1 **Innolysins: A novel approach to engineer endolysins to kill Gram-negative bacteria**

2 Athina Zampara^a, Martine C. Holst Sørensen^a, Dennis Grimon^b, Fabio Antenucci^a, Yves Briers^b and
3 Lone Brøndsted^{a#}

4

5 Department of Veterinary and Animal Sciences, University of Copenhagen, Stigbøjlen 4, 1870

6 Frederiksberg C, Denmark^a; Department of Biotechnology, Ghent University, Valentin

7 Vaerwyckweg 1, 9000 Gent, Belgium^b

8

9 Running Head: *Innolysins target Gram-negative bacteria*

10 #Address correspondence to Lone Brøndsted: lobr@sund.ku.dk

11 Keywords: endolysins, receptor binding proteins, engineering, outer membrane, Gram-negative
12 bacteria

ABSTRACT

Bacteriophage-encoded endolysins degrading the essential peptidoglycan of bacteria are promising alternative antimicrobials to handle the global threat of antibiotic resistant bacteria. However, endolysins have limited use against Gram-negative bacteria, since their outer membrane prevents access to the peptidoglycan. Here we present Innolysins, a novel concept for engineering endolysins that allows the enzymes to pass through the outer membrane, hydrolyse the peptidoglycan and kill the target bacterium. Innolysins combine the enzymatic activity of endolysins with the binding capacity of phage receptor binding proteins (RBPs). As our proof of concept, we used phage T5 endolysin and receptor binding protein Pb5, which binds irreversibly to the phage receptor FhuA involved in ferrichrome transport in *Escherichia coli*. In total, we constructed twelve Innolysins fusing endolysin with Pb5 or the binding domain of Pb5 with or without flexible linkers in between. While the majority of the Innolysins maintained their muralytic activity, Innolysin#6 also showed bactericidal activity against *E. coli* reducing the number of bacteria by 1 log, thus overcoming the outer membrane barrier. Using an *E. coli fhuA* deletion mutant, we demonstrated that FhuA is required for bactericidal activity, supporting that the specific binding of Pb5 to its receptor on *E. coli* is needed for the endolysin to access the peptidoglycan. Accordingly, Innolysin#6 was able to kill other bacterial species that carry conserved FhuA homologs such as *Shigella sonnei* and *Pseudomonas aeruginosa*. In summary, the Innolysin approach expands recent protein engineering strategies allowing customization of endolysins by exploiting phage RBPs to specifically target Gram-negative bacteria.

33 **IMPORTANCE**

34 The extensive use of antibiotics has led to the emergence of antimicrobial resistant bacteria
 35 responsible for infections causing more than 50,000 deaths per year across Europe and the US. In
 36 response, the World Health Organization has stressed an urgent need to discover new antimicrobials
 37 to control in particular Gram-negative bacterial pathogens, due to their extensive multi-drug
 38 resistance. However, the outer membrane of Gram-negative bacteria limits the access of many
 39 antibacterial agents to their targets. Here, we developed a new approach, Innolysins that enable
 40 endolysins to overcome the outer membrane by exploiting the binding specificity of phage receptor
 41 binding proteins. As proof of concept, we constructed Innolysins against *E. coli* using the endolysin
 42 and the receptor binding protein of phage T5. Given the rich diversity of phage receptor binding
 43 proteins and their different binding specificities, our proof of concept paves the route for creating an
 44 arsenal of pathogen specific alternative antimicrobials.

45 INTRODUCTION

46 The limited permeability of the outer membrane is a major obstacle for development of novel
 47 antimicrobials against Gram-negative pathogens preventing many compounds from reaching their
 48 intracellular targets (1). Bacteriophages (phages), viruses that infect bacteria, have naturally
 49 evolved mechanisms to overcome the outer membrane to infect their bacterial hosts (2, 3). In the
 50 first step of infection, phages bind to host cells and inject their genetic material across the outer and
 51 inner membrane of the bacterial cells into the cytoplasm. Also, during the final stage of the lytic
 52 infection cycle, phages produce proteins within the cell, which destroy the bacterial cell wall,
 53 leading to cell lysis. Thus, the molecular tools developed during phage evolution may be exploited
 54 to develop novel phage-based antimicrobials that are able to pass the outer membrane and to kill
 55 Gram-negative bacteria.

56 Phages recognize their host bacteria by binding to specific surface receptors that may be outer
 57 membrane proteins, lipopolysaccharides or components of bacterial capsules, pili and flagella (4).
 58 The adhesion specificity is mediated by receptor binding proteins (RBPs) that form fibers or spikes
 59 at the distal phage tail. A well-characterized RBP is Pb5, located at the tail tip of the phage T5,
 60 which specifically binds to the bacterial receptor FhuA during infection of the *E. coli* host (5, 6).
 61 FhuA is an outer membrane protein that actively transports siderophore-ferrichrome and allows *E.*
 62 *coli* to take up iron from the environment. The crystal structure of FhuA reveals two domains, a 22-
 63 stranded anti-parallel barrel that forms a hollow channel with eleven surface-exposed loops and a
 64 globular domain, known as the plug, which blocks the channel in its inactive state (7, 8). Previously
 65 it was shown that Pb5 of phage T5 binds irreversibly to the extracellular loop L4 and the plug of
 66 FhuA. In addition, it was proposed that FhuA acts as an anchor for phage T5 binding to *E. coli*,
 67 subsequently allowing the tail fiber composed of Pb2 to transverse the outer membrane and inject
 68 the DNA (9, 10).

69 During the last stage of phage infection, phages produce endolysins to lyse the bacterial cells and
70 release progeny phages. Endolysins are hydrolytic enzymes that, after gaining access to the
71 periplasm, degrade the peptidoglycan, leading to cell lysis (11). Endolysins may have different
72 catalytic activities, depending on the bond that they target in the peptidoglycan, and are classified as
73 glycosidases, amidases or endopeptidases. The endolysin encoded by phage T5 is a well
74 characterized endopeptidase that hydrolyzes the bond between L-alanine and D-glutamic acid of the
75 peptidoglycan in *E. coli* (12). While native endolysins have been successfully applied as
76 antimicrobials against Gram-positive bacteria including *Staphylococcus aureus* and streptococci,
77 the outer membrane of Gram-negative bacteria generally prevents access to the peptidoglycan layer
78 (13). To enable killing of Gram-negative pathogens using endolysins, a number of engineering
79 strategies have been proposed. Recently it was shown that the fusion of endolysins to polycationic
80 or amphipathic peptides enables the endolysins to pass the outer membrane and kill multi-drug
81 resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (14). Furthermore, recombinant
82 T4 lysozyme carrying the binding domain of pesticin, targeting the outer-membrane protein FyuA,
83 has been shown to kill *Yersinia* and pathogenic *E. coli* strains (15). Similarly, fusion of the *E. coli*
84 phage endolysin Lysep3 with the translocation and receptor-binding domain of another bacteriocin
85 colicin A, targeting the outer membrane protein BtuB could kill *E. coli* (16). Thus, these studies
86 have demonstrated that endolysins can be engineered to pass the outer membrane and subsequently
87 kill Gram-negative bacteria.

88 Here, we expand on the concept of delivering endolysins across the Gram-negative outer membrane
89 by combining the specificity of phage RBPs with the antimicrobial activity of endolysins into RBP-
90 endolysin hybrids (Innolysins). To provide a proof of concept, we utilize the endolysin and the RBP
91 (Pb5) of phage T5. Pb5 was selected among many phage RBPs due to the specific and stable
92 binding of Pb5 to its cognate protein receptor allowing us to target the fused phage T5 endolysin to

93 the periplasm, where it can degrade the peptidoglycan. This engineering approach has the potential
94 to utilize multiple distinct phage-derived RBPs to customize the bactericidal spectrum of Innolysins
95 against diverse Gram-negative bacteria.

