Int.J.Curr.Microbiol.App.Sci (2021) *10*(10): 142-155

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 10 Number 10 (2021)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2021.1010.017

Potential Application of Phage ϕ11 Lytic Proteins in Rapid Detection and Elimination of *Staphylococcus aureus*

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Keywords

Staphylococcus aureus, Phage ϕ11, endolysin, VAPGH, antibiotic resistant, MRSA, antimicrobials

Article Info

Accepted:
10 September 2021
Available Online:
10 October 2021

A B S T R A C T

The increasing antibiotic resistance conferred by *Staphylococcus aureus* to multiple potential antibiotics has become a serious issue of concern and threat to mankind worldwide. In light of this, phage lytic proteins have been reported which show potential antimicrobial activity against pathogenic microorganisms that could be a promising alternative to antibiotics to eradicate the antibiotic resistant problems. This review discusses the various applications of *S. aureus* phage lytic proteins and the potentiality of aureophage phi 11 endolysin and virion associated peptidoglycan hydrolase (VAPGH) against staphylococcus strains. Phage Phi11 endolysin harbors two enzymatically active domain; cysteine and histidine-dependent amidohydrolase/peptidase (CHAP) and Amidase 2 at the N-terminus and a cell wall binding domain (CBD) SH3 5 at the C-terminus, while virion associated peptidoglycan hydrolase (VAPGH) has two catalytic domains, CHAP and Glucosaminidase (Mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase) at its N-terminal and C-terminal, respectively.

Introduction

Dating back to the 19th century when phages were first discovered by the Englishman Frederick Twort in 1915 (Twort,1915) and the French-Canadian Felix d'Hérelle in 1917 (D'Herelle,1917), many countries like Russia, Georgia and Poland immediately recognized the potential antimicrobial ability of bacteriophages and phage therapy was researched and developed extensively during World War II to treat wounded soldiers and other bacterial infections until the antibiotic penicillin G was successfully developed in 1942. However, over the years, due to ill-usage of antibiotics, antibiotic resistance exhibited by many pathogenic microorganisms such as *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Bacillus anthracis*, *Streptococcus pneumoniae*, etc., is

a major clinical challenge worldwide. *Staphylococcus aureus* is distinctively one of the most important human pathogenic bacterium that is found normally in the mucous membranes and on the skin, causing minor skin infections to even more fatal disease conditions. A significant emergence of *S. aureus* strains resistant to one or combination of antibiotics, such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), and multiple-drug-resistant *S. aureus* (MDRSA) have been reported (Tong *et al.,* 2015). With this concerning scenario, phage lytic proteins that are involved in the phage's lytic life cycle are being researched again envisaging that it might play a significant role in eradicating the antibiotic resistant problem. Bacteriophages are the most abundant organisms, more than any other biological entity (10-100 million per gram of soil or cc of water) on the earth that infects a bacterial cell to replicate and produce its progeny. In the beginning of its infection, it lyses the bacterial cell from outside with its tail/virion associated peptidoglycan hydrolase (VAPGHs) and at the end of their lytic cycle, its progeny virions are released from inside of the host bacterial cell by lysing the cell wall with a phage encoded protein known as endolysin.

Phage lytic proteins have one or more enzymatic domains in the N-terminal that cleave specific peptidoglycan bond in the bacterial cell wall and can be distinguished into *N*-acetylmuramoyl-L-alanine amidase, interpeptide bridge endopeptidase, L-alanyl-D-glutamate endopeptidase, *N*-acetyl-β-D-muramidase, *N*-acetyl-β-D-glucosaminidase depending on their specific peptidoglycan target, and a cell wall binding domain (CBD) in the C-terminal in case of endolysin. CBD is essential for recognition and binding to specific cleavage sites on the cell wall of specific bacterial strains, and also help attain its maximum catalytic activity (Schmelcher *et al.,* 2012a). Truncated endolysin with no intact CBD showed minimum or no catalytic functions on SDS cell walls without the cell wall binding domain even in the presence of the two N-terminal catalytic domains (Sass & Bierbaum, 2007). Staphylococcal phage lytic proteins generally have either CHAP domain or Amidase-2/3 or both domains together at the N-terminal and the C-terminal end of endolysin contains a cell wall binding domain (CBD) that are most generally SH3 5 and SH3b in Staphylococcal phage endolysins. SH3 domains are generally small proteins 60 amino acid residues, recognition domains mostly involved in signaling pathways.

