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Cloning of bacteriophage lysins and evaluation of their antibacterial activity against *Enterococcus faecalis*

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Abstract

Enterococci are the causative agents of several serious infections. In addition, antibiotic-resistant enterococci are becoming increasingly prevalent due to the extensive use of antibiotics in the community and in hospitals. In this context, new antimicrobials acting by different mechanisms are highly desired. Lysins are bacteriophage-derived molecules that are produced when the bacteriophage (or phage) progeny needs to escape the bacterial host. Lysins very rapidly lyse the bacterial peptidoglycan. They can reach their target when added exogenously to Gram-positive bacteria, thanks to the lack of an impermeable outer membrane. In contrast, they are inactive when added externally to Gram-negative bacteria, which are surrounded by bilayer outer membrane. The action of lysins is normally restricted to bacterial hosts of the phages from which they were isolated, but some lysins have a broader spectrum of activity. In the present work, new lysins with a potentially lytic action against enterococci were identified by consulting a database of prophage sequences within bacterial genomes. Their amino acid sequences were analyzed in order to highlight both functional domains and possible differences with the previously described lysins. Three new lysins (plyEF, plyHH22 and plyE613) and the previously described enterococcal lysin plyV12 were studied. Cloning and purification were performed using several bacterial expression vectors with and without a C-terminal six-histidine tag. The expression and activity of lysins were screened by different methods. Recombinant host bacteria (*E. coli* BL21) were grown in various media and lysin expression was induced with IPTG. Induction of *plyEF*, *plyHH22* and *plyE613* readily blocked the growth of *E. coli* recipients, suggesting that they were toxic for this particular host. Thus, these enzymes could not be further purified. In contrast, induction of *plyV12* did not block *E. coli* growth and the enzyme could be further studied. The latter results confirm the validity of our protocol. Moreover, we could also confirm the rapid lytic activity of plyV12 against *E. faecalis* ATCC 29212. The reason of the toxicity of plyEF, plyHH22 and plyE613 toward *E. coli* is as yet unclear and needs to be further explored.

Introduction

Enterococcus spp. are Gram-positive bacteria that occur singly, in pairs or as short chains, and are difficult to differentiate from *Streptococcus spp.* considering only physical characteristics. They were considered as group D streptococci before the introduction of molecular method for studying microorganisms. (1) Enterococci are able to survive and grow under a range of adverse environments, such as extreme temperatures (5-65°C), variations of pH (4.5-10.0) and high NaCl concentrations. This characteristic allows them to colonize numerous environmental surfaces, such as mucosal surfaces of humans and animals, soil, water and foodstuffs. (2) Enterococci are part of the intestinal commensal flora and are found in the feces of most healthy adults. The colonization of other sites, such as oral cavity and vagina, is less common. (3) Less than 1% of the human microbiome from healthy people consists of *Enterococcus spp*. (4) *Enterococcus faecalis* and *Enterococcus faecium* are opportunistic pathogens; they cause invasive infections after disruption of the commensal relationship with the host, particularly in elderly patients and other immunocompromised patients. *E. faecalis* is the most frequent enterococcal species associated with clinical infections, representing 80-90% of the isolates, followed by *E. faecium* that is present in 5-10% of cases. Enterococci can be the causative agent of various infections from relatively mild infections, like urinary tract infections and wound infections, to more severe infections, like bacteremia and endocarditis. (1) Furthermore, enterococci are a major cause of healthcare associated infections (HAIs), such as bloodstream infections, peritonitis (usually in mixt infections), catheter-associated urinary tract infections, ventilator-associated pneumonia and surgical site infections. The prevalence of patients infected by nosocomial pathogens is estimated at 6% in acute care hospital in Europe. *Enterococcus spp.* is the third most frequent pathogen causing HAIs (in 9.6% of cases) after *Escherichia coli* (15.9%) and *Staphylococcus aureus* (12.3%). (5) In hospital setting, contamination of fomites (such as bed rails and sheets) and patient skin contributes to nosocomial transmission. (6) Hygiene, disinfection and isolation measures have been introduced to reduce transmission of bacteria in hospitals. (7)

Antibiotic-resistant Enterococci are becoming increasingly prevalent due to the extensive use of antibiotics in both the community and in the hospitals. In addition of acquiring new resistance genes, such as in vancomycin-resistance (see below), enterococci have intrinsic resistance to a large range of antibiotics such as cephalosporins, sulphonamides and low concentrations of aminoglycosides. The low affinity of the penicillin binding proteins (PBP) of enterococci to beta-lactams makes these antibiotics poorly effective against these pathogens. Moreover, enterococci can acquire additional resistances to antibiotics by genetic modifications (acquired resistances). The resistance to beta-lactam antimicrobials uses two mechanisms: the production of beta-lactamase and the modification of the PBPs to further reduce the affinity. (8) Beta-lactamase production for antibiotic resistance in enterococci was for the first time discovered in *E. faecalis* HH22 (strain used in this study). (9) Resistance to aminoglycosides is more prevalent in *E. faecalis* and is caused by a mutation within a protein of the 30S ribosomal subunit, the target of this class of antibiotics. (8) Resistance to glycopeptides, especially to vancomycin, is more prevalent in *E. faecium* and is due to the acquisition of a whole transposon (Tn_{1546}) carrying a set of genes (mainly *vanA*, *vanB* and *vanC*) that replace the ultimate D-alanine of the peptidoglycan precursor with a D-lactate, thus impeding binding of vancomycin to it. (10) (11) Vancomycin resistant enterococci (VRE) were observed for the first time just 15 years after the drug was put on the market. (2) The prevalence of aminoglycoside-resistant and glycopeptide-resistant enterococci is stabilizing or decreasing throughout Europe due to a careful use of these antibiotics and a better control of the infections. (8) The prevalence of VRE is higher in the USA because of the widespread use of oral vancomycin against *Clostridium difficile* diarrhea in US hospitals. (10) Resistance to antibiotics varies according to the bacterium and the hospital. It is important to know the local epidemiology of resistance of specific bacteria in a given hospital before beginning a therapy with antibiotics. In the University Hospital of Lausanne (CHUV) in 2012, the susceptibilities of *E. faecalis* to aminoglycosides and *E. faecium* to vancomycin reach 79% and 99%, respectively (Figure 1). (12) These antibiotics have to be used with parsimony to maintain a low rate of resistance in our country.

