectively. Alternatively, samples were taken from EGD-*prfA**-FLAG treated with the highest concentration possible of SA (SA+) or PAL (PAL+). As controls, samples were drawn from untreated cells (C) or cells exposed to 0.01% (Et) or 0.1% (Et+) of ethanol. For the experiment presented in (**C**), samples were taken from *L. monocytogenes* EGD wild type and EGD-*prfA** exposed to subinhibitory concentrations of EPA, GLA or PA for 3 h. As controls, cells were left untreated (C) or exposed to a final concentration of 0.01% ethanol (Et). LLO and ActA were detected using α-LLO and α-ActA antibodies, respectively, whereas PrfA*-FLAG was detected using α-FLAG antibody. As loading controls, all proteins on the membranes were stained with amino black (Fig. S3A, S3B and S4A, respectively). Relative levels of LLO, ActA and PrfA*-FLAG (normalized to control) are shown below the lanes.

compare lane Et with lanes EPA, GLA, PA and LA). Addition of the non-inhibitory FFAs SA and PAL did not affect the level of these virulence factors (Fig. 5B; compare lane Et with SA and PAL, and Et+ with SA+ and PAL+). As the expression of PrfA is autoregulated in a positive manner, we would expect to see a decrease in the level of PrfA*-FLAG protein in response to the antimicrobial FFAs. Indeed, a minor decrease in the level of FLAG-tagged PrfA* protein was detected after 3 h of FFA exposure, when comparing the samples treated with the antimicrobial FFAs, to the controls (C and Et) (Fig. 5B). However, after 1 h of FFA exposure, the levels of PrfA*-FLAG were comparable under all conditions tested (Fig. 5A).

To eliminate involvement of the FLAG-tag on the results obtained in the western blot analysis, the cellular levels of LLO and ActA was also examined in strains expressing wild type PrfA and PrfA*. In the wild type strain, the cellular levels of ActA and LLO were below the detection limit, whereas both proteins were readily detected in the EGD-*prfA** strain expressing constitutively active PrfA* protein (Fig. 5C; S4A and S4B). Notably, in the EGD-*prfA** strain, ActA and LLO levels were clearly reduced following 3 h of exposure to subinhibitory concentrations of EPA, GLA and PA (Fig. 5C).

Collectively, the western blot analyses demonstrate that subinhibitory concentrations of antimicrobial FFAs act to downregulate the cellular levels of the PrfA-regulated virulence factors LLO and ActA. Importantly, this effect is observed in strains expressing the constitutively active variant of PrfA, PrfA*.

3.4. PrfA-dependent transcription initiation is repressed by antimicrobial FFAs

The experiments performed so far suggest that FFA stress leads to a reduced expression of PrfA-dependent virulence genes. In order to test if antimicrobial FFAs prevent virulence gene expression at the level of transcription initiation, we tested the β-galactosidase activity of wild type EGD, EGDΔ*prfA* and EGD-*prfA** strains carrying the p*hly-lacZ* fusion plasmid. In this pTCV-lac-derived plasmid, transcription of the reporter gene *lacZ* is controlled by the PrfA-dependent promoter of the virulence gene *hly*. For the β-galactosidase assays, strains were grown in BHI medium to early exponential phase. Then, the cultures were split and cells were exposed to subinhibitory concentrations of EPA, GLA, PA or LA. Alternatively, the cells were exposed to comparable levels of the non-inhibitory FFAs SA or PAL, or the highest concentration possible to add without causing precipitations. As controls, cells were left untreated or were exposed to two relevant concentrations of ethanol. After 20 h of growth, cells were harvested and β-galactosidase activity was determined. For EGD-*prfA** containing the *hly-lacZ* fusion plasmid, the β-

