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REVIEW

Recombinant Endolysins as Potential Therapeutics against Antibiotic-Resistant *Staphylococcus aureus*: Current Status of Research and Novel Delivery Strategies

Hamed Haddad Kashani,[a] Mathias Schmelcher,[b] Hamed Sabzalipoor,[c] Elahe Seyed Hosseini,[a] Rezvan Moniri[a,d]

[a]Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan, Iran
[b]Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland
[c]Department of Nanobiotechnology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[d]Department of Immunology and Microbiology, Kashan University of Medical Sciences, Kashan, Iran

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SUMMARY *Staphylococcus aureus* is one of the most common pathogens of humans and animals, where it frequently colonizes skin and mucosal membranes. It is of major clinical importance as a nosocomial pathogen and causative agent of a wide array of diseases. Multidrug-resistant strains have become increasingly prevalent and represent a leading cause of morbidity and mortality. For this reason, novel strategies to combat multidrug-resistant pathogens are urgently needed. Bacteriophage-derived enzymes, so-called endolysins, and other peptidoglycan hydrolases with the ability to disrupt cell walls represent possible alternatives to conventional antibiotics. These lytic enzymes confer a high degree of host specificity and could potentially replace or be utilized in combination with antibiotics, with the aim to specifically treat infections caused by Gram-positive drug-resistant bacterial pathogens such as methicillin-resistant *S. aureus*. LysK is one of the best-characterized endolysins with activity against multiple staphylococcal species. Various approaches to further enhance the antibacterial efficacy and applicability of endolysins have been demonstrated. These approaches include the construction of recombinant endolysin derivatives and the development of novel delivery strategies for various applications, such as the production of endolysins in lactic acid

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Address correspondence to Mathias Schmelcher, mathias.schmelcher@hest.ethz.ch, or Rezvan Moniri, hamedir2010@gmail.com.

bacteria and their conjugation to nanoparticles. These novel strategies are a major focus of this review.

KEYWORDS antibiotic resistance, endolysin, *Staphylococcus aureus*, probiotic bacteria, nanoparticles, infectious diseases

INTRODUCTION

Staphylococcus aureus is a coccoid Gram-positive bacterium with a thick peptidoglycan layer (1), also known as a murein layer (Fig. 1). This murein layer consists of long ionic polymers comprising the alternating amino sugars *N*-acetylglucosamine and *N*-acetylmuramic acid that make up the glycan chains and peptide linkers connecting these glycan chains to a three-dimensional network. In the case of *S. aureus*, these peptide linkers consist of conserved stem peptides, which are interconnected through five consecutive glycines, the so-called pentaglycine bridge (2). Another characteristic feature of *S. aureus* peptidoglycan is O-acetylation at the C_6-OH position of muramic acid, rendering the bacterium resistant to lysozyme (3). Teichoic acids covalently linked to the peptidoglycan function as regulators of cell growth, as phage receptors, epitopes, for attracting cations, and as tools for pathogens to communicate with the environment (1, 4).

S. aureus is known to be a part of the normal microflora. This bacterium resides primarily on the nares, skin, and mucosal membranes of humans and animals and poses no threat to the host species under normal circumstances (5). The colonization rate in healthy adults is between 5 and 30%, and 10 to 20% of individuals exhibit permanent colonization (6). Conversely, bacteria from healthy individuals can pose a risk of transmission to the immunocompromised population, yielding detrimental effects on those infected (7, 8). A variety of diseases can be caused by staphylococcal strains, ranging from rather harmless localized skin infections to systemic infections upon the entry of bacteria into the blood as well as acute and chronic infections of various organs such as heart, bones, and lungs (9, 10). Sepsis, endocarditis, and toxic shock syndrome are examples of life-threatening diseases caused by *S. aureus* (11). One of the critical risk factors for the development of hospital-acquired (HA) and community-acquired (CA) infections is *S. aureus* nasal carriage (12). Increased colonization rates have been reported to lead to increased infection rates in the community and hospitals (13, 14).

ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

Drug resistance in *S. aureus* can be acquired by different mechanisms, including horizontal gene transfer via plasmids or other mobile genetic elements as well as spontaneous mutations and selection (15, 16). This led to the emergence of multiple strains that demonstrate resistance to one or a combination of antibiotics, such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), and multiple-drug-resistant *S. aureus* (MDRSA) (6). Over the past 20 years, there have been fluctuations in the prevalence of MRSA. Although overall rates of *S. aureus* infections may have stabilized (8) and the prevalence of MRSA is slightly decreasing in some Western countries, numbers are still alarmingly high on a worldwide scale. According to U.S. and Dutch prevalence data, between 2 million and 53 million people are conservatively estimated to carry MRSA worldwide (17).

Although comparison of epidemiological data has proven difficult because of differences in populations sampled and study designs, countries in North and South America and Asia as well as Malta have been reported to have the highest MRSA rates, exceeding 50%. Exceptionally high rates have been reported for Sri Lanka (86.5%), South Korea (77.6%), Vietnam (74.1%), and Taiwan (65.0%). In contrast, India and the Philippines have much lower rates of 22.6% and 38.1%, respectively. China, Australia, African countries, and some Southern and Eastern European countries, such as Portugal, Greece, Italy, and Romania, have intermediate rates ranging from 25 to 50%. MRSA is generally less prevalent in many Western and Northern European countries, including The Netherlands and Scandinavian countries (18).

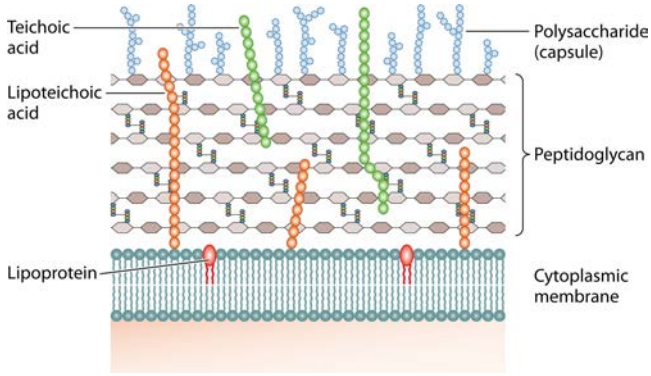

FIG 1 The cell wall structure of Gram-positive bacteria contains a thick layer of peptidoglycan that resides beyond the cytoplasmic membrane.

Mechanisms of Antibiotic Resistance

Beta-lactams, aminoglycosides, macrolides, tetracyclines, and glycopeptide antibiotics lyse or inhibit the growth of antibiotic-susceptible staphylococcal cells by disturbing peptidoglycan synthesis or other vital functions of the cell. Penicillin and its analogues act by recognizing and binding to penicillin-binding protein (PBP), thereby inhibiting the cross-linking of pentaglycine chains (19). Macrolides, aminoglycosides, and tetracyclines inhibit bacterial protein synthesis by binding to and inhibiting ribosome function (20). By binding to the C-terminal D-alanyl–D-alanine of the peptidoglycan stem peptides, glycopeptides such as dalbavancin, oritavancin, teicoplanin, telavancin, and vancomycin inhibit the transpeptidation of *S. aureus* peptidoglycan (21, 22). The rapid evolution of antibiotic-resistant microbes became apparent when resistant strains of *S. aureus* emerged only 2 years after the discovery of penicillin in 1940 (23). A plasmid-encoded penicillinase (beta-lactamase) was responsible for this resistant phenotype (24). The development of penicillinase-resistant methicillin was a response to penicillin-resistant *S. aureus* in 1961, but certain strains showed resistance to methicillin shortly thereafter, which led to the identification of the methicillin resistance gene *mecA* (25). By the genomic integration of this gene, *S. aureus* became resistant to a broader range of beta-lactams. *mecA* encodes a mutated PBP, termed PBP2′ (26), which displays a low binding affinity for beta-lactams, and as a consequence, peptidoglycan cross-linking is no longer affected by methicillin and related agents (27). After methicillin, vancomycin emerged as the new antibiotic of last resort to treat the infections by *S. aureus* (28). Vancomycin acts by binding to the terminal D-alanyl–D-alanine moieties of *N*-acetylmuramic acid and *N*-acetylglucosamine peptide monomers, thereby preventing peptidoglycan cross-linking and proper cell wall synthesis (29). However, the overprescription of vancomycin led to either complete or partial disruption of vancomycin susceptibility of *S. aureus* strains, termed VRSA and vancomycin-intermediate *S. aureus* (VISA), respectively (28). VISA was first identified from a clinical specimen in Japan in 1997 (30), while the first VRSA strain was identified 5 years later in the United States (31). Some isolates of VRSA were also resistant to beta-lactam antibiotics, thus making these isolates multidrug resistant (31). VISA strains were since identified to harbor mutations in several regulatory loci, i.e., the *walRK*, *clpP*, *graRS*, and *vraSR* genes (32). The *graRS* and *vraSR* genes encode cell wall synthesis-regulatory proteins that confer intermediate vancomycin resistance to VRSA strains (33). The synthesis of the peptidoglycan is upregulated following mutations of the *clpP*, *walRK*, *vraSR*, and *graRS* genes to produce an abnormally thick cell wall, thereby hindering effective vancomycin penetration into the cell (21). Foreign genetic material containing the *vanA* gene has also been revealed by genetic studies of VRSA; the presence of this gene results in an alteration within the peptidoglycan, replacing the terminal D-alanyl–D-alanine with D-alanyl–D-lactate, and consequently results in the abrogation of vancomycin binding (31).