(a) Aminoglycoside resistant *E. faecalis*

(b) Vancomycin resistant *E. faecium*

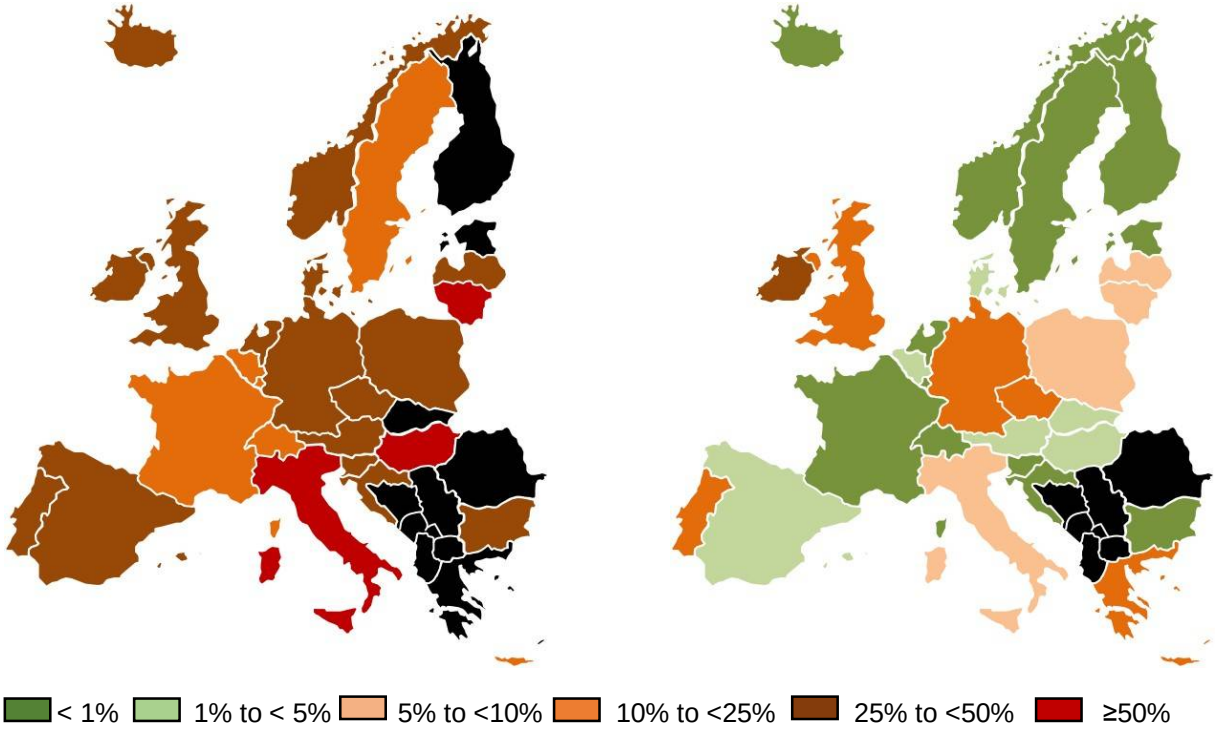


Figure 1. Proportion (in percent) of invasive enterococcal isolates resistant to aminoglycoside and vancomycin in Europe according to the Annual report of the European Antimicrobial Resistance Surveillance network (EARS-Net) 2012 (8) for the European Union and the antimicrobial resistance statistics from CHUV 2012 (12) for Switzerland. (a) Aminoglycoside resistant *E. faecalis* (mean percentage: 26.5%). (b) Vancomycin resistant *E. faecium* (mean percentage: 8.1%). In general, the proportion of resistance has stabilized or decreased over the last years in Europe. No data are available for the countries filled in black. Adapted from (8) and (12)

Antibiotics start to show their limits in the treatment of infectious diseases with the emergence of multi-drug resistant bacteria. Moreover, pharmaceutical companies are not interested in developing new antibiotics as it is not financially profitable. In this context, new antimicrobials acting by different mechanisms are highly desired.

Bacteriophages (phages) are bacterial viruses that can be found in extremely different environments, probably everywhere where there's life. (13) They are the most abundant microorganisms on the planet and they form a heterogeneous group with numerous species and a large genomic diversity. (14) Bacteriophages, as obligate intracellular pathogen, need to invade their bacterial hosts to produce progeny virions. The phage infection process (Figure 2) begins with the specific adsorption of the phage to a receptor on the surface of the bacterial host and the injection of its DNA in the host cell. Then the host vital processes are blocked in order to replicate the DNA and synthetize the phage structural components. Finally the progeny

phages are assembled and released from the host cell after lysis. Lysis is performed in non-filamentous phages by expressing two proteins: holin and endolysin. Holins are synthesized at a specific time to permeabilize the cytoplasmic membrane, allowing the endolysin to be funneled to and interact with the peptidoglycan wall. Endolysins recognize and hydrolyze specific peptidoglycan bonds resulting in bacterial lysis. (15) Filamentous phages replicate without killing the host and are continuously extruded from bacterial cell. (16)

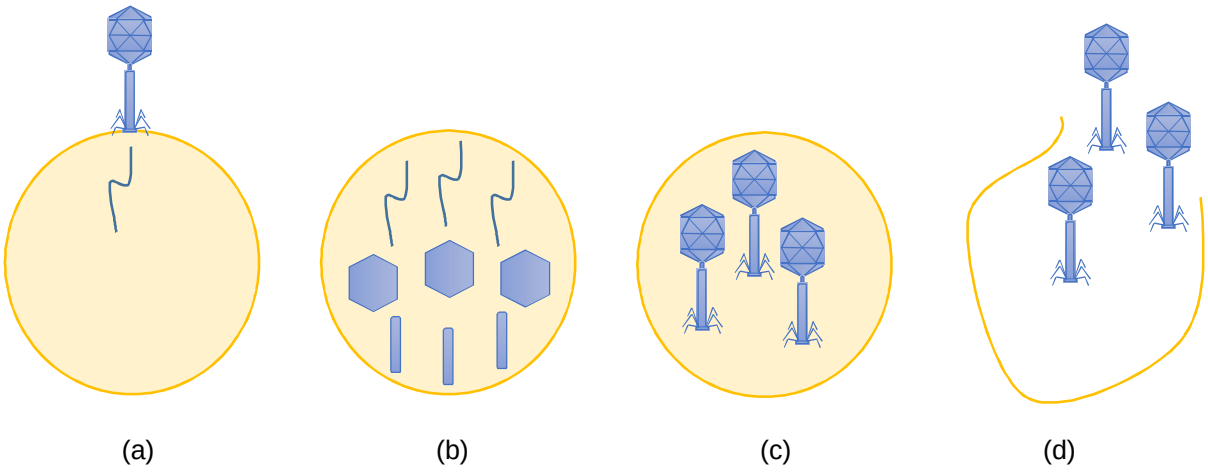


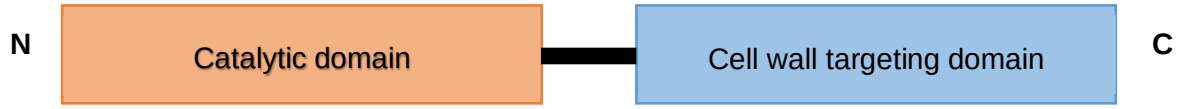
Figure 2. Schematic representation of the bacteriophage infection process: (a) Adsorption of the bacteriophage and injection of DNA in the host cell, (b) DNA replication and synthesis of phage structural components, (c) assembly of progeny phages, (d) bacterial cell lysis and release of progeny phages. Adapted from (23)

Bacteriophage (phage) therapy was used to treat various infections in Western countries before the advent of antibiotics in the 1940s. Nevertheless, in Eastern Europe and in the former Soviet Union, phages continued to be used alone or in association with antibiotics. A renewed interest in phage therapy is re-emerging in the western scientific community; finding alternative treatments against multi-drug resistant bacteria became necessary. During the long history of phages being used as antimicrobials in Eastern Europe and in the former Soviet Union, no report of serious complications has been published. (17) Clinical trials have been prepared to assess the safety and the effectiveness of this class of anti-infectives in humans. (18) People increasingly moved to Eastern Europe to benefit from phage therapy. Phage cocktails (several phages in one preparation) are used to avoid the development of phage resistance during the treatment. Indeed, bacteria quickly develop resistances in presence of phages. The modification of surface receptors with the reduction of affinity to phage adsorption is a well-documented mechanism. Alternatively, the presence of an already inserted prophage in the bacterial chromosome may impede the entry of a new phage of the same class (referred to as phage immunity). Many more phage resistance mechanisms are likely to be uncovered. (19)

Recently, researchers have shown an increased interest in bacteriolytic proteins (lysins) encoded by phages. (20)

Lysins are peptidoglycan hydrolases that are generally greater than 25 kDA in size and its structure is generally composed of two functional domains: an N-terminal catalytic domain and a C-terminal cell wall binding domain (Figure 3). The N-terminal catalytic domain cleaves specific peptidoglycan bonds: N-acetyl-β-D-glucosaminidases and N-acetylmuramidases (glycosidases) hydrolyze glycosidic bonds in the glycan strand, endopeptidases cleave the cross-bridge between peptidoglycan stem peptides and N-acetylmuramoyl-L-alanine amidases cleave amid bond connecting the glycan moiety and the stem peptide. The C-terminal cell wall binding domain provides the high specificity of the binding. (16) Lysins quickly kill Gram-positive bacteria by destabilizing the cell wall through peptidoglycan digestion. As mentioned, when administered therapeutically from the outside of the bacterium they are only active against Gram-positive bacteria, because lack an outer membrane. Lysins have specific properties: they rapidly kill bacteria after contact with highly conserved peptidoglycan substrates, they are efficient against biofilm-based infections such as infective endocarditis (IE), they have a synergistic lethal activity with cell-wall-active antibiotics and they are able to resensitize bacteria to non-susceptible antibiotics. (21) The high molecular affinity to bind their substrate in cell wall suggests that lysins are “one-use” enzymes that do not disengage from the cell wall after binding and cleavage. (22) No specific resistance mechanism to lysins have been discovered, likely because lysins target peptidoglycan, an essential structure for the bacterial viability in which mutations induce cell death. (23) Additionally, the formation of biofilm by bacteria does not affect the ability of lysin to kill bacteria. (24) In general, lysins have bactericidal activity against the bacterial host of the phages from which they were isolated. (22) They only kill targeted bacteria with little to no effect on the unrelated commensal flora. Some lysins have a broader spectrum of activity such as the enterococcal phage lysin PlyV12 with lytic activity not only against enterococci but also against *Streptococcus agalactiae* and *Streptococcus pyogenes*. (25)