96 **RESULTS**

97 **Strategy for construction of Innolysins**

98 To construct Innolysins, we combined the RBP of phage T5 (Pb5) with the phage T5 endolysin (T5
 99 Lys) for targeted delivery of the endolysin. The binding domain of Pb5 has previously been shown
 100 to be located in the N-terminus (488aa) of the protein (17). To determine whether the binding
 101 domain of the Pb5 is sufficient to enable the endolysin to pass through the outer membrane, we
 102 fused T5 endolysin with both the entire Pb5 and with the Pb5 binding domain (Pb5₁₋₄₈₈). We
 103 anticipated that antimicrobial activity of an Innolysin requires both that Pb5 is able to bind to the
 104 outer membrane protein FhuA, and that the fused phage T5 endolysin is translocated across the
 105 outer membrane and remains active to degrade the peptidoglycan. Thus, to ensure that the joined
 106 domains remain functional after fusion, we either fused them directly or added linkers in between.
 107 We used flexible linkers composed of small non-polar amino acids, glycine and alanine, providing a
 108 certain degree of movement of the fused domains (18). To optimize the two-domain cooperation,
 109 we used linkers of two different sizes, L1 composed of six amino acids and L2 that consists of
 110 fourteen amino acids. In addition, to investigate the optimal orientation of the endolysin and RBP
 111 domains, we constructed the Innolysins in two directions with and without linkers (Fig. 1). As such,
 112 a total of twelve Innolysins were constructed.

113 **Innolysins show muralytic activity**

114 To demonstrate that the muralytic activity of the endolysin is maintained after the fusion with the
 115 RBP and a linker, the fused proteins were expressed in *E. coli* BL21 and supernatants of cell lysates
 116 were tested for muralytic activity. A standardized assay for analysis of the muralytic activity of
 117 endolysins acting against peptidoglycan of Gram-negative bacteria was used, based on outer
 118 membrane permeabilized *P. aeruginosa* cells, which share a common peptidoglycan chemotype
 119 (A1γ) with *E. coli* (19). The majority of Innolysins (nine out of twelve) were active with enzymatic

activity ranging between 126-771 U/ml. Innolysin#9 (Pb5-L1-T5Lys) showed the highest activity per ml cleared lysate, reaching the muralytic activity of phage T5 endolysin alone (795 U/ml) (Fig. 2). Although expression was confirmed for all constructs, three of the Innolysins (#1, #3 and #12) and Pb5 alone did not show any significant activity compared to the negative control (muralytic activity of supernatant cell lysates carrying the empty vector, pVTSD2). Interestingly, both Innolysin#1 and #3 were composed of phage T5 endolysin and the whole Pb5 protein, fused in two opposite directions without a linker. Overall, the majority of hybrid endolysins showed muralytic activity after fusion with Pb5 either in the N- or C-terminus, while the presence of a flexible linker enhanced the activity of the engineered proteins.

Innolysin#6 inhibits *E. coli* growth

To assess whether binding of Pb5 allowed the fused endolysin to get access to the peptidoglycan, we screened the muralytically active Innolysins for their ability to inhibit growth of the phage T5 bacterial host *E. coli* ATCC11303. ATCC11303 was mixed with supernatants of cell lysates of strains expressing Innolysins, and bacterial growth was measured spectrophotometrically after 18h (Fig. 3). Growth inhibition was determined as the lack of growth of a start inoculum treated with an Innolysin compared to the negative control (growth of ATCC11303 cells treated with supernatant lysates of cells carrying the empty vector, pVTSD2). A significant inhibitory activity was noticed when *E. coli* ATCC11303 was treated with Innolysin#6 similar to the positive control, Art-175, an engineered endolysin that was previously shown to have both an inhibitory and bactericidal effect against *E. coli* (20, 21). The remaining eight Innolysins, Pb5 or endolysin alone did not significantly affect the *E. coli* growth compared to the negative control (Fig. 3). The screening resulted in one promising antimicrobial candidate out of the twelve Innolysins that could inhibit *E. coli* growth, indicating that domains have to be fused in a specific order and with a specific linker to acquire antibacterial activity after fusion. The active variant was composed of T5 endolysin in the N-

terminus linked by a six amino-acid linker to the Pb5₁₋₄₈₈ in the C-terminus. This result indicates that the binding domain of Pb5 alone can target the bacterial cells and allow phage T5 endolysin to overcome the outer membrane and inhibit *E. coli* growth.

Killing efficiency of Innolysin#6 requires FhuA, but not energy

To determine whether binding of Pb5 to FhuA is required for the antimicrobial activity of Innolysin#6, killing efficiency of the purified hybrid protein was tested on a *fhuA* deletion mutant in *E. coli* BL21 and compared to the wild type *E. coli* BL21 and *E. coli* ATCC11303. Bacterial cells were incubated with the purified Innolysin at a final concentration of 0.2 mg/ml and the reduction in cell counts was compared to the non-treated cells (Fig. 4). *E. coli* BL21 cell counts were reduced by 1.22 ± 0.12 log after Innolysin treatment and a similar decrease was noticed in *E. coli* ATCC11303 cells (1.09 ± 0.15 log reduction), without prior optimization. No significant effect of the Innolysin was shown in *E. coli* BL21 Δ *fhuA*, supporting our hypothesis that the Innolysin leads to peptidoglycan degradation when cells harbor the Pb5 receptor FhuA. While FhuA uptake of ferrichrome requires energy from the cytoplasmic proton motive force transduced to the outer membrane via the TonB protein, phage T5 interacts with FhuA independent of TonB (8, 22). To determine whether activity of Innolysin#6 requires such energy, bactericidal activity was tested against an *E. coli* *tonB* deletion mutant (ECOR4 Δ *tonB*) and the wild type ECOR4 as a positive control (Fig 4). As expected, TonB was not required for bactericidal activity of the Innolysin (Fig. 4). Our combined data demonstrate that Innolysin#6 requires the presence of FhuA in order to be transported passively through the outer membrane and target the peptidoglycan, leading to killing of *E. coli*.

Morphological changes in cells treated with Innolysin

Transmission Electron Microscopy (TEM) analysis was performed to determine the effects of Innolysin#6 on cell morphology and viability. The morphology of both *E. coli* BL21 and *E. coli*

BL21 Δ fhuA was visualized after incubation with Innolysin#6 for 15 min and compared to untreated cells (Fig. 5). Almost all untreated cells were intact with normal cell envelope morphology. In contrast, treatment of *E. coli* BL21 with Innolysin#6 led to cell integrity damage in the majority of cells with cytosol leakage occurring mainly at the poles. Furthermore, periplasmic space appeared to widen with the outer membrane disconnecting from the inner membrane and cell debris could be detected likely due to cell lysis. This dramatic effect on cell morphology was not observed on *E. coli* BL21 Δ fhuA treated with Innolysin#6, where only a low increase in damaged cells was noticed compared to the untreated cells. These observations demonstrate that Innolysin#6 acts rapidly by interfering with membrane integrity, while presence of FhuA is required for Innolysin to exert high antimicrobial activity.

Innolysin targets FhuA homologs of other than *E. coli* species

To investigate whether the constructed Innolysin#6 could target FhuA homologs in other species, we tested the killing activity of the purified engineered protein against *Shigella sonnei*, *Salmonella* Typhimurium LT2c and *Pseudomonas aeruginosa* PAO1 (Fig. 6). These bacteria carry FhuA homologs with identity ranging between 22.6 and 99.6% to the FhuA of *E. coli* BL21 (see Table S2 in the supplemental material). The Innolysin killed *S. sonnei* (99.6% FhuA identity at the protein level) and *P. aeruginosa* PAO1 (less than 39% FhuA identity), leading to average log reductions in cell number of 1.52 ± 0.14 and 1.03 ± 0.25 , respectively. In contrast, no significant reduction of *Salmonella* Typhimurium LT2c was noticed, despite the high similarity between the FhuA homolog in this strain and FhuA of *E. coli* BL21 (76.53% FhuA identity). Alignment of FhuA homologs showed that in comparison to FhuA of *S. sonnei* and *E. coli*, FhuA of *Salmonella* Typhimurium LT2c lacks 17 amino acids which are part of the surface-exposed L4 loop of *E. coli* FhuA (Fig. 7) (8). *P. aeruginosa* PAO1 lacks FhuA, however, it harbors other outer membrane proteins involved in the bacterial iron-uptake, including FpvA, FpvB, FptA, FiuA and FoxA (23). Alignment of *E.*

192 *coli* FhuA with these proteins revealed a low percent identity ranging between 22.61 and 38.52%
 193 (see Table S2). Notably, the amino-acid sequence responsible for L4 loop formation of FhuA
 194 aligned to parts of the outer membrane proteins FptA and FpvA of *P. aeruginosa* (Fig. S1) which,
 195 based on their tertiary structure predictions, form surface-exposed loops (24, 25). This observation
 196 suggests that such loops may interact with the Innolysin. In summary, we conclude that the
 197 Innolysin can target FhuA homologs in other than *E. coli* species.

