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# Lichenicidin Biosynthesis in *Escherichia coli*: *licFGEHI* Immunity Genes Are Not Essential for Lantibiotic Production or Self-Protection▿

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**This study demonstrated, for the first time, that immunity genes *licFGEHI* are not essential for self-protection and production of the two-component lantibiotic lichenicidin in the Gram-negative heterologous host *Escherichia coli* BLic5. Additionally, it was experimentally demonstrated that lichenicidin lantibiotics are active against the *E. coli imp4213* strain, a mutant strain possessing a permeable outer membrane.**

Lichenicidin is a two-component lantibiotic consisting of the peptides Bliα and Bliβ, produced by *Bacillus licheniformis*, with activity against several clinical important Gram-positive strains such as methicillin-resistant *Staphylococcus aureus* (MRSA). All the machinery involved in the expression, modification, transport, and regulation of the two peptides Bliα and Bliβ are encoded in 14 contiguous open reading frames (ORFs; *lic* gene cluster) on the *B. licheniformis* chromosome. Recently, the *lic* gene cluster was cloned and successfully expressed in *Escherichia coli* (BLic5 strain). This constituted the first report of fully active lantibiotic production totally *in vivo* by a Gram-negative host (3). This expression system was further exploited to investigate the role of all the ORFs of the *lic* gene cluster through the deletion of each of the genes, except those putatively involved in immunity. Lantibiotic self-protection mechanisms of the producer strain usually involve an individual immunity protein (LanI) and/or an ABC transporter, usually composed of two or three subunits (LanFE[G]). Also, an ancillary protein for the assembly of a functioning ABC transporter has been described (LanH) (5). These five proteins are putatively represented in the *lic* gene cluster by the *licFGEHI* genes (9, 13). Herein, the role of the five ORFs in lichenicidin self-protection and production by *E. coli* BLic5 was investigated. Lichenicidin peptides interact synergistically at a ratio of 1:1 within the nanomolar concentration range to exert their full activity (11). Individually, the action of each peptide requires much higher concentrations (11). Thus, the replacement of the *licFGEHI* ORFs by an apramycin resistance cassette was performed in the pLic5 fosmid (containing the complete lichenicidin gene cluster) in which either *licA1* or *licA2* structural genes were deleted (pLic5Δ*A1* and pLic5Δ*A2*, respectively) to reduce the possibility of bacterial self-killing by the lichenicidin peptides. For this purpose, the λ Red redirect system was used as previously described by Caetano et al. (3). The constructed fosmids pLic5Δ*A1*Δ*FGEHI* and pLic5Δ*A2*Δ*FGEHI* conferring resistance to chloramphenicol (12.5 μg/ml) and apramycin (50 μg/ml) were transformed into chemically competent *E. coli* BL21-Gold (DE3) (Agilent). The obtained strains, *E. coli* BLic5Δ*A1*Δ*FGEHI* and *E. coli* BLic5Δ*A2*Δ*FGEHI* (Table 1), were characterized by the absence of bioactivity against the indicator strain *Micrococcus luteus* ATCC 9341 (Fig. 1). These mutants were subjected to cross-feeding agar diffusion experiments, either with Bliα (through the strain BLic5Δ*A2*) or Bliβ (through the strain BLic5Δ*A1*) as previously described by Caetano et al. (3). Bioactivity was completely restored, confirming that *E. coli* BLic5Δ*A1*Δ*FGEHI* retained the capacity to produce Bliβ and that *E. coli* BLic5Δ*A2*Δ*FGEHI* retained the capacity to produce Bliα (Fig. 2). Likewise, such results were further confirmed by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis of butanol extracts (Fig. 1) as described in Caetano et al. (3). In order to understand if survival of these mutants retaining biosynthetic capabilities for one of the two peptides was due to the absence of the complementary peptide, Bliα or Bliβ, deletion of *licFGEHI* genes was performed in the pLic5 fosmid possessing both the *licA1* and *licA2* structural genes. The resulting plasmid, pLic5Δ*FGEHI*, was constructed and transformed in *E. coli* as described above. Remarkably, *E. coli* BLic5Δ*FGEHI* mutants producing both the Bliα and Bliβ peptides were obtained, as confirmed by bioassay (Fig. 1) and LC-ESI-MS analysis (Fig. 1). The relative abundance of both peptides was further investigated by LC-ESI-MS analysis of butanol extracts of three independent cultures of *E. coli* BLic5 and BLic5Δ*FGEHI* after 24 h of growth. The values obtained from the TIC (total ion current) peak integration of each peptide were generally higher for BLic5 cultures (Bliα, 585.7 ± 64.0 arbitrary units [AU]; Bliβ, 236.0 ± 16.6 AU) than for BLic5Δ*FGEHI* cultures (Bliα, 400.7 ± 169.5 AU; Bliβ, 125.7 ± 41.8 AU). These results demonstrate undoubtedly that *E. coli* is able to produce both the Bliα and Bliβ peptides, even if the immunity genes *licFGEHI* are not present. Nevertheless, it is not clear if such genetic modification can negatively affect BLic5Δ*FGEHI* bacterial fitness and consequently the lichenicidin production. The susceptibility of the BLic5 and BLic5Δ*FGEHI* strains to lichenicidin was also investigated. The agar plates containing each of these strains were prepared as done previously for those used for the bioassays of *M. luteus* (3). The lichenicidin sample was obtained from butanol extraction of a BLic5 cul-