General Antibiotic Therapies for *S. aureus*

The antibiotic of choice for the treatment of *S. aureus* infections initially was penicillin; however, penicillin resistance is extremely common in most countries (34). As a result, a penicillinase-resistant β-lactam antibiotic such as flucloxacillin or oxacillin is commonly used for first-line therapy; these antibiotics have the same mechanism of action as penicillin (35, 36). *S. aureus* strains that are methicillin resistant are also resistant to other β-lactam antibiotics, including penicillins (penicillin V, penicillin G, ampicillin, oxacillin, carbenicillin, and amoxicillin), carbapenems (imipenem-cilastatin [Primaxin]), cephalosporins (cephalothin), and monobactams (37).

Treatment of serious infections may utilize combination therapy with various antibiotics; however, because this strategy bears a high risk of damage to the kidneys, its use is controversial (38). Therefore, aiming to combat MRSA, vancomycin, which is a glycopeptide antibiotic, is commonly used. However, some treatment failures with vancomycin have been reported, even in patients infected with vancomycin-susceptible MRSA (39, 40). Linezolid, which belongs to the oxazolidinone class of drugs, has been reported to have bacteriostatic activity against *S. aureus*. This antibiotic is approved to treat complicated soft tissue and skin infections and also pneumonia in children and adults. It has oral and parenteral formulations, and its oral bioavailability is good (41), but *S. aureus* strains that are resistant to this antibiotic have been reported as well (42).

The antibiotic daptomycin, belonging to the new class of cyclic lipopeptides, shows activity against MRSA and methicillin-sensitive *S. aureus* (MSSA). This antibiotic is a powerful bactericidal agent against *S. aureus in vitro*, and it has received approval for the treatment of adults with complicated soft tissue and skin infections (43, 44). Although some clinics have reported daptomycin resistance, the use of high concentrations of the antibiotic is still effective against *S. aureus* (45).

One promising class of broad-spectrum antibiotics is quinolones, assuming a critical role in the treatment of hospital-acquired infections caused by strains that are resistant to older antibacterial classes. For example, JNJ-Q2 and delafloxacin, both of which are quinolone derivatives, are now undergoing clinical trials (46). Fluoroquinolones, the most commonly used subclass of quinolones in hospitals, can be effective against MRSA (47). However, as for the above-mentioned antibiotics, resistance to fluoroquinolones has also been observed in *S. aureus* (48), and this class of antibiotics is associated with an increased risk of MRSA colonization, for which reason its use is discouraged (49). There are additional antibacterial compounds that have entered clinical development, but the majority of these candidate drugs have failed to reach the market (50).

Treatment Costs of *Staphylococcus aureus* Infections

Based on the drug susceptibility of the infecting *S. aureus* strain, the costs of treatment can vary considerably. In the United States, a 6-month treatment regimen for a methicillin-sensitive *S. aureus* infection may cost approximately $16,000; however, costs for an otherwise identical MRSA infection treatment amount to nearly $36,000 (51). In addition to the increase in treatment costs, antimicrobial resistance has led to prolonged hospital stays and increases in morbidity and mortality rates. There are currently very few antibacterial agents present on the market for a number of common antibiotic-resistant strains (52). As some strains show resistance to multiple common antibiotics, there are few therapeutic options left to treat these multidrug-resistant infections, and those options that are available tend to be expensive, toxic to the patient, and/or less effective in treatment (53, 54).

BACTERIOPHAGE

History of Phage Therapy

Bacteriophages are considered the most abundant biological replicating entities on earth (55). As viruses with the ability to infect and lyse bacteria, bacteriophages were discovered separately by Frederick Twort and Félix d'Herelle (56, 57). Soon after this discovery, d'Herelle realized the potential of using bacteriophages to treat human and

animal bacterial infections (58). In 1919, a phage cocktail was prepared for the treatment of a child suffering from dysentery, and after its safety was tested by administering it to several healthy individuals, it was administered to the child. The boy recovered after a single administration of the phage preparation. Three more patients were treated in the same way, and all of them recovered soon after administration; however, these results were not published (59). Phage therapy was soon abandoned by western countries due to the discovery of antibiotics, but in the former Soviet Union, bacteriophages were used from 1920 to 1940 for treating open wounds and intestinal *Salmonella* and *Shigella* infections. Maintenance of phages in the body 3 to 10 days after their administration was used as prophylaxis in regions where infections could spread rapidly (60). In 1921, bacteriophages were used by Joseph Maisin and Richard Bruynoghe for the treatment of staphylococcal skin infections, which resulted in improvement within 24 to 48 h of administration (61). Several similar studies were carried out, and phages were used to treat cholera and bubonic plague in several Asian countries (62, 63). Besides phage therapy, bacteriophages have found a number of applications in different fields, such as for sterilizing processed food, treating crops, diagnosing infections, typing bacteria, and developing molecular biology assays (64–67). However, there is still some reticence in using a biological replicating entity for human phage therapy (68).

Bacteriophage-Derived Lytic Proteins

Many bacteriophages encode tail spike proteins that bind to receptors on the host cell surface. The tail spikes often include enzymatic components (virion-associated peptidoglycan hydrolases, also termed lytic structural proteins), which locally degrade the peptidoglycan in the bacterial cell wall, thereby facilitating the injection of phage DNA at the beginning of the infection process (58, 69). At the end of the phage lytic cycle, progeny virions escape from the host cell by the help of another type of phage-encoded peptidoglycan hydrolase, so-called endolysins. These enzymes, which are not part of the phage virion, accumulate in the cytoplasm, from where they gain access to the peptidoglycan through the action of a second phage protein, named holin, in a highly time-controlled process (70). When the holin concentration reaches a certain threshold, these initially monomeric transmembrane proteins are assembled into pore-forming oligomers in the cytoplasmic membrane. As a result, the membrane is depolarized, and the endolysins are allowed to diffuse through the membrane and degrade their peptidoglycan target. Destabilization of the peptidoglycan sacculus in combination with the internal turgor present in bacterial cells consequently results in lysis and cell death ("lysis from within"). In the case of the exogenous application of endolysins to Gram-positive pathogens, which do not have a protective outer membrane, rapid and effective "lysis from without" is caused by these enzymes, which makes endolysins interesting as potential antimicrobial agents (71, 72). The structure and function as well as the application of phage endolysins as therapeutics are discussed below.

ENDOLYSINS

Endolysin Structure and Function

Endolysins are categorized by their catalytic specificity, i.e., their target bonds within the peptidoglycan substrate. The bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine in the sugar strands of the peptidoglycan are the targets for glucosaminidases, muramidases, and lytic transglycosylases. The same bond is cleaved by lytic transglycosylases and muramidases, but their cleavage mechanisms are different (nonhydrolytic and hydrolytic, respectively). In contrast, endopeptidases cleave various bonds within the peptide portion of the peptidoglycan, and amidases hydrolyze amide bonds that link the sugar and peptide moieties in the peptidoglycan layer (71).

In the case of endolysins acting against Gram-negative bacteria, the catalytic function is usually located within one single globular domain (73). In contrast, endo-

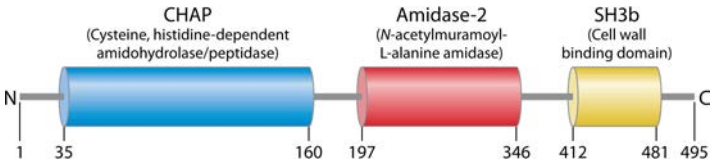

FIG 2 The modular structure of the LysK endolysin, consisting of 2 enzymatically active domains (CHAP and amidase-2) and one bacterial SH3 (SH3b) cell wall-binding domain.

lysins from a Gram-positive background typically show a modular design in which catalytic function and specific cell wall recognition are separated in two or more functional domains. In the simplest (and most frequent) case, they are composed of one N-terminal enzymatically active domain (EAD) and one C-terminal cell wall-binding domain (CBD), both of which are usually connected by a short linker region. However, there are more complex architectures, featuring multiple EADs and/or CBDs in different positions (65, 74–76). Most endolysins derived from staphylococcal phages, which are described in more detail below, consist of a C-terminal SH3b-type CBD, an amidase in a central location, and an N-terminal endopeptidase (Fig. 2) (77, 78). Also, endolysins derived from a streptococcal phage, such as the B30 and lambda SA2 endolysins, feature a dual-EAD architecture with muramidase and endopeptidase domains, in addition to one or several CBDs (79). However, it has been shown for several dual-EAD endolysins by deletion analysis and site-directed mutagenesis that one of these EADs is virtually inactive, whereas the other one is dominant and exhibits high lytic activity in a lysis-from-without scenario (74, 78). While the lytic activity of some endolysins depends strictly on the presence of a CBD (77, 80, 81), this is not always the case. There are other examples of endolysins whose activity is not compromised or is even increased after the removal of the CBD (65, 74). The answer to the question of whether a CBD is required or not has been suggested to depend on both the assay used to determine the activity and the net charge of the EAD, with positively charged catalytic domains being less dependent on a CBD (82). Among all endolysins described to date, the lysin PlyC from streptococcal phage C1 is particularly unique, because its structure is multimeric, consisting of two separate gene products, named PlyCA and PlyCB, of 50 kDa and 8 kDa, respectively. These gene products self-assemble into a complex consisting of 1 PlyCA subunit and 8 PlyCB subunits (83). Although rare, there are a few examples of modular endolysins from phages infecting Gram-negative bacteria, such as the two-domain lysin KZ144 from a *Pseudomonas* phage (73).

