UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA
DEPARTAMENTO DE MICROBIOLOGIA E IMUNOLOGIA

BACTERIOPHAGE LYTIC ENZYMES AND THEIR ENGINEERING TOWARDS IMPROVED ANTIBACTERIAL EFFICACY

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Daniela Sofia Moreira Proença

Tese orientada pelos Professores Doutores Carlos Jorge Sousa de São-José (orientador universitário) e Miguel Ângelo da Costa Garcia (orientador empresarial), elaborada para a obtenção do grau de doutor em Farmácia (Microbiologia)

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ABBREVIATIONS

GENERAL

CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
dsDNA	double stranded DNA
dsRNA	double stranded RNA
EFS	*Enterococcus faecalis* strain
His	Histidine tag
NCBI	National Center for Biotechnology Information
OD	Optical Density
CDD	Conserved Domain Database
RNA	Ribonucleic Acid
ssDNA	single stranded DNA
Pfam	Protein family

TECHNIQUES

ELISA	Enzyme-linked immunosorbent assay
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field Gel Electrophoresis
SDS-PAGE	Sodium Dodecyl Sulphate - Poli-Acrilamide Gel Electrophoresis
SEC	Size-Exclusion Chromatography
SEC-MALS	SEC-Multi Angle Light Scatering

REAGENTS

BHI	Brain Heart Infusion
BS3	bis(sulfosuccinimidyl)suberate
DTT	Ditiotreitol
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HRP	Horseradish peroxidase
NaCl	Sodium Chloride

TSB	Tripton Soy Broth

UNITS

µg	microgram
µL	microliter
µM	micromolar
µmol	micromol
kb	kilo base
kDa	kilo Dalton
LB	Lysogeny broth
mg	miligram
mL	mililiter
pmol	picomol
mM	milimolar

SUMMARY

Increasing antibiotic resistance among bacterial pathogens has been promoting the study of bacteriophage (phage) lytic enzymes (bacterial cell wall hydrolases) as alternatives/complements to antibiotics. Phages can employ two types of these enzymes during their life cycle: i) virion-associated lysins (VALs), which promote a local cleavage of cell wall bonds to facilitate phage genome entry into the host cell; and ii) endolysins that destroy the wall at the end of infection, leading to cell burst and release of virion progeny. We studied the lytic activity of two enterococcal endolysins, Lys168 and Lys170, towards clinical isolates of different Gram-positive bacterial pathogens. In the conditions tested, both enzymes showed broad antimicrobial activity against *E. faecalis*, including vancomycin-resistant strains, and to less extent against *E. faecium*.

We show that *lys170* expression results in the production of the expected full length polypeptide (Lys170FL, 32.6 kDa) and of a C-terminal fragment of the enzyme (CWB170, 12 kDa), with both proteins co-eluting in the purification steps. Further analysis revealed that CWB170 corresponded to the Lys170 cell wall binding domain, which is independently produced from an in-frame, secondary translational start site. Biochemical and biophysical analysis indicated that the fully active Lys170 is a complex most likely corresponding to one subunit of Lys170FL associated to three of CWB170. Study of Lys170 has thus uncovered a new strategy of increasing the number of CWB domains in this type of enzymes.

A frequently reported problem when working with phage lytic enzymes is their propensity to become insoluble. Further, the activity of endolysins is rarely studied in conditions that promote robust growth of target bacteria. With the goal of supplanting these limitations we engineered a chimerical lysin, EC300, aimed at lysing *E. faecalis* growing in rich culture media. EC300 resulted from the fusion of a M23 endopeptidase domain of a VAL to the CWB170 domain of Lys170. The bacteriolysin-like protein exhibited a clear enhanced lytic activity when compared to the parental endolysin, particularly when assayed in a rich culture medium, thus having the potential to be used as an anti-*E. faecalis* therapy.

Keywords: Endolysin, M23 peptidase, chimeric lysin, cell wall binding domain, *Enterococcus.*

RESUMO

A emergência de bactérias patogénicas resistentes a antibióticos e a consequente limitação de antibióticos eficazes na eliminação destes microrganismos tem sido o motor para a pesquisa de alternativas ao uso da terapia antimicrobiana convencional. Nesse sentido, tem sido intensamente estudado o potencial das hidrolases do peptidoglicano da parede celular bacteriana produzidas por vírus que infetam bactérias (bacteriófagos ou, mais simplesmente, fagos), como alternativas e/ou complementos aos antibióticos. Existem dois tipos de enzimas líticas que participam em etapas distintas do ciclo de infeção bacteriofágico: i) as lisinas associadas ao virião (VALs), responsáveis por uma clivagem controlada e não-letal do peptidoglicano (PG) para facilitar a entrada do genoma viral na célula bacteriana hospedeira; e ii) as endolisinas, que destroem a camada de PG no final do ciclo de reprodução do fago, o que leva à rutura (lise) da célula hospedeira com consequente libertação da descendência viral. As endolisinas em particular têm sido muito estudadas e exploradas como terapêutica antimicrobiana, uma vez que têm a capacidade de lisar rapidamente bactérias alvo Gram-positivas quando aplicadas exogenamente na forma de enzimas recombinantes.

Este trabalho iniciou-se com a identificação e estudo da actividade lítica de duas endolisinas produzidas pelos fagos de *Enterococcus faecalis* F168/08 e F170/08. A endolisina Lys168 apresenta um domínio catalítico (CD) da família das amidohidrolases / peptidases dependentes de cisteína-histidina (CHAP), enquanto Lys170 apresenta um CD da família Amidase_2. Ambas as proteínas foram heterologamente produzidas em fusão com uma extensão C-terminal de 6 histidinas e subsequentemente purificadas na forma de proteínas solúveis. A atividade lítica destas proteínas foi testada contra uma vasta coleção de isolados clínicos, que incluía diferentes espécies bacterianas Gram-positivas. Ambas as enzimas mostraram uma elevada especificidade contra isolados de *E. faecalis*, ainda que com capacidade de atuação em alguns isolados de *E. faecium*. Numa primeira fase, Lys168 e Lys170 foram testadas numa coleção não tipada de isolados clínicos e exibiram capacidade lítica em 81% e 97% das estirpes de *E. faecalis* (n = 73) e 42% e 54% das estirpes de *E. faecium* (n = 26), respetivamente. Numa segunda coleção de estirpes geneticamente caracterizadas composta por 30 estirpes clínicas de *E. faecalis* e 21 de *E. faecium*, incluindo enterococos resistentes à vancomicina (VRE), as lisinas Lys170 e

Lys168 exibiram atividade lítica em 93% e 73% das estirpes de *E. faecalis*, respetivamente. Curiosamente, neste segundo grupo de isolados apenas 10% das estirpes de *E. faecium* foram sensíveis à ação de ambas as enzimas. Contrastando com o espectro de ação de endolisinas de fagos de *E. faecalis* reportado anteriormente, Lys168 e Lys170 apresentaram uma atividade quase exclusiva sobre *E. faecalis*. Num ensaio controlado em meio líquido, ambas as lisinas foram eficazes na eliminação de células da estirpe VRE modelo *E. faecalis* V583.

Durante os ensaios de expressão heteróloga da endolisina Lys170 observou-se sistematicamente a produção de um fragmento C-terminal de Lys170 com cerca de 12 kDa, para além do polipéptido esperado correspondente à totalidade da proteína (Lys170FL, 32,6 kDa). Ambas as proteínas foram co-purificadas através de cromatografia de afinidade em colunas de níquel e subsquentemente submetidas a uma cromatografia de exclusão molecular (SEC) com o objetivo de as separar. Inesperadamente, os dois polipéptidos foram co-eluídos durante a SEC, sugerindo uma associação entre Lys170FL e o polipeptídeo de 12kDa. Análises genéticas e bioquímicas provaram que o polipéptido de menor dimensão correspondia essencialmente ao domínio que se previa mediar a ligação de Lys170 à parede celular (domínio CWB170). Demonstrou-se que este é produzido de forma independente a partir de um segundo sinal de tradução interno ao gene *lys170*. A eliminação deste sinal resultou na produção de uma única proteína (mLys170) de tamanho idêntico ao de Lys170FL, mas com a metionina de iniciação interna substituída por uma leucina. Surpreendentemente, a atividade lítica de mLys170 revelou ser muito reduzida quando comparada com a de Lys170 nativa (Lys170FL + CWB170). Notavelmente, a incubação de mLys170 com quantidades crescentes de CWB170 purificada permitiu melhorar progressivamente a atividade lítica de mLys170. Observou-se que CWB170 *per se* não produziu atividade lítica detetável contra *E. faecalis*, apesar de se ter demonstrado a sua afinidade para a superfície bacteriana. Análises bioquímicas e biofísicas suportam um modelo em que a forma ativa de Lys170 corresponde a um complexo constituído por uma subunidade de Lys170FL associada a três de CWB170. Complementarmente, ensaios de infeção com o fago F170/08 revelaram que os polipéptidos Lys170FL e CWB170 são igualmente produzidos neste contexto, descartando a possibilidade de produção artificial durante a sua expressão heteróloga. A endolisina Lys170 define assim uma nova família estrutural de hidrolases de PG, até à

data desconhecida, revelando uma nova estratégia de aumento do número de subunidades de ligação à parede neste tipo de enzimas.

Um problema frequentemente relatado quando se trabalha com este tipo de enzimas é a sua baixa solubilidade e/ou propensão para precipitarem durante a produção em larga escala, concentração ou armazenamento. Além disso, e com base nos estudos publicados atualmente, pode-se concluir que a atividade das hidrolases do PG de origem fágica é raramente estudada em condições que promovem o crescimento ativo da bactéria alvo. Com base nesta observação, construiu-se uma lisina quimérica, designada por EC300, com capacidade para eliminar células de *E. faecalis* em fase de crescimento activo em meios ricos em nutrientes. EC300 resultou da fusão de um domínio com atividade de endopeptidase do tipo M23 da VAL Orf73, também codificada pelo fago F170/08, com o domínio de ligação à parede CWB170 da endolisina Lys170. A estrutura hétero-oligomérica descrita para a endolisina Lys170 foi também observada para a quimera EC300, ou seja, a forma ativa desta proteína também corresponde a um complexo multimérico entre EC300FL e CWB170. Além de demonstrar uma elevada solubilidade, esta proteína, que apresenta uma organização de domínios funcionais semelhante a uma bacteriolisina, exibiu uma atividade lítica bastante superior à exibida pela endolisina parental, particularmente quando ambas são testadas em condições que permitem o crescimento robusto de *E. faecalis*. Em contraste com a Lys170, a lisina quimérica demonstrou ter a capacidade de eliminar eficazmente um painel de estirpes de *E. faecalis* geneticamente caracterizadas e com elevado nível de resistência a antibióticos, quando estas se encontravam em fase ativa de crescimento. A EC300 é a primeira enzima semelhante a uma bacteriolisina construída a partir de proteínas fágicas com elevada atividade antimicrobiana, constituindo assim um potencial agente terapêutico para a eliminação de infecções causadas por *E. faecalis*.

Palavras-chave: endolisina, peptidase M23, lisina quimérica, domínio de ligação à parede celular, *Enterococcus*.

THESIS OUTPUTS

The research work described in this thesis was performed in TechnoPhage SA laboratories, headquartered at Instituto de Medicina Molecular, Lisbon, Portugal, from October 2010 until November 2014, under the supervision of Dr. Carlos Jorge Sousa São-José from Faculdade de Farmácia da Universidade de Lisboa, Lisbon, Portugal, and Dr. Miguel Ângelo da Costa Garcia, president and CEO of Technophage, SA, Lisbon, Portugal.

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CHAPTER 1

GENERAL INTRODUCTION

BACTERIOPHAGES: THE VIRUSES OF BACTERIA

Bacteriophages, or phages, are viruses that infect bacteria. Phages are frequently described as the most abundant and diverse biological entity on earth and they are estimated to outnumber bacteria by a factor of ten (Hendrix, 2003; Pedulla *et al*., 2003). Phages were first discovered by Twort (1915) and d´Herelle (1917) in independent experiments and it was soon realized that these viruses could be explored as antibacterial agents (Chanishvili, 2012). Yet, the decisive impetus to the role of phages in Biology came up with M. Delbrück, that together with other scientists such S. Luria and A. Hershey, formed a research group that went by the name "phage group". The research conducted by this group and its followers on realizing the mechanisms of phage infection and bacterial lysis are at the very foundations of the field that later came to be known as Molecular Biology (Pennazio, 2006). Bacteriophages are ubiquitous forms, found wherever bacteria reside, but they are most frequently isolated from aquatic environments. Phages are not able to infect eukaryotic cells, requiring specific target bacterial cells for replication. This specificity can be highly refined, with each phage attacking just one bacterial species and, in some cases, a few strains of a given species (Hanlon, 2007).

The International Committee for Taxonomy of Viruses (ICTV) presently classifies viruses into 7 orders, 103 families, 455 genera and 77 families with unassigned order (http://ictvonline.org/taxonomyReleases.asp). Bacteriophages presently constitute 20 families (Table 1).

Table 1. Major characteristics of bacteriophage families.

Family	*Description*	*Examples*
Double-stranded (ds) DNA phages		
Myoviridae	Contractile long tail	T4
Siphoviridae	Non-contractile long tail	λ
Podoviridae	Short tail	T7
Corticoviridae	Lipid-containing phages with icosahedral capsid	PM2
Rudiviridae	Non-enveloped, straight rod-shaped phages	SIRV-1
Tectiviridae	Phages with internal lipoprotein vesicle icosahedral capsid	PRD1
SH1, group*		SH1
STV1 group*	Icosahedral with protruding vertices	STV1
Fuselloviridae	Lipid-containing with lemon-shape phages	SSV1
Globuloviridae	Enveloped, lipid-containing, spherical phages	PSV
Plasmaviridae	Enveloped, lipid-containing, no capsid phages	L2
Guttaviridae	Droplet-shaped phages	SNDV
Lipothrixiviridae	Enveloped, filamentous or rod-shaped phages	TTV1
Ampullaviridae	Bottled-shaped phages with helical nucleocapsid	ABV
Bicaudaviridae	Two-tailed, oval phages with helical nucleocapsid	ATV
Salteprovirus**	Short-tails, spindle-shaped phages	His1
Single-stranded (ss) DNA phages		
Inoviridae	Non-enveloped	Fd, MVL1
Microviridae	Non-enveloped, icosahedral phages	ΦX174
Double-stranded (ds) RNA phages		
Cystoviridae	Enveloped icosahedral phages	Φ6
Single-stranded (ss) RNA phages		
Leviviridae	Non-enveloped icosahedral phages	MS2

*Preliminary designation
**No family assigned

There is a variety of bacteriophage morphological types (Table 1 and Fig. 1A), although about 96% of those reported in the literature belong to the order *Caudovirales* (tailed phages, Fig. 1B). Phages from this order are composed by a double-stranded (ds) DNA-

containing icosahedral head, which is attached to a tail involved in the phage DNA delivery to host cells. Figure 1 and Table 1 illustrate the morphological diversity of phages and highlight some of their most typical features. The capsid is a protein shell that contains the viral nucleic acid; when present, the tail may or may not be a contractile structure, and connected to this are usually fibers or analogous structures involved in the recognition of specific receptors of the bacterial cell surface (Fig. 1B) (Hanlon, 2007). Tailed phages are classified into three families according to the morphological features of the tail: *Myoviridae*, *Siphoviridae* and *Podoviridae* (Table1 and Fig. 1A). These three families comprise the order *Caudovirales* (Ackermann, 2007; Maniloff, 2012). The other 4% of phages are distributed into 17 families, that comprise the polyheadral, filamentous, and pleomorphic phages. The nucleic acid material of phages can be made of ds or single-stranded (ss) DNA or RNA.

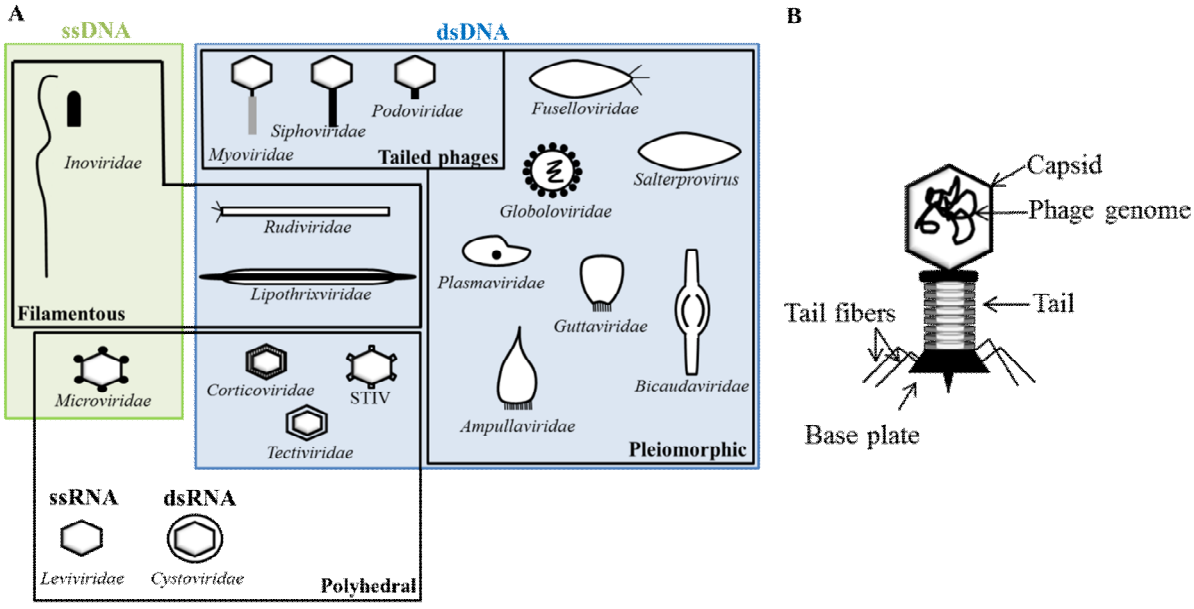

Fig. 1. (A) Schematic representation of the major bacteriophage families. (B) *Caudovirales* prototype here illustrated by the typical myovirus morphology.

Bacteriophages, like all obligate parasites, cannot complete their life cycle by themselves and depend on host bacterial cells to replicate and maintain. The phage extracellular form, the virion, is a supramolecular structure that has evolved to maximize viral propagation by protecting the phage genome and by promoting its efficient delivery to host bacteria. When phages encounter suitable bacterial cells during random motion, they adsorb to their cell surface (Fig. 2, adsorption step) *via* specific receptor sites. These may be a wide

variety of cell surface components, such as proteins, oligosaccharide, teichoic acids, peptidoglycan, lipopolysaccharides, or even bacterial structures like cell capsule, flagella or conjugative pilli (Hanlon, 2007; Rakhuba *et al*., 2010). After adsorbing, the phage injects its genome into the bacterial cell. This step can be mediated by different mechanisms, but in *Caudovirales* it usually involves major structural rearrangements of the tail and the formation of a conduit across the bacteria cell envelope (wall and membrane(s)), through which the genome is delivered to the host cell cytoplasm. After genome injection, two different lifestyles can be followed depending on whether the phage as a temperate or virulent (strictly lytic) nature: the lysogenic and/or the lytic pathways (Fig. 2). The lytic pathway, which may immediately follow viral genome entry of either temperate or virulent phages, has as major role: the multiplication and spread of the virus particle. It starts with an intense viral DNA replication and viral protein synthesis, taking advantage of bacterial synthetic machinery. Later in the infection process the viral genome is encapsidated and the virion progeny assembled within the host cell. During this process, lytic functions that include the holin and endolysin proteins (in dsDNA phages) accumulate within the infected cell and, at a specific time, both proteins cooperate in killing and disrupting the bacterial cell and consequently enabling the release of the newly formed virions (Catalão *et al.*, 2013; Young, 2014). Alternatively, temperate phages can follow the lysogenic circuit where the viral genome normally integrates into the bacterial chromosome. In some cases though, the phage genetic material can be maintained in the host cell cytoplasm as an extrachromosomal element (*e.g.* plasmid). In both situations the phage genome (prophage) is perpetuated as part of that of the host bacterium, with each daughter bacterial cell inheriting the viral DNA (Fig. 2, lysogenic pathway). Eventually, and generally in response to environmental factors, the prophage can be induced to enter the lytic pathway, leading to virion production and escape from infected bacteria through cell lysis, as described above.

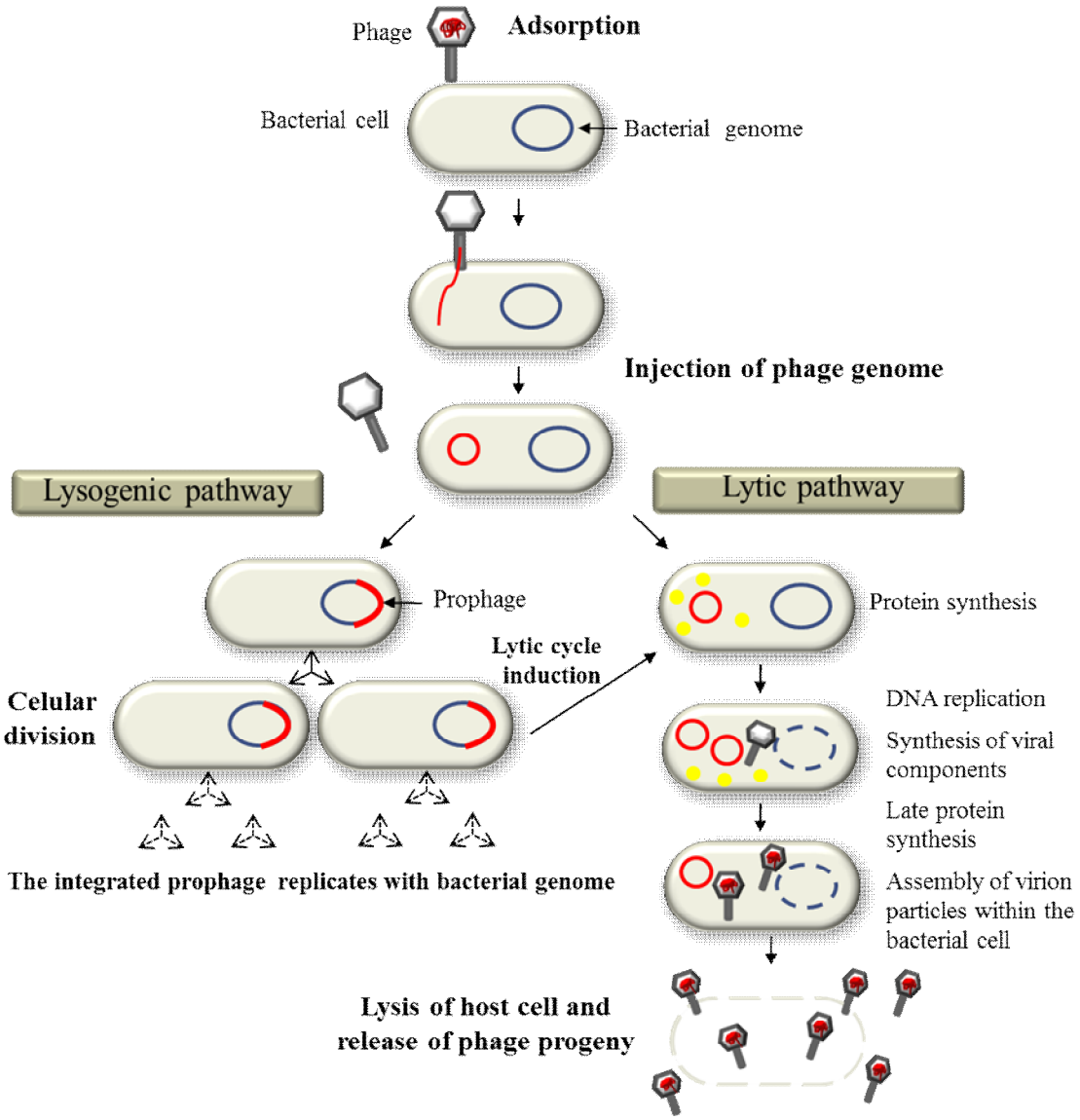

Fig. 2. Schematic representation of the two major bacteriophage life styles (adapted from Thiel, 2004).

THE BACTERIAL CELL ENVELOPE: A BARRIER TO PHAGE ENTRY AND EXIT FROM HOST CELLS

Like all viruses, phages need to deliver their genome to the site of replication within the host cell, in this case the bacterial cytoplasm. However, in contrast to eukaryotic viruses, the genome of the vast majority of bacteriophages enters naked, or accompanied by only a few virion proteins, to the host cell cytoplasm; the emptied virion structure remains at the cell surface (Vinga *et al.*, 2006). This most certainly reflects the rather rigid structure of the bacterial cell wall, which basically works as tight physical barrier to the passage of

most virus particles. Phages evolved mechanisms to deliver their genome into bacteria without compromising the integrity and functions of the cell envelope (Vinga *et al.*, 2006 and see below). In contrast, and exception made for filamentous phages, escape of the viral offspring from infected bacteria typically involves extensive disintegration of the envelope structure upon the action of phage lytic functions (Catalão *et al.*, 2013, see also below).

The complex and multilayer cell envelope of bacteria consists of a cytoplasmic membrane (CM), a cell wall (CW) and, an outer membrane (OM) in the case of Gram-negative bacteria and mycobacteria (Fig. 3).

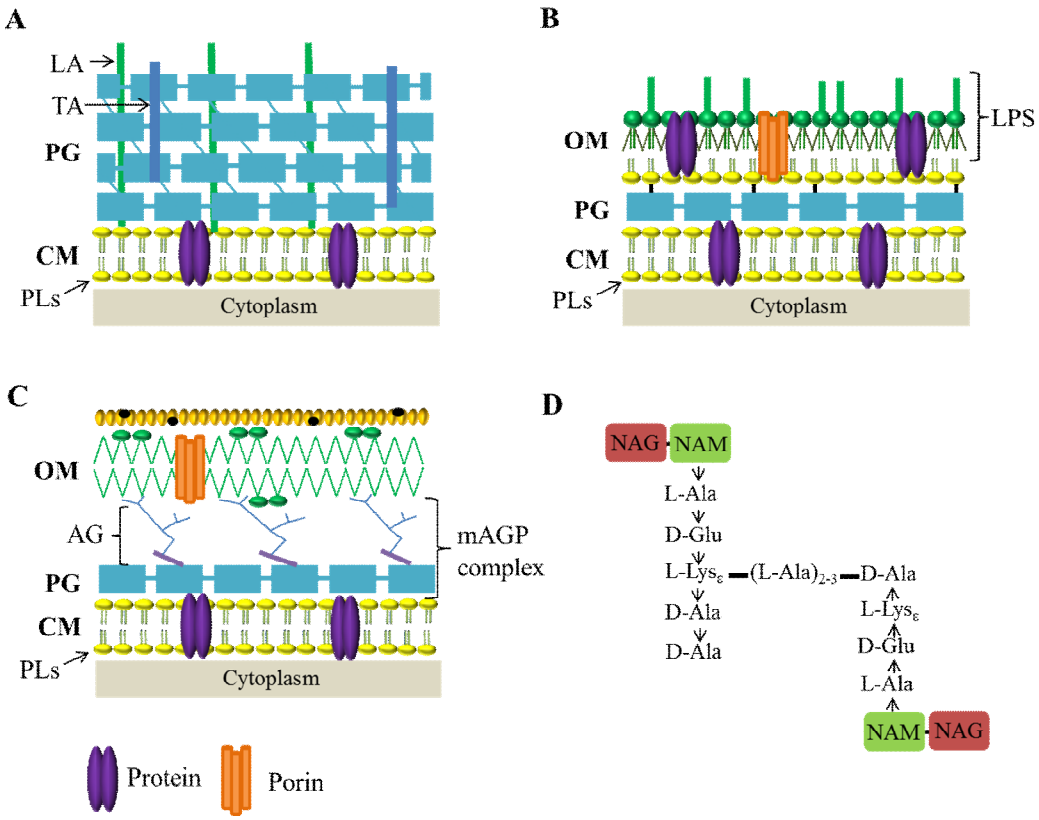

Fig. 3. Bacterial cell envelopes. (A) Gram-positive bacteria, (B) Gram-negative, and (C) mycobacteria. (D) *Enterococcus faecalis* peptidoglycan prototype structure (Schleifer and Kandler, 1972). OM, outer membrane; PG, peptidoglycan; CM, cytoplasmaic membrane; LA, lipoteichoic acids; TA, teichoic acids; LPS, lipopolysaccharides; PLs, phospholipids; AG, arabinogalactan; mAGP, complex arabinogalactan-peptidoglycan; NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid. Adapted from Catalão *et al.*, 2013.

The bacterial CM is a hydrophobic phospholipid bilayer imbedded with proteins, which surrounds and contains the cytoplasm and is common to all bacteria. It is the structure responsible of energy production, lipid biosynthesis, protein secretion, and transport, and acts as a semi-permeable barrier preventing leakage of hydrophilic constituents from the cytoplasm and protecting this cell compartment from external aggressions (Silhavy *et al.*, 2010). The CM is impermeable to protons and other ions, allowing the cell to sustain an electron-chemical gradient across the membrane and thus generating the so-called proton motive-force (PMF) (Weiner and Rothery, 2007).

Bacteria do not lyse when put into distilled water due to a rigid CW composed of peptidoglycan (PG), which protects the cells from osmotic pressure. PG is a large polymer made of repeating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), which are cross-linked by peptide side chains attached to NAM via amide bonds; due to its rigidity the CW also confers shape do the cell (Vollmer *et al.*, 2008a). The overall variation in the PG structure of the different bacteria resides in the amino acid sequence of stem peptides and, most importantly, in that forming the interpeptide cross-bridge (Schleifer and Kandler, 1972; Vollmer *et al.*, 2008a). Most Gram-positive bacteria have a stem peptide consisting of L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (L-Lys in position 3, Lys-type PG). Stem peptides of adjacent strands are cross-linked with an interpeptide bridge from the ε-amino group of the L-Lys residue of one strand to the carboxyl group of D-Ala in position 4 of the adjacent strand. This covalent modification results in the removal of the terminal D-Ala residue at position 5 (Fig. 3D) (Hancock *et al.*, 2014). The exact nature of these cross-bridges can be species-specific and accounts for more than 100 different PGs (Schleifer and Kandler, 1972). For most species in the genus *Enterococcus*, which was central to the work presented in this thesis (see next chapters), this cross-bridge is comprised of a single D-Asp residue (Kilpper-Bälz and Schleifer, 1987). *Enterococcus faecalis* appears to be an exception to this theme, as it possesses a cross-bridge of 2-3 L-Ala residues (Schleifer and Kandler, 1972) (Fig. 3D). In Gram-negative bacteria and some Gram-positive bacilli, peptide side chains are usually directly cross-linked, with the position 4 D-Ala of one chain being linked to the opposite meso-diaminopimelic acid (m-Dap) at position 3 (Dap-type PG) (Schleifer and Kandler, 1972; Vollmer *et al.*, 2008a).

Gram-positive bacteria are surrounded by several layers of PG that form a cell wall thicker than that found in Gram-negative bacteria. Inside the PG mesh of Gram-positive bacteria are long anionic polymers, the teichoic (TA) and lipoteichoic acids (LTA), which

can correspond to 60% of the mass of the cell wall, making them major contributors to envelope structure and function (Neuhaus, 2003; Dramsi *et al.*, 2008) (Fig. 3A).
In Gram-negative bacteria the thinner PG layer is surrounded by the OM, which is absent from Gram-positive organisms (Fig. 3B). This structure plays a major role in protecting Gram-negative bacteria from the environment by excluding toxic molecules and providing an additional stabilizing layer around the cell. Because the OM indirectly helps stabilize the inner membrane, the peptidoglycan mesh surrounding Gram-negative is covalently linked to the OM. The OM is a lipid bilayer composed by phospholipids (PLs) in the inner leaflet and lipophospholipids and lipopolysaccharides (LPS) in the outer leaflet (Ruiz *et al*., 2006).

Mycobacteria also have an OM, of distinct composition from that of Gram-negative bacteria, and, in these particular bacteria, the OM is surrounded by a capsule which is composed by proteins, polysaccharides and a small amount of lipids (Lemassu and Daffé, 1994; Lemassu et *al et*., 1996; Sani *et al*., 2010). Interestingly, in mycobacteria the PG is covalently attached to OM via arabinogalactan (AG), which is esterified to mycolic acids, forming the complex arabinogalactan-peptidoglycan (mAGP) (Brennan, 2003) (Fig. 3C).

Double-stranded DNA bacteriophages follow the most drastic strategy to overcome the host cell barriers and release their virion progeny, that is, they induce bacterial lysis. As detailed below, lysis is accomplished through specialized and regulated functions that compromise the physical integrity of the different layers composing the bacterial cell envelope.

PHAGE RELEASE FROM INFECTED CELLS: LYSIS-MECHANISMS OF dsDNA BACTERIOPHAGES

The culmination of the bacteriophage lytic cycle coincides with the lysis of the host cell to allow the release of the virion progeny. Lysis of bacterial hosts mediated by dsDNA phages seems to require at least two partners for efficient cell burst: a PG hydrolase, known as endolysin and a small hydrophobic protein designated by holin. Endolysins (see next section) are responsible for the breakdown of the PG network composing the cell wall and are essential for rapid and efficient host cell lysis. Holins are typically small

proteins (<150 amino acids) displaying 1 to 3 transmembrane domains and a hydrophilic C-terminus (Wang *et al*., 2000; Young 2002). Generally, holin and endolysin genes are clustered with the same transcription orientation in the phage genomes (São-José *et al*., 2003; Catalão *et al*., 2013).

The coordinated action of these two proteins in the lysis mechanism of *Escherichia coli* phage λ is, by far, the best studied and still serves as a model for most dsDNA phages employing this lysis strategy (São-José *et al.*, 2003; Young and Wang, 2006; São-José *et al.*, 2007). According to this model, phage endolysins accumulate in their active state in the host cell cytoplasm during phage replication, while holins are progressively embedded in CM. After reaching a critical concentration in the CM, the holins suddenly trigger to form a pore that dissipates the membrane PMF, thus killing the cell. In the case of the phage λ model system, this pore also constitutes the passage through which the endolysin gains access to the cell wall, which rapidly leads to its digestion, and hence to cell lysis (Young, 2013; Savva *et al*., 2014) (Fig. 4A).

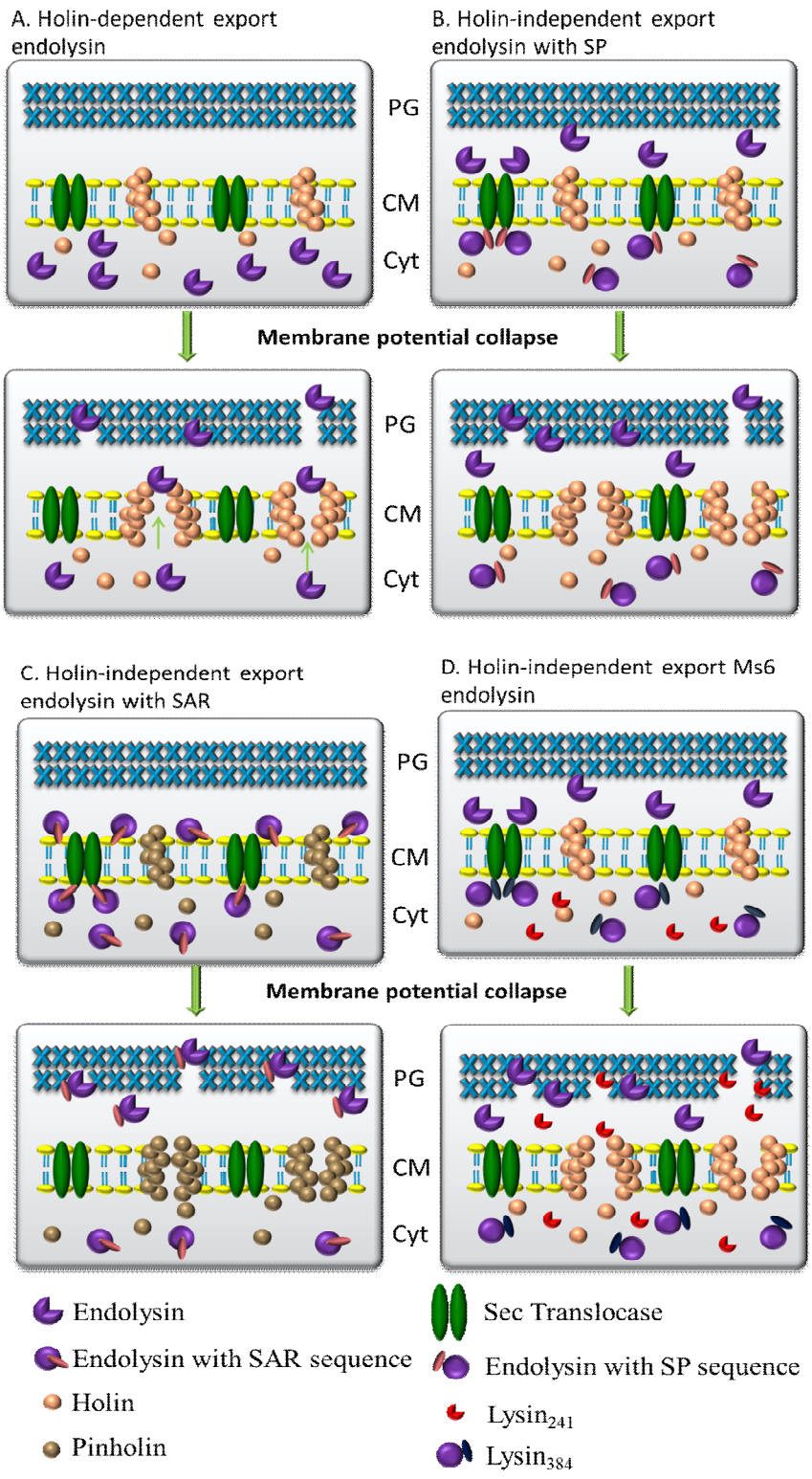

Fig. 4. Models for export and activation of endolysins. (A) The endolysin is exported to the periplasm through the holin pores (*e.g.* phage λ). Holin independent, Sec-mediated export of endolysin: (B) endolysins with typical signal peptides (SP) (*e.g.* fOg44); (C) endolysins with signal-arrested-release sequence (SAR) (*e.g.* P1); and (D) mycobacteriophage Ms6 endolysin, were the endolysin export is assisted by a chaperone protein. PG, peptidoglycan; CM, cytoplasmic membrane; Cyt, cytoplasm. Adapted from Catalão *et al.*, 2013.

The confinement of endolysins in the host cell cytoplasm during phage development was for long regarded as an imperative of any lysis strategy of dsDNA phages, simply because premature cell lysis, before the entire assembly of viral progeny, would not make biological sense. Today, however, there is an increasing awareness that phage lysis mechanisms can be diverse, with at least subtle deviations to the λ paradigm. For instance, it is now known that some dsDNA phages instead of making use of the holin holes to export their endolysins, they engage the host cell secretion machinery (Sec system) to carry these enzymes to the periplasm, way before the completion of the viral life cycle (São-José *et al*., 2000; Young, 2005; São-José *et al*., 2007). These phages produce endolysins with secretion signals, that is, typical signal peptides (SP) or signal-arrested-release (SAR) sequences, or synthesize chaperon-like proteins that interact with endolysins and target them to the Sec translocase (São-José *et al*., 2000; Xu *et al*., 2004; Catalão *et al*., 2010) (Fig 4. B, C and D).