(a) Structure



(b) Cleavage sites

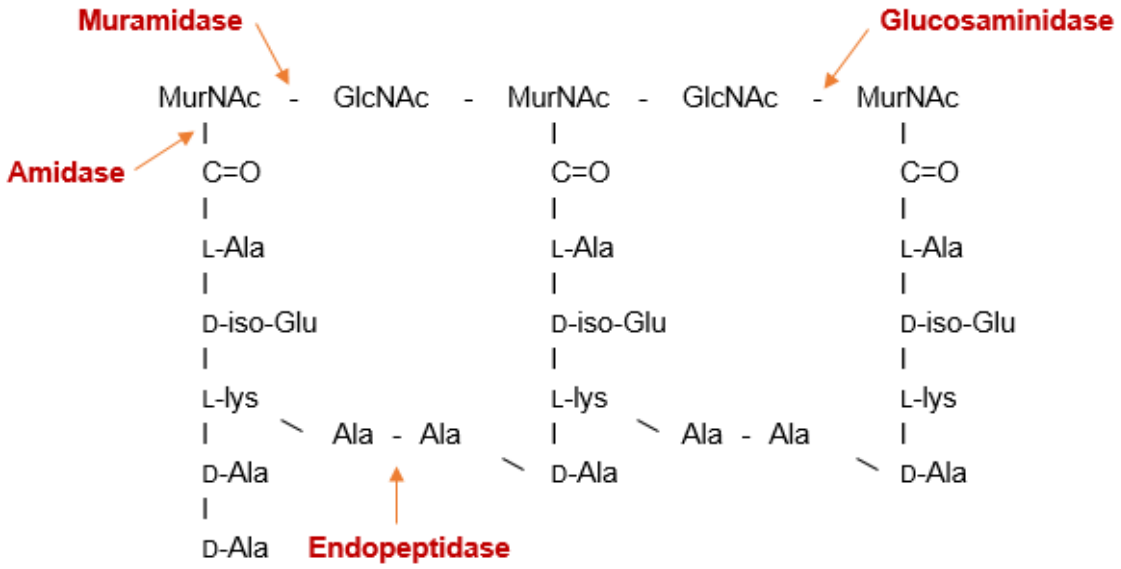


Figure 3. Schematic representations of the structure and the cleavage sites of lysins. (a) The two functional domains of lysins. (b) Sites in the peptidoglycan sensitive to cleavage by lysins. Adapted from (23)

Lysin therapy is being evaluated in humans to assess the safety and the pharmacokinetic properties of this new class of anti-infectives. (26) Lysins have already been evaluated in animal models of pneumonia, endocarditis and sepsis. (27) (28) (29) (30) These trials have shown the ability of lysins to eliminate bacteria from mucosal surface and from deeper tissues, including bacteria in biofilms. (23) Lysins might be used in situations where the pathogen is known (because they are very specific) or in combination with antibiotics. Combined use of these two molecules may reduce the emergence of antibiotic-resistant bacteria and enable the use of antibiotics for shorter durations at possibly lower doses. The lysin therapy potentially appears to be safe. Bacteriophage are indeed part of the normal commensal flora and humans are constantly in contact with lysin without discernible consequences. The use of lysin as therapeutics could have potential limitations. First, lysins, as protein molecules, may induce the production of antibodies. However, in vitro and in vivo studies have shown that lysin specific antibodies are not neutralizing. (31) (32) Second, lysin therapy may induce inflammatory

response when the bacterial content is released after lysis. Finally, some adverse events, such as reaction at infusion site, may develop. It is important to determine the proper doses and dosing schedules of lysins to administer to humans in order to be safe and effective.

The purpose of the present set or experiments was to attempt identifying new original phage lysins active against *Enterococcus spp.* in general and *E. faecalis* in particular. The methodology followed a step-by-step procedure starting with the screening of putative new lysins using bioinformatics means, followed by physically amplifying, cloning and expressing these putative lysins in *E. coli* and eventually testing them in lysis and killing assays against the target organisms. As a control, cloning purification and lysis assays were performed with a described anti-enterococcal lysin (namely plyV12) (25), in order to validate the overall method.

Materials and methods

Bacteriophage lysins identification. Prophage lysin genes were identified initially using the PHage Search Tool (PHAST), a web server that identifies, annotates and displays prophage sequences within bacterial genomes or plasmids. (33) Then the corresponding amino acid sequences were deduced using the Basic Local Alignment Search Tool (BLAST) in order to identify functional domains (catalytic domains (CD) and cell wall binding domains (CWBD)) and differences with previously described lysins. (34)

Cloning of *plyEF*, *plyHH22*, *plyE613* and *plyV12*. Cloning was performed in several bacterial expression vectors (pET28a, pET15b and pIN-IIIA) (see Table 1). Since fusing a six-histidine tag to a protein greatly simplifies subsequent purification but can lead to the inactivation of the protein enzymatic activity, different constructs with and without a six-histidine tag were used in order to identify any effect of the tag on protein activity.

PlyEF, plyE613 and plyV12 genes were synthetized and subcloned into the pUC57 plasmid (GenScript, Piscataway, NJ, USA). Then pUC57-plyEF, pUC57-plyE613 and pUC57-plyV12 were transformed into *E. coli* DH5α (Life Technologies, Carlsbad, CA, USA) in order to store them. *PlyHH22* was obtained from *E. faecalis* strain HH22 using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) to isolate chromosomal DNA.

All lysin genes were PCR amplified with specific primers (Microsynth, Balgach, Switzerland), digested with corresponding restriction endonucleases (Promega, Madison, WI, USA) and ligated into corresponding bacterial expression vectors. Constructs with the plasmid pET28a (pET28a-plyEF, pET28a-plyHH22, pET28a-plyE613 and pET28a-plyV12) contain a C-terminal six-histidine tag. Constructs with the plasmids pET15b (pET15b-plyEF) and pIN-IIIA (pIN-IIIA-plyEF, pIN-IIIA-plyHH22, pIN-IIIA-plyE613 and pIN-IIIA-plyV12) do not contain a histidine tag. All constructs were transformed in BL21(DE3)pLysS (*E. coli* BL21) chemically competent *E. coli* (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and a colony PCR was achieved to determine the presence or absence of the lysin genes in the plasmids. Restriction digestion of recombinant plasmids was also performed to confirm the insertion of lysin genes in the plasmids. Finally, DNA sequencing from recombinant plasmids, using the universal T7 primers for pET vectors and pIN-IIIA primers for pIN-IIIA vectors, was performed to confirm that the lysin genes were ligated at the precise planned position within the plasmids and to identify potential mutations.