Several studies have elucidated the crystal structures of both globular and modular endolysins as well as individual functional domains thereof. Examples include the T7 lysin (84), the T4 lysin (85), the streptococcal phage lysins Cpl-1 (86) and PlyC (87), the *Bacillus* phage lysins PlyL (88) and PlyB (89), the *Listeria* phage endolysin PlyPSA (90), and the staphylococcal phage endolysin PlyGH15 (91). The crystal structure of Cpl-1 has been investigated in both free and choline-bound states, suggesting that the choline-binding CBD helps the N-terminal catalytic domain to be correctly positioned (86, 92). It should be noted that the inherent flexibility of modular endolysins (afforded by the flexible linker regions connecting the individual domains) often prevents the crystallization of full-length enzymes, for which reason the majority of reported endolysin crystal structures comprise only single domains (65).

Endolysins as Potential Antimicrobials

As the treatment of bacterial infections proves to be increasingly difficult with the growing incidence of antibiotic-resistant strains, phage endolysins have been suggested as promising alternative antibacterial therapeutics (93, 94, 228). These phage-based enzymes have proven effective in various animal models of bacterial infection as well as in the decolonization of mucosal membranes (summarized in Table 1).

Although endolysins with broad-range lytic activity have been identified (95), most phage endolysins show near species specificity, and this is believed to be one of their

TABLE 1 Selection of antimicrobial applications of endolysins and engineered derivatives[a]

Target pathogen(s)	Endolysin(s) and/or derivative(s)	Model type (animal[s])	Delivery method(s)	Description and/or outcome	Reference
Streptococcus pyogenes, MRSA	PlySs2	*In vivo* (mouse)	Intraperitoneal	Protection of 92% of mice from *S. pyogenes* and MRSA bacteremia	201
Streptococcus suis	PlySs2	*In vivo* (mouse)	Intranasal	High killing activity against multiple serotypes and strains of *S. suis* and survival of mice	202
S. pyogenes	PlyPy	*In vivo* (mouse)	Intraperitoneal	Protection of mice from death (90–95%) in a bacteremia model	203
S. pyogenes	PlyC	*In vivo* (mouse)	Oral, nasal	Elimination of the pathogen in orally colonized mice	204
S. aureus	$CHAP_K$	*In vivo* (mouse)	Intranasal	Truncated endolysin construct eliminates *S. aureus* in the nares of mice	144
Streptococcus agalactiae	PlySK1249	*In vivo* (mouse)	Intraperitoneal	Protection of 80% of mice from systemic infection	205
S. agalactiae, *S. dysgalactiae*, *S. uberis*	B30, λSA2	*In vivo* (mouse)	Intramammary	Reduction of intramammary bacterial loads (1.5–4.5 logs) in a mouse model of bovine mastitis	127
Streptococcus pneumoniae	Cpl-1	*In vivo* (mouse)	Aerosol	Reduction in mice mortality due to pneumonia by 80%	206
S. pneumoniae	Cpl-1	*In vivo* (mouse)	Intravenous, nasal, oral	Reduction of bacterial loads in nasopharyngeal and bacteremia models; longer survival of animals	207
S. pneumoniae	Cpl-1	*In vivo* (mouse)	Intraperitoneal	Total protection of mice from pneumonia when endolysin was administered within 24 h postinfection; reduced protection with delayed treatment (42% survival)	208
S. pneumoniae	Cpl-1	*In vivo* (rat)	Intravenous	Elimination of pneumococci causing endocarditis from blood within 30 min with high-dose treatment	209
MRSA	PlyGH15	*In vivo* (mouse)	Intraperitoneal	Protection of mice from bacteremia	145
MRSA	SAL-1	*In vivo* (mouse)	Intravenous	Increased survival rate of mice and reduction of bacterial counts in the bloodstream	137
MRSA	MV-L	*In vivo* (mouse)	Intraperitoneal, nasal	Elimination of MRSA from nares of mice and protection against death from systemic infection	125
MRSA	8 different endolysins and lysostaphin	*In vivo* (mouse)	Intraperitoneal	100% protection from death by the majority of the tested enzymes; no clinical signs of disease at the end of the treatment	78
B. anthracis	PlyG	*In vivo* (mouse)	Intraperitoneal	Rescue of mice from fatal septicemia	210
MRSA	SAL-1	*In vivo* (rat, dog)	Intravenous	Absence of adverse effects upon administration of endolysin	146
MRSA	ClyH	*In vivo* (mouse)	Intraperitoneal	100% protection of mice from death	211
MRSA	ClyS	*In vivo* (mouse)	Intraperitoneal	Protection of mice from death with synergy between the endolysin and oxacillin	121
S. aureus	ClyS	*In vivo* (mouse)	Topical	Better performance (3-log CFU reduction on skin) than that of the antibiotic mupirocin	126
S. aureus	λ SA2-E-Lyso-SH3b, λ SA2-E-LysK-SH3b	*In vivo* (mouse)	Intramammary	Reduction of 3.36 log CFU with combined treatment with lysostaphin in a mouse model of bovine mastitis	101
S. aureus	Several chimeric peptidoglycan hydrolases	*In vivo* (mouse, rat)	Intranasal, intramammary, intramuscular	Better activity than that of the parental protein in models of nasal infection, mastitis, and osteomyelitis	114
S. pneumoniae, *S. pyogenes*	Cpl-7	*In vivo* (zebrafish embryos)	Immersion	Increased survival rate of 95–99% of animals; combination with carvacrol was effective at killing Gram-negative bacteria	212
Pseudomonas aeruginosa	Artilysin	*In vivo* (*Caenorhabditis elegans*)	Immersion	Improved survival of nematodes treated with an artilysin (63%) active against the Gram-negative pathogen compared to the native endolysin (40%)	213
MRSA	MR-10	*In vivo* (mouse)	Subcutaneous	Combined application with minocycline effectively reduced bacterial burdens in localized and systemic burn wound infections	214

(Continued on next page)

TABLE 1 (Continued)

Target pathogen(s)	Endolysin(s) and/or derivative(s)	Model type (animal[s])	Delivery method(s)	Description and/or outcome	Reference
S. aureus	PlyGRCS	*In vivo* (mouse)	Intravenous	PlyGRCS was effective at rescuing 30% of mice from death induced by *S. aureus* bacteremia after 3.5 days	120
MRSA, MSSA	LysGH15	*In vivo* (mouse)	Subcutaneous	LysGH15 did not induce resistance in MRSA, and its *in vitro* and *in vivo* efficacy was not compromised in the presence of antibodies generated against the endolysin	215
MRSA	SAL200 (SAL-1)	*In vivo* (monkey)	Intravenous	Repeated SAL200 injections over a period of <1 wk or single high-dose injections were well tolerated	138
MRSA	SAL200 (SAL-1)	*In vivo* (human)	Intravenous	Phase 1 clinical study evaluating pharmacokinetics, pharmacodynamics, and tolerance of SAL200 in humans; no serious adverse effects were observed	140
MRSA	Trx-SA1	*In vivo* (cow)	Intramammary	Significant reductions in pathogen levels and somatic cell counts in milk from udder quarters with mild clinical *S. aureus*-induced mastitis	216
MSSA, MRSA	SA.100	*In vivo* (human)	Topical	Case report describing successful treatment of 3 different *S. aureus*-related dermatoses by topical application of the endolysin-based therapeutic Staphefekt SA.100	217
S. pyogenes	PlyC	*In vitro*	NA	PlyC is able to cross epithelial cell membranes, making it a promising agent to control intracellular streptococci	218
S. suis	LySMP	*In vitro*	NA	Efficacy against biofilms	219
Streptococcus equi	PlyC	*In vitro*	NA	1,000-fold-higher activity against the causative agent of equine strangles than the commercial disinfectant Virkon-S	220
Streptococcus sp.	Ply700	*In vitro*	NA	Activity against *S. uberis* in milk	221
MRSA	$CHAP_K$	*In vitro*	NA	Thermally triggered release of a synergistic staphylolytic cocktail consisting of lysostaphin and $CHAP_K$ from poly(*N*-isopropylacrylamide) nanoparticles	222
E. coli	Bacteriophage T5 endolysin	*In vitro*	NA	Synergistic effect of T5 endolysin in combination with membrane-permeabilizing compounds against Gram-negative bacteria	223
MSSA, MRSA, and ceftaroline-ceftobiprole- and borderline oxacillin-resistant staphylococcal isolates	HY-133	*In vitro*	NA	Activity of a chimeric endolysin construct against a collection of African staphylococcal strains	133
Multidrug-resistant *Acinetobacter baumannii*	ABgp46	*In vitro*	NA	Activity of the novel ABgp46 endolysin against *Acinetobacter* and other Gram-negative bacteria is enhanced by citric and maleic acids	224
S. aureus, *S. epidermidis*	LysH5	*In vitro*	NA	Activity in milk against *S. aureus*	225
S. aureus	LysH5	*In vitro*	NA	Expression and secretion of an active staphylococcal endolysin in lactic acid bacteria	157
L. monocytogenes	Ply511	*In vitro*	NA	Expression and secretion of an active endolysin by lactic acid bacteria	111
A. baumannii	LysAB2	*In vitro*	NA	LysAB2 exhibited broad bacteriolytic activity against a number of Gram-negative and Gram-positive bacteria	226
S. aureus	SAL-2	*In vitro*	NA	Activity against planktonic bacteria and biofilms	227