In contrast to what was expected, it was proved that the export of these endolysins to the periplasm at early stages of virus replication had no major impact in the bacterial cell wall. This implied that the endolysins are kept inactive in the cell wall compartment, "waiting" for the exact moment for lysis to occur. An interesting observation is that the phages producing holin-independent exported endolysins also encode a holin. In fact, it has been demonstrated that even in the systems employing secreted endolysins the holins still maintain the key role of defining the lysis timing. In addition, the holin PMF-dissipating action is responsible for the activation of the pre-secreted endolysins (Nascimento *et al*., 2008; Young, 2013; Savva *et al*., 2014). It was speculated (São-José *et al*., 2000) and latter demonstrated (Frias *et al*., 2009) that the holin membrane-depolarizing function can also trigger the bacterial autolytic machinery, which contributes to fast and effective lysis of host cells. Interestingly, at least for phages relying on SAR endolysins, it has been shown that the cognate holins produce small-sized pores, as these need only to allow the passage of ions and depolarize the CM in order to fulfil their role in lysis (Park *et al*., 2006, 2007). These holins have been coined as 'pinholins' given their small-hole (pinhole)-forming character when compared to canonical holins like that of phage λ, which forms micron-scale holes (Park *et al*., 2007; Dewey *et al*., 2010).

In addition to the fundamental holin and endolysin players, dsDNA phages seem to have evolved auxiliary functions that contribute to the regulation and effectiveness of bacterial lysis. Well- known examples are the antiholin protein, whose role is to tune the timing of

the holin action, spanins that weaken the OM barrier of Gram-negative hosts and lipases that are thought to compromise the mycolyl-arabinogalactan external layer of the mycobacterial cell envelope (Catalão *et al.*, 2013; Young, 2014).

Besides endolysins, dsDNA bacteriophages can also encode cell wall lytic functions that are associated with the virus particle. These often make part of multidomain, virion structural proteins that are here designated as virion-associated lysins (VALs, see next section). In addition to their role in virion morphogenesis, VALs are thought to act at the onset of phage infection by promoting a local, controlled cleavage of cell wall bonds to facilitate phage genome transference to the host bacterial cell. Since endolysins and VALs were the main object of the studies presented in this thesis, the next sections will provide a detailed description of their fundamental features.

PHAGE-ENCODED PEPTIDOGLYCAN HYDROLASES

Due to the relatively conserved structure of PG, there are limited types of covalent bonds available for cleavage by phage PG hydrolases (Fig. 5). Independently of their prokaryotic or eukaryotic origin, PG hydrolases are generally classified into five major classes depending on the specific bond they attack and/or the reaction mechanism (Vollmer *et al.*, 2008b): 1) lysozymes; 2) lytic transglycosylases; 3) *N*-acetyl-*β*-*D*-glucosaminidases (glucosaminidases); 4) N-acetylmuramoyl-L-alanine amidases (amidases); and 5) peptidases (carboxi- or endopeptidases, but with the latter being much more relevant in the context of phage PG hydrolases) (Fig. 5). Lysozymes and lytic transglycosylases are also collectively known as *N*-acetyl-*β*-*D*-muramidases (muramidases).

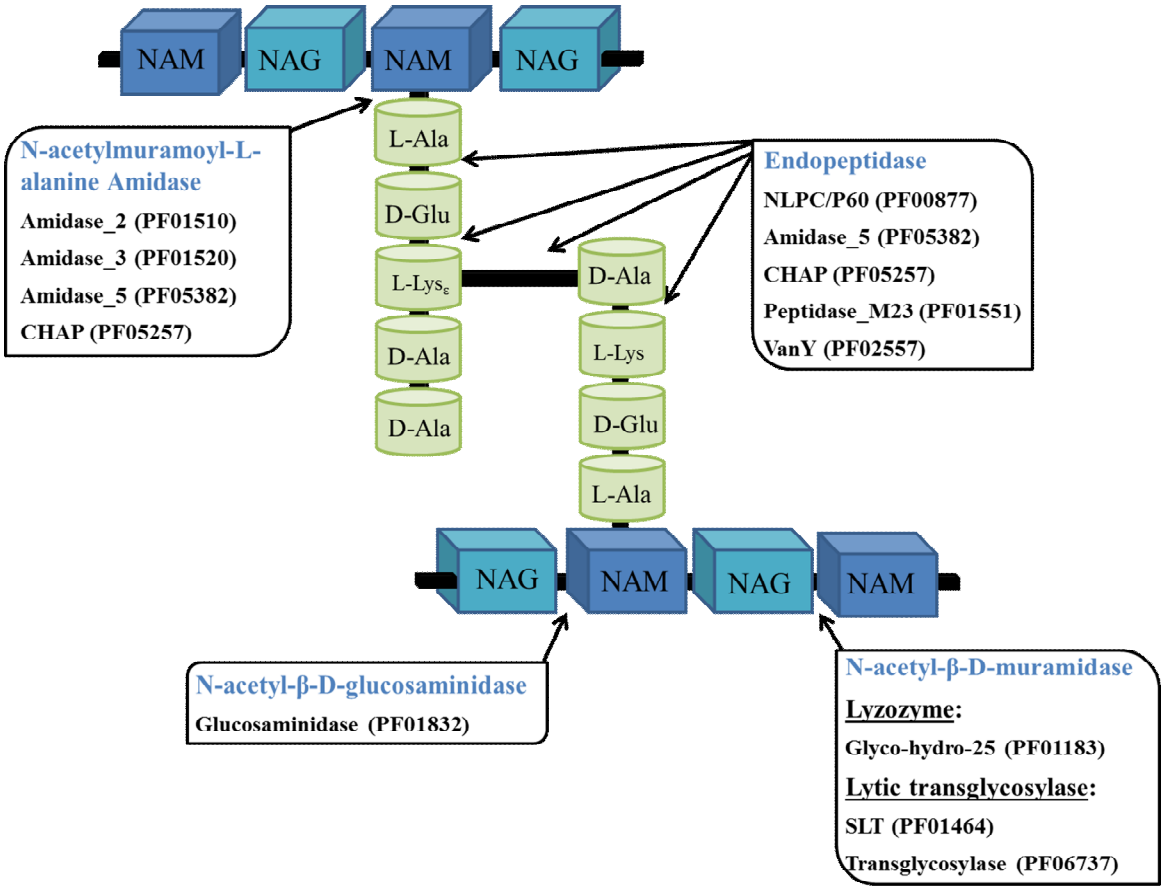

Fig. 5. Basic structure of peptidoglycan with indication of the bonds that are targeted by the five main types of enzymatic domains found in phage PG hydrolases. The predominant families of catalytic domains within each class of PG hydrolases are indicated according to the Pfam database nomenclature. Note that the genesis of these families is essentially based on primary sequence relatedness; this explains why different cleavage specificities can be displayed by a single family (*e.g.* CHAP and Amidase_5). SLT, soluble lytic transglycosylase; CHAP, cysteine, histidine-dependent amidohydrolases/peptidases; NAM, N-acetylmuramic acid; NAG, N-acetylglucosamine.

Lysozymes and lytic transglycosylases breakdown the *β*-1,4-glycosidic bond between NAM and NAG. However, the latter differ from true lysozymes in that they cleave the bond with concomitant formation of an intramolecular 1,6-anhydro ring at NAM by an intramolecular transglycosylation reaction (Holtje *et al.*, 1975; Thunnissen *et al.*, 1994). These glycosidase activities are frequently found in phage-encoded PG hydrolases, including VALs, like the tail-associated lysin CwlP from *Bacillus subtilis* phage SP-β (Sudiarta *et al.*, 2010), and endolysins, such as the pneumococcal lysozyme Cpl-1 (Garcia *et al.*, 1987) and the phage λ lytic transglycosylase R (Taylor and Gorazdowska, 1974). Lysozyme and lytic transglycosylase activities are also common in PG hydrolases

produced by bacteria, either in enzymes involved in cell wall metabolism or in bacteriolysins (PG hydrolases released to the extracellular media to attack bacterial competitors, formerly class III bacteriocins, Cotter *et al.*, 2005), and also in enzymes produced by eukaryotic cells (Scheurwater *et al*., 2008; Callewaert and Michiels, 2010)

Glucosaminidases cleave the glycan component of the PG on the reducing side of NAG. This kind of activity is much more frequent in autolysins, such as AltA from *E. faecalis* (Mesnage *et al*., 2008), but it has also been described for the streptococcal LambdaSa2 endolysin (Pritchard *et al*., 2007).

The amidases target the amide bond between the C2 on the NAM and the primary L-Ala of the stem peptide. This activity is among the most frequently found in PG hydrolases and seems to be associated quite often with endolysins, maybe because this bond is highly conserved in the bacterial cell wall PG (Nelson *et al*., 2012). In addition, since hydrolysis of this bond separates the glycan polymer from the stem peptides, it may be more destabilizing to the PG mesh than the cleavage of other bonds and thus it may have been favored evolutionarily by bacteriophages for rapid lysis of host cells (Nelson *et al*., 2012). This activity has been demonstrated for the amidase domain of the staphylococcal phage ϕ11 endolysin (Navarre *et al*., 1999), the phage K endolysin, LysK (Becker *et al*., 2009;) and the Listeria phage endolysins Ply511 (Loessner *et al*., 1995), just to give a few examples.

Finally, endopeptidases are the lytic enzymes that cleave any of the peptide bonds within or between the peptide stems. As referred to above, the most important variation among the bacterial cell wall PG resides in the interpeptide cross-bridges. Therefore, the activity of a given endopeptidase tends to be restricted to a particular type of PG. The listerial Ply500 and Ply118 endolysins display L-alanyl-D-glutamate endopeptidase activity (Loessner *et al*., 1995). The endolysin of the staphylococcal phage ϕ11 is a bifunctional enzyme, cleaving the bond between position 4 D-Ala and the first Gly residue of the pentaglycine cross-bridge (D-alanyl-glycine endopeptidase) in addition to its amidase activity (Navarre *et al*., 1999). The also bifunctional endolysin of the streptococcal phage B30 exhibits D-alanine-L-alanine endopeptidase and lysozyme activities (Pritchard *et al*., 2004). The bacteriolysin lysostaphin from *Staphylococcus simulans* cleaves the *S. aureus* pentaglycine cross-bridge (Iversen and Grov, 1973). Besides a lytic transglycosylase activity (see above), the VAL CwlP from *B. subtilis* phage SP-β also harbors a peptidase domain of the M23 family (Sudiarta *et al*., 2010).

Phage lytic enzymes, *i.e.*, endolysins and VALs, harbour at least one of the five muralytic activities just described but, as perceived from examples given above, some have been reported that comprise two distinct enzymatic specificities, which are generally attributed to two separate catalytic domains (CDs). In addition to these, the endolysins of phages infecting Gram-positive bacteria and mycobacteria typically harbor a C-terminal cell wall binding (CWB) domain, which mediates substrate recognition and enzyme anchoring (Nelson *et al.*, 2012; Payne and Hatfull, 2012; Schmelcher *et al.*, 2012; Oliveira *et al.*, 2013). Bioinformatics and structural studies have been evidencing the diversity of catalytic and CWB domains present in PG hydrolases, when considering their primary sequence and fold, with the same PG bond being cleaved by CDs of distinct configurations. Despite this, the wealth of enzyme sequences deposited in databases has been enabling, through bioinformatics analysis, the organization of CDs and CWB motifs into different superfamilies and/or families (López and García, 2004; Firczuk and Bochtler, 2007; Layec *et al.*, 2008a,b; Scheurwater *et al.*, 2008; Payne and Hatfull, 2012; Oliveira *et al.*, 2013). This, complemented with the development of sequence analysis tools (*e.g.* Marchler-Bauer *et al.*, 2011), generally allows the inclusion of the functional domains of a given PG hydrolase in known superfamilies/families. This type of analysis though should be taken with caution when trying to assign the cleavage specificities of lytic enzymes as it can lead to erroneous conclusions. For example, some CHAP CDs have been shown to specify amidase activity (Nelson *et al.*, 2006), others are endopeptidases (Navarre *et al.*, 1999; Pritchard *et al.*, 2004), and there is at least one example where a single CHAP displays both amidase and endopeptidase activities (Linden *et al.*, 2014).

Endolysins

Structural diversity

Analysis of the overall structure of known phage endolysins generally leads to a distinction of those targeting Gram-positive and mycobacteria from those acting on Gram-negative bacteria, which again probably reflects the major differences in the cell wall architecture of these major bacterial groups.

In Gram-negative bacteria, the PG lies between the OM and the CM and is a relatively thin layer. Endolysins from phages that infect this type of bacteria are usually single domain, globular proteins that typically harbor a single CD and range in mass from 15 to 20 kDa (Nelson *et al.*, 2012). Exceptions have been described, such as the Gram-negative endolysins KJ144 and EL188, both from *Pseudomonas* phages, which have been shown to carry a catalytic domain and an N-terminal CWB domain (Briers *et al.*, 2007; Fokine *et al.*, 2008) (Fig. 6).

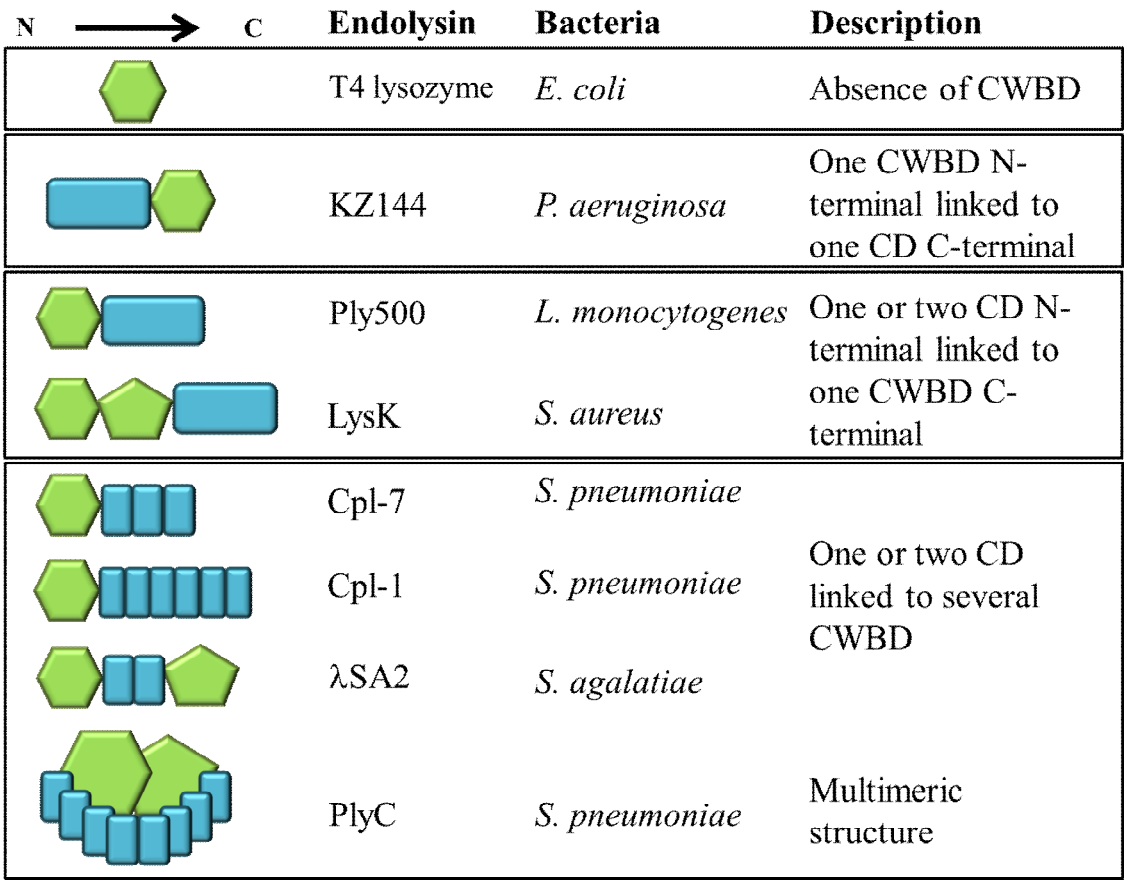

Fig. 6. Domain architecture of Gram-negative and Gram-positive endolysins. Functional domains not drawn to scale. Green boxes correspond to catalytic domains (CD); blue boxes represent cell wall binding domains (CWBD); N, N-terminus; and C, C-terminus.

Gram-positive organisms lack the OM and the PG is a highly cross-linked multilayer followed by the CM. As referred to above, Gram-positive endolysins show a modular structure (Diaz *et al.*, 1991) (Fig. 6) and are usually composed by one or two N-terminal CDs connected to one to several repeats of CWB motifs at the C-terminus, which specifically recognize the host PG or other cell wall components (López and García,

2004; Nelson *et al.*, 2012; Schmelcher *et al.*, 2012). The two endolysin functional domains are usually linked by a flexible peptide chain (Korndorfer *et al.*, 2006). The staphylococcal lysin LysK is an example of a bifunctional endolysin, which bears a CHAP endopeptidase and an amidase CD in the N-terminal region linked to a SH3b CWB domain (SH3_5 family, (Pfam08460) (O'Flaherty *et al.*, 2005; Horgan *et al.*, 2009).

The cell wall binding domain can have a significant impact in the activity range of endolysins. Several conserved CWB motifs have been described in the literature such as: the LysM domain (Visweswaran *et al.*, 2011), which is the most common CWB domain in PG hydrolases and has been shown to bind to NAG residues in the sugar backbone of the PG (Ohnuma *et al.*, 2008); the bacterial SH3b domain (Whisstock and Lesk, 1999), which is also present in some bacteriolysins; the choline-binding modules of Cpl-1 and other pneumococcal lysins (Lopez and Gracía, 2004), which specifically recognize the choline-containing theichoic acids in the cell wall of *S. pneumonia*; and the Cpl-7 biding domain, which binds to ethanolamine molecules present in the pneumococcal cell walls (Bustamante *et al.*, 2010).

The recognition specificity of a CWB domain in many cases encompasses an entire bacterial genus, as observed in studies using various GFP-tagged staphylococcal SH3b binding domains (Gu *et al.*, 2011), and is in general broader than the spectrum of the respective phage. This indicates recognition of a rather conserved ligand such as the pentaglycine interpeptide bridge shared by the most staphylococcal strains (Schleifer and Kandler, 1972). Other interesting feature about CWB motifs is that frequently they appear in multiple copies. Cpl-1 endolysin bears 6 tandem copies of the choline-binding repeats and its lytic activity depends on activation through choline binding (Garcia *et al.*, 1990). The related pneumococcal endolysin Cpl-7 harbors 3 tandem repeats of a different CWB motif and appears to lyse bacteria both exhibiting choline and ethanolamine at the cell wall (Diaz *et al.*, 1991).

Gram-positive endolysins are generally described as being monomeric proteins and are thus the product of a single gene. A remarkable exception is the pneumococcal endolysin PlyC, which is composed of two different subunits, PlyCA and PlyCB encoded by separate genes. PlyCA is a two CD-containing polypeptide that associates with eight PlyCB subunits with CWB activity (Nelson *et al.*, 2006; McGowan *et al.*, 2012) (Fig. 6).

Endolysin mode of action

The degradation of the PG layer by the action of endolysins in the context of phage infection leads to lysis of the bacterial cell. As revealed by thin-section electron microscopy, endolysins seem to display their lethal effects by forming holes in the cell wall through PG digestion. The high intracellular osmotic pressure causes extrusion of the cytoplasmic membrane, ultimately leading to hypotonic lysis (Fischetti, 2008; Fischetti, 2005). In principle, a single endolysin molecule should be sufficient to cleave several numbers of bonds. However, Loessner and collegues (2002) showed that a listerial phage endolysin had a binding affinity approaching that of an IgG molecule for its substrate, suggesting that phage proteins are one-use enzymes, probably requiring several molecules attacking the same region to efficiently weaken the cell wall.

Virion-associated lysins of dsDNA bacteriophages

As described above, bacteriophages must transport their genome across the bacterial cell envelope to initiate infection. The common obstacles to phage genome transit are the PG and CM layers but additional barriers like an OM and/or a polysaccharidic capsule may be present depending on the host. While the OM is generally traversed by puncturing (for example by a device of the tail), crossing of capsule and PG layers generally benefit from depolymerizing activities carried in the virion structure (Casjens and Molineux, 2012). Most phage particles carry at least one protein with cell wall degrading activity (the VAL) that allows access of the tail tube to the CM (Moak and Molineux, 2004). Traffic through this last barrier likely involves pore formation and/or membrane fusion events but its molecular details remain the less understood in the process of virus entry (Letellier *et al.*, 2004). VALs are designed to promote a “surgical” lesion in the cell wall without leading to cell death. However, if a VAL-carrying phage adsorbs at very high multiplicities to a host cell, it can culminate in cell destruction. This phenomenon is denominated by “lysis from without”, as it is a lysis that does not rely on phage infection (Abedon, 2011).

VALs seem to be quite common in both Gram-negative and Gram-positive infecting phages (Moak and Molineux, 2004). These enzymes are typically associated to the phage DNA injection machinery and are most frequently incorporated in the tail structure (Fokine and Rossmann, 2014). The P7 VAL of the tail-less, dsDNA phage PRD1 which

infects various Gram-negative bacteria, such as *E. coli*, *Salmonella enterica* and *Pseudomonas aeruginosa*, is associated with the membrane beneath the icosahedral capsid (Rydman and Bamford. 2000). VALs are much less studied compared to endolysins. Very often they correspond to multifunctional proteins that, in addition to the PG hydrolase activity, play a role in the assembly of the phage tail. Known examples of this are the tape measure proteins (TMP), which determine the length of the tail and at the same time may display PG hydrolase activity (Piuri and Hatfull, 2006; Boulanger *et al.*, 2008). VALs may also make part of central tail knobs, fibers or spikes (Moak and Molineux, 2000; Kanamaru *et al.*, 2002; Kenny *et al.*, 2004; Xiang *et al.*, 2008). They are usually larger than cognate endolysins, present high sequence diversity and variable domain organization (Rodriguez-Rubio *et al.*, 2012) (Fig. 7).

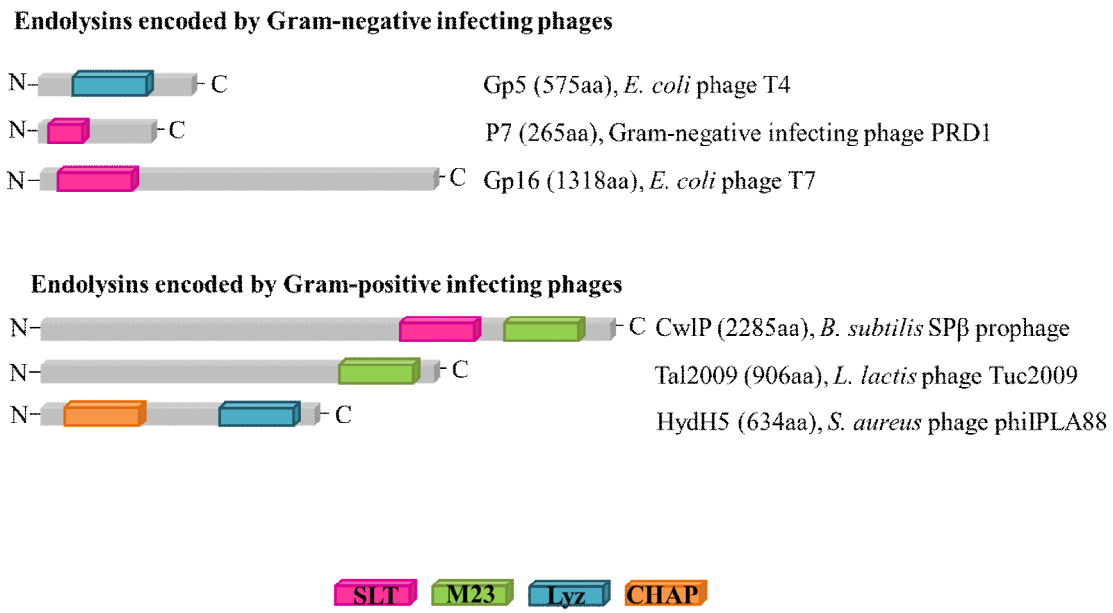

Fig. 7. VALs domain organization and diversity of PG cleavage specificities. Three illustrative examples (not to scale) of known VALs targeting Gram-negative and Gram-positive bacteria are shown. CD families: SLT, soluble lytic transglycosylase; M23, peptidase M23; Lyz, lysozyme; CHAP, cysteine, histidine-dependent amidohydrolases/peptidases.

The domains of VALs responsible for PG hydrolase activity are related to those of endolysins and bacterial PG hydrolases. Yet, in contrast to the endolysins acting on Gram-positive bacteria, the VALs targeting this group of bacteria usually lack a domain responsible for cell wall binding (Rodriguez-Rubio *et al.*, 2012). The lack of a CWB

domain is not surprising given the context of action of these proteins. Receptor binding proteins (RBP) carried in the phage tail distal end are responsible for recognition and attachment to bacterial surface receptors. This RBP/receptor interaction triggers major conformational changes in the tail structure that ultimately places the VAL it in close contact with its substrate (Fig. 8), thus becoming unnecessary the presence of a specific domain to direct the enzyme, as it happens with Gram-positive endolysins (Veesler and Cambillau, 2011; Rodriguez-Rubio *et al.*, 2012; Fokine and Rossmann, 2014). One exception to this rule seems to be the staphylococcal phage 68 VAL P17, which shows a typical endolysin domain organization composed by an N-terminal CD and a C-terminal CWB domain (Takac *et al.*, 2005). Interestingly, Rodriguez and collaborators (2011) showed that the two CDs of the VAL HydH5, encoded by the *S. aureus* phage phiIPLA88, had the ability to bind target cells. In fact, VALs acting on Gram-positive bacteria frequently display two CDs (Rodriguez-Rubio *et al.*, 2013). To date, there is no described VAL from a Gram-negative infecting phage that harbors more than one CD.

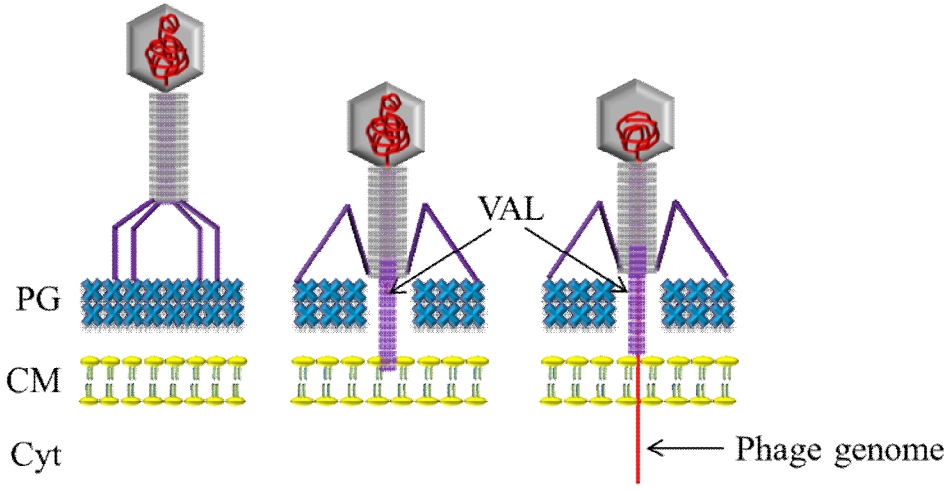

Fig. 8. Schematic representation of the mode of action of a virion-associated lysin (VAL) of a prototype Gram-positive *Myoviridae* bacteriophage.

A curious observation is the apparent abundance of VALs of Gram-positive phages carrying a CD of the peptidase M23 family in (our analysis). As far as we know, such CD has never been observed in VALs from Gram-negative phages. The M23 peptidase CD is also present in other PG hydrolases that, similarly to VALs, “attack” the bacterial cell from the outside. This is the case of the bacteriolysins lysostaphin (Shindler and

Shuhardt, 1964) and Enterolysin-A (Nielsen *et al*., 2003). In contrast, the peptidase M23 is rarely found in endolysins, with an exception being the staphylococcal phage 2638A endolysin (Abaev *et al.*, 2013).

THE USE OF PHAGE-BASED PRODUCTS TO CONTROL PATHOGENIC BACTERIA

The increasing antibiotic resistance among some important bacterial pathogens, associated to the short pipeline of new antibiotic drugs (Gould, 2008; Boucher *et al*., 2009), has been calling for the urgent need of developing alternative antibacterials. Among the different pursuit approaches, one that has drawn particular attention actually goes back into history in the search for potential solutions: the use of bacteriophages and their lytic enzymes as antibacterial agents (Thiel, 2004; O'Flaherty *et al*., 2009; Viertel *et al*., 2014).

Phage therapy

At its origin, the concept of phage therapy proclaimed the use of bacteriophages to treat bacterial infections (Harper *et al*., 2011), although currently the term is commonly used when referring to any application of phages with the goal of reducing the density of target bacteria with therapeutic, prophylactic or sanitary purposes. The use of these viruses as therapies appear to offer a number of advantages over ”conventional” antibiotics: (1) bacteriophages generally kill target bacteria with high specificity, being harmLess to the natural commensal microbiome; (2) they are effective in eliminating multiple antibiotic resistance bacteria given their completely different mode of action; (3) phages replicate in presence of the target pathogen, which may limit the number of required doses and time of treatment; (4) very few cases of side effects have been reported as result of phage administration in humans and animals; and (5) new phages are relatively easy to isolate and produced at low costs (Hanlon, 2007; Gorski *et al*., 2009).

The first attempts to treat bacterial infections in humans with live phages were conducted around 1920, just a few years after the discovery of bacteriophages (Dublanchet and Bourne, 2007). Since then, a great number of ‘‘clinical’’ studies have been carried out to

evaluate the efficacy and safety of phage therapy (Sulakvelidze and Kutter, 2005) and phage therapy was in fact established to considerable extent in Eastern Europe and countries of the former Soviet Union, where it has been applied to treat infections caused by *Staphylococcus*, *Pseudomonas*, *E. coli*, *Klebsiella* and *Salmonella* strains (O'Flaherty *et al*., 2009). One human clinical trial was performed in the Institute of Immunology and Experimental Therapy (founded in 1952 in Wroclaw, Poland) between 1981 and 1986 with 550 patients, with a wide range of bacterial infections, of which 518 had previously been unsuccessfully treated with antibiotics. These results demonstrated that 92.4% of the patients were cured, 6.9% patients showed an improvement in health condition, and in only 0.7% of patients phage therapy appeared ineffective (Slopek *et al*., 1987). In a more recent study made by the same group, 1307 patients were exposed to phage therapy and 85.9% fully recovered from the bacterial infection, 10.9% showed an improvement of condition and 10.8% showed no improvement (Weber-Dabrowska *et al*., 2000).

Despite the promising results of the use of bacteriophages as antimicrobial agents, there are plausible risks associated to phage therapy, which make essential a judicious choice of the viruses to be used as therapeutic agents. The chosen bacteriophages must: (1) have a narrow host range, to avoid an imbalance in natural human microflora; (2) be unable to establish any sort of lysogeny, as lysogens will become immune to the therapeutic phage; (3) not carry virulence/toxin genes in their genomes; (4) display minimal tendency to carry out DNA transduction between bacteria; and (5) be endotoxin-free manufactured (Gill and Hyman, 2010; Loc-Carrillo and Abedon, 2011). Even when meeting these requirements, a major concern related to the use of phages as antibacterials is the emergence of phage-resistant bacteria, which seems to be relatively frequent for some phage/host systems, at least in laboratory conditions. Although the development of phage cocktails might configure a solution to this problem (Drulis-Kawa *et al*., 2012), in the last years there has been an increasing interest in the study of the phage lytic enzymes as an alternative antibacterial strategy. One of the reasons motivating such interest is the apparent incapacity, or at least difficulty, of bacteria in developing resistance against these agents (Shuch *et al*., 2002; Fischetti, 2005; Rodríguez-Rubio *et al*., 2013).

Endolysins as antibacterials

An alternative to the killing action of phage particles themselves is the use of the lytic enzymes they produce. Endolysins seem to be highly potent enzymes when acting under their natural context, lysing the host cells from the inside within a reduced time frame. The exploration of these enzymes in the combat of pathogenic bacteria builds on the observation that such or similar lytic action can occur when endolysins reach the cell wall from the outside. Of course, without any special elaboration (see section 5.3) endolysins will need to have free access to the cell wall in order to act, and so their use has been mainly envisaged in the killing of Gram-positive bacteria because these lack an OM. The use of phage lytic proteins presents some advantages over the use of bacteriophages such as: (1) to date, and in the conditions tested, no bacterial resistance has been observed, even after repeated endolysin treatment (Fischetti, 2005; Rodríguez-Rubio *et al*., 2013); (2) lysins modular structure allows the engineering of enzymes with specific attributes; and (3) they can be identified and used from both temperate and lytic phages (O'Flaherty *et al*., 2009).

The first report of an *in vivo* assay showed that a single dose of the endolysin PlyC administered orally to mice, prior to the addition of group A streptococci, protected the animals from upper respiratory tract colonization (Nelson *et al*., 2001). The same group reported eradication of nasopharyngal colonization of mice by *Streptococcus pneumonia* by a single dose of the enzyme Pal within 5h, without affecting the commensal microorganisms (Loeffler *et al*., 2001). PlyGBS is another endolysin that is active against group A streptococci as well as B, C, G and L streptococci (Cheng *et al*., 2005). This protein was tested in a murine vaginal model of *Streptococcus agalactiae* (group B streptococci) colonization, aiming its potential use in pregnant women to prevent transmission of neonatal meningitis-causing streptococci to newborns. A single vaginal dose decreased colonization of group B streptococci by ~3logs. In addition, this enzyme was harmLess to the natural vaginal microflora.

Not only colonization of mucous membranes, but also systemic bacterial infections have been successfully eliminated with endolysins. The intraperitoneal administration of endolysin PlyG, encoded by the *Bacillus anthracis* phage γ, prevented death in 13 of the 19 infected mice, which were infected 15min before the treatment (Such *et al*., 2002).

Cpl-1 and Pal, two pneumococcal endolysins, were able to protect mice from pneumococcal bacteremia induced by intraperitoneal injection 1h prior to endolysins administration (Loeffler *et al*., 2003).

The prevalence of methicillin-resistant *S. aureus* (MRSA) in nosocomial and community-acquired infections is among the current serious threats to public health. This has generated a considerable amount of interest in identifying and study anti-staphylococcal endolysins. The first study using an *S. aureus* endolysin as therapeutic agent dates from 2007, in which the endolysin MV-L from phage *ϕ*MR11 was used to eliminate MRSA from mice nares. The intraperitoneal administration of MV-L 30min after a lethal MRSA inoculum (same route) also provided full protection to the challenged mice (Raschel *et al*., 2007). Recently, LysGH15, the endolysin from the *S. aureus* phage GH15, conferred 100% protection in a mouse model of septicemia (Gu *et al*., 2011).

In addition to phage-encoded endolysins, a large body of work has been devoted to the study of the antibacterial potential of the bacteriolysin lysostaphin, either alone or in combination with other *S. aureus* PG hydrolases. *In vitr*o studies with lysostaphin in combination with the endolysin LysK (from phage K) showed a strong synergism in eliminating MRSA strains (Becker *et al.*, 2008). A patent application (Kokai-Kun, 2003; US 20030211995) also indicates a synergistic action between lysostaphin and the *ϕ*11 endolysin against *S. aureus*. Other types of reported synergisms involve either the combined action of different endolysins or the co-treatment with endolysins and other antibacterial agents such as conventional antibiotics and bacteriocins. This is the case of the phage lytic enzyme Cpl-1, which was synergistic with gentamycin, penicillin and with the phage endolysin Pal against several penicillin-resistant and sensitive *S. pneumonia* strains (Loeffler and Fischetti, 2003). A strong synergistic effect was observed between the endolysin LysH5 and the bacteriocin nisin during elimination of *S. aureus* from pasteurized milk (García *et al*., 2010).

The majority of the studied endolysins only kill the species (or subspecies) of bacteria against which they were naturally designed to act on, although there are some exceptions. The endolysin PlyV12, from the *E. faecalis* infecting phage Φ1, showed a spectrum of activity outside that of the host and closely related bacterial strains. In addition of being active against *E. faecalis* and *E. faecium* strains, PlyV12 was also found to act against several disease-causing streptococcal and staphylococcal strains (Yoong *et al*., 2004).

Despite the numerous studies showing the great potential of endolysins as antibacterial agents, there are also potential problems associated to the exploration of these lytic enzymes. A frequently reported issue is the rather low solubility of endolysins during large scale production and/or concentration (Daniel *et al.*, 2010; García *et al.*, 2010; Fernandes *et al.*, 2012). In some cases, purified endolysins display poor lytic activity or spectrum (Mao *et al.*, 2013) and in others the good activity observed *in vitro* is not paralleled when endolysins are assayed in animal infection models, where the lytic enzymes have to be administrated soon after the inoculation of the bacterial agent to guarantee high levels of animal survival (Loeffler *et al.*, 2003; Gu *et al.*, 2011; Oechslin *et al.*, 2013). The next section presents some strategies that have been followed to solve the problems underlying the use of native endolysins or simply to improve their features.

Engineering of phage-lytic proteins

The modular structure of the majority of the endolysins allowed researchers to start exploiting the enzymes "promiscuous" structural arrangements by truncating and/or swapping functional domains, in order to create more active and stable enzymes, and in some cases with extended lytic spectrum. Table 2 presents a few examples of the type of engineering commonly done (for a detailed review see Nelson *et al.*, 2012; Schmelcher *et al.*, 2012a). The phage K endolysin LysK is the most intensively engineered phage lytic protein. As it was mentioned before, LysK harbors two catalytic domains, a CHAP ($CHAP_K$) and an amidase ($Amid_K$), linked to a SH3b CWB domain (CWB_K) (O'Flaherty *et al.*, 2005). Deletion analysis of LysK showed that the $CHAP_K$ domain is not only essential for activity, but when isolated it is more active than the wild-type protein (Becker *et al.*, 2009; Horgan *et al.*, 2009). *In vivo* assays showed that a single dose of $CHAP_K$ domain was sufficient to decolonize *S. aureus* from mice nares (Fenton *et al.*, 2010). When the peptidase domain of λSA2, a streptococcal endolysin, was fused to either the CWB domain of LysK or lysostaphin (CWB_{Lysos}), the two resulting lytic chimeras showed to be active not only *in vitro* but also in an *in vivo* mouse model of mastitis (Schmelcher *et al.*, 2012b). The chimeric endolysins Lys170-87 and Lys168-87 are another successful example of swapping functional domains of endolysins from different bacterial species. These proteins harbor a CD from two distinct enterococcal

endolysins fused to a CWB domain from the *S. aureus* endolysin Lys87. They showed to be active against more than 90% of the *S. aureus* clinical isolates from a large collection, which included a panel of the dominant MRSA and MSSA clones from different parts of the world (Fernandes *et al*., 2012).

Table 2. Molecular engineering and its effects on phage PG hydrolases properties.

