Enterococcal Cytolysin: A Novel Two Component Peptide System that Serves as a Bacterial Defense Against Eukaryotic and Prokaryotic Cells

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Abstract: The cytolysin is a novel, two-peptide lytic toxin produced by some strains of *Enterococcus faecalis*. It is toxic in animal models of enterococcal infection, and associated with acutely terminal outcome in human infection. The cytolysin exerts activity against a broad spectrum of cell types including a wide range of gram positive bacteria, eukaryotic cells such as human, bovine and horse erythrocytes, retinal cells, polymorphonuclear leukocytes, and human intestinal epithelial cells. The cytolysin likely originated as a bacteriocin involved with niche control in the complex microbial ecologies associated with eukaryotic hosts. However, additional anti-eukaryotic activities may have been selected for as enterococci adapted to eukaryotic cell predation in water or soil ecologies. Cytolytic activity requires two unique peptides that possess modifications characteristic of the lantibiotic bacteriocins, and these peptides are broadly similar in size to most cationic eukaryotic defensins. Expression of the cytolysin is tightly controlled by a novel mode of gene regulation in which the smaller peptide signals high-level expression of the cytolysin gene cluster. This complex regulation of cytolysin expression may have evolved to balance defense against eukaryotic predators with stealth.

Keywords: Cytolysin, toxin, bacteriocin, hemolysin, enterococcal pathogenesis.

INTRODUCTION

Enterococci are gram-positive cocci that naturally inhabit the gastrointestinal tracts of a wide variety of hosts ranging from mammals to insects [1]. They can also be found on plants, in soil, and in water [2-4]. In the human GI tract consortium they are present in numbers of approximately 10^6 colony forming units per gram of feces [5,6]. In addition to being commensal organisms, they are leading causes of hospital acquired surgical site infections (17.1%), blood stream infections (11.5%) and nosocomial urinary tract infections (14.3%) [7] resulting in health care costs in the $ 100 Millions [8,6]. Enterococci are also leading causes of community acquired subacute endocarditis [9]. Two enterococcal species are responsible for most enterococcal infections; 65 – 80% are caused by *Enterococcus faecalis*, and 20 – 35% are caused by *Enterococcus faecium* [6,10]. Enterococcal infections are especially problematic as they are often associated with resistance to multiple antibiotics, and clinical strains have emerged that are resistant to all available antibiotics [6]. Commensal strains of enterococci rarely possess antibiotic resistance beyond that intrinsic to the species [6].

THE ENTEROCOCCAL CYTOLYSIN

Some strains of *E. faecalis* produce a two-peptide lytic toxin, termed cytolysin because of its broad spectrum of conceivable that the cytolysin evolved as a defense system against both eukaryotic competitors, perhaps including activity against both eukaryotes and prokaryotes [11]. As enterococci inhabit highly competitive ecologies, it is species of grazing zooflagellates which have been suggested to feed on enterococci [12], and competing bacteria. Structural and functional attributes of the cytolysin place it as a distant relative of Streptolysin S, produced by *S. pyogenes*, and also of the lantibiotic class of bacteriocins [13,14,11]. The cytolysin, while having evolved the ability to target and lyse both bacterial and mammalian cells, likely originated within the lantibiotic class of bacteriocins, which are expressed by a wide variety of gram positive bacteria, as a niche control mechanism (see paper by Cotter *et al*., this issue). Molecular modifications to the basic lantibiotic design may have been selected for as enterococci adapted to niches occupied by eukaryotic competitors, or in response to eukaryotic cell predation. However, the exact identity of the eukaryotic cells that may have exerted this selective pressure is unknown. No other members of the lantibiotic family are known to possess natural toxin activity [15,16].

HISTORICAL SYNOPSIS

The first report examining the cytolytic activity of *Enterococcus* is credited to E.W. Todd [17] for work conducted in 1934 on the hemolytic properties of streptococci. Todd first observed that *E. faecalis* strains that were hemolytic on blood agar had no detectable hemolytic activity in filtrates prepared from liquid culture. This observation led him to classify cytolysin as a "pseudohaemolysin". Further examination led to the development of a horse flesh infusion medium that allowed for the production of hemolytic filtrates. Using these filtrates, Todd then went on to characterize the hemolysin

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activity in response to pH, and demonstrated that the hemolysin was acid sensitive and somewhat alkali labile after heating [17]. Hemolytic activity was completely lost after incubation at 37°C for two hours at pH 6.6, and reduced by five-fold after heating at 60°C for 30 minutes at pH 8.0. In this same study [17], Todd observed that the hemolysin was oxygen stable and lacked immunogenicity in rabbits.