[a]NA, not applicable.

most advantageous properties in this age of broad-range antibiotic resistance, by avoiding selective pressure on commensal populations (65). Furthermore, the development of resistance against endolysins is unlikely for several other reasons. Since the phage and the host bacteria are coevolving, endolysins have evolved to bind to and cleave highly conserved structures in the cell wall, the modification of which is believed to be detrimental to the host organism (96, 97). In addition, by being applied externally and acting on the cell wall, without having to enter the bacterial cell, endolysins used therapeutically avoid a majority of the possible resistance mechanisms (e.g., active efflux from the cell or decreased membrane permeability) that play a role in resistance to most conventional antibiotics (98, 99). Several endolysins also possess two catalytic domains that hydrolyze different bonds in the peptidoglycan, which is also believed to further reduce the chance of resistance development (1).

Finally, to enhance their therapeutic activity and to avoid the development of resistance, multiple endolysins may be used in combination with antibiotics to treat bacterial infections (1, 100). Synergistic effects among endolysins and between endolysins and other antimicrobial agents have been reported (101–103). Other topics related to the systemic application of endolysins, such as immunogenicity, toxicity, and serum half-life, have been discussed elsewhere (65) and are not a focus of this review.

Engineering of Endolysins

The modular structure of endolysins provides a unique opportunity for protein engineering in order to modify bacteriolytic activity, specificity, solubility, and other physicochemical properties of these enzymes. One prime example of an engineered enzyme with increased lytic activity is Ply187AN-KSH3b, a fusion of the endopeptidase domain of the staphylococcal Ply187 lysin (Ply187AN) with the SH3b CBD of another staphylococcal phage endolysin, LysK. The fusion construct displayed >10-fold-higher staphylolytic than that of Ply187AN and was also more active than LysK in multiple activity assays (104). Similarly, the chimeric enzyme ClyH has been created by the fusion of a non-SH3b CBD from phiNM3, another *S. aureus* phage, with the endopeptidase domain of Ply187, resulting in a 14-fold improvement in staphylolytic activity against the tested strains. In addition, a broader spectrum of activity has been shown for ClyH, including some staphylococcal strains that were not susceptible to native Ply187 (105). Changes in specificity, such as an expansion of the lytic spectrum of an endolysin, can be achieved by the exchange or addition of CBDs outside the native endolysin's serovar, species, or even genus specificity. Fusion of the endopeptidase domain of the streptococcal LambdaSa2 phage endolysin with the SH3b CBDs from either the staphylococcal phage endolysin LysK or lysostaphin drastically increased the staphylolytic activity of the chimeric enzyme, while the parental streptolytic activity was maintained (74, 106). The peptidoglycan hydrolase lysostaphin is a bacteriocin produced by *S. simulans* that exhibits lytic activity against *S. aureus* (107). It features a modular architecture similar to that of a bacteriophage endolysin, consisting of an N-terminal catalytic domain and a C-terminal binding module. However, as opposed to endolysins, lysostaphin targets the weakly conserved pentaglycine bridge of staphylococcal peptidoglycan. Consequently, mutant strains featuring modifications within this portion of the peptidoglycan and showing resistance to lysostaphin have been reported (108). For this reason, it is not recommended that lysostaphin be used as a single antimicrobial agent. However, combined applications with endolysin-derived proteins, which exploit synergistic effects due to the different peptidoglycan cleavage sites of these enzymes, have been reported and may represent a feasible strategy to decrease the chance of resistance (102, 109). Furthermore, components of lysostaphin have been used for the construction of chimeric peptidoglycan hydrolases (74, 109). The spectrum of specificity of an enzyme can also be extended by a combination of two heterologous CBDs; this has been shown by fusions of CBDs from *Listeria* phage endolysins with different binding specificities that were fluorescently tagged (110). Although the full lytic activity of most endolysins depends on a CBD, there are various examples where the deletion of a binding module resulted in enzymes with advantageous properties. When the CBD

of the *Bacillus anthracis* prophage endolysin PlyL was deleted, the species specificity of the parental enzyme was abolished, and its lytic activity was broadened to include a wider spectrum of *Bacillus* species (88). In some cases, the removal of the CBD may even increase endolysin potency. A truncated version of the *Listeria* phage endolysin Ply511 lacking a part of its CBD caused enhanced lysis zones compared to those with the full-length parental enzyme in a soft agar overlay assay, which was attributed to a more efficient diffusion of the truncated construct through this semisolid matrix (111). Similarly, truncation of the *Clostridium difficile* phage endolysin CD27L to its N-terminal domain (i.e., removal of its putative CBD) improved bacteriolytic activity and led to a broadened lytic range of the deletion construct (112). Besides CBDs, the removal of low-activity EADs may also improve the potency of an enzyme. The streptococcal prophage LambdaSa2 endolysin contains two enzymatically active domains, a C-terminal glycosidase that is poorly active and a highly active endopeptidase, in addition to 2 centrally located Cpl-7 CBDs (113). The removal of the entire glycosidase domain while retaining the CBDs led to an increased streptolytic activity of the truncated enzyme compared to that of the parental endolysin (106). Another significant report recently described the construction of artificial staphylococcal peptidoglycan hydrolases that combine three unique catalytic activities into single fusion proteins, thereby effectively avoiding the development of resistant strains. Additional modification of these constructs with so-called protein transduction domains enhanced their efficacy against staphylococcal biofilms and rendered them active against intracellular staphylococci, as demonstrated in cultured mammary gland epithelial cells and a mastitis mouse model (114). Besides the above-mentioned examples, molecular engineering of endolysins led to constructs with an increased binding affinity for the cell wall (110), enhanced solubility (115), and an altered ionic strength optimum (110).

Endolysins with Activity against *S. aureus*

In the light of the above-mentioned resistance problem, *S. aureus* has become a prime target for endolysin technology in recent years. Numerous studies describing cloning, recombinant production, and characterization of native and engineered staphylococcal peptidoglycan hydrolases *in vitro* and in animal models have been reported during the past decade (65, 116).

The vast majority of these enzymes feature a unique 3-domain architecture, with two EADs (one at the N terminus and a second one located centrally) and one C-terminal SH3b-type CBD (Fig. 2). In most cases, the N-terminal EAD is a cysteine-, histidine-dependent amidohydrolase/peptidase (CHAP), which cleaves the bond between D-Ala of the stem peptide and the pentaglycine bridge of the staphylococcal peptidoglycan. The centrally located EAD displays amidase activity (amidase 2 or amidase 3), cutting between the sugar strands and the stem peptide (78, 100, 117). The SH3b domain is known to require an intact pentaglycine bridge for full binding activity, which has been shown for the SH3b-like cell wall-targeting domain of the bacteriocin lysostaphin (118). There are exceptions to this conserved architecture, such as the 2638A endolysin, which harbors an M23 endopeptidase domain instead of a CHAP domain at its N terminus (119), as well as the P68 (115) and PlyGRCS (120) endolysins, which feature 2-domain architectures. A few staphylococcal endolysins containing a CBD unrelated to the common SH3b domain have been reported as well (121). For most of the dual-EAD staphylococcal endolysins, lytic activity relies mostly on the N-terminal CHAP endopeptidase domain (116, 122, 123), whereas the amidase domain is virtually inactive when applied externally. One exception is the 2638A endolysin, which features a highly active amidase domain (119).

Despite the highly similar modular design of the SH3b-containing enzymes, which form by far the largest subgroup among the staphylococcal phage endolysins, they can differ considerably at the amino acid sequence level. Becker et al. reported that these proteins fall into 5 homology groups that show more than 90% sequence identity within each group but less than 50% identity between groups. Six "stand-alone" proteins could not be assigned to any of the groups. It is noteworthy that SH3b

domains share the highest degree of conservation between the different groups and stand-alone enzymes (74). The remarkable diversity found among this group of endolysins at the sequence level is also reflected in the diverse enzymatic and antibacterial properties of these enzymes. This became apparent when representative peptidoglycan hydrolases from each of the above-mentioned homology groups were comparatively characterized (78). The 9 investigated enzymes (8 phage endolysins and the bacteriocin lysostaphin) differed in their lytic activities against live *S. aureus* cells, their optimum ionic strengths, their spectra of susceptible strains, and their efficacies against *S. aureus* biofilms. The endolysin with the highest staphylolytic activity in this study was LysK, which is derived from *S. aureus* phage K (124) and, at the same time, is one of the best-characterized staphylococcal peptidoglycan hydrolases described to date. Therefore, a separate section of this review is dedicated to LysK and its close homologues.