Type of modification	Effect	Examples	References
Truncation of functional domains	Increased lytic activity	$CHAP_K$	Becker *et al*., 2009; Horgan *et al*., 2009; Fenton *et al*., 2010
Exchange of CWBD	Swapped cell wall specificity	Lys170-87 Lys168-87	Fernandes *et al*., 2012
		λSA2-$CWBD_{Lysos}$ λSA2-$CWBD_K$	Schmelcher *et al*., 2012b
Fusion of two full-length enzymes	Increased activity and broader lytic spectrum	B30-443-Lysos	Donovan *et al*., 2006
		HydH5-Lysos	Rodríguez-Rubio *et al*., 2012
Fusion of a CD to a full-length enzyme		B30-182-Lysos	Donovan *et al*., 2006
Fusion of a full-length enzyme to a CWBD	Broader binding spectrum	LytA-$CWBD_{LYC}$	Croux *et al*., 1993a, b
		HydH5- $CWBD_{Lysos}$	Rodríguez-Rubio *et al*., 2012
Fusion of two CWBD	Increased CWB affinity	GFP-$CWBD_{Ply118}$-$CWBD_{Ply500}$	Schmelcher *et al*., 2011
Duplication of CWBD		GFP-2$CWBD_{Ply500}$	Schmelcher *et al*., 2011
Fusion of CD from a VAL to CWBD of an autolysin	Increased lytic activity	P128	Paul *et al*., 2011; George *et al*., 2012; Vipra *et al*., 2012
		$CHAP_{HydH5}$-$CWBD_{Lysos}$	Rodríguez-Rubio *et al*., 2012
	Gained CWB specificity	$CD_{Tuc2009}$-$CWBD_{LytA}$	Sheehan *et al*., 1996
Random mutagenesis	Increased lytic activity	PlyGBS90-1	Cheng and Fischetti, 2007
Site-directed mutagenesis	Increased lytic activity and tune specie specificity	L98WCD27L L98WCD27L1-179	Mayer *et al*., 2011
Enzyme dimerization	Increased molecular weight	Cpl-1	Resch *et al*., 2011a
PEGylation		Cpl-1	Resch *et al*., 2011b

CWBD, cell wall binding domain; CD, catalytic domain; Lysos, Lysostaphin; K, LysK; LYC, clostridial autolysin; GFP, green fluorescent protein; VAL, virion associated lysin.

The interest in discovering and engineering new phage-derived lytic enzymes with improved characteristics has also turned the attention to the exploration of the antimicrobial potential of VALs or their CDs. The VAL CwlP of *B. subtilis* SP-β prophage harbors two CDs: one with muramidase activity and the other with peptidase M23 activity. Both CDs were studied independently and showed to be active against the host cell wall (Sudiarta *et al.*, 2010).

The chimeric protein P128, designed to target *S. aureus*, results from the fusion of a CHAP CD from the phage K VAL *orf56* ($CHAP_{VALK}$) with the CWB domain of the bacteriolysin lysostaphin ($CWBD_{Lysos}$) (Table 2). P128 enzyme displayed higher activity than the truncated $CHAP_{VALK}$ *in vitro*, and revealed efficacy in decolonizing *S. aureus* from rat nares (Paul *et al.*, 2011; George *et al.*, 2012; Vipra *et al.*, 2012).

The last examples of lytic protein engineering were focused on producing enzymes with higher activity and/or different targets when compared to the parental proteins. Another issue transversal to endolysins is their reduced molecular weight, which might cause their rapid elimination from the body by the excretion system (short half-life). The pneumococcal endolysin Cpl-1 is another protein that has been extensively studied and manipulated. In order to increase its half-life, the enzyme was dimerized (Resch *et al.*, 2011a) and PEGylated (Resch *et al.*, 2011b). Comparing with the monomer, the dimeric form resulted in higher lytic activity and increased half-life in mice. However, PEGylation abolished lytic activity.

Because of the reasons referred to above, the study of phage PG hydrolases as potential antibacterials has been almost exclusively focused in Gram-positive systems. More recently however, several groups have been devising strategies to overcome the main physical barrier hindering endolysin access to the cell wall in Gram-negative bacteria, the OM. In fact, recent studies in the field of OM permeabilizers have raised hopes of expanding the use of phage PG hydrolases against important Gram-negative pathogens such as *Pseudomonas*, *E. coli* and *Salmonella* (reviewed in Nelson *et al.*, 2012). The OM permeabilizers can be divided into two groups according to their mechanism of action (Briers *et al.*, 2011): chelators, such as EDTA and other organic acids, which remove divalent cations leading to OM disruption; and polycationic agents like polymyxin and its derivatives, which competitively displace the cations, resulting in OM disorganization. The pseudomonad endolysins KZ144 and EL188 were shown to display lytic activity against a broad range of EDTA-treated Gram-negative species (Briers *et al.*, 2007).

Despite the promising results with these endolysins, the use of chelators limits the therapeutic application to topical use due to its blood coagulation properties.

The other approach focused in fusing PG hydrolases to cationic, polycationic or other membrane-disrupting peptides (including natural and synthetic peptides) to increase the efficacy of these enzymes when added exogenously to Gram-negative bacteria. The Artilysins are the product of the fusion of Gram-negative endolysins to a cationic peptide. They showed lytic activity against important Gram-negative pathogens, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella enterica* (Briers *et al*., 2014).

Another interesting strategy to exploit the use of endolysins to treat Gram-negative bacterial infections was described by Lukacik and collaborators (2012). They created a "hybrid lysin" fusing the colicin-like protein pesticin, responsible for the recognition of the membrane transporter FyuA, to the lysozyme domains of phage T4 endolysin. This chimera was able to transverse the OM and gain access to the cell wall. Importantly, this protein was harmLess to the commensal flora, due to its specific biding to the FyuA, which is a major virulence factor of several pathogenic bacterial species (Heesemann *et al*., 1993).

Other applications of phage lytic proteins

The use of PG hydrolases, more specifically endolysins, may not be limited to treat human bacterial infections. They seem also to have application in other areas such as in the detection and decontamination of environmental and food-borne pathogens, in veterinary settings, and in biofilm elimination (Nelson *et al*., 2012). For example, endolysins targeting MRSA may have utility in nursing homes, surgical suites, or athletic locker rooms. Endolysins against *Bacillus anthracis*, such as PlyG and its derivatives were effective in the detection of this agent (Such *et al*., 2002; Fujinami *et al*., 2007). Enzymes against group A streptococci could be used to reduce bacterial loads in child care settings (Nelson *et al*., 2012). The endolysin PlyC was specifically tested as environmental disinfectant (Hoopes *et al*., 2009). As mentioned before, PlyC displays lytic activity towards several streptococcal species, including *Streptococcus equi*, which causes equine strangles disease. This is a highly contagious disease of horses that is

transmitted through shedding of live bacteria from nasal secretions and abscess drainage onto common surfaces in barns. Chemical disinfectant's are toxic and damages the equipment. PlyC was found to eradicate or significantly reduce *S. equi* from equipment without spoiling it.

Several studies have demonstrated the potential use of phage PG hydrolases for the detection and control of food-borne pathogens. The major advantage of these enzymes over other decontaminating products is their higher specificity for the target pathogens. Application of the phiEA1h lysozyme on the surface of pears inhibited the negative impact of *Erwinia* inoculation (Kim *et al*., 2004), whereas the staphylococcal endolysin LysH5 eliminated *S. aureus* from pasteurized milk (Obeso *et al*., 2008) in synergy with the lactococcal bacteriocin nisin (Garcia *et al*., 2010). The chimeric protein resultant from the fusion of streptococcal endolysin B30 and lysostaphin was also shown to kill both streptococci and staphylococci in milk products (Donovan *et al*., 2006).

A very relevant role that endolysins play in food safety is based on the high specificity of their CWB domains. These recognition domains have also been used to develop rapid and sensitive identification, detection, and differentiation methods (Fujinami *et al*., 2007; Schmelcher *et al*., 2010). Magnetic beads coated with recombinant CWB domains enabled immobilization and recovery of more than 90% of *L. monocytogenes* cells from food samples (Kretzer *et al*., 2007; Walcher *et al*., 2010).

Endolysins have also been explored to eliminate bacterial biofilms, which is the predominant bacterial lifestyle associated to infections in humans (Lebeaux *et al*., 2014), and also a problem in food production and processing and various other industries (Van Houdt and Michiels, 2010). The high level of antimicrobial resistance makes biofilms difficult to eradicate. Yet, recent successes in studies with endolysins hold promise for future applications. These include: the removal of static *S. aureus* biofilms by staphylococcal endolysin ϕ11 (Sass and Bierbaum, 2007) and SAP-2 (Son *et al*., 2010), the destruction of *S. penumoniae, S. pseudopneumoniae* and *S. oralis* biofilms by various pneumococcal endolysins, with Cpl-1 acting synergistically with the autolysin LytA (Domenech *et al*., 2011), and the elimination of *Staphylococcus suis* biofilms by lysin LySMP alone or in combination with antibiotics (Meng *et al*., 2011).

The use of PG hydrolases in the treatment of human diseases or as chemical disinfectants, avoid several problems associated with antibiotics. By their enzymatic nature, these proteins do not rely on potentially toxic reactive groups utilized by chemical disinfectants. As proteins, they are inherently biodegradable and noncorrosive.

PHAGE-ENCODED LYTIC PROTEINS WITH ACTIVITY AGAINST *ENTEROCOCCUS* SP

Enterococci are robust Gram-positive bacteria that are ubiquitous in several ecological niches. They are found in soil, sand, water, food products and plants and they commensally colonize the lower intestinal tract, oral cavity, and vaginal tract of humans and animals (Klein, 2003). Despite this primary status as colonizing bacteria, some species have emerged in the last decades as important causes of nosocomial infections, notably *E. faecalis* and *E. faecium* (Gilmore *et al*., 2013). In healthy individuals, *E. faecalis* and *E. faecium* colonization normally has no adverse effect on the host; however, the acquisition of virulence factors and high-level antibiotic resistance by enterococci are causing these organisms to emerge as a leading source of nosocomial infections, particularly in immunocompromised patients (Jett *et al.*, 1994; Jonhson *et al*., 1994; Cetinkaya *et al*., 2000; Werner *et al*., 2013). Common diseases caused by enterococcal infections include endocarditis, abdominal abscesses, bacteremia and urinary tract infections (Schaberg *et al.*, 1991; Emori *et al.*, 1993; Poh *et al.*, 2006; Fisher *et al.*, 2009; Sava *et al.*, 2010).

Due to *E. faecalis* intrinsic antibiotic resistance, the use of phage lysins as anti-enterococcal agents has been proposed. At this moment, four endolysins have been described with activity against *E. faecalis*: PlyV12 (Yoong *et al*., 2004); Orf9 (Uchiyama *et al*., 2008), EFAL-1 (Son *et al*., 2010) and the endolysin of phage IME-EF1 (Zhang *et al*., 2013), which shares highly amino acid similarity to the uncharacterized endolysins of enterococcal phages SAP-6 (Lee and Park, 2012) and BC-611 (Horiuchi *et al*., 2012). When tested *in vitro* Orf9 and the phage IME-EF1 endolysin showed preferential lytic activity towards *E. faecalis*, but they could also lyse some *E. faecium* strains. PlyV12 and EFAL-1 had wider lytic spectrums, being able to act on different streptococcal and/or staphylococcal species besides *E. faecalis* and *E. faecium*. Despite carrying CDs of

different families, PlyV12 (Amidase-5), Orf9/EFAL-1 (Amidase-2) and the phage IME-EF1 endolysin (CHAP) were proposed or demonstrated to specify N-acetyl-muramoyl-L-alanine amidase activity (Yoong *et al.*, 2004; Uchiyama *et al.*, 2011; Son *et al.*, 2010; Zhang *et al.*, 2013).

Curiously, phage lysins from non-enterococcal bacteria have been shown to be effective in eliminating *Enterococcus* species. The endolysin Mur-LH from the temperate phage ϕ-0303 of *Lactobacillus helveticus* can lyse a diverse array of Gram-positive bacteria, including *E. faecium*, but surprisingly not *E. faecalis* (Deutsch *et al.*, 2004). In addition, chimeric lysins based on enterococcal endolysins, such as Plys187N-V12C, Lys168-87 and Lys170-87, designed to attack *S. aureus*, retain lytic activity against *Enterococcus* species independently of the origin of CD and/or CWBD (Fernandes *et al.*, 2012; Dong *et al.*, 2014). Until date, the endolysin of phage IME-EF1 was the only enterococcal lytic enzyme tested *in vivo* in a sepsis murine model, and showed to protect animals from sepsis shock (Zhang *et al.*, 2013).

The spread of multidrug-resistant *E. faecalis* strains over the community it's a huge concern, specially the interspecies dissemination in households promoted by the proximity of pets to their owners (Leite-Martins *et al.*, 2014). There is an urgent need to seek for alternatives to conventional antimicrobial therapy to control opportunistic infections caused by this pathogen, both in humans and animals. In the following 3 chapters it is described the antimicrobial potential of the endolysins Lys168 and Lys170 encoded by the enterococcal phages F168/08 and F170/08, respectively. In addition, we studied and characterized the enzyme Lys170 which revealed to be a multimeric protein with a novel configuration of endolysin functional domains. We also took advantages of Lys170 architecture special features to design a potent chimeric lysin, EC300, based on a novel lytic protein design technology. EC300 revealed to be a promising antibacterial agent with high lethality against actively growing *E. faecalis* cells (including vancomycin-resistant strains).

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CHAPTER 2

PHAGE ENDOLYSINS WITH BROAD ANTIMICROBIAL ACTIVITY AGAINST *ENTEROCOCCUS FAECALIS* CLINICAL STRAINS

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Author contributions:

The author of this dissertation performed all the experiments presented in this chapter, with the help of Sofia Fernandes in endolysin cloning, production and purification. Clara Leandro and Filipa Antunes Silva were responsible for phage isolation, purification and extraction of phage DNA. Clara Leandro carried out the phage genome analysis, whereas Sofia Santos was responsible for *Enterococcus* species identification. Experimental design, data analysis and manuscript preparation were done by the author of this thesis and by Clara Leandro, Miguel Garcia, Madalena Pimentel, Fátima Lopes, Rosario Mato, Patrícia Cavaco-Silva and Carlos São-José. Carlos São-José was the main supervisor of the work.

PHAGE ENDOLYSINS WITH BROAD ANTIMICROBIAL ACTIVITY AGAINST *ENTEROCOCCUS FAECALIS* CLINICAL STRAINS

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AUTHOR'S NOTE

In the following chapter it is highlighted the striking sequence similarity between the enterococcal phage F168/08 and a previously described phage named SAP6, which was in a first registry assigned in sequences databases as being from *Staphylococcus aureus*. The close relatedness between these two phages translated in almost identical endolysins both at the gene and protein levels. However, soon after the approval for publication of the work presented in this chapter, the SAP6 genome and endolysin entries in sequences databases, GenBank JF731128 and AEM24735, respectively, were corrected and it turned out that SAP6 appears also to be an *Enterococcus faecalis* phage. This should be taken into account when reading the following chapter.

ABSTRACT

Increasing antibiotic resistance of bacterial pathogens has drawn the attention to the potential use of bacteriophage endolysins as alternative antibacterial agents. Here we have identified, characterized and studied the lytic potential of two endolysins, Lys168 and Lys170, from phages infecting *E. faecalis*. Lys168 and Lys170 belong to the CHAP and Amidase_2 protein families, respectively. Lys168 is quite an unique enterococcal phage endolysin. It shares 95% amino acidic identity with the endolysin of *S. aureus* phage SAP6, which in turn is distantly related to all known CHAP endolysins of *S. aureus* phages. Lys170 seems to be a natural chimera assembling catalytic and cell wall binding domains of different origin. Both endolysins showed a clear preference to act against *E. faecalis* and they were able to lyse a high proportion of clinical isolates of this species. Specifically, Lys168 and Lys170 lysed more than 70 and 90% of the tested isolates, respectively, which included a panel of diverse and typed strains representative of highly prevalent clonal complexes. Lys170 was active against all tested *E. faecalis* VRE strains. The *quasi* specificity towards *E. faecalis* is discussed considering the nature of the enzymes' functional domains and the structure of the cell wall peptidoglycan.

INTRODUCTION

Gram-positive pathogens are responsible for a large number of community-acquired and health-care-associated bacterial infections. Staphylococci, enterococci, streptococci and *Clostridium difficile* are the most prevalent Gram-positive pathogens of clinical interest (Rossolini *et al*., 2010).

Enterococci belong to the normal bacterial flora of the intestinal tract of humans and several animals and can be found in environmental soil, water, plants, and food. Although they are considered commensal bacteria, at least *Enterococcus faecalis* and *Enterococcus faecium* species are regarded as relevant opportunistic pathogens, being associated with nosocomial, and to a lesser extent, community-acquired infections. Typical enterococcal infections occur in hospitalized patients with underlying conditions. Both species have been described as the second most common cause of wound and urinary tract infections, and the third most common cause of bacteremia (Schaberg *et al*., 1991), and can also be involved in neonatal sepsis (Poh *et al*., 2006), peritonitis, device-related infections, and endocarditis (Schaberg *et al*., 1991; Emori and Gaynes, 1993; Fisher and Philips, 2009). The massive use of antibiotics in human health care systems and animal production has increased the incidence of antibiotic-resistant enterococci (Rossolini *et al*., 2010), some of which are already intrinsically resistant to a broad range of antibiotics including cephalosporins, sulphonamides and low concentrations of aminoglycosides (French, 2010). In the last decades there has been a dramatic increase of *E. faecalis* and *E. faecium* infections due to resistant strains to vancomycin (VRE), for long considered the last resource when all other classes of antibiotics failed; therefore the search for alternative antibacterials to combat these pathogens has become an immediate need.

Enzybiotics are an example of new potential antibacterials and among these, bacteriophage endolysins have been one of the most intensively explored (O'Flaherty *et al.*, 2009; Fenton *et al.*, 2010; Fischetti, 2010). Endolysins are enzymes encoded by double-stranded DNA bacteriophages that cleave the bacterial cell wall peptidoglycan. This activity is essential to promote bacterial host cell lysis at the end of phage life cycle thus allowing efficient escape of the viral progeny from infected cells (São-José *et al*., 2007). The vast majority of known endolysins from phages infecting Gram-positive bacteria feature well conserved domain architecture, in which the N-terminal region carries one or two enzymatically active catalytic domains (CD) and the C-terminus motifs

responsible for cell wall binding domain (CWBD) (Fischetti, 2008). These enzymes are designed to attack one or two of the five major bonds in the peptidoglycan network (Loessner, 2005). The rationale behind utilization of endolysins as antibacterial agents is that, in principle, they should retain their lytic potential when added exogenously as recombinant enzymes.

Three different *E. faecalis* phage endolysins, belonging to two Amidase families, have been reported before and their killing efficacy towards *Enterococcus* studied *in vitro*. These are: PlyV12, encoded by phage Φ1 (Yoong *et al.*, 2004), EFAL-1 produced by phage EFAP-1 (Son *et al.*, 2010), and ORF9 from phage ϕEF24C (Uchiyama *et al.*, 2008). In addition to the capacity to lyse their natural target, *E. faecalis*, the enzymes were also reported to act on the related species *E. faecium*. Moreover, EFAL-1 could also lyse some streptococcal isolates, whereas PlyV12 showed the broadest lytic spectrum by also acting against several streptococcal and staphylococcal strains (Yoong *et al.*, 2004).

In this study we have identified, produced and purified two phage endolysins, Lys168 and Lys170, encoded in the genome of two *E. faecalis* phages, F168/08 and F170/08, respectively. Lys168 represents a novel endolysin among enterococcal phages as it carries a CD from the CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) family. We have studied the lytic action of both endolysins against different Gram-positive pathogenic bacteria, which included a panel with representatives of the most prevalent VRE clonal complexes in nosocomial infection. The results obtained with Lys170 call for a reappraisal of those obtained with ORF9, since these two endolysins are virtually identical.

MATERIALS AND METHODS

Bacteria, phages, culture media and growth conditions

The *E. coli* cloning strain XL1-Blue MRF' and its derivatives were grown at 37 ºC with aeration in Luria-Bertani (LB) medium (Sambrock and Russell, 2001). The *E. coli* expression strain CG61(São-José *et al.*, 2000) and its derivatives were grown in LB in the same conditions, except that incubation temperature was 28 ºC before induction of protein

production, and 37 ºC afterwards. When appropriate, LB medium was supplemented with kanamycin (30 μg/mL) and/or ampicillin (100 μg/mL) for plasmid selection.

Lytic action of enterococcal phage endolysins was assayed in 193 bacteria clinical isolates (Tables 1, S1, S3 and S5). Table 1 lists a panel of 28 *E. faecalis* and 21 *E. faecium* typed strains recovered from patients of a Portuguese hospital between 2004 and 2006 (Mato *et al*., 2009) (see Table S3 for a detailed description of these strains). Table 1 also includes the two model *E. faecalis* VRE strains V583 and MMH594. Table S1 corresponds to 99 clinical isolates from Technophage's collection, 73 *E. faecalis* and 26 *E. faecium*, which were obtained from different Portuguese community and hospital settings between 2005 and 2007. Lytic action of recombinant enzymes was also tested in clinical isolates of other bacterial species from Technophage's collection, namely against *Streptococcus pneumoniae* (n = 10), *Streptococcus pyogenes* (n = 8), *Streptococcus agalactiae* (n = 8), *Staphylococcus aureus* (n = 9), *Staphylococcus haemolyticus* (n = 4) and *Staphylococcus* epidermidis (n = 4) (Table S5).

Table 1. Typed enterococcal clinical strains used in this study.

Strain ID	PFGE pattern	Vancomycin	Reference	Strain ID	PFGE pattern	Vancomycin	Reference
***E. faecalis* (n=30)**				***E. faecium* (n=21)**			
EHCP 3	AO6	resistant	27	EHCP 5	c10	resistant	27
EHCP 13	S	susceptible	27	EHCP 6	a1	resistant	27
EHCP 24	AO5	susceptible	27	EHCP 14	d2	susceptible	27
EHCP 31	A2	susceptible	27	EHCP 36	a2	resistant	27
EHCP 55	AW	susceptible	27	EHCP 40	d9	susceptible	27
EHCP 73	J	susceptible	27	EHCP 65	o	susceptible	27
EHCP 78	A3	susceptible	27	EHCP 88	c2	susceptible	27
EHCP 92	AR	susceptible	27	EHCP 149	d6	susceptible	27
EHCP 93	AX	susceptible	27	EHCP 161	t	susceptible	27
EHCP 94	AM	susceptible	27	EHCP 178	p	susceptible	27
EHCP 107	K	susceptible	27	EHCP 181	d8	susceptible	27
EHCP 118	AT	susceptible	27	EHCP 184	f	susceptible	27
EHCP 143	AU	susceptible	27	EHCP 211	c12	susceptible	27
EHCP 151	H	susceptible	27	EHCP 264	e	susceptible	27
EHCP 164	B	susceptible	27	EHCP 302	c5	susceptible	27
EHCP 193	BC	susceptible	27	EHCP 341	u	susceptible	27
EHCP 225	R	susceptible	27	EHCP 358	i	susceptible	27

Table 1, *cont.*

Strain ID	PFGE pattern	Vancomycin	Reference	Strain ID	PFGE pattern	Vancomycin	Reference
EHCP 237	AO2	susceptible	27	EHCP 361	c16	resistant	27
EHCP 241	O	susceptible	27	EHCP 378	w	susceptible	27
EHCP 267	AO2	resistant	27	EHCP 407	d7	resistant	27
EHCP 271	A11	susceptible	27	EHCP 459	s	susceptible	27
EHCP 279	T	susceptible	27				
EHCP 281	U	susceptible	27				
EHCP 292	A4	susceptible	27				
EHCP 332	I	susceptible	27				
EHCP 339	AO1	susceptible	27				
EHCP 389	AO1	resistant	27				
EHCP 391	M	susceptible	27				
MMH594	NA	resistant	16,45				
V583	NA	resistant	31,38				

Abbreviations: NA, Not Applied; PFGE, Pulse Field Gel Electrophoresis

The growth media for these bacteria were purchased from Biokar Diagnostics, Beauvais, France. Enterococcal and staphylococcal strains were cultured either in Brain Heart Infusion (BHI) or Tryptic Soy Broth (TSB) whereas streptococci were propagated in Todd Hewitt Yeast broth (THY). Liquid cultures of *Enterococcus* and *Streptococcus* species were grown at 30 ºC and/or 37 ºC, without aeration, while those of *Staphylococcus* were incubated at 37 ºC with aeration.

When necessary, culture media were supplemented with 1.5% or 0.7% agar to obtain solid or soft-agar plates, respectively. *E. faecalis* phages were isolated, purified and propagated by standard methods (Kutter and Sulakvelidze, 2004; Clokie and Kropinski, 2009) either in soft-agar media or liquid broth supplemented with $CaCl_2$ and $MgCl_2$ (5 mM each). Phage F168/08 and F170/08 propagation hosts were *E. faecalis* clinical isolates 1518/05 and 926/05, respectively (Table S1).

Identification and bioinformatics analysis of phage endolysins

Genomes from *E. faecalis* phages F168/08 and F170/08 were extracted from CsCl-purified lysates (Vinga *et al.*, 2012) and their complete nucleotide sequence determined (service purchased to Macrogen, Seoul, Korea). DNA homology searches were carried out with BLASTN (Zhang *et al.*, 2000), using the NCBI's non-redundant nucleotide sequences database. Recognition of phage putative genes was performed by integrating

the results obtained with GeneMark.hmm and MetaGeneAnnotator web software (Besemer and Borodovsky, 2005; Noguchi *et al*., 2008). Identification of F168/08 and F170/08 endolysin genes was based on BLASTP homology searches (Altschul *et al*., 1997) with deduced gene products against the NCBI's nonredundant protein sequence database, and on prediction of protein functional domains using NCBI's CDD (Marchler-Bauer *et al*., 2011) and Pfam (http://pfam.sanger.ac.uk/). Assignment of putative linkers connecting protein functional domains was performed with SVM (Ebina *et al*., 2009), using the SVM-joint output. Multiple protein sequence alignments were performed with ClustalW2 (Larkin *et al*., 2007).

Cloning of Lys168 and Lys170 endolysin genes

The coding sequence of endolysins Lys168 and Lys170 was amplified Polymerase Chain Reaction (PCR) from phage DNA using a high fidelity *Pfu* DNA Polymerase (Fermentas Molecular Biology Tools, Thermo Scientific). The forward and reverse primers used to amplify *lys168* carried at their 5' end the restriction sites N*co*I and X*ma*I, respectively, whereas the corresponding primers for *lys170* amplification carried B*sp*I and X*ma*I sites. Both products were purified using the *High Pure PCR Product Purification Kit* (Roche Applied Science), double-digested with the appropriate restriction enzymes and ligated to the pIVEX2.3d expression vector (Roche Applied Science), which had been previously restricted with N*co*I and X*ma*I. This vector is designed to drive the expression of cloned genes under the control of the phage T7 ϕ10 promoter and to allow production of the corresponding proteins C-terminally fused to a hexahistidine tag. Ligations were used to transform the *E. coli* strain XL1-Blue MRF' as previously described (Chung *et al*., 1989). Transformants were selected in presence of 100 μg/mL ampicillin and screened for the presence of the desired recombinant plasmids by PCR using insert and vector complementary primers. Plasmid DNA from positive clones was extracted (*Pure Link Quick Plasmid Miniprep Kit*, Invitrogen) and the correct DNA structure confirmed by endonuclease restriction and DNA sequencing (Macrogen, Seoul, Korea). The constructs pDP1 and pDP2 are pIVEX2.3d derivatives carrying *lys168* and *lys170*, respectively.

Production and purification of the endolysins Lys168 and Lys170

E. coli strain CG61, which overproduces phage T7 RNA polymerase upon temperate upshift (São-José *et al.*, 2000) was transformed with plasmids pDP1 and pDP2 and transformants selected at 28 ºC in presence of 100 μg/mL ampicillin and 30 μg/mL kanamycin. The ability of CG61 derivatives to produce soluble and active Lys168 and Lys170 was firstly checked by their culturing over a dense lawn of autoclavated enterococcal cells, incorporated in soft-agar LB medium, and confirming the presence of lysis halos around *E. coli* colonies (Fig. S2).

Selected clones of each endolysin were grown at 28 ºC until an optical density at 600 nm (OD_{600}) of 0.3-0.5, after which protein production was induced by moving cultures to a shaking water bath set to 42 ºC. After 45 min induction, cultures were transferred to an incubator at 37 ºC and agitated for an additional period of 3 h. Cells from induced cultures were pelleted by centrifugation (8,000xg, 30 min, 4 ºC) and resuspended in 1/50 volume of lysis buffer (20 mM Hepes-Na, 500 mM NaCl, 20 mM imidazole, 1% glycerol, 1 mM DTT, pH 8.0) supplemented with 1x Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Applied Science). Cells were kept on ice and disrupted by sonication (Vibra Cell MS2T, Sonic Materials) by performing about 10 bursts of 1 min (amplitude 5, pulse 3, 30–40 W) intercalated with pauses of 1 min. Insoluble material was sedimented by centrifugation (10,000xg, 30 min, 4 ºC). The supernatant corresponding to the total soluble protein extract was filtered through a 0.22 μm and endolysins purified by affinity chromatography using *HisTrap™ HP* columns (GE Healthcare) coupled to an AKTA-Prime system (GE Healthcare). The column and elution buffers had the same composition of the lysis buffer, except that the imidazole concentration in the elution buffer was 500 mM. Eluted fractions were analyzed by SDS-PAGE and Coomassie blue staining (LaemmLi, 1970). Endolysins from pure fractions were pooled, concentrated and changed to an imidazole-free, phosphate-based endolysin buffer (50 mM phosphate-Na, 500 mM NaCl, 25% glycerol, 1 mM DTT, pH 8.0) using *HiTrap™ Desalting* columns (GE Healthcare). Protein concentrations were determined by the Bradford method (Bio-Rad Laboratories) using bovine serum albumin as standard. The enzymes were divided in small aliquots and kept at -80 ºC.

Evaluation of endolysin lytic action against bacterial pathogens

The capability of endolysins Lys168 and Lys170 to induce lysis of clinical strains from different bacterial species was evaluated by two different assays. The endolysins were tested against a large number of bacterial isolates by spotting different enzyme quantities in dense lawns of viable target cells, which were prepared as follows. Enterococcal and streptococcal strains were cultured overnight at 30 ºC, without aeration. Typically, these cultures reached an OD_{600} of about 0.8-1.0. Staphylococcal cultures at this OD_{600} were prepared after 1:200 dilution of overnight cultures and growth at 37 ºC with aeration. Cells from these cultures were recovered by centrifugation and resuspended in 1/100 volumes of the correspondent growth medium. A 300 µL sample of these cell suspensions was incorporated in lysis assay buffer (25 mM phosphate-Na, 250 mM NaCl, 1% glycerol, 1 mM DTT, pH 8.0), supplemented with 0.7% agar and poured in a Petri dish. Four protein quantities of each endolysin (5, 1, 0.2 and 0.04 µg, in 10 mL final volume) were spotted on each strain lawn and, after overnight incubation at 37 ºC, checked for the presence of lysis halos. These were evaluated and scored (- to +++) according to their relative diameter and transparency (Fig. S3).

Bacterial cell lysis was also studied in liquid medium. Selected strains were grown until an OD_{600} of 0.3-0.4, centrifuged and cells recovered in 1/2 volume of lysis assay buffer. Cell suspensions were challenged with the indicated endolysin concentrations and OD_{600} variation followed over time. At the end of each assay the surviving colony forming units (CFU) /milliliter was determined. Negative controls were equally prepared except that endolysin buffer was added instead of endolysin.

Identification of bacterial species

When necessary discrimination between *E. faecalis* and *E. faecium* was performed by a PCR based approach, using species specific primers targeting the *ddl* gene. Primers for *E. faecalis* were fw: CACCTGAAGAAACAGGC and rv: ATGGCTACTTCAATTTCACG, with an amplicon size of 475 bp (Depardieu *et al.*, 2004). For *E. faecium* the amplicon size was 1091 bp using primers fw: GAGTAAATCACTGAACGA and rv: CGCTGATGGTATCGATTCAT (Jackson *et al.*, 2004). For identification purposes, *Enterococcus* type-strains obtained from the Deutsch SammLung von Mikroorganismen

and Zellkulturen collection (DSMZ; Braunschweig, Germany) were used as references, namely *Enterococcus faecalis* DSM 20478 and *Enterococcus faecium* DSM 20477.

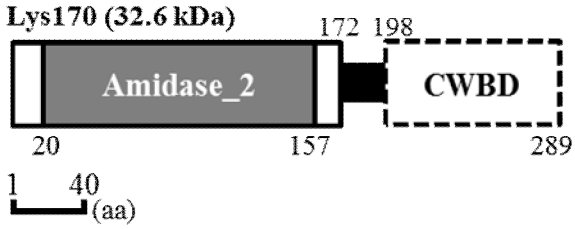
RESULTS

Bioinformatics of enterococcal phage endolysins Lys168 and Lys170

We have recently determined the nucleotide sequence of the genome of two *E. faecalis* phages from Technophage's collection, F168/08 and F170/08. Sequence analysis by bioinformatics tools identified an open reading frame in each phage genome, whose deduced amino acid sequences had high sequence identity with known or putative phage endolysins, and which featured conserved domains involved in the hydrolysis of bacterial cell wall peptidoglycan. Therefore, these proteins were assigned as the endolysins of phages F168/08 and F170/08 and were designated as Lys168 and Lys170, respectively.

Lys170 is basically identical to the previously described endolysin ORF9 of *E. faecalis* phage ϕEF24C (Uchiyama *et al*., 2008; Uchiyama *et al*., 2011), showing a single amino acid substitution over its 289 amino acid sequence. Both enzymes carry in their amino terminal region a CD of the Amidase_2 family (Figs 1A and S1), whose members include zinc amidases that have N-acetylmuramoyl-L-alanine amidase activity (Cheng *et al*., 1994). This type of activity was confirmed experimentally for ORF9 (Uchiyama *et al*., 2008). Lys170 (and ORF9) appears to be a natural chimera of intergeneric origin since its N-terminal CD is highly similar to that of lactobacilli amidases whereas its C-terminal region, probably containing the CWBD, reveals high sequence identity to that of enterococcal amidases (Fig. 1B).

A



B

Lys170 N-terminal region

```
Lys170              1 MAGEVFSSLITSVNPNPMNAGSRNGITIDTIILHHNATTNKDVAMNTWLLGGGAGTSAHY 60
L. parafarraginis   1 MANPTFSKLITSLNPKKMNASSRNGIKIDRIVIHHNAMTDADKAMNIWIAGGPANTSAHY 60
                      **. .**.****:**: ***.*****.** *::**** *: * *** *: ** *.*****

Lys170             61 ECTPTEIIGCVGEQYSAFHAGGTGGIDVPKIANPNQRSIGIENVNSSGAPNWSVDPRTI 119
L. parafarraginis  61 EVTPTEIIGCVGEQYAAWHAGGIGQADPPKMANPNQRSIGIENVNSTGAPEWLVDPRTV 119
                      * *************:*:**** *  * **:***************:***:* *****:

Lys170            120 TNCARLVADICTRYGIPCDRQHVLGHNEVTATACPGGMDVDEVVRQAQ      167 (289)
L. parafarraginis 120 QNCARLVADICTRYQIPLDRQHILAHREVTNTACPGGLDVDEVVRLAR      167 (179)
                       ************* ** ****:*.*.*** ******:******* *:
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Lys170 C-terminal region

```
Lys170               192 NNKN---KEGVATMYCLYERPINSKTGVLEWNGDAWTVMFCNGVNCRRVSH 239
E. faecalis HIP11704 185 NNQNNIEKGGETTMQCLYERPINSKTGALEWNGDAWTVMFCNGVNTRRVYH 235
                         **:*   * * :** ************.***************** *** *

Lys170               240 PDEMKVIEDIYRKNNGKDIPFYSQKEWNKNAPWYNRLETVCPVVGITKKS  289
E. faecalis HIP11704 236 PDEMKVIEKVYKDNNGRSIPFYSQKEWNKNAPWYNRLESMFPVVK-----  280
                         ********.:*:.***:.**********************:: ***
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Fig. 1. Domain architecture and sequence relatedness of Lys170. (**A**) Schematic representation of Lys170 domain organization. The dashed rectangle delimits the C-terminal region that must contain the CWBD. The amino acidic coordinates of functional domains are indicated above and below the scheme. (**B**) ClustalW2 alignment of Lys170 N- and C-terminal primary structures with those of its closest homologues (*Lactobacillus parafarraginis*, Acc. N0. WP_008215034; *E. faecalis* HIP11704, Acc. N0. EEU69620). Asterisk, fully conserved residues; colon, conservation of residues with strongly similar properties; period, conservation of residues with weakly similar properties.

In silico analysis of Lys168 identified in the first half of the protein a conserved domain of the CHAP family (Bateman and Rawlings, 2003; Rigden *et al.*, 2003; Layec *et al.*, 2008) (Figs 2A and S1). This protein family includes enzymes that cleave different amide bonds in the peptidoglycan network. Unexpectedly, Lys168 shared 95% identity with a protein assigned as "amidase" from *Staphylococcus aureus* phage SAP6 (GenBank AEM24735.1). In addition, the F168/08 genome shared between 80 and 94% sequence identity over 68% of the SAP6 genome (BLASTN analysis), which translated into a high sequence similarity between the products encoded by the homologous portions of both

genomes. In addition to its close relationship to the SAP6 endolysin, the Lys168 CD shared significant identity with the CHAP domain of a single *E. faecalis* protein (strain TX0104) and with that of other *S. aureus* phage proteins (Fig. 2B). The later however are ~600aa, multifunctional proteins associated with the virion structure and which are thought to assist DNA entry into host cells at the initial steps of infection (Rashel *et al.*, 2008; Rodríguez *et al.*, 2011). Lys168 C-terminal region had no equivalent homologues besides that of the already mentioned endolysin from phage SAP6.