	Characteristics	References
Bacterial strains		
E. coli DH5α	Competent cells	Life Technologies
E. coli BL21(DE3)pLysS	Expression competent cells, Cam^r	Life Technologies
E. coli BL21/pET28a-plyEF	BL21 transformed with pET28a-plyEF	This study
E. coli BL21/pET28a-plyV12	BL21 transformed with pET28a-plyV12	This study
E. coli BL21/pET28a-plyHH22	BL21 transformed with pET28a-plyHH22	This study
E. coli BL21/pET28a-plyE613	BL21 transformed with pET28a-plyE613	This study
E. coli BL21/pET15b-plyEF	BL21 transformed with pET15b-plyEF	This study
E. coli BL21/pIN-IIIA-plyEF	BL21 transformed with pIN-IIIA-plyEF	This study
E. coli BL21/pIN-IIIA-plyV12	BL21 transformed with pIN-IIIA-plyV12	This study
E. coli BL21/pIN-IIIA-plyHH22	BL21 transformed with pIN-IIIA-plyHH22	This study
E. coli BL21/pIN-IIIA-plyE613	BL21 transformed with pIN-IIIA-plyE613	This study
E. faecalis ATCC 29212	Clinical isolate from patient urine	ATCC
E. faecalis HH22	Unknown	B. Murray
Plasmids		
pET28a	Bacterial expression vector, Kan^r	Novagen
pET15b	Bacterial expression vector, Amp^r	Novagen
pIN-IIIA	Bacterial expression vector, Amp^r	V. Fischetti
pET28a-plyEF	pET28a containing the plyEF gene	This study
pET28a-plyV12	pET28a containing the plyV12 gene	This study
pET28a-plyHH22	pET28a containing the plyHH22 gene	This study
pET28a-plyE613	pET28a containing the plyE613 gene	This study
pET15b-plyEF	pET15b containing the plyEF gene	This study
pIN-IIIA-plyEF	pIN-IIIA containing the plyEF gene	This study
pIN-IIIA-plyV12	pIN-IIIA containing the plyV12 gene	This study
pIN-IIIA-plyHH22	pIN-IIIA containing the plyHH22 gene	This study
pIN-IIIA-plyE613	pIN-IIIA containing the plyE613 gene	This study
Oligonucleotides		
plyEFpET28aFwNcoI	5'-GGGCATGCCATGGACTACTCCCAAAAAGCGATTGATCTGTG-3'	This study
plyEFpET28aRevBamH1	5'-CGCGGATCCTTACGCAACATAACGACGCAGACCCGAGCC-3'	This study
plyV12pET28aFwNcoI	5'-CATGCCATGGCAAGTAACATTAACATGGAAACCG-3'	This study

plyV12pET28aRevXhoI	5'-CCGCTCGAGCTTAAATGTACCCCATGC-3'	This study
plyHH22pET28aFwNcoI	5'-CATGCCATGGCTTTTTTAAGAAAGGAGC-3'	This study
plyHH22pET28aRevXhoI	5'-CCGCTCGAGCGTAGCAAATGATCCCCATG-3'	This study
plyE613pET28aFwNcoI	5'-CATGCCATGGGACAACGAGAAACTGCAAAG-3'	This study
plyE613pET28aRevXhoI	5'-CCGCTCGAGTTAATTAAAGAAACCTGTCCCCC-3'	This study
plyEFpET15bFwNcoI	5'-GGGCATGCCATGGACTACTCCCAAAAAGCGATTGATCTGTG-3'	This study
plyEFpET15bRevXhoI	5'-CCGCCGCTCGAGCGCAACATAACGACGCAGACCCGAGCC-3'	This study
plyEFpIN-IIIAFwXbaI	5'-CTAGTCTAGAGGTGGAATAATGAACTACTCCCAAAAAGCGATTGATCT-3'	This study
plyEFpIN-IIIARevHindIII	5'-CCCCAAGCTTACGCAACATAACGACGCAGACCCGAGCCAGCC-3'	This study
plyV12pIN-IIIAFwXbaI	5'-CTAGTCTAGAGGTGGAATAATGAGTAACATTAACATGGAAACCGC-3'	This study
plyV12pIN-IIIARevBamHI	5'-CGCGGATCCTTACTTAAATGTACCCCATGC-3'	This study
plyHH22pIN-IIIAFwXbaI	5'-CTAGTCTAGAGGTGGAATAATGCCTTTTTTAAGAAAGGAGCATGC-3'	This study
plyHH22pIN-IIIARevHindIII	5'-CCCAAGCTTAGCTAGCAAATGATCCCCATG-3'	This study
plyE613pIN-IIIAFwXbaI	5'-CTAGTCTAGAGGTGGAATAATGGGACAACGAGAAACTGC-3'	This study
plyE613pIN-IIIARevHindIII	5'-CCCAAGCTTAATTAAAGAAACCTGTCCCCC-3'	This study
T7 primer #69348-3	5'-TAATACGACTCACTATAGGG-3'	Novagen
T7 primer #69337-3	5'-GCTAGTTATTGCTCAGCGG-3'	Novagen
pIN-IIIAFw	5'-CACACAGGAAACAGCTATGACC-3'	G. Resch
pIN-IIIARev	5'-GCCATAGCCTGCAATCCATAGAG-3'	G. Resch

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study. Cam^r: chloramphenicol resistant, Kan^r: kanamycin resistant, Amp^r: ampicillin resistant, ATCC: American Type Culture Collection (ATCC, Manassas, Virginia, USA), restriction sites are underlined.

Lysin expression and activity screening. Transformed *E. coli* BL21 selected by colony PCR and restriction digestion were used in this experiment. BL21/pET28a-plyEF, BL21/pET28a-plyHH22, BL21/pET28a-plyE613 and BL21/pET28a-plyV12 were plated onto Lysogeny Agar (LA) plates with kanamycin (30µg/mL), chloramphenicol (25µg/mL) and 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for protein synthesis induction. BL21/pET15b-plyEF, BL21/pIN-IIIA-plyEF, BL21/pIN-IIIA-plyHH22, BL21/pIN-IIIA-plyE613 and BL21/pIN-IIIA-plyV12 were plated onto LA plates with ampicillin (100µg/mL), chloramphenicol (25µg/mL) and 0.4 mM IPTG. Recombinant bacteria were also plated onto LA plates without IPTG to screen the effect of protein synthesis induction on bacteria. The plates were incubated overnight at 37°C. Then the colonies were exposed to chloroform vapors to permeabilize the bacteria allowing the release of lysins. To this end, chloroform was poured in the lids of the petri plates and the dishes were placed upside down on lids during 20 minutes. The plates were dried under the hood during 10 minutes before 15 mL of molten soft agar containing autoclaved *E. faecalis* was poured on the colonies. The agar/bacteria suspension was prepared with an overnight culture of *E. faecalis* ATCC 29212 which was centrifuged, washed with one volume of NaCl 0.9% and resuspended in 0.25 volume of lysis buffer (40 mM phosphate buffer, 200 mM NaCl, 1 mM EDTA, pH 7.4). The bacterial suspension was supplemented with agar (7.5 g/L) and autoclaved for 15 minutes at 120°C. Before use, the agar/bacteria suspension was melted in a microwave and equilibrated in a water bath set to 55°C. The plates were incubated overnight at 37°C in order to allow the formation of clearing zones surrounding the colonies. (35)

Lysin expression and solubility assessment. Recombinant *E. coli* BL21 selected by colony PCR and validated by DNA sequencing were used in this experiment. BL21/pET28a-plyEF, BL21/pET28a-plyHH22 and BL21/pET28a-plyV12 were grown overnight at 37°C and 250 revolutions per minute (rpm) in Lysogeny Broth (LB) with kanamycin (30µg/mL) and chloramphenicol (25µg/mL). BL21/pET15b-plyEF, BL21/pIN-IIIA-plyEF, BL21/pIN-IIIA-plyHH22 and BL21/pIN-IIIA-plyV12 were grown overnight in LB with ampicillin (100µg/mL) and chloramphenicol (25µg/mL). Then 10 mL aliquots of overnight cultures were diluted in 200 mL of pre-heated LB and the Erlenmeyer flasks were placed in the incubator set to 37°C and 250 rpm. The bacterial growth was monitored by observing the increase in optical density (turbidity) with a spectrophotometer. At OD_{600nm} of 0.7, 20 mL aliquots were collected in small Erlenmeyer flasks as non-induced controls and 0.4 mM IPTG was added to the rest of the solution in order to induce protein synthesis. All Erlenmeyer flasks were placed in the incubator, 10 mL aliquots were collected in 50 mL Falcon™ tubes (Becton Dickinson, Franklin Lakes, NJ, USA) 1, 2, 3 and 4 hours after induction and a single 10 mL aliquot was collected at 4 hours for the non-

induced controls. Samples were kept on ice, centrifuged, resuspended in 0.25 volume of lysis buffer (40 mM phosphate buffer, 200 mM NaCl, 1 mM EDTA, pH 7.4) and frozen overnight at -80°C. Samples were thawed and sonicated on ice three times for 20 seconds at 80% of maximum power using a SONOPULS HD2070 sonicator (BANDELIN electronic, Berlin, Germany) to form a mixture of all proteins from E. coli cytoplasm and some additional macromolecules, cofactors and nutrients. For each sample, a total fraction aliquot was taken and the rest of the sample was centrifuged at 13000 rpm during 15 minutes to collect the supernatant (i.e. the soluble fraction or crude extract).

