LlpB represents a second subclass of lectin-like bacteriocins

Maarten G. K. Ghequire* and René De Mot
Centre of Microbial and Plant Genetics, KU Leuven, Kasteelpark Arenberg 20 bus 2460, 3001 Heverlee, Belgium.

Summary
Bacteriocins are secreted bacterial proteins that selectively kill related strains. Lectin-like bacteriocins are atypical bacteriocins not requiring a cognate immunity factor and have been primarily studied in Pseudomonas. These so-called LlpAs are composed of a tandem of B-lectin domains. One domain interacts with D-rhamnose residues in the common polysaccharide antigen of Pseudomonas lipopolysaccharide (LPS). The other lectin domain is crucial for interference with the outer membrane protein assembly machinery by interacting with surface-exposed loops of its central component BamA. Via genome mining, we identified a second subclass of Pseudomonas lectin-like proteins, termed LlpB, consisting of a single B-lectin domain. We show that these proteins also display bactericidal activity. Among LlpB-resistant transposon mutants of an LlpB-susceptible Pseudomonas strain, a major subset was hit in an acyltransferase gene, predicted to be involved in LPS core modification, hereby suggesting that LlpBs equally attach to LPS for surface anchoring. This indicates that LPS binding and target strain specificity are condensed in a single B-lectin domain. The identification of this second subclass of lectin-like bacteriocins further expands the toolbox of antibacterial warfare deployed by bacteria and holds potential for their integration in biotechnological applications.

Introduction
Bacteriocins are secreted ribosomally encoded antibacterial peptides, proteins or multi-protein complexes that selectively kill phylogenetically related strains, thus facilitating the colonization of competitive environments. Among Gram-negative bacteria, bacteriocins from Escherichia coli (colicins) and Pseudomonas aeruginosa (pyocins) serve as model systems for studying receptor binding, cell import mechanisms and toxin-immunity interactions (Cascales et al., 2007; Papadakos et al., 2012; Ghequire and De Mot, 2014; Chassaing and Cascales, 2018). These compounds are potent antibacterials and their use in food and therapeutic applications is currently being investigated (Schulz et al., 2015; Paškevičius et al., 2017; Scholl, 2017; Schneider et al., 2018). Major advantages of bacteriocins include biodegradability, selective killing and eligibility (of some bacteriocins) for large-scale production in plants (Behrens et al., 2017; Ghequire and De Mot, 2018).

To date, four main classes of Pseudomonas bacteriocins have been described, highly diverse in molecular architecture and killing mechanism: R- and F-type tailocins (Ghequire and De Mot, 2015; Scholl, 2017), modular (or S-type) bacteriocins (Jamet and Nassif, 2015), B-type microcins (Metelev et al., 2013) and lectin-like bacteriocins (Ghequire et al., 2018b). The latter set of bacteriocins (also called LlpAs) are composed of two B-lectin domains followed by a short carboxy-terminal extension and share structural similarity with lectins from monocot plants (Ghequire et al., 2013; McCaughey et al., 2014). The carboxy-terminal lectin domain of these antibacterial proteins binds to D-rhamnose (McCaughey et al., 2014), the major constituent of the common polysaccharide antigen in the lipopolysaccharide (LPS) layer (Lam et al., 2011), in contrast to B-lectins from plants which show a much higher affinity for D-mannose (Barre et al., 1996). The amino-terminal lectin domain selectively interacts with the essential outer membrane protein BamA (Ghequire et al., 2018a). The latter protein acts as an insertase responsible for the...
integration of new proteins in the outer membrane (Noinaj et al., 2015). It remains unclear how LlpA interacts with the surface-exposed loops of BamA and how cellular killing is achieved. Given the lack of a distinct toxin domain and cognate immunity factor as found in modular bacteriocins (Sharp et al., 2017), LlpA killing is likely initiated upon contact with the outer membrane. This way no subsequent bacteriocin import, as is the case for modular bacteriocins, would be required (White et al., 2017). Several other hypothetical prokaryotic proteins in which a B-lectin domain is combined with (an)other domain(s) have been identified (Ghequire et al., 2012b). For a protein with an amino-terminal B-lectin domain fused to a putative peptidase domain, bacteriocin activity has been described: albusin B from ruminal bacterium Ruminococcus albus 7 kills Ruminococcus flavefaciens (Chen et al., 2004). However, how these domains contribute to bacteriocin activity has not been studied. Homologues of this bacteriocin gene are present in some other strains of this Firmicutes species (Azevedo et al., 2015). In Mycobacterium smegmatis MCstä155, a protein consisting of a B-lectin and a LysM domain has been described (Patra et al., 2011), though it remains unclear whether this compound serves a role in bacterial antagonism.