198 **DISCUSSION**

199 Phages have developed unique and complex mechanisms to infect and lyse bacteria. In the first
 200 stage of infection, phages utilize receptor binding proteins (RBPs) to target specific bacterial host
 201 receptors on the cell surface, whereas at later stages phage endolysins degrade the peptidoglycan
 202 layer inducing lysis and progeny release. Here, we exploit the binding capacity of a phage RBP to
 203 enable an endolysin to bypass the outer membrane of Gram-negative bacteria and to gain access to
 204 peptidoglycan. As proof of concept, we have utilized the phage T5 endolysin and its receptor
 205 binding protein Pb5 and constructed twelve Innolysins by fusing the endolysin with the whole Pb5
 206 or with only the binding domain of Pb5 in different orientations, with or without linkers. The
 207 majority of the novel Innolysins maintained their muralytic activity and Innolysin#6 also displayed
 208 antimicrobial activity against *E. coli*. Our work expands the concept of developing alternative
 209 antimicrobials based on enzyme fusions and demonstrates that a phage RBP efficiently can be used
 210 to target antimicrobial enzymes selectively to Gram-negative bacterial species.

211 Here we showed that the bactericidal activity of Innolysin#6 consisting of T5 endolysin, a small
 212 linker and the binding domain of Pb5, is dependent on FhuA receptor of phage T5 (5, 6). Similar to
 213 phage T5 binding and DNA injection (22, 26, 27), the antimicrobial activity of Innolysin#6 is
 214 independent of energy provided by TonB, indicating that Innolysin#6 is passively transported to the
 215 peptidoglycan. Microscopic analysis revealed that Innolysin#6 causes cytosol leakage mainly from
 216 the bacterial poles of wild type *E. coli* encoding FhuA. Interestingly, several phages have been
 217 shown to preferentially adsorb at the bacterial poles, including *E. coli* phage ϕ 80 that uses FhuA as
 218 receptor (28). Thus, Innolysin#6 may bind to FhuA of *E. coli* in a similar manner as phage T5
 219 causing specific lysis of cells carrying the FhuA receptor of Pb5.

220 Since FhuA is the binding target of Innolysins and works as a transport channel for uptake of

221 ferrichrome, it is tempting to speculate that Innolysin is transported passively through the channel.
 222 Yet, it has been shown that Pb5 binding to FhuA does not allow the channel to open (5, 9) and
 223 similarly binding of Innolysin may not provide the conformational changes needed for the FhuA
 224 channel to open. In addition, the size of the unplugged channel (2.5 nm) appears to be too narrow
 225 for the Innolysin (67.62 kDa) to pass (29), indicating that partial unfolding is required for the
 226 Innolysin to overcome the size restraint by FhuA. Lukacik and co-workers showed that a hybrid
 227 lysin consisted of the phage T4 lysozyme and the binding domain of pesticin targeting FyuA could
 228 reach the peptidoglycan (15). But similar to Innolysins, the dimensions of the FyuA pore do not
 229 permit passage of the hybrid T4 lysozyme without unfolding. Yet, it was demonstrated that
 230 unfolding of the enzymatic domain is not required for activity (15), indicating that such hybrid
 231 endolysins may overcome the outer membrane by other means than passing through the targeted
 232 channels.

233 Alternatively, Innolysins may access the peptidoglycan by interfering with the membrane integrity.
 234 FhuA has been proposed to work as an anchor for phage T5 by an irreversible binding of Pb5 to the
 235 FhuA receptor (10). In Innolysins we combine Pb5 with phage T5 endolysin that is a globular
 236 endolysin carrying a single enzymatically active domain with limited intrinsic antimicrobial activity
 237 (30). Thus, we equipped a globular endolysin with a binding domain using a phage RBP and
 238 transformed a globular endolysin into a novel engineered modular endolysin. In comparison to
 239 globular endolysins, modular endolysins composed of catalytic domain(s) and a cell wall binding
 240 domain display up to five times higher enzymatic activity (31, 32). Thus, by irreversible binding to
 241 FhuA (5), Pb5 may bring the Innolysin in close proximity to the outer membrane, enhancing the
 242 antibacterial activity of the endolysin. The binding of the Innolysin could interfere with phosphates
 243 in the outer membrane lipopolysaccharides and displace the stabilizing Mg^{2+}/Ca^{2+} ions due to the
 244 high isoelectric point of phage T5 endolysin (7.91), thus destabilizing the ionic forces in the outer

245 membrane (33). A similar mode of action has been described for Artilyns, endolysins engineered
 246 with polycationic or amphipathic peptides that destabilize the outer membrane and target the
 247 endolysins to peptidoglycan (14). Furthermore, we found that a linker between the T5 endolysin
 248 and Pb5₁₋₄₈₈ components is essential for bactericidal activity. Similarly, modular endolysins contain
 249 flexible interdomain linkers, which are important for enzyme activity (34). Thus, the linker may be
 250 necessary for the Innolysin to properly bind to the receptor FhuA, interfere with the membrane
 251 integrity and overcome the outer membrane.

252 FhuA is conserved in bacterial species including *Escherichia*, *Shigella* and *Salmonella* and is
 253 structurally homologous to other Ton-B dependent outer membrane receptors involved in bacterial
 254 iron uptake (35). The Innolysin showed antimicrobial activity towards *Shigella sonnei* and
 255 *Pseudomonas aeruginosa*, whereas *Salmonella* Typhimurium LT2c was not sensitive to the
 256 Innolysin. While *S. sonnei* and *P. aeruginosa* encode homologs or equivalents of FhuA and share a
 257 conserved L4 loop, shown to be the binding target of phage T5 (22, 26), *Salmonella* FhuA lacks the
 258 L4 loop. Thus, the L4 loop of FhuA may be required for the antimicrobial activity of the Innolysin,
 259 enabling efficient binding of Pb5 to its cognate receptor. However, since FhuA is a phage receptor,
 260 common phage adsorption blocking mechanisms, such as receptor masking by lipopolysaccharides
 261 or downregulation of receptor expression (36, 37) could also play a role in Innolysin resistance in
 262 *Salmonella*.

263 In this study, we used the binding specificity of the phage T5 receptor binding protein to its cognate
 264 FhuA receptor to deliver an endolysin across the outer membrane and target the peptidoglycan.
 265 Compared to bacteriocin-derived fusions with endolysins, the natural diversity of phage RBPs
 266 greatly expands the potential of designing novel antimicrobials targeting a wide range of Gram-
 267 negative bacterial species. As opposed to some bacteriocins and traditional antibiotics, Innolysins

268 can target non-actively growing bacteria, since they bypass the outer membrane passively (38).
 269 Additionally, Innolysins can be designed to target important virulent determinants such as OmpX in
 270 *Escherichia coli* and Ail in *Yersinia pestis*, in order to modulate virulence (39, 40). Although
 271 further experiments are needed for optimizing the activity of such antimicrobials and for elucidating
 272 the exact mechanism by which Innolysins bypass the outer membrane, our findings provide a
 273 promising approach to design novel therapeutic agents with a highly customizable bactericidal
 274 spectrum.

