How can naturally occurring fatty acids neutralize *Listeria*?

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Listeria monocytogenes is a Gram-positive bacterial pathogen causing severe foodborne disease listeriosis [1]. It thrives in a broad range of environments and grows under conditions traditionally used for food preservation, such as low temperature, low pH and high salt concentrations. After ingestion of contaminated food products, the bacterium enters the GI tract. In at-risk populations (pregnant individuals and elderly or otherwise immunocompromised individuals), *Listeria* may cross multiple protective barriers within the body, causing severe complications such as spontaneous abortion, septicemia or meningitis [1]. The high lethality rates of infected individuals (up to 30%), even with antibiotic treatment, clearly demonstrate that listeriosis represents a severe clinical challenge. Most recently, alternative options to the traditional antibiotics have been proposed, such as inhibitory compounds reducing the virulence of *Listeria* [2–4]. One of these options relies on the observation that naturally occurring medium- and long-chain free fatty acids (FFAs) efficiently prevent *Listeria* from expressing its key virulence factors [4]. In this editorial, recent examples of antivirulence agents acting on *Listeria* will be discussed.

The intracellular lifestyle of *Listeria*

During infection, *L. monocytogenes* gains access to the cytoplasm of a variety of host cells [1]. The bacterium multiplies intracellularly and spreads to neighboring cells through host actin polymerization. Key virulence factors mediating invasion and intracellular replication by *L. monocytogenes* are well-characterized [1]. Specific surface proteins (e.g., InlA and InlB) are essential for bacterial entry into nonphagocytic cells, whereas listeriolysin O (LLO) and phospholipases (PlcA and PlcB) mediate the escape from the host cell phagosome. Once inside the host cell cytosol, the surface protein ActA promotes actin polymerization and cell-to-cell movement. Expression of these and other virulence factors in *Listeria* is regulated by PrfA, a transcriptional activator protein belonging to the Crp/Fnr family of regulators [1,5]. To activate transcription, PrfA binds as a homodimer to a specific DNA sequence, the PrfA box, found in the promoter region of PrfA-regulated genes. When *Listeria* resides in the external environment, PrfA most often takes up an ‘inactive’ conformation, which binds to the PrfA box albeit with low affinity [1,5]. In the intracellular environment, the activity of PrfA increases dramatically, mainly due to the binding of bacterial- and host-derived glutathione [6]. Upon binding of this cofactor, PrfA undergoes a conformational change in its ‘active’ form, holding an optimal conformation for PrfA-DNA interaction [7]. Mutant variants of PrfA, called PrfA*, are known to lock the protein in its active conformation [1,5,7]. When *Listeria* carries such a *prfA** mutation, PrfA-regulated virulence genes are constitutively expressed irrespective of the growth conditions. Importantly, a mutant *Listeria* lacking *prfA* (e.g., $\Delta prfA$) is avirulent.

Antivirulence strategies against *Listeria*?

Due to the rapid increase in antibiotic resistance, alternatives to classical antibiotics are receiving increasing attention, such as drugs that intervene with bacterial virulence [8,9]. Antivirulence drugs act by disarming the pathogen of its virulence factors and may be used either alone or together with antibiotics. In the case of *Listeria*,

PrfA represents an obvious target for antivirulence compounds. Indeed, several recent studies focused on the identification and characterization of compounds acting to prevent PrfA-dependent virulence gene expression. First, a study demonstrated that ring-fused 2-pyridone molecules attenuate the virulence of *Listeria* by reducing the expression of PrfA-activated virulence genes [2]. More specifically, these drugs bind directly to PrfA to reduce its DNA binding activity. Importantly, a PrfA* mutant was not affected, suggesting that ring-fused 2-pyridones could act to lock PrfA wt in its inactive form. Indeed, structural characterization of the interaction between PrfA and a ring-fused 2-pyridone confirmed that the drug binds to regions important for PrfA activation and DNA binding [2]. A purine analog (6-*N*-hydroxylaminopurine [6-*N*-HAP]) represents another recent example of an antivirulence drug acting on *Listeria* [3]. 6-*N*-HAP was shown to decrease virulence gene expression in *Listeria* by reducing the level as well as the activity of PrfA wt. In addition to its antivirulence effect, 6-*N*-HAP also acts as a potent mutagen in *Listeria*, thereby reducing its viability. At last, naturally occurring medium- and long-chain FFAs, such as the omega-3 fatty acid eicosapentaenoic acid (C20:5), were shown to prevent PrfA-dependent activation of virulence genes in *Listeria* [4]. As a foodborne pathogen, *Listeria* encounters a variety of FFAs in foods and in the GI tract. Some naturally occurring FFAs exhibit potent antimicrobial activity against *Listeria*, but the exact mechanism underlying their antimicrobial effect is not well understood. Additionally, specific medium- and long-chain FFAs have antivirulence effects on *Listeria*. First, the invasive efficiency of *Listeria* in enterocyte-like cells is strongly decreased in the presence of subinhibitory concentrations of medium- and long-chain FFAs naturally present in milk [10]. Second, the expression of LLO, ActA and other PrfA-dependent virulence factors is strongly downregulated upon exposure to subinhibitory concentrations of specific medium- and long-chain FFAs, including eicosapentaenoic acid [4]. Thus, some FFAs not only exhibit antibacterial activity against *Listeria*; they also act to prevent *Listeria* from expressing its key virulence factors.

