Susceptibility of *Listeria monocytogenes* to antimicrobial peptides

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Abstract

We assessed the susceptibility of several pathogenic and non-pathogenic *Listeria* species to antimicrobial peptides of animal and plant origin. Human defensins and thionins were highly inhibitory, whereas protamine, snakin and magainin showed an intermediate effect. A temperature dependence in the activity of potato defensin was observed for *Listeria monocytogenes* and *Listeria ivanovii*. PrfA* L. monocytogenes* mutants, that overexpress constitutively PrfA-dependent genes, were sensitive to the peptide independently of the temperature whereas isogenic PrfA− derivatives were constitutively resistant. These data indicate that the thermoregulated transcription factor PrfA controls the expression of bacterial products that influence the susceptibility or resistance to some antimicrobial peptides.

Keywords: Plant–bacteria interaction; Gram-positive bacterium; Plant antimicrobial peptide

1. Introduction

The foodborne pathogen *Listeria monocytogenes* is a highly versatile organism able to survive and proliferate in a wide range of substrates. The primary habitat of these Gram-positive bacteria is decaying plant matter-rich soil. From this habitat, they reach the intestine of man and animals to form part of the fecal microflora [1]. Due to its widespread distribution, *L. monocytogenes* is a common contaminant of the raw materials of animal and plant origin used by the food industry. Consumption of *L. monocytogenes*-contaminated food may lead to a severe illness called listeriosis, characterized by abortion, septicemia and meningoencephalitis [2].

In all its habitats, whether soil, food or infected host tissues, *Listeria* are presumably confronted to antimicrobial peptides. More than 500 of these peptides have been discovered in plants and animals, where they are believed to play a major role as components of the innate defense against invading microorganisms [3,4]. These peptides usually have a broad antimicrobial spectrum, including Gram-positive and Gram-negative bacteria. In most cases, the mechanism of action is related to the permeabilization of the cytoplasmic membrane [5]. Antimicrobial peptides are usually found in those surfaces of animals and plants which are most likely to come into contact with pathogens from the environment, such as epithelial surfaces [4] or plant cell walls [3]. Antimicrobial peptides are also produced by phagocytes and appear to be involved in bacterial killing by these cells [6].

Eight antimicrobial peptides of animal and vegetal origin were chosen for this study. Defensins are a family of structurally related peptides found both in animals and plants [3,7] and are probably ubiquitous in both kingdoms. In humans, defensins constitute the major antimicrobial peptides in phagocytic cells [7]. Thionins, lipid transfer proteins (LTPs) and snakins have been isolated, so far, only from plant tissues. Thionins are the first and best-characterized family of antimicrobial peptides and are abundant in the leaves and seeds of many plant species. The so-called non-specific LTPs are a family of peptides previously thought to be involved in lipid shuttling between organelles and its antimicrobial activity has been proved against a broad range of microorganisms [8]. Snakins are a recently described family of antimicrobial peptides found in potato tubers [9]. Protamine is a polyca-
togenic peptide found in the nuclei of sperm cells of different animal species [10] and magainins were initially isolated from amphibian’s skin [11]. Information about the effect of antimicrobial peptides on *Listeria* spp. is scarce. In this study, we have examined the susceptibility of *L. monocytogenes* to a variety of natural antimicrobial peptides of animal and plant origin.

2. Materials and methods

2.1. Strains and culture conditions

The strains used in this work are described in Table 1. For preparation of inocula, *Listeria* were grown overnight in BHI (Biomerieux) at 37°C with shaking, then washed twice with sterile saline solution (v/v) and resuspended in an appropriate volume of BHI to give 1 \( \times 10^3 \) colony forming units (cfu) per 20 μ. Serial dilutions of the final suspension were plated to assess the number of cfu for each experiment.

2.2. Purification of antimicrobial peptides

Thionin was purified from wheat flour as described [12]. LTPs were purified as previously described [8]. Defensin-Pth1 and snakin-1 were purified from potato tuber as follows: 20 g of frozen material was ground to powder in liquid nitrogen, using a mortar and pestle, and extracted once with 80 ml buffer (0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) and twice with 80 ml H2O. The resulting pellet was then extracted with 50 ml 1.5 M LiCl at 4°C for 1 h, and the extract dialyzed against 5 l of water using a Spectra/Por 6 (MWCO: 3000) membrane, and freeze-dried. The extract was subjected to reverse-phase HPLC as previously described [8]. Collected peaks were analyzed by SDS-PAGE in preformed gradient gels (4–20%; Bio-Rad). Salmon-sperm protamine and human defensins were purchased from Sigma (St. Louis, MO, USA). Magainin was a kind gift from Magainin Pharmaceuticals Inc. (Plymouth Meeting, PA, USA).