In this paper, we report on the bacteriocin activity of a distinct type of Pseudomonas lectin-like protein, termed LlpB, consisting of a single B-lectin domain and a short carboxy-terminal extension. Characterization of transposon mutants resistant to an LlpB from a Pseudomonas fluorescens strain indicates that target recognition involves LPS of susceptible cells.

**Results and discussion**

**LlpB: a distinct type of lectin-like protein in Pseudomonas**

Using proteobacterial B-lectin modules (Pfam PF01453) of Pseudomonas LlpAs as search queries, BlastP homology searches previously revealed a second group of lectin-like proteins in pseudomonads (Ghequire et al., 2012b; Loper et al., 2012; Ghequire and De Mot, 2014). These proteins (~19.8 kDa) consist of a single B-lectin domain and a carboxy-terminal extension of ~32 AA. The latter stretch is poorly conserved but typified by a number of basic and aromatic residues (Fig. S1), similarly to Pseudomonas LlpAs (Ghequire et al., 2013). Phylogenetic analysis shows that the predicted lectin modules of these proteins, further called LlpBs, cluster with the amino-terminal domains of LlpAs, acting as target selectivity determinants in these bacteriocins (Ghequire et al., 2013). The LlpB sequences fall apart in two distinct branches, of which the smaller one is exclusively populated by representatives belonging to the P. fluorescens species group (Fig. 1). As seen for LlpAs, the putative sugar-binding motifs in LlpBs display strongly differing degrees of sequence conservation, with the first and last of the tree pockets being well conserved (Fig. S1).

**Fig. 1.** Maximum likelihood phylogenetic tree of B-lectin domains from LlpA and LlpB proteins in Pseudomonas, characterized LlpA and B-lectin domain-containing proteins retrieved in other bacteria, and select B-lectin mono-domain proteins in other bacteria. The domain architecture is specified by a schematic representation, and domains are coloured according to function (see colour legend in box). Amino-terminal and carboxy-terminal lectin domains from LlpAs and lectin domains from LlpBs are shown on a yellow, red and blue background, respectively. B-lectin domains from LlpBs cluster with the amino-terminal domain of LlpAs. In the case of LlpAs, the B-lectin domain shown in the respective cluster is highlighted by a glowing background. Highly similar sequences (>75% pairwise amino acid sequence identity for full length LlpA/LlpB proteins) are represented by one sequence only. Previously characterized proteins with a B-lectin domain are labelled in blue, and LlpBf proteins containing the respective cluster (a) and (b). Phylogenetic analysis was performed with PhyML, using the JTT substitution model. Bootstrap values (percentages of 1000 replicates) higher than 50 are shown at the branches. The tree is rooted to the amino-terminal B-lectin domain of the LlpA from Burkholderia ambifaria MEX-5. Scale bar represents 0.5 substitutions per site. Bamb, Burkholderia ambifaria; Bcer, Bacillus cereus; Bsp, Burkholderia sp.; Cvac, Chromobacterium vaccinii; Ksp, Kitasatospora sp.; Msme, Mycobacterium smegmatis; Paer, Pseudomonas aeruginosa; Pasp, Paraburkholderia sp.; Pcon, Pseudomonas congelaens; Ppel, Paenibacillus elginii; Pfl, Pseudomonas florensis; Pfli, Pseudomonas fluorescens; Pfre, Pseudomonas frederiksbergensis; Pgpa, Pseudomonas gamsinis; Plib, Pseudomonas libanensis; Pmos, Pseudomonas mosselii; Pory, Pseudomonas oryzihabitans; Ppr, Pseudomonas protegens; Ppsy, Pseudomonas psychrophila; Pput, Pseudomonas putida; Psap, Pseudomonas sp.; Psyr (syr), Pseudomonas syringae (pathovar syringae); Pvv, Pseudomonas viridiflava; Rabl, Ruminococcus albus; Rsp, Rathayibacter sp.; Sanm, Streptacidiphilus anmyonensis; Ssp, Sphingobium sp.; Ssp, Streptomyces sp.; Tavi, Tumebacillus avium; Xcit, Xanthomonas citri.