Analysis of target cell susceptibility to the enterococcal hemolysin was conducted by Kobayashi in 1940 [18]. It was observed that erythrocytes from several different mammalian species possessed differing degrees of sensitivity to the hemolysin. It was shown that rabbit, cow, horse, and human erythrocytes were vulnerable to cytolysin-mediated destruction [18]. Sheep and goat erythrocytes were shown to be resistant to lysis under the same experimental conditions, an outcome that would later be suggested by Roelofsen *et al.* [19] to be the result of the relatively higher levels of phosphatidylcholine present in the outer leaflet of rabbit, human and horse erythrocytes.

In 1949, Sherwood *et al.* [20] first hinted at the bacteriocidal properties of cytolysin. While no hypothesis was put forth to suggest a connection between their hemolytic and bacteriocidal properties, it was reported that five out of eight beta-hemolytic group D streptococci examined were found to produce "antibiotic substances" [20].

In 1963, a wide assortment of enterococcal isolates obtained from various culture collections were examined by Brock *et al.* [21], who found that over 50% of the strains evaluated produced some type of bacteriocin. These bacteriocins were categorized by determining their sensitivity to heat, chloroform and proteolytic enzymes and grouped into five different types. The authors observed that type 1 bacteriocin was produced by all strains of *Streptococcus zymogenes* tested (now recognized as hemolytic *E. faecalis*) and found that it had broad spectrum activity against a variety of other Gram positive bacteria [21].

Brock and Davie [22] provided the first evidence suggesting that the bacteriocin and hemolysin produced by *E. faecalis* were the same entity. It was demonstrated that after UV irradiation, the hemolysin and bacteriocin activities were concomitantly lost, and could be simultaneously regained by reversion. With these findings, it was first suggested that the bacteriocin activity of cytolysin might function as a virulence factor, and that it might provide an advantage to cytolytic strains over other, non-cytolytic Gram positive organisms in the gastrointestinal tract [22].

The first evidence suggesting that the enterococcal cytolysin is composed of multiple components was reported in 1969 by Granato and Jackson [23]. Using nitrosoguanidine mutagenesis, the authors were able to generate non-cytolytic mutants that, when plated on blood agar, were observed to produce zones of hemolysis only between select closely spaced mutant colonies, an outcome that suggests extracellular complementation. These results, combined with studies of the kinetics of the interactions between mutants, prompted the authors [23] to conclude that cytolysin is composed of a lytic component and an activator component, which they termed "L" and "A", respectively. Later studies [24,25] attempted to purify components A and L by gel filtration and ion-exchange chromatography, and their molecular sizes were estimated. The A component was found to have a molecular mass of 27,000 Da [24]. The L component was estimated to have a mass of 11,000 Da [25].

Detailed analysis of the genetics of cytolysin production first began in the mid 1970s with the work of Dunny and Clewell [26] and Jacob *et al.* [27]. Both groups made similar observations of the transmissibility of the cytolysin determinant by conjugation. Jacob *et al.* [27] inferred the presence of the cytolysin determinant on a plasmid with experiments that involved the curing of strains JH1 and JH3 of their hemolysin and bacteriocin phenotypes. They reported a 0.5% loss of this phenotype in overnight cultures grown at 37° and 45°C. A mixture of JH1 or JH3 with plasmid-free recipients in liquid culture revealed that cytolytic exconjugates were produced within four hours, at a rate of 10^{-1} to 10^{-2} per donor cell [27]. This was followed by the observation that bacteriocin resistance was transferred with a 58 kb plasmid conferring the cytolysin determinant, and that the plasmid could be maintained, conferring bacteriocin resistance in non-cytolytic recipients. Dunny and Clewell [26] reported similar findings which showed transmission of resistance after two hours at a frequency of 4.3 per 10^5 donors. Interestingly, they also reported the dramatic killing of recipients (only 3 to 50% of recipients, in separate experiments, survived prolonged incubation) after several hours of incubation in mating mixtures, and speculated that this apparent bacteriocin effect was enhanced by the significant growth of donor cells early in the incubation period. Later work by Dunny *et al.* [28,29] went on to demonstrate that conjugative transfer of the plasmid encoding the cytolysin determinant is pheromone responsive.

