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Lytic Activity of Recombinant Bacteriophage ϕ11 and ϕ12 Endolysins on Whole Cells and Biofilms of *Staphylococcus aureus*▽

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The recombinant ϕ11 endolysin hydrolyzed heat-killed staphylococci as well as staphylococcal biofilms. Cell wall targeting appeared to be a prerequisite for lysis of whole cells, and the combined action of the endopeptidase and amidase domains was necessary for maximum activity. In contrast, the ϕ12 endolysin was inactive and caused aggregation of the cells.

Infections caused by *Staphylococcus aureus* and *Staphylococcus epidermidis* still play a major role in human and animal disease. In particular staphylococcal biofilms on indwelling devices are difficult to treat due to their inherent antibiotic resistance (5, 7, 26). In this context, it is important to develop alternative treatment strategies to combat staphylococcal infections, particularly in view of the ability of staphylococci to acquire resistance to commonly used antibiotics (10, 23, 29). Phage lysins, or endolysins, have received considerable attention as possible antimicrobial agents against gram-positive bacteria and have been applied to a variety of pathogens, such as *Bacillus anthracis* (21), *Streptococcus pneumoniae* (9), and *S. aureus* (25). Endolysins are active against dead cells and even living, planktonic cells (9, 25), but their ability to lyse the complex structure of staphylococcal biofilms has not yet been investigated. In this approach, we cloned and heterologously overexpressed the lysis genes of the bacteriophages ϕ11 and ϕ12 of *S. aureus* NCTC8325 in *Escherichia coli* for subsequent analysis of the lytic activity of the enzymes and their single subdomains on cell walls, whole cells, and biofilms. Knowledge of the lytic activity of both endolysins is limited. Their nucleotide sequences have been published (16), and the ϕ11 endolysin has been shown to possess a D-alanyl-glycyl endopeptidase and an *N*-acetylmuramyl-L-alanine amidase activity on crude cell walls of *S. aureus* OS2 (24).

Sequence comparison, cloning, and overexpression of ϕ11 and ϕ12 endolysins. The ϕ11 and ϕ12 endolysin sequences were BLAST searched against the NCBI protein database. Both endolysins are modular enzymes which consist of three distinct domains coding for an N-terminal CHAP (*c*ysteine, *h*istidine-dependent *a*midohydrolases/*p*eptidases) domain with hydrolytic function, a central amidase domain (*N*-acetylmuramyl-L-alanine amidase), and a C-terminal SH3b domain, which is involved in cell wall recognition (1). In spite of their similar domain architecture, these endolysins show low sequence identity (26.9%).

The endolysin genes of the bacteriophages ϕ11 (open reading frame [ORF] 53; 1,473 bp; accession number NC_004615) and ϕ12 (ORF 49; 1,455 bp; accession number NC_004616) were amplified by PCR from genomic DNA of *S. aureus* NCTC8325, using the primers listed in Table 1. In order to test the activity of the ϕ11 endolysin subunits, the endopeptidase unit (ϕ11endo, amino acids [aa] 1 to 180) and the amidase unit (ϕ11ami, aa 180 to 371) as well as each unit plus the cell wall binding domain (ϕ11endo/CBD, aa 1 to 180/371 to 490, and ϕ11ami/CBD, aa 180 to 490) were constructed separately (Fig. 1). In addition, the cell wall binding module was deleted from the ϕ11 endolysin (ϕ11endo/ami, aa 1 to 371). The amplification products were cloned into the multiple cloning site of the expression vector pET22b (Novagen) without the *pelB* leader tag to inhibit protein transport to the periplasm of the expression host. The resulting plasmids, pETerΔ11, pETerΔ12, pETendo11, pETendoCBD11, pETami11, pETamiCBD11, and pETendo/ami11, were used to overexpress each endolysin as a C-terminal six-His-tagged fusion protein. After subcloning of the plasmids in *E. coli* JM109, *E. coli* BL21(DE3) was used as a host for expression of each six-His-tagged endolysin. Expression cultures were grown in Luria-Bertani (LB) broth containing ampicillin (40 μg/ml) to an optical density at 600 nm (OD_{600}) of 0.6. Then protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Expression cultures were harvested after 4 h followed by protein purification steps under native conditions via nickel-nitrilotriacetic acid affinity chromatography (Fig. 2). Protein purification was also performed with cells harboring the empty vector, and the eluate served as a control in the activity tests.