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genes are organized in tandem, whereas \( \textit{lpA} \) genes in strains carrying two representatives usually appear at distant loci (Ghequire and De Mot, 2014). As noted for other (mid-sized) bacteriocins (Ghequire \textit{et al.}, 2015, 2017a,b; Dingemans \textit{et al.}, 2016; Sharp \textit{et al.}, 2017), \( \textit{lpB} \) genes are typified by a lower GC content than the genomic average (~47% versus ~60%), pointing towards foreign origin. Yet another similarity with \( \textit{lpA} \) genes is that some of these \( \textit{lpB} \) genes arise in prophage/tailocin clusters (Ghequire \textit{et al.}, 2015), for example in a Rp3

\[\text{B-lectin} \quad \text{Peptidase M15} \quad \text{C-terminal extension} \quad \text{LysM}\]

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tailorin gene cluster of *Pseudomonas libanensis* DSM 17149. Such association is confined to the minor branch of *llpB*-carrying isolates (Fig. 1). In contrast, in the large clade they mainly occur at two other loci: downstream of sulphate adenyllytransferase *cysN* or downstream of a flavin monoamine oxidase gene (data not shown). In some strains, a modular bacteriocin-immunity gene tandem is integrated between *cysN* and *llpB*, for example in *P. putida* MTCC 5279 (putative HNH DNase toxin), underlining the plasticity of the locus. Taken together, the striking parallels of *LlpBs* with other bacteriocins suggest that these proteins may also exert an antibacterial function. To explore this further, representative and divergent *LlpBs* (Fig. 1) from biocontrol strain *P. fluorescens* A506 (Loper *et al.*, 2012) and plant growth-promoting rhizobacterium *Pseudomonas* sp. UW4 (Duan *et al.*, 2013) were selected for further characterization.

**Bacteriocin activity of LlpBs**

*llpB* genes from strains A506 (locus_tag PfIA506_2041) and UW4 (locus_tag PPUTUW4_RS25815, codon-optimized) were PCR-amplified, digested and cloned in pET28a to encode an amino-terminal His6-tagged protein (primers in Table S1), resulting in pCMPG6205 and

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![Fig. 2. SDS-PAGE electrophoresis of purified recombinant LlpB proteins from strains *P. fluorescens* A506 and *Pseudomonas* sp. UW4. Lane 1, Precision Plus Dual Xtra size marker (kDa); lane 2, LlpBPfIA506 (~19 kDa, predicted size 20.6 kDa); lane 3, LlpBPPUTUW4 (~21 kDa, predicted size 21.4 kDa).](image)

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### Table 1. Antibacterial activity of purified recombinant LlpBs against *Pseudomonas* isolates.