2.3. Inhibition assays

The sensitivity of *Listeria* strains to antimicrobial peptides was assessed by a growth inhibition assay in microtiter plates in a final volume of 75 μ. Wells contained appropriate dilutions of the peptide and 1 \( \times 10^3 \) cfu of bacteria in 0.25× BHI broth. Inoculated plates were incubated with shaking at 200 rpm for 24 h at 37°C and bacterial growth was monitored by measuring the absorbance at 600 nm using an automated reader.

3. Results

3.1. Susceptibility of *L. monocytogenes* to animal and plant antimicrobial peptides

Eight antimicrobial peptides from various sources were tested for their antilisterial activity (Table 1). *Listeria innocua*, a species closely related to *L. monocytogenes*, with which it shares the same environmental habitats but which is unable to colonize animal host tissues, and *Listeria ivanovii*, a second pathogenic species of the genus *Listeria* [2], were also tested using the same procedure (Table 1).

Human defensins and thionins were the most active peptides against the three *Listeria* spp. tested, with a minimum inhibitory concentration (MIC) of 2–5 μg ml\(^{-1}\). Thionin has been reported to be active against several bacteria and fungi at a concentration interval of 5–50 μg ml\(^{-1}\) [3], and the α-defensins from non-human primates have been shown to be active against *L. monocytogenes* strain EGD at 7–15 μg ml\(^{-1}\) [13]. Protamine, snakin and magainin showed an intermediate effect, with MICs of 5–10 μg ml\(^{-1}\). LTP and potato defensin were the less effective peptides, with MICs between 25 and 35 μg ml\(^{-1}\) or above. No striking differences in the susceptibility of the three *Listeria* species tested were observed at 37°C, except that potato defensin exhibited inhibitory activity against the two pathogenic *Listeria* spp. whereas it was inactive against the non-pathogenic species *L. innocua* (Table 2; see also Fig. 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P14</td>
<td>Serovar 4b wild-type clinical isolate</td>
<td>[16,22]</td>
</tr>
<tr>
<td>P14-A</td>
<td>PrfA* phenotype. Isogenic mutant of P14 carrying a Gly145Ser substitution in PrfA leading to constitutive overexpression of PrfA-regulated genes</td>
<td>[16,22]</td>
</tr>
<tr>
<td>MS5</td>
<td>PrfA* phenotype. PrfA-deficient isogenic derivative of P14-A resulting from transposon-mediated disruption of prfA transcription</td>
<td>[16,22]</td>
</tr>
<tr>
<td><em>L. ivanovii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 19119</td>
<td>Type strain of the subsp. <em>ivanovii</em></td>
<td>Collection</td>
</tr>
<tr>
<td><em>L. innocua</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 33090</td>
<td>Type strain of the species</td>
<td>Collection</td>
</tr>
</tbody>
</table>
3.2. Effect of temperature on listerial susceptibility to antimicrobial peptides

The transition from the environmental habitat to the mammalian host is accompanied by a temperature up-shift which is sensed by pathogenic *Listeria*, resulting in upregulation of virulence factors, among which are surface-associated proteins [2,14,15]. We assessed whether an eventual temperature-associated change in the listerial surface properties affects the sensitivity to selected antimicrobial peptides by carrying out bacterial inhibition assays at 20 and 37°C, representative of environmental and mammalian body temperatures, respectively.

There were no major temperature-dependent differences in the susceptibility to human defensins 1 and 2, except for a slight decrease in sensitivity of *L. ivanovii* at 37°C (Fig. 1). For thionin and at a concentration of 2 μg ml⁻¹, a differential response was observed for *L. monocytogenes* and *L. ivanovii*. A shift from 20°C to 37°C made *L. monocytogenes* to become fully sensitive to that peptide whereas the converse was observed for *L. ivanovii*. A clear effect of temperature on bacterial susceptibility was observed with potato defensin. Interestingly, whereas at 20°C the three bacteria tested were all resistant, at 37°C the two pathogenic species, *L. monocytogenes* and *L. ivanovii*, but not the non-pathogenic *L. innocua*, exhibited some degree of sensitivity to the peptide (Fig. 1).