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002

galactosidase activity was strongly reduced in cells exposed to the antimicrobial FFAs, relative to the controls (Fig. 6A; compare C or Et to EPA, GLA, PA and LA). In contrast, addition of the non-inhibitory FFAs SA and PAL did not repress the β-galactosidase activities (Fig. 6A; compare Et with SA and PAL; compare Et + with SA+ and PAL+). As expected, the β-galactosidase activities measured in wild type EGD and EGDΔ*prfA* containing the p*hly-lacZ* fusion plasmid were very low under all conditions tested, corresponding to the background levels expressed from the empty vector pTCV-lac (Fig. S5A and S5B). To determine if the observed effect was specific to the PrfA-regulated promoter of *hly*, the β-galactosidase activity in EGD wild type, EGDΔ*prfA* and EGD-*prfA** containing the control plasmid p*lhrA36-lacZ* was assessed. In this plasmid, the PrfA-independent core promoter of *lhrA*, named *lhrA36*, was fused to *lacZ* in pTCV-lac [30]. For all three strains containing the *lhrA36-lacZ* fusion plasmid, comparable levels of β-galactosidase activities were measured under FFA treated and non-treated conditions (Fig. 6B). Collectively, the results of the β-galactosidase experiments demonstrate that, at subinhibitory levels, antimicrobial FFAs act to prevent PrfA-dependent activation of transcription from the *hly* promoter.

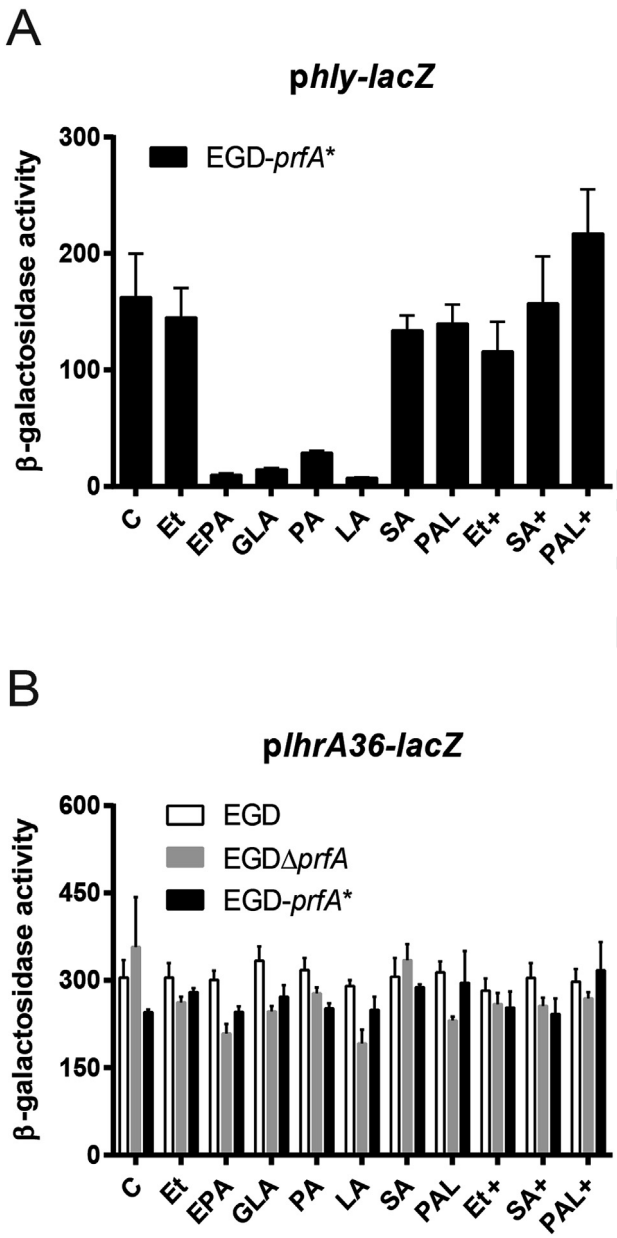

Fig. 6. β-galactosidase assay of *hly-lacZ* and *lhrA36-lacZ* expression in response to FFAs. Samples were taken from *L. monocytogenes* EGD-*prfA** containing p*hly-lacZ* (**A**) or *L. monocytogenes* EGD wild type, EGDΔ*prfA* and EGD-*prfA** containing p*lhrA36-lacZ* (**B**). Cells were exposed to subinhibitory concentrations of EPA, GLA, PA, LA or comparable concentrations of SA or PAL. In addition, cells were treated with the highest concentration possible of SA or PAL (indicated as SA+ and PAL+, respectively). As controls, cells were left untreated (C) or treated with two relevant final concentrations of ethanol (Et: 0.08%; Et+: 0.96%). Results are the average of three biologically independent experiments, each carried out in technical duplicates.