The SH3 fold consists of mainly beta-sheets arranged in a two anti-parallel β sheets manner that lie at right angles to each other as predicted in its structure and it binds to ligands that are rich in proline motifs (**P--P**) (Zarrinpar *et al.,* 2003). *Staphylococcus aureus* Phage ϕ11 is a temperate phage and it is one of the most genetically characterized *S. aureus* phages because it exhibits very high transducing efficiency and is often used to study transfer gene mutations between *S. aureus* strains. The phage genome is arranged into six functional modules: lysogeny, DNA replication, packaging, head, tail, and lysis. Virion-associated peptidoglycan hydrolases (VAPGHs) is located upstream of phage's two-component lysis module, while ORF 53 in the lytic system encodes the protein endolysin (Xia & Wolz, 2014Among the phage encoded lytic proteins reported till date, phage phi 11 endolysin has also been identified as a potent antistaphylococcal lytic protein although no work has been done on its VAPGH so far.

This review encompasses the multi-functional ability of *S. aureus* phage lytic proteins against *S. aureus*, emphasizing mainly on *S. aureus* phage ϕ11 lysins as another potential candidate.

Applications of Staphylococcus phage lytic proteins

Anti-staphylococcal applications against *S. aureus* infections

Phage encoded endolysin and virion associated cell wall hydrolase (VAPGHs) as well as chimeric lysins are gaining considerable interest as an alternative in eradicating antibiotic resistant strains to conventional antibiotics. Among the pathogenic bacteria, *S. aureus* is one of the most harmful human pathogenic bacteria that confer resistance to many potential antibiotics as mentioned earlier. Plenty of reports in support to phage lytic proteins as possible potential anti staphylococcal agent in vitro and in vivo have been established against *S. aureus* infections. Some of the well characterized and potent bactericidal phage encoded endolysin includes, MV-L from phage phiMR11 (Rashel *et al.,* 2007), Lysk from phage K (O'Flaherty *et al.,* 2005a; O'Flaherty *et al.,* 2005b), PlyGRCS from phage GRCS (Linden *et al.,* 2015), Lys-phiSA012 from phage phiSA012 (Fujiki *et al.,* 2018) and LysGH15 from phage phiGH15(Gu *et al.,* 2011a; Gu *et al.,* 2011b; Zhang *et al.,* 2016), have shown to exhibit efficient lytic activity against *S. aureus* strains including antibiotic resistant strains (VRSA, MRSA, VISA and teicoplanin-resistant) invitro as well as in infected mouse models. An ointment has also been formulated with LysGH15 and Apigenin (LysGH15-api-Aquaphor ointment) which showed bactericidal activity against *S. aureus* and anti-hemolysis (Cheng *et al.,* 2018).

The modular domain structure of endolysin also provides an advantage to recombine the catalytic domain and cell wall binding domain of different phages and construct an engineered lysin that exhibits higher bacteriolytic activity, specificity, solubility, and other physicochemical nature of these enzymes which is prerequisite of a potential antimicrobial. For example, ClyS(Daniel *et al.,* 2010), ClyH (Yang *et al.,* 2017), ClyF (Yang *et al.,* 2014), Ply187AN-KSH3b (Singh *et al.,* 2014; Mao *et al.,* 2013), these chimeric lysins have demonstrated efficient lytic activity against many antibiotic resistant *S. aureus* strains in vitro as well as in vivo. Also, a topical ointment containing ClyS has been reported to be more effective than mupirocin on a *S. aureus* infected skin (Pastagia *et al.,* 2011).

Although, only few works have been reported on the lytic activity of *S. aureus* phages' VAGPHs against *S. aureus*, it has also shown promising effects in vitro. Some examples include, gp61 of phage MR11 (Rashel *et al.,* 2008), truncated-fusion and whole HydH5, which have shown efficient lysis of live *S. aureus* cells and *S. aureus* Sa9 cells, bovine and human *S. aureus* strains, MRSA strain N315, and human *Staphylococcus epidermidis* strains in three different studies by Rodriguez *et al.,* (Rodríguez *et al.,* 2011; Rodríguez *et al.,* 2012; Rodríguez *et al.,* 2013). Also, some other chimeric peptidoglycan hydrolases against *S. aureus* strains such as; P16-17 of phage P68 (Manoharadas *et al.,* 2009; Takác and Blas, 2005) and P128 of phage K against global clinical isolates of *S. aureus* including drug resistant strains in vitro as well as in vivo (Sundarrajan *et al.,* 2014; Channabasappa *et al.,* 2018; Paul *et al.,* 2011) have been reported.

