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Brief note

Bioengineering of lanthipeptides in *Escherichia coli*: assessing the specificity of lichenicidin and haloduracin biosynthetic machinery

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Abstract

The lichenicidin and haloduracin biosynthetic machinery specificity was investigated *in vivo* in *Escherichia coli*. Unlike previous reports using different hosts, it was found that the biosynthetic machineries of lichenicidin and haloduracin are highly specific to their dedicated peptide precursors. Likewise, the substitution of lichenicidin structural genes by chimeras of lichenicidin leader sequences and haloduracin core peptides did not yield mature haloduracin peptides. Despite these restrictions, it was found that the bifunctional enzyme HalT was able to process and export lichenicidin peptides. These findings corroborate the promiscuity of LanT enzymes reported for other lantibiotics, such as nukacin ISK-1 and lacticin 481.

Keywords: *Bacillus*; Bacteriocins; Chimeras; *In vivo*; Lantibiotics

1. Introduction

Considering the increasing ineffectiveness of classical antimicrobials against some clinically relevant Gram positive bacteria, lanthipeptides with potent antimicrobial activity are currently under investigation as viable alternatives to antibiotics presently commercialized [1–5]. These compounds, also known as lantibiotics, are gene encoded, representing an advantage over non-ribosomal or polyketide antibiotics to implement bioengineering strategies [6]. Lanthipeptides result from extensive post-translational modifications of their precursor peptides to reach a mature and active form. Therefore, it is essential to understand the flexibility and/or specificity of the biosynthetic machinery responsible for this processing [7–10].

Lichenicidin is a class II lanthipeptide composed by two peptides (Bliα and Bliβ) that act synergistically to inhibit the growth of several Gram positive bacteria, including *Staphylococcus aureus* and *Listeria monocytogenes* [11,12]. Previously, the study of lichenicidin biosynthesis in *Escherichia coli* revealed that the modification modifying enzymes, LicM1 and LicM2, tolerate various amino acid substitutions within the LicA1 and LicA2 core peptides [13]. Most excitingly, analogs of active Bliα and Bliβ containing non-canonical amino acids were produced *in vivo* in *E. coli* [14]. Among the two-component peptide lantibiotics already characterized, lichenicidin is more related to haloduracin (Halα and Halβ), which is produced by *Bacillus halodurans* C-125 from modification of HalA1 and HalA2 by the dedicated enzymes HalM1 and HalM2, respectively [15,16]. The LicA1 and LicA2 precursor peptides have similarities of approximately 52% and 50% with HalA1 and HalA2, respectively. Considering their corresponding enzymes dedicated to modification and transport, it was found that: i) LicM1 and LicM2 each display 35% similarity with modifying enzymes HalM1 and

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HalM2, and ii) LicT shares 50% similarity with its counterpart in the haloduracin biosynthetic cluster, HalT. Regarding their sequence and the functional similarities of haloduracin and lichenicidin, the present study aims to assess the flexibility of the modifying enzymes (LanM) and transporter (LanT) to produce fully active peptides, using *E. coli* as the producer organism.

2. Results and discussion

To achieve our purpose, the system based on the strains produced in the previous study performed by Caetano et al. (2011) was used. Briefly, five different fosmids were obtained by deleting the genes *licA1*, *licA2*, *licM1*, *licM2* or *licT* from the parental pLic5 fosmid containing the complete lichenicidin (*lic*) biosynthetic gene cluster (Fig. 1A) and transformed into *E. coli* BL21Gold (Table 1). Subsequently, chemically competent cells of each of these strains were transformed with a complementation plasmid, containing the deleted gene, or its counterpart in the haloduracin (*hal*) biosynthetic gene cluster (Table 1; Fig. 1A). The antibacterial activity was assessed by deferred antagonism assays against the indicator strain *Micrococcus luteus* ATCC 9341, as previously published for the mutagenesis studies of Bliα and Bliβ [13]. Moreover, the presence of lichenicidin and/or haloduracin peptides was investigated in the culture supernatant extracts by LC-ESI-MS, as described by Caetano et al. [17].