4. Discussion

As a foodborne and facultative intracellular pathogen, *L. monocytogenes* senses and responds to a variety of signals from the environment to express its virulence genes at the appropriate locations in the infected host. SigB plays a major role in adaptation during the intestinal phase of infection, where it acts to upregulate a number of genes important for in vivo survival, including those encoding the invasion proteins InlA and InlB and the bile salt hydrolase Bsh [12,16,17]. After crossing the intestinal barrier, the major virulence regulator PrfA activates the expression of core virulence genes supporting the intracellular lifestyle of *L. monocytogenes* [2–4,17]. In this work, we show that selected antimicrobial medium- and long-chain FFAs commonly found in our diet and as an active component of bile, act to repress PrfA-dependent virulence gene expression. Importantly, these FFAs inhibit transcription activation of virulence genes in a strain expressing the constitutively active variant of PrfA, PrfA*. PrfA-dependent expression of virulence genes was strongly inhibited at the RNA level after 1 h of FFA exposure (Fig. 4), whereas at this same timepoint, the level of PrfA* protein itself was unaffected (Fig. 5). Since the PrfA* protein is clearly present during exposure to the medium- and long-chain FFAs, they most likely act by generating a signal that efficiently prevents the PrfA* protein from turning on virulence gene expression.

The virulence of bacteria may be influenced by FFAs through various mechanisms. Studies on branched-chain fatty acids (BCFAs) in *L. monocytogenes* suggested a link between the membrane fatty acid composition and virulence [35,36]. When grown in BHI medium at 37 °C, the membrane of *L. monocytogenes* contains very high levels of BCFAs. Interestingly, exposure of *L. monocytogenes* to fatty acid precursors, which leads to a decrease in the proportion of anteiso-BCFA in the membrane, had profound effects on the expression of *hly* and *inlA* [36]. It was hypothesized that a decrease in the content of anteiso-BCFAs in the membrane suppresses either the level or function of PrfA [36]. However, it still remains to be demonstrated if a signaling event

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002

affecting PrfA activity is taking place upon perturbations of the fatty acid composition of the membrane. Interestingly, studies in *V. cholerae* and *S. enterica* have demonstrated a direct inhibitory effect of unsaturated or saturated long-chain FFAs on the activity of major virulence transcription regulators in these intestinal pathogens. The transcription regulator ToxT in *V. cholerae* activates expression of the two major virulence factors cholera toxin (CT) and toxin-coregulated pilus (TCP). Antimicrobial unsaturated long-chain FFAs present in bile, such as oleic (C18:1), linoleic (C18:2) and arachidonic (C20:4) acids, repress ToxT-dependent activation of *ctx* and *tcp* genes, encoding CT and TCP, respectively [19,37,38]. In contrast, the non-inhibitory saturated long-chain FFAs palmitic (C16:0) and stearic acids (C18:0) had no significant effect on CT production [19]. It has been proposed that unsaturated long-chain FFAs act by preventing ToxT dimerization, or by preventing monomeric ToxT from binding to DNA [37–39]. Indeed, resolution of the ToxT crystal structure revealed that palmitoleic acid (C16:1) binds directly to ToxT [37]. Furthermore, palmitoleic acid was shown to reduce CT and TCP expression and prevent ToxT binding to DNA in vitro [37]. Likewise, the presence of oleic or linoleic acid decreases the DNA binding activity of ToxT in vitro [37,38]. In *S. enterica*, a screening of extracts from native plant species led to the identification of unsaturated long-chain FFAs that downregulate the activity of the PhoP/PhoQ two-component regulatory system orchestrating the expression of virulence phenotypes in this pathogen [21]. More specifically, the presence of unsaturated long-chain FFAs is detected by the sensor protein PhoQ, leading to an inhibition of PhoQ autokinase activity [21]. This leads to inactivation of the regulator PhoP, followed by a down-regulation of PhoP-activated genes. Most recently, saturated and unsaturated long-chain FFAs oleic (C18:1), myristic (C14:0) and palmitic (16:0) acid have been shown to reduce expression of the Salmonella pathogenicity island 1 (SPI 1) type III secretion system in *S. enterica*, primarily acting through the transcription activator HilD [20]. In this case, the authors demonstrated that degradation of long-chain FFAs is not required for this regulation and that oleic acid affects the DNA binding activity of HilD [20]. Thus, in *Vibrio* and *Salmonella* species, medium- and long-chain FFAs act as signaling molecules to downregulate virulence expression by a mechanism that involves direct binding of FFAs to key virulence regulators. Future studies in *L. monocytogenes* should focus on elucidating whether medium- and long-chain FFAs control the activity of PrfA by generating a signaling event via the membrane or by direct binding to the PrfA protein. Notably, of the six FFAs tested in the present study, four (EPA, GLA, PA and LA) displayed an antimicrobial effect on *L. monocytogenes* and were furthermore capable of inhibiting the activity of PrfA. In contrast, SA and PAL had no effect on bacterial growth and were unable to repress the activity of PrfA. This observation indicates a putative correlation between the antimicrobial activity of a specific FFA and its ability to repress PrfA-dependent virulence gene expression that should be explored further in future work.