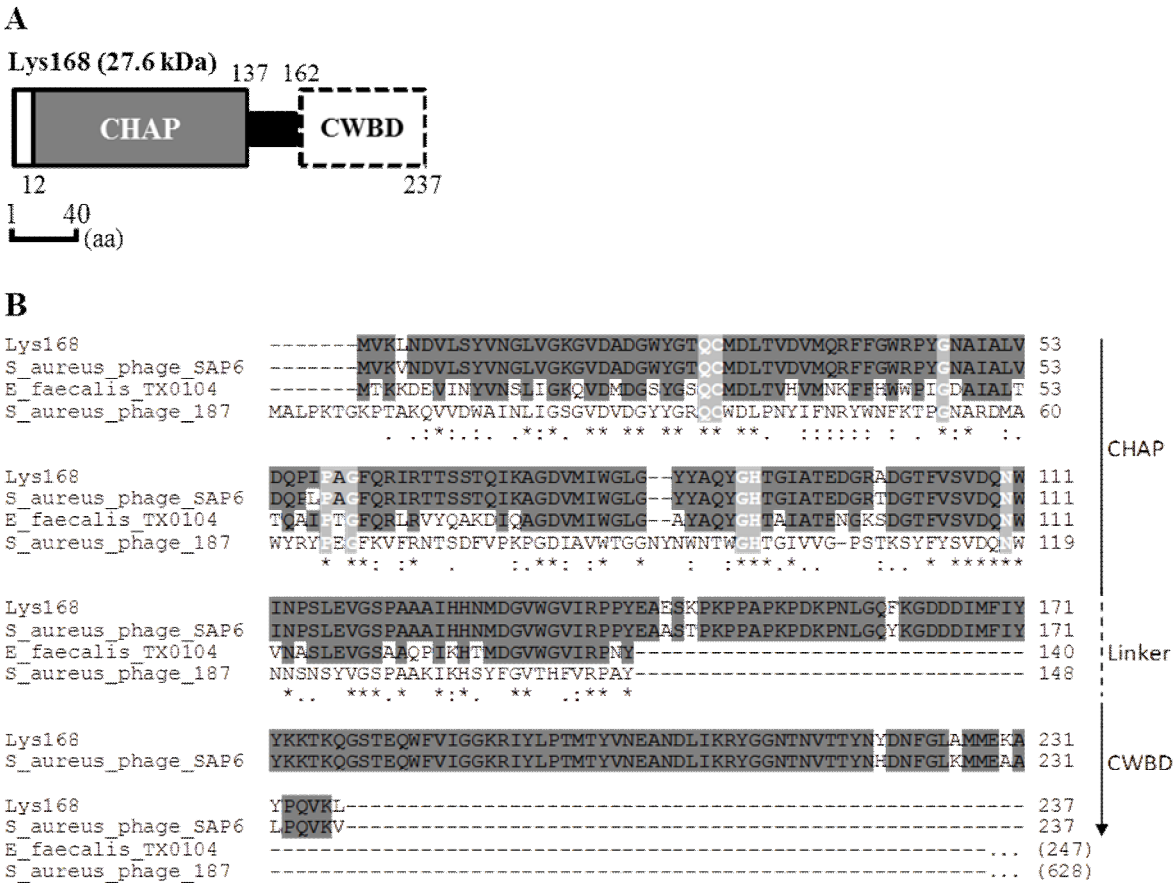

Fig. 2. Domain architecture and sequence relatedness of Lys168. (**A**) Schematic representation of Lys168 domain organization. The dashed rectangle delimits the C-terminal region that must contain the CWBD. The amino acidic coordinates of functional domains are indicated above or below the scheme. (**B**) ClustalW2 alignment of Lys168 primary sequence with that of its closest homologues (phage SAP6 endolysin, Acc. N0. AEM24735; *E. faecalis* TX0104, Acc. N0. EEI10842). Dark gray shading highlights the close identity between Lys168 and the endolysin from *S. aureus* phage SAP6 and between these and the CD of one CHAP-like protein from *E. faecalis* strain TX0104. Highly conserved residues of the CHAP domain are depicted in white with light gray shading (Bateman and Rawlings, 2003). Note that the C-terminal halves of Lys168 and SAP6 endolysins are unrelated to the CHAP-like proteins from *E. faecalis* TX0104 and *S. aureus* phage 187 (Acc. N0. YP_239513). Asterisk, fully conserved residues; colon,

conservation of residues with strongly similar properties; period, conservation of residues with weakly similar properties.

Endolysins from phages infecting Gram-positive bacteria display a typical domain architecture in which N-terminal CD and C-terminal CWBD are connected by a linker sequence (Fischetti, 2008). Although the CDs of Lys168 and Lys170 could be delimited in their N-terminal portion using bioinformatics tools (see above), these failed to recognize any known CWBD in their C-terminal region. We could however predict the location of the central linker domain in each endolysin, and based on this we inferred the probable position of CWBD (Figs 1A, 2A and S1).

Heterologous production and purification of endolysins Lys168 and Lys170

The genes encoding Lys168 and Lys170 were PCR-amplified and cloned in *E. coli* expression vector pIVEX2.3d, which allowed production of the endolysins C-terminally fused with a hexahistidine tail (see Materials and Methods). *E. coli* clones producing the enzymes in their active form were initially selected by growing transformants on a dense lawn of autoclavated *E. faecalis* target cells and checking for the presence of lysis halos around the *E. coli* colonies (Fig. S2). Medium scale protein production from selected clones allowed us to obtain substantial amounts of soluble Lys168 and Lys170 with the expected molecular weight, which were subsequently purified by affinity chromatography using nickel columns. Endolysins of pure fractions from the affinity chromatography were changed to an imidazole-free, sodium phosphate-based buffer by performing a desalting step (Fig. 3).

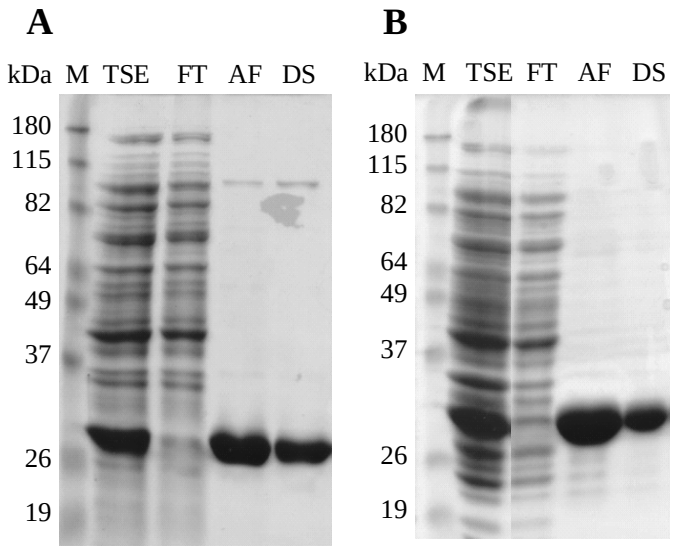

Fig. 3. Analysis of endolysins Lys168 (**A**) and Lys170 (**B**) purification by SDS-PAGE and Coomassie blue staining. Lanes: M, molecular weight marker; TSE, total soluble protein extract; FT, flowthrough of the HisTrap column; AF, fraction of the HisTrap affinity peak; DS, fraction of the HiTrap desalting peak. Lys168, 27.6 kDa; Lys170, 32.6 kDa.

Lytic action of Lys168 and Lys170 against enterococcal clinical strains

In a preliminary assay we assessed the lytic action of purified Lys168 and Lys170 against a panel of enterococcal clinical isolates from Technophage's collection, which were provided by different Portuguese clinical settings and isolated from different infection contexts. This panel was composed of 73 *E. faecalis* and 26 *E. faecium* isolates (Table S1). Four different amounts of each endolysin (5, 1, 0.2 and 0.04 μg) were spotted on a dense lawn of viable cells from each isolate, which was produced by incorporating cells from exponentially growing cultures in a soft-agar, phosphate-buffered medium (see Material and Methods). Lytic activity was qualitatively evaluated by scoring the relative diameter and turbidity of the lysis halos produced after overnight incubation at 37 ºC (Fig. S3).

When applied in its highest quantity (5 μg) Lys170 produced a discernible lysis halo in 97 and 54% of *E. faecalis* and *E. faecium* isolates, respectively, whereas Lys168 lysed 81 and 42% of these. When we scored the percentage of susceptible isolates for the lower amounts of each endolysin, it became clear that Lys170 had higher lytic action compared to Lys168 (Fig. S4). In addition, for each tested enzyme quantity, Lys170 almost always

produced clearer and larger lysis halos than Lys168 (Table S2). The results from this preliminary study indicated that Lys170 had better lytic performance than Lys168 and suggested that both endolysins preferentially lysed *E. faecalis* strains.

The isolates from the panel referred to above were not typed and thus the diversity within each *Enterococcus* species was unknown. To gain more insight on the lytic potential of each endolysin against these enterococcal species, the enzymes were equally assayed in a panel of 51 multi-resistant typed strains, 30 *E. faecalis* and 21 *E. faecium* (Tables 1 and S3), 49 of which were the cause of infections in a Portuguese hospital, over a 3-year period. These strains displayed high-level resistance to gentamicin and included VREs of clonal complexes *E. faecalis*-CC2 and *E. faecium*-CC17, which are highly prevalent in nosocomial settings and disseminated worldwide (Mato *et al*., 2009).

We observed that 5 μg of Lys168 and Lys170 were still able to induce lysis of more than 70 and 90% of the *E. faecalis* strains, respectively, but only up to 10% of *E. faecium* strains were susceptible to the endolysins. The percentage of lysed strains decreased just slightly when the quantity of applied Lys170 was lowered to 0.04 μg. In contrast, this percentage was significantly diminished when Lys168 quantity dropped to 0.2 and 0.04 μg (Fig. 4). As described above, Lys170 produced clearer and larger lysis halos than Lys168 (Table S4). These results confirmed the highest lytic capacity of Lys170 and the clear preference of both endolysins towards *E. faecalis* when compared to *E. faecium*.

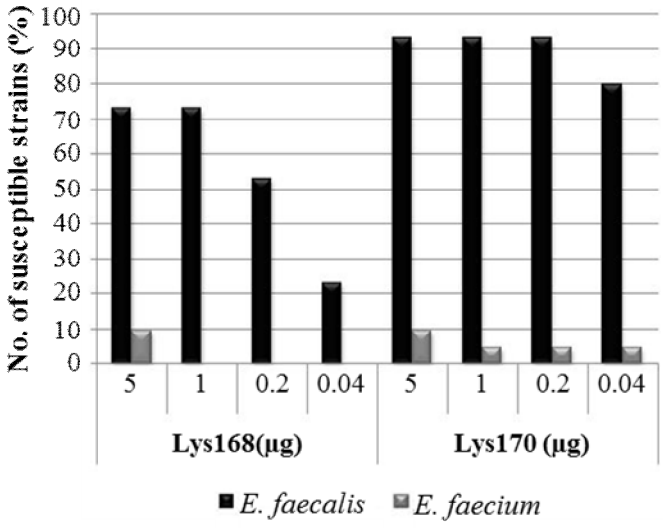

Fig. 4. Lytic action of Lys168 and Lys170 against a panel of diverse, typed clinical strains of *E. faecalis* (n=30) and *E. faecium* (n=21). The percentage of strains that presented lysis halos is plotted as a function of each endolysin quantity.

Lytic action of Lys168 and Lys170 against E. faecalis in liquid medium

The enterococcal endolysins also induced lysis of dense suspensions of viable *E. faecalis* cells prepared from exponentially growing cultures. The examples of figure 5 show the lytic action of both endolysins against two target strains, one that was only susceptible to Lys170 in the spot assay (see above), the *E. faecalis* VRE strain V583 (Fig. 5A), and another that was similarly lysed by both endolysins, the *E. faecalis* strain 1915/05 (Fig. 5B). VRE strain V583 was challenged with 5 μg/mL of each endolysin or with a mixture of both enzymes, each at a concentration of 2.5 μg/mL (Fig. 5A). As expected, Lys170 induced fast and extensive cell lysis, with the OD_{600} of the suspensions decreasing to about 10% of the initial value within 30 min. At the end of the assay (t = 90 min) the CFU/mL dropped to 1% of the initial value. Interestingly, although V583 seemed to be resistant to Lys168 in the spot assays, in liquid medium this endolysin could still produce a rather gradual cell lysis, leading to a 60% reduction of the initial OD_{600} and to ~80% cell killing during the time course of the assay. No significant synergistic effect was observed when cell suspensions were treated with a mixture of both enzymes, as the lysis profile and loss of cell viability were very similar to those observed with Lys170 alone.

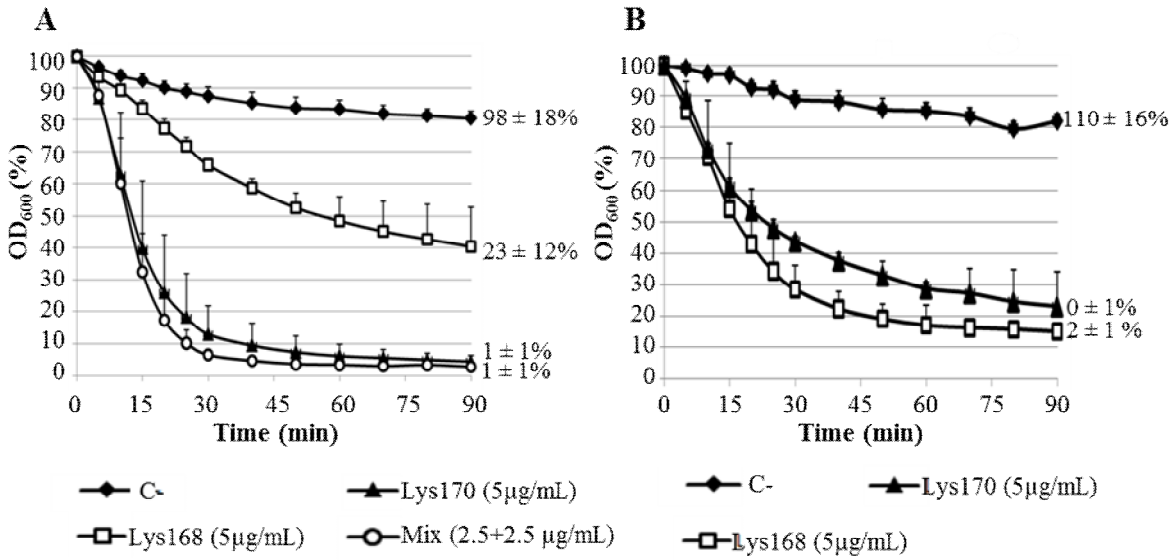

Fig. 5. Lytic action of Lys168 and Lys170 in a turbidity assay using *E. faecalis* strains V583 (**A**) and 1915/05 (**B**). The control (C-) was performed under the same conditions but with added lysin buffer instead of endolysin. The "Mix" curve in **A** results from the combined action of both endolysins. Values are the means of three independent experiments with indication of standard deviation. The values on the right side

of the curves indicate the percentage of the initial CFU/mL after 90 min of enzymes action and the corresponding standard deviation.

The apparent similar efficacy of Lys168 and Lys170 in lysing strain 1915/05 in soft-agar medium basically correlated with lysis induced by each endolysin in liquid medium (Fig. 5B). Both enzymes produced similar lysis curves with the OD_{600} decreasing to about 20% of the initial after 90 min, although in this case Lys168 seemed to induce slightly faster and more extensive lysis than Lys170. Both endolysins were capable of killing ≥98% of the initial CFU/mL by the end of the assay.

Overall, we observed that the lysis profile of a particular *E. faecalis* strain when challenged in liquid medium with the enterococcal endolysins, essentially correlated with the lysis efficiencies observed in the spot assay.

Activity of enterococcal endolysins against other Gram-positive pathogenic bacteria

The lytic activity of Lys168 and Lys170 was also evaluated in a few clinical isolates of other common Gram-positive pathogenic cocci (Table S5) by performing the enzyme spot assay as described above. No obvious lysis halo could be discernible in any of the tested isolates even for the highest protein amount spotted (5 μg). The results suggest that Lys168 and Lys170 are evolutionarily designed to specifically act against *Enterococcus* species, particularly *E. faecalis* if we consider the results described above.

DISCUSSION

In this work we have characterized two endolysins, Lys168 and Lys170 from phages infecting *E. faecalis* and have evaluated their bacterial cell lysis activity. As far as we know, only three additional *E. faecalis* phage endolysins have been described in the literature, PlyV12, EFAL-1 and ORF9 (Yoong *et al.*, 2004; Uchiyama *et al.*, 2008; Son *et al.*, 2010). ORF9 is virtually identical to Lys170 and thus it will be omitted from this

discussion, except in the part where we compare the lytic spectrum we obtained with Lys170 with that reported for ORF9 (see below).

Analysis of the primary sequence of these endolysins uncovered interesting features. The enzymes are clear examples of modular architecture by assembling different CDs and CWBDs of heterologous origin, thus generating endolysin diversity (Fig. 6). In fact, the four distinct endolysins referred to above have distantly related amino acid sequences, even when sharing CDs of the same family, as it is the case of Lys170 and EFAL-1 (Amidase_2 family). Remarkably, although designed to act on the same bacterial cell wall each endolysin seems to carry a distinct CWBD, suggesting that several different ligands of that cell compartment might be targeted by the endolysins. These enzymes are completely unrelated to those identified in 8 sequenced *E. faecalis* temperate phages, which encode endolysins with CD and CWBD of the Glyco_hydro_25 and LysM families, respectively (Yasmin *et al*., 2010). Another striking feature is the lack of close similarity between the CD of Lys170, EFAl-1 and PlyV12 and that of *E. faecalis* peptidoglycan hydrolases of the same family. BLASTP analysis showed that the closest homologues of the endolysin CDs are those carried by enzymes from different bacterial species, some from different genera (Fig. 6). This is in clear contrast to other known phage/bacteria systems, where the CD of endolysins is closely related to that of bacterial host autolysins (López and García, 2004; Zou and Hou, 2010). Lys168 CD was found to be closely related to a single *E. faecalis* peptidoglycan hydrolase encoded by strains TX0104 and TX1341 (Fig. 6).

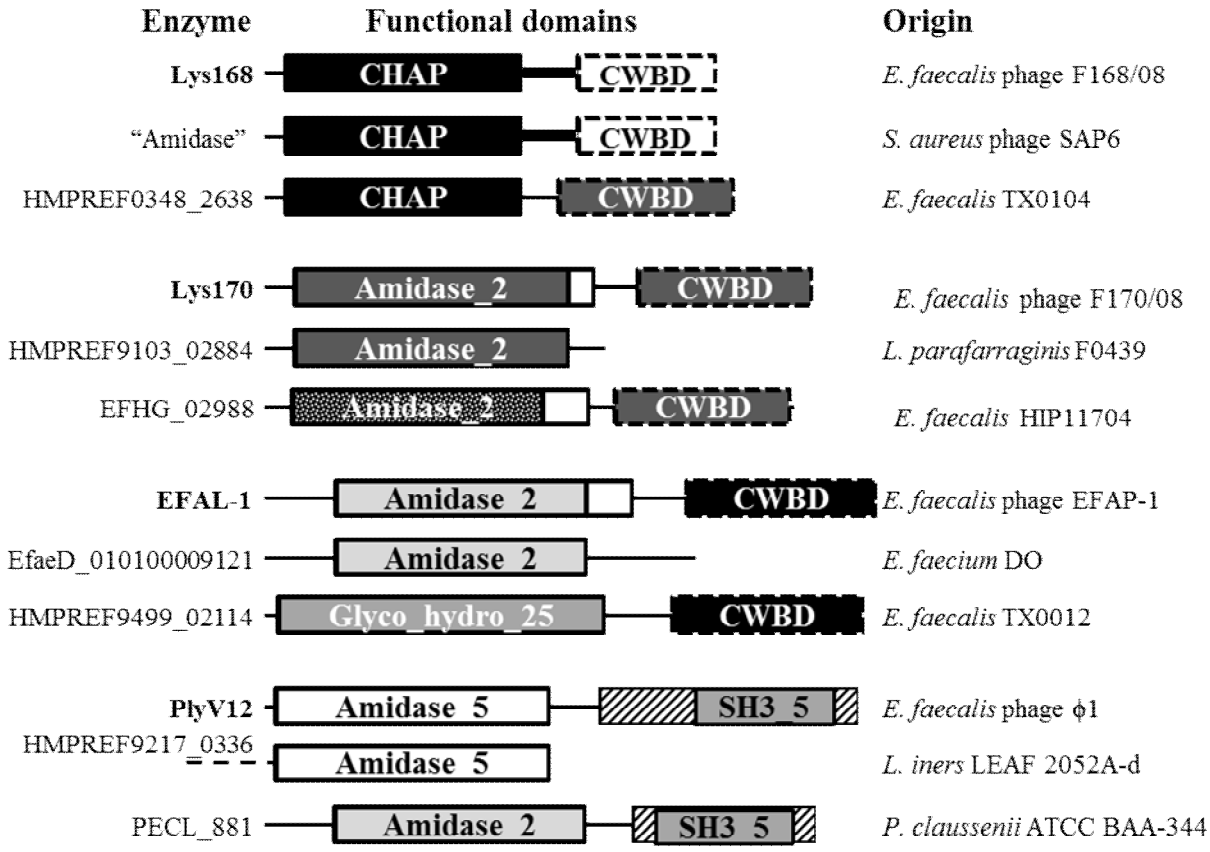

Fig. 6. Nature organization and sequence relatedness of *E. faecalis* phage endolysin functional domains. The sequence similarity between functional domains is evidenced by using identical filling patterns. *L.*, *Lactobacillus*; *P.*, *Pediococcus.*

Uchiyama *et al*. (2008) reported for ORF9 (identical to Lys170) a lytic spectrum of 97% and 60% against 35 and 10 non-typed *E. faecalis* and *E. faecium* isolates, respectively, which is very close to the results we obtained when Lys170 was tested in 73 and 26 clinical isolates of these species (Fig. S4). However, when assayed in a panel of distinct and typed *E. faecalis* and *E. faecium* strains, Lys170 lytic range against *E. faecium* dropped to about 10% while maintaining that against *E. faecalis* (Fig. 4). The results show the importance of testing the lytic spectrum of endolysins on a reasonable number of strains with different known genetic backgrounds. We have thus concluded that Lys170 has a strong preference to act against *E. faecalis*. Both ORF9 and Lys170 were unable to lyse bacterial species outside the *Enterococcus* genus (Uchiyama *et al.*, 2008) (Table S5).

Lys170 is most likely an N-acetylmuramoyl-L-alanine amidase as this was the activity experimentally determined for ORF9 (Uchiyama *et al.*, 2008). Amidases cleave the amide bond that links the N-acetyl muramic acid of glycan strands to the L-alanine residue of peptide stems. This bond and the nature of the linked residues are common to the vast majority of bacterial cell wall peptidoglycans, including that of *E. faecium* (Schleifer and Kandler, 1972). Why is then the *E. faecalis* cell wall the preferred substrate of Lys170? The ability of a given endolysin to cleave the bacterial cell wall depends on the integration of four major factors: i) binding of the CWBD to a specific ligand of the cell wall; ii) dependence of CD activity on CWBD binding; iii) CD affinity to its substrate; and iv) the presence of the peptidoglycan bond that is specifically cleaved by the CD (Low *et al.*, 2011). BLASTP analysis of Lys170 CWBD showed that this domain shares only significant similarity with those from *E. faecalis* enzymes. This suggests that Lys170 CWBD binds to an epitope that is predominantly found in the *E. faecalis* cell wall and that this binding is important for endolysin lytic action. This epitope eventually exists in a few strains of the related species *E. faecium*, explaining why some strains of this species are susceptible to Lys170.

Lys168 also displayed preferred lytic action against *E. faecalis* cells, acting poorly and in a much reduced number of *E. faecium* typed strains (Fig. 4). As referred to above, Lys168 CWBD is unrelated to that of Lys170, and thus the CHAP endolysin must recognize an epitope different from that targeted by Lys170. The peptidoglycan hydrolases of the CHAP family cleave different bonds of the murein structure, although a recent survey of the literature suggests that when present in bacterial autolysins the CHAP domain specifies amidase activity, whereas in phage endolysins it seems to confer endopeptidase activity (Layec *et al.*, 2008). The later activity typically cleaves the amino acidic bridges that cross-link the peptidoglycan stem peptides (Navarre *et al.*, 1999; Pritchard *et al.*, 2004), which can be different among bacterial species as it happens, for example, in *E. faecalis*, *E. faecium*, *S. aureus* and *S. agalactiae* (Schleifer and Kandler, 1972). Assuming this type of activity for Lys168 it could be easily explained the specificity of the endolysin towards *E. faecalis* cell wall. However, a recently constructed chimera composed of the Lys168 CHAP domain and the CWBD of a *S. aureus* endolysin proved to be very efficient in lysing several bacterial species, including a large number of *S. aureus* clinical strains (Fernandes *et al.*, 2012) (see below). It is therefore more likely that

the Lys168 CHAP domain specifies amidase activity and that the enzyme specificity towards *E. faecalis* cell wall is conferred by its CWBD.

The lytic spectrum of the other two putative amidases, EFAL-1 and PlyV12 (Fig. 6), has been also studied. In contrast to what we have observed with Lys168 and Lys170, PlyV12 was reported to have a broad lytic spectrum, displaying different degrees of activity against *E. faecium* and several streptococcal and staphylococcal strains (Yoong *et al*., 2004). The authors provided a possible explanation for the broad lytic spectrum of PlyV12, which relied on some sequence relatedness between the enzyme CD and that of endolysins from phages infecting the susceptible bacterial species (Yoong *et al*., 2004), although these endolysins are not the closest PlyV12 homologues, as mentioned above (Fig. 6). It was also suggested that PlyV12 CWBD might target a cell wall epitope that is common to the different bacteria (Yoong *et al*., 2004).

The significant sequence relatedness observed between the PlyV12 CD and that of streptococcal and staphylococcal phage endolysins was not verified for Lys170. Lys168 though, exhibited 95% sequence identity with the endolysin of *S. aureus* phage SAP6 and significant similarity with virion-associated lysins of staphylococcal phages (Figs 2 and 6). Despite this fact, Lys168 failed completely to induce lysis of all tested staphylococcal isolates, including those of *S. aureus* (Table S5). This suggests that the few differences observed between Lys168 and SAP6 endolysins (Fig. 2) are on key residues that determine the specificity of these enzymes and that these most likely reside in the CWBD (see above). In fact, and as referred before, when we exchanged the Lys168 CWBD by that of a *S. aureus* phage endolysin the resulting chimera could efficiently lyse *S. aureus* (Fernandes *et al*., 2012).

The endolysin EFAL-1 was also reported to display a broad lytic spectrum against *E. faecalis* and *E. faecium* (Son *et al*., 2010). Although this enzyme was tested in a reduced number of isolates (13 *E. faecalis* and 7 *E. faecium*) and no information was provided about their diversity, the fact is that the enzyme seems to be a natural chimera assembling a CD and a CWBD closely related to those from *E. faecium* and *E. faecalis* cell wall lytic enzymes, respectively (Son *et al*., 2010) (Fig. 6). This may explain the ability of EFAL-1 in lysing these two bacterial species. No significant sequence similarity was observed between the CD of Lys170 and Lys168 and that of *E. faecium* lytic enzymes (Fig. 6).

In conclusion, the results here presented indicate that endolysins Lys168 and Lys170 are good candidates for the specific elimination of *E. faecalis*, including VRE strains, either for sanitation or therapeutic purposes. The efficacy of these endolysins in animal models of *E. faecalis* infections is currently under study.

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SUPPLEMENTARY MATERIAL

Lys168

```
1    MVKLNDVLSYVNGLVGKGVDADGWYGTQCMDLT   33
34   VDVMQRFFGWRPYGNAIALVDQPIPAGFQRIRT   66
67   TSSTQIKAGDVMIWGLGYYAQYGHTGIATEDGR   99
100  ADGTFVSVDQNWINPSLEVGSPAAAIHHNMDGV   132
133  WGVIRPPYEAESKPKPPAPKPDKPNLGQFKGDD   165
166  DIMFIYYKKTKQGSTEQWFVIGGKRIYLPTMTY   198
199  VNEANDLIKRYGGNTNVTTYNYDNFGLAMMEKA   231
232  YPQVKLPGGGSHHHHHH                   248
```

Lys170

```
1    MAGEVFSSLITSVNPNPMNAGSRNGITIDTIIL   33
34   HHNATTNKDVAMNTWLLGGGAGTSAHYECTPTE   66
67   IIGCVGEQYSAFHAGGTGGIDVPKIANPNQRSI   99
100  GIENVNSSGAPNWSVDPRTITNCARLVADICTR   132
133  YGIPCDRQHVLGHNEVTATACPGGMDVDEVVRQ   165
167  AQQFMAGGSNNAVKPEPSKPTPSKPSNNKNKEG   198
199  VATMYCLYERPINSKTGVLEWNGDAWTVMFCNG   231
232  VNCRRVSHPDEMKVIEDIYRKNNGKDIPFYSQK   264
265  EWNKNAPWYNRLETVCPVVGITKKSPGGGSHHH   297
298  HHH                                 300
```

Fig. S1. Bioinformatics analysis of endolysins Lys168 and Lys170 primary sequence. According to CDD and Pfam analysis (Marchler-Bauer *et al*., 2011 and http://pfam.sanger.ac.uk/), Lys168 and Ly170 harbor N-terminal CDs of the CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) and Amidase_2 families, respectively, which are evidenced by the gray shading. Putative linker segments (boldface residues) were predicted with SVM tool (Ebina *et al*., 2009). Inferred CWBDs are dash-underlined and plasmid-born, hexahistidine-containing sequences are in italics.

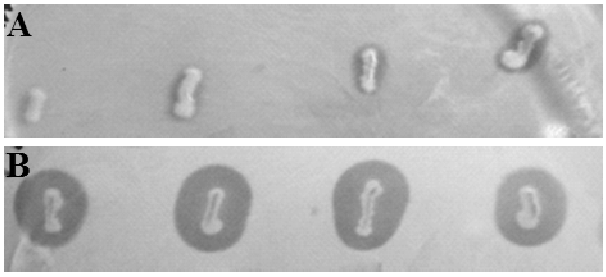

Fig. S2. "TritonX-100-induced lysis halo assay" (Yokoi *et al*., 2005) used for confirming production of active endolysins by *E. coli* CG61 derivatives. Transformants were grown overnight at 30 ºC on an LB soft-agar plate containing 2% (w/v) of autoclavated cells from the host strains of phages F168 and F170 (*E. faecalis* 926/05 and 1518/05, respectively), 0.1% Triton-X100, 100 µg/mL ampicillin and 30 µg/mL

kanamycin. Subsequently the plates were incubated at 4 ºC for 24 h. Lysis halos around *E. coli* CG61 colonies expressing Lys168 (A) or Lys170 (B) are shown.

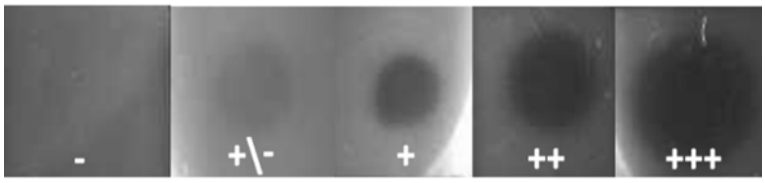

Fig. S3. Representative lysis halos resulting from endolysin lytic action in the spot lytic assay. The examples shown resulted from the application of 5 µg of Lys170 on lawns of different *E. faecalis* clinical isolates. Lysis halos were scored (- through +++) according to their relative size and transparency.

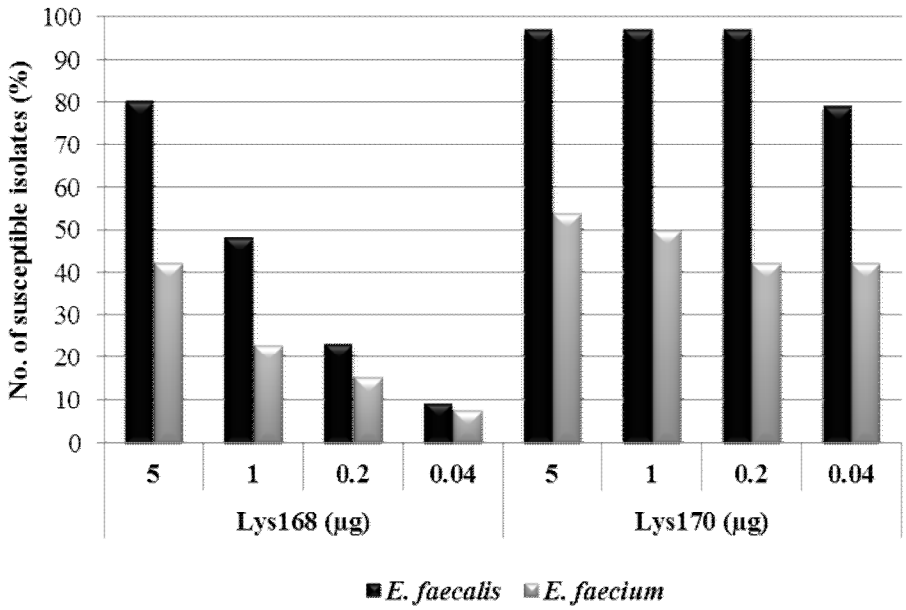

Fig. S4. Lys168 and Lys170 lytic action against 73 *E. faecalis* and 26 *E. faecium* non-typed clinical isolates (Tables S1 and S2). Each isolate was grown until an OD_{600} of 0.8-1.0 at 30 ºC without aeration. Cells were recovered by centrifugation and concentrated 100-fold in fresh culture medium. Samples of 300 µL of these cell suspensions were incorporated in lysis assay buffer (25 mM phosphate-Na, 250 mM NaCl, 1% glycerol, 1 mM DTT, pH 8.0) supplemented with 0.7% agar and poured in a Petri dish. The indicated amounts of each endolysin were spotted on this cell lawn and plates incubated at 37 ºC for 16-18 h. The percentage of isolates that presented lysis halos is plotted as a function of each endolysin quantity.

Table S1. Non-typed *E. faecalis* and *E. faecium* clinical isolates used in this study.

Isolate code	Clinical specimens	Isolate code	Clinical specimens	Isolate code	Clinical specimens
***E. faecalis*, n=73**				***E. faecium*, n=26**	
1/06	Pus	926/06	Urine	1000/05	Urine
1902/05	Ascites fluid	958/05	Urine	1131/05	Pus
344/06	Scab exudate	992/06	Urine	1132/05	Pus
363/06	Pleural fluid	1113/06	Blood culture	1607/05	Scab exudate
46/06	Pus peritoneal	127/06	Urine	1729/05	Urine
497/06	Blood culture	1408/05	Urine	1793/05	Pus
834/06	Blood culture	1409/05	Urine	1795/05	Urine
838/05	Urine	1551/05	Urine	1866/05	Urine
911/05	Urine	1554/05	Urine	1903/05	Pus
953/06	Exudate	1558/05	Urine	969/05	Urine
1130/06	Exudate	1853/05	Urine	1793$^{(VRE)}$	Pus
1451/05	Urine	2/06	Urine	184/06	Respiratory product
1665/05	NK	2093/05	Urine	185/06	Catheter
1915/05	Urine	3/06	Urine	186/06	Respiratory product
2092/05	Urine	307/06	Urine	187/06	Respiratory product
45/06	Blood culture	43/06	Urine	188/06	Blood culture
604/07	Urine	44/06	Pus	198/06	Bile
71/07	Urine	563/07	Blood culture	226/06	Urine
786/06	Blood culture	750/06	Blood culture	267/06	Anal exudate
993/06	Pus	751/06	Blood culture	268/06	Respiratory product
1040/06	Urine	81/06	Urine	269/06	Blood culture
1041/06	Urine	952/06	Blood culture	388/06	Blood culture
1271/06	Urine	954/06	Blood culture	389/06	Blood culture
1285/06	Urine	263/06	Urine	390/06	Blood culture
140/07	Urine	1/07	Urine	729/06	Pleural fluid
1403/06	Ascites fluid	110/07	Urine	515/07	Vaginal exudate
1404/06	Urine	139/07	Urine		
1405/06	Urine	158/07	Blood culture		
1553/05	Urine	310/07	Urine		
1654/05	Bile	311/07	Urine		
1710/05	Catheter	328/07	Urine		
264/06	Urine	332/07	Urine		
470/06	NK	514/07	Urine		
556/06	Urine	606/07	Urine		
73/07	Urine	1518/05	Pus		
857/05	Urine	926/05	Blood culture		
882/06	Urine				

Abbreviations: NK, Not Known; VRE, Vancomycin Resistant *Enterococcus*

Table S2. Lytic action of Lys168 and Lys170 against the group of non-typed enterococcal clinical isolates.[1]

Isolate	Lys168 (µg)				Lys170 (µg)			
	5	1	0.2	0.04	5	1	0.2	0.04
***E. faecalis*, n=73**								
1/06	+/-	-	-	-	+++	+++	+	+/-
1902/05	+	+	-	-	+++	+++	++	++
344/06	-	-	-	-	+++	+++	+/-	-
363/06	+/-	-	-	-	+++	+++	++	++
46/06	++	+/-	-	-	+++	+++	+++	-
497/06	+/-	-	-	-	+++	+/-	+/-	-
834/06	+/-	-	-	-	+++	+++	++	-
838/05	+/-	-	-	-	+++	++	+/-	-
911/05	-	-	-	-	++	++	++	+++
953/06	+/-	+/-	-	-	+++	+++	+++	+/-
1130/06	+	+	+	-	+++	+++	+++	+++
1451/05	+/-	-	-	-	+++	++	+	-
1665/05	+/-	-	-	-	+++	++	+	+/-
1915/05	+++	+++	+++	++	+++	+++	+++	+++
2092/05	-	-	-	-	+++	++	+	+/-
45/06	-	-	-	-	+++	+++	++	+
604/07	+	+	+	+	+++	+++	+++	+++
71/07	++	++	+	-	+++	+++	++	++
786/06	+	+/-	-	-	+++	+++	++	+
993/06	+	+/-	+/-	-	++	++	+++	+++
1040/06	+/-	+/-	-	-	+++	+++	++	+
1041/06	+	+/-	-	-	+++	+++	+++	++
1271/06	+	+/-	-	-	+++	+++	++	+
1285/06	+/-	-	-	-	+++	+++	++	-
140/07	+/-	+/-	-	-	+++	+++	++	+
1403/06	-	-	-	-	+/-	-	-	-
1404/06	+/-	-	-	-	+++	++	+	-
1405/06	+	+	-	-	+++	+++	++	-
1553/05	-	-	-	-	+++	+++	+++	++
1654/05	++	++	+	+/-	+++	+++	+++	+++
1710/05	+/-	-	-	-	+++	+++	+++	++
264/06	+	+/-	-	-	+++	+++	+++	+
470/06	+	+	+	+/-	+++	+++	+++	+
556/06	+	-	-	-	+++	+++	+++	++
73/07	+	+/-	-	-	+++	+++	+++	+
857/05	+/-	-	-	-	+++	+++	++	++
882/06	+/-	-	-	-	+++	+++	+++	+
926/06	+	+/-	-	-	+++	+++	+++	+
958/05	+/-	-	-	-	+++	+++	+++	++
992/06	+	+/-	-	-	+++	++	+	+/-
1113/06	+	-	-	-	+++	+++	+	-
127/06	+	+/-	-	-	+++	+++	++	+

Table S2, *cont.*

Isolate	Lys168 (μg)				Lys170 (μg)			
	5	1	0.2	0.04	5	1	0.2	0.04
E. faecalis, cont.								
1408/05	+/-	-	-	-	+++	+++	++	++
1409/05	+/-	+/-	-	-	+++	++	+	-
1551/05	+	+/-	-	-	+++	+++	+++	++
1554/05	-	-	-	-	+++	+++	++	+/-
1558/05	+/-	-	-	-	+++	+++	++	+
1853/05	++	++	+	+/-	++	++	+	+/-
2/06	-	-	-	-	+++	+	+/-	+/-
2093/05	-	-	-	-	+++	+++	++	+
3/06	+/-	-	-	-	+++	++	+	+
307/06	-	-	-	-	+++	++	+/-	-
43/06	+	+/-	-	-	+++	+++	+	+/-
44/06	+/-	-	-	-	+++	+++	+++	++
563/07	+	+	+/-	-	+++	+++	++	+
750/06	+	+	+/-	-	+++	+++	++	+
751/06	+	-	+/-	-	+++	+++	++	++
81/06	+/-	+/-	-	-	+++	+++	+++	+
952/06	+/-	-	-	-	+++	+++	++	+/-
954/06	-	-	-	-	+++	++	+	+/-
263/06	-	-	-	-	+++	++	+	+/-
1/07	+/-	+/-	-	-	++	++	+	+/-
110/07	+	+	+/-	-	+++	+++	++	+/-
139/07	-	-	-	-	-	-	-	-
158/07	+	+/-	+/-	-	+++	+++	++	+
310/07	-	-	-	-	+++	+++	+++	++
311/07	+/-	-	-	-	+++	+++	++	+/-
328/07	+/-	-	-	-	+++	+++	++	+/-
332/07	-	-	-	-	-	-	-	-
514/07	+	+	+	+/-	+++	+++	+++	+
606/07	++	+/-	+/-	-	+++	+++	+	-
1518/05	+	+	+	+/-	+++	+++	+++	+/-
926/05	++	++	+	-	+++	+++	+++	+
***E. faecium*, n=26**								
1000/05	-	-	-	-	-	-	-	-
1131/05	-	-	-	-	-	-	-	-
1132/05	-	-	-	-	-	-	-	-
1607/05	-	-	-	-	-	-	-	-
1729/05	-	-	-	-	-	-	-	-
1793/05	-	-	-	-	-	-	-	-
1795/05	-	-	-	-	-	-	-	-
1866/05	-	-	-	-	-	-	-	-
1903/05	-	-	-	-	-	-	-	-
969/05	-	-	-	-	-	-	-	-
1793$^{(VRE)}$	-	-	-	-	-	-	-	-

Table S2, *cont.*

Isolate	Lys168 (µg)				Lys170 (µg)			
	5	1	0.2	0.04	5	1	0.2	0.04
E. faecium, cont.								
184/06	+/-	-	-	-	+++	+++	++	++
185/06	-	-	-	-	+++	+++	+	+
186/06	+	+/-	-	-	+++	+++	+	++
187/06	+	+	+/-	-	+++	+++	+	+
188/06	++	+	+/-	-	+++	+++	+++	++
198/06	+/-	-	-	-	+++	+++	++	+
226/06	+/-	+/-	-	-	+++	+++	-	-
267/06	+/-	-	-	-	+/-	+++	+/-	+/-
268/06	+/-	-	-	-	+++	+++	+/-	+/-
269/06	+	+	+/-	+/-	+++	+++	+/-	+/-
388/06	-	-	-	-	+++	+++	-	-
389/06	+	+	+	+/-	+++	+++	++	++
390/06	+/-	-	-	-	+++	+++	+/-	+/-
729/06	-	-	-	-	-	-	-	-
515/07	-	-	-	-	-	-	-	-

[1]Four different quantities of each endolysin were spotted on a dense cell lawn of each isolate, which was prepared by incorporating cells from exponentially growing cultures in a soft-agar, phosphate-buffered medium. Lytic activity was scored (- to +++) according to the relative size and transparency of lysis halos after overnight incubation at 37 ºC.
Abbreviations: VRE, Vancomycin Resistant *Enterococcus*

Table S3. Detailed characterization of the typed enterococcal clinical strains used in this study.