Application in food safety

Food-borne diseases in humans cause by *S. aureus* and its derived toxins are also a concerning global issue and therefore its detection and eradication in foods and biofilm destruction on food surfaces and utensils is an emergent need. Purified lytic proteins have been demonstrated as a safe and stable

additive into food products to control the contamination of food borne pathogens. For example, LysH5 have shown rapid lysis of *S. aureus* present in pasteurized milk after 4 h of incubation at 37˚C (Obeso *et al.,* 2008) and also biofilm removal. Purified LysSA97 in synergy with carvacrol have shown anti staphylococcal activity in skimmed milk, whole milk and lean beef (Chang *et al.,* 2016). Purified LysSA11 endolysin have also shown significant reduction in MRSA colony in milk and ham as well as on the surfaces of polypropylene plastic cutting boards and stainless-steel knives (Chang *et al.,* 2017). Three lytic proteins (HydH5Lyso, HydH5SH3b, CHAPSH3b) constructed by fusion of different domains of *Staphylococcus aureus* phage vB_SauS-phiIPLA88 VAPGH HydH5 and lysostaphin have also demonstrated antimicrobial activity against *S. aureus* strain Sa9 in milk and extension of commercial whole milk shelf life (Rodríguez *et al.,* 2013).

Synergistic application of phage lytic proteins with antibiotics

Potential synergistic ability of endolysin can be achieved by combination with VAPGHs or other phage's endolysin or antibiotics. This synergism can lead to improved lytic efficacy, requirement of low antimicrobial dosage and reduces resistance by targeting different multiple cleavage sites or the cleavage of a particular site by the first catalytic domain which indirectly enhances the lytic activity of the conjugated endolysin catalytic domain or VAGPHs or antibiotic. Becker *et al.,* (2008) have reported the synergistic effects observed in vitro of *S. aureus* endolysin LysK CHAP domain with lysostaphin glycylglycine endopeptidase (Becker *et al.,* 2008). Schmelcher *et al.,* (2012b) have also investigated the synergistic effect of two chimeric proteins of the streptococcal λSA2 endolysin endopeptidase domain conjugated with cell wall binding domains of lysostaphin and LysK and both the constructs showed lysis of 16 different *S. aureus* mastitis isolates, including penicillin-resistant strains in vitro and in vivo in mouse model (Schmelcher *et al.,* 2012b). Endolysin MV-L from *S. aureus* phage phi MR11 have also showed synergistic staphylocidal effect with glycopeptide antibiotics vancomycin and teicoplanin against VISA and prevention from MRSA septic death by intraperitoneal administration of MV-L without any observed side effects (Rashel *et al.,* 2007). Chimeric staphylococcal endolysin ClyS have also shown synergistic effect in combination with vancomycin and oxacillin by protecting mice from MRSA induced septicemia (Daniel *et al.,* 2010). Also, LysH5 endolysin encoded by the staphylococcal bacteriophage phi-SauS-IPLA88, in combination with nisin increased the lytic activity of LysH5 by 8-fold and showed a strong synergistic effect in eliminating *S. aureus* Sa9 present in pasteurized milk (Garcia *et al.,* 2010).

Application in detection of *S. aureus* strains

S. aureus infections are mostly mediated by the production of its virulence factors such as staphylococcal exotoxins (SEs), among which SEA and SEB are most commonly found and characterized for pathogen detection (Pinchuk *et al.,* 2010). Systematic and rapid detection of the bacterium or its toxins is a critical step in order to eradicate the pathogen efficiently. Wu *et al.,* (2016) have also reviewed different types of conventional and modern techniques, and its limitations for detection of *S. aureus* toxins. Early conventional methods include direct tests on animal models which involves negative ethical issues. More reliable, rapid, sensitive and specific methods which include, antigen-antibody binding based serological tests such as gel diffusion, agglutination assays and ELISA, and immunoassays coupled with sensitive detecting elements;

molecular biological techniques such as specific hybridization and PCR based methods and chromatographic techniques, have been developed, however, it requires highly stable antibodies, expensive and complicated instruments, detection elements and PCR machines as well as skilled trained professionals to handle.