The mutant strain lacking *prfA* displayed an increased tolerance to antimicrobial FFAs, whereas the *prfA** mutant strain behaved similarly to the wild type. It is not known why the loss of *prfA* increases the tolerance to antimicrobial FFAs. However, in addition to the virulence locus in *L. monocytogenes*, PrfA also affects expression of multiple genes encoding transporters, stress response proteins and proteins of unknown function [2,40,41]. Future investigation into how medium- and long-chain FFAs modulate global gene expression in *L. monocytogenes* will likely extend our understanding of how PrfA contributes to the response to these antimicrobial agents. Such studies may also lend insight into the mechanisms by which *L. monocytogenes* uses specific FFAs as cues to optimize the production of PrfA-dependent virulence factors.

Collectively, our studies suggest that selected antimicrobial FFAs act to block PrfA-dependent activation of virulence genes under conditions where their induction is not required, such as in food and during the gastrointestinal phase of infection [16,17]. In line with this, antimicrobial medium- and long-chain FFAs are known to inhibit *L. monocytogenes* invasion of enterocyte-like cells [22]. Since FFAs act on the otherwise constitutively active variant of PrfA, this indicates that FFAs hold the capacity to overrule other signals that might lead to increased activity of PrfA. By improving our understanding of how FFAs affect the growth and virulence of *L. monocytogenes* and other intestinal pathogens, we may elaborate new knowledge on how to use such compounds to improve food safety and prevent foodborne infections.

Acknowledgments

This work was supported by The Danish Council for Independent Research/Natural Sciences (grant number 12-124735 to B.H.K.) and the University of Southern Denmark. Q2

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2017.03.002.

References

[1] Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, Goebel W, et al. *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev 2001;14:584–640.

[2] Freitag NE, Port GC, Miner MD. *Listeria monocytogenes* - from saprophyte to intracellular pathogen. Nat Rev Microbiol 2009;7:623–8.

[3] Scortti M, Monzo HJ, Lacharme-Lora L, Lewis DA, Vazquez-Boland JA. The PrfA virulence regulon. Microbes Infect 2007;9:1196–207.

[4] de las Heras A, Cain RJ, Bielecka MK, Vazquez-Boland JA. Regulation of *Listeria* virulence: PrfA master and commander. Curr Opin Microbiol 2011;14:118–27.

[5] Johansson J, Mandin P, Renzoni A, Chiaruttini C, Springer M, Cossart P. An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. Cell 2002;110:551–61.

[6] Reniere ML, Whiteley AT, Hamilton KL, John SM, Lauer P, Brennan RG, et al. Glutathione activates virulence gene expression of an intracellular pathogen. Nature 2015;517:170–3.

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002

[7] Reniere ML, Whiteley AT, Portnoy DA. An in vivo selection identifies *Listeria monocytogenes* genes required to sense the intracellular environment and activate virulence factor expression. PLoS Pathog 2016;12: e1005741.

[8] Hall M, Grundstrom C, Begum A, Lindberg MJ, Sauer UH, Almqvist F, et al. Structural basis for glutathione-mediated activation of the virulence regulatory protein PrfA in *Listeria*. Proc Natl Acad Sci USA 2016;113: 14733–8.