BACTERIOCIN ACTIVITIES OF THE ENTEROCOCCAL CYTOLYSIN

The spectrum of bacteriocidal activity against other bacteria by the enterococcal cytolysin extends to a wide variety of Gram positive organisms. Brock *et al.* [21] characterized bacteriocin activity against a wide range of different organisms and found that the majority of organisms were sensitive with the exception of *Bacillus polymyxa* and two Gram negative strains, *Escherichia coli* and *Proteus vulgaris*. More recently Jett and Gilmore [30] have shown that this spectrum of activity also extends to the oral streptococci [30]. Table **1** is a current list of known Gram positive organisms that display sensitivity to the enterococcal cytolysin.

In a study by Davie and Brock [31] to elucidate the mode of action and characterize the mechanism of resistance to the cytolysin, a D-alanyl ribitol teichoic acid was implicated in resistance. While it is unclear how modification of teichoic acids facilitates resistance to the cytolysin, recent work [32] with *Staphylococcus aureus* has shown that D-alanine esterified techoic acids in the cell wall confer resistance to a number of cationic antimicrobial peptides, including the human defensin HNP1-3, mammalian-derived protegrins and tachyplesins, as well as to the bacterial peptides gallidermin and nisin. These studies suggest that the addition of D-

Table 1. Spectrum of Bacteriocin Activity of *E. faecalis* Cytolysin

Organism	Sensitivity	Reference
Enterococcus faecalis FA2-2	S	[30]
Enterococcus faecalis FA2-2 (pAM714)	R	[30]
Enterococcus faecalis X-98	R	[30]
Streptococcus mutans strain (Ingbritt)	SS	[30]
Streptococcus sanguis	S	[30]
Streptococcus salivarius	S	[30]
Streptococcus milleri	S	[30]
Streptococcus anginosus	S	[30]
Streptococcus rattus	S	[30]
Streptococcus sobrinus	R	[30]
Streptococcus mitis	S	[30]
Streptococcus pyogenes	S	[30]
Streptococcus agalactiae	S	[30]
Streptococcus lactis	S	[21]
Streptococcus mastitidis	S	[21]
Staphylococcus aureus	S	[21]
Staphylococcus aureus RN2442 (pE194)	S	[30]
Staphylococcus epidermidis	S	[21]
Leuconostoc citrovorum	S	[21]
Lactobacillus fermenti	S	[21]
Lactobacillus plantarum	S	[21]
Bacillus subtilis	WS	[21]
Bacillus cereus	S	[21]
Bacillus cereus var. *mycoides*	S	[21]
Bacillus megaterium	S	[21]
Clostridium sporognes	S	[21]
Clostridium difficile	S	[30]
Clostridium perfringens	S	[30]
Micrococcus roseus	S	[21]
Micrococcus lysodeikticus	S	[21]
Sarcina lutea	S	[21]
Corynebacterium hoagii	S	[21]
Corynebacterium pseudodiphtheriticum	S	[21]
Bacillus polymyxa	R	[21]
Escherichia coli	R	[21]
Proteus vulgaris	R	[21]

S = Sensitive; **SS** = Strongly Sensitive; **WS** = Weakly Sensitive; **R** = Resistant

alanine to teichoic acid reduced the over all negative charge of the cell wall, thus reducing the electrostatic attraction for cationic peptides. It is uncertain whether addition of D-alanine to teichoic acids is simply providing a charge barrier to the cytolysin, and/or whether the cytolysin utilizes unmodified teichoic acids as docking molecules.

Basinger and Jackson [33] demonstrated that bacterial membranes were approximately 3 orders of magnitude more sensitive than erythrocyte ghosts in inhibiting cytolysin activity. This suggests that a specific receptor on the bacterial surface may be utilized by the cytolysin, whereas binding to the erythrocyte membrane may be less specific. Because of the broad range of gram-positive species affected by the cytolysin, the putative surface receptor would have to be ubiquitous such as membrane-associated teichoic acids. Additionally, another potential candidate for such a receptor is the peptidoglycan synthesis intermediate, lipid II. Recent data has shown that the initial interaction of the lantibiotic nisin with the bacterial membrane is specific and requires lipid II [34,35, and paper by Cotter *et al.* this issue], and these experiments serve as a model for the study of the cytolysin. Studies are underway to determine the mode of antibacterial action of the cytolysin, and whether a specific bacterial receptor is required.