Recently, aptamer (short ssDNA or RNA and peptides) based biosensors proves to be a better alternative to counter the associated limitations with traditional techniques and assays which employ antigen/antibody based for microbial detection. Aptamers generally are shorter and possess smaller molecular weight than antibodies which make them superior in stability. It can be easily synthesized in vitro and also allows flexible modification with various chemical tags such as fluorophores and nanoparticles that do not interfere with efficient target recognition (Wu *et al.,* 2016). Phage lytic proteins can be modeled as short aptamers, owing to its structure characterized by different functional domains that can be reconstructed. The C-terminal cell wall binding domain binds specifically to its host bacteria's peptidoglycan layer as reported in many studies. This specific affinity of endolysin CBDs to bacterial cell walls can be exploited in different ways to detect infectious bacteria in clinical and food safety as shown in Fig. 1.

One of the simplest and mostly reported method is fluorescent microscopic detection of target pathogens by phage endolysin CBDs tagged with fluorescent proteins, which can be detected upon specific binding to targeted cell walls. Gu *et al.,* (2011c) have reported the specific binding of a fusion endolysin protein, LysGH15B with green fluorescent protein (LysGH15B-GFP) to staphylococcal isolates, including MRSA, while the unbound GFP did not display any fluorescence when scanned under laser scanning confocal microscope (Gu *et al.,* 2011c). Listeria strains from a mixed bacterial culture were rapidly differentiated by conjugating listeria infecting phage endolysin with fluorescent markers (Schmelcher *et al.,* 2010). Loessner *et al.,* (2002) have also demonstrated specific binding of two Listeria monocytogenes phage endolysins Ply118 and Ply500 CBDs fused with GFP to listeria cells of different serovar groups (Loessner *et al.,* 2002). Similarly, The CBD domain from a streptococcal phage was fused with GFP which showed that the recombinant CBD was able to bind to living cells of *Staphylococcus aureus* and *Streptococcus agalactiae* (Jarabkova *et al.,* 2020).

Secondly, surface immobilization of target cells on magnetic beads or magnetic nanoparticles coated with CBDs can be employed for efficient recovery and detection of pathogens in food, water and blood samples. Chibli *et al.,* (2014) have exploited the specific binding ability of phage endolysins LysK and phi11, and the bacteriocin lysostaphin, coated on silicon wafers to detect staphylococcal strains wherein it specifically binds to eight tested clinical isolates of *S. aureus* and *S. epidermidis* (Chibli *et al.,* 2014).

Paramagnetic beads coated with recombinant Listeria phage endolysin-derived CBDs have also shown efficient and sensitive detection than conventional standard procedures of *L. monocytogenes* cells from suspended cultures as well as artificially and naturally contaminated food samples (Kretzer *et al.,* 2007). Similarly, a novel strategy has been developed by Yi *et al.,* (2019) for sensitive detection of *S. aureus*, which employs PlyV12 CBD-functionalized magnetic beads for separation and detection by fluorescence system of horseradish peroxidase (HRP), hydrogen peroxide and Amplex Red (Yi *et al.,* 2019). Park *et al.,* (2018) performed an ATP bioluminescence assay for *B. cereus* detection

using CBD-conjugated magnetic nanoparticles (CBD-MNPs) which showed sensitive detection up to as few as 10 CFU/ml and 103 CFU/ml in buffer and blood, with no cross-reactivity with other microflora (Park *et al.*, 2018).