275 MATERIAL AND METHODS

276 Bacterial strains

277 *E. coli* BL21-CodonPlus-(DE3)-RIL (Agilent Technologies) was used for expression of
 278 recombinant proteins. Inhibition assays were conducted with *E. coli* ATCC11303 (Leibniz
 279 Institute). *E. coli* BL21 (DE3) (Agilent Technologies), *E. coli* ECOR4 (STEC Center at Michigan
 280 State University), *Shigella sonnei* (41), *Pseudomonas aeruginosa* PAO1 (42) and *Salmonella*
 281 *Typhimurium* LT2c (43) were used for the bactericidal assay. Deletions of the *fhuA* and *tonB* genes
 282 on the chromosomes of *E. coli* BL21 (DE3) and *E. coli* ECOR4, respectively, were previously
 283 generated by the Lambda red recombination system as described before (44). Briefly, the
 284 kanamycin cassette from plasmid pKD4 was amplified (*Pfu* polymerase; Thermo Fisher Scientific,
 285 Waltham, MA) with oligonucleotides carrying ~39 nucleotide extensions homologous to regions
 286 adjacent to the target gene (custom primers, Table S1). The amplicon was purified with QIAquick
 287 PCR Purification Kit (Qiagen) and electroporated into competent cells. Kanamycin-resistant clones
 288 were selected and insertion of the kanamycin cassette was verified by PCR.

289 **Cloning of constructs**

290 Genomic material was purified from phage T5 (Leibniz Institute) and used as a template for
 291 amplification (*Pfu* polymerase; Thermo Fisher Scientific, Waltham, MA) of the genes encoding T5
 292 endolysin (YP_006868.1) or Pb5 (YP_006985.1) with specific primers (Table S1). The linkers were
 293 created as primer cassettes. The specific primers were mixed, heated to 95°C and gradually cooled
 294 down for hybridization. Each assembly encoding the different fragments of the fusion proteins was
 295 constructed by Type IIs cloning. Amplicons or primer cassettes (each 50 ng/μl) were mixed with
 296 100 ng/μl pNICBsa4, in which the N-terminal His-tag was exchanged for a C-terminal His-tag, and
 297 BsaI and T4 DNA ligase were added. The mixture was incubated alternatingly at 37°C and 16°C
 298 (50 cycli) and heat inactivated at 80°C. Transformation of *E. coli* BL21-CodonPlus-(DE3)-RIL was
 299 conducted and transformants were selected in the presence of kanamycin (100 μg/ml) and
 300 chloramphenicol (50 μg/ml).

301 **Protein expression and purification**

302 Recombinant proteins were simultaneously expressed in a 96 deep-well plate. Freshly transformed
 303 colonies were resuspended in 500 μl of auto-induction medium (93% ZY medium, 0.05% 2M
 304 MgSO₄, 2% 50x 5052, 5% 20x NPS) and incubated at 37 °C for 5 hours, followed by 16 °C for 40
 305 hours, both at 900 rpm. Cells were spun down by centrifugation (3,200 × *g*, 30 min, 4°C) and the
 306 supernatants were removed. Cell pellets were lysed by exposure to chloroform vapor, by placing the
 307 deep-well plate upside-down in a glass petri dish containing chloroform-saturated filters for 2 h.
 308 Cell lysates were resuspended in 20 mM HEPES-NaOH (pH 7.4), and 1 U DNase I, followed by
 309 incubation (100 × rpm, 1 hour, 30 °C). Insoluble fractions of cell lysates were removed by
 310 centrifugation (3,200 × *g* for 30 min, at 4°C) and the supernatants containing the soluble fractions
 311 of the lysates were screened for both muralytic and inhibitory activities.

312 For large-scale expression of the Innolysin#6, a 1 L expression culture (LB medium) was induced
 313 with 1 mM isopropyl-beta-D-thiogalactopyranoside (Thermo Fisher Scientific) in the mid-
 314 logarithmic phase ($OD_{600}=0.6$). Incubation of the culture was followed at 16°C for eighteen hours at
 315 120 rpm. Cells were harvested by centrifugation ($8,000 \times g$, 10 min, 4°C) and the cell pellet was
 316 resuspended in 10 ml of lysis buffer (20 mM NaH_2PO_4 -NaOH, 0.5 M NaCl, 50 mM imidazole, pH
 317 7.4), followed by sonication (Bandelin Sonopul HD 2070 homogeniser) with 10 bursts of 30 sec
 318 (amplitude of 50%) with 30 sec intervals. Protein lysate was double-filtered using filters with pore
 319 size of 0.22 μm . His GraviTrapTM gravity flow columns (GE Healthcare) were used for His-tagged
 320 protein purification according to the manufacturer's instructions. Buffer exchange was performed
 321 with 20 mM HEPES-NaOH (pH 7.4) by using Amicon^R Ultra - 4 centrifugal filters with 50 kDa
 322 cutoff (Merck Millipore) and protein concentration was measured by QubitTM Protein Assay Kit
 323 (Q33211) in Qubit 2.0 Fluorometer (Invitrogen, Q32866).

324 **Muralytic assay**

325 Analysis of the muralytic activity was conducted as described before (14, 45) using outer membrane
 326 permeabilized *P. aeruginosa* PAO1 cells as substrate. Briefly, exponentially growing cells ($OD_{600} =$
 327 0.6) were harvested by centrifugation ($3,200 \times g$, 30 min, 4°C) and permeabilized by resuspension
 328 in chloroform-saturated 0.05 M Tris-HCl (pH 7.7) and gentle shaking for 45 min. To remove
 329 chloroform traces, cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and
 330 further concentrated to $OD_{600} = 1.5$. 30 μl of the soluble lysate fractions was added on top of 270 μl
 331 of the substrate. Supernatants of lysates of cells expressing the phage T5 endolysin or carrying an
 332 empty vector (pVTSD2) were used as positive and negative controls, respectively. Turbidities were
 333 measured spectrophotometrically at 655 nm every 30s for one hour by a Microplate Reader 680
 334 system (Bio-Rad). Muralytic activities were calculated by a previously described standardized

335 method (46).

336 **Growth inhibition assay**

337 *E. coli* ATCC11303 cells were used for the growth inhibition assay. Overnight cell cultures
338 prepared in Mueller Hinton (MH) broth were adjusted to OD₆₀₀= 0.1 in 2xMH and further 100-fold
339 diluted in 2xMH. 50 µl of the cell suspension was mixed with 50 µl of the soluble lysate fraction.
340 Art-175 (0.1 mg/ml) and the soluble lysate fraction of cells carrying an empty vector were mixed
341 with cells as positive and negative controls, respectively. Endpoint measurement was performed
342 spectrophotometrically at 655 nm after exactly 18 h incubation at 37°C. All experiments were done
343 in triplicate.

344 **Antibacterial assay**

345 Gram-negative, exponentially growing cells (OD₆₀₀=0.6) were diluted 100-fold in 20 mM HEPES-
346 NaOH (pH 7.4). 100 µl of the diluted cell suspensions were mixed with 100 µl of the pure Innolysin
347 at a final concentration of 0.2 mg/ml. 100 µl of 20 mM HEPES-NaOH (pH 7.4) was added to the
348 cells as a negative control. The samples were incubated for 30 min at 20°C, and appropriate cell
349 dilutions were plated on LB (Lysogeny Broth) agar plates in triplicate. After overnight incubation at
350 37°C, colony forming units (CFU) were counted and cell concentrations (CFU/ml) were calculated.
351 Experiments were conducted three independent times. The antibacterial activity was determined
352 based on the difference of the average logarithmic cell concentrations of the treated samples
353 compared to the negative control.

354 **Transmission Electron Microscopy**

355 1 ml of exponentially growing cells were harvested by centrifugation (10000 rpm, 5 min) and
356 resuspended in 200 µl of HEPES buffer (pH 7.4). This washing procedure was repeated 3 times,

after which the cell pellets were resuspended in either 50 µl of HEPES buffer or the Innolysin#6 and incubated for 15 minutes at 20°C. Samples were negatively stained with 2% uranyl-acetate on glow-discharged continuous carbon-coated 300-mesh copper grids (EM Resolutions Ltd). Transmission electron microscopy was performed on a Philips CM100 (Tungsten emitter) electron microscope operating at 100 kV. Images were recorded on a side-mounted Olympus Veleta (2048 x 2048 pixels) charge-coupled device camera via the iTEM software.