Lytic activity of ϕ11 endolysin modules. The lytic activities of the full-length ϕ11 endolysin and its deletion variants (each applied in a concentration of 20 μg/ml) were examined photometrically at 600 nm employing *Staphylococcus simulans* 22 cell walls (purified with sodium dodecyl sulfate [SDS]) (4) resuspended in incubation buffer (50 mM Tris-HCl, 100 mM NaCl; pH 7.5) to an OD_{600} of ~0.3. All lysis experiments were performed in triplicate. The full-length enzyme showed efficient lysis of the peptidoglycan compared to the control (empty-vector eluate), and both the endopeptidase and the amidase module plus cell wall binding domain (ϕ11endo/CBD and ϕ11ami/CBD) were active and able to lyse cell walls. However, the full-length enzyme was more active than the isolated sub-

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s) or sequence[a]	Reference(s) or source
Strains		
E. coli		
K-12 JM109	Subcloning host for pET22b derivatives	34
BL21(DE3)	λDE3 lysogen, T7 RNA polymerase gene; expression host	30
S. aureus		
NCTC8325	Biofilm-positive, laboratory strain, lysogenic for bacteriophages ϕ11 and ϕ12	16
Cowan 1	MSSA, catheter infections by binding of fibrinogen and fibronectin	6, 18
Newman	MSSA	18, 32
Wood 46	Protein A-deficient, alpha-toxin-producing strain	6
S. simulans 22	Penicillin resistant, lysostaphin susceptible	19
S. epidermidis O-47	Biofilm-positive, clinical isolate causing CVC-associated infections	13, 28
Plasmids		
pET22b	C-terminal six-His-tagged expression vector, T7 *lac* promoter, β-lactamase gene (Amp^r); in this study, without *pelB* leader tag	30
pETerΔ11	pET22b + ORF 53 of ϕ11 (aa 1 to 490, 56.5 kDa) (I/II)	This study
pETendo/ami11	pET22b + truncated ORF 53 of ϕ11 (aa 1 to 371, 43.2 kDa) (I/IX)	This study
pETendo11	pET22b + truncated ORF 53 of ϕ11 (aa 1 to 180, 22.1 kDa) (I/V)	This study
pETendoCBD11	pET22b + truncated ORF 53 of ϕ11 (Δaa 181 to 370, 35.2 kDa) (I/V; VI/VII)	This study
pETami11	pET22b + truncated ORF 53 of ϕ11 (aa 180 to 371, 22.8 kDa) (VIII/IX)	This study
pETamiCBD	pET22b + truncated ORF 53 of ϕ11 (aa 180 to 490, 36.1 kDa) (VIII/II)	This study
pETerΔ12	pET22b + ORF 49 of ϕ12 (aa 1 to 484, 55.3 kDa) (III/IV)	This study
Primer no. and name		
I; phi11F	5′ TTTGGATCCAATGAGTATCATCATGGAGGTG 3′ (BamHI)	
II; phi11R	5′ GTCAAGCTTACTGATTTCTCCCCATAAGTCA 3′ (HindIII)	
III; phi12F	5′ GTGGGATCCAATGTTGATAACAAAAAACCAA 3′ (BamHI)	
IV; phi12R	5′ TAAAAGCTTAATCGTGCTAAACTTACCAAAAC 3′ (HindIII)	
V; endo11R	5′ TTGAAGCTTAGCTGTTTCTTTTTTAGGT 3′ (HindIII)	
VI; CBD11F	5′ ATGAAGCTTAAAATACCGGTTGCCACTGT 3′ (HindIII)	
VII; CBD11R	5′ GTCCTCGAGACTGATTTCTCCCCATAAGTCA 3′ (XhoI)	
VIII; ami11F	5′ GAACATATGAAGCCACAACCTAAAGCAGTAG 3′ (NdeI)	
IX; ami11R	5′ CGGAAGCTTACCATCCATGTACGCCCTAA 3′ (HindIII)	