That the bacteriocin activity of the cytolysin might serve as a colonization niche clearance and/or control factor within the gastrointestinal tract and allow enterococci to take control of areas colonized by noncytolytic, sensitive enterococci or other gram-positive bacteria was first suggested by Brock and Davie [22]. Functioning as a colonization factor may potentiate infection by allowing overgrowth of enterococci at newly colonized sites within the intestine and subsequent invasion of extra-intestinal sites. Brock and Davie [22] observed that cytolytic strains were able to outgrow noncytolytic strains *in vitro* even when the noncytolytic strain was initially 100-fold higher in concentration. To test this hypothesis *in vivo*, Huycke *et al.* fed mice pretreated with spectinomycin and streptomycin a 1:1 mixture of cytolytic and noncytolytic strains of *E. faecalis*, and enumerated bacteria in stool samples after 1 or 7 days. Surprisingly, the authors did not observe a significant difference in the numbers of cytolytic and non-cytolytic *E. faecalis* recovered from stool specimens, even though it was shown that the cytolytic strain outcompeted and overgrew the non-cytolytic strain *in vitro*. While this experimental system failed to show a growth advantage for cells expressing cytolysin in the intestinal tract, it is difficult to know whether microecological conditions existed in this model that would be identical to those encountered naturally by cytolytic strains.

CYTOLYTIC ACTIVITIES OF THE ENTEROCOCCAL CYTOLYSIN

The genes encoding the cytolysin are encoded on mobile elements, and are found only among a subset of enterococcal strains [36,37,38,39]. The fact that not all enterococcal strains possess the cytolysin indicates that either cytolysin production is not beneficial in all ecologies, or that the mobile elements that bear this trait have not fully penetrated the species. Toxins may lyse target cells to release nutrients [40], but whether this was an important driving force for the evolution of the cytolytic activity of the enterococcal cytolysin is speculative. At the site of infection the ability to lyse target cells and release nutrients must also be expected to be carefully balanced with evasion of the immune system. The complex regulation of cytolysin expression [41] may have evolved to balance toxin activity with stealth. That the cytolysin displays toxin activity for mammals to invertebrates suggests that it targets a highly conserved feature of the eukaryotic membrane. As shown in Table **2**, the cytolysin is active against human, bovine, equine, and rabbit erythrocytes [18], and is toxic for retinal cells [42,43,44], macrophages and polymorphonuclear leukocytes [45], as well against intestinal epithelial cells [46].

Examination of the contribution of cytolysin to retinal cell toxicity [42], utilizing a rabbit endophthalmitis model, revealed extensive neural and retinal tissue destruction, measured as a loss of retinal function as a result of infection, over a 24 to 72 hour time frame. Infection with cytolytic *E. faecalis* resulted in a 99% loss of retinal function. However, infections caused by isogenic, non-cytolytic *E. faecalis* displayed a loss of only 74.2%. Another study by the same investigators reported a failure to attenuate cytolysin toxicity to retinal tissue by either antibiotics or anti-inflammatory therapy, while infections caused by isogenic, non-cytolytic strains were completely resolved in response to the same combination therapy [44].

In studies published by Miyazaki *et al.* [45], it was suggested that, in addition to cytolytic activity against erythrocytes, cytolytic strains of *E. faecalis* were also able to target mouse polymorphonuclear neutrophils and macrophages. However, whether cytolysin contributes to enterococcal resistance to PMN-mediated killing *in vivo* is unclear. *In vitro* opsonophagocytosis assays found no difference in kinetics of survival between a cytolytic strain and an isogenic noncytolytic mutant, however cytolysin production under the specific assay conditions used was not controlled [47].

Cytolytic *E. faecalis* have been shown to be active against the human intestinal epithelial cell line HT29 [11]. Recent experiments involving trypan blue staining of HT29 cells incubated with cytolytic *E. faecalis*, suggest a significant level of sensitivity when compared to cells incubated with an isogenic, non-cytolytic control strain, although 2 to 3-fold less so than human erythrocytes [11].