| Indicator strain | LlpBPfIA506 | LlpBPPUTUW4 |
|------------------|-------------|-------------|
| *P. aeruginosa* group |             |             |
| *P. aeruginosa* LMG 1242 | –            | –           |
| *P. aeruginosa* ATCC27853 | –            | –           |
| *P. aeruginosa* PA01 | –            | –           |
| *P. aeruginosa* UCBPP-PA14 | –            | –           |
| *P. resinovorans* LMG 2274 | –            | –           |
| *P. fluorescens* complex |             |             |
| *P. chlororaphis* subsp. aureofaciens LMG 1245 | –            | –           |
| *P. chlororaphis* subsp. chlororaphis LMG 5004 | –            | –           |
| *P. fluorescens* 2-79 | –            | –           |
| *P. fluorescens* 13-79 | –            | –           |
| *P. fluorescens* A1-8 | –            | –           |
| *P. fluorescens* CC-B84406-E | –            | –           |
| *P. fluorescens* F113 | –            | –           |
| *P. fluorescens* LMG 1794 | –            | +           |
| *P. fluorescens* LMG 2210 | –            | –           |
| *P. fluorescens* OE 39.4 | –            | –           |
| *P. fluorescens* OE 48.2 | –            | –           |
| *P. fluorescens* P10-1 | –            | –           |
| *P. fluorescens* PGSB 7705 | +            | T           |
| *P. fluorescens* PGSB 7716 | –            | –           |
| *P. fluorescens* PGSB 7947 | –            | –           |
| *P. fluorescens* PGSB 8301 | –            | +           |
| *P. fluorescens* PGSB 8472 | –            | –           |
| *P. fluorescens* SBW25 | –            | –           |
| *P. fluorescens* WCS141 | –            | –           |
| *P. fluorescens* WCS365 | T            | –           |
| *P. protegens* CHA0 | –            | –           |
| *P. tolaasii* CH36 | –            | –           |
| *P. tolaasii* LMG 2342 | –            | –           |
| *P. tolaasii* LMG 2344 | –            | –           |
| *P. putida* group |             |             |
| *P. putida* KT2440 | –            | –           |
| *P. putida* LMG 2257 | –            | –           |
| *P. putida* OE 53.2 | –            | –           |
| *P. putida* WCS358 | –            | –           |
| *P. stutzeri* group |             |             |
| *P. stutzeri* LMG 11199 | –            | +           |
| *P. stutzeri* LMG 1228 | +            | +           |
| *P. syringae* group |             |             |
| *P. cichorii* LMG 2162 | T            | –           |
| *P. savastanoi* LMG 2209 | –            | –           |
| *P. savastanoi* LMG 5154 | –            | –           |
| *P. savastanoi* LMG 5485 | –            | –           |
| *P. savastanoi* LMG 6768 | –            | –           |
| *P. savastanoi* LMG 17581 | –            | –           |
| *P. syringae* GR12-2R3 | +            | +           |
| *P. syringae* pv. glycinea LMG 5066 | +            | +           |
| *P. syringae* pv. syringae LMG 1247 | –            | –           |
| *P. syringae* pv. tabaci LMG 5192 | –            | –           |
| *P. syringae* pv. tomato DC3000 | –            | –           |
| *P. viridiflava* LMG 2352 | +            | +           |
| Other *Pseudomonas* spp. |             |             |
| *P. agarici* LMG 2112 | –            | –           |
| *P. mendocina* LMG 1223 | –            | –           |

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a. Growth inhibition due to Lbp bacteriocin activity was scored as follows: +, clear halo; T, turbid halo; –, no zone of growth inhibition. Running buffer was used as a negative control.

b. Of the strains used in the test panel (and for which genome sequence information is available), only *P. protegens* CHA0 carries an *llpA* gene in its genome. No strain contains an *llpB* gene.

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pCMGP6207, respectively. Sequence-verified plasmids (GATC Biotech, Constance, Germany) were transformed to *E. coli* BL21(DE3). Cells grown in 500-ml LB erlenmeyers were induced with isopropyl-β-D-thiogalactopyranoside and incubated overnight, as described earlier (Ghequire *et al.*, 2012a). After, cells were harvested, dissolved and sonicated, and soluble proteins isolated via centrifugation. His-tagged proteins were purified via affinity chromatography on Ni-NTA agarose. The presence of recombinant protein in the imidazole-eluted fractions was confirmed via SDS-PAGE, and samples were further polished by gel filtration. The calculated molecular weights of His6-tagged LlpBs (20.6 kDa LlpBP*<i>*sp*UW4*; 21.4 kDa LlpBP*<i>*P.*aeruginosa* PA506*) match well with the apparent sizes of the recombinant proteins as estimated by SDS-PAGE (Fig. 2).