			Virulence determinants							Antibiotic									
Strain ID	**PFGE pattern**	**Vancomycin**	***esp***	***cylA***	***asaI***	***gelE***	***hyl***	**citolysin**	**gelatinase**	**Amp**	**HLG**	**Van**	**Teic**	**Q/D**	**Cip**	**Te**	**E**	**DA**	**LZD**
***E. faecalis*, n=28**																			
EHCP 3	AO6	resistant	-	+	+	+	-	β	+	S	R	R	R	R	R	R	R	R	ND
EHCP 13	S	susceptible	-	-	+	+	-	-	-	S	R	S	S	S	R	S	R	R	ND
EHCP 24	AO5	susceptible	-	-	+	+	-	-	+	S	R	S	S	R	R	R	R	R	ND
EHCP 31	A2	susceptible	-	+	+	-	-	β	-	S	R	S	S	R	S	R	R	R	ND
EHCP 55	AW	susceptible	-	+	+	+	-	β	+	S	R	S	I	R	S	S	R	R	ND
EHCP 73	J	susceptible	-	-	+	+	-	-	-	S	R	S	S	R	S	I	R	R	ND
EHCP 78	A3	susceptible	+	+	+	-	-	β	-	S	R	S	S	R	S	R	R	R	ND
EHCP 92	AR	susceptible	-	-	+	+	-	α	-	S	R	S	S	S	R	R	I	R	ND
EHCP 93	AX	susceptible	-	-	+	+	-	α	-	S	R	S	S	S	R	R	R	R	ND
EHCP 94	AM	susceptible	+	-	+	+	-	α	-	S	R	S	S	S	R	R	R	R	ND
EHCP 107	K	susceptible	+	-	-	-	-	α	-	S	R	S	S	S	R	S	R	R	S
EHCP 118	AT	susceptible	-	-	+	+	-	-	-	S	R	S	S	R	R	R	R	R	S
EHCP 143	AU	susceptible	+	-	+	+	-	α	-	S	R	S	S	S	R	R	R	R	ND
EHCP 151	H	susceptible	-	+	+	+	-	β	-	S	R	S	S	S	R	S	R	R	S
EHCP 164	B	susceptible	+	+	+	+	-	β	+	S	R	S	S	R	R	S	R	R	S
EHCP 193	BC	susceptible	-	-	+	+	-	-	-	S	R	S	S	I	R	S	I	R	S
EHCP 225	R	susceptible	-	-	+	+	-	-	-	S	R	S	S	I	R	S	I	R	S
EHCP 237	AO2	susceptible	-	-	+	+	-	α	+	S	R	S	S	R	R	R	R	R	S
EHCP 241	O	susceptible	-	+	+	+	-	β	-	S	R	S	S	I	R	S	I	R	S
EHCP 267	AO2	resistant	+	+	+	+	-	β	+	S	R	R	R	R	R	R	R	R	S
EHCP 271	A11	susceptible	+	+	+	-	-	β	-	S	R	S	S	R	S	R	R	R	S
EHCP 279	T	susceptible	+	-	+	+	-	-	+	S	R	S	S	R	S	R	I	R	S
EHCP 281	U	susceptible	+	+	+	+	-	β	-	S	R	S	S	R	R	R	R	R	S
EHCP 292	A4	susceptible	+	+	+	+	-	β	+	S	R	S	S	R	S	R	R	R	S
EHCP 332	I	susceptible	-	-	+	+	-	α	-	S	R	S	S	I	R	S	R	R	S
EHCP 339	AO1	susceptible	+	+	+	+	-	α	+	S	R	S	S	R	R	R	R	R	S
EHCP 389	AO1	resistant	-	+	+	+	-	β	+	S	R	R	R	R	R	R	R	R	S
EHCP 391	M	susceptible	-	+	+	+	-	β	+	S	R	S	S	R	S	R	R	R	S

Table S3, *cont.*

Strain ID	PFGE pattern	Vancomycin	Virulence determinants							Antibiotic									
			esp	*cylA*	*asaI*	*gelE*	*hyl*	citolysin	gelatinase	Amp	HLG	Van	Teic	Q/D	Cip	Te	E	DA	LZD
***E. faecium*, n=21**																			
EHCP 5	c10	resistant	+	-	-	-	-	α	-	R	R	R	R	S	R	R	R	R	ND
EHCP 6	a1	resistant	-	-	-	-	-	α	-	R	R	R	R	S	R	S	R	R	ND
EHCP 14	d2	susceptible	-	-	-	-	+	α	-	R	R	S	S	S	R	R	R	R	ND
EHCP 36	a2	resistant	-	-	-	-	-	α	-	S	S	R	R	S	R	S	R	R	ND
EHCP 40	d9	susceptible	+	-	-	-	+	α	-	R	R	S	S	S	R	R	R	R	ND
EHCP 65	o	susceptible	-	-	-	-	+	α	-	R	R	S	S	S	R	R	R	R	ND
EHCP 88	c2	susceptible	+	-	-	-	-	α	-	R	R	S	S	S	R	R	R	R	ND
EHCP 149	d6	susceptible	-	-	-	-	+	α	-	R	R	S	S	S	R	R	R	R	S
EHCP 161	t	susceptible	+	-	-	-	+	α	-	R	R	S	S	S	R	R	R	S	S
EHCP 178	p	susceptible	-	-	-	-	+	α	-	S	R	S	S	S	R	S	R	R	S
EHCP 181	d8	susceptible	+	-	-	-	+	α	-	R	R	S	S	S	R	R	I	R	S
EHCP 184	f	susceptible	-	-	-	-	+	α	-	R	R	S	S	S	R	R	R	R	S
EHCP 211	c12	susceptible	+	-	-	-	-	α	-	R	R	S	S	S	R	S	R	R	S
EHCP 264	e	susceptible	-	-	-	-	-	α	-	R	R	S	S	I	R	R	R	R	S
EHCP 302	c5	susceptible	+	-	-	-	-	**α**	-	R	R	S	S	S	R	S	R	R	S
EHCP 341	u	susceptible	+	-	+	+	-	α	-	R	R	S	S	S	R	R	R	S	S
EHCP 358	i	susceptible	+	-	-	-	+	α	-	R	R	S	S	S	R	R	R	R	S
EHCP 361	c16	resistant	+	-	-	-	-	α	-	R	R	R	R	S	R	S	R	R	S
EHCP 378	w	susceptible	-	-	-	-	+	α	-	R	R	S	S	S	R	R	R	R	S
EHCP 407	d7	resistant	-	-	-	-	-	α	-	R	S	R	R	S	R	S	R	R	ND
EHCP 459	s	susceptible	-	-	-	-	-	α	-	R	R	S	S	S	R	S	R	R	S

Abbreviations: Amp, Ampicillin; HLG, High-Level-Gentamicin; Van, Vancomycin; Teic, Teicoplanin; Q/D, Quinupristin/Dalfopristin, Cip, Ciprofloxacin; Te, Tetracyclin; E, Erythromycin; Da, Clindamycin; LZD, Linezolid; R, Resistant; S, Susceptible; I, Intermediate; ND, Not Determined.

Table S4. Lytic action of Lys168 and Lys170 against the panel of typed enterococcal clinical strains.[1]

Strain ID	Lys168 (μg)				Lys170 (μg)			
	5	1	0.2	0.04	5	1	0.2	0.04
***E. faecalis*, n=30**								
EHCP 3$^{(VRE)}$	+/-	+	+/-	-	+++	++	+	+/-
EHCP 13	+++	++	++	+/-	-	+	++	+/-
EHCP 24	++	+	-	-	+++	++	+	+
EHCP 31	+	+	+/-	-	+++	++	+	+/-
EHCP 55	+	+	+/-	-	+++	++	++	+
EHCP 73	+++	++	+	+	+++	++	+	+
EHCP 78	+/-	+/-	-	-	+++	++	+	+/-
EHCP 92	-	-	-	-	+++	+++	++	+
EHCP 93	++	-	-	-	+	+++	++	+/-
EHCP 94	++	+	+/-	-	+++	++	+	-
EHCP 107	-	-	-	-	-	-	-	-
EHCP 118	+++	++	+	+/-	+++	+++	++	+/-
EHCP 143	+/-	+/-	-	-	+++	++	+	+/-
EHCP 151	+++	++	+	+/-	+++	++	+	+/-
EHCP 164	+/-	+/-	-	-	+++	++	+	+/-
EHCP 193	+/-	+/-	-	-	+/-	-	+/-	-
EHCP 225	+++	++	-	-	++	++	+	-
EHCP 237	-	-	-	-	+++	+/-	++	+
EHCP 241	-	-	-	-	++	+/-	+/-	-
EHCP 267$^{(VRE)}$	-	-	-	-	+++	++	++	+/-
EHCP 271	+/-	+/-	+/-	-	+++	++	++	++
EHCP 279	+	+	+	+	+++	+++	+++	+
EHCP 281	++	+/-	+/-	-	+++	++	+	+/-
EHCP 292	+/-	+/-	+/-	-	+++	++	++	+
EHCP 332	+++	++	+	+/-	+++	+++	+++	+
EHCP 339	+/-	+/-	+/-	-	+++	++	++	+/-
EHCP 389$^{(VRE)}$	-	+/-	+/-	-	+++	+	+	++
EHCP 391	-	-	-	-	+++	++	++	+/-
MMH 594	+++	+	+	+/-	+++	++	+	+
V583$^{(VRE)}$	-	-	-	-	++	+	-	-
***E. faecium*, n=21**								
EHCP 5$^{(VRE)}$	-	-	-	-	-	-	-	-
EHCP 6$^{(VRE)}$	-	-	-	-	-	-	-	-
EHCP 14	-	-	-	-	-	-	-	-
EHCP 36$^{(VRE)}$	-	-	-	-	-	-	-	-
EHCP 40	-	-	-	-	-	-	-	-
EHCP 65	-	-	-	-	-	-	-	-
EHCP 88	-	-	-	-	-	-	-	-
EHCP 149	-	-	-	-	-	-	-	-
EHCP 161	-	-	-	-	-	-	-	-
EHCP 178	-	-	-	-	-	-	-	-
EHCP 181	-	-	-	-	-	-	-	-
EHCP 184	-	-	+/-	-	-	+/-	-	-
EHCP 211	-	-	+/-	-	-	+	-	-
EHCP 264	-	-	-	-	-	-	-	-
EHCP 302	-	-	-	-	-	-	-	-
EHCP 341	-	-	-	-	-	-	-	-

Table S4, *cont.*

Strain ID	Lys168 (µg)				Lys170 (µg)			
	5	1	0.2	0.04	5	1	0.2	0.04
E. faecium, cont.								
EHCP 358	-	-	-	-	-	-	-	-
EHCP 361$^{(VRE)}$	-	-	-	-	-	-	-	-
EHCP 378	-	-	-	-	-	-	-	-
EHCP 407$^{(VRE)}$	-	-	-	-	-	-	-	-
EHCP 459	-	-	-	-	-	-	-	-

[1]Four different quantities of each endolysin were spotted on a dense cell lawn of each strain, which was prepared by incorporating cells from exponentially growing cultures in a soft-agar, phosphate-buffered medium. Lytic activity was scored (- to +++) according to the relative size and transparency of lysis halos after overnight incubation at 37ºC.
Abbreviations: VRE, Vancomycin Resistant *Enterococcus*

Table S5. Non-typed clinical isolates from other Gram-positive pathogenic cocci used in this study.

Species	Isolate code	Clinical specimens
Streptococcus pneumoniae, n=10	01/08	Sputum
	07/08	Sputum
	63/08	Sputum
	04/08	Ocular exudate
	02/08	Blood culture
	03/08	Blood culture
	05/08	Blood culture
	65/08	Bronchial secretions
	68/08	Bronchial secretions
	73/08	Cerebrospinal fluid
Streptococcus pyogenes, n=8	12/08	Pharyngeal exudate
	13/08	Pharyngeal exudate
	14/08	Pharyngeal exudate
	15/08	Pharyngeal exudate
	16/08	Pharyngeal exudate
	191/08	Pharyngeal exudate
	192/08	Pharyngeal exudate
	193/08	Pharyngeal exudate
Streptococcus agalactiae n=8	516/07	Vaginal exudate
	517/07	Vaginal exudate
	518/07	Vaginal exudate
	519/07	Vaginal exudate
	520/07	Vaginal exudate
	521/07	Vaginal exudate
	522/07	Blood culture
	595/07	Blood culture
Staphylococcus aureus, n=9	1020/05$^{(MSSA)}$	Synovial fluid
	1538/05$^{(MSSA)}$	Exudate
	1039/06$^{(MSSA)}$	Exudate
	662/07$^{(MSSA)}$	Exudate
	53/08$^{(MRSA)}$	Exudate
	1011/05$^{(MRSA)}$	Wound exudate
	1018/05$^{(MRSA)}$	Wound exudate
	1035/06$^{(MRSA)}$	Exudate
	1037/06$^{(MRSA)}$	Wound exudate
Staphylococcus haemolyticus, n=4	1930/05	Catheter
	06/06	Urine
	1318/05	Sputum
	1703/05	Sputum
Staphylococcus epidermidis, n=4	158/08	Exudate
	114/08	Exudate
	110/08	Face exudate
	1736/05	Catheter exudate

Abbreviations: MSSA, Methicillin-sensitive *Staphylococcus aureus*; MRSA, Methicillin-resistant *Staphylococcus aureus.*

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CHAPTER 3

A TWO-COMPONENT, MULTIMERIC ENDOLYSIN ENCODED BY A SINGLE GENE

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Author contributions:

The author of this dissertation performed all the experiments presented in this chapter with the help of Clara Leandro in a few assays. Experimental design, data analysis and manuscript preparation were done by the author of this thesis and by Clara Leandro, Miguel Garcia, Madalena Pimentel and Carlos São-José. Carlos São-José was the main supervisor of the work

A TWO-COMPONENT, MULTIMERIC ENDOLYSIN ENCODED BY A SINGLE GENE

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ABSTRACT

Bacteriophage endolysins are bacterial cell wall degrading enzymes whose potential to fight bacterial infections has been intensively studied. Endolysins from Gram-positive systems are typically described as monomeric and as having a modular structure consisting of one or two N-terminal catalytic domains (CD) linked to a C-terminal region responsible for cell wall binding (CWB). We show here that expression of the endolysin gene *lys170* of the enterococcal phage F170/08 results in two products, the expected full length endolysin (Lys170FL) and a C-terminal fragment corresponding to the CWB domain (CWB170). The latter is produced from an in-frame, alternative translation start site. Both polypeptides interact to form the fully active endolysin. Biochemical data strongly support a model where Lys170 is made of one monomer of Lys170FL associated with up to three CWB170 subunits, which are responsible for efficient endolysin binding to its substrate. Bioinformatics analysis indicates that similar secondary translation start signals may be used to produce and add independent CWB170-like subunits to different enzymatic specificities. The particular configuration of endolysin Lys170 uncovers a new mode of increasing the number of CWB motifs associated to CD modules, as an alternative to the tandem repetition typically found in monomeric cell wall hydrolases.

INTRODUCTION

Like all viruses, bacteriophages need to parasite host cells, in this case bacteria in order to multiply. After replication, newly formed virus particles need to escape from infected cells to disseminate. To accomplish this, double-stranded DNA phages have evolved protein systems that compromise the integrity of the bacterial cell envelope in order to cause host cell lysis. These systems may comprehend a set of functions targeting the different cell envelope barriers, but the hallmark of lysis functions is the well-known holin-endolysin dyad (Catalão *et al.*, 2013; Young, 2014). Holins are hydrophobic proteins that oligomerize in the cytoplasmic membrane and induce the formation of holes in a tightly scheduled, saltatory manner. Canonical holins produce micron-scale holes large enough to allow the passage of cytoplasm-accumulated endolysins to the cell wall compartment, whereas pinholins form small channels that serve to depolarize the membrane and activate previously secreted endolysins (Nascimento *et al.*, 2008; Young, 2013; Savva *et al.*, 2014). Endolysins are enzymes that cleave the peptidoglycan (PG) network of the bacterial cell wall. They have been classified into five major functional types according to the bonds of the PG they cleave: N-acetylmuramidases (lysozymes), endo-β-N-acetylglucosaminidases and lytic transglycosylases cleave bonds of the N-acetylmuramic acid (NAM)/N-acetylglucosamine (NAG) moiety of the PG, but with different specificities and/or end products; N-acetyl-muramoyl-L-alanine amidases hydrolyze the amide bond between NAM and L-alanine residues in the oligopeptide chains, and endopeptidases attack the peptide bonds within or between these chains (São-José *et al.*, 2003; Loessner, 2005). Within each major group, endolysins have been subdivided into families according to the sequence relatedness of their functional domains (Oliveira *et al.*, 2013).

The vast majority of endolysins produced by phages of Gram-positive bacteria and of mycobacteria seem to display a conserved modular architecture (Diaz *et al.*, 1990; Payne and Hatfull, 2012) of two separated functional regions: an N-terminus carrying one to three catalytic domains (CD) and a C-terminus segment harboring one or several repeats of cell wall binding (CWB) motifs (Fischetti, 2008; Schmelcher *et al.*, 2012). With the exception of the multimeric endolysin PlyC, which is composed of a two CD-containing polypeptide (PlyCA) associated to eight PlyCB subunits with CWB activity, with A and

B subunits codified by separate genes (Nelson *et al*., 2006; McGowan *et al*., 2012), all studied endolysins are encoded by a single gene and seem to be monomeric when purified.

Some endolysins have the capacity to degrade the cell wall PG and cause cell lysis when added in the form of recombinant proteins to Gram-positive bacteria. This has stimulated intense research to exploit the potential of endolysins as antibacterial agents (for recent reviews see Fischetti, 2010; Nelson *et al*., 2012; Schmelcher *et al*., 2012). In one of these studies we showed that the endolysin Lys170 from the enterococcal phage F170/08 exhibited broad lytic activity against *Enterococcus faecalis* clinical strains (Proença *et al*., 2012). Lys170 is a typical modular endolysin displaying an N-terminal amidase CD linked to a putative C-terminal CWB region. Lys170 is virtually identical to the previously described endolysin of *E. faecalis* phage ϕEF24C (Uchiyama *et al*., 2011), with both enzymes showing a single substitution over their 289 amino acid sequence.

We show now that expression of *lys170* systematically results in the production of a *ca*. 12 kDa small protein, basically corresponding to the predicted Lys170 CWB domain, in addition to the expected full length polypeptide (Lys170FL, 32.6 kDa). We have studied the interaction between these two endolysin components and their contribution to lytic activity. We present data supporting that fully active Lys170 is a multimeric endolysin resulting from the association of ~12 kDa CWB subunits with Lys170FL. We speculate that such endolysin architecture may be quite common and we discuss its potential advantages in terms of lytic efficacy.

RESULTS

Expression of endolysin gene lys170 results in two stable polypeptides

Production of a C-terminal His_6-tagged version of Lys170 in *E. coli* systematically resulted in the accumulation of two polypeptides, one corresponding to expected full length protein (Lys170FL, 32.6 kDa) and the other with an apparent mass of about 12 kDa. We have missed this smaller protein in previous works because of too long SDS-PAGE runs (Proença *et al*., 2012). The two proteins co-purified during metal chelate affinity chromatography (AFC) and were immunodetected with anti-His_6 specific

antibodies (not shown), which indicated that the ~12 kDa polypeptide was a C-terminal fragment of Lys170FL; its mass suggested that it would essentially correspond to the predicted Lys170 CWB domain (Proença *et al.*, 2012) and therefore it was designated CWB170 (Fig. 1A).

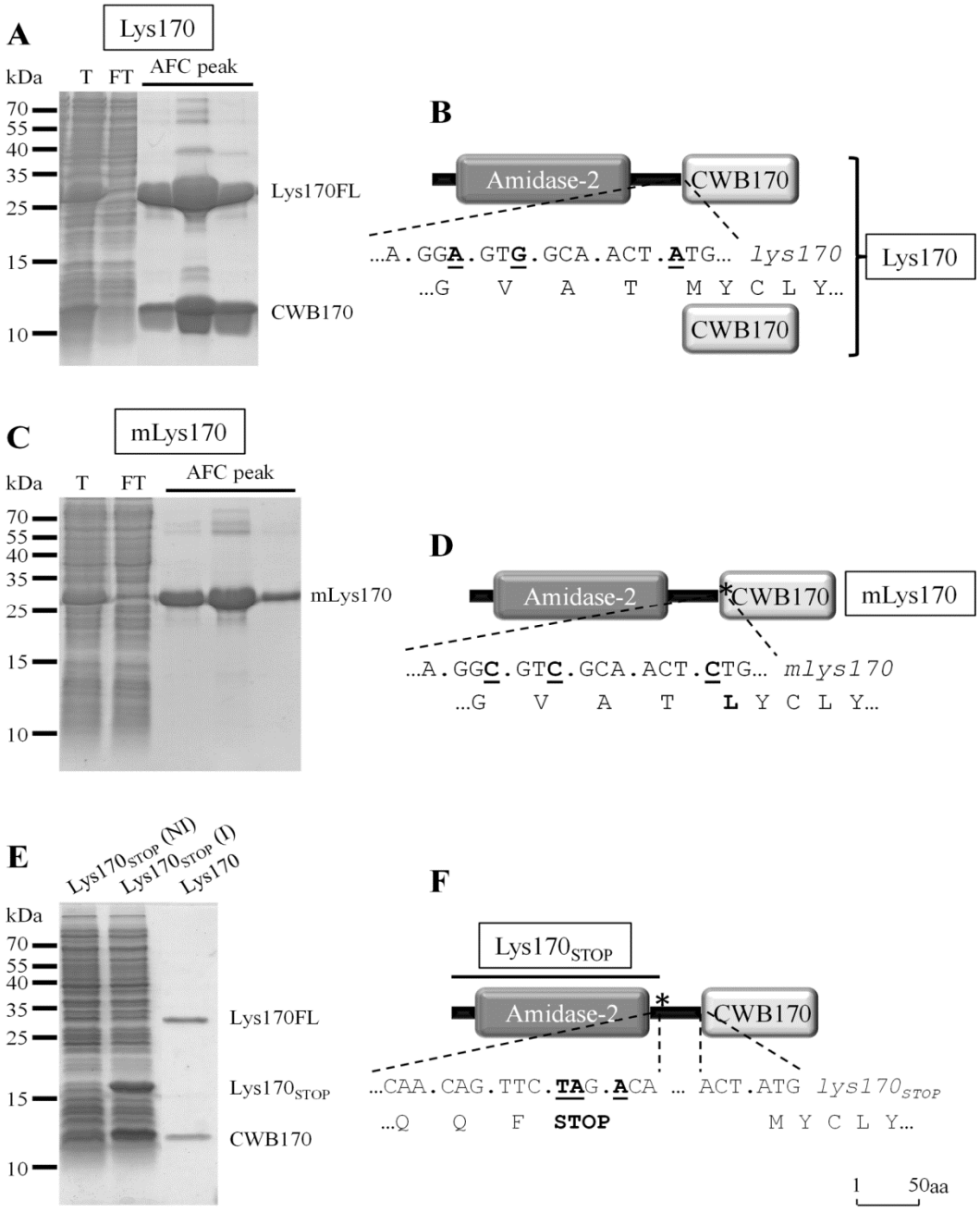

Fig. 1. The two polypeptides of endolysin Lys170. A) SDS-PAGE analysis of the Lys170 overproduction in *E. coli* and of the peak fractions resulting from the endolysin purification by metal chelate affinity chromatography (AFC). T, total soluble protein extract; FT, affinity column flowthrough. The protein bands corresponding to the full length endolysin (Lys170FL) and to its C-terminal product CWB170 are indicated. B) Schematic representation of the predicted Lys170 domain architecture. Details of the putative internal translation start site driving the independent production of CWB170 are shown below the endolysin

scheme. The nucleotide positions mutagenized to eliminate the putative ribosome binding site and start codon (Met_{202}) are underlined. C) SDS-PAGE analysis of the mLys170 overproduction in *E. coli* and of the peak fractions resulting from the protein purification by AFC. T and FT as in panel A. The protein band corresponding to mLys170 is indicated. D) Domain architecture of mLys170. Below the endolysin scheme it is shown the nucleotide and amino acid sequences resulting from the site-directed mutagenesis of the putative *lys170* internal translation start site. E) SDS-PAGE analysis of the Lys170-derived polypeptides produced after substitution of the *lys170* Met_{170} codon by a stop codon. The gel shows total protein extracts produced from non-induced (NI) and induced (I) *E. coli* cultures carrying $lys170_{STOP}$. The lane from the induced culture shows the truncated Lys170 ($Lys170_{STOP}$; 17.8 kDa) and CWB170 polypeptides. A control lane with purified Lys170 shows the positions of Lys170FL and CWB170 proteins. F) Generation of $Lys170_{STOP}$. Details of the nucleotide and amino acid sequences resulting from the substitution of the *lys170* Met_{170} codon by a stop codon (**TA**G) are shown below the endolysin scheme. Note that the mutagenesis also changed the Ala_{171} codon (GCA) to a Thr codon (**A**CA), which generated an *XbaI* site (TC.**TA**G.**A**) used for screening purposes. The starting Met_{202} codon and N-terminal amino acid sequence of CWB170 is also shown.

Inspection of *lys170* nucleotide sequence revealed the possibility of an internal and in-frame translational start site located at a position compatible with the production of a ~12 kDa protein (Fig. 1B). Elimination of the putative ribosome binding site (RBS) and methionine start codon (Met_{202}) through site-directed mutagenesis resulted in the production of a single protein (mLys170) with the same apparent mass of Lys170FL (Fig. 1C), in agreement with these sequences being a translation signal in *E. coli*. The polypeptide mLys170 has the same amino acid sequence of Lys170FL, except that the internal start methionine was substituted by a leucine residue (Fig. 1D). N-terminal sequencing of a CWB170 band obtained after SDS-PAGE separation of a sample from the Lys170 AFC peak (Fig 1A) revealed the sequence MY(?)LY, which basically matches the N-terminal sequence MYCLY(…) expected for a protein initiated at the putative secondary start site (cysteine residues could not be determined by the method used). To discard the possibility of CWB170 being generated by some sort of cleavage mechanism, which could have been inhibited by the $M_{202}L$ alteration we have substituted the Met_{170} codon of *lys170* by a stop codon (TAG). Insertion of this stop codon upstream of the putative starting Met_{202} resulted in the production and accumulation of the expected truncated product ($Lys170_{STOP}$) of 17.8 kDa (Fig. 1E,D). Yet, this premature stop in translation had no obvious impact on the synthesis of CWB170 (Fig. 1E), as it would be expected if the latter polypeptide resulted from processing of Lys170FL. We have thus concluded that CWB170 was produced from independent translation initiation at the

internal Met_{202} codon, given rise to a product of 11.6 kDa as deduced from the Lys170 primary sequence.

The Lys170 peak fraction from the AFC step (Fig. 1A) was subjected to size-exclusion chromatography (SEC) in an attempt to separate Lys170FL from CWB170. Strikingly, the two polypeptides essentially co-eluted in a single peak during SEC despite their markedly different predicted masses, 32.6 and 11.6 kDa, respectively (Fig. 2). Based on the elution volume of the peak we estimated a mass of about 62 kDa (see methods), assuming for the species composing the peak a homogeneous and globular nature analogous to that of the standard proteins run in the same conditions. Interestingly, when a C-terminally His_6-tagged CWB170 independently produced and purified by AFC was subjected to the same SEC, it resulted in a profile clearly distinguishable from that of Lys170, eluting with an apparent mass of ~37 kDa (Fig. 2A). mLys170 also peaked at a different elution volume during SEC, in this case with an estimated mass of ~50 kDa (Fig. 2A). Although the masses estimated from simple SEC analysis can be influenced by the proteins Stokes radii, the results suggested that: i) Lys170 corresponded to a complex of Lys170FL + CWB170 and ii) CWB170 and mLys170 oligomerized and/or formed elongated structures conferring them apparent masses higher than those expected for monomeric proteins (Erickson, 2009).

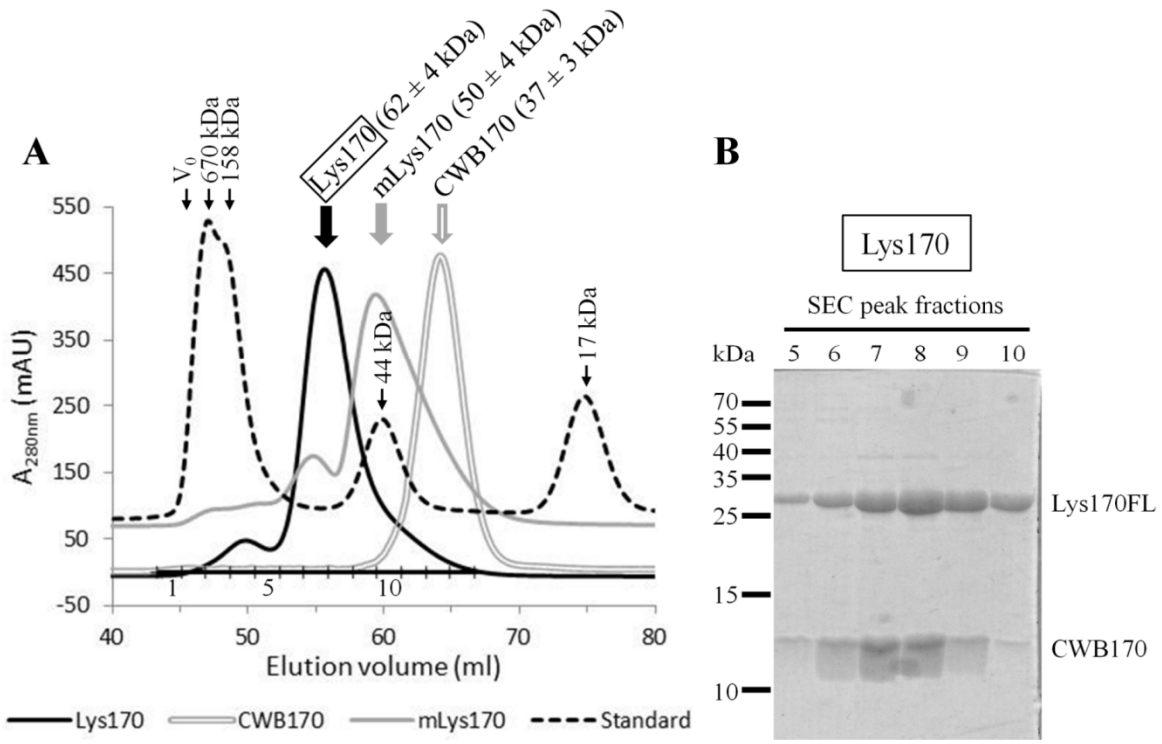

Fig. 2. Size-exclusion chromatography of the Lys170, mLys170 and CWB170 proteins from the affinity purification step. A) The eluting profile of the proteins was monitored by taking absorbance measurements at 280 nm ($A_{280\ nm}$). Representative UV curves were combined in a single graph. The apparent protein masses derived from the experimentally-determined partition coefficients (K_{av}, see methods) are indicated for each protein. The column void volume (V_0), the masses of standard proteins and the fractionation range of Lys170 are also indicated. B) SDS-PAGE analysis of the SEC peak fractions of Lys170, confirming the co-elution of Lys170FL and CWB170.

Lys170FL and CWB170 polypeptides are required for full endolysin lytic activity in vitro

We showed above that the CWB170 polypeptide seemed to associate with Lys170FL, a result that could hint for a role of the C-terminal fragment in endolysin activity. In fact, when assays of cell suspension turbidity reduction were performed to compare the lytic activity of Lys170 and mLys170 against *E. faecalis* cells, we observed that the latter protein could not elicit any detectable lysis, in clear contrast to the two-component endolysin (data not shown). We have thus reasoned that co-incubation of purified mLys170 and CWB170 might generate active endolysin complexes, resulting in a visible enhancement of lytic activity. To test this hypothesis we have empirically fixed an amount of mLys170 (10 µg, 0.31 nmol) and varied the quantity of the smaller protein to

obtain mLys170:CWB170 molar ratios of 1:0.25, 1:0.5, 1:1, 1:2, 1:4 and 1:6. The different mixtures were spotted on a dense lawn of *E. faecalis* cells (see methods) and enterolytic activity evaluated based on the presence and relative diameter of the lysis halos developed after overnight incubation. Lys170, mLys170 and CWB170 were also spotted alone and at the maximum concentration used in the different combinations. The results confirmed the much reduced lytic activity of mLys170 when compared to Lys170 and showed that CWB170 was unable to elicit any detectable lysis by itself (Fig. 3, bottom row). However, when mLys170 was pre-incubated with increasing amounts of CWB170 the lytic activity was progressively restored, with the ratio 1:6 producing a lytic effect apparently similar to that of Lys170 (Fig. 3). The results indicated that CWB170 is required for full endolysin activity and again supported an interaction between Lys170FL and CWB170. Somewhat unexpectedly, when we tried to evaluate the lytic effect of the mLys170/CWB170 mixtures in a more quantitative way, by determining lysis kinetics of dense cell suspensions, we could not measure any obvious lysis, even with 1:6 mLys170:CWB170 molar ratio mixtures. We believe though that this apparently contradictory result can be explained by a low efficiency of production of active complexes upon mLys170/CWB170 co-incubation, which are still sufficient to be detected by the highly sensitive spot test assays (Fig. 3), but not enough to elicit lysis of dense cell suspensions (see discussion).

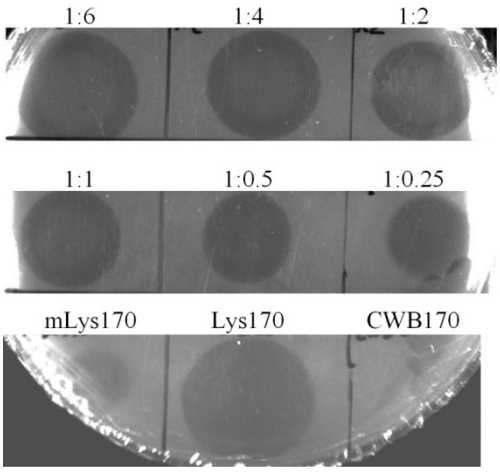

Fig. 3. Impact of CWB170 polypeptide in endolysin activity. Purified mLys170 and CWB170 were co-incubated at the indicated mLys170:CWB170 molar ratios for 1 h at room temperature. After this period, each protein mixture was spotted on a dense lawn of *E. faecalis* cells. The image shows the lysis halos developed after overnight incubation at 37 ºC. Lysis halos from individually spotted mLys170 (0.31 nmol), Lys170 (0.31 nmol) and CWB170 (1.86 nmol) are shown in the bottom row.

The two endolysin polypeptides are produced in the phage infection context

Since efficient lytic activity of endolysin Lys170 seemed to require the presence of Lys170FL and CWB170, we have anticipated that both polypeptides should be produced in *E. faecalis* cells during infection by phage F170/08. To test this, a mid-log culture of *E. faecalis* strain 926/05 was infected with the phage at an input multiplicity of 2 and samples collected every 10 min for production of total protein extracts. These were separated by SDS-PAGE, followed by Coomassie blue-staining (to confirm even loading, Fig. 4A) and Western blot analysis with anti-Lys170 antibodies (Fig. 4B). The results showed that both Lys170FL and CWB170 started to accumulate at t = 20 min, reaching their peak 60 min after phage infection. In contrast to what happened with *lys170* expression in *E. coli* (Fig. 1A), in the phage infection context CWB170 seemed to accumulate in great excess when compared to Lys170FL. The results confirmed that the two Lys170 polypeptides produced in *E. coli* are also synthesized during the phage F170/08 infection cycle, excluding the possibility of an artifact resulting from heterologous expression.