While the above mentioned studies all focus on the activity of cytolysin against various human and mammalian cells, it is also possible that the expression of cytolysin evolved as a defense against predatory eukaryotic organisms. Hartke *et al*. [12] observed that the elimination of *E. faecalis* from samples of seawater is mediated by several species of nanoflagellates. Zooflagellates were observed "grazing" on chains of *E. faecalis* by EM, and interestingly, no multiplication of the protozoa were observed in cultures devoid of enterococci [12]. Various species of zooflagellates may have exerted the initial predation pressure necessary to select for anti-eukaryotic activity. Additionally, Garsin *et al*. observed that *C. elegans* fed on lawns of a cytolytic *E. faecalis* strain were killed significantly more rapidly than

Table 2. Spectrum of Cytolytic Activity of *E. faecalis* Cytolysin

Target cell type	Sensitivity	Reference
Erythrocytes		
Human	S	[18]
Horse	S	[18]
Bovine	S	[18]
Sheep	R	[18]
Goat	R	[18]
Retinal tissue	S	[42]
Polymorphonuclear leukocytes	S	[45]
Human intestinal epithelial cells (HT29)	S	[11]

S = Sensitive; **R** = Resistant

worms fed on lawns of an isogenic, noncytolytic mutant strain [48]. It is uncertain whether the cytolysin is active against some or all of these aquatic organisms. However, it is conceivable that these predators for prokaryotes represent vestiges of an early environmental selection pressure for the evolution of an enterococcal defense peptide.

CYTOLYTIC ACTIVITY IS TOXIC IN MODELS OF INFECTION

Several models of enterococcal infection have been described and range from the first investigations by MacCallum and Hastings [49] of enterococcal infection using murine and rabbit models, to recent work by Garsin *et al*. [48] utilizing *Caenorhabditis elegans* to screen for virulence traits in ingested enterococci. Ike *et al*. [50] contributed the first report of the toxic ability of the enterococcal cytolysin in murine lethality tests. They observed that *E. faecalis* strains expressing the cytolysin are an order of magnitude more toxic for mice than isogenic, non-cytolytic strains, an observation confirmed by Singh *et al.* [51] who also demonstrated that the cytolysin led to a significant decrease in the LD_{50}. Dupont *et al*. [52] further expanded on these results in a different mouse strain, with an examination of the LD_{50} of several strains of *E. faecalis* expressing either the cytolysin or aggregation substance (a mating response-related surface protein), or a combination of both factors. Expression of cytolysin decreased the LD_{50} by one order of magnitude [52], but aggregation substance was found to have little or no effect on toxicity.

Wells *et al.* [53] have reported development of a murine orogastric feeding model to examine the ability of exogenously acquired *E. faecalis* to colonize and establish intestinal overgrowth, leading to transcytosis and systemic dissemination. In this study, they were able to recover *E. faecalis* from mesenteric lymph nodes, spleens, and livers of orally inoculated mice [53]. Using immunofluorescence microscopy, they were able to detect *E. faecalis* within the submucosa, columnar epithelial cells, lamina propria and muscularis externa the mouse intestinal tract. Transmission electron microscopy also indicated *E. faecalis* within vacuoles in the cytoplasm of intact epithelial cells.

The cytolysin contributes to lethality of *E. faecalis* in endocarditis [54]. Using a rabbit model, Chow *et al.* [54] established endocarditis by catheterization of animals, followed by intravenous administration of various *E. faecalis* strains defective in expression of either cytolysin or aggregation substance. Co-expression of cytolysin and aggregation substance resulted in the formation of lethal vegetations in 55% of animals, whereas lethality was observed in only 15% when aggregation substance, but not cytolysin, was produce [54]. This suggests a possible synergism between cytolysin and aggregation substance, as no mortality was observed among rabbits injected with strains expressing only the cytolysin.

Epidemiological data supports a role for the cytolysin as a toxin in human infection. Enrichment of the cytolysin among infection-derived isolates of *E. faecalis*, particularly those that cause multiple infections in a hospital ward, has been observed [36,37]. Moreover, the cytolysin has been associated with lethality to humans following analysis of an outbreak of multiple antibiotic resistant *E. faecalis* [55]. Patients infected with cytolytic, gentamicin/kanamycin resistant strains were observed to be at a five-fold increased risk of acutely terminal outcome [55].

Model systems, and analyzing infection outcome in humans, have been valuable in enhancing our understanding of the cytolysin in enterococcal virulence, but none have thus far shown it to be a colonization factor *in vivo*. As colonization was recently proposed [56] to be the initial event in a two-step sequence leading to enterococcal disease, future examination of the role of the cytolysin in colonization will require the development of new, tractable systems that will allow for a detailed analysis of the interplay between the enterococcal cytolysin and the various host factors involved in the maintenance of the commensal flora and defense against infection.