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TABLE 1. List of *E. coli* strains used in the present study

| Strain | Fosmid | Observation(s)[a] |
| --- | --- | --- |
| BLic5 | pLic5 | *E. coli* BL21-Gold (DE3) cells transformed with the pLic5 fosmid containing the complete lichenicidin gene cluster; production of Bliα and Bliβ |
| BLic5Δ*A1* | pLic5Δ*A1* | Deletion of *licA1* gene in the pLic5 fosmid; single production of Bliβ |
| BLic5Δ*A2* | pLic5Δ*A2* | Deletion of *licA2* gene in the pLic5 fosmid; single production of Bliα |
| BLic5Δ*FGEHI* | pLic5Δ*FGEHI* | Replacement of *licFGEHI* genes in the pLic5 fosmid by an Apra$^r$ cassette; production of Bliα and Bliβ |
| BLic5Δ*A1*Δ*FGEHI* | pLic5Δ*A1*Δ*FGEHI* | Replacement of *licFGEHI* genes in the pLic5Δ*A1* fosmid by an Apra$^r$ cassette; single production of Bliβ |
| BLic5Δ*A2*Δ*FGEHI* | pLic5Δ*A2*Δ*FGEHI* | Replacement of *licFGEHI* genes in the pLic5Δ*A2* fosmid by an Apra$^r$ cassette; single production of Bliα |
| BWLic5Δ*FGEHI* | pLic5Δ*FGEHI* | *E. coli* BW25113 cells transformed with the pLic5Δ*FGEHI* fosmid |
| BWΔ*tolC*Lic5Δ*FGEHI* | pLic5Δ*FGEHI* | *E. coli* BW25113Δ*tolC*::*kan* cells transformed with the pLic5Δ*FGEHI* fosmid |

[a] Apra$^r$, apramycin resistance.

ture grown for 24 h. The pellet obtained after butanol evaporation was dissolved in aqueous acetonitrile (70%) solution and used in the bioactivity test. After an overnight incubation at 37°C, no inhibition zones were observed on BLic5 and BLic5Δ*FGEHI* strain plates, suggesting again that *licFGEHI* are not essential for the lichenicidin self-protection mechanism in Gram-negative *E. coli*.

In analogy with the mechanism of action of haloduracin and lacticin 3147 reported previously (8, 14), it is believed that lichenicidin exerts its activity by inhibiting the cell wall biosynthesis accompanied by pore formation in the cytoplasmic membrane. Lantibiotics such as nisin are generally not active against Gram-negative bacteria. It has been shown that this is due to the presence of the outer membrane (OM), since the alteration of its permeability by chelators or some physical treatments resulted in nisin sensitivity by several Gram negatives (1, 6, 12). Likewise, an *E. coli* strain with a permeable OM should be sensitive to the extracellular presence of a mixture of Bliα and Bliβ peptides. To prove this hypothesis, a culture of *E. coli* BLic5 in medium M (3) was performed, and its cell-free supernatant (containing both Bliα and Bliβ) was bioassayed against the *E. coli imp4213* strain and also its wild-type strain *E. coli*