The first staphylococcal phage endolysin tested in animal models was MV-L, which is derived from phage ΦMR11 and is able to kill various strains of *S. aureus*, including VISA, MRSA, and VRSA variants. Conversely, MV-L is harmless to several other commensal species that have been tested, such as *Staphylococcus epidermidis*, which is an inhabitant of skin and mucus membranes that is able to competitively inhibit *S. aureus* colonization (125). The nasal cavity in humans and animals is considered a primary site of colonization by *S. aureus*. Intranasal treatment using MV-L was shown to effectively eliminate *S. aureus* from artificially inoculated nares of mice (125). Nasal decolonization ability has since also been demonstrated for engineered endolysin constructs such as ClyS, a fusion protein consisting of the CHAP domain of the phage Twort endolysin and the non-SH3b CBD of the phiNM3 endolysin. This chimera effectively decreased the numbers of bacteria of an MRSA strain by 2 logs within 1 h, 24 h after inoculation in the nasal passage (121). When applied in a murine skin infection model, a topical ointment containing ClyS was demonstrated to be more effective than mupirocin, the commonly prescribed antibiotic for topical application against *S. aureus* infections (126). Besides nasal decolonization and topical applications on the skin, some staphylococcal endolysins have also been shown to be effective against systemic *S. aureus* infections in animal models. The survival of mice intraperitoneally infected with MRSA was improved by 100% after a single 0.05-mg (~2-mg/kg of body weight) intraperitoneal injection of MV-L within 30 min after infection. However, when the same treatment was delayed by 1 h, mouse survival decreased by 40% or more when the treatment was further postponed. In addition, MV-L was not able to eradicate all *S. aureus* bacteria from the bloodstream (125).

More recently, the above-mentioned 9 unique peptidoglycan hydrolases were compared for their efficacies at rescuing mice from MRSA-induced bacteremia, with the antibiotics vancomycin and oxacillin being used as controls (78). Mice were infected intraperitoneally with MRSA together with an immunosuppressant. A total of 0.2 mg of the enzymes (~8 mg/kg) was administered via the same route 30 min later. Within 2 days, 75% of the infected and buffer-treated control mice died in this model. In contrast, the endolysins phi11, LysK, 80α, WMY, and 2638A were able to save 100% of the mice, similarly to vancomycin and lysostaphin. Mice treated with these agents temporarily developed clinical signs indicative of slight to moderate illness but completely recovered within 40 h postinfection. The endolysins phiSH2 and Twort rescued only about 60% of the animals, while no protection was offered by the endolysin P68, likely due to solubility issues (78).

Not only do endolysins hold promise as individually applied antibacterials, they also have been reported to act synergistically when used in combination with each other (i.e., two endolysins with different cleavage specificities) or with other antimicrobial agents (127). The efficacy of less efficient or overused antibiotics can even be revived by utilizing a combination of endolysins and antibiotics. For instance, intraperitoneal administration of the fusion enzyme ClyS alone (0.96 mg [~38.4 mg/kg]) increased survival rates of MRSA-infected mice to 88% (121). However, when used in combination with 100 μg of oxacillin (a dose that, when individually administered, saved only 40% of the animals), a similar efficacy (~80% survival) could be achieved with a reduced

dose of ClyS of only 0.17 mg (~6.8 mg/kg). This synergistic effect has been attributed to the enhanced production of staphylococcal autolysins in the presence of oxacillin, an inhibitor of peptidoglycan cross-linking. These autolytic enzymes may be responsible for the observed synergy (121).

While the above-mentioned intraperitoneal infection and treatment studies may be valid as important first steps toward evaluating the *in vivo* efficacy of endolysins, they inadequately model localized infections such as endocarditis or osteomyelitis, which involve deeper tissue penetration and the formation of abscesses (6). However, there are a few studies that have also described the efficacy of (engineered) endolysins in more specific animal models of *S. aureus* infection. One example is the treatment of *S. aureus*-induced endophthalmitis using the chimeric staphylococcal endolysin Ply187AN-KSH3b, and also, the possibility of using other endolysins for the treatment of bacterial eye infections has been reported (128, 129). *S. aureus* represents the leading cause of this disease, which is characterized as a vision-threatening complication of ocular trauma. Intravitreal injection of Ply187AN-KSH3b into the eyes of mice that had been infected with *S. aureus* 6 to 12 h prior to treatment significantly reduced bacterial burdens in the eye and improved the clinical outcome, preserving retinal function.

Another example is the treatment of *S. aureus*-induced mammary gland infections in a mouse model of bovine mastitis by chimeric phage endolysins in combination with lysostaphin (101). The combination treatment reduced the numbers of intramammary bacteria by >3 log units and markedly decreased inflammation, as measured by tumor necrosis factor alpha concentrations and mammary gland wet weights.

Of note, a first series of staphylococcal phage endolysin-based antibacterial products is already available on the market. These products include Staphefekt XDR.300, a solution that is active against *S. aureus* (including MRSA) on human skin, and the Gladskin series of skin care products for the treatment of various skin conditions with an infectious component, such as acne, eczema, rosacea, and skin irritation. Both products contain the active ingredient Staphefekt and are marketed by Micreos Human Health BV (The Netherlands).

LysK AND ITS HOMOLOGUES

Structure, Function, and Physicochemical Properties

The endolysin LysK is derived from bacteriophage K, a virulent broad-range phage from the family of *Myoviridae* whose genome length is 127,395 bp. Interestingly, LysK, a 54-kDa protein of 495 amino acids, is encoded by two open reading frames interrupted by an intron, and the mature endolysin is created from these two separated parts through a splicing reaction (130). The modular architecture of LysK is that of a typical 3-domain staphylococcal phage endolysin as described above, i.e., featuring a CHAP domain, an amidase-2 domain, and an SH3b domain (Fig. 2) (130, 131, 229). The cleavage sites of the CHAP and amidase-2 domains within the staphylococcal peptidoglycan (D-Ala–Gly endopeptidase and MurNAc–L-Ala amidase, respectively) have been elucidated by mass spectrometry analysis of peptidoglycan digestion products (100) and are identical to those reported for other staphylococcal phage endolysins (78, 117) (depicted in Fig. 3). While the mere presence of amidase activity was demonstrated in those experiments, several studies predict that the midprotein amidase-2 domain contributes little to the overall staphylolytic activity of the endolysin and is dispensable in a lysis-from-without setting (131, 132).

Deletion studies showed that upon the removal of the amidase-2 and SH3 domains, the construct retaining only the CHAP domain of LysK (termed $CHAP_K$), comprising the first 165 amino acids, remained highly active and in addition showed increased solubility compared to that of the parental full-length endolysin (131). Similarly, Becker et al. generated a series of LysK deletion constructs and compared their *in vitro* activities against *S. aureus*. As opposed to $CHAP_K$, a CHAP-only construct produced in that study (termed LysK221, comprising the first 221 amino acids) retained only marginal activity, possibly due to improper folding at the C-terminal end. However, another deletion construct consisting of the CHAP and SH3b domains (i.e., a deletion

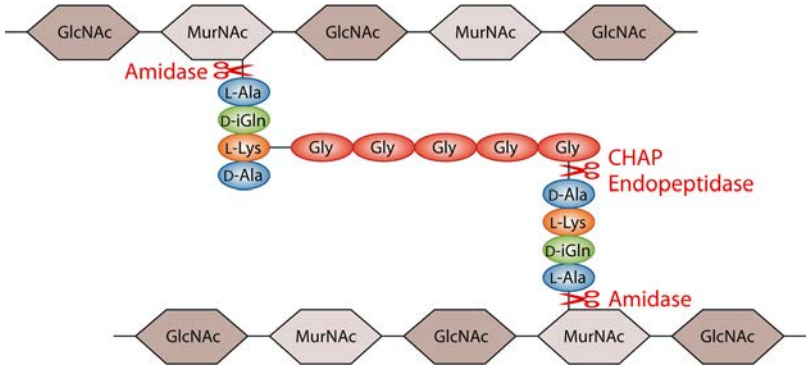

FIG 3 Schematic representation of *S. aureus* peptidoglycan and LysK cleavage sites. The peptidoglycan is made up of sugar strands consisting of two alternating units (*N*-acetylmuramic acid and *N*-acetylglucosamine) and peptide linkers connecting these strands. Five units of glycine (Gly) act as a cross-bridge between stem peptides.

of the amidase domain) exhibited staphylolytic activity similar to that of the full-length enzyme (100). Another construct, named HY-133 (owned by Hyglos GmbH, Germany), and its progenitor, PRF-119, which consist of the CHAP domain of LysK and the SH3b domain of lysostaphin, were reported to display pronounced bactericidal activity against multiple staphylococcal strains (132, 133).