STRUCTURE OF THE CYTOLYSIN AND ITS OPERON

The active enterococcal cytolysin is now known to consist itself of two unique peptide subunits, a 38 amino acid, 3437.98 Da peptide designated $CylL_L$", and a smaller 21 amino acid, 2031.81 Da peptide designated $CylL_S$" [14]. These are amphipathic peptides which are broadly similar in size to eukaryotic defensins [57]. While the structural details for cytolysin are largely unexplored, amino acid analysis of purified enterococcal cytolysin has revealed the presence of lanthionine residues [14], which fall into the class of type A lantibiotics. Unlike most eukaryotic defensins, which are highly cationic, the active cytolysin subunits are only slightly cationic due the presence of a single lysine residue in both $CylL_S$" and $CylL_L$" subunits, and additionally a histidine residue in $CylL_L$".

Cytolysin expression requires the products of eight genes that are organized into two divergently expressed cistrons Fig. (**1**) [38,58,13,59,60,41]. The *cylL*$_L$ and *cylL*$_S$, genes specify the cytolysin structural subunit peptides, and cylM, cylB, cylA, and cylI are proteins involved in introducing posttranslational modifications into each peptide subunit, cytolysin peptide secretion, extracellular activation, and producer immunity.

$CylL_L$ and $CylL_S$ are ribosomally synthesized as 68 and 63 amino acid peptides, respectively. These peptides are then posttranslationally modified by CylM [13], which introduces modifications typical of the lantibiotic bacteriocins, including but not limited to, dehydrated residues and lanthionine moieties. CylB is required to affect secretion of both subunits across the membrane, a process that apparently requires the energetics of ATP binding and hydrolysis for $CylL_L$ transport, but not $CylL_S$ [58,13]. During transit, CylB catalyzes an initial proteolytic trimming of both subunits, a reaction catalyzed by an N-terminal cysteine protease domain [61]. A 24 and 36 amino acid leader sequence is removed from the N-terminus of $CylL_L$ and $CylL_S$ respectively, generating $CylL_L$' and $CylL_S$' [14]. The secreted forms do not possess measurable activity, and require an additional proteolytic processing reaction to generate peptides competent to affect target cell lysis. CylA, a subtilisin-like serine protease, removes an identical six amino acid sequence from the N-terminus of each subunit, generating $CylL_L$" and $CylL_S$" [59,14].

CylI has been demonstrated to encode a protein necessary and sufficient to provide protection from the bactericidal affects of the cytolysin. Cytolysin immunity represents a novel mechanism of producer self-protection in that CylI has no homologues in the current database and is not related to other lantibiotic immunity proteins [60]. However, the mechanism of producer self-protection has not been elucidated.

Finally, regulation of cytolysin expression is dependent on two small open reading frames, designated *cyl*R1 and *cyl*R2, neither of which encode products related to members of the family of histidine kinase sensor/response regulator bacterial two-component systems. The *cylR1* gene product is predicted to contain 94 amino acids and three putative transmembrane domains. The product of *cylR2* is predicted to be 66 amino acids, contains a putative helix-turn-helix DNA binding motif, and appears somewhat related to transcriptional repressor proteins. Transcriptional analysis using *lacZ* reporter gene constructs have defined the cytolysin promoter region [41], and revealed that the *cylR1*and *cylR2* genes reduce expression from the cytolysin promoter by nearly 40-fold. However, these gene products do not completely silence the promoter because activity is still 65-fold higher when compared to the promoterless *lacZ* gene. Both *cylR*1 and *cylR2* are required for repression as mutations in either *cylR* gene restored high-level reporter gene activity. Recently, CylR2 has been purified and shown by gel-shift analysis to bind specifically to an inverted repeat sequence overlapping the –35 sequence of the cytolysin structural gene promoter [62]. Additionally, CylR2 has recently been crystallized, which has revealed dimerization and the presence of a helix-turn-helix DNA binding domain. Furthermore, NMR spectroscopy data has revealed the contact point between CylR2 and DNA as a positively charged region [62].