A protein gel was made to assess lysin expression and solubility. To this end, all aliquots were prepared according to the manufacturer's instructions and electrophoresed on NuPAGE® Bis-Tris Gels (Life Technologies, Carlsbad, CA, USA). A pre-stained protein standard (Novex® Sharp Protein Standard, Life Technologies) was used to determine the protein sizes.

Lysin antibacterial activity evaluation. A bacterial suspension was prepared with *E. faecalis* ATCC 29212. To this end, bacteria were grown overnight in Brain Heart Infusion (BHI) medium. Then 2 mL aliquot of the overnight culture was diluted in 150 mL of pre-heated BHI and the mixture was placed in the incubator set to 37°C and 250 rpm. The bacterial growth was followed by spectrophotometry. At OD_{600nm} of 0.4, 40 mL aliquots were centrifuged in 50 mL Falcon™ tubes (7 minutes, 4000 rpm), washed with NaCl 0.9% and resuspended in 20 mL of lysis buffer (40 mM phosphate buffer, 200 mM NaCl, 1 mM EDTA, pH 7.4) to reach an OD_{600nm} of 0.9. The bacterial suspension was placed on ice before the use.

The bacterial suspension was diluted with the protein mixtures obtained in the previous experiment within a Corning® 96 well plate (Corning Incorporated, Corning, NY, USA). The bacterial suspension and the protein mixtures were first put separately in different columns of the plate; odd column's wells were filled with 150 µL of protein mixture, even column's wells were filled with 150 µL of bacterial suspension and the last odd column's wells were filled with 150 µL of lysis buffer, as control, to confirm that the lysis buffer has no lytic effect on bacteria. The content of the odd column's wells was collected using a multichannel pipette and mixed with the content of the neighboring even column's wells. The decrease in OD_{600nm} was immediately monitored with EL808 absorbance microplate reader run with Gen5™ software (BioTek, Winooski, VT, USA) set to 37°C and 3 seconds shaking before each reads. The OD_{600nm} was read every minute during 1 hour.

Results

Bacteriophage lysins identification. Three different *E. faecalis* lysins have already been described and trials have shown their efficacy in killing bacteria in vitro (Figure 4). (36) PlyV12 encoded by phage Φ1 was identified by Yoong et al. in 2004. (25) EFAL-1 encoded by phage EFAP-1 was described by Son et al. in 2009 (37) And ORF9 encoded by phage ΦEF24C was characterized by Uchiyama et al. in 2011. (38) All these lysins are active against their natural target *E. faecalis* and the related species *E. faecium*. PlyV12 has the broadest spectrum of lytic activity by acting on several streptococcal and staphylococcal strains.

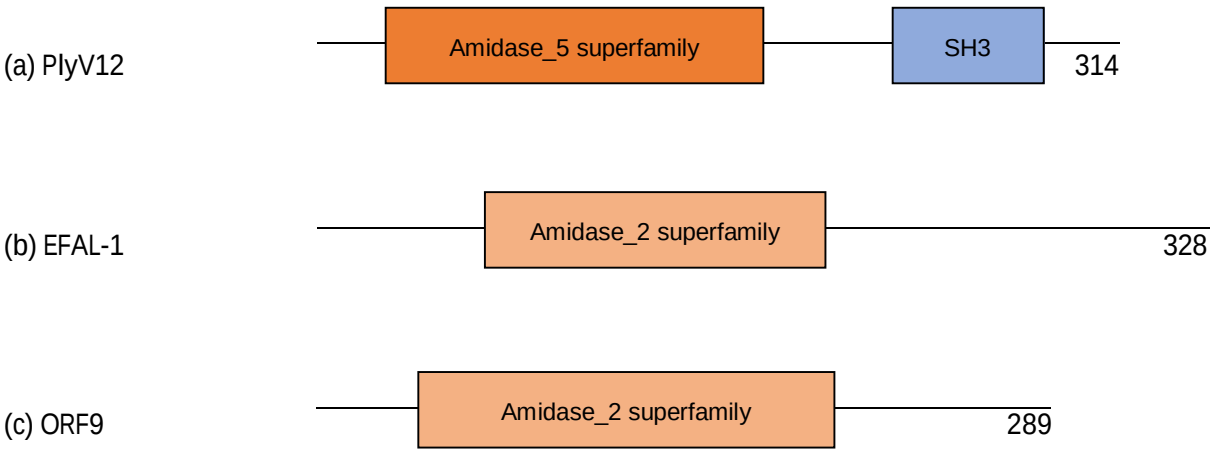


Figure 4. Schematic representation of previously described lysins. (a) PlyV12 has an N-terminal catalytic domain with N-acetylmuramyl-L-alanine amidase activity (amidase_5 superfamily) and a C-terminal cell wall targeting domain (SH3). (b) EFAL-1 contains a conserved N-terminal domain with N-acetylmuramyl-L-alanine amidase activity that belongs to the amidase_2 superfamily and a C-terminal domain of unknown function. (c) ORF9 has a conserved N-terminal catalytic domain (amidase_2 superfamily) and a C-terminal domain with a probable binding function. (protein sizes in amino acids)

Three new lysin genes were identified from the genome of three different *E. faecalis* strains in the database regrouping prophage sequences within bacterial genomes (Figure 5). The amino acid sequences were deduced using bioinformatics methods. Potential functional domains (CD and CWBD) and differences with previously described lysins were highlighted. Two of those lysins have a unique structure; plyEF has a supplementary lysozyme like domain potentially involved in wall hydrolysis and plyE613 contains three SH3 domains potentially involved in binding the peptidoglycan substrate. The structure of plyHH22 has similarities with the one's of plyV12, but the sequences of the CD and the CWBD are different.

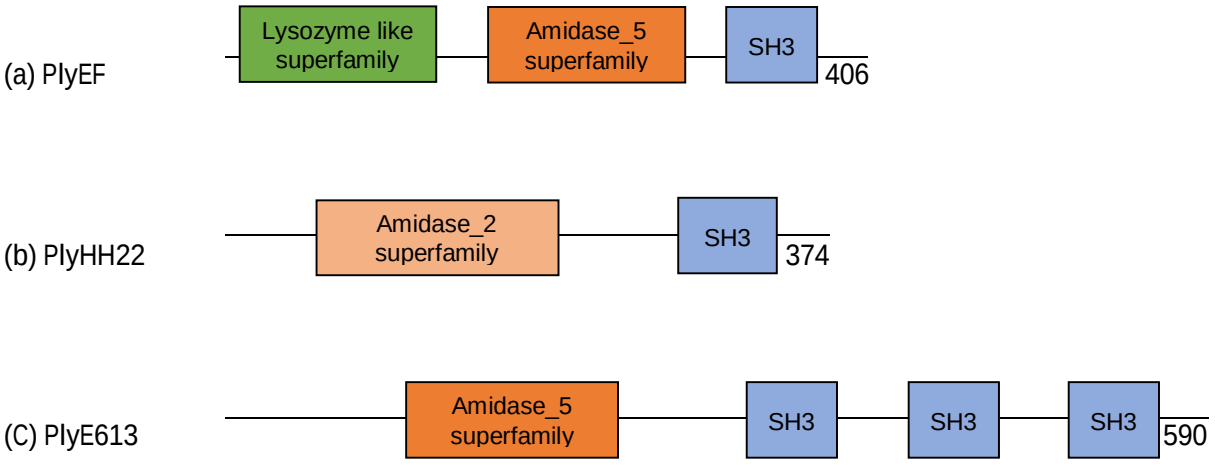


Figure 5. Schematic representation of lysins selected for this study**.** (a) PlyEF is a 43 kDa protein with three different functional domains: a lysozyme like domain involved in the hydrolysis of beta -1, 4- linked polysaccharides, an amidase domain with N-acetylmuramoyl-L-alanine amidase activity and a SH3 domain for the binding to the peptidoglycan. (b) PlyHH22 is a 41 kDa protein with two different functional domains: a PeptidoGlycan Recognition Protein (PGRP) that can bind and, in certain cases, hydrolyze peptidoglycan due to an N-acetylmuramoyl-L-alanine amidase activity (amidase_2 superfamily), and SH3 domain for the binding with the peptidoglycan. (c) PlyE613 is a 66 kDa protein with four functional domains: an amidase domain with N-acetylmuramoyl-L-alanine amidase activity and three SH3 domains for the binding with the peptidoglycan. (protein sizes in amino acids)