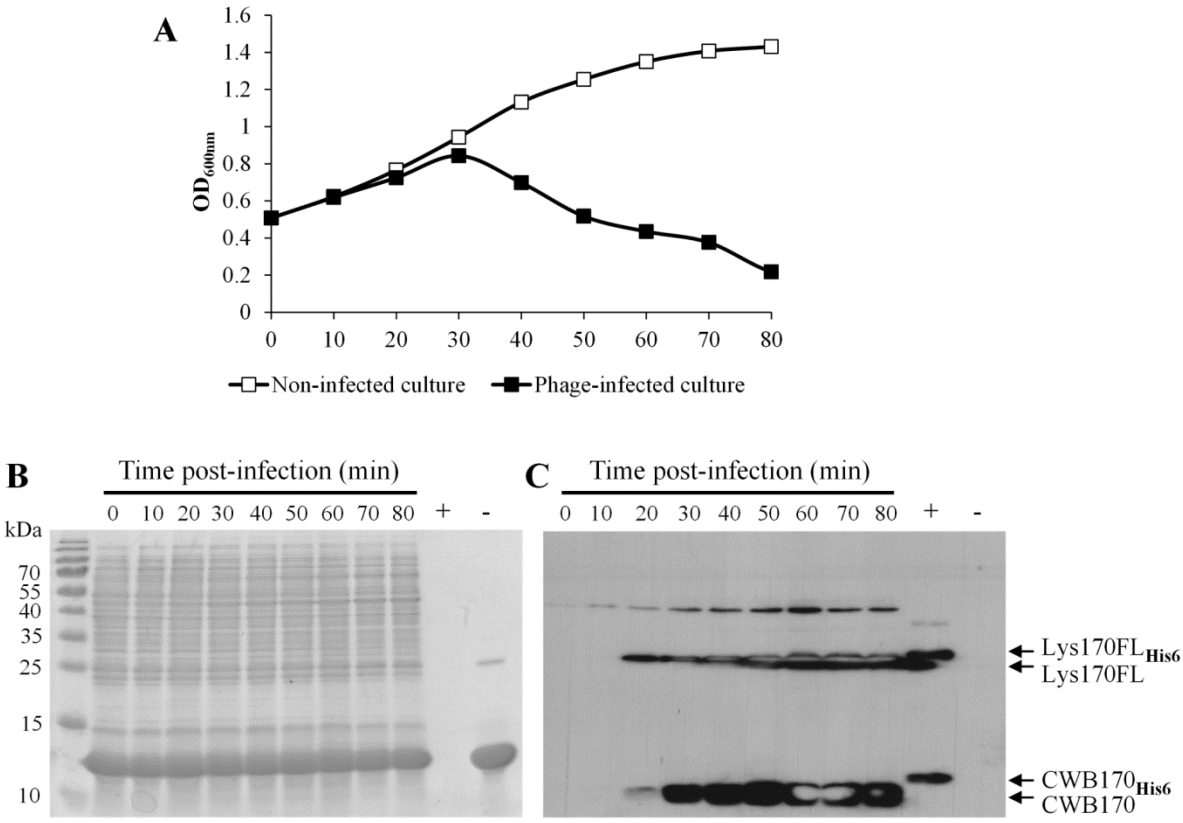

Fig. 4. Time course of Lys170FL and CWB170 synthesis during *E. faecalis* infection by phage F170/08. Total protein extracts were prepared from samples of an infected culture (A) collected every 10 min after infection, during 80 min. Ten micrograms of each extract were separated by SDS-PAGE and analyzed by Coomassie blue-staining (B) and Western blotting with anti-Lys170 antibodies (C). Purified Lys170 (50 ng) was used as positive control (+) and 10 µL of lysis buffer was used as negative control (-). The very intense band observed in B between positions 10 and 15 kDa of the molecular weight marker is from lysozyme, which is present in the lysis buffer at a 2.5 mg mL^{-1} concentration.

CWB170 promotes endolysin binding to target cells

Based on the typical modular structure of endolysins from Gram-positive systems and also on bioinformatics analysis, we hypothesized that the C-terminal polypeptide CWB170 would mediate the binding of endolysin Lys170 to the cell wall of target bacteria. To study the binding capacity of the different endolysin polypeptides we have incubated *E. faecalis* cells with purified Lys170, mLys170 and CWB170. After 30 min incubation, the mixtures were centrifuged and we have evaluated how the proteins distributed between the supernatant and cell pellet fractions. In the absence of target cells the three proteins were essentially present in the supernatant fraction (B_s lanes in Fig. 5), discarding major protein precipitation and consequent sedimentation (B_p lanes in Fig. 5)

during the assay. The larger component of Lys170 (Lys170FL) seemed to be more associated with the *E. faecalis* pellet fraction than the mutagenized form mLys170, which was mainly detected in the supernatant fraction (compare fractions S and P for the two polypeptides). The CWB170 portion of Lys170 was only faintly detected in the supernatant, being most of it found in the pellet fraction. The capacity of the CWB170 polypeptide to bind *E. faecalis* was confirmed when the protein was tested alone, as again a much higher fraction of the protein was present in the pellet fraction. The results clearly showed that CWB170 bound to *E. faecalis* cells and strongly suggested that this independent domain was responsible for promoting binding of Lys170FL. The weak binding of mLys170 to the bacterial cell wall, probably due to the absence of independently-produced CWB170, may be the cause underlying the decrease of mLys170 activity (see above).

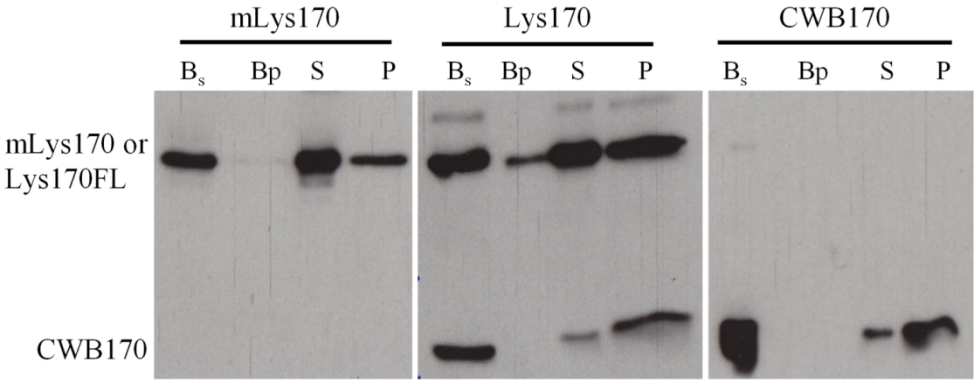

Fig. 5. Binding of purified mLys170, Lys170 and CWB170 to *E. faecalis* cells. Binding reactions were prepared by adding 1µg of each protein to 100 µL of a concentrated *E. faecalis* cell suspension, followed by 30 min incubation on ice. Each reaction was centrifuged and the relative distribution of the proteins in the supernatant and pellet fractions analyzed by Western blot using anti-Lys170 antibodies. Negative controls consisting in the addition of the proteins to endolysin buffer only (no target cells) were equally prepared and processed. B_s and B_p, supernatant and pellet fractions of negative controls, respectively; S and P, supernatant and pellet fractions of test assays, respectively.

Composition of the Lys170 complex

The elution profiles of Lys170 and CWB170 during SEC (Fig. 2) and the relative lytic activity exhibited by Lys170, mLys170 and mLys170+CWB170 (Fig. 3) strongly suggested that Lys170FL and the CWB170 polypeptide associated to form the fully active Lys170. The mass estimations of Lys170 (62 kDa) and CWB170 (37 kDa) derived from SEC (Fig. 2A) seemed to be incompatible with Lys170 being a simple 1:1 Lys170FL (32.6 kDa):CWB170 (11.6 kDa) heterodimer.

To gain insight on the nature of the putative Lys170FL:CWB170 complex we determined the molar masses of purified Lys170, mLys170 and CWB170 by Size-Exclusion Chromatography - Multi Angle Light Scattering (SEC-MALS), a technique that allows determination of molar mass independently of the protein Stokes radii. SEC-MALS analysis of CWB170 produced a single UV peak (Fig. 6), with the protein eluting in SEC as monodisperse species at 16.1 mL and with a mass of 46 kDa, which indicated that purified CWB170 was a tetramer in solution (theoretical mass of 4CWB170 = 46.4 kDa). Most mLys170 showed its UV peak at 15.1 mL elution volume, with a corresponding molar mass of 34.1 kDa. This mass fits well that expected for a mLys170 monomer (predicted mass = 32.6 kDa) but deviates significantly from that estimated by the simple SEC analysis of Fig. 2 (~50 kDa). Such discrepancies between the masses predicted by conventional SEC and those determined by more accurate methods have been observed previously, and they generally derive from the extended, non-globular nature of the proteins under analysis (São-José *et al*., 2006; Ruggiero *et al*., 2009). Two minor peaks were also detected for mLys170 at around 12.6 and 13.7 mL, corresponding to molar masses of 98.8 and 62.4 kDa, respectively, suggesting that a fraction of mLys170 could form homotrimers and homodimers (predicted masses of 97.8 and 65.2 kDa, respectively). Finally, the vast majority of Lys170 (Lys170FL+CWB170) produced an UV peak centered at 14.2 mL elution volume, with monodisperse species detected at 13.9 mL, to which corresponded a molar mass of 70.3 kDa. A minor peak eluted at 15.1 mL, with a measured mass (32 kDa) compatible with monomeric Lys170FL.

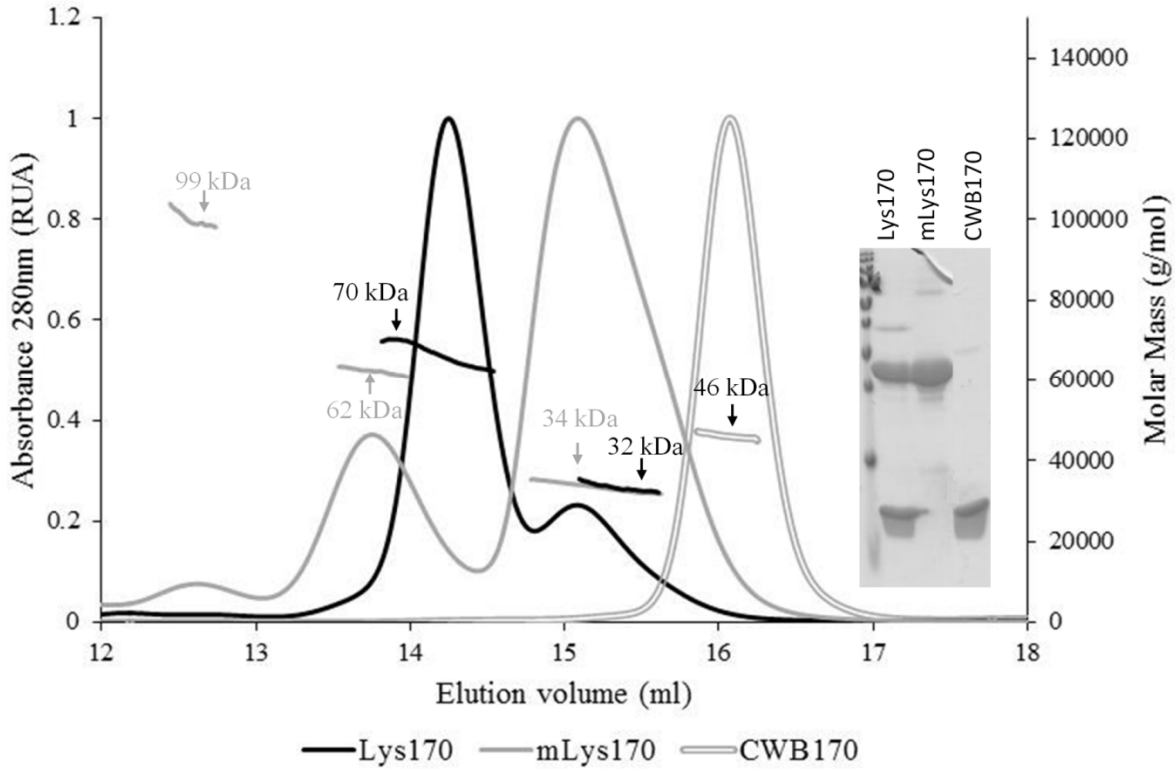

Fig. 6. SEC-MALS analysis of Lys170, mLys170 and CWB170. The relative UV and molar mass (M) curves of the three proteins were combined in a single graph; the refractive index (dRI) and light-scattering (LS) curves were omitted for clarity. The molar masses measured for the detected peaks are indicated. The inset shows a SDS-PAGE loaded with 10 µg samples of each protein preparation used in the SEC-MALS analysis.

Considering that the major Lys170 peak would necessarily contain Lys170FL associated to CWB170, the best fitting model for the measured molar mass of 70.3 kDa was a 1Lys170FL:3CWB170 complex (67.4 kDa). Interestingly, this 1:3 stoichiometry hypothesis was coherent with the tetrameric state found for isolated CWB170 (see above). Of course the molar mass obtained for the peak could also accommodate Lys170FL dimers (65.2 kDa), but in this case we would expect a hydrodynamic radius (and hence an elution volume) similar to that of mLys170 dimers (62 kDa peak of mLys170, Fig. 6). Thus, the presence of a significant fraction of Lys170FL dimers would be expected to produce a “shoulder” in the Lys170 UV curve, something which was not observed.

To try sorting out the most likely stoichiometry for the Lys170 complex we extracted from the SEC-MALS analysis the UV_{280nm} extinction coefficients (ε_p) of the peaks and

compared them to the predicted ε_p of the different multimer models, which was computed based on the amino acid sequence of each multimer subunit (see methods). Table 1 shows the results of this analysis. We observed that the experimental ε_p for the 70 kDa peak of Lys170 (1.953 mL mg^{-1} cm^{-1}) was very close to that predicted for a 1Lys170FL:3CWB170 complex (1.983 mL mg^{-1} cm^{-1}) and quite distinct from the ε_p expected for a Lys170FL dimer (2Lys170FL, ε_p = 1.379 mL mg^{-1} cm^{-1}). The experimental ε_p for all the other species detected in SEC-MALS (monomeric Lys170FL/mLys170, dimeric mLys170 and tetrameric CWB170) showed a very good match to the corresponding theoretical ε_p (Table 1), supporting the robustness of the analysis.

Table 1. Analysis of the UV_{280nm} extinction coefficient (ε_p) of Lys170 multimer models.

Protein	SEC-MALS M peaks (kDa) Fig. 6	Experimental ε_p, mL mg^{-1} cm^{-1}	Multimer model	Computed molar mass (kDa)	Computed ε_p, mL mg^{-1} cm^{-1}
Lys170	70	**1.953**	**1**Lys170FL:**3**CWB170	67.4	1.**983**
			2Lys170FL	65.2	1.379
	32	1.440	**1**Lys170FL	32.6	1.379
mLys170	62	1.417	**2**mLys170	65.2	1.379
	34	1.410	**1**mLys170	32.6	1.379
CWB170	46	2.457	**4**CWB170	46.4	2.552

In summary, from the presented data we have concluded that the most likely stoichiometry for the 70 kDa Lys170 complex was 1Lys170FL:3CWB170.

Cross-linking of endolysin multimers

To independently confirm the presence of multimers identified in the SEC-MALS analysis, we used the water-soluble, noncleavable cross-linking agent BS^3, which reacts with primary amines, to treat purified Lys170, CWB170 and mLys170 at micromolar range concentrations (see methods). Cross-linked and control samples were separated by SDS-PAGE, followed by Western blotting analysis of the resulting products with anti-Lys170 antibodies. Note that the cross-linking reaction conditions were optimized to allow simultaneous detection of the free monomers and of the different multimeric forms of the proteins under analysis in the ensemble of reactions. Several products were detected in the cross-linked sample of Lys170 that were compatible with different homo- and heterooligomeric states (Fig. 7A). In addition to a band expected for the complex 1Lys170FL:3CWB170 (67.4 kDa), other bands most probably corresponding to different association/dissociation states of the complex were observed: 2CWB170 (23.2 kDa), 3CWB170 (34.8 kDa), 1Lys170FL:1CWB170 (44.2 kDa) and 1Lys170FL:2CWB170 (55.8 kDa). Note that the ~55 kDa band in the cross-linking profile of Lys170 can only be explained by a 1Lys170FL:2CWB170 multimer, further supporting interaction between the two Lys170 subunits. We detected molecular species corresponding to dimers, trimers and tetramers (46.4 kDa) in the cross-linked sample of purified CWB170 (Fig. 7B), while in the corresponding sample of mLys170 we detected monomeric (32.6 kDa), dimeric (65.2 kDa) and a faint band of trimeric (97.8 kDa) species (Fig. 7C). In essence the results were coherent with those obtained in the SEC-MALS analysis, although in the particular assay shown in figure 7 the signal of the CWB170 tetramer was weaker than those obtained in other analogous experiments using higher concentrations of BS^3 (data not shown).

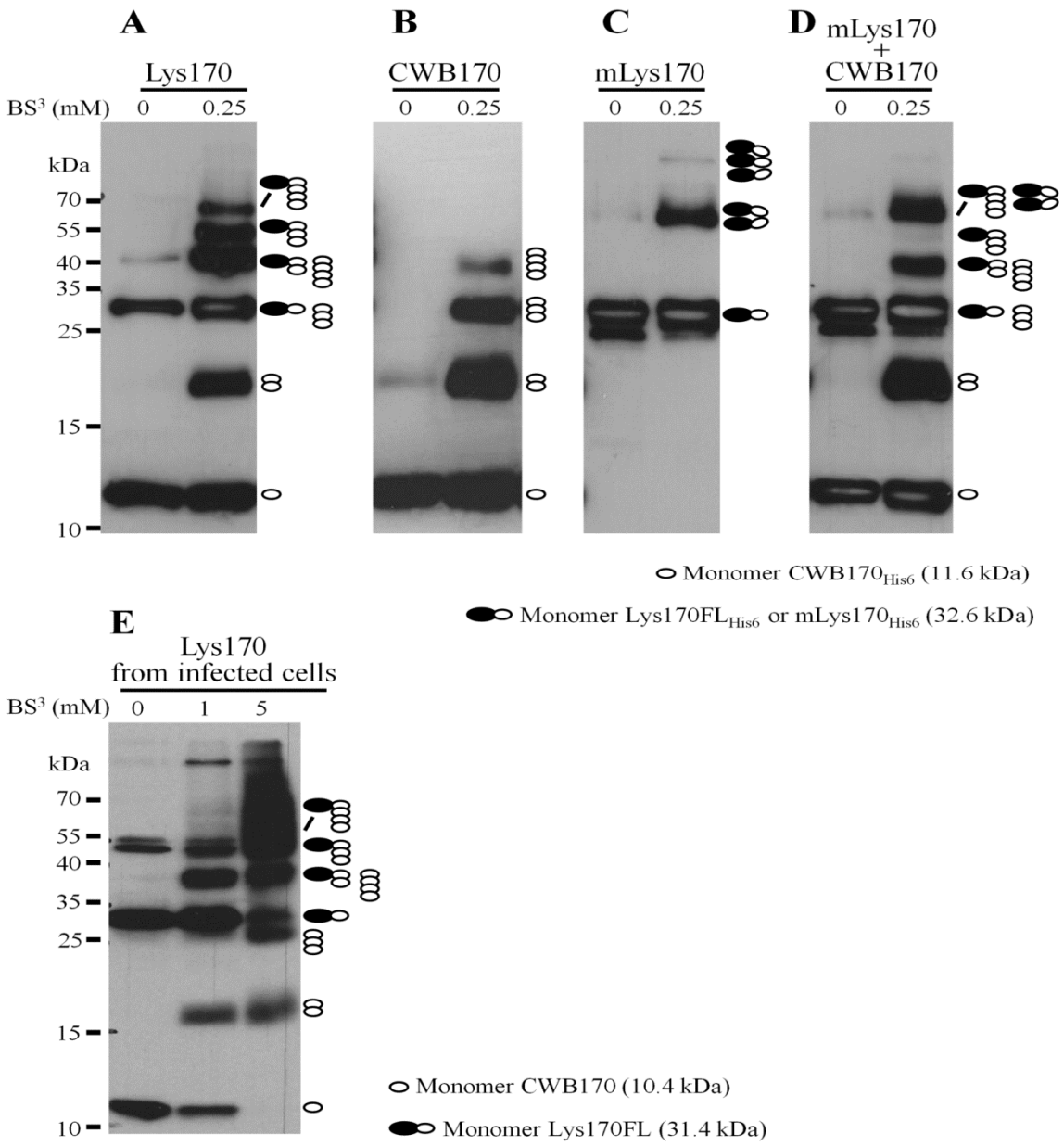

Fig. 7. Cross-linking analysis of endolysin multimers. Purified Lys170 (A), CWB170 (B), mLys170 (C), a pre-incubated mixture of CWB170 with mLys170 (D) and a protein sample from the time-point t = 60 min of the assay shown in figure 4 (E) were cross-linked with the indicated concentrations of BS^3 and the reaction products analyzed by Western blot with anti-Lys170 antibodies. When possible, the protein species deduced to compose each band is indicated following the depicted cartoon codes; bands compatible with more than one composition/oligomeric state are marked accordingly.

We have also prepared a reaction where pre-incubated mLys170 and CWB170 were cross-linked in the same conditions (Fig. 7D). The cross-linking of the mixture resulted in the appearance of new bands and in the apparent intensification of others when compared to the independent cross-linking of each protein (interpreted as formation of the 44.2 kDa

1Lys170FL:1CWB170, the 55.8 kDa 1Lys170FL:2CWB170, and eventually the 67.4 kDa 1Lys170FL:3CWB170 complexes). The cross-linking pattern resembled that of Lys170, although with altered band intensities. In an attempt to unambiguously identify protein bands resulting from cross-linking of mLys170 and CWB170 subunits we have generated and purified a version of the latter protein with the hemagglutinin (HA) epitope inserted just upstream of the His_6 tag. Unfortunately, this CWB170-HA protein revealed to be non-competent to enhance lysis mediated by mLys170 in experiments like that of figure 3. In addition, CWB170-HA seemed to be inhibited in its self-association capacity as judged by SEC-MALS analysis (data not shown). We have thus decided not use this protein in cross-linking assays since it seemed affected in its normal biological activity.

To check if the Lys170 cross-linking pattern could be reproduced with endolysin synthesized during phage infection, we used a sample of the t = 60 min protein extract of figure 4 and cross-linked it with 1 and 5 mM BS^3. The results obtained (Fig. 7E) were consistent with those derived from the cross-linking of purified Lys170 and again supported CWB170 self-association and interaction with Lys170FL. However, caution should be taken when analyzing cross-links of crude protein extracts because of the presence of a high number of other proteins; this may explain the smear observed in presence of 5 mM BS^3 (Fig. 7E).

DISCUSSION

We presented genetic and enzymatic activity studies indicating that maximum lytic activity of endolysin Lys170 requires the full length enzyme (Lys170FL) and a small polypeptide corresponding to the last 88 amino acid residues of Lys170FL. This C-terminal fragment is produced from an in-frame, secondary translation start site and basically matches the predicted cell wall binding domain of the endolysin (CWB170). In addition, biochemical and biophysical characterization of the endolysin and derived proteins strongly suggests that functional Lys170 corresponds to a Lys170FL:CWB170 heterooligomer, being 1:3 the most likely stoichiometry for the higher order endolysin complex. Lys170 is thus the first two-component multimeric endolysin described to date, whose subunits are produced from a single gene.

The other known multimeric endolysin, PlyC from the streptococcal phage C_1, is composed of a two CD-containing polypeptide (PlyCA) associated to eight PlyCB subunits with CWB activity, but in this case the A and B subunits are encoded by separate genes (Nelson *et al*., 2006). The ring-like PlyCB assembly, which contains eight potential binding sites for cell wall components, is crucial for PlyC function as the isolated PlyCA module has only residual activity (Nelson *et al*., 2006; McGowan *et al*., 2012). The poor lytic activity of the mutant endolysin mLys170, which is unable to produce independent CWB170 subunits, could be at least partially rescued upon incubation with purified tetramers of the latter protein (Fig. 3). This implies that, despite the apparent stability of the CWB170 tetrameric form, the equivalent module in mLys170 can still interact with and displace subunits of the tetramer, something that seems also to be supported by the cross-linking experiments presented in figure 7. Yet, the same cross-linking results also suggest that the efficiency of formation of mLys170:CWB170 complexes during co-incubation of the purified subunits is low. Such indication emerges when comparing the intensity of the bands of cross-linked heterooligomers present in Lys170 and in the mLys170/CWB170 mixtures, like for example the ~55 kDa cross-linking product corresponding to a 1Lys170FL(or mLys170):2CWB170 complex (compare panels A and D of Fig. 7). We could not detect also any obvious new peak (at most, we detected slight peak shifts) during analytical SEC of mLys170/CWB170 mixtures, again suggesting low efficiency of complex formation (data not shown). This should explain why mLys170/CWB170 mixtures could not induce detectable lysis of dense cell suspensions, despite being able to produce the lysis halos of figure 3. In conclusion, the production of Lys170FL:CWB170 heterooligomers, that is, Lys170, seems to be much facilitated when the two subunits are produced concomitantly within the same cell.

Our results indicate that the full length, monomeric endolysin needs to increase its number of CWB motifs for optimal lytic activity, a requirement that is fulfilled by the self-association capacity of the CWB170 subunit (a total of 4 CWB170 motifs present in the 1:3 complex). The assembly of CWB170 modules at the Lys170FL C-terminus seems to increase the endolysin affinity to cells (Fig. 5); it might also contribute to the proper folding/orientation of the enzyme, namely of its N-terminal CD, as suggested for the CWB repeats of the pneumococcal endolysin Cpl-1 (Hermoso *et al.*, 2003). The extended configuration of mLys170 inferred from its “abnormal” SEC profile (Fig. 6) might be an indication of this.

It has been shown for several PG hydrolases of Gram-positive systems that the number and type of CWB motifs can play a critical role on the catalytic regulation and/or affinity of the enzymes to their target cell walls (López and Garcia 2004; Steen *et al*., 2005; Mesnage *et al*., 2014; Wong *et al*., 2014). This has been particularly studied for the CWB segments of pneumococcal autolysins (bacterial PG hydrolases responsible for the phenomenon of autolysis) and their related phage endolysins, whose tandemLy-arranged repeats of CWB motifs seem to provide a mechanism to improve cell wall recognition (López and Garcia 2004; Bustamante *et al.*, 2010). For several of these pneumococcal PG hydrolases the CWB repeats recognize the choline residues of cell wall teichoic acids. The number of choline-binding repeats (ChBRs) of autolysins can vary from 7 to up to 18, but a minimum of 4 ChBRs appears to be required for efficient binding to the cell wall (Garcia *et al*., 1994; López and Garcia 2004; Moscoso *et al*., 2005). Interestingly, in presence of choline the C-terminal ChBRs mediate the dimerization of the LytA autolysin, being this dimerization crucial for catalytic activity (Usobiaga *et al*., 1996; Fernández-Tornero *et al.*, 2001).

The LytA-like pneumococcal phage amidases Ejl and Pal can exist in solution in a monomer↔dimer equilibrium (and additionally ↔tetramer in case of Ejl) depending on the choline concentration in the media, with high concentrations favoring the dimeric (and tetrameric Ejl) state (Sáiz *et al*., 2002; Varea *et al*., 2004). The particular ability of Ejl to tetramerize upon substrate binding was proposed as a mechanism to compensate the relatively low affinity of the enzyme for choline (Sáiz *et al*., 2002). Our results do not exclude the possibility of co-existence of the 1Lys170FL:3CWB170 complex (67.4 kDa) with intermediate molecular species like for example 1Lys170FL:2CWB170 (55.8 kDa) or even 1Lys170FL:1CWB170 (44.2 kDa). In fact, in the SEC-MALS analysis of figure 6 we could observe that elution of the ~70 kDa monodisperse species corresponding to the 1:3 complex was followed by a gradual decrease of the molar mass curve, suggesting the presence of species with lower molar mass. The occurrence of this intermediate species is also compatible with the cross-linking studies (Fig. 7).

We wondered if this particular endolysin CWB domain could be found associated to different CDs, other than the Amidase-2 family CD present in Lys170. We performed BLASTP homology searches using the CWB170 primary sequence and, remarkably, the results showed that CWB170-like modules can be found in the C-terminus of PG hydrolases harboring CDs with diverse enzymatic specificities, including lysozymes,

glucosaminidases and peptidases, in addition to different families of amidases (Fig. 8). This observation reinforces the independent functional character of this CWB module. Another interesting feature emerging at the DNA level was that all CWB170-like domains analyzed started with a methionine codon preceded by a putative RBS (Fig. 8), suggesting that translation initiation at this internal start sites should be responsible for the independent production of this domain.

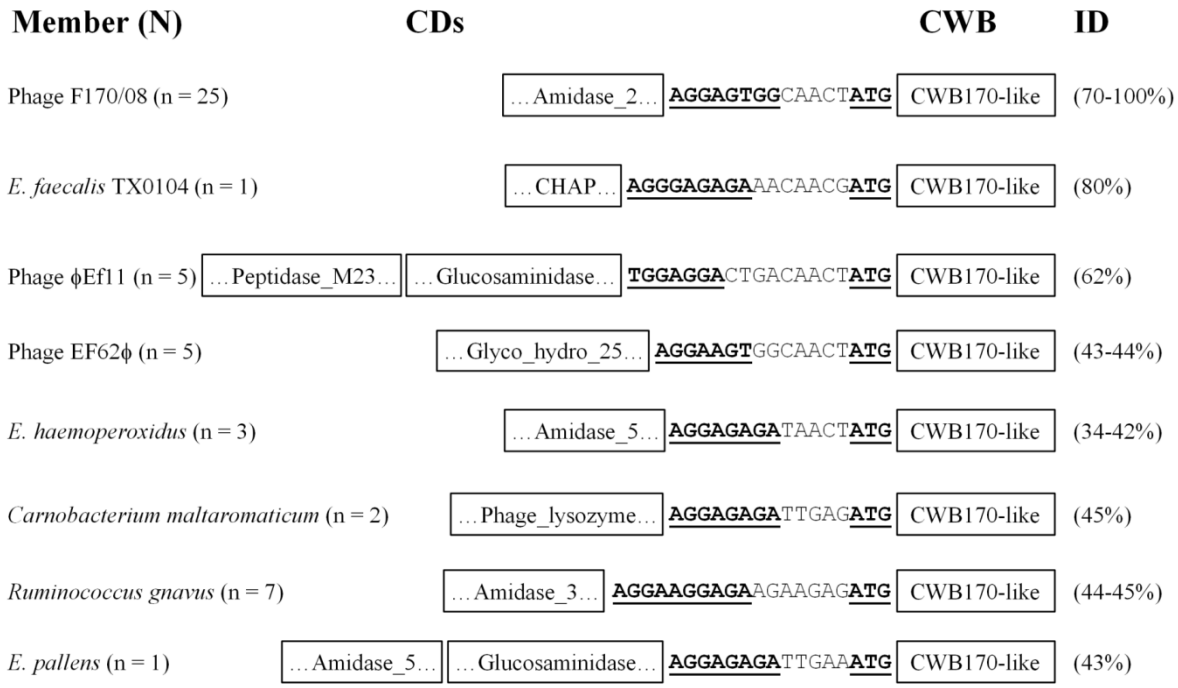

Fig. 8. PG hydrolases with CWB170-like domains. BLASTP searches with CWB170 sequence retrieved several PG hydrolases displaying CDs of different enzymatic specificities. The PG hydrolases are organized according to their domain architecture and only one member representative of each group is shown. The "N" value denotes the number of single, non-redundant protein sequences within each group, but each sequence may have been described in many different sequenced genomes (*e.g.*, the represented *E. faecalis* TX0104 sequence is identical to that of 25 different database entries). The GenBank Acc. N0. of the members shown are: *E. faecalis* TX0104, EEI10842; Phage ϕEf11, ACV83371; Phage EF62ϕ, ADX81356; *E. haemoperoxidus*, EOH93425; *Carnobacterium maltaromaticum*, CCO10928; *Ruminococcus gnavus*, EDN76763; *E. pallens*, EOH88591. CDs families are according to Pfam database: Amidase_2, pfam01510; CHAP, pfam05257; Peptidase_M23, pfam01551; Glucosaminidase, pfam0183; Glyco_hydro_25, pfam01183; Amidase_5, pfam05382; Phage_lysozyme, pfam00959; Amidase_3, pfam01520). The in-frame ATG codon defining the beginning of CWB170-like domains and the putative RBS upstream are depicted in bold (also present in many other members of each group, but we have not confirmed if in all of them). ID, sequence identity of the CWB170-like domains to the CWB170 of Lys170, within each group of PG

hydrolases (a range or single ID values are presented depending on weather CWB170-like sequences vary or not within a given group, respectively).

While we were preparing this article for publication, Dunne *et al*. (2014) reported on the *E. coli* expression of *Clostridia* endolysins CD27L and CTP1L and the concomitant production of endolysin C-terminal fragments that functioned as a trigger/release factor for these amidases. As for Lys170, these C-terminal fragments essentially correspond to the predicted CWB domains of the endolysins. Moreover, the N-terminal residue of the CD27L fragment was identified as a methionine, whose corresponding codon is preceded by putative RBS (**TGAGGGAGT**TAAACAG.*ATG*). The CTP1L C-terminal fragment was deduced to start with a valine residue (GTG codon), also preceded by a putative RBS (**AGGGGGA**AGATGAA.*GTG*). Substitution of the initiating Met and Val residues by a proline ceased the production of the C-terminal CWB domains. Strangely, the authors never refer to the hypothesis of CD27L and CTP1L C-terminal fragments being produced from translation initiation at the putative internal start sites. Instead, they propose that the Met and Val residues are critical for an autocleavage event that is responsible for the generation of the C-terminal fragments. The authors also argue that the cleavage mechanism is triggered by one of two possible dimerization modes of the C- terminal fragments and that release of the CWB domain is necessary to activate CTP1L, whereas in the case of CD27L it might simply facilitate endolysin cell wall penetration.

In the case of endolysin Lys170, we have excluded cleavage as the mechanism generating the independent CWB170 module and proved the functionality of the internal translation site. In addition, in none of the experiments presented in this work we could obtain evidences for the presence of a 21 kDa N-terminal fragment of Lys170, which would result from a cleavage event at Met_{202}. Such N-terminal endolysin fragment is never shown also in the work of the *Clostridia* endolysins. In contrast to the CD27L endolysin that apparently exhibited fast and continuous cleavage (Dunne *et al*., 2014), independent incubation of Lys170, mLys170 and CWB170 for 3 days at 4 ºC or at room temperature did not reveal any obvious alteration in the polypeptides composition, apart from the slight formation of high molecular weight SDS-resistant aggregates at room temperature (data not shown). The fact that in a great number of PG hydrolases the putative CWB170-like domain is initiated by an in-frame methionine preceded by a properly spaced RBS (Fig. 8) is also suggestive of the independent production of the C-terminal module by

translation initiation at these internal sites. Thus, in our opinion the autocleavage mechanism proposed to explain the CD27L and CTP1L C-terminal fragments deserves confirmation, namely by studying the effect of inserting a stop codon upstream of the Met_{186} and Val_{195} putative internal starts, respectively.

It is relatively common in phage genomes the existence of fully or partially overlapped genes, some of which encoding lysis proteins that are known to interact. The best studied examples are provided by the *E. coli* phage λ lytic functions. The λ holin *S* gene has a dual translation start that enables the synthesis of the holin and anti-holin functions (Bläsi *et al*., 1989). The last step of λ virus particles release from infected *E. coli* involves the disruption of the host cell outer membrane. This is accomplished by a spanin complex whose components are encoded by genes *Rz* and *Rz1*, with the latter being fully embedded in the +1 reading frame of *Rz* (Berry *et al*., 2012). Endolysin genes encoding the expected full length and truncated products through alternative translation initiation have been described previously for the staphylococcal and mycobacterial phages 2638A and Ms6, respectively. It was speculated for the 2638A endolysin that interaction between the full length and the truncated product could explain the higher activity observed when the two polypeptides were present (Abaev *et al*., 2013). The two products of the Ms6 endolysin gene were shown to be necessary for the normal timing, progression and completion of host cell lysis during phage infection, but the possible interaction between the two proteins remains elusive (Catalão *et al*., 2011).

In conclusion, we believe that the enterococcal endolysin Lys170 represents the first described example of a class of PG hydrolases, whose optimal lytic activity depends on the assembly of independent CWB subunits at the corresponding module of the full length monomer. We speculate that this may constitute a new strategy of increasing the number of CWB motifs in these enzymes, as an alternative to the CWB tandem repetition commonly found in monomeric PG hydrolases. Considering the data of figure 8 and the discussion above about the *Clostridia* endolysins, we think that this class of lytic enzymes might be more widespread than previously anticipated.

MATERIALS AND METHODS

Bacteria, plasmids, phage and growth conditions

E. coli strains XL1-Blue and XL1-Blue MRF' (Stratagene), used for plasmid isolation and propagation, were grown at 37 ºC in Luria Bertani (LB) medium (Sambrook and Russell, 2001). The *E. coli* expression strain CG61 (São-José *et al.*, 2000) and its derivatives were grown in LB at 28 °C before induction of protein production and at 37 °C afterward. Protein production was induced by heat shock at 42 °C in a water-bath with shaking for 30 min. When necessary LB was supplemented with ampicillin (100 µg mL^{-1}), kanamycin (30 µg mL^{-1}) and/or tetracycline 10 µg mL^{-1}. The *E. faecalis* strains 926/05 and 1518/05 (Proença *et al*., 2012) were grown in Trypton Soy Broth (TSB). All culture media components were purchased from Biokar Diagnostics. The expression vector pIVEX2.3d (Roche Applied Science), used for protein overproduction in *E. coli*, allows the expression of cloned genes under the control of the phage T7 ϕ10 promoter and the production of the corresponding proteins C-terminally fused to a hexahistidine tag. Phage F170/08 was propagated in *E. faecalis* 926/05 as described previously (Proença *et al*., 2012).

General DNA techniques

Phage F170/08 DNA was extracted from CsCl-purified lysates as described by Vinga *et al.* (2012). DNA polymerase KOD hot start master mix (Novagen) was used for high fidelity Polymerase Chain Reaction (PCR), whereas screenings by PCR were performed with DNA polymerase NzyTaq green 2x master mix (NZYTech). Extraction of *E. coli* plasmid DNA and purification of PCR products was performed with the commercial kits QIAprep Spin Miniprep (QIAGEN) and High Pure PCR Product Purification (Roche Applied Science), respectively, following the manufacturers' instructions. Restriction endonucleases and T4 DNA ligase were from Fermentas Molecular Biology Tools (Thermo Scientific). DNA restriction, ligation and conventional agarose gel electrophoresis were carried out essentially as described by Sambrook and Russell (2001). Development of competence and transformation of *E. coli* strains was according to the

method of Chung *et al*. (1989). All recombinant plasmids were confirmed by DNA sequencing (Macrogen, Seoul, Korea).

General protein techniques

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by LaemmLi (1970). Western blotting analysis was carried out basically as described by Renart *et al*. (1979). Endolysin immunodetection was performed using a rabbit anti-Lys170 polyclonal serum (see section *Rabbit immunization with pure Lys170*) as primary antibody, and horseradish peroxidase (HPR)-conjugated goat anti-rabbit Fc polyclonal antibody (Pierce, Thermo Scientific) as secondary antibody. Antigen/antibody complexes were detected by chemiluminescence using the RapidStep ECL Reagent (Calbiochem). Protein quantification was carried out with the Bradford reagent (Bio-Rad Laboratories) using bovine serum albumin (BSA, Bio-Rad Laboratories) as standard. PageRuler Prestained Protein Ladder (Thermo Scientific) was used as protein marker in SDS-PAGE.

Construction of lys170 derivatives

Plasmid pDP2, a pIVEX2.3d derivative carrying gene *lys170* was described previously (Proença *et al.*, 2012). Gene *lys170* in pDP2 was subjected to site-directed mutagenesis by using the Quick Change II Site directed mutagenesis kit (Stratagene Agilent Technologies), resulting in plasmid pDP3 carrying *mLys170* gene. The introduced nucleotide substitutions eliminated a putative secondary translation start site internal to *lys170* (see text for details). Mutagenic primers were also used to substitute the Met_{170} and Ala_{171} codons of *lys170* by stop and Thr codons, respectively, with the concomitant creation of an *XbaI* restriction site (see text for details). The pIVEX2.3d derivative harboring this mutated gene ($lys170_{STOP}$) was designated pDP5 The DNA segment encoding the endolysin C-terminal region CWB170 was PCR amplified with a primer pair that added *Nde*I and *Xma*I restriction sites to the 5' and 3' ends of the coding sequence, respectively. The PCR product was cloned into pIVEX2.3d cut with the referred enzymes, yielding plasmid pDP4.