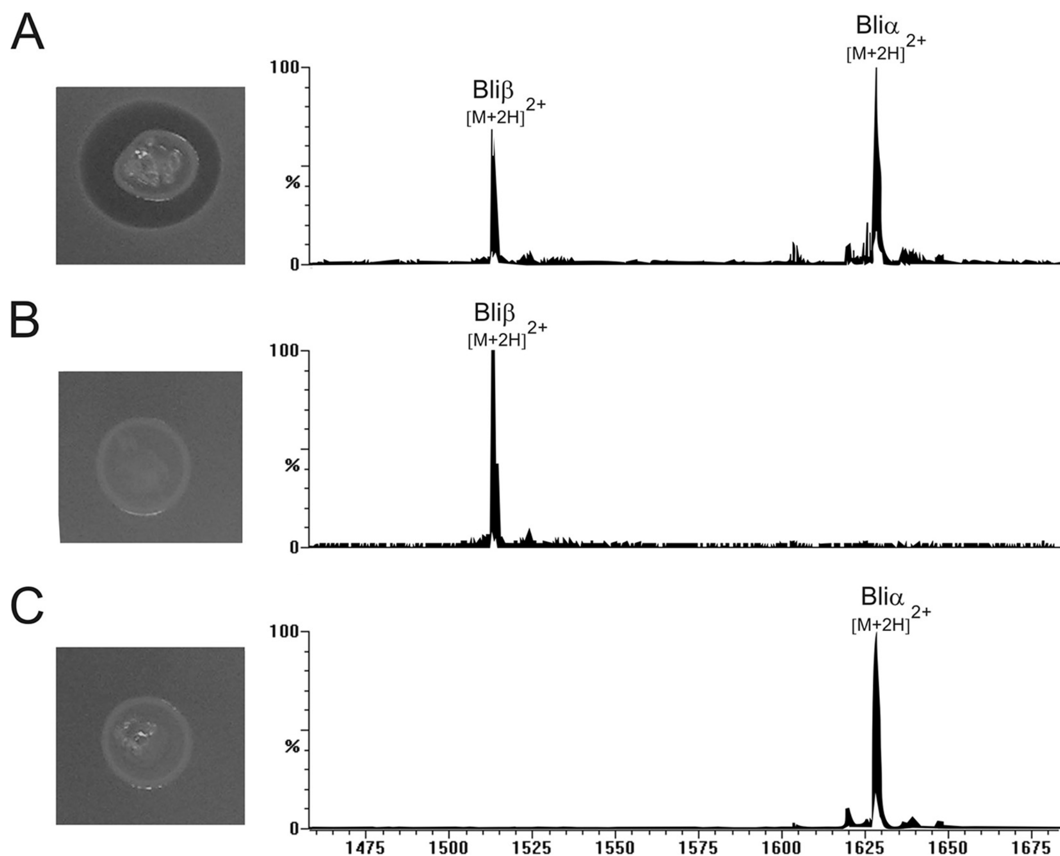


FIG. 1. Bioassay of *E. coli* mutants BLic5Δ*FGEHI* (A), BLic5Δ*A1*Δ*FGEHI* (B), and BLic5Δ*A2*Δ*FGEHI* (C) against the *M. luteus* indicator strain. LC-ESI-MS analyses of butanol extracts from the respective mutant liquid culture are also shown.

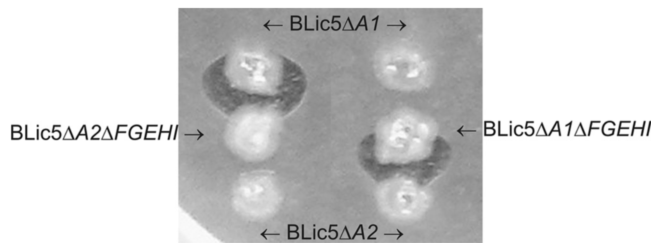


FIG. 2. Cross-feeding agar diffusion assay of the *E. coli* BLic5Δ*A1*Δ*FGEHI* and BLic5Δ*A2*Δ*FGEHI* mutants with the Bliα (BLic5Δ*A2*) and Bliβ (BLic5Δ*A1*) peptides. The bacterial activity restored between BLic5Δ*A1* and BLic5Δ*A2*Δ*FGEHI* suggested that this mutant was able to produce the Bli( peptide. Similarly, the *M. luteus* inhibition zone observed between BLic5Δ*A2* and BLic5Δ*A1*Δ*FGEHI* suggested that this mutant was able to produce the Bliβ peptide.