Conjugation of CBDs to nanoparticles is another feasible method which can be exploited in many pathogen detection assays. Nanoparticles can be of different types such as magnetic nanoparticles, fluorescent nanoparticles and metallic nanoparticles, for example, gold or silver nanoparticles, which are advantageous owing to their large surface area for immobilization of different molecules that can bind specifically to their target molecules, and also the ability to emit signals upon binding. Kong *et al.,* (2017) have developed a nitrocellulose-based lateral flow assay employing CBD conjugated colloidal gold nanoparticle for the detection of *Bacillus cereus.* The result showed sensitivity up to 1×104 CFU/ml in 20 minutes and superior to conventional antibody-antigen based assay (Kong *et al.,* 2017). Kim *et al.,* (2018) have also demonstrated the selective killing of *Bacillus anthracis* from a mixed culture including *B. subtilis* and *S. aureus* by silver nanoparticle conjugated with fluorescent protein tagged-CBD of *B. anthracis* (Kim *et al.,* 2018).

Staphylococcus aureus phage phi 11 lytic proteins

Till date, few studies have been reported only on *S. aureus* phage ϕ11 endolysin as a potent anti-staphylococcal lysin. Navarre *et al.,* (1999) first reported the lytic activity of its N-terminal catalytic domains, CHAP and Amidase 3 on Staphylococcal cell wall and solubilized surface protein from staphylococcal cell wall (Navarre *et al.,* 1999). Phi 11 endolysin has also shown potential in vitro lysis and anti-biofilm activity against a number of staphylococcal strains including *S. epidermidis* and *S. simulans,* cell surface mutants and MRSA by whole endolysin as well as truncated parental endolysin as efficient as lysostaphin. Also, in vivo treatment of systemic MRSA infected mouse models with phi 11 endolysin, showed 100% protection from death (Schmelcher *et al.,* 2015; Sass & Bierbaum, 2007). In another study, the whole phi 11 endolysin and truncated part that possessed only its CHAP domain have also shown great anti staphylococcal activity against live whole cells of mastitis pathogens, *Staphylococcus aureus* and coagulase-negative staphylococci (*Staphylococcus chromogenes, Staphylococcus epidermidis, Staphylococcus hyicus, Staphylococcus simulans, Staphylococcus warneri* and *Staphylococcus xylosus*) as well as in contaminated milk (Donovan *et al.,* 2006).

Staphylococcus aureus bacteriophage ϕ11 endolysins

Phage Phi 11 endolysin is 481 amino acid residues long encoded by ORF 53, with GenBank accession no. AAL82281.2. It possesses two enzymatically active domains (EAD); CHAP (D-alanyl-glycyl endopeptidase) and Amidase 2 (N-acetylmuramyl-L-alanine amidase) at its N-terminal and a CBD (cell wall binding domain) at its C-terminal site (Fig 2a), as predicted by PFAM 33.1 server (El-Gebali *et al.,* 2019). Phi 11 endolysin cleaves at two sites of *S. aureus* peptidoglycan layer, the D-Ala-Gly peptide bond by CHAP and the *N*-acetylmuramyl-L-Ala amide bond by Amidase-2 as suggested by Navarre, *et al.,* (1999). The C-terminal cell wall binding site belongs to SH3 5 (SRC Homology) CBD group that bears close homology to the cell wall targeting domain of lysostaphin (Navarre *et al.,* 1999).

Fig.1 Schematic diagram showing different phage endolysin cell wall binding domain's conjugates for efficient pathogen detection

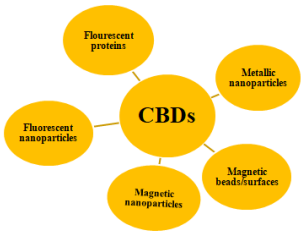


Fig.2 Conserved domain analysis by PFAM 33.1. (a) Three domains recognized on Phi 11 endolysin; CHAP (20 to 113 amino acids), Amidase 2 (196 to 324 amino acids) and SH3 5 CBD (395 to 460 amino acids). (b) Two domains identified on Phi 11 VAPGH; CHAP (34 to 120 amino acids) and Glucosaminidase (494 to 616 amino acids)