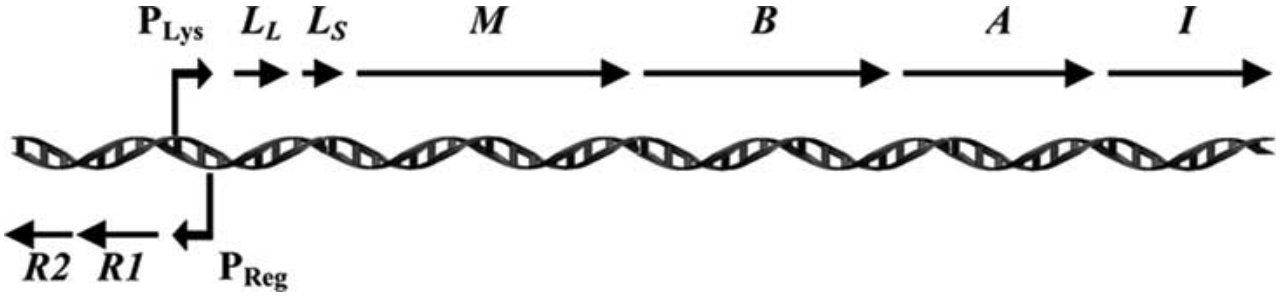

Fig. (1). Genetic organization of the cytolysin operon. P_{Lys} is the promoter for the structural genes, all of which are read in the rightward direction. *CylL*$_L$ and *CylL*$_S$ encode the initial precursor forms of the large and small cytolysin structural subunits, respectfully, which are post-translationally modified by the *cylM* gene product. *CylB* encodes a C-terminal ATP-binding cassette transporter, responsible for secretion of both subunits across the cytoplasmic membrane. Additionally, CylB possesses an N-terminal cysteine protease domain, which, upon secretion, further modifies both subunits by removing 24- and 36-amino-acid leader sequences, the end-products of which are termed $CylL_L$' and $CylL_S$'. *CylA* encodes a serine protease which removes an identical 6-amino-acid sequence from the N-terminus of both $CylL_L$' and $CylL_S$' to yield the fully active $CylL_L$" and $CylL_s$". *CylI* encodes a polypeptide which protects the cytolysin producer from the bactericidal activity of the cytolysin. P_{Reg} is the promoter for the cytolysin regulatory genes *cylR1* and *cylR2* that are involved in regulation of cytolysin expression by quorum sensing.

A NOVEL MECHANISM OF GENE REGULATION GOVERNS CYTOLYSIN EXPRESSION

Cytolysin expression is governed by a novel mode of gene regulation requiring both *cylR1* and *cylR2* gene products to cooperatively silence the cytolysin promoter. Activation of cytolysin expression was shown to require the activated form of $CylL_S$ ($CylL_S$") [41]. A transcriptional fusion of *cyl*R1/R2, the cytolysin promoter region, and a promoterless *lacZ* gene was used in reporter gene assays to evaluate conditions that would result in β-galactosidase expression. Neither preactivated $CylL_S$' or $CylL_L$', or the activated form of $CylL_L$ ($CylL_L$") was capable of eliciting a response from the reporter strain. Only $CylL_S$" was capable of inducing β-galactosidase expression, and a quorum-sensing mechanism was confirmed by real-time polymerase chain reaction experiments which indicated a threshold concentration of $CylL_S$" for maximal activation of cytolysin gene expression. Quorum sensing regulation requires that the autoinducer molecule reach a critical concentration, as a result of increases in bacterial density, to activate transcription of responsive genes [63]. A quorum-sensing, autofeedback type of regulatory mechanism would entail low-level expression from the cytolysin structural gene promoter, allowing accumulation of $CylL_S$" during bacterial replication. This assumes that cooperative repression by CylR1 and CylR2 is leaky and that a basal level of transcription occurs from the cytolysin structural gene promoter, an assumption supported by reporter gene data [41].