3.3. PrfA dependence of susceptibility to potato defensin

The above data suggest that pathogenic *Listeria* possesses thermoregulated factors mediating susceptibility to potato defensin. In pathogenic *Listeria*, the only known regulatory mechanism that responds to a change in temperature from 20°C to 37°C is that mediated by the central virulence regulator, PrfA. At low temperature, PrfA-dependent genes are repressed whereas at 37°C they are induced [2,14]. To test whether the PrfA transcription factor is involved in the observed temperature-dependent susceptibility to potato defensin, we used two isogenic mutants in the *prfA* allele of *L. monocytogenes*. In one of these mutants, PrfA-dependent expression is abolished (PrfA⁻ phenotype) [17] (Table 1). The inhibitory effect of 25 μg ml⁻¹ of potato defensin on these mutants was assessed at 20 and 37°C.

As shown in Fig. 2, the PrfA* phenotype was associated with constitutive susceptibility to the peptide whereas the PrfA⁻ strain was constitutively resistant. These results demonstrate that PrfA-regulated factors affect the susceptibility of *L. monocytogenes* to potato defensin. Interestingly, PrfA has no effect in susceptibility to human defensins at the concentrations assayed (not shown).

Table 2

<table>
<thead>
<tr>
<th>Antimicrobial peptide</th>
<th>MIC (μg ml⁻¹)</th>
<th><em>L. monocytogenes</em></th>
<th><em>L. innocua</em></th>
<th><em>L. ivanovii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Human defensin 1</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Human defensin 2</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Potato defensin</td>
<td>&gt; 25</td>
<td>NA</td>
<td>&gt; 25</td>
<td></td>
</tr>
<tr>
<td>Thionin</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Snakin</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>LTP</td>
<td>NA</td>
<td>NA</td>
<td>&gt; 25</td>
<td></td>
</tr>
<tr>
<td>Protamine</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Magainin</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*a* An inhibitory effect was observed, but total inhibition was not achieved.

*b* NA, not active (no inhibitory effect observed).

4. Discussion

We showed that *L. monocytogenes* and its cognate species *L. innocua* and *L. ivanovii* were strongly inhibited by human defensins, protamine and magainin. This is consistent with previous observations on the susceptibility of *L. monocytogenes* to antimicrobial peptides of animal origin [18]. Plant-derived peptides such as thionin and snakin...
had also a strong inhibitory effect on the Listeria species tested. However, potato defensin and LTP were only weakly inhibitory. This heterogeneity in Listeria susceptibility to antimicrobial peptides from plants is consistent with the observed differential fate of L. monocytogenes in vegetable substrates, some of which support its growth and/or survival (e.g. potatoes) while others do not (e.g. carrots) [19]. Further work will be required to determine whether differences in the amounts and specific activities of the antimicrobial peptides found in vegetables may account for differences in their capacity to support or inhibit growth of Listeria spp.

Our data have revealed an interesting effect of the ambient temperature on the susceptibility of the pathogenic species L. monocytogenes and L. ivanovii to potato defensin. While these bacteria exhibited some degree of sensitivity to the peptide at 37°C, they became totally resistant at 20°C, i.e. the temperature found by these bacteria when living free in the environment. Although this observation may be merely coincidental, it could also reflect an adaptive response aimed at improving survival in the plant-dominated habitat in which Listeria carry out their environmental saprophytic life. Consistent with this view, we showed that this temperature-dependent change in susceptibility to the antimicrobial peptide is mediated by PrfA, a transcription factor which acts as a main switch turning on and off Listeria genes in response to physicochemical environmental stimuli, including temperature [2,14,17]. For virulence genes, PrfA acts as a transcriptional activator, but there is evidence that it can also negatively regulate other genes, such as the stress response mediator clpC [20] and the motility-associated genes motA [21] and flaA [22]. Thus, the observed increase in susceptibility in a PrfA* background may result from PrfA-dependent induction of a susceptibility factor or, alternatively, repression of an antimicrobial peptide resistance factor. Our observations further illustrate that PrfA not only controls virulence functions but that it is a pleiotropic regulator with a more general regulatory role in L. monocytogenes physiology. Changes in peptide susceptibility associated with virulence gene regulatory networks have been reported for other bacteria. Thus, in Salmonella enterica serovar Typhimurium the virulence regulator PhoP-PhoQ induces the acylation of the lipid A, a major component of the lipopolysaccharide (LPS) of the outer membrane, increasing bacterial sensitivity to several antimicrobial peptides [23].

The high susceptibility found for L. monocytogenes to certain antimicrobial peptides opens the possibility of using such molecules as food preservatives. Such a strategy has been already considered for an antimicrobial peptide, the bacteriocin nisin [24,25]. Thionin is a particularly promising molecule, since it is very active and it is abundant in cereal grains, which are common components of food.

Acknowledgements

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