Production and purification of endolysin polypeptides

Protein overproduction in *E. coli CG61* and subsequent purification by metal chelate affinity chromatography was as previously described (Proença *et al.*, 2012). Peak fractions from the affinity chromatography step were further purified by size-exclusion chromatography using a HiLoad 16/600 superdex 75 prep grade column (GE Healthcare Life Sciences), which was equilibrated and run with imidazole-free endolysin buffer (20 mM HEPES-Na, 500 mM NaCl, 1% glycerol and 1 mM DTT, pH 8.0). Fractions containing the purified proteins were pooled, concentrated when necessary and stored at -80 °C as small aliquots. Experimentally-determined partition coefficients (K_{av}) of proteins were used to estimate Stokes radii and the corresponding relative molecular masses by extrapolation from a plot of Stokes radii of standard proteins versus $(-\log K_{av})^{1/2}$ (Cabré *et al*., 1989). The column void volume (V_0) was determined with blue dextran 2000 (GE Healthcare Life Sciences). The standard proteins (Bio-Rad Laboratories) were thyroglobulin (molecular mass = 670 kDa; Stokes radius = 8.6 nm), γ-globulin (158 kDa; 4.8 nm), ovalbumin (44 kDa; 2.73 nm), myoglobin (17 kDa; 2.08 nm) and vitamin B12 (1.35 kDa; 0.85 nm) (Cabré *et al*., 1989; Talmard *et al*., 2007).

Protein N-terminal sequencing

N-terminal sequencing by the Edman reaction was performed by the Analytical Services Unit, ITQB (Oeiras, Portugal) in a Procise 491 HT Protein Sequencer (Applied Biosystems).

Rabbit immunization with purified Lys170

The service of raising a rabbit polyclonal anti-serum against endolysin Lys170 was purchased to ACIVET, FMV-UTL (Lisbon, Portugal). One New Zealand white rabbit was treated with a total of five subcutaneous injections, where the first one contained 220 μg of purified Lys170 in 1 mL emulsion of Freund's Adjuvant Complete (Sigma-Aldrich) and the remaining four contained each 110 μg of endolysin in 1 mL emulsion of Freund's Adjuvant Incomplete (Sigma-Aldrich). The injections were administered at 2-3 week intervals. The anti-Lys170 reactivity/specificity of the different sera collected from the animal throughout the protocol was analyzed by Enzyme-Linked Immunosorbent Assay

(ELISA) using HRP-conjugated goat anti-rabbit Fc polyclonal antibody as secondary antibody. Five days after the final boost, the total serum was recovered, aliquoted and stored at -80 °C.

Lytic activity of Lys170 and its derivatives

The lytic activity of Lys170, mLys170 and CWB170, alone or in combination was evaluated by spotting the indicated protein quantities on a dense lawn of viable target cells, which was prepared as follows. The enterococcal strain 1518/05 was grown overnight at 30 °C without aeration, reaching an OD_{600} of approximately 0.8-1.0. Cells were harvested by centrifugation and concentrated 100-fold in fresh TSB. A sample of 300 µL of this bacterial suspension was incorporated in endolysin buffer supplemented with 0.7 % agar and poured in a Petri dish. Lysis halos developed during overnight incubation at 37 °C. Negative controls were equally prepared by spotting endolysin buffer.

Time course of endolysin production during phage infection

To study the synthesis of Lys170 polypeptides in *E. faecalis* 926/05 during infection by phage F170/08, an exponentially growing culture of the strain was infected with the phage at an input multiplicity of ~2 and incubated at 37 °C for 80 min. One-milliliter samples were collected every 10 min, cells were pelleted by centrifugation and stored at -80 °C. After thawing, cells were resuspended in 40 µL TE buffer (Sambrook and Russell, 2001) supplemented with 2.5 mg mL^{-1} lysozyme, 10 µg mL^{-1} DNase I and 1x Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Applied Science), and incubated for 80 min at 37 °C for cell lysis. Ten micrograms of total protein from each time point were separated by SDS-PAGE, followed by Western blotting analysis with anti-Lys170 polyclonal serum prepared at 1:10,000 dilution in 1 % skim milk in PBS-T (PBS 1x supplemented with 0.02 % Tween-20) and HRP-conjugated goat anti-rabbit Fc polyclonal antibody diluted 1:5,000 in 3 % skim milk in PBS-T.

Binding of endolysin polypeptides to E. faecalis cells

Protein samples used in the following experiments were centrifuged (16,000 *g*, 20 min, 4 ºC) just before their use to ensure elimination of eventual protein aggregates/precipitates. *E. faecalis* strain 1518/05 was grown until OD_{600} 0.5 at 37 °C with aeration, pelleted by centrifugation and concentrated 10-fold in endolysin buffer. Samples of 100 µL of this cell concentrate were incubated with 1 µg of endolysin polypeptides (Lys170, mLys170 or CWBD170) for 30 min on ice to minimize cell lysis. The reactions were prepared in microcentrifuge tubes pre-coated with 3 % BSA (Sigma-Aldrich) to avoid unspecific protein binding to plastic. Controls were equally prepared with endolysin buffer added instead of target cells. The mixtures were centrifuged (16,000 *g*, 10 min, 4 ºC) and 10 µL of both supernatant and resuspended pellet fractions (same initial volume) were analyzed by Western blot with anti-Lys170 antibodies as described above.

Size-Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS)

Purified Lys170, mLys170 and CWB170 were analyzed using an HPLC-MALS system (Shimadzu), a light scattering detector (mini DAWN TREOS system, Wyatt Technology) and refractive index detector (Optilab T-rEX, Wyatt Technology). A 120 µg sample of each protein was injected in a Superdex 200 10/300 GL Increase column (GE Healthcare Life Sciences) equilibrated in endolysin buffer and run at a flow rate of 0.5 mL min^{-1}. Molar masses of proteins were calculated using ASTRA 6.1 software (Wyatt Technology) using a refractive index increment (dn/dc) value of 0.183 mL g^{-1}.

Protein stoichiometry analysis was performed using the multisignal detection system of SEC-MALS (Nelson *et al*., 2006). The "UV extinction from RI peak" method of ASTRA software allows the determination of UV extinction coefficient (ε_p) in units of mL mg^{-1} cm^{-1}. By using the dn/dc value and the UV and RI signals of the protein peaks, experimental ε_p values were calculated and compared with those predicted from the protein amino acid sequences. Theoretical ε_p were computed with ProtParam tool (http://web.expasy.org/protparam/; Gasteiger *et al*., 2005), which calculates protein extinction coefficients using the Edelhoch method (Edelhoch, 1967), but with the extinction coefficients for Trp and Tyr residues determined according to Pace *et al*. (1995). We have considered the output values assuming reduced Cys residues.

Protein Cross-linking experiments

Working solutions of the cross-linking agent bis(sulfosuccinimidyl) suberate (BS^3, Thermo Scientific) were prepared immediately before use to decrease the extent of hydrolysis. BS^3 was first dissolved in ultra-pure water to a final concentration of 20 mM and then diluted to 5 mM in endolysin buffer. Purified Lys170, mLys170 and CWB170 were set to a final concentration of 50 ng μL^{-1} (monomer molar concentrations of 1.5 and 4.3 µM for mLys170 and CWB170, respectively). Protein samples were treated with 250 µM of BS^3 for 30 min at room temperature (RT), after which the reactions were stopped with 50 mM Tris-HCl at pH 7.5 for 15 min at RT. In the control samples the cross-linking agent was substituted by endolysin buffer. Two hundred nanograms of each reaction were separated by SDS-PAGE followed by Western blot analysis as described above, except that anti-Lys170 antibodies were diluted 1:40,000. To cross-link Lys170FL and CWBD170 produced during phage F170/08 infection, 5 µg of total protein from time point t = 60 min (see section *Time course of endolysin production during phage infection*) were treated with 1 or 5 mM BS^3. Cross-linking conditions were as above and subsequent analysis by Western blot was with a 1:10,000 dilution of anti-Lys170 antibodies.

Bioinformatics analysis

Protein homology searches were carried out with BLASTP (Altschul *et al.*, 1997) using the NCBI's nonredundant protein sequence database. Protein conserved domains were predicted with NCBI's tool CDD (Marchler-Bauer *et al.*, 2011) and Pfam (http://pfam.xfam.org/). Multiple protein sequence alignments were performed with ClustalW2 (Larkin *et al.*, 2007).

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CHAPTER 4

EC300: A PHAGE-BASED, BACTERIOLYSIN-LIKE PROTEIN WITH ENHANCED ANTIBACTERIAL ACTIVITY AGAINST *ENTEROCOCCUS FAECALIS*

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The author of this dissertation performed all the experiments presented in this chapter with the help of Clara Leandro in a few assays. Experimental design, data analysis and manuscript preparation were done by the author of this thesis and by Clara Leandro, Miguel Garcia, Madalena Pimentel and Carlos São-José. Carlos São-José was the main supervisor of the work.

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EC300: A PHAGE-BASED, BACTERIOLYSIN-LIKE PROTEIN WITH ENHANCED ANTIBACTERIAL ACTIVITY AGAINST *ENTEROCOCCUS FAECALIS*

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ABSTRACT

Bacteriophage lytic enzymes, either endolysins or virion-associated lysins, have been receiving considerable attention as potential antibacterial agents, particularly for the combat of antibiotic-resistant Gram-positive pathogens. A common obstacle in the exploration of these enzymes is their low solubility during large scale production. In addition, a conclusion that easily emerges from the careful analysis of a great number of reports on the field is that the activity of phage lytic enzymes is rarely studied in conditions that support robust growth of the target bacteria. Here we report the construction and study of a chimerical lysin, EC300, which was designed to target and kill *Enterococcus faecalis* in conditions supporting vigorous bacterial growth. EC300 resulted from the fusion of a predicted M23 endopeptidase domain of a virion-associated lysin to the putative cell wall binding domain of a previously characterized amidase endolysin, both produced by the *E. faecalis* phage F170/08. In addition to display high solubility, this bacteriolysin-like protein exhibited a clear enhanced lytic activity over the parental endolysin, when both were assayed in a rich bacterial growth medium. We demonstrate the killing efficacy of EC300 against growing cells of a panel of typed *E. faecalis* clinical strains with high level of antibiotic-resistance. The possible reasons for the marked difference between the lytic performance of EC300 and that of the amidase are discussed.

INTRODUCTION

Enterococci are commensal bacteria in the intestines of humans and several animals, and can also be found in soil, water and plants (Klein, 2003). In the recent years, the species *Enterococcus faecalis* and *Enterococcus faecium* have become increasingly important opportunistic pathogens worldwide, especially because of life-threatening nosocomial infections (Gilmore *et al.,* 2013). They have been associated to several human infections, such as neonatal sepsis, peritonitis, device-related infections and infective endocarditis, being described as the second most common cause of wound and urinary tract infections and the third most common cause of bacteraemia (Schaberg *et al.,* 1991; Emori *et al.,* 1993; Poh *et al.,* 2006; Fisher *et al.,* 2009; Sava *et al.,* 2010). Enterococci exhibit intrinsic resistance to several first-line antimicrobial agents; they show low-level resistance to β-lactams and aminoglycosides, and resistance to cephalosporins (Hammerum, 2012). In addition, enterococci show high propensity to acquire resistance to other antimicrobial agents, including quinolones, macrolides, tetracyclines, streptogramins and glycopeptides (Murray, 1990; Arias *et al.,* 2008; French *et al.,* 2010).

The reduced susceptibility to antibiotics can make extremely difficult the treatment of an infection caused by enterococci and the therapeutic options are limited (Werner *et al.,* 2013). Equally worrying is the fact that new antibiotics are not being developed at a rate sufficient to replace those drugs that are becoming ineffective (Theuretzbacher, 2012). Therefore, there is a growing need to find therapeutic alternatives to fight infections caused by these multidrug resistant enterococci.

Bacteriophages, or simply phages, are viruses that specifically infect bacteria. During their life cycle, most double-stranded DNA phages seem to employ two types of enzymes that degrade de peptidoglycan (PG) moiety of the bacterial cell wall: i) virion-associated lysins (VALs), which are typically carried in the virus particle and are thought to promote a local cleavage of PG bonds to facilitate phage DNA transference into the host bacterial cell, and ii) endolysins, which act at the end of the phage reproductive cycle to destroy the cell wall PG mesh leading to cell burst and to the consequent release of the virion progeny. The potential of endolysins, and more recently of VALs as antibacterial agents towards Gram-positive bacterial pathogens has been intensively studied (for recent reviews see Fenton *et al.,* 2010; Nelson *et al.,* 2012; Schmelcher *et al.,* 2012a; Rodríguez-

Rubio *et al.;* 2013). Phage endolysins from Gram-positive systems typically display a modular architecture where one or more catalytic domains (CDs) responsible for PG cleavage are connected by a flexible linker to a cell wall binding (CWB) domain (Schmelcher *et al.,* 2012a). VALs from phages infecting Gram-positive bacteria are frequently multidomain proteins and are usually larger than cognate endolysins; similarly to these they can display multiple CDs (Rodríguez-Rubio *et al.,* 2013).

In spite of the increasing number of reports in the last 10-15 years supporting the antibacterial activity of phage PG hydrolases, the fact is that in the vast majority of the studies the lytic enzymes are tested in conditions that do not support robust bacterial growth; most commonly, *in vitro* experiments are performed with target cells washed and suspended in buffered solutions. In fact, the high lytic activity observed in these conditions frequently does not translate to the expected results when assays are transposed to animal infection models. In most cases, satisfactory levels of animal survival are observed only when lytic enzymes are administrated to animals soon after the injection of the deadly bacterial inoculum, which is also prepared in a buffer (Loeffler *et al.,* 2003; Gu *et al.,* 2011; Oechslin *et al.,* 2013). The observation that metabolically active, growing bacteria are able to mount at least a certain level of resistance to endolysin attack from the outside is somewhat expected, since in the context of phage infection endolysins always act after cells had been killed by another phage-encoded protein, the holin (Catalão *et al.,* 2013). Regarding this aspect, VALs might be viewed as having the advantage of being naturally "designed" to act on actively growing bacteria. Another group of proteins sharing this feature are bacteriolysins (formerly class III bacteriocins, Cotter *et al.,* 2005) like the M23-like endopeptidases lysostaphin and enterolysin A, which are known to display potent lytic activity in growth promoting conditions (Kumar, 2008; Khan *et al.,* 2013).

The goal of this work was to design an enzyme with effective anti-*E. faecalis* activity in growth supporting conditions. For that, we have assumed the theoretical advantages of VALs and bacteriolysins referred to above and have generated an artificial, bacteriolysin-like enzyme (EC300) by fusing the M23 endopeptidase CD of the VAL Orf73 to the CWB domain of the previously characterized endolysin Lys170 (Proença *et al.,* 2012; Proença *et al.,* 2014), both produced by the *E. faecalis* phage F170/08. The results show the superior lytic activity of EC300 when compared to the endolysin Lys170.

MATERIALS AND METHODS

Bacteria, phage and growth conditions

Escherichia coli strain XL1-Blue MRF' (Stratagene), used for plasmid isolation and propagation, was grown at 37 °C in LB medium (Sambrook and Russell, 2001). *E. coli* expression strain CG61 (São-José *et al.*, 2000) and its derivatives were grown in LB at 28 °C before thermal induction of protein production (heat-shock at 42 °C for 30 min in a wet bath), and at 37 °C afterward. When necessary, LB was supplemented with ampicillin (100 µg/mL), kanamycin (30 µg/mL) and/or tetracycline (10 µg/mL). The antibacterial activity of EC300 was tested against a panel of typed, multiresistant enterococcal clinical strains (Table 1), which was composed of 28 *E. faecalis* and 21 *E. faecium* isolates from patients of a Portuguese hospital between 2004 and 2006 (Mato *et al.*, 2009), plus the two model *E. faecalis* clinical strains MMH594 and V583 (Sahm *et al.*, 1989; Huycke *et al.*, 1991; Shankar *et al.*, 2002; Paulsen *et al.*, 2003). These strains and the *E. faecalis* clinical isolates 1518/05 and 926/05 from TechnoPhage collection were grown in Trypton Soy Broth (TSB). When required, media were supplemented with 1.4 or 0.7 % agar to obtain solid or soft-agar plates, respectively. All culture media components were purchased from Biokar Diagnostics. *E. faecalis* phage F170/08 was propagated in *E. faecalis* strain 926/05 as described previously (Proença *et al.*, 2012).

Table 1. Typed enterococcal clinical strains used in this work.

Strain ID	PFGE pattern	Vancomycin resistance	Other relevant resistances[1]	References
E. faecalis, n=30				Mato *et al.*,, 2009
EHCP 3	AO6	**resistant**	HLG, Teic, Q/D, Cip, Te, E, DA	
EHCP 13	S	susceptible	HLG, Cip, E, DA	
EHCP 24	AO5	susceptible	HLG, Q/D, Cip, Te, E, DA	
EHCP 31	A2	susceptible	HLG, Q/D, Te, E, DA	
EHCP 55	AW	susceptible	HLG, Teic, Q/D, E, DA	
EHCP 73	J	susceptible	HLG, Q/D, Te, E, DA	
EHCP 78	A3	susceptible	HLG, Q/D, Te, E, DA	
EHCP 92	AR	susceptible	HLG, Cip, Te, E, DA	
EHCP 93	AX	susceptible	HLG, Cip, Te, E, DA	
EHCP 94	AM	susceptible	HLG, Cip, Te, E, DA	
EHCP 143	AU	susceptible	HLG, Cip, Te, E, DA	
EHCP 107	K	susceptible	HLG, Cip, E, DA	
EHCP 151	H	susceptible	HLG, Cip, E, DA	
EHCP 118	AT	susceptible	HLG, Q/D, Cip, Te, E, DA	
EHCP 164	B	susceptible	HLG, Q/D, Cip, E, DA	
EHCP 193	BC	susceptible	HLG, Q/D, Cip, E, DA	
EHCP 225	R	susceptible	HLG, Q/D, Cip, E, DA	
EHCP 241	O	susceptible	HLG, Q/D, Cip, E, DA	
EHCP 237	AO2	susceptible	HLG, Q/D, Cip, Te, E, DA	
EHCP 267	AO2	**resistant**	HLG, Teic, Q/D, Cip, Te, E, DA	
EHCP 271	A11	susceptible	HLG, Q/D, Te, E, DA	
EHCP 279	T	susceptible	HLG, Q/D, Te, E, DA	
EHCP 292	A4	susceptible	HLG, Q/D, Te, E, DA	
EHCP 281	U	susceptible	HLG, Q/D, Cip, Te, E, DA	
EHCP 339	AO1	susceptible	HLG, Q/D, Cip, Te, E, DA	
EHCP 391	M	susceptible	HLG, Q/D, Te, E, DA	
EHCP 332	I	susceptible	HLG, Q/D, Cip, E, DA	
EHCP 389	AO1	**resistant**	HLG, Teic, Q/D, Cip, Te, E, DA	
MMH594	NA	susceptible	HLG, E	Huycke *et al.*,, 1991; Shankar *et al.*, 2002
V583	NA	**resistant**	HLG, E	Sahm *et al.*, 1989 ; Paulsen *et al.*, 2003

Table 1, *cont*

Strain ID	PFGE pattern	Vancomycin resistance	Other relevant resistances[1]	References
E. faecium, n=21				Mato *et al.*, 2009
EHCP 5	c10	**resistant**	Amp, HLG, Teic, Cip, Te, E, DA	
EHCP 6	a1	**resistant**	Amp, HLG, Teic, Cip, E, DA	
EHCP 14	d2	susceptible	Amp, HLG, Cip, Te, E, DA	
EHCP 40	d9	susceptible	Amp, HLG, Cip, Te, E, DA	
EHCP 36	a2	**resistant**	Teic, Cip, E, DA	
EHCP 65	o	susceptible	Amp, HLG, Cip, Te, E, DA	
EHCP 88	c2	susceptible	Amp, HLG, Cip, Te, E, DA	
EHCP 178	p	susceptible	HLG, Cip, Te, E, DA	
EHCP 149	d6	susceptible	Amp, HLG, Cip, Te, E, DA	
EHCP 161	t	susceptible	Amp, HLG, Cip, Te, E	
EHCP 181	d8	susceptible	Amp, HLG, Cip, Te, E, DA	
EHCP 184	f	susceptible	Amp, HLG, Cip, Te, E, DA	
EHCP 211	c12	susceptible	Amp, HLG, Cip, E, DA	
EHCP 264	e	susceptible	Amp, HLG, Q/D, Cip, Te, E, DA	
EHCP 341	u	susceptible	Amp, HLG, Cip, Te, E	
EHCP 358	i	susceptible	Amp, HLG, Cip, Te, E, DA	
EHCP 361	c16	**resistant**	Amp, HLG, Teic, Cip, E, DA	
EHCP 302	c5	susceptible	Amp, HLG, Cip, E, DA	
EHCP 407	d7	**resistant**	Amp, Teic, Cip, E, DA	
EHCP 459	s	susceptible	Amp, HLG, Cip, E, DA	
EHCP 378	w	susceptible	Amp, HLG, Cip, Te, E, DA	

[1]**Amp**- Ampicillin; **HLG**- High-level gentamicin; **Teic**- Teicoplanin; **Q/D**- Quinupristin/Dalfopristin; **Cip**- Ciprofloxacin; **Te**- Tetracyclin; **E**- Erythromycin; **Da**- Clindamycin; **LZD**- Linezolid
NA: Not applied.

General DNA techniques

Phage F170/08 DNA was extracted from CsCl-purified lysates (Vinga *et al.*, 2012). Preparation of *E. coli* plasmid DNA and purification of Polymerase Chain Reaction (PCR) products were performed with the commercial kits QIAprep Spin Miniprep kit (QIAGEN) and High Pure PCR Product Amplification kit (Roche Applied Science),

respectively, following the manufacture instructions. The restriction enzymes used were purchased to Fermentas (Thermo Scientific). Recombinant plasmids were confirmed by DNA sequencing (Macrogen, Seoul, Korea). Restriction endonuclease digestions, DNA ligations, and conventional agarose gel electrophoresis were carried out essentially as described by Sambrook and Russell (2001). Development of competence and transformation of *E. coli* strains was according to the method of Chung *et al.*, (1989).

General protein techniques

The Bradford reagent (Bio-Rad Laboratories) was used for protein quantification using bovine serum albumin as standard. After Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), gels were either stained with Coomassie blue or transferred to 0.45 µm nitrocellulose membranes (Bio-Rad Laboratories) for Western blotting analyses. EC300 polypeptides were immunodetected using a horseradish peroxidase-conjugated anti-His6 monoclonal antibody (Roche Applied Science). PageRuler Prestained Protein Ladder (Thermo Scientific) was used as protein marker in SDS-PAGE.

Construction and cloning of EC300 chimeric gene and its derivatives

The coding sequence including the peptidase M23 CD of the VAL Orf73 from the enterococcal phage F170/08 was PCR amplified from the phage DNA using the KOD hot start master mix (Novagen). The sequence encoding the C-terminal region of the cognate endolysin Lys170, harbouring its CWB domain (CWB170), was similarly amplified in a separate reaction. The 3' and 5' ends of the M23 and of the CWB domain PCR products, respectively, carried a 28-bp complementary segment that allowed fusing both fragments by overlap-extension-PCR (Ho *et al.*, 1989), using the M23 forward and the CWB domain reverse primers. These primers added *Nde*I and *Xma*I restriction sites for cloning of the chimerical gene in the expression vector pIVEX2.3d (Roche Applied Science), originating the recombinant plasmid pDPEC300. Gene *EC300* in pDPEC300 was subjected to site-directed mutagenesis by using the Quick Change II Site directed mutagenesis kit (Stratagene Agilent Technologies), resulting in plasmid pDPmEC300 carrying *mEC300* gene. The introduced nucleotide substitutions eliminated the internal translation start site known to drive the independent synthesis of the CWB170 domain

(Proença *et al.*, 2014, see text also). The pIVEX vectors allow the expression of genes under the control of the phage T7 ϕ10 promoter and the production of the corresponding proteins C-terminally fused to a hexahistidine tag. Details of the primary sequence of EC300, mEC300 and of the parental lytic enzymes are presented in Figure S1. The pIVEX2.3d derivatives were used to transform *E. coli* strain CG61, which produces the phage T7 RNA polymerase upon temperate upshift. The production of active EC300 by CG61 clones was confirmed by growing them over a dense lawn of autoclaved enterococcal cells and by checking the development of lysis halos around *E. coli* colonies (Proença *et al.*, 2012, Fig. S2).

Protein Production and purification

EC300 and mEC300 production and purification by metal chelate affinity chromatography was as described by Proença, *et al.*, (2012). Fractions eluted from the HisTrap HP columns (GE Healthcare) were analysed by SDS-PAGE and those containing the partially purified enzymes were subjected to size-exclusion chromatography using a Hi-load 16/600 superdex 75 prep grade column (GE Healthcare), equilibrated and run in protein buffer (20 mM HEPES-Na, 500 mM NaCl, 1 % glycerol and 1 mM DTT, pH8.0) at a flow rate of 1 mL/min. Purified enzymes were divided in small aliquots and stored at – 80 °C until use. Experimentally-determined partition coefficients (K_{av}) of proteins were used to estimate Stokes radii and the corresponding relative molecular masses by extrapolation from a plot of Stokes radii of standard proteins versus $(-\log K_{av})^{1/2}$ (Cabré *et al.*, 1989). The column void volume (V_0) was determined with blue dextran 2000 (GE Healthcare Life Sciences). The standard proteins (Bio-Rad Laboratories) were thyroglobulin (molecular mass = 670 kDa; Stokes radius = 8.6 nm), γ-globulin (158 kDa; 4.8 nm), ovalbumin (44 kDa; 2.73 nm), myoglobin (17 kDa; 2.08 nm) and vitamin B12 (1.35 kDa; 0.85 nm) (Cabré *et al.*, 1989; Talmard *et al.*, 2007). Proteins Lys170 and CWB170, also used in this work were produced from pIVEX2.3 derivatives pDP2 (Proença *et al.*, 2012) and pDP4 (Proença *et al.*, 2014) and purified as described above.

Lytic activity in liquid media

The lytic activity of EC300 and Lys170 was studied against selected *E. faecalis* strains actively growing in TSB. The strains were grown until OD_{600} of 0.3-0.4, centrifuged and resuspended in ½ volume of fresh TSB. Cell suspensions were challenged with the indicated concentrations of EC300, Lys170 and/or nisin (Sigma Aldrich) and OD_{600} variations followed over time. Lytic activity was also tested with *E. faecalis* cells recovered in ½ volume of protein buffer. Negative controls were similarly prepared, except that protein buffer was added instead of the lytic proteins.

Evaluation of EC300 antibacterial activity in solid medium

The bacterial growth inhibition potential of EC300 and Lys170 was evaluated against the panel of typed *E. faecalis* and *E. faecium* clinical strains (Table 1) on double-layer agar TSA plates as follows. A 200 µL sample of each target bacteria in exponential growth phase (OD_{600} = 0.3-0.4) was incorporated in 5 mL of TSA soft-agar and poured over a TSA solid bottom. Plates were allowed to dry for 30 min in a laminar flow class 2 biological safety cabinet and then 4 different amounts of purified EC300 (10, 3.3, 1.1 and 0.37 µg in a final volume of 10 µL) were spotted on each strain lawn. The plates were incubated overnight at 37 °C and the anti-enterococcal activity was evaluated and scored (- to +++) according to relative diameter and transparency of the growth inhibition halos. Lys170 endolysin was only tested at the maximum amount (10 µg). EC300, mEC300, Lys170 and CWB170, alone or in combination, were also tested on dense lawns of viable *E. faecalis* strain 1518/05 prepared in agarized protein buffer as described previously (Proença *et al.*, 2014). Negative controls were prepared by spotting 10 µL of protein buffer.

Bioinformatics tools

Phage F170/08 putative genes were recognized by integrating results obtained with GeneMark.hmm and MetaGeneAnnotator web software (Besemer *et al.*, 2005; Noguchi *et al.*, 2008). Identification of phage F170/08 putative VALs were based on BLASTP homology searches (Altschul *et al.*, 1997) and on the prediction of protein functional domains using NCBI's CDD (Marchler-Bauer *et al.*, 2011) and Pfam

(http://pfam.xfam.org/). Putative linkers connecting protein functional domains were assigned with SVM (Ebina *et al.*, 2008), using the SVM-joint output.

RESULTS

Rationale for the generation of the chimeric lysin EC300

We have characterized previously the lytic activity of the endolysin Lys170 from the *E. faecalis* phage F170/08 (Proença *et al.*, 2012). Analysis of the primary sequence of the enzyme indicated that it carried an N-terminal CD of the Amidase_2 family (pfam01510) linked to a C-terminal CWB domain. Interestingly, and in contrast to the vast majority of described endolysins, was showed recently that Lys170 corresponds to a multimer composed of one subunit of the expected full length protein (Lys170FL) associated with up to three copies of the enzyme CWB domain (CWB170), which is simultaneously and independently produced from an in-frame, secondary translation start site (Proença *et al*., 2014). Lys170 displayed a broad spectrum of lytic activity against *E. faecalis* clinical strains when these were collected from exponentially growing cultures and resuspended in a physiologic buffer before enzyme addition (Proença *et al.*, 2012). However, Lys170 exhibited very poor lytic or killing activity when added directly to logarithmic phase cultures in rich media like TSB or Brain Heart Infusion (BHI), even at concentrations of several tenths of micrograms per milliliter (Proença *et al.*, unpublished). Strikingly, the lytic capacity of the endolysin could be fully restored in these conditions if *E. faecalis* cells were treated with nisin (see below), a well know lantibiotic that induces lipid II-mediated pore formation in the bacterial cytoplasmic membrane (Hasper *et al.*, 2004; Wenzel *et al.*, 2012). These results indicated on the one hand that Lys170 activity was not being inhibited by growth media components, and from the other hand that actively growing *E. faecalis* exhibits intrinsic resistance to exogenously-added Lys170.

As mentioned above, the ability of actively growing cells to resist endolysin attack may simply reflect the fact that during phage infection endolysins always act in cells previously killed by the holin function. In contrast, other PG hydrolases such as VALs and bacteriolysins are meant to act against bacteria in this physiologic state. Curiously, some PG hydrolysis CDs seem to be shared by VALs and bacteriolysins, like for example the endopeptidase CD of the M23 family (Fig. 1A), which is found in several VALs and

in the bacteriolysins lysostaphin and enterolysin A (Thumm and Götz 1997; Sudiarta *et al.*, 2010; Rodríguez-Rubio *et al.*, 2012a; Khan *et al.*, 2013; Stockdale *et al.*, 2013).

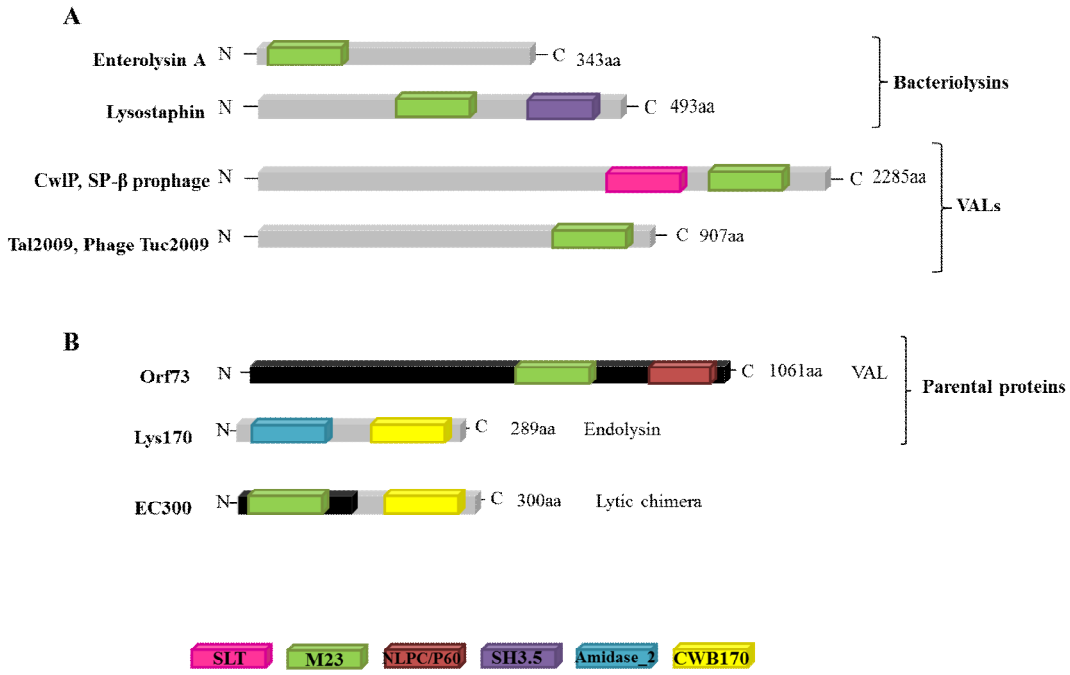

Fig. 1. Rationale behind the construction of the lytic chimera EC300. (A) Examples of bacteriolysins and virion associated lysins (VALs) harbouring the endopeptidase catalytic domain (CD) of the M23 family (M23). Note that the bacteriolysin schemes represent the direct, non-processed products of translation. (B) Domain architecture of EC300 and of the parental proteins Orf73 and Lys170 of phage F170/08. CWB170 is the CWB domain of endolysin Lys170. CD families (Pfam database entries): M23, peptidase family (PF01551); SLT, soluble lytic transglycosylase (PF01464); NLPC/P60, peptidase family (PF00877); SH3b, cell wall binding domain of the SH3_5 family (PF08460); Amidase_2, amidase family (PF01510).

In silico analysis of phage F170/08 genome sequence allowed the identification of two putative genes, *orf72* and *orf73*, encoding VALs. We focused on *orf73*, whose deduced product (1061 amino acid residues; 118 kDa) harbours two putative PG hydrolysis CDs, a peptidase M23 (residues 687 to 787) and a NLPC/P60 (residues 926 to 1058; Anantharaman and Aravind, 2003) (Fig. 1B and Fig. S1A). We have reasoned that by fusing the peptidase M23 CD of Orf73 to the CWB domain of Lys170 (CWB170) we would generate a bacteriolysin-like chimera with the capacity of inducing lysis of actively growing *E. faecalis*, thus overcoming the limitation described for Lys170. The resulting anti-*Enterococcus faecalis* chimera of 300 amino acids (EC300) is schematically represented in Figure 1B and details of its primary sequence are presented in Figure S1.

Production and Purification of EC300

EC300 was produced in *E. coli* C-terminally fused to a hexahistidine tag (His6), which allowed its purification by affinity chromatography (AFC). During protein production we systematically detected in Coomassie-stained SDS-PAGE gels and in anti-His6 Western blots a C-terminal fragment of EC300 of about 12kDa, in addition to the expected full length EC300 (Fig. 2). As mentioned above, this fragment is also produced during Lys170 synthesis and we showed it is essential for robust lytic activity of endolysin Lys170 (Proença *et al.,* 2014). The C-terminal product (CWB170) results from an in frame, secondary translational start site lying at the beginning of the CWB170 coding sequence; this secondary start site is present both in the parental endolysin Lys170 and in the chimera EC300 (Fig. S1). We have shown that CWB170 oligomerizes and associates to the full length Lys170 via CWB170-CWB170 interactions (Proença *et al.,* 2014). Analysis of the major size-exclusion chromatography (SEC) peak of EC300 (SE1 peak in Fig. 2) indicates that the chimeric protein is an analogous heterooligomer, which also results from the association of the full length EC300 with the CWB170 subunit. As for Lys170 (Proença *et al.*, 2014), elimination of the internal start site in EC300 coding sequence, with the consequent abolishment of synthesis of the extra CWB170-containing ~12 kDa polypeptide, resulted in a dramatic decrease of the lytic activity of the mutated protein (mEC300).

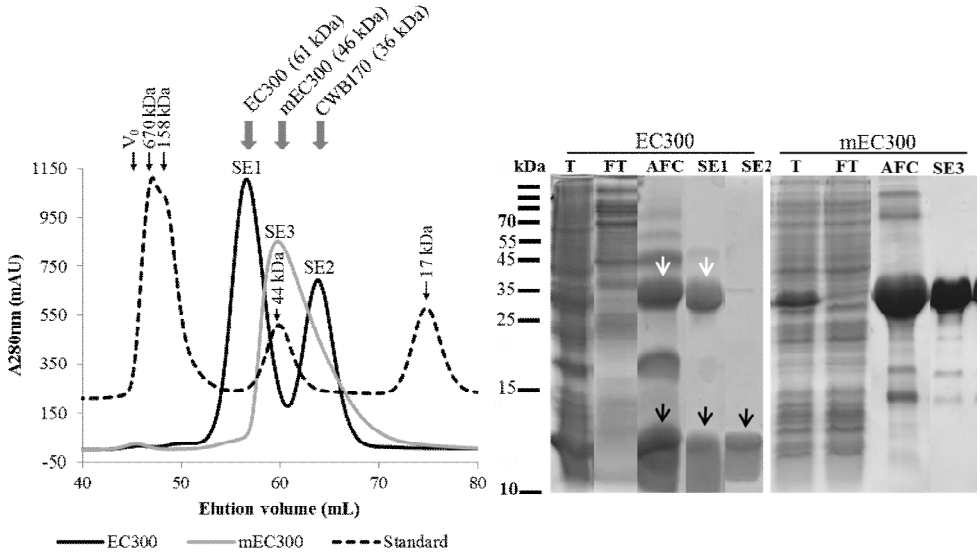

Fig. 2. EC300 and mEC300 purification. (A) EC300 and mEC300 fractions from the corresponding affinity chromatography (AFC) purification steps were subjected to size-exclusion chromatography (SEC). The eluting profile of the proteins was monitored by taking absorbance measurements at 280 nm (A280 nm).

Representative UV curves were combined in a single graph. Note the two-peak elution profile of EC300 corresponding to the full length EC300/CWB170 complex and to the free CWB170 module, respectively. The apparent protein masses derived from the experimentally-determined partition coefficients (K_{av}, see methods) are indicated for each protein. The column void volume (V_0) and the masses of standard proteins are also indicated. (B) SDS-PAGE analysis of the AFC and SEC steps of EC300 and mEC300. Lanes: T, total protein extract; FT, AFC flowthrough; AF1 and AF2, EC300 and mEC300 AFC peak fractions, respectively; SE1 and SE2, EC300 SEC peak fraction; SE3, mEC300 SEC peak fraction. The full length EC300 (34 kDa) and the CWB170 (12 kDa) polypeptides are indicated by white and black arrows, respectively.

Yet, as observed also for the corresponding mLys170 mutant, co-incubation of mEC300 with increasing amounts of independently purified CWB170 progressively restored at least part of the lytic activity lost by the mutated protein (Proença *et al.*, 2014 and Fig. 3), further supporting the heterooligomeric nature of the fully active EC300.