[a] Roman numerals in parentheses are primer pairs used for amplification of ORFs (forward/reverse). Restriction site sequences are underlined, and the sites are given in parentheses. MSSA, methicillin-susceptible *S. aureus*; CVC, central venous catheter.

domains, and only the combination of ϕ11endo/CBD and ϕ11ami/CBD (20 μg/ml each) restored full lytic activity (Fig. 3). The effect of the truncation was even more pronounced with whole cells. To this end, cells of *S. aureus* NCTC8325 were grown overnight, diluted in incubation buffer to an OD_{600} of approximately 0.3, and pasteurized for 10 min at 80°C. After addition of equivalent amounts of endolysin (20 μg/ml), the lytic activity was determined as before. In contrast to the full-length protein (Fig. 4), the isolated amidase or endopeptidase domains were hardly active against heat-killed cells (data not shown). In conclusion, the two catalytic domains of the enzyme have to be combined with each other to cleave the cell wall of intact cells. Thus, the ϕ11 endolysin seems to belong to a group of endolysins which act as multifunctional hydrolases (2). Recently, the lytic enzyme of phage ϕWMY of *Staphylococcus warneri* M (LysWMY) was reported to show strong similarities in its domain architecture to the ϕ11 endolysin (35). In contrast to the ϕ11 endolysin, LysWMY retained its full activity even when both the amidase and cell wall binding domains had been deleted. This result indicates that combined action of different domains does not seem to be an obligatory characteristic even among closely related murein hydrolases.

Purified cell walls were a much more sensitive substrate for the enzymes than heat-killed cells were, since they showed the residual activities of the single subdomains much more clearly. In spite of this, the ϕ12 endolysin did not show any activity with *S. simulans* 22 cell walls (data not shown).

Substrate recognition is essential for full lytic activity. Substrate recognition mediated by the cell wall binding domain, which is homologous to the C-terminal domain of lysostaphin (20), appeared to be necessary for high catalytic efficiency. Lytic activity of the ϕ11 endolysin on heat-killed staphylococcal cells was abolished after deletion of the cell wall binding domain (ϕ11endo/ami) (Fig. 4), while it retained nearly all of its lytic activity on SDS cell walls (data not shown). The single domains, ϕ11endo and ϕ11ami, showed significantly reduced lytic activity on SDS cell walls in the absence of the cell wall binding domain (Fig. 3). These findings are similar to the results obtained with lysostaphin and ALE-1, a glycyl-glycine endopeptidase homologous to lysostaphin, which showed a