Antagonistic activity of the LlpBs was evaluated via spot assay against a panel of pseudomonads, including several *Pseudomonas* reference strains. Ten-μl drops of recombinant protein (concentration 1 mg ml<sup>−1</sup>) were applied onto bacterial cell lawns, incubated overnight, and scored for the presence of zones of growth inhibition the following day (Hockett and Baltrus, 2017). For both LlpBs, eight out of 49 strains in the *Pseudomonas* test panel proved susceptible (Table 1), confirming the bactericidal function of these proteins. Five strains were killed by both LlpBs, despite their low sequence identity (~34%). As seen for other *Pseudomonas* bacteriocins (LlpAs and other (non-*P.*aeruginosa) bacteriocins) (Ghequire et al., 2012a, 2015), LlpB activity surpasses species boundaries: the bacteriocins from *P. fluorescens* AS06 and *Pseudomonas* sp. UW4 (*P.*jessenii group (Gomila *et al.*, 2015; Garrido-Sanz *et al.*, 2016)) both also kill strains from the *P.*stutzeri and *P.*syringae groups.

**Genes affected in LlpB-resistant mutants indicate a key role of LPS in target cell susceptibility**

The first and last of the three sugar-binding motifs in LlpBs show sequence similarity with the consensus motif accounting for α-mannose binding in plant lectins, OxDxNxVxY (Ghequire *et al.*, 2012b). Given the role assigned to α-rhamnose as a ligand for LlpAs, we hypothesized that one or both of these lectin motifs in LlpBs may bind to carbohydrates from lipopolysaccharides as well, enabling target cell attachment in a similar way.

In search for susceptibility determinants of LlpB killing, a mutant library was created in *P. fluorescens* LMG 1794<sup>T</sup> (sequenced as NCTC10038<sup>T</sup>) using transposon delivery vector pRL27 (Larsen et al., 2002), via tri-parental conjugation. Transposon mutants were pooled, supplemented with concentrated LlpBP<sub>P.*aeruginosa* PA506</sub> (~5 mg ml<sup>−1</sup>), and subsequently plated. Following day, colonies were selected, verified for bacteriocin resistance and transposon insertion sites determined, as described earlier (Ghequire et al., 2017b). Interestingly, of the 34 (independent) LlpB-resistant mutants isolated, 24 were hit in an acyltransferase gene oatA (NCTC10038_05872) (Fig. 3). The encoded protein shares 27% amino acid identity with oatA, previously studied in *Salmonella* Typhimurium and shown to function as an O-antigen acetylase (Slauch *et al.*, 1996). Gene synteny and significant sequence similarity (48% pairwise amino acid identity) can be noted for PA5238 from *Pseudomonas aeruginosa* PAO1. Lipopolysaccharide acetylation activity has been proposed for the latter enzyme (King *et al.*, 2009), but remains to be verified. The repeating units constituting the O-specific polysaccharide chains of LPS in *P. fluorescens* LMG 1794 have been determined and consist of α-rhamnose and N-acetyl-α-fucose (Vere-meirinko *et al.*, 2005). Given that PA5238 was suggested to play a role in O-acetylation of the LPS core and not of the repeating units (King *et al.*, 2009), we thus do not expect these two carbohydrate residues to interact with LlpBP<sub>P.*aeruginosa* PA506</sub>. It remains to be assessed whether other LlpBs equally depend for killing on the activity of this acyltransferase gene in target cells, which would be expected if these lectin-like bacteriocins share a common LPS moiety as receptor. It should be emphasized that polar effects on the two genes downstream of NCTC10038_05872 cannot be excluded a priori, though the multiple transposon insertions independently hitting...
oatA argue against this. When evaluating our strain panel for the presence of oatA and oatA-like genes, we found that the majority of the strains (for which a full or draft genome is available, 23/30) encodes such an acyltransferase, including all the strains killed by one or both of the LipBs.

A second set of seven transposon mutants were hit in an operon that is possibly involved in LPS biogenesis as well (Fig. 3). This cluster is conserved in *Pseudomonas* species, but apparently lacks from *P. aeruginosa* genomes. In LPS of *P. fluorescens NCTC10038*, different amino sugars have been detected (Wilkinson, 1972), which may require dat aminotransferase activity. Whether this second cluster plays a role in LPS biosynthesis remains speculative however. In the nearby future, chemical characterization of LPS constituents of different mutants obtained in this study will shed further light on the carbohydrates interacting with LipB. Whether BamA or (an)other outer membrane protein(s) contribute to LipB killing also remains to be investigated.

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**Conflict of interest**

None declared.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Multiple sequence alignment of LlpBs included in Figure 1.  
**Table S1.** Primers used in this study.