How do naturally occurring free fatty acids sabotage *Listeria* virulence?

The PrfA regulator is likely to be the target of the medium- and long-chain FFAs acting by an antivirulence mechanism. Notably, the inhibitory effect of FFAs on virulence gene expression is also observed in *Listeria* encoding the constitutively active mutant variant PrfA* [4]. After 1 h of FFA exposure, transcription of PrfA-dependent virulence genes is strongly reduced, but the level of PrfA* protein remains unaffected at this time point. These findings suggest that FFAs act by blocking the activity of PrfA, possibly by generating an inhibitory signaling event via the membrane, or by direct binding to the PrfA protein itself [4]. Interestingly, unsaturated long-chain FFAs present in bile are known to inhibit the expression of two primary virulence genes in *Vibrio cholera*, encoding the cholera toxin and toxin-coregulated pilus [11]. The transcription factor ToxT, belonging to the AraC family, directly activates expression of cholera toxin and toxin-coregulated pilus, but the presence of unsaturated long-chain FFAs inhibits ToxT-dependent activation. Structural and functional analyses of ToxT revealed that unsaturated long-chain FFAs bind directly to a regulatory region in ToxT, suggesting that they prevent ToxT dimerization and/or DNA binding [12–14]. Importantly, unsaturated long-chain FFAs have been shown to also inhibit the activity of another member of the AraC family, the HilD virulence regulator in *Salmonella enterica* [15]. Structural and functional studies on PrfA in *Listeria* could show if FFAs also bind directly to Crp/Fnr family members. If so, structural analyses of the binding of FFA to PrfA might reveal the mechanism underlying the inhibitory effect of FFAs in *Listeria* and could form the basis for the future design of antivirulence compounds against *Listeria* and potentially other pathogens encoding virulence regulators of the Crp/Fnr family. Indeed, the x-ray structure of the unsaturated FFA palmitoleic acid (C16:1) bound to ToxT has served as a template for the design of a new class of highly effective ToxT inhibitors [16]. These small-molecule inhibitors were designed to resemble the folded fatty acid and bind very tightly to ToxT. Importantly, the compounds inhibit virulence gene expression even more efficiently than unsaturated FFAs and inhibit ToxT-DNA binding interaction [16]. These findings clearly demonstrate how detailed studies of the inhibitory action of FFA on virulence gene expression can elaborate new knowledge on how to develop antivirulence strategies against bacterial pathogens.

Conclusion

Naturally occurring medium- and long-chain FFAs represent an interesting and very useful group of antibacterial and antivirulence agents. In nature, they may act as signaling molecules to prevent activation of virulence gene expression under conditions where their induction is not required, such as in food. Studies on how these FFAs affect the growth and virulence of *Listeria* and other bacterial pathogens will most likely provide new ideas on how to combat bacterial pathogenic disease. The most encouraging potential clearly lies in the ability of subinhibitory

concentrations of specific FFAs to inhibit virulence gene expression in important bacterial pathogens. Structural and functional studies on the inhibitory action of such FFAs on virulence will serve as inspiration for the future development of more efficient antivirulence agents. Most importantly, compounds acting by targeting virulence mechanisms represent a promising alternative to traditional antibiotics [8,9]. In contrast to antibiotics, agents acting by an antivirulence mechanism could reduce the pressure for the development of resistance by simply disarming the bacteria of its virulence factors while not preventing bacterial growth [8,9].

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