Cloning of *plyEF*, *plyHH22*, *plyE613* and *plyV12*. PlyEF gene was initially cloned in pET28a and pET15b using a rapid cloning protocol. However, this technique failed as no plyEF genes were detected in the plasmids as confirmed by DNA sequencing. The cloning protocol was modified; the digestion and ligation times were prolonged and pET15b was replaced by pIN-IIIA, another plasmid without C-terminal six-histidine tag. The presence of plyEF gene in the plasmids was confirmed by DNA sequencing and the absence of mutations in plyEF gene was confirmed by comparing the sequences with ClustalW2, the multiple sequence alignment program for DNA. (39) Then plyHH22 and plyE613 were cloned using the same protocol. The insertion of lysin genes without mutations in the plasmids was confirmed by DNA sequencing. The positive control plyV12 was processed in parallel to confirm the validity of the cloning protocol.

Lysin expression and activity screening. Genetically modified *E. coli* BL21 were grown on two different LA plates containing specific antibiotics; one with IPTG for protein synthesis induction and the other without IPTG. After an overnight incubation, the colonies were permeabilized with chloroform vapors and overlaid with autoclaved *E. faecalis* ATCC 29212 supplemented with agar.

Recombinant *E. coli* BL21, obtained with the modified cloning protocol, were tested for confirmation of the presence of the lysin genes in the plasmids by DNA sequencing. BL21/pET28a-plyEF, BL21/pIN-IIIA-plyEF, BL21/pET28a-plyHH22, BL21/pIN-IIIA-plyHH22, BL21/pET28a-plyE613 and BL21/pIN-IIIA-plyE613 were plated onto LA plates with and without IPTG. After an overnight incubation, all bacteria have grown on the plates without IPTG, but only certain of them were found on both plain and IPTG containing plates (see Figure 6). This suggest already the possibility of a toxic effect of the expressed lysins. The plates were nevertheless overlaid with the agar/bacteria suspension, but no lysis zone surrounding the colonies was observed. Two types of bacteria were highlighted by DNA sequencing; those that have integrated an empty plasmid and that have grown on both types of plates and those that have integrated plasmids containing the lysin gene and that have not grown when protein synthesis was induced by IPTG. A second method to induce protein synthesis was tested. The colonies were grown overnight on LA plates without IPTG and then IPTG was vaporized on the plates during 1 hour using a nebulizer. The colonies were overlaid with the agar/bacteria suspension and again no lysis zone was observed.

Finally, plyV12 was used as positive control in order to confirm if the cloning protocol was accurate. BL21/pET28a-plyV12 and BL21/pIN-IIIA-plyV12 have grown likewise on the plates with and without IPTG. The colonies were overlaid with the agar/bacteria suspension and lysis zones were observed around some of the colonies already after 1 hour of incubation. The presence of plyV12 gene in the bacteria surrounded by a lysis zone was confirmed by DNA sequencing, whereas the bacteria that was not surrounded with a lysis zone contained empty plasmids.

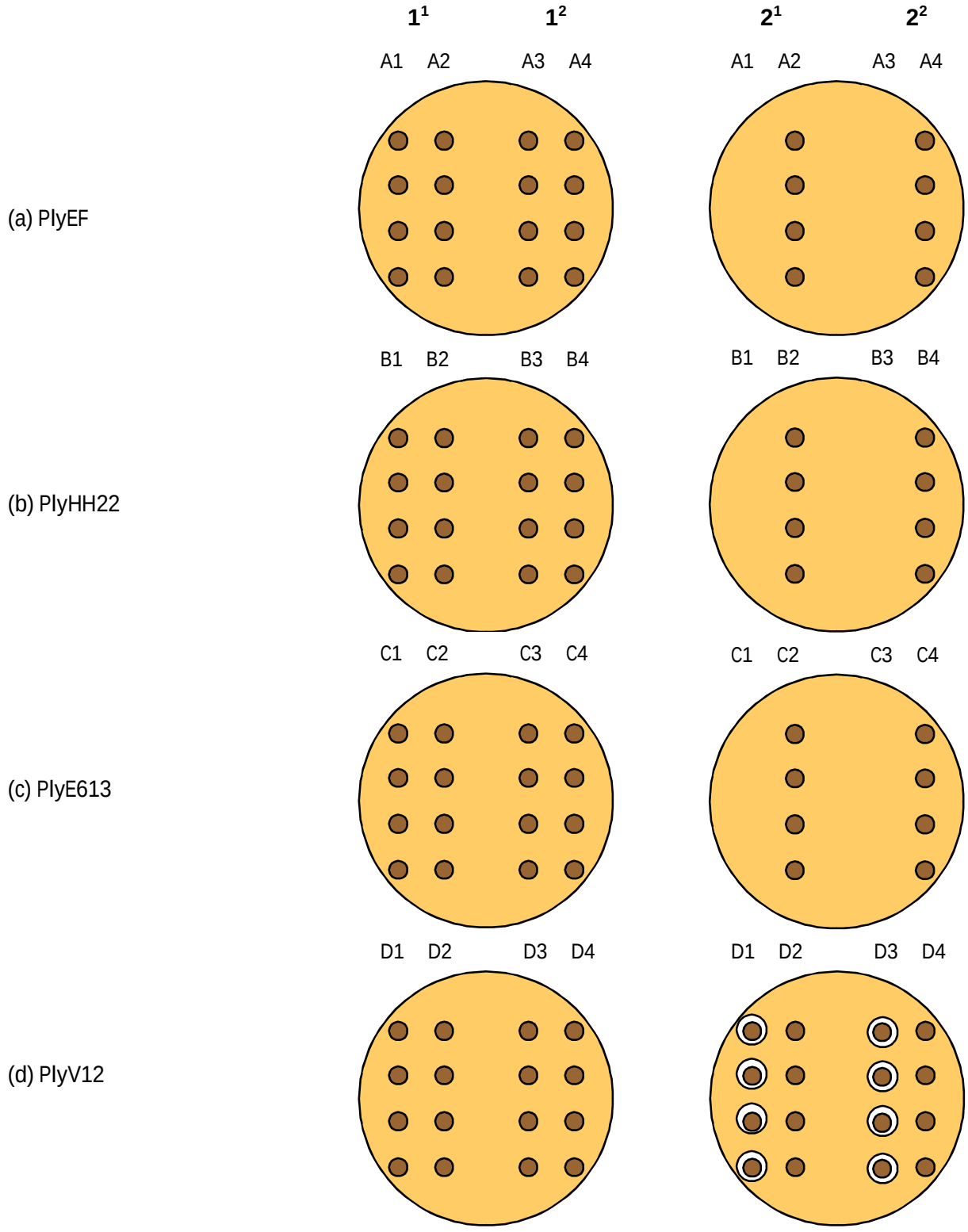


Figure 6. Schematic representation of the plates used to screen lysins expression and activity. (1^1) LA plates supplemented with Kan and Cam containing BL21/pET28a-plyEF (A1), BL21/pET28a-plyHH22 (B1), BL21/pET28a-plyE613 (C1), BL21/pET28a-plyV12 (D1) and E. coli BL21 with empty pET28a plasmids (A2, B2, C2 and D2). (1^2) LA plates supplemented with Amp containing BL21/pIN-IIIA-plyEF (A3), BL21/pIN-IIIA-plyHH22 (B3), BL21/pIN-IIIA-plyE613 (C3), BL21/pIN-IIIA-plyV12 (D3) and *E.coli* BL21 with empty pIN-IIIA plasmids (A4, B4, C4 and D4). (2^1/2^2) LA plates with the corresponding antibiotics plus IPTG, overlaid with *E. faecalis* ATCC 29212 supplemented with agar.