As stated above, two-peptide lantibiotics act synergistically to inhibit other bacteria. Accordingly, the gene-inactivation strains constructed in this study presented no antibacterial activity, due to the lack of one or both of the complementary lichenicidin peptides. Nonetheless, the bioactivity was restored after their transformation with plasmids expressing each one of the complementary *lic* genes. In addition, the molecular masses of Bliα and Bliβ were detected by LC-ESI-MS in supernatant extracts of all of the strains (Table 1). These results demonstrated that the *trans* complementation system herein used was fully functional. The proteins essential for the biosynthesis of haloduracin were also successfully expressed with *E. coli* BL21 (DE) and the pET-system in previous studies [15,18]. However, when the same experiment was conducted by replacing the missing *lic* genes by their counterparts of the haloduracin biosynthetic cluster, bioactivity was only restored in the presence of the *halT* gene (BLic5ΔTp*halT*; Table 1). This phenotype indicated that fully active Bliα and Bliβ were produced and secreted by strain BLic5ΔTp*halT*, which was confirmed by the detection of their molecular masses using LC-ESI-MS (Table 1). The bifunctional transporters/proteases of class II lanthipeptides like LicT or HalT, commonly referred as LanT, are characterized by a dual role: i) removal of the leader peptide and ii) secretion of the modified and active core peptide [19]. Thus, our results show that HalT is able to replace LicT in these two biosynthetic steps in lichenicidin production. This observation is corroborated by a previous report, whereupon the lacticin 3147 transporter LtnT was able to proteolytically process and transport nisin as well as the hormone angiotensin, when fused to the leader peptide of lacticin LtnA2 [20]. However, in the present study, HalT was able to play its role in the absence of haloduracin leader peptides. A similar outcome was also reported by Nagao et al. (2007), which described the processing of the lantibiotic nukacin ISK-1 by the transporter of lacticin 48s1 (LctT) in the *Lactococcus lactis* NZ9000 strain. However, the inhibition radius of the Lic5ΔTplicT (0.68 mm ± 0.04) and Lic5ΔTphalT (0.45 mm ± 0.05) colonies demonstrate that HalT protein should not be as efficient as LicT. The analysis of the four leader sequences recognized by HalT (LicA1, LicA2, HalA1 and HalA2), revealed that LicA1 and HalA1 share the highest similarity (53%), while LicA1 and HalA2 share the lowest (29%). Together, these results suggest that LanT proteins are extremely flexible regarding the amino acid residues of the leader peptides that they recognize. Focusing only in the proteolysis reaction, it was determined that the Glu residue of the consensus sequence ELXXBX (B = V, L or I), characteristic of class II leader peptides, is a possible recognition site [21]. In fact, this residue is found in all of the leader sequences processed by HalT (Fig. 1). Interestingly the consensus sequence of LicA1 and HalA1 is longer than expected ($ELX_5(X)BXX$; Fig. 1B). Therefore, the distance between the conserved residue Glu and the double-glycine motif should not affect the recognition and removal of leader peptides by LanT proteins. In addition, it

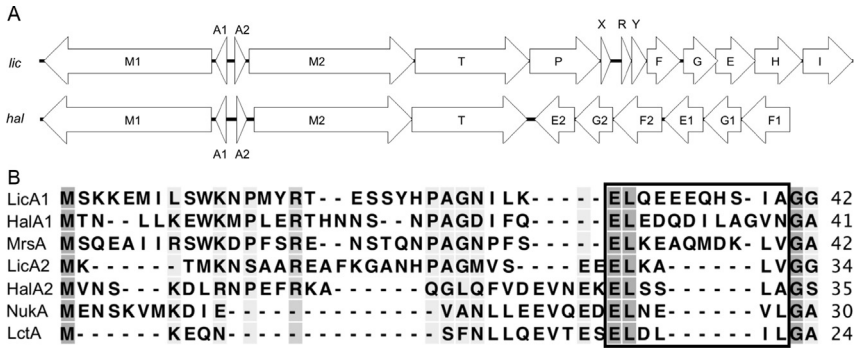

Fig. 1. Schematic representation of the lichenicidin and haloduracin gene clusters (A) and ClustalW alignment of the leader peptides of mersacidin (MrsA), lichenicidin (LicA1 and LicA2), haloduracin (HalA1 and HalA2), lacticin 481 (LctA) and nukacin ISK-1 (NukA). The consensus sequence characteristic of the leader peptides of class II lanthipeptides (ELXXBX (B = V, L or I)) is highlighted with a black box (B).