Since LysK has been identified as an endolysin, researchers have conducted several studies to determine its physicochemical properties. The biological stability of an enzyme being considered for antimicrobial applications is particularly important, as stability directly affects the efficacy of the enzyme. The stability of LysK was found to be increased 100-fold by the addition of sucrose, glycerol, or other low-molecular-weight additives to the storage buffer. The addition of such low-molecular-weight additives enables LysK to maintain 100% of its activity for up to a month at room temperature, which is remarkable and very uncommon for most enzymes. The addition of divalent cations such as Ca^{2+} also increased the stability of LysK. At temperatures over 40°C, a dramatic loss in stability was observed and attributed to secondary-structure changes, as measured by circular dichroism spectroscopy (134). The same group reported that LysK displayed increased stability in complexes with polycationic polymers (poly-L-lysines [PLLs]) and block copolymers of these PLLs with polyethylene glycol (135).

LysK was also one of the first staphylococcal endolysins for which structural information was available. Sanz-Gaitero et al. solved the crystal structure of $CHAP_K$, which showed a papain-like topology with the catalytic triad located inside a hydrophobic cleft. Two metal ions were found to be associated with the structure: a structural calcium ion and a zinc ion within the active site (122, 136). Shortly before that, the structure of all three functional domains of the LysGH15 endolysin was reported (91). Of note, LysGH15 is almost identical to LysK, differing in only 4 amino acids. Accordingly, structural features described for the CHAP domain of LysGH15 are in agreement with the $CHAP_K$ structure. In addition, those authors reported that the calcium ion-binding site of the CHAP domain is located in a so-called "EF hand-like" motif in close proximity to the active-site cleft and that the presence of the calcium ion is critical for the lytic activity of the enzyme. The addition of calcium to the EDTA-inactivated CHAP domain completely restored enzymatic activity, whereas other divalent metal cations (including zinc) failed to do so. The amidase domain of LysGH15 was found to feature a shallow groove on its surface, which would support the accommodation and cleavage of the highly cross-linked peptidoglycan structure. A zinc ion is bound at the center of the groove in an arrangement typical of zinc-dependent metalloenzymes. The important role of the zinc ion is supported by the finding that mutation of all zinc-binding residues abolished the activity of the amidase domain. The SH3b domain of LysGH15 was found to be structurally very similar to that of the lysostaphin homologue ALE-1.

Nuclear magnetic resonance (NMR) titration experiments using the peptide "AGGGGG," mimicking the pentaglycine bridge of staphylococcal peptidoglycan, revealed the interaction of this peptide with residues of the presumptive peptidoglycan-binding groove of the SH3b domain albeit at a relatively low affinity. Mutation of the respective residues abolished the binding of the CBD to *S. aureus* peptidoglycan (91). Besides LysGH15, another LysK homologue, named SAL-1, was recently described and extensively characterized (137–139). This endolysin differs from LysK in only 3 amino acids; however, it was claimed by those authors to exhibit higher activity against multiple *S. aureus* strains than LysK (139). SAL-1 is the first endolysin that has been intravenously administered to humans (140), which is discussed in more detail below.

In Vivo Studies with LysK and Its Derivatives and Homologues

Although quantitative comparison of results between different laboratories is difficult, LysK and its homologues are among the most potent staphylococcal phage endolysins described to date. In direct comparison with 7 endolysins from different homology groups, LysK revealed the highest staphylolytic activity, as determined by turbidity reduction assays, and also proved most effective at removing *S. aureus* biofilms from polystyrene surfaces (78). Furthermore, this endolysin has a broad spectrum of activity, killing a variety of *S. aureus* strains, including MRSA, VRSA, and teicoplanin-resistant strains; surface mutant strains; as well as coagulase-negative staphylococci (78, 124, 141). In addition, LysK has been demonstrated to exhibit strong synergy when applied in combination with lysostaphin, presumably due to the different peptidoglycan cleavage sites of these two enzymes (102). All these properties make LysK and its derivatives and close homologues highly interesting as potential antistaphylococcal therapeutics and have led researchers to investigate their efficacy in various *in vivo* models.

In a mouse model of systemic MRSA infection, which was part of the above-mentioned comparative endolysin characterization, LysK (injected intraperitoneally 30 min after infection at 200 μg/mouse) was one of the most effective enzymes, saving 100% of the animals from death. MRSA was not detected in the blood of any of the LysK-treated mice at the conclusion of the experiment, but 67% of control animals surviving after 48 h tested positive for bacteremia, with an average concentration in the blood of 2.48 log CFU/ml (78).

Besides full-length LysK, the truncated construct $CHAP_K$ has also been shown to be a potent antimicrobial both *in vitro* and *in vivo*. Its lytic spectrum is even broader than that of LysK and, besides numerous species of *Staphylococcus*, includes members of the genera *Streptococcus*, *Micrococcus*, *Arthrobacter*, *Nesterenkonia*, *Carnobacterium*, and *Leuconostoc*. The active pH range of the enzyme is 6 to 11, with optimum activity at pH 9, and it shows activity at temperatures between 5 and 40°C, suggesting potential applications in various fields such as medicine and food production (142). $CHAP_K$ also proved effective at preventing *S. aureus* biofilm formation and removing preformed biofilms from artificial surfaces (143). Biofilms play an important role in bacterial infections of both humans and animals and as a source of contamination in food production and processing. Finally, nasal and oral administration of $CHAP_K$ to mice that had been inoculated in the nostrils with *S. aureus* caused an effective reduction in bacterial concentrations by 2 logs (144).

Similar to the full-length LysK endolysin, its close homologue LysGH15 has also been evaluated in a mouse model of systemic MRSA infection. Mice infected intraperitoneally with two times the minimum lethal dose of an MRSA strain were treated with different concentrations of the endolysin injected via the same route at various time points after infection. One hundred percent protection of the mice was achieved when the protein was administered at 1 h postinfection at a concentration of 50 μg/mouse. Numbers of MRSA bacteria in the blood of mice treated with this dose were decreased by 2 log units within 2.5 h, whereas those of control mice were increased to $>10^7$ CFU/ml (145). Besides its *in vivo* efficacy, LysGH15 was found to exhibit higher average *in vitro* activity against a tested set of MRSA strains than against a selection of MSSA strains. Those

authors hypothesized that this could be due to a tighter binding of LysGH15 to cell wall structures predominantly present in strains with methicillin resistance (145).

SAL-1, the second well-characterized homologue of LysK, is derived from the *Staphylococcus* phage SAP-1 (139). In an effort to develop this enzyme as a therapeutic agent for human application, those authors demonstrated that a stabilizing formulation containing poloxamer 188 and Ca ions increased its lytic activity against *S. aureus* planktonic cells, encapsulated forms, and biofilms *in vitro*. The intravenous administration of this preformulation, which was designated SAL200, to mice once a day for 3 days after infection with MRSA significantly reduced the mortality of the animals. Furthermore, no bacteria were recovered from blood and splenic tissue of SAL200-treated mice, whereas concentrations between 10^5 and 10^7 CFU/ml were found in the control groups (137). SAL200 is the first endolysin-based therapeutic that underwent a good laboratory practice (GLP)-compliant safety evaluation, including single- and repeated-dose toxicity studies and central nervous system, respiratory, and cardiovascular function tests in rats and dogs (146). Rats tolerated repeated doses of 100 mg/kg over 4 weeks, and no signs of abnormal behavior or toxicity were observed. Two weeks of repeated dosing showed no changes related to the treatment in dogs, including changes in food consumption, body weight, electrocardiography, ophthalmology, hematology, urinalysis, organ weight, or serum biochemistry. However, mild and transient (i.e., lasting for 30 min to 1 h after injection) clinical signs such as irregular respiration, subdued behavior, and vomiting were observed in dogs starting after 10 days of repeated administration. Upon further investigation, an immune response was elicited after repeated exposure to the enzyme for 14 and 28 days in dogs and rats, respectively, as measured by the presence of anti-SAL-1 antibodies. The exposed dogs also showed a reduction in the level of blood C3 complement, considered representative of the complement system (146). It is unclear whether this response was due to the enzyme itself or due to residual lipopolysaccharide endotoxin in the protein preparation. Nonetheless, after the injection of SAL200, the symptoms were observed only transiently and were considered mild (146). In a further safety and pharmacokinetic study conducted in monkeys, SAL200 was well tolerated and did not cause any adverse effects when injected as a single dose (up to 80 mg/kg body weight) or as multiple doses, up to 40 mg/kg per day (138). Most recently, SAL200 became the first endolysin-based drug applied to humans by intravenous infusion as part of a phase 1 clinical trial (140). Single ascending doses (up to 10 mg/kg) were applied to healthy male volunteers, and pharmacokinetics, pharmacodynamics, and tolerance of the drug candidate were evaluated. No serious adverse effects or clinically significant values were observed for any of the participants as a consequence of administration up to the highest tested dose. Mild and temporarily observed effects included fatigue, headache, rigors, and myalgia.