Bioinformatic analysis

To identify FhuA homologs, we used the FhuA protein sequence of *E. coli* BL21 (WP_000124402.1) to search homologous proteins in the National Center for Biotechnology Information (NCBI) genome database through BLASTP (47). Homologous proteins with different levels of identity in other than *E. coli* species were selected and aligned by the multiple sequence alignment Clustal Omega (48), through which the percent identity was obtained. To further identify the conserved homologs of the proteins we used Jalview sequence alignment tool (49).

Statistical analysis

Analysis of the data was conducted by using GraphPad Prism 7 software (Version 7.0d). For muralytic assays, the activity of each soluble lysate fraction was tested in triplicate and means of activity was compared with the average activity of soluble lysate fraction of cells carrying an empty vector. The significance of muralytic activity was assessed with Unpaired-Samples t-test using 95% confidence interval for the mean difference. The same software was used to analyze results from the growth inhibition assays, using the optical density (OD₆₅₅) of the bacterial growth after incubation with the soluble lysate fraction of each protein compared to the optical density of cells grown after treatment with soluble lysate fraction of cells carrying an empty vector. For the bactericidal assay, all bacterial counts were converted to log-scale and means and standard deviations were calculated

380 afterwards. Bactericidal activity was tested in triplicate in three independent experiments. Decimal
 381 reductions of cells were calculated by the difference between the average logarithmic
 382 concentrations of cells treated with Innolysin#6 and untreated cells, after incubation. The
 383 significance of the decimal reductions of cells was assessed with Paired-Samples t-test using 95%
 384 confidence interval percentage.

385 **ACKNOWLEDGMENTS**

386 This work was supported by the Danish Council for Independent Research (4184-00109B). We
 387 sincerely thank Lysando AG for supplying Artilysin-175 and our colleague Stephen Ahern for
 388 supplying the *fhuA* and *tonB* mutants of *E. coli*. Furthermore, we would like to thank Yilmaz Emre
 389 Gencay and Hans Gerstmans for fruitful discussions.

REFERENCES

1. **Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ.** 2015. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* **13**:42-51. <https://doi.org/10.1038/nrmicro3380>.
2. **Chaturongakul S, Ounjai P.** 2014. Phage–host interplay: examples from tailed phages and Gram-negative bacterial pathogens. *Frontiers in Microbiology* **5**:442. <https://doi.org/10.3389/fmicb.2014.00442>.
3. **Samson JE, Magadan AH, Sabri M, Moineau S.** 2013. Revenge of the phages: defeating bacterial defences. *Nat Rev Micro* **11**:675-687. <https://doi.org/10.1038/nrmicro3096>.
4. **Rakhuba DV, Kolomiets EI, Dey ES, Novik GI.** 2010. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Pol J Microbiol* **59**:145-155.
5. **Plancon L, Janmot C, Le Maire M, Desmadril M, Bonhivers M, Letellier L, Boulanger P.** 2002. Characterization of a high-affinity complex between the bacterial outer membrane protein FhuA and the phage T5 protein pb5. *J Mol Biol* **318**:557-569. [https://doi.org/10.1016/S0022-2836\(02\)00089-X](https://doi.org/10.1016/S0022-2836(02)00089-X).
6. **Mondigler M, Vögele RT, Heller KJ.** 1995. Overproduced and purified receptor binding protein pb5 of bacteriophage T5 binds to the T5 receptor protein FhuA. *FEMS Microbiol Lett* **130**:293-300. <https://doi.org/10.1111/j.1574-6968.1995.tb07734.x>.
7. **Ferguson AD, Hofmann E, Coulton JW, Diederichs K, Welte W.** 1998. Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. *Science* **282**:2215-2220. <https://doi.org/10.1126/science.282.5397.2215>.
8. **Locher KP, Rees B, Koebnik R, Mitschler A, Moulinier L, Rosenbusch JP, Moras D.** 1998. Transmembrane Signaling across the Ligand-Gated FhuA Receptor: Crystal

Structures of Free and Ferrichrome-Bound States Reveal Allosteric Changes. *Cell* **95**:771-778. [https://doi.org/10.1016/S0092-8674\(00\)81700-6](https://doi.org/10.1016/S0092-8674(00)81700-6).

9. **Flayhan A, Wien F, Paternostre M, Boulanger P, Breyton C.** 2012. New insights into pb5, the receptor binding protein of bacteriophage T5, and its interaction with its *Escherichia coli* receptor FhuA. *Biochimie* **94**:1982-1989. <https://doi.org/10.1016/j.biochi.2012.05.021>.

10. **Böhm J, Lambert O, Frangakis AS, Letellier L, Baumeister W, Rigaud JL.** 2001. FhuA-mediated phage genome transfer into liposomes: A cryo-electron tomography study. *Curr Biol* **11**:1168-1175. [https://doi.org/10.1016/S0960-9822\(01\)00349-9](https://doi.org/10.1016/S0960-9822(01)00349-9).

11. **Oliveira H, Melo LD, Santos SB, Nobrega FL, Ferreira EC, Cerca N, Azeredo J, Kluskens LD.** 2013. Molecular aspects and comparative genomics of bacteriophage endolysins. *J Virol* **87**:4558-4570. <https://doi.org/10.1128/JVI.03277-12>.

12. **Mikoulinskaia GV, Odinkova IV, Zimin AA, Lysanskaya VY, Feofanov SA, Stepnaya OA.** 2009. Identification and characterization of the metal ion - dependent l - alanoyl - d - glutamate peptidase encoded by bacteriophage T5. *FEBS journal* **276**:7329-7342. <https://doi.org/10.1111/j.1742-4658.2009.07443.x>.

13. **Schmelcher M, Loessner MJ.** 2016. Bacteriophage endolysins: applications for food safety. *Curr Opin Biotechnol* **37**:76-87. <https://doi.org/10.1016/j.copbio.2015.10.005>.

14. **Briers Y, Walmagh M, Van Puyenbroeck V, Cornelissen A, Cenens W, Aertsen A, Oliveira H, Azeredo J, Verween G, Pirnay J-P.** 2014. Engineered endolysin-based “Artilynsins” to combat multidrug-resistant gram-negative pathogens. *MBio* **5**:e01379-01314. <https://doi.org/10.1128/mBio.01379-14>.

- 436 15. **Lukacik P, Barnard TJ, Keller PW, Chaturvedi KS, Seddiki N, Fairman JW, Noinaj**
437 **N, Kirby TL, Henderson JP, Steven AC.** 2012. Structural engineering of a phage lysin
438 that targets Gram-negative pathogens. *Proceedings of the National Academy of Sciences*
439 **109**:9857-9862. <https://doi.org/10.1073/pnas.1203472109>.
- 440 16. **Yan G, Liu J, Ma Q, Zhu R, Guo Z, Gao C, Wang S, Yu L, Gu J, Hu D, Han W, Du R,**
441 **Yang J, Lei L.** 2017. The N-terminal and central domain of colicin A enables phage lysin to
442 lyse *Escherichia coli* extracellularly. *Antonie Van Leeuwenhoek* **110**:1627-1635.
443 <https://doi.org/10.1007/s10482-017-0912-9>.
- 444 17. **Mondigler M, Holz T, Heller KJ.** 1996. Identification of the Receptor-Binding Regions of
445 pb5 Proteins of Bacteriophages T5 and BF23. *Virology* **219**:19-28.
446 <https://doi.org/10.1006/viro.1996.0218>.
- 447 18. **Argos P.** 1990. An investigation of oligopeptides linking domains in protein tertiary
448 structures and possible candidates for general gene fusion. *J Mol Biol* **211**:943-958.
449 [https://doi.org/10.1016/0022-2836\(90\)90085-Z](https://doi.org/10.1016/0022-2836(90)90085-Z).
- 450 19. **Schleifer KH, Kandler O.** 1972. Peptidoglycan types of bacterial cell walls and their
451 taxonomic implications. *Bacteriol Rev* **36**:407-477.
- 452 20. **Briers Y, Walmagh M, Grymonprez B, Biebl M, Pirnay J-P, Defraigne V, Michiels J,**
453 **Cenens W, Aertsen A, Miller S, Lavigne R.** 2014. Art-175 Is a Highly Efficient
454 Antibacterial against Multidrug-Resistant Strains and Persisters of *Pseudomonas*
455 *aeruginosa*. *Antimicrobial Agents and Chemotherapy* **58**:3774-3784.
456 <https://doi.org/10.1128/AAC.02668-14>.
- 457 21. **Schirmeier E, Zimmermann P, Hofmann V, Biebl M, Gerstmans H, Maervoet VE,**
458 **Briers Y.** 2017. Inhibitory and bactericidal effect of Artilysin((R)) Art-175 against colistin-

resistant mcr-1-positive *Escherichia coli* isolates. Int J Antimicrob Agents.