[9] Xayarath B, Alonzo 3rd F, Freitag NE. Identification of a peptide-pheromone that enhances *Listeria monocytogenes* escape from host cell vacuoles. PLoS Pathog 2015;11:e1004707.

[10] Shetron-Rama LM, Mueller K, Bravo JM, Bouwer HG, Way SS, Freitag NE. Isolation of *Listeria monocytogenes* mutants with high-level in vitro expression of host cytosol-induced gene products. Mol Microbiol 2003;48:1537–51.

[11] Bruno Jr JC, Freitag NE. Constitutive activation of PrfA tilts the balance of *Listeria monocytogenes* fitness towards life within the host versus environmental survival. PLoS One 2010;5:e15138.

[12] Chaturongakul S, Raengpradub S, Wiedmann M, Boor KJ. Modulation of stress and virulence in *Listeria monocytogenes*. Trends Microbiol 2008; 16:388–96.

[13] Rauch M, Luo Q, Muller-Altrock S, Goebel W. SigB-dependent in vitro transcription of *prfA* and some newly identified genes of *Listeria monocytogenes* whose expression is affected by PrfA in vivo. J Bacteriol 2005;187:800–4.

[14] Schwab U, Bowen B, Nadon C, Wiedmann M, Boor KJ. The *Listeria monocytogenes prfAP2* promoter is regulated by sigma B in a growth phase dependent manner. FEMS Microbiol Lett 2005;245:329–36.

[15] Kazmierczak MJ, Mithoe SC, Boor KJ, Wiedmann M. *Listeria monocytogenes* sigma B regulates stress response and virulence functions. J Bacteriol 2003;185:5722–34.

[16] Gahan CG, Hill C. Gastrointestinal phase of *Listeria monocytogenes* infection. J Appl Microbiol 2005;98:1345–53.

[17] Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, Balestrino D, et al. The Listeria transcriptional landscape from saprophytism to virulence. Nature 2009;459:950–6.

[18] Desbois AP, Smith VJ. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol 2010;85:1629–42.

[19] Chatterjee A, Dutta PK, Chowdhury R. Effect of fatty acids and cholesterol present in bile on expression of virulence factors and motility of *Vibrio cholerae*. Infect Immun 2007;75:1946–53.

[20] Golubeva YA, Ellermeier JR, Cott Chubiz JE, Slauch JM. Intestinal long-chain fatty acids act as a direct signal to modulate expression of the *Salmonella* pathogenicity island 1 type III secretion system. mBio 2016; 7. e02170-15.

[21] Viarengo G, Sciara MI, Salazar MO, Kieffer PM, Furlan RL, Garcia Vescovi E. Unsaturated long chain free fatty acids are input signals of the *Salmonella enterica* PhoP/PhoQ regulatory system. J Biol Chem 2013; 288:22346–58.

[22] Petrone G, Conte MP, Longhi C, di Santo S, Superti F, Ammendolia MG, et al. Natural milk fatty acids affect survival and invasiveness of *Listeria monocytogenes*. Lett Appl Microbiol 1998;27:362–8.

[23] Wang LL, Johnson EA. Inhibition of Listeria monocytogenes by fatty acids and monoglycerides. Appl Environ Microbiol 1992;58:624–9.

[24] Bockmann R, Dickneite C, Middendorf B, Goebel W, Sokolovic Z. Specific binding of the *Listeria monocytogenes* transcriptional regulator PrfA to target sequences requires additional factor(s) and is influenced by iron. Mol Microbiol 1996;22:643–53.

[25] Brondsted L, Kallipolitis BH, Ingmer H, Knochel S. kdpE and a putative RsbQ homologue contribute to growth of *Listeria monocytogenes* at high osmolarity and low temperature. FEMS Microbiol Lett 2003;219:233–9.

[26] Schaferkordt S, Chakraborty T. Vector plasmid for insertional mutagenesis and directional cloning in *Listeria* spp. BioTechniques 1995;19: 720–2. 724–725.

[27] Mollerup MS, Ross JA, Helfer AC, Meistrup K, Romby P, Kallipolitis BH. Two novel members of the LhrC family of small RNAs in *Listeria monocytogenes* with overlapping regulatory functions but distinctive expression profiles. RNA Biol 2016;13:895–915.