ENDOLYSIN PRODUCTION IN LACTIC ACID BACTERIA FOR FOOD SAFETY APPLICATIONS

Besides its role as an infectious agent, *S. aureus* is also an important foodborne pathogen. It causes food poisoning through the production of heat-stable enterotoxins during growth in contaminated food products. When ingested, even in the absence of the bacterium, these toxins cause classical symptoms of foodborne intoxication such as vomiting and diarrhea (147, 148). The addition of purified endolysins to certain high-risk food products is being discussed as one possibility to prevent the growth of pathogenic organisms and thereby the production of toxins in food. An alternative approach is the use of starter organisms used in the production of fermented food for the expression and secretion of active endolysins into the product (93). Lactic acid bacteria have been utilized for years in different food preservation and fermentation processes (149). Both lactococci and lactobacilli have been characterized as probiotic bacteria, based on their "generally recognized as safe" (GRAS) status, and they have been exploited for heterologous protein production (150, 151). Furthermore, lactococci are found as commensals in the gastrointestinal tract, oral cavity, and female reproductive tract. *Lactococcus*

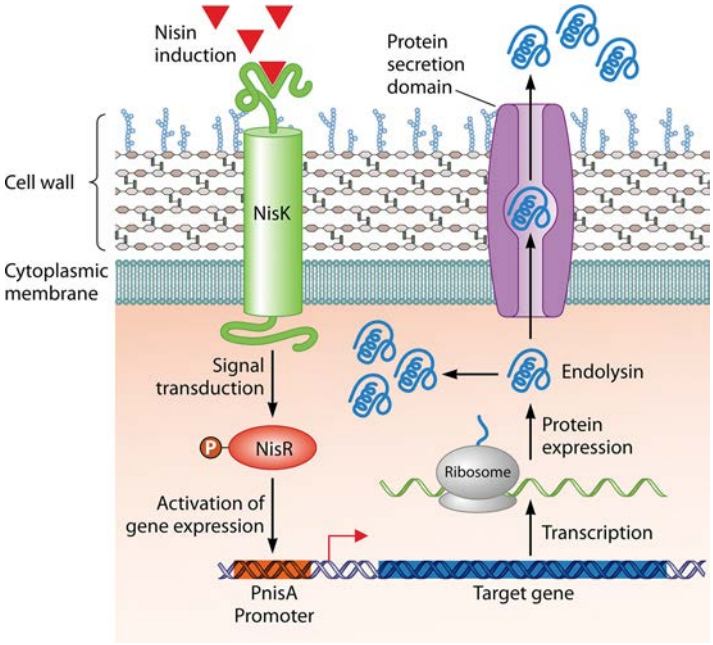

FIG 4 Protein secretion pathway in the nisin-controlled gene expression system in *L. lactis*. After nisin is detected by the sensor histidine kinase protein located in the membrane (NisK), autophosphorylation of this protein occurs, followed by the transfer of its phosphatase group to the cytoplasmic response regulator NisR. NisR, which is now activated, then activates transcription via the P*nisA* promoter, followed by the production of a target protein, which in this case is a recombinant endolysin. Depending on the presence or absence of a specific signal peptide, the protein either is secreted into the external medium or accumulates in the cytoplasm.

lactis in particular is a nonpathogenic, Gram-positive, spherical bacterium that has been widely used by the food industry, e.g., in the production of fermented dairy products (152). Also, biotechnology has widely used this bacterium as an expression system in order to produce heterologous proteins on a large scale (153, 154). The *Listeria* phage endolysins Ply118 and Ply511, which are an L-alanoyl–D-glutamate peptidase and an *N*-acetylmuramoyl–L-alanine amidase, respectively, were the first endolysins successfully produced and secreted by *L. lactis*. That study was conducted in an effort to develop dairy starter strains with biopreservation properties for enhanced food safety (111). Similarly, Ply511 and lysostaphin were produced and secreted in various different lactic acid bacteria, one of which (a *Lactobacillus plantarum* strain secreting lysostaphin) was able to reduce numbers of *S. aureus* bacteria by >7 log units in a coculture study (155).

O'Flaherty and colleagues were the first ones to produce a staphylococcal phage endolysin (LysK) in *L. lactis* (124), using a nisin-inducible expression system (Fig. 4) (156), in order to overcome solubility problems encountered when attempting expression in *Escherichia coli* at that time. In this case, the active endolysin was not secreted, but its staphylolytic activity was detected in lysates of the *L. lactis* culture (124). The crude lysates caused a 99% reduction in numbers of MRSA bacteria within 1 h, demonstrating that recombinantly produced LysK in *L. lactis* is able to eradicate live cells of pathogenic staphylococci (124).

More recently, the same expression system was used for the production of the staphylococcal phage endolysin LysH5 in *L. lactis* (157). In addition to the P*nisA* promoter, various other constitutive and inducible lactococcal promoters were tested in order to optimize production. As opposed to the previous study with LysK, a signal peptide was fused to the LysH5 endolysin, which resulted in its secretion into the culture supernatant (Fig. 4). However, secretion was found to occur with low efficiency, and most of the protein was still found in the cellular fraction. In conclusion, the production and secretion of recombinant endolysins in lactic acid bacteria could be a

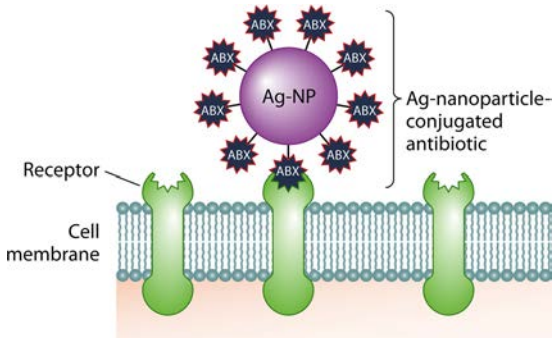

FIG 5 Schematic structure of an antibiotic-nanoparticle conjugate reacting with receptors within the cytoplasmic membrane. Ag-NP, silver nanoparticle; ABX, any kind of natural, synthetic, or recombinant antibiotic.

viable method to deliver the enzymes into food products, but the efficiency of the expression and secretion systems still needs to be improved.

NANOTECHNOLOGY AS A NEW STRATEGY FOR ENDOLYSIN DELIVERY

Considering the continuous evolution of resistance mechanisms against multiple commercial antibiotics, regimes of traditional therapies need to be overhauled and developed into novel strategies (158). Nanoparticles (NPs) are now being used extensively for the delivery of therapeutic agents such as anticancer drugs to eukaryotic cells (159, 160), and they also offer possibilities to effectively target bacterial cells (161, 162). Since surfaces of various NPs can be modified to allow conjugation with biological molecules via different functional groups, these particles can be utilized as efficient vehicles that are able to deliver a predetermined amount of antibiotic molecules to the target pathogens (163, 164).

Several research groups are now investigating nanotechnology-based methods to design delivery vehicles for antibiotics and other antimicrobial compounds (159, 160). Similarly, there are possibilities for engineering surfaces or interiors of NPs with fluorophores in order to achieve quantitative detection (165). By utilizing these properties in combination, NPs could be capable of improving diagnostics and the treatment of bacterial infections at the same time (166, 167).

Aiming to increase therapeutic efficacy, one or several morphological and antibacterial features of NPs can be exploited. This includes the application of NPs as (i) membrane-penetrating agents in order to effectively transduce antimicrobials into bacterial cells (168–171), (ii) drug-concentrating agents enabling polyvalent effects through tight packing of antimicrobials on the NP surface (172, 173), and (iii) attacking agents that are able to specifically act against biological targets, either through inherent antimicrobial properties or through coupling with antimicrobial agents (174–176).

Some NPs, such as those consisting of copper, silver, and selenium, have intrinsic antimicrobial properties, which could possibly be further enhanced by combining or coating them with more specific antibacterial agents, taking advantage of synergistic effects (177, 178). For example, several reports described enhanced antimicrobial effects of antibiotics against various bacterial pathogens, including *S. aureus*, when used in combination with silver NPs (Fig. 5) (179–181). Besides NPs with intrinsic antimicrobial properties, there are other types of NPs that can be used in therapeutic applications that do not exhibit any antimicrobial effect themselves but are able to enhance the effect of other therapeutic agents or approaches. Gold nanoparticles (Au-NPs), for instance, are employed in several antimicrobial applications and have been reported to enhance the killing efficacy of antimycobacterial peptides (182).

There are various examples in the literature describing the conjugation or combined application of phage endolysins or individual domains thereof with different types of nanoparticles. The *Listeria* phage endolysin Ply500 is a reportedly strong antimicrobial

agent that is active against strains of the foodborne pathogen *Listeria monocytogenes* (183). A conjugate of Ply500 with silica nanoparticles (SNPs) retained the activity of the endolysin and was able to reduce numbers of *Listeria* bacteria in suspension by almost 5 log units within 24 h. A complete eradication (~4-log reduction) of *Listeria* bacteria was achieved on iceberg lettuce (184). Surprisingly, the conjugation of SNPs with Ply500 significantly enhanced the stability of the endolysin. When incubated at 25°C for 15 days, the Ply500-SNP conjugates retained >90% of their activity, whereas the unconjugated enzyme was completely inactivated over the same period of time (184). In that same study, Ply500 was immobilized on edible cross-linked starch NPs via maltose-binding protein (MBP). Both engineered Ply500-MBP fusion proteins alone and the Ply500-MBP-NP conjugates caused a >2.5-log reduction of the number of *Listeria* bacteria in suspension. These results demonstrate that the immobilization of lytic enzymes on NPs can enhance protein stability and thereby the efficacy of the enzymes in food applications, where lytic activity during extended periods of storage is desired. Similarly, the conjugation of SNPs with LysK or other *S. aureus*-specific endolysins could improve the efficacy of these enzymes in certain niche applications.