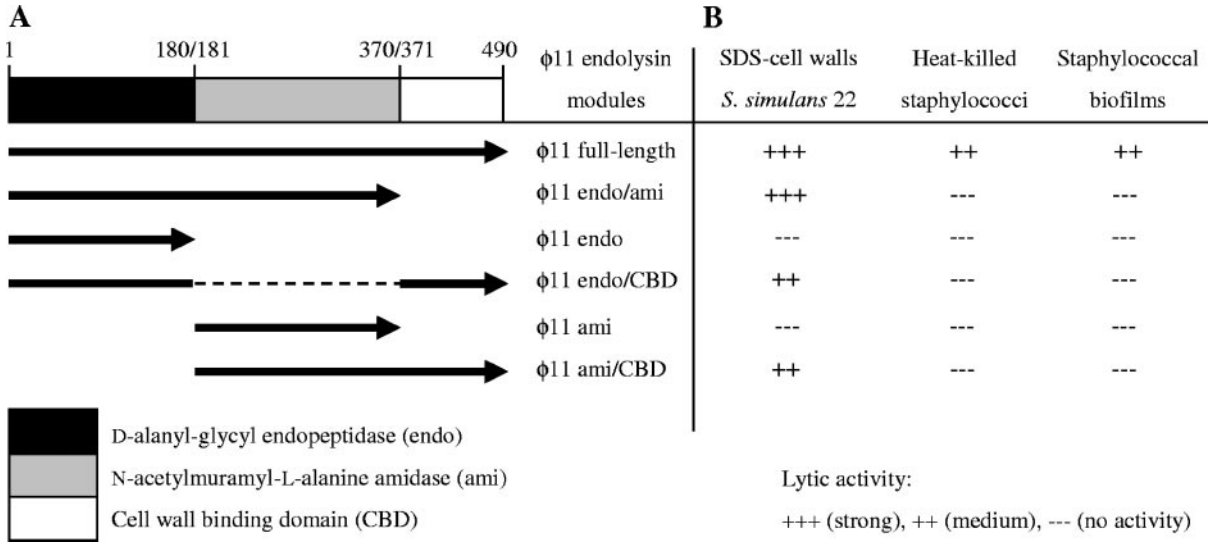

FIG. 1. Recombinant murein hydrolases of the staphylococcal bacteriophage ϕ11. A. Schematic overview of the ϕ11 endolysin modules illustrating the constructs tested in this study. The ϕ11 endolysin features a modular design which consists of an N-terminal endopeptidase domain (endo), a central amidase domain (ami), and a C-terminal cell wall binding domain (CBD). The full-length and deletion constructs of the ϕ11 endolysin were overexpressed as six-His fusion proteins. B. Lytic activity of the staphylococcal bacteriophage ϕ11 endolysin domains.

significant reduction in lytic activity on autoclaved staphylococci or viable cells, respectively, after deletion of their C-terminal cell wall targeting domains (1, 22), which bind to cross-linked peptidoglycan and recognize the Gly_5 interpeptide cross bridge (12, 22). Our results suggest that a similar targeting mechanism exists for the ϕ11 endolysin and that, in this respect, the phage enzyme closely resembles its staphylococcal counterparts.

Activities of the endolysins with different staphylococcal strains. We next analyzed the lytic activities of the ϕ11 and ϕ12 endolysins on whole cells of several staphylococcal strains. In addition to *S. aureus* NCTC8325, the full-length ϕ11 endolysin rapidly lysed heat-killed cells of *S. aureus* Wood 46, *S. aureus* Cowan 1, *S. aureus* Newman, *S. epidermidis* O-47, and *S. simulans* 22 (Fig. 4). Its lytic activity was equivalent to that of highly purified lysostaphin (5 μg/ml) (Dr. Petry Genmedics GmbH, Reutlingen, Germany).

Unlike ϕ11, the ϕ12 endolysin was not able to hydrolyze heat-killed cells of *S. aureus* Wood 46, *S. aureus* Cowan 1, or *S. aureus* Newman. However, after addition of the ϕ12 endolysin an increase in optical density of the cultures was observed (Fig. 5). This was accompanied and most probably caused by an aggregation of the cells that was macroscopically visible. This phenomenon was earlier described by Takano et al. (31), who investigated the influence of synthetic peptides derived from

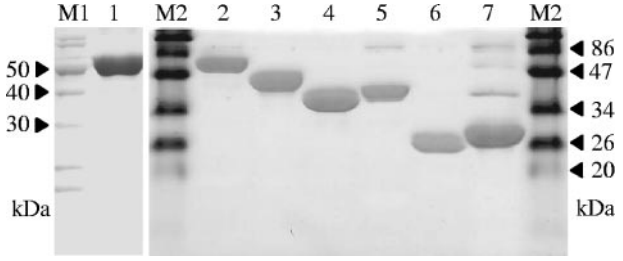

FIG. 2. SDS-polyacrylamide gel electrophoresis analysis of ϕ12 and ϕ11 endolysins and derived proteins purified by nickel-nitrilotriacetic acid affinity chromatography. The purified proteins were analyzed by 15% SDS-polyacrylamide gel electrophoresis and stained with PageBlue protein staining solution (Fermentas) according to the manufacturer's instructions. Lane M1, Fermentas Page Ruler unstained protein ladder; lanes M2, Fermentas prestained protein molecular weight marker; lane 1, ϕ12 endolysin (full length); lane 2, ϕ11 endolysin (full length); lane 3, ϕ11endo/ami; lane 4, ϕ11endo/CBD; lane 5, ϕ11ami/CBD; lane 6, ϕ11endo; lane 7, ϕ11ami.