[28] Christiansen JK, Larsen MH, Ingmer H, Sogaard-Andersen L, Kallipolitis BH. The RNA-binding protein Hfq of *Listeria monocytogenes*: role in stress tolerance and virulence. J Bacteriol 2004;186: 3355–62.

[29] Larsen MH, Kallipolitis BH, Christiansen JK, Olsen JE, Ingmer H. The response regulator ResD modulates virulence gene expression in response to carbohydrates in *Listeria monocytogenes*. Mol Microbiol 2006;61:1622–35.

[30] Nielsen JS, Larsen MH, Lillebaek EM, Bergholz TM, Christiansen MH, Boor KJ, et al. A small RNA controls expression of the chitinase ChiA in *Listeria monocytogenes*. PLoS One 2011;6:e19019.

[31] Nielsen JS, Lei LK, Ebersbach T, Olsen AS, Klitgaard JK, Valentin-Hansen P, et al. Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in *Listeria monocytogenes*. Nucleic Acids Res 2010;38:907–19.

[32] Sheehan B, Klarsfeld A, Msadek T, Cossart P. Differential activation of virulence gene expression by PrfA, the *Listeria monocytogenes* virulence regulator. J Bacteriol 1995;177:6469–76.

[33] Sievers S, Sternkopf Lillebaek EM, Jacobsen K, Lund A, Mollerup MS, Nielsen PK, et al. A multicopy sRNA of *Listeria monocytogenes* regulates expression of the virulence adhesin LapB. Nucleic Acids Res 2014; 42:9383–98.

[34] Poyart C, Trieu-Cuot P. A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to beta-galactosidase in gram-positive bacteria. FEMS Microbiol Lett 1997;156:193–8.

[35] Sun Y, O'Riordan MX. Branched-chain fatty acids promote *Listeria monocytogenes* intracellular infection and virulence. Infect Immun 2010; 78:4667–73.

[36] Sun Y, Wilkinson BJ, Standiford TJ, Akinbi HT, O'Riordan MX. Fatty acids regulate stress resistance and virulence factor production for *Listeria monocytogenes*. J Bacteriol 2012;194:5274–84.

[37] Lowden MJ, Skorupski K, Pellegrini M, Chiorazzo MG, Taylor RK, Kull FJ. Structure of *Vibrio cholerae* ToxT reveals a mechanism for fatty acid regulation of virulence genes. Proc Natl Acad Sci USA 2010;107: 2860–5.

[38] Plecha SC, Withey JH. Mechanism for inhibition of *Vibrio cholerae* ToxT activity by the unsaturated fatty acid components of bile. J Bacteriol 2015;197:1716–25.

[39] Childers BM, Cao X, Weber GG, Demeler B, Hart PJ, Klose KE. N-terminal residues of the *Vibrio cholerae* virulence regulatory protein ToxT involved in dimerization and modulation by fatty acids. J Biol Chem 2011;286:28644–55.

[40] Milohanic E, Glaser P, Coppee JY, Frangeul L, Vega Y, Vazquez-Boland JA, et al. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. Mol Microbiol 2003;47:1613–25.

[41] Port GC, Freitag NE. Identification of novel *Listeria monocytogenes* secreted virulence factors following mutational activation of the central virulence regulator, PrfA. Infect Immun 2007;75:5886–97.

[42] Bohne J, Sokolovic Z, Goebel W. Transcriptional regulation of *prfA* and PrfA-regulated virulence genes in *Listeria monocytogenes*. Mol Microbiol 1994;11:1141–50.

[43] Dramsi S, Kocks C, Forestier C, Cossart P. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator *prfA*. Mol Microbiol 1993;9:931–41.

[44] Lingnau A, Domann E, Hudel M, Bock M, Nichterlein T, Wehland J, et al. Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. Infect Immun 1995;63:3896–903.

Please cite this article in press as: Sternkopf Lillebæk EM, et al., Antimicrobial medium- and long-chain free fatty acids prevent PrfA-dependent activation of virulence genes in *Listeria monocytogenes*, Research in Microbiology (2017), http://dx.doi.org/10.1016/j.resmic.2017.03.002