<https://doi.org/10.1016/j.ijantimicag.2017.08.027>.

22. **Killmann H, Videnov G, Jung G, Schwarz H, Braun V.** 1995. Identification of receptor binding sites by competitive peptide mapping: phages T1, T5, and phi 80 and colicin M bind to the gating loop of FhuA. J Bacteriol **177**:694-698. <https://doi.org/10.1128/jb.177.3.694-698.1995>.

23. **Llamas MA, Sparrius M, Kloet R, Jimenez CR, Vandenbroucke-Grauls C, Bitter W.** 2006. The heterologous siderophores ferrioxamine B and ferrichrome activate signaling pathways in *Pseudomonas aeruginosa*. J Bacteriol **188**:1882-1891. <https://doi.org/10.1128/JB.188.5.1882-1891.2006>.

24. **Cobessi D, Celia H, Pattus F.** 2005. Crystal Structure at High Resolution of Ferric-pyochelin and its Membrane Receptor FptA from *Pseudomonas aeruginosa*. J Mol Biol **352**:893-904. <https://doi.org/10.1016/j.jmb.2005.08.004>.

25. **Cobessi D, Celia H, Folschweiller N, Schalk IJ, Abdallah MA, Pattus F.** 2005. The crystal structure of the pyoverdine outer membrane receptor FpvA from *Pseudomonas aeruginosa* at 3.6 angstroms resolution. J Mol Biol **347**:121-134. <https://doi.org/10.1016/j.jmb.2005.01.021>.

26. **Bonhivers M, Ghazi A, Boulanger P, Letellier L.** 1996. FhuA, a transporter of the *Escherichia coli* outer membrane, is converted into a channel upon binding of bacteriophage T5. The EMBO Journal **15**:1850-1856. <https://doi.org/10.1002/j.1460-2075.1996.tb00535.x>.

27. **Killmann H, Benz R, Braun V.** 1993. Conversion of the FhuA transport protein into a diffusion channel through the outer membrane of *Escherichia coli*. The EMBO Journal **12**:3007-3016.

- 482 28. **Edgar R, Rokney A, Feeney M, Semsey S, Kessel M, Goldberg Marcia B, Adhya S,**
483 **Oppenheim Amos B.** 2008. Bacteriophage infection is targeted to cellular poles. *Mol*
484 *Microbiol* **68**:1107-1116. <https://doi.org/10.1111/j.1365-2958.2008.06205.x>.
- 485 29. **Erickson HP.** 2009. Size and Shape of Protein Molecules at the Nanometer Level
486 Determined by Sedimentation, Gel Filtration, and Electron Microscopy. *Biol Proced Online*
487 **11**:32-51. <https://doi.org/10.1007/s12575-009-9008-x>.
- 488 30. **Shavrina MS, Zimin AA, Molochkov NV, Chernyshov SV, Machulin AV,**
489 **Mikoulinskaia GV.** 2016. In vitro study of the antibacterial effect of the bacteriophage T5
490 thermostable endolysin on *Escherichia coli* cells. *J Appl Microbiol* **121**:1282-1290.
491 <https://doi.org/10.1111/jam.13251>.
- 492 31. **Walmagh M, Briers Y, Santos SBd, Azeredo J, Lavigne R.** 2012. Characterization of
493 Modular Bacteriophage Endolysins from Myoviridae Phages OBP, 201φ2-1 and PVP-SE1.
494 *PLoS One* **7**:e36991. <https://doi.org/10.1371/journal.pone.0036991>.
- 495 32. **Walmagh M, Boczkowska B, Grymonprez B, Briers Y, Drulis-Kawa Z, Lavigne R.**
496 2013. Characterization of five novel endolysins from Gram-negative infecting
497 bacteriophages. *Appl Microbiol Biotechnol* **97**:4369-4375. [https://doi.org/10.1007/s00253-](https://doi.org/10.1007/s00253-012-4294-7)
498 [012-4294-7](https://doi.org/10.1007/s00253-012-4294-7).
- 499 33. **Gerstmans H, Criel B, Briers Y.** 2017. Synthetic biology of modular endolysins.
500 *Biotechnology Advances*. <https://doi.org/10.1016/j.biotechadv.2017.12.009>.
- 501 34. **Hermoso JA, Monterroso B, Albert A, Galán B, Ahrazem O, García P, Martínez-**
502 **Ripoll Mn, García JL, Menéndez M.** 2003. Structural Basis for Selective Recognition of
503 Pneumococcal Cell Wall by Modular Endolysin from Phage Cp-1. *Structure* **11**:1239-1249.
504 <https://doi.org/10.1016/j.str.2003.09.005>.

- 505 35. **Pawelek PD, Croteau N, Ng-Thow-Hing C, Khursigara CM, Moiseeva N, Allaire M,**
506 **Coulton JW.** 2006. Structure of TonB in complex with FhuA, *E. coli* outer membrane
507 receptor. *Science* **312**:1399-1402. <https://doi.org/10.1126/science.1128057>.
- 508 36. **Shin H, Lee JH, Kim H, Choi Y, Heu S, Ryu S.** 2012. Receptor diversity and host
509 interaction of bacteriophages infecting *Salmonella enterica* serovar Typhimurium. *PLoS*
510 *One* **7**:e43392. <https://doi.org/10.1371/journal.pone.0043392>.
- 511 37. **Labrie SJ, Samson JE, Moineau S.** 2010. Bacteriophage resistance mechanisms. *Nature*
512 *Reviews Microbiology* **8**:317. <https://doi.org/10.1038/nrmicro2315>.
- 513 38. **Yang JH, Bening SC, Collins JJ.** 2017. Antibiotic efficacy—context matters. *Curr Opin*
514 *Microbiol* **39**:73-80. <https://doi.org/10.1016/j.mib.2017.09.002>.
- 515 39. **Meng X, Liu X, Zhang L, Hou B, Li B, Tan C, Li Z, Zhou R, Li S.** 2016. Virulence
516 characteristics of extraintestinal pathogenic *Escherichia coli* deletion of gene encoding the
517 outer membrane protein X. *The Journal of Veterinary Medical Science* **78**:1261-1267.
518 <https://doi.org/10.1292/jvms.16/0071>.
- 519 40. **Atkinson S, Williams P.** 2016. Yersinia virulence factors - a sophisticated arsenal
520 for combating host defences. *F1000Research* **5**:F1000 Faculty Rev-1370.
521 <https://doi.org/10.12688/f1000research.8466.1>.
- 522 41. **Aabo S, Rasmussen OF, Roseen L, Sørensen PD, Olsen JE.** 1993. *Salmonella*
523 identification by the polymerase chain reaction. *Mol Cell Probes* **7**:171-178.
524 <https://doi.org/10.1006/mcpr.1993.1026>.
- 525 42. **Holloway BW.** 1955. Genetic Recombination in *Pseudomonas aeruginosa*. *Microbiology*
526 **13**:572-581. <https://doi.org/10.1099/00221287-13-3-572>.