Other nanotechnology-based approaches employ certain polymers as controlled drug release vehicles, which are able to initiate a "burst response" triggered by certain host- or pathogen-produced stimuli. These stimuli may include temperature or pH changes; consequences of a host immune response, such as increased cytokine concentrations (185, 186); and molecules released from infecting bacteria, such as enzymes, secreted toxins, and signaling molecules (187–189). Various polymers have been studied as potential drug release vehicles for research and clinical purposes, including poly(*N*-isopropylacrylamide) (PNIPAM) (190), chitosan (CS) (191, 192), fucoidan (F) (192), PLLs (193), and poly(lactic-co-glycolic acid) (PLGA) (194). PNIPAM is a thermostatic polymer that is able to reversibly undergo a phase transition at its lower critical solution temperature (LCST), which leads to water expulsion, followed by a polymer volume change. By controlling the polymer concentration as well as surfactant and copolymer concentrations, the LCST of PNIPAM and the structures derived from this polymer (NPs, nanogels, and micelles, etc.) can be manipulated and adjusted to a clinically relevant temperature, resulting in a wide range of possible biomedical applications, such as wound healing, cell cultivation, bioscaffolding, and cancer therapy (195–197). NPs of PNIPAM copolymerized with allylamine have been used for the controlled release of bacteriophage K as an active antistaphylococcal agent, in which NP collapse was thermally controlled (198).

More recently, that same group used PNIPAM NPs for the coadministration of the endolysin construct $CHAP_K$ and lysostaphin via a thermally triggered release event. Aiming to develop this system for the treatment of *S. aureus* skin infections, the temperature at which the controlled expulsion of $CHAP_K$ and lysostaphin from the polymer occurs was adjusted to 37°C, corresponding to the threshold temperature of an infected wound in the skin. While no bacterial lysis in the presence of the NPs occurred at 32°C (the temperature of healthy skin), numbers of *S. aureus* bacteria were reduced by >4 log units when the temperature was increased to 37°C, owing to the release of the active enzyme mix. $CHAP_K$ and lysostaphin were chosen for these experiments due to their demonstrated synergistic effect when used in combination (199).

The generation of complexes consisting of endolysins and cationic polymers (PLLs) has also been reported as a possible way to produce antimicrobial agents with high activity against the target bacteria and enhanced stability at both storage and physiological temperatures. The stability of LysK in complexes with PLLs and their block copolymers with polyethylene glycol (PEG) was improved by both hydrophobic and electrostatic mechanisms (193). The stabilizing effect was attributed to enhanced structural ordering and breaking of aggregates due to electrostatic interactions. In

addition, the lytic activity of LysK increased 3- to 3.5-fold when complexed with cationic polymers compared to the free form (193).

Another interesting NP-based approach relies on the thermal ablation of pathogens by Au-NPs functionalized with CBDs from endolysins instead of the antimicrobial properties of the endolysins themselves. The CBDs immobilized on the surface of the Au-NPs ensure their specific binding to the surface of the target pathogens. Once in the proximity of the bacteria, the NPs can be heated to high temperatures by infrared (IR) irradiation to kill the organisms. This principle was successfully demonstrated with Au-NPs functionalized with the CBD of PlyC, PlyCB, which reduced numbers of streptococci by several log units upon IR irradiation, while irradiation alone had only a minor effect (200). This approach may also be extended to other bacterial genera such as staphylococci by using CBDs specific for the target pathogen.

CONCLUDING REMARKS

Strains of pathogenic bacteria that are resistant to one or multiple antibiotics are increasingly prevalent, and since this has become a problem of global significance, alternative solutions are urgently needed. Endolysins show great potential as a possible replacement for or an addition to conventional antibiotics. Their high antibacterial activity and specificity and the low chance of resistance development make them promising candidates as antimicrobials against multiple bacterial pathogens and for various fields of application, including medicine, food safety, and agriculture. Most staphylococcal phage endolysins show a broad activity spectrum for the genus *Staphylococcus*. After the first *in vitro* characterization of a purified staphylococcal phage endolysin in the 1990s and the first *in vivo* studies 1 decade ago, research on these potent enzymes has intensified, and most recently, the first clinical study in humans was reported.

LysK and its close homologues LysGH15 and SAL-1 are among the best-studied staphylococcal phage endolysins to date. Several attributes of these enzymes, including their high staphylolytic activity; the presence of 2 catalytic domains, which further reduces the chance of resistance formation; and the finding that they act synergistically with other antimicrobials support their further exploitation as valuable and robust antibacterial agents for the treatment of *S. aureus* infections. Moreover, enzymatic activity and stability may be increased by the addition of low-cost additives such as glycerol and calcium ions, and novel delivery strategies may further enhance the efficacy of these endolysins in various types of applications. The ability of lactic acid bacteria to express and secrete functional endolysins opens new possibilities for applications in food production and processing.

Furthermore, researchers have recently focused on the development of novel nanotechnology-based delivery vehicles for antimicrobial agents, including phage endolysins. Controlled-release systems, which allow the delivery of endolysins to infection sites depending on parameters indicative of inflammation, may be of particular interest for future developments.

In conclusion, staphylococcal phage endolysins such as LysK and its derivatives hold great promise for being used as novel therapeutic agents against infections by *S. aureus* in the future, presenting several important advantages over traditional antibiotics. The first endolysin-based products have already hit the market, and in the light of ongoing clinical trials, more developments can be expected in the near future.

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Hamed Haddad Kashani obtained his Biochemistry (M.Sc.) degree from JH University of New Delhi, India, in 2007. Between 2008 and 2012, he worked as a researcher in several research centers in different fields of molecular biology, including the production of vaccines, natural drugs, and recombinant proteins. Since 2012, he has been a Ph.D. student of the Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan, Iran, in the field of Biotechnology under the supervision of Prof. Rezvan Moniri, where he has focused on the development of endolysin-based therapeutics against methicillin-resistant *Staphylococcus aureus* (MRSA). During the Ph.D. program, he has published several research papers related to the recombinant endolysins.

Mathias Schmelcher studied Biology at the Technical University of Munich, Germany, and obtained his Ph.D. from ETH Zurich, Switzerland, in 2008, working on the engineering of bacteriophage endolysins for detection and control of the foodborne pathogen *Listeria monocytogenes*. Between 2009 and 2012, he was employed at the Agricultural Research Service, U.S. Department of Agriculture, in Beltsville, MD, as a postdoctoral associate, where he focused on the development of endolysin-based therapeutics for treatment of bovine mastitis. After his return to ETH Zurich as a senior scientist and lecturer, he has continued research on endolysins and their possible applications against bacterial pathogens in medicine, food safety, and agriculture, with a strong focus on *Staphylococcus aureus*.

Hamed Sabzalipoor obtained his biotechnology (M.Sc.) degree from the department of biotechnology at Tarbiat Modares University, Tehran, Iran, in 2014 and specializes in genetic engineering in crop plants. He is currently a Ph.D. student of Nanobiotechnology, at the same university, working on designing and fabrication of optical biosensors under the supervision of Dr. Maryam Nikkhah. His current research interests have focused on the development and application of metal-enhanced fluorescence-based nanobiosensors for the detection of breast cancer biomarkers, particularly trace amounts of microRNAs, which can predict the aggressiveness of the disease and drive better treatment decisions.

Elahe Seyed Hosseini obtained her biotechnology (M.Sc.) degree from the Biotechnology Research Center at Tehran, Iran, in 2013, where she focused on photoprotein purification by a self-cleavage method. Since 2014, she has been a Ph.D. student of molecular biotechnology at the Kashan University of Medical Sciences, Kashan, Iran, under the supervision of Prof. Hossein Nikzad. Her current focused area of research is on the effect of dendrosomal nanocurcumin on apoptosis induction and expression of MEG3, H19, and HOTAIR long noncoding RNAs in SKOV3 and OVCAR3 ovarian cancer cell lines.

Rezvan Moniri is a professor of medical microbiology. She received a doctorate degree in Veterinary Medicine from the School of Veterinary Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. She was awarded a Ph.D. in Medical Microbiology from the Tehran University of Medical Sciences for her studies on the comparison of different methods for diagnosis of *Helicobacter pylori* infection with Dr. Parviz Adibfar. She received a postdoctoral fellow in Virology from the laboratory of Dr. Chetankumar Tailor in 2003 at Sick Kids Hospital, Toronto, Canada. Since 2010, she has joined the Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan, Iran, as a Molecular Biology Researcher. She has continued her research on endolysins against methicillin-resistant *S. aureus*. Dr. Moniri was member of the Iranian Ministry of Health and the Medical Education Board of Medical Virology from 2005 to 2011, and she has been a member of the Board of Medical Microbiology since 2014.