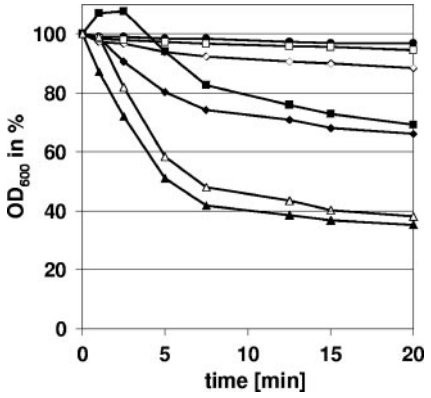

FIG. 3. Activity of the domains of the multifunctional ϕ11 endolysin. The lytic activities of purified enzyme samples of the full-length and truncated ϕ11 endolysin (20 μg/ml each) were examined using purified cell walls of *S. simulans* 22. The full-length enzyme (▲) was able to lyse the substrate efficiently compared to the protein eluate of the empty-vector control (●). The truncated enzymes ϕ11endo (□) and ϕ11ami (◇) did not show mentionable lytic activity. The truncated enzymes containing the cell wall binding domain, ϕ11endo/CBD (■) and ϕ11ami/CBD (◆), showed enhanced lytic activity but still worked less effectively than the full-length enzyme. The lytic activity could be restored by the parallel use of ϕ11endo/CBD and ϕ11ami/CBD (△).