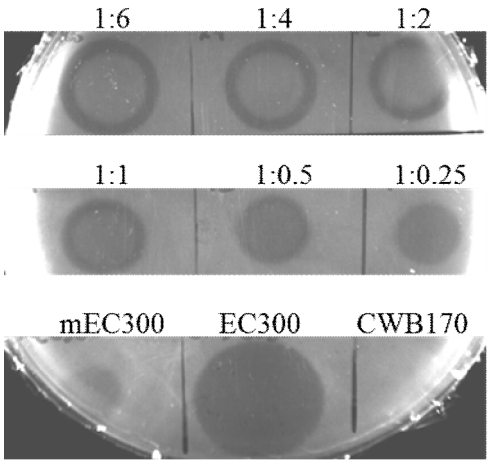

Fig. 3. Impact of CWB170 polypeptide in EC300 activity. A fixed amount of purified mEC300 (10 µg, 0.31 nmol) was co-incubated with CWB170 at the indicated mEC300:CWB170 molar ratios for 1 h at room temperature. After this period, each protein mixture was spotted on a dense lawn of live *E. faecalis* cells prepared in agarized protein buffer. The image shows the lysis halos developed after overnight incubation at 37 ºC. Lysis halos from individually spotted mEC300 (0.31 nmol), EC300 (0.31 nmol) and CWB170 (1.86 nmol) are shown in the bottom row.

During EC300 heterologous production the CWB170 fragment seemed to accumulate in large excess when compared to the amount detected during Lys170 synthesis in the same conditions (Proença *et al.*, 2014); this is probably the cause of the second peak observed during EC300 SEC, which is composed of free CWB170 (SE2 in Fig. 2). The elution

profile of this non-associated form of CWB170, with an apparent mass of 36 kDa (Fig. 2A) is very similar to that of an independently-expressed, recombinant form of CWB170, which we have shown to form homotetramers (Proença *et al*., 2014).

All the experiments described below were carried out with EC300 from factions of SEC peak SE1 (Fig. 2), which in fact corresponds to a complex of full length EC300 associated with CWB170 subunits. Data from SEC (Fig. 2A) and cross-linking experiments (not shown) indicate that the stoichiometry of the EC300 complex should be identical to that of Lys170 (Proença *et al*., 2014), that is, one full length EC300for three CWB170 subunits. In addition, mEC300 seems monomeric in solution but, as described for mLys170 (Proença *et al*., 2014), the protein appears to exhibit an extended conformation as it elutes during SEC with an apparent mass higher (46 kDa) than the expected for the monomer (34 kDa) (Fig. 2A).

EC300 has superior lytic activity when compared to Lys170

Lys170 and EC300 could lyse viable *E. faecalis* cells that were collected from exponentially growing cultures and suspended in a buffered solution before enzyme addition, with EC300 provoking faster and more extensive lysis than the endolysin (Fig. 4A). However, Lys170 could neither induce lysis nor even arrest growth of cell suspensions prepared in TSB culture medium, in clear contrast to EC300 that was able to elicit lysis in these conditions (Fig. 4B). To rule out the possibility of selective inhibition of Lys170 by TSB components, cell suspensions were simultaneously treated with the endolysin and the lantibiotic nisin, which induces membrane pore formation and consequently cell death. Cells killed by the nisin action revealed to be fully susceptible to the lytic action of Lys170, indicating that TSB components do not significantly interfere with endolysin activity (Fig. 4B). The results also indicated that in nutritional media *E. faecalis* cells are intrinsically resistant to Lys170 attack from the outside, but still susceptible to the chimeric enzyme.

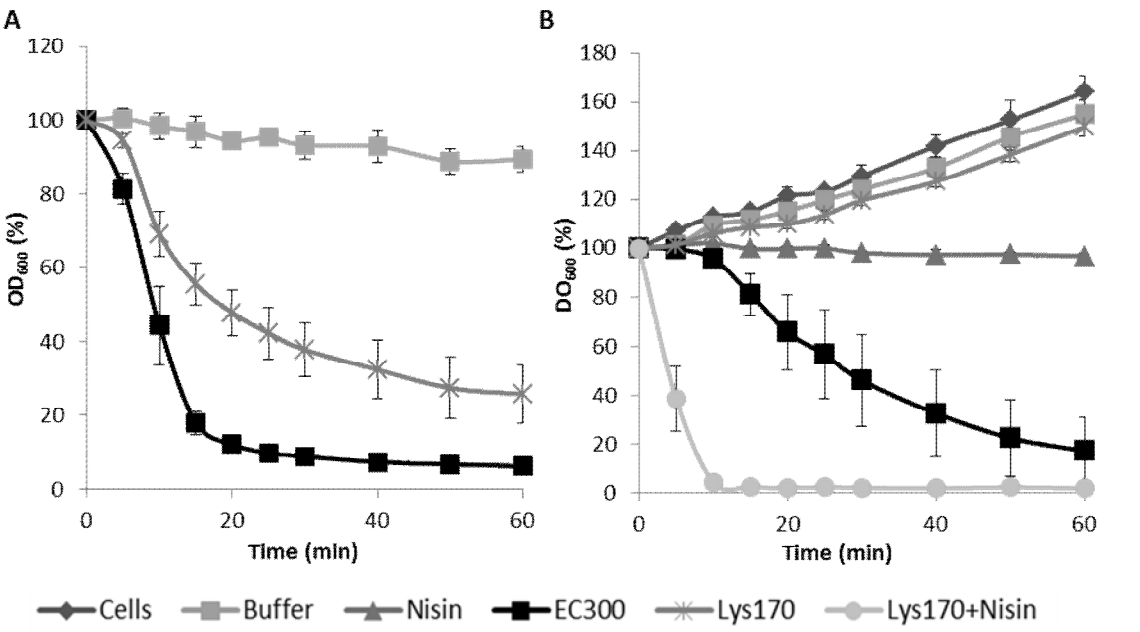

Fig. 4. Comparison of EC300 and Lys170 lytic activities in liquid medium. Cells from exponentially growing *E. faecalis* strain 1518/05 were suspended either in a buffered solution (A) or in TSB (B) and OD_{600} variation followed after addition of the lytic enzymes. EC300 and Lys170 were added at 10 µg/mL in (A) and 50 µg/mL in (B). Nisin concentration, either alone or in combination with Lys170 was 2 µg/mL. Curves "Cells" and "Buffer" correspond to controls with no additions or with added protein buffer, respectively.

EC300 spectrum of activity against enterococcal clinical strains

The spectrum of EC300 antibacterial activity was evaluated on a panel of typed, multidrug-resistant *E. faecalis* and *E. faecium* clinical strains (Table 1). These strains displayed a high-level resistance to gentamicin and included vancomycin-resistant enterococci (VRE) of clonal complexes *E. faecalis*-CC2 and *E. faecium*-CC17, which have been described as highly prevalent in nosocomial settings and disseminated worldwide (Top *et al.*, 2008; Mato *et al.*, 2009; Kuch *et al.*, 2012;). Four quantities of EC300 (10, 3.3, 1.1 and 0.37 µg) were spotted on soft-agar TSA lawns that had been inoculated with cells from exponentially growing cultures of each strain of the panel (see methods). A lawn of *E. faecalis* clinical isolate 1518/05 served as positive control for EC300 activity. Growth of *E. faecium* strains appeared unaffected by any of the spotted EC300 amounts. In contrast, growth inhibition could be detected in 97% of *E. faecalis* strains for the highest tested quantity of the chimeric lysin and 40% for the lowest (Fig. 5).

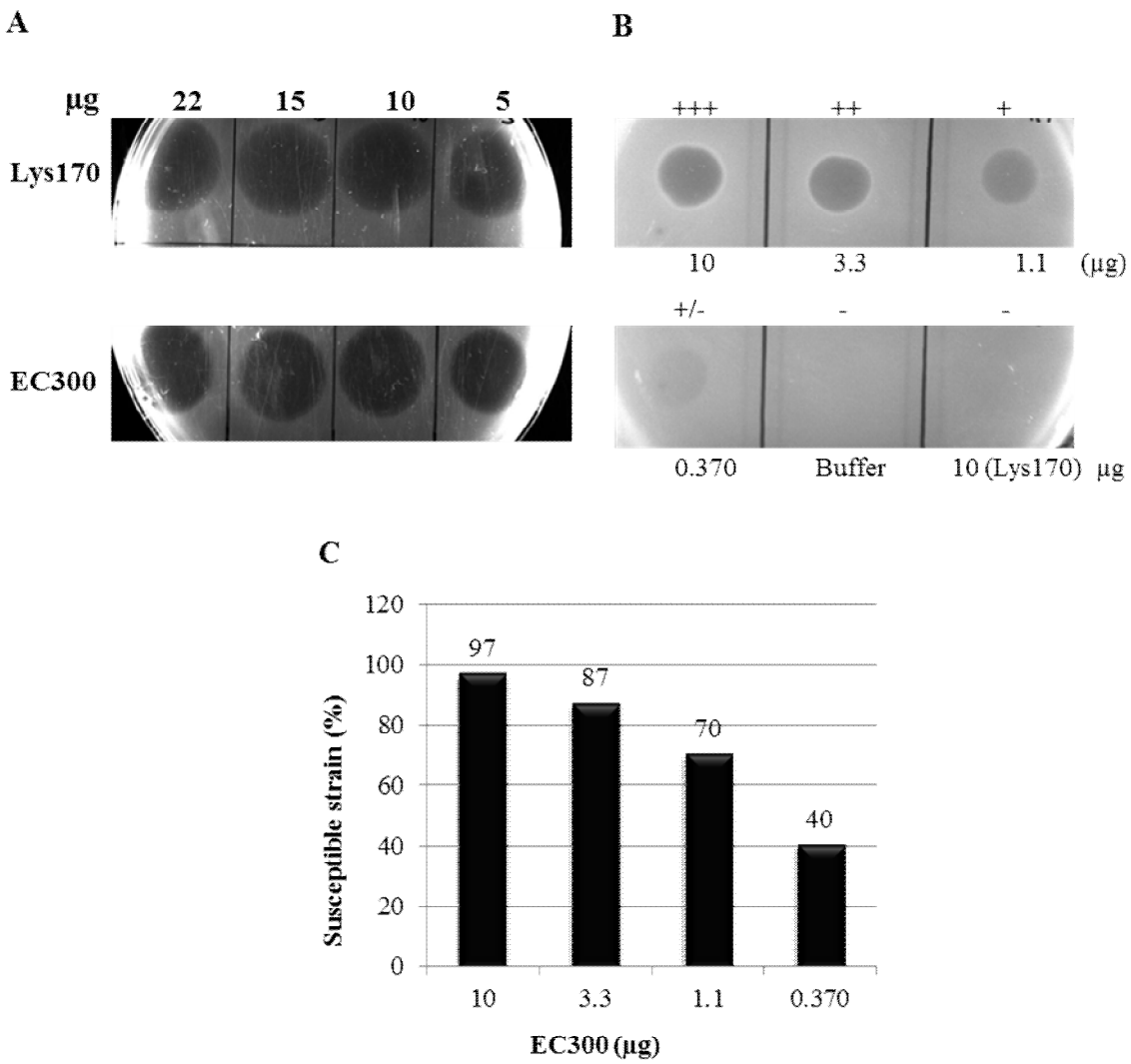

Fig. 5. Susceptibility of a panel of typed *E. faecalis* strains to the EC300 growth inhibition activity. (A) Lytic activity of Lys170 and EC300 against live cells of strain 1518/05 incorporated as a dense lawn in an agarized buffer. (B) Representative growth inhibition halos, classified (-) to (+++), obtained in a soft-agar TSA lawn of strain EHCP 78 after spotting the indicated EC300 quantities. A spot of 10 µg of Lys170 and 10 µL of protein buffer were also tested. (C) Percentage of strains susceptible to the different amounts of EC300 (growth inhibition evaluated on soft-agar TSA lawns).

Of the four vancomycin-resistant *E. faecalis* tested strains, two (EHCP 267 and EHCP 389) seemed to be much more susceptible to EC300 than the others (V583 and EHCP 3) (Fig. 6). Given the reduced number of tested VRE strains, and considering that a wide spectrum of relative activity was also observed for vancomycin-susceptible strains (Table S1), we could not establish any obvious correlation between glycopeptide resistance and susceptibility to EC300. Note that in these assay conditions 10 µg of the endolysin Lys170 produced only a very slight growth inhibition in a couple of *E. faecalis* strains (Table S1). In a previous study, where the endolysin was tested against dense lawns of

bacteria prepared in a soft-agar physiologic buffer, more than 90% of the same strains were susceptible to 5 µg of the endolysin (Proença *et al.*, 2012). The two lytic enzymes produce indistinguishable lysis halos on dense lawns of the control *E. faecalis* strain 1518/05 prepared in agarized assay buffer (Fig. 5A).

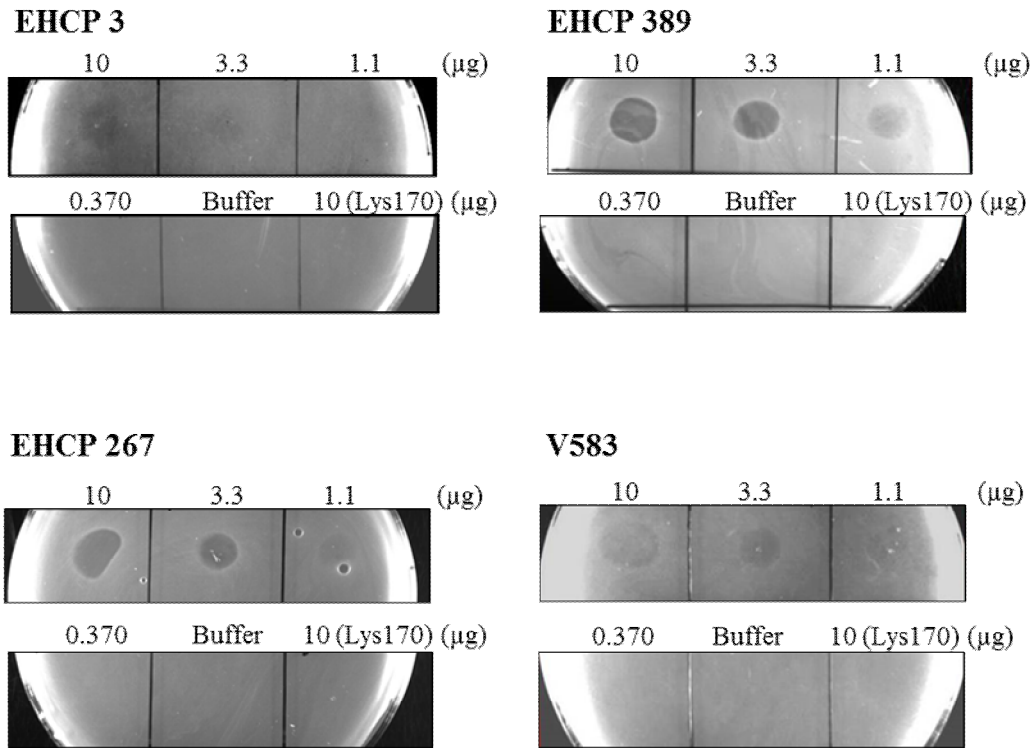

Fig. 6. Evaluation of EC300 capacity to inhibit growth of four vancomycin-resistant *E. faecalis* strains (EHCP 3, EHCP 389, EHCP 267 and V583). The amounts of tested EC300 and the assay conditions were as in figure 4. A spot of 10 µg of Lys170 and 10 µL of protein buffer were also tested as controls. ´

DISCUSSION

The work here presented was prompted by a couple of observations with a few endolysins we have studied recently: 1) the endolysins were able to lyse target bacteria suspended in media that keep cell viability without supporting growth (*e.g.* buffered solutions); and 2) the same endolysins could only induce efficient lysis of target cells suspended in growth-promoting media (*e.g.* culture media) if bacteria were first or concomitantly killed by another agent, like for example the lantibiotic nisin. Nisin was previously shown to

dramatically enhance the lytic activity of endolysins (Nascimento *et al.*, 2008; Catalão *et al.*, 2010; García *et al.*, 2010) and to trigger the activity of bacterial autolysins (Severina *et al.*, 1998; Frias *et al.*, 2009; Lansa *et al.*, 2012). These facts, associated to the observation that most studies on the lytic action of endolysins are performed in conditions where target bacteria are in a state of reduced metabolic activity, led us to raise the hypothesis that, in the natural context of phage infection, the killing action of holins may be required to fully sensitize cells to the activity of at least some endolysins. In other words, at least some endolysins may not be suited to attack well-fitted bacteria from the outside. Although related to endolysins, phage VALs and bacteriolysins may stand as better suited alternatives as they are naturally adapted to act against dividing bacteria. In fact, VALs are responsible for the phenomenon of 'lysis from without', which is characterized by the ability of some phages to induce premature lysis when added to host cells at high multiplicities (Abedon, 2011).

The idea of taking advantage of the particular features of bacteriolysins and VALs has been explored recently, particularly when targeting *Staphylococcus aureus*. Lysostaphin and its functional domains have been fused to endolysin or VAL moieties to generate chimeras with improved antibacterial activity against *S. aureus* (Idelevich *et al.*, 2001; Donovan *et al.*, 2006; Paul *et al.*, 2011; Rodríguez-Rubio *et al.*, 2012b; Schmelcher *et al.*, 2012b; Saravanan *et al.*, 2013); in other approaches lysostaphin has been simply used synergistically with phage lytic enzymes (Becker *et al.*, 2008).

Although PG hydrolase activity has been demonstrated for several VALs, one important observation is that, with very few known exceptions (Takác and Bläsi, 2005), VALs seem to lack the CWB domain typically found in endolysins; this is probably because cell wall targeting is fulfilled by other proteins of the virion structure, such as the receptor binding proteins (Rodríguez-Rubio *et al.*, 2013). To overcome this limitation, VALs or their CDs have been fused to the CWB domain SH3b of lysostaphin (Paul *et al.*, 2011; Rodríguez-Rubio *et al.*, 2012b).

In line with the ideas explained above, we have for the first time engineered a bacteriolysin-like enzyme aimed at killing *E. faecalis* in growth supporting conditions. The chimera EC300, which combined a M23 peptidase CD from a VAL with an endolysin CWB domain (CWB170), showed increased lytic and killing properties when compared to the parental endolysin (Lys170). The superior performance of EC300 was particularly evident in growth media. M23-like peptidase domains are present in a wide

variety of proteins such as bacteriolysins, autolysins and eukaryotic cell proteins, but are rarely found in endolysins; one exception is the staphylococcal phage 2638A endolysin (Abaev *et al.*, 2013).

Another interesting feature of EC300 results from the fact that, similarly to Lys170, the fully active chimerical enzyme is a complex made of EC300 full-length polypeptide associated with independently produced CWB170 subunits. Although the exact stoichiometry of the EC300 multimer was not determined, the available evidences strongly suggest that it should have the same configuration of the Lys170 multimer (Proença *et al.*, 2014), that is, being made of one molecule of the full-length EC300 complexed with three of CWB170. This will means that EC300 assembles one M23 endopeptidase CD with four copies of the CWB170, which provide to the lytic enzyme high affinity to the cell wall (Proença *et al.*, 2014). In addition, due to its multimeric nature EC300 is a protein with almost 70 kDa; this will certainly be an advantage for the study of its effectiveness in animal infection models since, as observed for the dimeric form of the pneumococcal endolysin Cpl-1 (Resch *et al.*, 2011), it should reduce renal clearance (proteins smaller than 60-65 kDa tend to be rapidly eliminated by glomerular filtration in humans; Maack *et al.*, 1979).

The rather promiscuous modular structure of endolysins themselves has also been intensively explored to engineer chimeras with increased solubility and with changed and/or extended lytic spectra when compared to parental endolysins (Croux *et al.*, 1993; Daniel *et al.*, 2010; Pastagia *et al.*, 2011; Schmelcher *et al.*, 2011; Fernandes *et al.*, 2012; Mao *et al.*, 2013; Yang *et al.*, 2014). The results obtained with EC300 suggest that fusing CDs from VALs to CWB domains of cognate endolysins may constitute an additional strategy to generate enzymes with improved features. The next step will be to evaluate the therapeutic efficacy of EC300 in a murine model of enterococcal bacteraemia.

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SUPPLEMENTARY MATERIAL

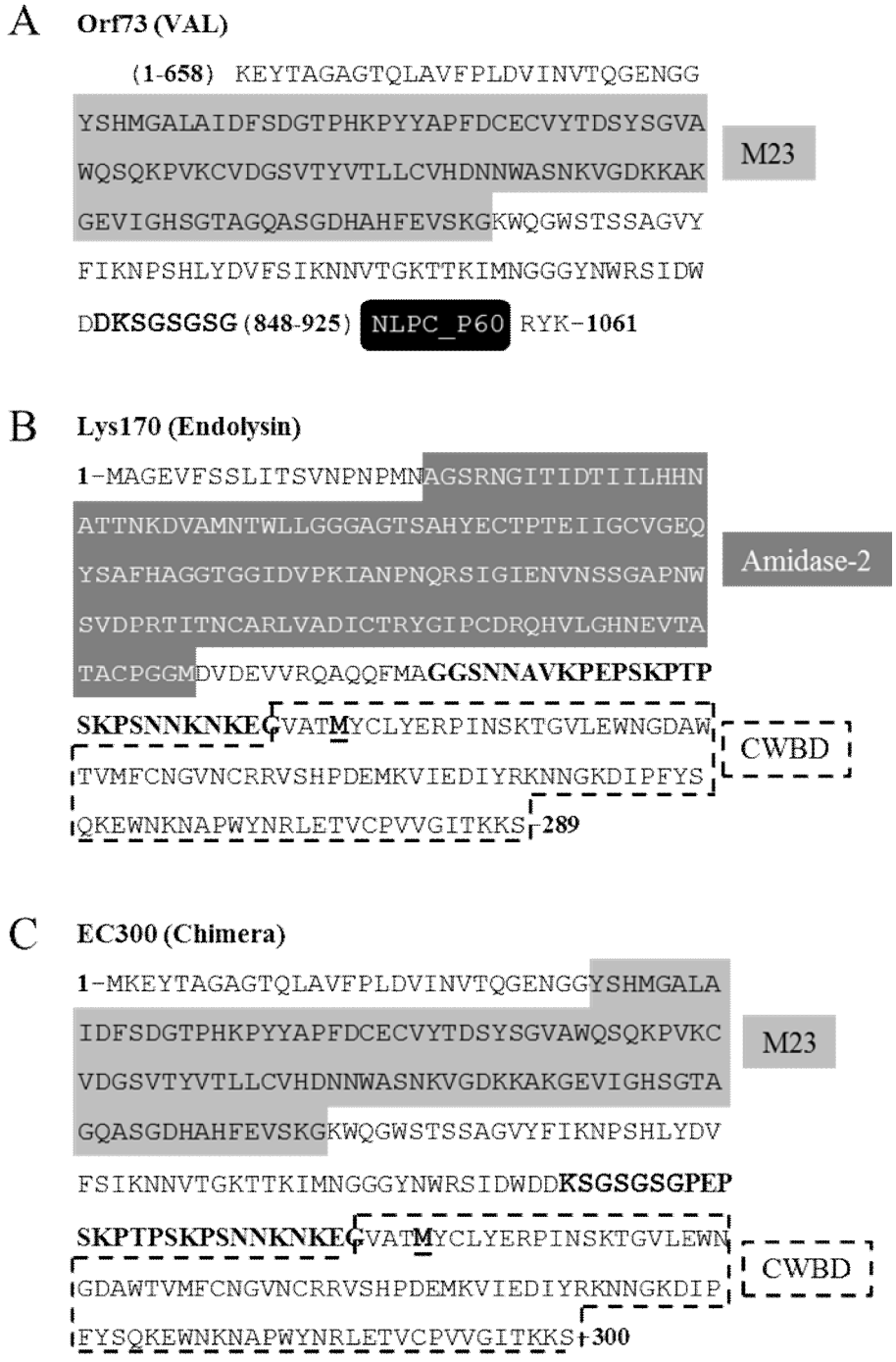

Fig. S1. Primary sequence details and domain architecture of the VAL Orf73 (**A**), the endolysin Lys170 (**B**) and of the chimera EC300 (**C**). M23 peptidase and Amidase_2 CDs were defined by CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and Pfam (http://pfam.xfam.org/) analysis. Putative linker segments (boldface residues) were predicted with SVM tool (http://domserv.lab.tuat.ac.jp/dlpsvm.htmL). Inferred cell wall binding domain (CWBD) is delimited by a dashed box with indication of the secondary translation starting Met_{202} (**M**), which initiates the independent CWB170 module. The protein mEC300 carries the single amino acid substitution $M_{202}L$.

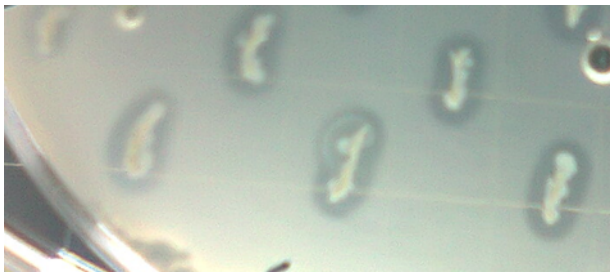

Fig. S2. "TritonX-100-induced lysis halo assay" (Ebina *et al*., 2009) used for confirming production of active EC300 chimera by *E. coli* CG61. Transformants were grown overnight at 30 ºC on an LB soft-agar plate containing 2% (w/v) of autoclavated cells from the *E. faecalis* strain 1518/05, 0.1% Triton-X100, 100 µg/mL ampicillin and 30 µg/mL kanamycin. Subsequently the plates were incubated at 4 ºC for 24 h. Lysis halos around *E. coli* CG61 colonies expressing EC300 are shown.

Table S1. Growth inhibition of typed *E. faecalis* clinical strains by EC300.[1]

Specie	Strain[2]	Vancomycin	EC300 (µg)				Buffer	Lys170 (µg)
			10	**3.3**	**1.1**	**0.370**		**10**
E. faecalis	**V583**	**R**	+/-	+/-	-	-	-	-
	MMH594	**S**	+++	+++	+	-	-	-
	EHCP 3	**R**	+/-	+/-	-	-	-	-
	EHCP 13	S	+++	+++	++	+	-	-
	EHCP 24	S	+++	++	+	+/-	-	+/-
	EHCP 31	S	+++	+++	++	+	-	-
	EHCP 55	S	+/-	+/-	-	-	-	-
	EHCP 73	S	++	++	+	+/-	-	-
	EHCP 78	S	+++	++	+	+/-	-	-
	EHCP 92	S	+	+/-	-	-	-	-
	EHCP 93	S	+++	++	+/-	-	-	-
	EHCP 94	S	+++	++	+	+/-	-	-
	EHCP 107	S	-	-	-	-	-	-
	EHCP 118	S	+++	++	+	-	-	-
	EHCP 143	S	+/-	-	-	-	-	-
	EHCP 151	S	++	+	+/-	-	-	-
	EHCP 164	S	+++	++	+/-	-	-	-
	EHCP 193	S	+++	++	+/-	+/-	-	-
	EHCP 225	S	+++	+++	+++	+++	-	+
	EHCP 237	S	+/-	+/-	-	-	-	-
	EHCP 241	S	++	+	+/-	+/-	-	-
	EHCP 267	**R**	++	+	+/-	-	-	-
	EHCP 271	S	+++	+++	++	+	-	-
	EHCP 279	S	+++	+++	++	++	-	+/-
	EHCP 281	S	+/-	-	-	-	-	-
	EHCP 292	S	+++	+++	+	+/-	-	-
	EHCP 332	S	++	+	+/-	-	-	-
	EHCP 339	S	+++	++	+/-	-	-	-
	EHCP 389	**R**	+++	++	+	-	-	-
	EHCP 391	S	+/-	-	-	-	-	-
E. faecalis	**1518/05**[3]	S	+++	+++	++	+/-	-	-

[1]Growth inhibition was qualitatively evaluated by scoring as (–) to (+++) the relative diameter and transparency of the lysis halos produced by each EC300 quantity, after overnight incubation at 37 ºC.
[2]See Table 1 for additional features of the strains
[3]Positive control for EC300 activity.

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CONCLUDING REMARKS AND FUTURE PRESPECTIVES

The general increase of antibiotic resistance among some of the most relevant bacterial pathogens has been prompting the development of new approaches to tackle this major public health problem. One strategy receiving particular attention preconizes the use of recombinant forms of phage PG hydrolases to eliminate bacteria, particularly those that are Gram-positive. Search of such antimicrobial agents to combat *E. faecalis* infections remains poorly developed when compared to other infectious agents, like for example *S. aureus*.

We report in this thesis the study of the lytic activity of the endolysins Lys168 and Lys170 encoded by the enterococcal phages F168/08 and F170/08, respectively (Proença *et al*., 2012). In contrast to other reported enterococcal endolysins (Yoong *et al*., 2004; Son *et al*., 2010), Lys168 and Lys170 were active almost exclusively against *E. faecalis* and ineffective in lysing other Gram-positive bacteria (Proença *et al*., 2012). In the conditions tested Lys170 exhibited better lytic performance than Lys168 and showed a broader lytic spectrum against *E. faecalis* strains. In a study developed by our group, the CDs of these two endolysins were fused to a CWB domain from a staphylococcal endolysin. In addition to retain the action against *E. faecalis*, the host-range of the chimerical endolysins was expanded not only to *Staphylococcus* species but also to *S. pyogenes* (Fernandes *et al*., 2012). These results showed that the CDs of the enterococcal endolysins are not species specific; however, changing the CWB domains was crucial for shifting the bacterial target as Lys170 and Lys168 could not lyse non-enterococcal species. The fact that the Lys168 CD of could degrade the PG from different species might indicate that, rather than displaying the most commonly observed peptidase activity, the CHAP domain of Lys168 displays in fact amidase activity, as proposed for the enzyme of phage IME-EF1 (Zhang *et al*., 2013). Lys168 digests of purified *E. faecalis* PG could be analyzed by HPLC coupled to mass spectrometry to determinate the cleavage specificity of the endolysin.

The fact that CWB170 and the putative CWB domain of Lys168 are unrelated at the primary sequence level might suggest that they recognize different epitopes of the

enterococcal cell surface, which are not present either in *Staphylococcal* species or in *S. pyogenes*. In this work we have neither explored the specificity of each CWB domain nor to which cell wall components they might bind to, but at least for Lys170 we could provide evidences that the CWB170 module greatly increases the binding efficiency of the endolysin to the bacterial surface.

The established idea of endolysins being monomeric in solution and having a highly conserved modular architecture, where the two functional regions of the enzymes are linked by a peptide linker, was challenged by the unusual arrangement of the pneumococcal endolysin PlyC functional domains (Nelson *et al*., 2006). The multimeric nature and the particular two-component assembly described in this thesis for the enterococcal endolysin Lys170 corresponds to another deviating example. In contrast to PlyC, where CD and CWB subunits are produced by separated genes (Nelson *et al*., 2006), the extra monomers of CWB170 in Lys170 result from a secondary translational start site internal to *lys170*, *i.e*. both subunits are produced by the same gene.

A great number of PG hydrolases of Gram-positive systems carry (or are predicted to) tandem repetitions of CWB motifs. For some of these, such as the choline-binding repeats and the LysM motifs, it has been shown that their cooperative action increases the affinity and/or activity of the enzymes towards their respective substrates (López and Garcia 2004; Steen *et al*., 2005; Mesnage *et al*., 2014; Wong *et al*., 2014). PlyC and Lys170 represent a distinct class of PG hydrolases where the number of CWB domains in each functional unit is increased through multimerization of this module, instead of relying on the repetition of their coding sequence in the corresponding genes. One question that remains open after our studies concerns the mechanism mediating Lys170 assembly, namely what are the interacting segments involved in Lys170FL and CWB170 association. In an *in silico* analysis of the Lys170FL/CWB170 primary sequences we could not obtain any obvious hints about the possible interaction interface(s). Note that all the biochemistry work with Lys170 and its derivatives was carried out in reducing conditions; thus, disulfide bond formation between Lys170 Cys residues is not expected to be involved in Lys170FL/CWB170 association. Further protein biochemical and biophysical characterization, such as x-ray crystallography could be attempted to disclose how the Lys170 multimer is assembled.

In chapter 3 we provided clues for the presence of CWB170-like modules in different PG hydrolases and speculated that the previously described *Clostridia* endolysins CD27L and

CTP1L may in fact undergo a multimerization process analogous to that of Lys170. In other words, we believe that the production of endolysin (of other PG hydrolases) functional units through the assembly of different subunits may be more common than expected. These subunits may be produced from separate genes, from alternative translation initiation and perhaps even from proteolytic processing from the full length products (for example autocleavage of peptidases). It is interesting to note that expression of several endolysin genes has been described to result in the production of truncated products through alternative translation initiation. These products may correspond to small C-terminal polypeptides of unknown function (Wittmann *et al.*, 2010; Shearman *et al.*, 1994) or be larger proteins carrying both CD and putative CWB domains (Catalão *et al.*, 2011; Abaev *et al.*, 2013). The latter situation is very interesting as in case of demonstration of heterooligomer formation the endolysin functional units would display repeated CD and CWB domains.

A transversal observation that comes out from the studies of endolysins relates to the *in vitro* conditions usually employed to evaluate their lytic potential. With the goal of having the most controlled assay conditions possible, lytic activity of phage PG hydrolases is typically studied in buffered environments of defined chemical composition. Most frequently, target cells are washed from their culture medium and suspended in buffer that keeps bacterial viability, but which does not support cell proliferation. These were the type of conditions we employed to study the lytic potential of the enterococcal endolysins Lys168 and Lys170 (Proença *et al.*, 2012). However, when exerting their action during phage infection, endolysins always act from the inside of host cells and, most importantly, after these had been killed by the holin-mediated membrane pores (São-José *et al.*, 2007; Catalão *et al.*, 2013; Young, 2014). We have raised the hypothesis that there might be a certain misconception when assuming that recombinant endolysins added from the outside to metabolically active cells will act as efficiently as in the natural context of an infection. In fact, our experience with enterococcal and other endolysins has been telling us that actively growing bacteria (like those in a rich culture medium) are much more resistant (sometimes fully resistant) to endolysin attack from without, when compared to the same bacteria lying in nutrient-depleted media (like physiologic buffers). This may constitute a limitation to the use of endolysins as antibacterial therapy.

With these assumptions in mind, in Chapter 4 we describe the development of a chimerical lytic enzyme active against *E. faecalis* based on a novel design that aimed to

overcome the limitations referred to above. The rationale behind this new technology was to fuse CDs from virion-associated lysins (VALs), which are phage proteins naturally designed to act on the bacterial cell wall from the outside (for more detailed information see VALs characteristics in Chapter 1), to CWB domains of endolysins, mimicking the natural domain arrangement of some bacteriolysins (previously considered as bacteriocins with PG hydrolase activity). The most promising chimera obtained based on this design was the anti-enterococcal protein EC300, which bears as CD a peptidase M23 domain from a VAL of phage F170/08 and the well characterized CWB170 as cell binding domain. This enzyme showed better stability and solubility in comparison to the parental endolysin Lys170 (not shown) and, most relevantly, exhibited much higher lytic action against *E. faecalis* cells challenged in a rich culture medium (see RESULTS in Chapter 4). EC300 is the first bacteriolysins-like, 100% phage-based protein reported in the literature and the first enterococcal chimeric lysin capable of killing live cells of *E. faecalis* in growth promoting media). Another interesting feature of this chimera is the fact that it inherited the oligomerization features discovered for Lys170 (Proença *et al.*, 2014), since both enzymes share the same C-terminal domain. This characteristic is expected to confer to EC300 high affinity to target cells, because of the multiple copies of CWB170 carried in each EC300 functional unit, and to be advantageous in future *in vivo* assays of animal infection models, since the EC300 mass (~ 70 kDa) should contribute to extend its half-life within animals.

Enterococci are just one example of Gram-positive bacterial pathogens that currently pose serious problems in the context of antibiotic resistance and healthcare-associated infections. *Staphylococcus aureus* is by far the most worrying bacterial species, more specifically because of its methicillin resistant strains. It is also the most extensively studied in terms of seeking alternatives to the conventional antibiotherapy, including the search of PG hydrolases with potential antimicrobial action. We have also tried the EC300 approach to develop bacteriolysin-like chimeras targeting *S. aureus*. During this project we constructed 4 anti-*S. aureus* chimerical enzymes, which were generically denominated as *S. aureus* chimeras (SC). Figure 1 represents the pipeline of the 5 bacteriolysin-like enzymes constructed during this PhD project. The four SC proteins harbour the CWB domain of the *S. aureus* endolysin Lys87, previously used to construct the chimeric endolysins Lys170-87 and Lys168-87 (Fernandes *et al.*, 2012). Unfortunately, none of the 4 SC constructs reached the final goal: SC248 failed to be

produced; SC91 and SC87 were very active in preliminary lytic assays, but become insoluble during large scale production; and, SC170, become inactive after its purification, although being stable in solution and active in preliminary lytic assays. Thus, despite our new approach we still faced the commonly reported issue of the low solubility of anti-*S. aureus* PG hydrolases , whether endolysins or VALs (Daniel *et al.*, 2010; García *et al.*, 2010; Fernandes *et al.*, 2012).

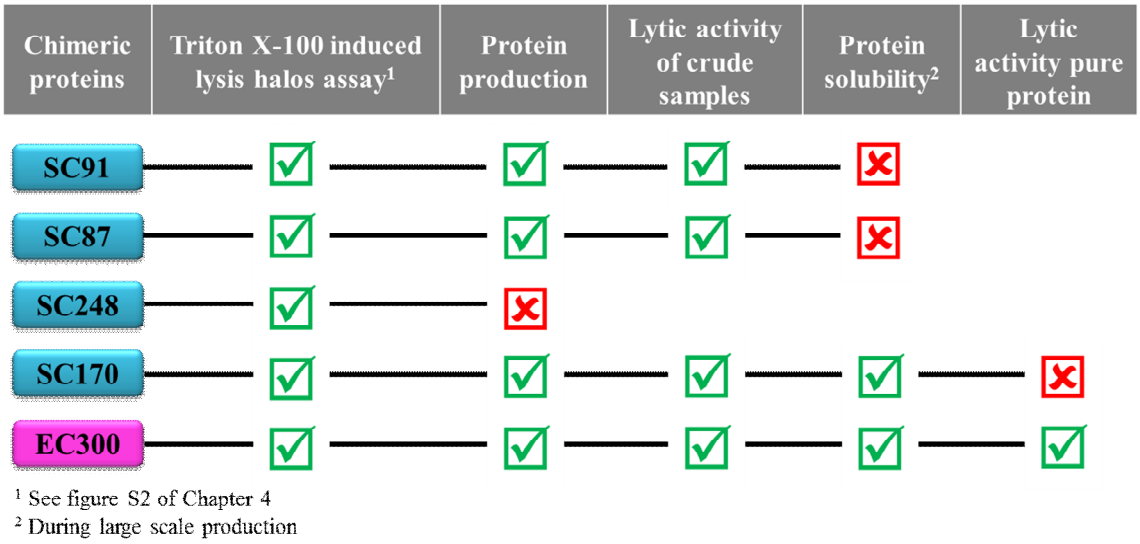

Fig. 1. Pipeline of bacteriolysins-like proteins developed during this thesis. The blue boxes correspond to *S. aureus* targeting lytic chimeras (SC) and the pink box corresponds to *E. faecalis* targeting lytic chimera (EC). For more detailed information about "Triton X-100 induced lysis halos assay" see Fig. S2 Chapter 2.

Although extension of the new lytic enzyme design to other pathogens still requires optimization, the technology proved successful in the development of a product (EC300) with improved lytic action against *E. faecalis*, leading to the filling of patent (Provisional national application patent No. 20141000060398).

Regarding the engineering of artificial lytic proteins with improved antibacterial features, the chimeric lysin EC300 is a good example of the importance of studying the fundamental biochemical properties of the parental proteins.

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