- 527 43. **Kim M, Kim S, Park B, Ryu S.** 2014. Core Lipopolysaccharide-Specific Phage SSU5 as
528 an Auxiliary Component of a Phage Cocktail for *Salmonella* Biocontrol. Appl Environ
529 Microbiol **80**:1026-1034. <https://doi.org/10.1128/AEM.03494-13>.
- 530 44. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in
531 *Escherichia coli* K-12 using PCR products. Proceedings of the National Academy of
532 Sciences **97**:6640-6645. <https://doi.org/10.1073/pnas.120163297>.
- 533 45. **Lavigne R, Briers Y, Hertveldt K, Robben J, Volckaert G.** 2004. Identification and
534 characterization of a highly thermostable bacteriophage lysozyme. Cell Mol Life Sci
535 **61**:2753-2759. <https://doi.org/10.1007/s00018-004-4301-v>.
- 536 46. **Briers Y, Lavigne R, Volckaert G, Hertveldt K.** 2007. A standardized approach for
537 accurate quantification of murein hydrolase activity in high-throughput assays. J Biochem
538 Biophys Methods **70**:531-533. <https://doi.org/10.1016/j.jbbm.2006.10.009>.
- 539 47. **NCBI Resource Coordinators.** . 2013. Database resources of the National Center for
540 Biotechnology Information. Nucleic Acids Res **41**:D8-D20.
541 <https://doi.org/10.1093/nar/gks1189>.
- 542 48. **Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H,**
543 **Remmert M, Söding J, Thompson JD, Higgins DG.** 2011. Fast, scalable generation of
544 high - quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol **7**.
545 <http://doi.org/10.1038/msb.2011.75>.
- 546 49. **Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ.** 2009. Jalview
547 Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics
548 **25**:1189-1191. <http://doi.org/10.1093/bioinformatics/btp033>.

549

550 **FIGURE LEGENDS**

551 **FIG 1** Visual representation of Innolysins. Phage T5 endolysin (T5 Lys) was fused with the whole
552 phage T5 receptor binding protein, Pb5 or the binding domain of Pb5 (Pb5₁₋₄₈₈). Fusion was
553 conducted without linkers (1 - 4) or with linkers (L1 or L2) (5 - 12) with T5 Lys located in the N-
554 terminus (1-2; 5 - 8) or C-terminus (3 - 4; 9 - 12) of the engineered proteins.

555 **FIG 2** Muralytic activity of Innolysins. Soluble fractions of the cleared lysate of the engineered
556 proteins (1-12, see Fig. 1) were screened for ability to degrade peptidoglycan of *P. aeruginosa*
557 PAO1 and compared to the activity of soluble lysate of cells carrying the empty vector, pVTSD2.
558 Phage T5 endolysin and receptor binding protein, Pb5 were used as a positive and a negative
559 control, respectively. Average muralytic activities (U/ml) were estimated based on triplicates. *
560 Significant muralytic activity at $P < 0.05$.

561 **FIG 3** Growth of *E. coli* after treatment with Innolysins. Soluble induced fractions of muralytically
562 active engineered proteins (see Fig. 2) were screened for the ability to inhibit growth of *E. coli*
563 ATCC11303. Art-175 was provided by Lysando AG (20) and used as a positive control. Phage T5
564 endolysin and receptor binding protein, Pb5 were used as negative controls. Growth inhibition was
565 detected as the absence of growth (OD_{655nm}) of cells treated with an Innolysin in comparison to the
566 negative control (growth of ATCC11303 cells treated with supernatant lysates of cells carrying the
567 empty vector, pVTSD2) after incubation for 18 hours at 37°C. * Significant bacterial growth
568 inhibition at $P < 0.05$.

569 **FIG 4** Bactericidal activity of the Innolysin against *E. coli*. Average logarithmic reductions of
570 different *E. coli* strains, after incubation with Innolysin#6 for 30 min at 20°C, compared to the
571 untreated cells. The average reduction was calculated based on triplicates of three independent
572 experiments. * Significant reduction at $P < 0.05$.

FIG 5 Morphological changes of *E. coli* treated with Innolysin#6. The effect of Innolysin#6 on BL21 (Fig 5B-D) and BL21 Δ fhuA (F) was compared to the untreated BL21 (Fig 5A) and BL21 Δ fhuA (Fig 5E), respectively. Cytosol leakage mainly at poles (Fig. 5B, arrow 1); cell debris likely due to cell lysis (Fig. 5C, arrow 2) and separation of the outer membrane (Fig. 5D, arrow 3) and inner membrane (Fig. 5D, arrow 4) were observed in the majority of BL21 cells treated with the Innolysin. Such dramatic changes were not observed in BL21 Δ fhuA cells after incubation with Innolysin#6 (Fig. 5F).

FIG 6 Bactericidal spectrum of the Innolysin. Average logarithmic reductions of different species, after incubation with Innolysin#6 for 30 min at 20°C, compared to the untreated cells. The average reduction was calculated based on triplicates of three independent experiments. * Significant reduction at $P < 0.05$.

FIG 7 Conservation of FhuA homologs in Gram-negative bacteria. FhuA of *E. coli* BL21 (WP_000124402.1) was used as a reference to search for homologous proteins in other than *E. coli* species. FhuA of *Shigella sonnei* (WP_094317049.1) and *Salmonella* Typhimurium LT2c (NP_459196.1) are aligned with FhuA of *E. coli* BL21 (WP_000124402.1). FhuA protein sequence of *E. coli* ECOR4 (locus tag ESC_AA8779AA_AS_03052, PRJEB2879) is also aligned with FhuA of *E. coli* BL21 to confirm the high conservation between the FhuA of both *E. coli* strains that were used. Amino acids are colored based on the level of conservation with transition of the orange to grey to represent increased conservation. A 5.5 threshold was used for illustrating the conservation of homologs by Jalview sequence alignment tool. The red box highlights the amino acids responsible for the formation of the surface-exposed part of loop L4 of FhuA (WP_000124402.1) in *E. coli* BL21.

595 SUPPLEMENTAL MATERIAL

596 FIG S1 Alignment of *E. coli* BL21 FhuA with homologous proteins of *P. aeruginosa*.

597 FiuA (NP_249161.1), FoxA (NP_251156.1), FptA (NP_252911.1), FpvA (NP_251088.1) and FpvB
598 (NP_252857.1) of *Pseudomonas aeruginosa* PAO1 were aligned with *E. coli* BL21 FhuA
599 (WP_000124402.1). Amino acids are colored based on the level of conservation with transition of
600 orange to grey to represent higher conservation. Conservation with no threshold was used by
601 Jalview sequence alignment tool. The red box highlights the alignment of *P. aeruginosa*
602 homologous proteins to the amino acid sequence of FhuA responsible for the formation of the
603 surface-exposed part of loop L4 in *E.coli* BL21.

604 Table S1: Primers used for PCR amplification in cloning

605 For generating mutations in *E. coli* strains, primers were designed with regions of homology to the
606 target genes (indicated in bold). To engineer recombinant proteins, *oad* and *lys* genes of phage T5
607 encoding Pb5 and phage T5 endolysin, respectively, were used for fusion, whereas linkers L1:
608 AGAGAG and L2: GAGAGAGAGAGAGA were created as primer cassettes. The recognition sites
609 of BsaI (highlighted in grey) were used for cloning into pNICBsa4 vector.

610 Table S2: Percent matrix identity of BL21 FhuA homologs

611 Percent identity between homologous proteins and *E. coli* BL21 FhuA (WP_000124402.1) was
612 calculated by sequence alignment Clustal Omega. Full-length FhuA protein sequences of *E. coli*
613 BL21 (WP_000124402.1), *E. coli* ECOR4 (locus tag ESC_AA8779AA_AS_03052, PRJEB2879),
614 *Shigella sonnei* (WP_094317049.1) and *Salmonella* Typhimurium LT2c (NP_459196.1) were used
615 for constructing the identity matrix. Furthermore, homologous proteins of *Pseudomonas aeruginosa*
616 PAO1, including FiuA (NP_249161.1), FoxA (NP_251156.1), FptA (NP_252911.1), FpvA
617 (NP_251088.1) and FpvB (NP_252857.1), were tested for the identity level compared to *E. coli*
618 BL21 FhuA (WP_000124402.1).