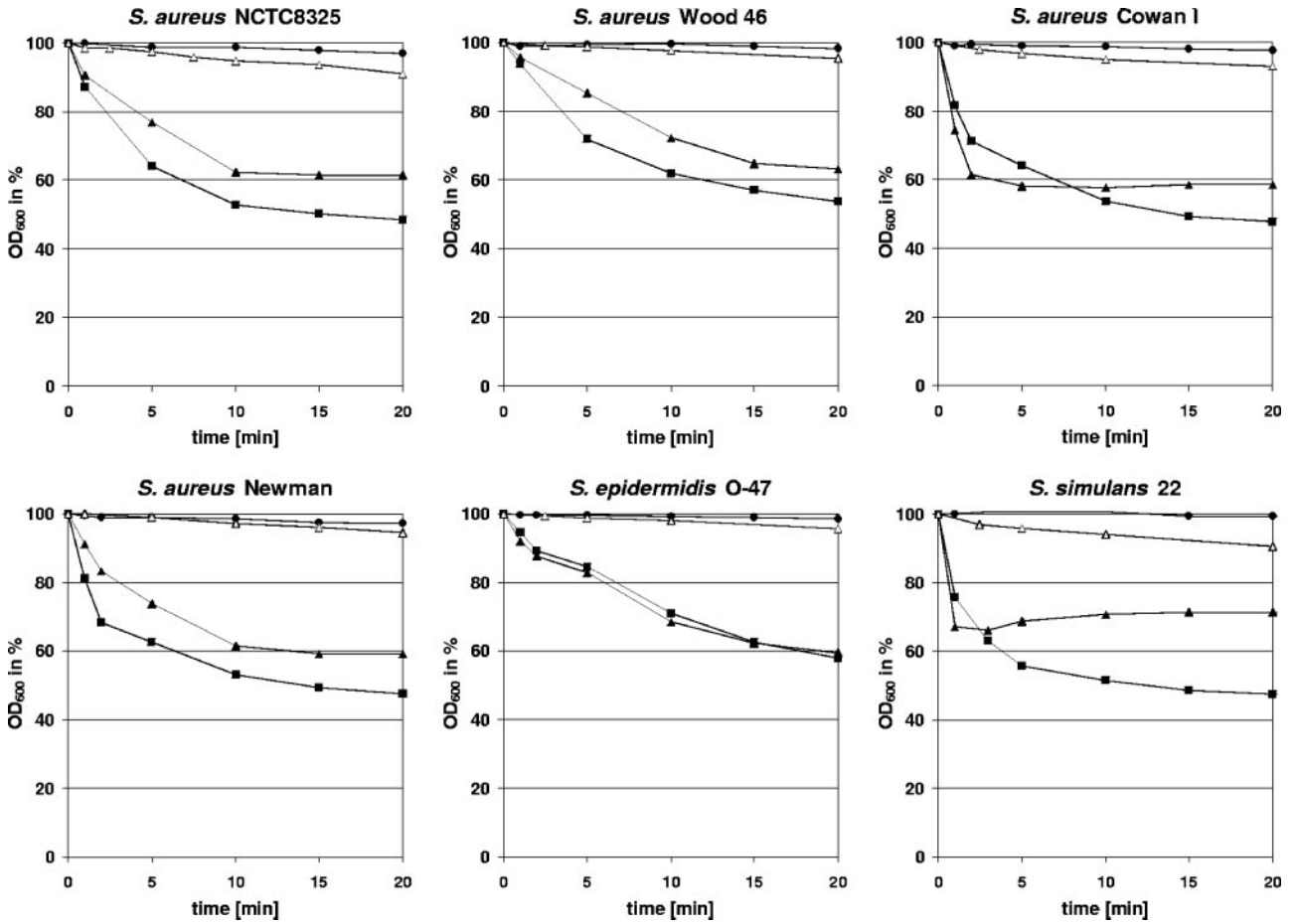

FIG. 4. The ϕ11 endolysin lyses whole cells of several staphylococci. Light scattering data showed that the ϕ11 endolysin (▲; 20 μg/ml) rapidly decreased the optical density of heat-inactivated *S. aureus* NCTC8325, *S. aureus* Wood 46, *S. aureus* Cowan 1, *S. aureus* Newman, *S. epidermidis* O-47, and *S. simulans* 22 cultures compared to the protein eluate of the empty-vector control (●). Its lytic activity was comparable to the effect observed after addition of lysostaphin (■; 5 μg/ml), which is a highly efficient glycyl-glycine endopeptidase. After deletion of the cell wall targeting domain, the ϕ11 enzyme (ϕ11endo/ami, △; 20 μg/ml) showed a significantly reduced activity, which indicates a fundamental role of the cell wall binding domain in efficient cell lysis.

the *S. aureus* major autolysin Atl on autolysis. Sequence comparison with the homologous amidase-3 domains of the *S. aureus* bacteriophages ϕSA 2MW, L54a, ϕSLT, PVL, 96, 3a, 53, 77, ROSA, and ϕETA and amidases of *Staphylococcus haemolyticus* JCSC1435 and *Staphylococcus epidermidis* RP62a identified an amino acid exchange at position 260 in the amidase-3 domain of the ϕ12 endolysin. This exchange introduces a histidine and therefore an additional positive charge in a position that is occupied by glutamine, glutamate, or asparagine in the other staphylococcal enzymes. Whether this amino acid exchange or an inadequate folding of the His-tagged protein is involved in the loss of hydrolytic activity is part of current investigations. Most probably, the ϕ12 endolysin in our test system is unable to exert efficient hydrolytic activity on staphylococcal murein, while retaining its ability to bind the cell wall. The substrate binding ability of the enzyme may then lead to the enhanced adhesion between the cells.

ϕ11 endolysin eliminates *S. aureus* NCTC8325 biofilm on artificial surfaces. Finally, we determined the influence of the ϕ11 and ϕ12 endolysins on staphylococcal biofilms by a modified biofilm plate assay (33). To this end, an overnight culture was diluted 1:200 in tryptic soy broth medium supplemented with 0.25% D-(+)-glucose to a final volume of 200 μl in each well of a 96-well polystyrene microtiter plate (Nunc, Wiesbaden, Germany). The plates were incubated for 24 h or 48 h at 37°C under aerobic and O_2-limited conditions, respectively. Afterwards, the wells were washed twice with incubation buffer. The biofilm-containing wells were then filled with incubation buffer plus endolysin. Fresh medium and incubation buffer alone or incubation buffer plus lysostaphin or lysozyme was employed as a control. Lysostaphin was used as a positive control, since it is able to lyse *S. aureus* and *S. epidermidis* biofilms (33). Following incubation for 2 hours at 37°C, the wells were washed again and stained with 0.1% safranin.