Lysin expression and solubility assessment. A protein gel was made to assess lysin expression and solubility. To this end, transformed *E. coli* BL21 harboring proper inserts were grown at 37°C to an OD_{600nm} of 0.7 and induced with 0.4 mM IPTG during 1, 2, 3 or 4 hours. Then samples were frozen overnight and sonicated to form a mixture of all proteins from *E. coli* cytoplasm including lysins, if they are synthesized. The total and soluble fractions of induced cultures and the total fractions of non-induced cultures were run on NuPAGE® Bis-Tris Gels.

Protein mixtures derived from BL21/pET28a-plyEF and BL21/pET15b-plyEF were run on the protein gel, but no supplementary band between 40 and 50 kDa (plyEF molecular weight: 43 kDa) was observed.

Then BL21/pET28a, BL21/pET28a-plyHH22, BL21/pIN-IIIA-plyHH22, BL21/pET28a-plyV12 and BL21/pIN-IIIA-plyV12 were grown at 37°C to an OD_{600nm} of 0.7 and the bacterial growth was followed by spectrophotometry after IPTG was added. A weak decrease in turbidity was observed for induced BL21/pET28a-plyHH22 whereas non-induced bacteria continued to proliferate reaching an OD_{600nm} of 0.981 after 4 hours. An increase in turbidity (i.e. a bacterial growth) was observed for BL21/pET28a-plyV12 for both induced and non-induced fractions. The bacterial growth was maintained for *E. coli* BL21 containing empty pET28a plasmids (Figure 7). The protein mixtures derived from BL21/pET28a-plyHH22 were nevertheless run on the protein gel, but no supplementary band between 40 and 50 kDa (plyHH22 molecular weight: 41 kDa) was observed. Finally protein mixtures derived from BL21/pET28a-plyV12 were tested. A supplementary band with a low intensity between 30 and 40 kDa (plyV12 molecular weight: 34 kDa) was observed for the total induced fraction only.

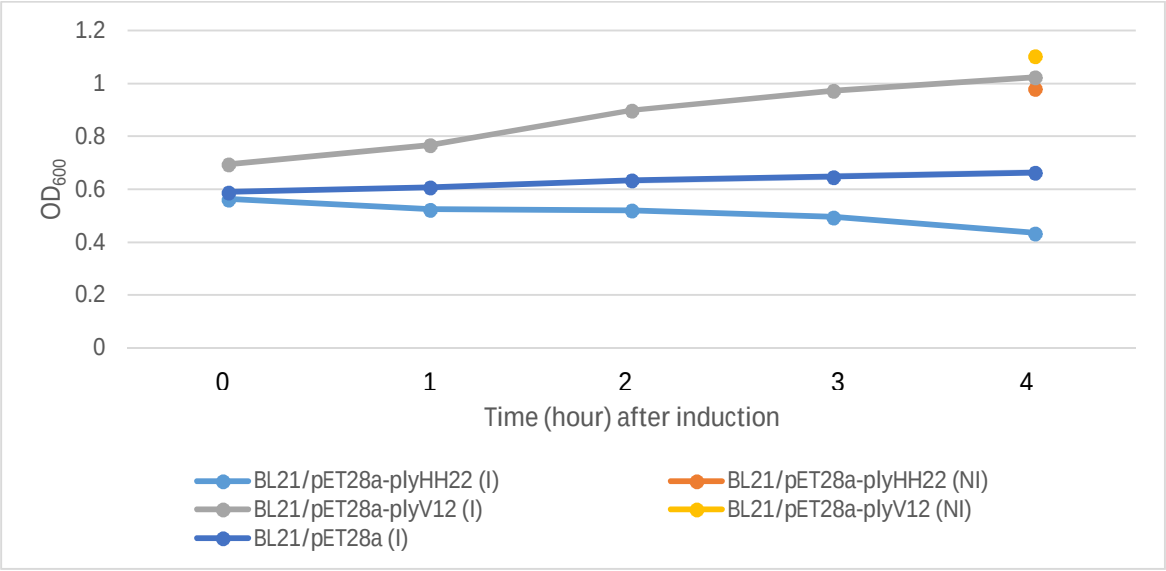


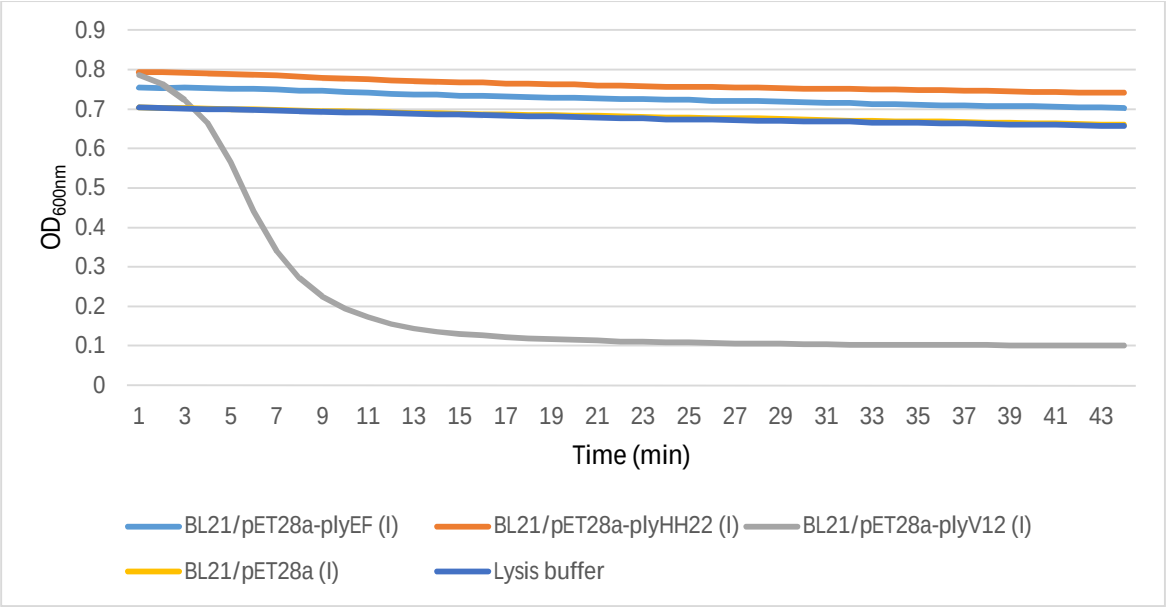
Figure 7. Evolution of the turbidity after protein synthesis induction with IPTG. (I) Single measure of the turbidity after 4 hour for the non-induced bacteria. (NI)

Lysin antibacterial activity evaluation. The antibacterial activity of lysins was evaluated by following the decrease in turbidity of a suspension of *E. faecalis* ATCC 29212 after the same volume of a mixture containing all proteins from recombinant *E. coli* cytoplasm was added. Lysis buffer and protein mixture from *E. coli* transformed with empty pET28a were also mixed with the bacterial suspension to ensure that no other molecule had antibacterial activity.

Protein mixtures derived from BL21/pET28a-plyEF and BL21/pET28a-plyHH22 were mixed with the bacterial suspension. A weak decrease in turbidity of the bacterial suspension was observed. The decrease in turbidity was similar with the protein mixture coming from BL21/pET28a and with the lysis buffer. No differences were observed between the total, soluble and non-induced fractions.

Protein mixtures derived from BL21/pET28a-plyV12 induce a rapid decreased in turbidity of the bacterial suspension with both induced and non-induced fractions. After 5 minutes (9 minutes for the non-induced fraction) the turbidity is reduced by one half and after 35 minutes the turbidity reaches a plateau with an OD_{600nm} near to 0.110. For the non-induced fraction, the steady-state was reached after 52 minutes with an OD_{600nm} near to 0.120 (Figure 8).