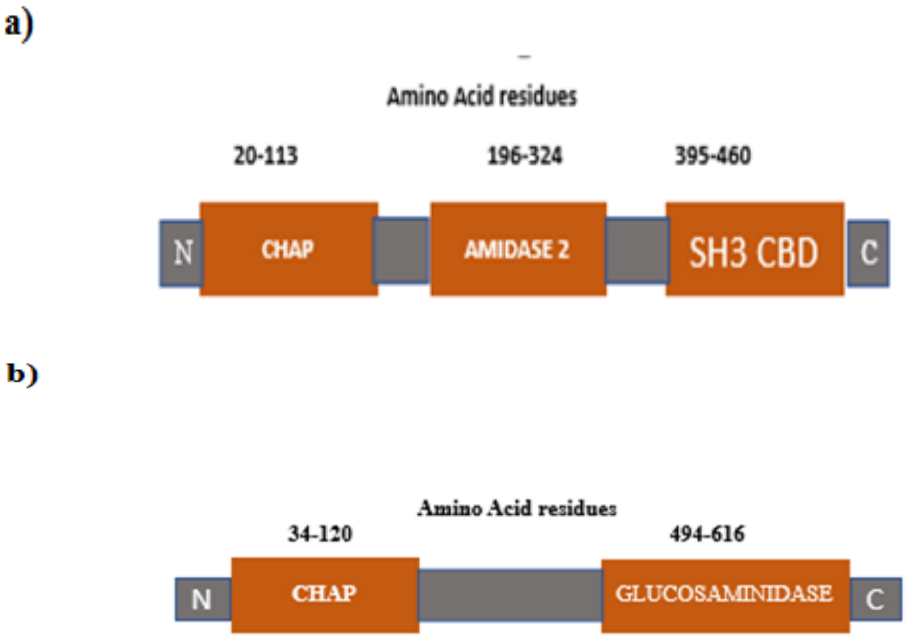
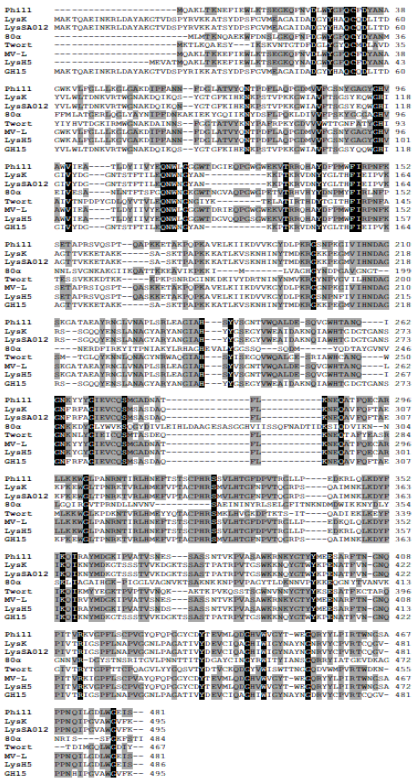
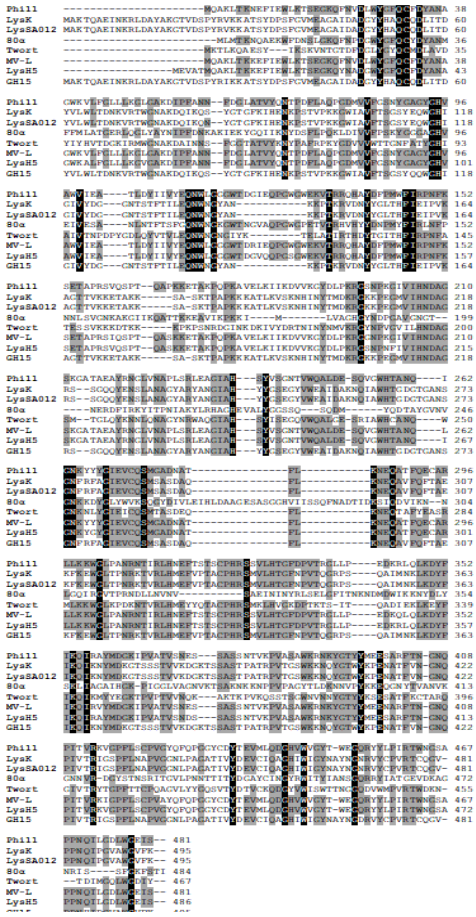


Fig.3 Multiple sequence alignment by CLUSTAL OMEGA; amino acid residues conserved between the aligned protein sequences are highlighted by black (100%) and grey (up to 50%). (a) Phi 11 endolysin aligned with other seven reported endolysins from *S. aureus* phages; LysK (YP_009041293.1); PhiSA012 (YP_009006722.1); 80α-LYT2 YP_001285385.1);Twort(YP_238716.1); MV-L (YP_001604156.1); LysH5 (ACE77796.1); and GH15 (ADG26756.1). (b) Phi 11 cell wall hydrolase (VAPGH) aligned with other three reported VAPGHs from *S. aureus* phages; GP61 **(**ACJ64589.1); HydH5 (ACJ64586); and 80α (YP_001285381.1).