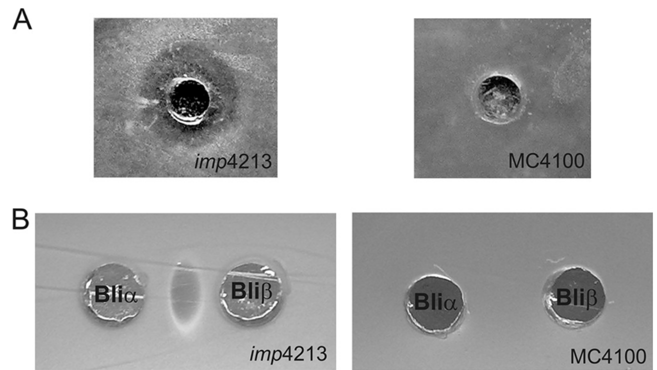


FIG. 3. Bioassay of lichenicidin peptides against the *E. coli imp4213* and MC4100 strains. (A) The *E. coli imp4213* strain is susceptible to *E. coli* BLic5 cell-free supernatant, containing both the Bliα and Bliβ peptides. (B) *E. coli imp4213* strain growth is inhibited only if both peptides are present. The extracts of the BLicΔ*A1* and BLicΔ*A2* strains include only Bliβ or Bli(, respectively.

MC4100. The *E. coli imp4213* strain possesses a mutation on the essential *lptD* (*imp*) gene, which causes an OM permeability defect (2) and thus sensitivity to a variety of antibiotics, including vancomycin, which is otherwise not active against Gram-negative strains (10, 15). An inhibition zone caused by the BLic5 supernatant was observed on the agar plates containing the *imp4213* strain, contrary to what was registered in the plates inoculated with the wild-type MC4100 strain, where no inhibition of growth was observed (Fig. 3). The activity of the individual peptides on these two strains was also examined. To that end, *E. coli* BLic5Δ*A1* (producing exclusively Bliβ) and *E. coli* BLic5Δ*A2* (producing exclusively Bliα) cell-free supernatants were bioassayed side by side on plates containing either the *E. coli imp4213* strain or the *E. coli* MC4100 strain. It was observed that *E. coli imp4213* strain growth was affected only if both Bliα and Bliβ peptides were present (Fig. 3). These findings proved that in fact, similarly to nisin, once both the Bliα and Bliβ peptides reach the *E. coli* periplasmic space, they could exert their activity. It was recently demonstrated that the TolC OM protein is related with the presence of the active lichenicidin peptides in the *E. coli* BLic5 supernatant (3). However, it was not determined whether their transport should involve a periplasmic sublocation. Together, our results suggest that either the peptides are exported through a type I system or at least one of the lichenicidin peptides needs to be inactive in the periplasmic space. In this case, the cooccurrence of Bliα and untrimmed Bliβ′ can be considered, where Bliβ′ would require extracellular processing by the LicP protease to achieve its Bliβ active form (3). The fact that it was possible to transform a *tolC*-deficient *E. coli* BW25113 with the pLic5Δ*FGEHI* fosmid (BWΔ*tolC*Lic5Δ*FGEHI* strain) (Table 1) also supports this hypothesis. After the bioassay, BWΔ*tolC*Lic5Δ*FGEHI* was not able to inhibit *M. luteus* growth, in contrast with the control strain (BWLic5Δ*FGEHI*).

Currently, little information is available concerning the lantibiotics' immunity systems. Nevertheless, for lacticin 3147, it was demonstrated that LtnI is an essential protein for the *Lactococcus lactis* immune phenotype most probably by preventing the insertion of the bacteriocins in the membrane or by interacting directly with the peptides, inactivating them (7). More recently, it was shown that both components of the ABC transporter, LtnFE, are also involved in lacticin 3147 self-protection. In this case, LtnFE should be involved in the transport of Ltnα and Ltnβ peptides that have crossed the cell wall of the Gram-positive producer (4). The application of such models to the *E. coli* lichenicidin expression system could help to explain the results herein reported. If Bliα and Bliβ are not exported simultaneously through the periplasmic space and once outside the cell, the peptides are unable to enter the cell, then at least the LicFEI proteins would be dispensable in such a system. Therefore, BLic5 and BLic5Δ*FGEHI* lichenicidin self-protection is more likely due to the Gram-negative cell wall structure rather than to the expression of *licFGEHI* immunity genes.

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