In the current model, based on the requirement of both CylR1 and CylR2 for repression, and recent data demonstrating CylR2 binding to cytolysin promoter DNA [62], repression is predicted to require the binding of CylR2 to the cytolysin structural gene promoter DNA. As CylR1 is also required, it is predicted that CylR1 interacts with CylR2 to stabilize a repressor complex bound to the cytolysin promoter. Basal transcription through a "leaky" repressor complex would result in expression of the cytolysin structural and auxiliary genes, ultimately leading to accumulation of $CylL_S$". High concentrations of $CylL_S$" are predicted to cause destabilization of the repressor complex, and allow high-level transcription of the cytolysin genes. Recently, reporter gene assays have suggested that $CylL_L$" also plays a key role in controlling cytolysin expression [64]. It was observed that wildtype strains elaborating both $CylL_L$" and $CylL_S$" elicited less β-galactosidase expression from the reporter strain than a strain elaborating only $CylL_S$". Furthermore, addition of purified $CylL_L$" to a strain producing $CylL_S$" inhibited β-galactosidase expression from the reporter strain. Preincubation of the purified $CylL_L$" with erythrocytes was found to significantly alleviate this inhibition. Interestingly, surface plasmon resonance studies have demonstrated that the $CylL_L$" subunit binds to phosphatidylcholine:cholesterol lipid bilayers with a significantly higher affinity than $CylL_S$". Based on these studies, a model for the regulation of cytolysin that links sensing of the inducer molecule $CylL_S$" to the presence or absence of a suitable target cell has been proposed [64]. In the absence of a target cell, $CylL_L$" and $CylL_S$" are maintained only at basal levels. We have shown that the subunits interact to form an insoluble oligomeric complex that effectively reduces the concentration of free inducer $CylL_S$". Thus, $CylL_L$" acts to titrate the level of free $CylL_S$" in solution to below the threshold level necessary to trigger high-level cytolysin production. However, in the presence of a target cell, $CylL_L$" binds preferentially to the membrane, thus reducing the concentration of $CylL_L$" in solution, and allowing the free accumulation of $CylL_S$" to the level necessary to activate high-level cytolysin expression [64]. Thus, this would provide a mechanism whereby the bacterium produces high levels of cytolysin only when required by simultaneously detecting both the accumulating inducer $CylL_S$" molecule and a target cell via the membrane sensor $CylL_L$". Furthermore, this would provide an additional fail safe mechanism for protecting the producer from the bactericidal affects of the cytolysin.

The Current Understanding of Structure/Function Relationships: Toxin, Bacteriocin, and Signaling Activities Reside in Distinct but Overlapping Faces of each Peptide

Site-directed mutagenesis studies have provided insight into the requirement of modified, as well as various unmodified amino acids for the bacteriocin activity displayed by the lantibiotic peptides (reviewed in [15]). Based on these types of studies, it has been hypothesized that certain amino acid alterations may render a bacteriocin toxic to eukaryotic cells. For the cytolysin, certain amino acids may be involved in only bacteriocin and not toxin activity. However, less critical amino acids within the ancestral bacteriocin may have been altered to confer toxin activity. A systematic approach to dissecting the relationship between toxin, bacteriocin, and also signaling activities within the cytolysin, and for determining the importance of modified and unmodified amino acids to these activities is being taken to examine structure and function releationships. Alanine-scanning mutagenesis is being used to alter each amino acid throughout both the $CylL_L$ and $CylL_S$ subunits, and the resultant alanine replacement mutants are being analyzed for altered toxin, bacteriocin, and signaling activity. Given the hydrophobic nature of the cytolysin, alanine served as a conservative starting point for these types of studies. Somewhat surprisingly, cytolysin and bacteriocin activities are distinct, although both require an overlapping set of amino acids. Hemolysin, bacteriocin, and signaling activities are separable activities, i.e., mutants of both $CylL_L$" and $CylL_S$" were observed that retained hemolysin activity but not bacteriocin activity, and vice versa, when combined with the complementary wildtype subunit. $CylL_S$" mutants that were devoid of signaling activity, but still retained toxin and bacteriocin activity when combined with wildtype $CylL_L$", were also observed [65].

CONCLUSIONS

The enterococcal cytolysin is a unique, two-peptide lytic toxin that has evolved multiple activities in a single system. Expression of cytolysin not only affords enterococci the ability to evade eukaryotic predatory assailants, but also conveys an advantage over prey target cells in the context of a niche control system, which possibly evolved in the complex microbial ecology associated with a broad range of eukaryotic host organisms. While structurally it has been

classified as a distant relative of Streptolysin S and the lantibiotic family of bacteriocins, cytolysin shares several attributes with eukaryotic host defensins. Cytolysin's similar small size, bacteriocidal activity against a broad range of gram positive species, and likely insertional lysis of target membranes are strikingly similar to the known eukaryotic host defense peptides. As current research continues to unravel the details of cytolysin regulation, structural characteristics, and mode of action against multiple prokaryotic and eukaryotic cell types, new, tractable systems designed to evaluate the role of cytolysin in the interactions between enterococci, the host innate immune system, and members of the commensal flora, are currently being developed. These studies and others should continue to extend our understanding of cytolysin as a unique antimicrobial peptide that serves as a bacterial host defensin.

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