Bactericidal activity
(log reduction)

Accession	Protein	Sequence	Position
S_t_LT2c/1-729	1	MARLKTAAQPNSSLRKIAVVVATAVSGMSVYAQAQAVQPKEDTITVTAAPAPQESAWGPAATIAAKRSATTTKTDTPIEKTPQSVSVVTNEEMQMHPQFSVKEALGYTPGVTVSS	113
S_sonnei/1-747	1	MARSKTAQPKHSLRKIAVVVATAVSGMSVYAQAQAVEPKEDTITVTAAPAPQESAWGPAATIAAKRSATTTKTDTPIQKVPQSI SVVTAEEEMALHQP KSVKEALSYPGVSVGT	113
E_c_BL21/1-745	1	MARSKTAQPKHSLRKIAVVVATAVSGMSVYAQAQAVEPKEDTITVTAAPAPQESAWGPAATIAAKRSATTTKTDTPIQKVPQSI SVVTAEEEMALHQP KSVKEALSYPGVSVGT	113
ECOR4/1-747	1	MARSKTAQPKHSLRKIAVVVATAVSGMSVYAQAQAVEPKEDTITVTAAPAPQESAWGPAATIAAKRSATTTKTDTPIQKVPQSI SVVTAEEEMALHQP KSVKEALSYPGVSVGT	113
S_t_LT2c/1-729	114	RGASNTYDFV IIRGFSSVGLSQNNYLDGLKLQGNFYNDAVIDPYMLERVELMRGPTSVLYGKSNPGGIISMVSKRPTTEPLKEIQFKMGTDNLFQTGFDFSDSLDDNGEF SYR	226
S_sonnei/1-747	114	RGASNTYDHL IIRGFAAEGQSQNNYLNGLKLQGNFYNDAVIDPYMLERAEIMRGPVSVLYGKSSPGGLLNMVSKRPTTEPLKEVQFKAGTDSL FQTGFDFSDSLDDDG VYSYR	226
E_c_BL21/1-745	114	RGASNTYDHL IIRGFAAEGQSQNNYLNGLKLQGNFYNDAAIDPYMLERAEIMRGPVSVLYGKSSPGGLLNMVSKRPTTEPLKEVQFKAGTDSL FQTGFDFSDALDDDG VYSYR	226
ECOR4/1-747	114	RGASNTYDHL IIRGFAAEGQSQNNYLNGLKLQGNFYNDAVIDPYMLERAEIMRGPVSVLYGKSSPGGLLNMVSKRPTTEPLKEVQFKAGTDSL FQTGFDFSDALDDDG VYSYR	226
S_t_LT2c/1-729	227	LTGLARSTNEQQKSSSEQRYAIAPAFTWRPDDKTNFTFLSYFQNEPETGGYGGWLPKEGTVPEPLNGKRLPTDFNEGASNNNTYSRNEKMGVGSFDHEFNDFTFIVRQNLRFVEMK	339
S_sonnei/1-747	227	LTGLARSANAQQKGSEEQRYAIAPAFTWRPDDKTNFTFLSYFQNEPETGGYGGWLPKEGTVPEPLNGKRLPTDFNEGAKNNNTYSRNEKMGVGSFDHEFNDFTFIVRQNLRFVEMK	339
E_c_BL21/1-745	227	LTGLARSANAQQKGSEEQRYAIAPAFTWRPDDKTNFTFLSYFQNEPETGGYGGWLPKEGTVPEPLNGKRLPTDFNEGAKNNNTYSRNEKMGVGSFDHEFNDFTFIVRQNLRFVEMK	339
ECOR4/1-747	227	LTGLARSANAQQKGSEEQRYAIAPAFTWRPDDKTNFTFLSYFQNEPETGGYGGWLPKEGTVPEPLNGKRLPTDFNEGAKNNNTYSRNEKMGVGSFDHEFNDFTFIVRQNLRFVEMK	339
S_t_LT2c/1-729	340	TAQKSVYGTGIAAD-----GHTLNRGTIVDNERLQNFSDVTQLQSKFATGDIIDHTLLTGVDFMRMNRNDINATFGSAPSIDLYNNYHPPEYFAF--GGAEP--	431
S_sonnei/1-747	340	TSQNSVYGYGVCSDPANAYSQKQCAALAPADKGHYLARKYVVVDDEKLQNFSDVTQLQSKFATGDIIDHTLLTGVDFMRMNRNDINAWFGYDDSVPLLLNLYNPVNTDFDFNAKDPAN	452
E_c_BL21/1-745	340	TSQNSVYGYGVCSDPANAYSQKQCAALAPADKGHYLARKYVVVDDEKLQNFSDVTQLQSKFATGDIIDHTLLTGVDFM--RNDINAWFGYDDSVPLLLNLYNPVNTDFDFNAKDPAN	452
ECOR4/1-747	340	TSQNSVYGYGVCSDPANAYSQKQCAALAPADKGHYLARKYVVVDDEKLQNFSDVTQLQSKFATGDIIDHTLLTGVDFMRMNRNDINAWFGYDDSVPLLLNLYNPVNTDFDFNAKDPAN	452
S_t_LT2c/1-729	432	Y- QMNESKQTGLYVQDQAENKWVFTLGGRYDWSKQATTVRQNSTTPTEGVIERNDHQFTWRGGVNYVFDNGISPYFSYSQSSEFESSAFDLWSTPRVSXKPSKGEQYEAGV	540
S_sonnei/1-747	433	SGPYRI LNKQKQGTGVYVQDQAQWDKVLVTLGGRYDWDQESLNRVAGTTD-----KRDDKQFTWRGGVNYLFDNGVTPYFSYSSEFESS--QVGKDGNI-IFAPSKGKQYEVGV	558
E_c_BL21/1-745	433	SGPYRI LNKQKQGTGVYVQDQAQWDKVLVTLGGRYDWDQESLNRVAGTTD-----KRDDKQFTWRGGVNYLFDNGVTPYFSYSSEFESS--QVGKDGNI-IFAPSKGKQYEVGV	558
ECOR4/1-747	433	SGPYRI LNKQKQGTGVYVQDQAQWDKVLVTLGGRYDWDQESLNRVAGTTD-----KRDDKQFTWRGGVNYLFDNGVTPYFSYSSEFESS--QVGKDGNI-IFAPSKGKQYEVGV	558
S_t_LT2c/1-729	541	KYVPKDMPPVVVTGAVYQLTKTNLTADPTNPLAQVPAGEIRARGVELEAKAALTANINMTASYTYTDAEYTKDNLKGNTPAQVPEHMASLWGDYTFNEGPLSGLTLGTGGRF	653
S_sonnei/1-747	559	KYVPEDRPIVVGTGAVYNLTKNNNLMADPEGSFFSVEGGEIRARGVEIEAKAALSASVNVVGSYTYTDAEYTTDTTYKGNTPAQVPEHMASLWADYTFDGPPLSGLTLGTGGRY	671
E_c_BL21/1-745	557	KYVPEDRPIVVGTGAVYNLTKNNNLMADPEGSFFSVEGGEIRARGVEIEAKAALSASVNVVGSYTYTDAEYTTDTTYKGNTPAQVPEHMASLWADYTFDGPPLSGLTLGTGGRY	669
ECOR4/1-747	559	KYVPEDRPIVVGTGAVYNLTKNNNLMADPEGSFFSVEGGEIRARGVEIEAKAALSASVNVVGSYTYTDAEYTTDTTYKGNTPAQVPEHMASLWADYTFDGPPLSGLTLGTGGRY	671
S_t_LT2c/1-729	654	TGSSSYGDPANSEFKVGSAAVMDAVVKYDILARFGMAGSSIAVNVNNLLDREYVASCFTYGCFWGAERQVVATATFRF	729
S_sonnei/1-747	672	TGSSYGDPAANSFKVGSYTVVDALVRYDLARVGMAGSNVALHVNLLDREYVASCNTYGCFWGAERQVVATATFRF	747
E_c_BL21/1-745	670	TGSSYGDPAANSFKVGSYTVVDALVRYDLARVGMAGSNVALHVNLLDREYVASCNTYGCFWGAERQVVATATFRF	745
ECOR4/1-747	672	TGSSYGDPAANSFKVGSYTVVDALVRYDLARVGMAGSNVALHVNLLDREYVASCNTYGCFWGAERQVVATATFRF	747