(a) Comparison of the antibacterial activity of protein mixtures



(b) Screening of the antibacterial activity of plyV12

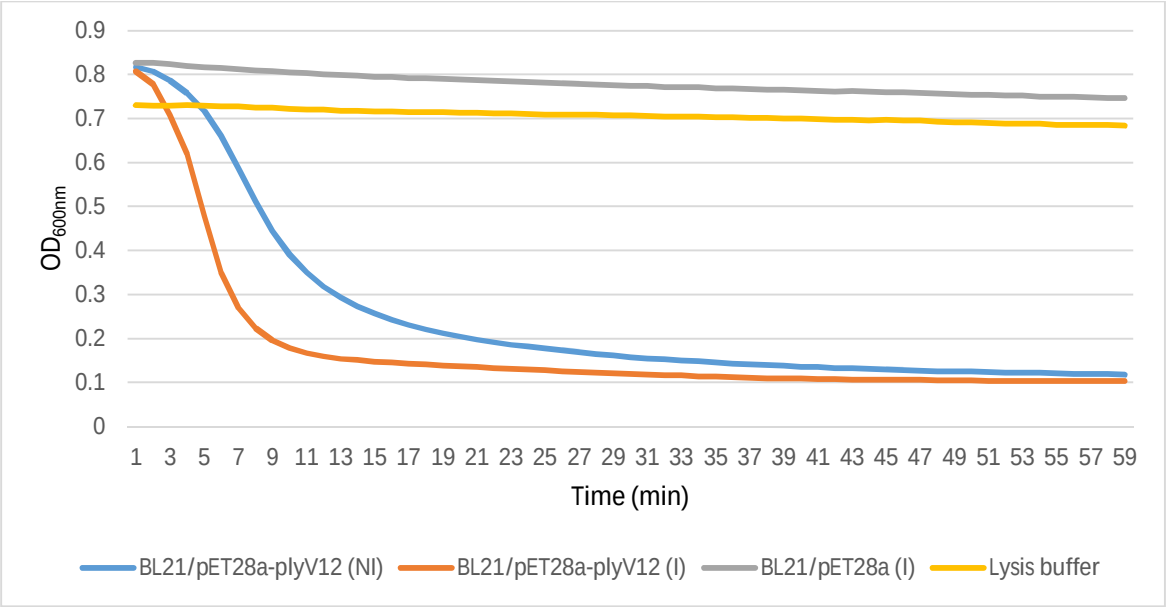


Figure 8. Protein mixtures antibacterial activity against *E. faecalis* ATCC 29212. (a) Comparison of the antibacterial activity of protein mixtures obtained from BL21/pET28a, BL21/pET28a-plyEF, BL21/pET28a-plyHH22 and BL21/pET28a-plyV12. The total fraction induced with IPTG during 4 hours was used in this experiment. (b) Screening of the antibacterial activity of plyV12. Comparison of the lytic activity of protein mixtures obtained from non-induced BL21/pET28a-plyV12 and from induced bacteria during 4 hour. The total fraction was used in this experiment. (I) stands for IPTG-induced and (NI) for non-induced.

Discussion

In this study, three new prophage lysin genes were identified in the genome of different *E. faecalis* strains by screening a database of prophage sequences from published bacterial genomes. After a literature search, three *E. faecalis* lysins that have already shown their efficacy were identified. Three new lysin genes were identified and analyzed using bioinformatics methods to ensure that they were not identical to previously described lysins.

First, the plyEF gene was cloned in pET28a and pET15b using a rapid cloning protocol. Recombinant bacteria selected by colony PCR and restriction digestion were plated onto LA plates to screen expression and activity of plyEF. All bacteria grew successfully on plates with and without IPTG, but no lysis zone surrounding the colonies was observed. This failure appeared to result from the absence of the plyEF gene in the plasmids as confirmed by DNA sequencing. This result was unexpected since the plyEF gene was amplified by colony PCR and isolated by restriction digestion. Moreover the same purified plasmids were used for the restriction digestion and the DNA sequencing. In view of these results, the cloning protocol was modified; the digestion and ligation times were prolonged to improve the insertion of the lysin genes in the plasmids and pET15b was replaced by pIN-IIIA.

Second, the modified cloning protocol was run with plyEF, plyHH22 and plyE613. Recombinant bacteria selected by colony PCR and restriction digestion were plated onto LA plates with and without IPTG in order to screen the expression and activity of lysins. However only some of the transformed bacteria appeared to grow on the plates supplemented with IPTG and no lysis zone surrounding the colonies was observed. Re-analyzing the content of plasmids indicated that only bacteria containing “empty” plasmids were able to grow on both plain and IPTG-induced plates. Moreover, growth curves in liquid media confirmed that the presence of plyEF, plyHH22 and plyE613 prevented the growth of BL21(DE3)pLysS chemically competent *E. coli* (see below). Therefore, these lysins must have some kind of toxic effect against the bacterial hosts and induce an interruption of the bacterial growth. A second method to induce protein synthesis was tested; recombinant bacteria were grown overnight and then vaporized with IPTG during 1 hours. The appearance of the colonies was not modified and no lysis zone surrounding the colonies was observed, although the presence of lysins genes was confirmed in all bacteria by DNA sequencing. It was not possible to screen the expression and the activity of these lysins, because they were probably not synthesized or synthesized in too small quantity to form lysis zones around the colonies.

An alternative method was tested to screen the expression of the lysins; protein mixtures derived from recombinant bacteria induced with IPTG were run on a protein gel. The bacterial growth was followed by spectrophotometry after the protein synthesis was induced. A decrease in turbidity of the solution that contains bacteria expressing plyHH22 was observed, whereas non-induced bacteria and bacteria containing plyV12 gene or empty plasmids continued to grow. The toxic activity of plyHH22 against *E. coli* BL21 was confirmed in this experiment. The weak decrease in turbidity does not allow to say if the synthesis of plyHH22 induces an interruption of the bacterial growth or a bacterial lysis. The protein mixtures were nevertheless electrophoresed and no supplementary band was observed in the protein gel. This result was expected, since the lysins was not synthesized in sufficient quantity to form a supplementary band in the protein gel.

Finally, the expression and activity of the lysins were screened by following the decrease in turbidity of a suspension of *E. faecalis* ATCC 29212 diluted with the protein mixtures obtained in the previous experiment. No significant decrease in turbidity (i.e. no lytic activity against *E. faecalis*) was observed. This result was expected since plyEF and plyHH22 were already not highlighted in the protein gel. The lysins were not synthesized or synthesized in too small quantity to kill bacteria, or maybe they had no lytic activity against *E. faecalis* ATCC 29212.

None of these three experiments has confirmed the expression and activity of our lysins. To confirm the feasibility of the cloning protocol, the previously described plyV12 was tested following the same steps. Recombinant bacteria have grown on LA plates supplemented with IPTG and clearing zone surrounding the colonies were observed rapidly after the agar/bacteria suspension was poured on the plates. The expression of plyV12 was then confirmed with the protein gel, but only for the induced total fraction. This means that plyV12 was insoluble in the conditions of the experiment. Finally, a rapid decrease in turbidity was observed after the protein mixture containing plyV12 was diluted with a suspension of *E. faecalis* ATCC 29212. This confirms that it is possible to synthetize lysins with this protocol.

In conclusion, we successfully identified three new phage lysins putatively active against *Enterococcus* spp. using bioinformatic tools. We also successfully devised a protocol to clone them in standard *E. coli* expression vectors, in which they could be expanded. The validity of the technique was attested by processing in parallel the described lysin plyV12, used as a control. Nevertheless, we failed to recover any of the three new lysins when it came to express them in the *E. coli* surrogate using IPTG induction. Nor could we recover limited amounts of the proteins that could carry out some anti-enterococcal lytic activity by expression leakage in IPTG non-induced cells. This indicates toxicity of the cloned lysins at either the transcriptional or translational levels. To this end we have not clue as to the responsible mechanisms.

However, there might be ways out to inquire about these issues. One would be to express truncated forms of the lysins containting either the main lytic domains or other domains, in order to map which part(s) of the gene is (are) responsible for *E. coli* toxicity. In the same experimental setting, if lytic domains were to be expressible alone, then they could be tested for their intrinsic lytic activity against target enterococci.

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