(a)



(b)



Sequence alignment with seven other well characterized *S. aureus* phage endolysin using CLUSTAL OMEGA software (Madeira *et al.,* 2019), showed significant sequence similarity with MV-L and LysH5 (97.7 % and 96.1% respectively) (Fig 3a). The stability of the recombinant phi11 endolysin at different storage (4°C and 22°C) and working temperatures (37°C) and pH level have also been studied. They have stated that secondary structures of the protein play the primary role in all the factors determining its stability. From the CD spectra of secondary structures, it has observed a sharp rise in the number of random coils (26.8 ± 4.2% to 43.1 ± 4.0%), with increasing temperature and pH. The report showed that an increase in pH from 7.5 to alkaline range (up to 9) and temperature from 4°C to 22°C to 37°C, the half inactivation time of the protein decreased from 140 days to 8 days to 10 mins, respectively (Filatova *et al.,* 2016).

Staphylococcus aureus phage phi 11 virion associated peptidoglycan hydrolase (VAPGH)

Phi 11 VAPGH has 632 amino acid residues (GenBank accession no. AAL82277.1) constituted of only two EADs (CHAP and Glucosaminidase) and no CBD(Fig 2b), as predicted by PFAM 33.1 server (El-Gebali *et al.,* 2019). Sequence alignment with other three reported S. *aureus* phage VAPGHs using CLUSTAL OMEGA software (Madeira *et al.,* 2019), have shown a significant sequence similarity with 80α and HydH5 (98.4 % and 90.1% respectively) followed by G61 which

shows only 57% similarity (Fig 3b). Although no work has been done on the cell lysis potency of phi 11 VAPGH, it shows significant sequence homology to HydH5, which have been well characterized and reported as a potent staphylococcal lytic protein which is also highly thermostable, retaining its activity up to 72% at 100°C (Rodriguez *et al.*, 2011).

The whole idea of lytic protein as an antibacterial is that it should be able to efficiently lyse the bacteria from outside when applied exogenously, which gives a bigger prospective to VAPGH considering its natural mode of action that lyse a bacterial cell from outside against different physiological and environmental conditions in contrast to the activity of phage encoded endolysin, which lyse the cell from within in a closed defined system that in most cases after holin-mediated cell membrane disruption.

Bacteriophages and its lytic proteins may be the new frontier in combating infectious and antibiotic resistant pathogenic bacteria. Notable progression has been made in recognition of phage lytic proteins as prospective antibacterial agents against *S. aureus*, however therapeutically it is still objectionable in most cases and some limitations associated particularly with systemic application still need to be addressed, which includes, stability (half-life inactivation time), immunogenicity, toxicity and efficient penetration into specific cell or tissues. Many potential aspects of bacterial phage lytic proteins as a whole or as engineered chimeric lysins have been discussed in this review. The potentiality of phi 11 endolysin have been reported by few studies although not much work has been done with phi 11 VAPGH, more distinctively on its lytic activity. As discussed earlier, VAPGH lyses a bacterial cell from outside during the initial infection cycle against different environmental constraints and specifically even without the presence of a cell wall binding domain. Considering all these propensities of a VAPGH and its significant homology with HydH5, phi 11 VAPGH can also be a potential prospect against *S. aureus* strains, including antibiotic resistant strains.

Acknowledgments

The authors gratefully acknowledge the logistic support of Department of Biotechnology, Nagaland University, India. Ms. Amenti is also thankful to Department of Biotechnology, Government of India for providing regular monthly Research Scholarship.

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How to cite this article:

Amenti and Rajkrishna Mondal. 2021. Potential Application of Phage ϕ11 Lytic Proteins in Rapid Detection and Elimination of *Staphylococcus aureus*. *Int.J.Curr.Microbiol.App.Sci.* 10(10): 142-155. doi: https://doi.org/10.20546/ijcmas.2021.1010.017