We found that the ϕ12 endolysin showed no hydrolytic activity on biofilms of *S. aureus* NCTC8325 and *S. epidermidis* O-47. In contrast, after addition of the ϕ12 endolysin we noticed an enhanced staining of the biofilms (Fig. 6). This effect is consistent with the results of the experiments performed with heat-killed cells and may be explained by a murein hydrolase-mediated adhesion of staphylococci (14, 15). In contrast, the purified ϕ11 endolysin was able to eliminate biofilms

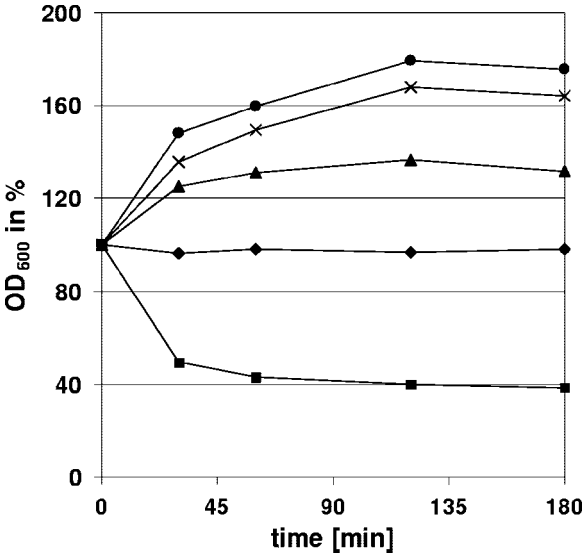

FIG. 5. Increase of optical density after addition of ϕ12 endolysin. Unlike the ϕ11 enzyme, the ϕ12 endolysin was not able to lyse heat-killed cells of any tested staphylococcal strain. Upon addition of ϕ12 endolysin (15 μg/ml) we detected an obvious increase of optical density of *S. aureus* Wood 46 (●), *S. aureus* Cowan 1 (×), and *S. aureus* Newman (▲). The protein eluate of empty-vector control (◆) showed no effect, whereas the control with lysostaphin (■; 5 μg/ml) lysed all staphylococcal strains.

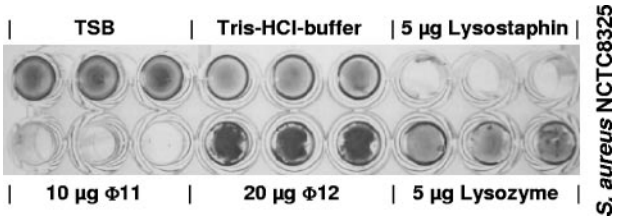

FIG. 6. Biofilm plate assay of *S. aureus* NCTC8325. The ϕ11 endolysin lysed biofilms of *S. aureus* NCTC8325 with an efficiency comparable to that of lysostaphin. In contrast, addition of the ϕ12 endolysin resulted in enhanced staining of the cells. TSB, tryptic soy broth.

of *S. aureus* NCTC8325, with an efficiency that was comparable to or slightly lower than that of lysostaphin (Fig. 6). However, there was no effect on biofilms of *S. epidermidis* O-47. To our knowledge, no phage lysin has been reported so far to disrupt staphylococcal biofilms.

Bacterial biofilm formation is part of a survival strategy to resist suboptimal environmental conditions such as limited nutrient availability or lethal concentrations of antibiotics. Antimicrobial agents often show significantly reduced effects on biofilms, which is thought to be due to several biofilm-inherent properties. For example, a low growth rate impedes the action of antibiotics (8, 29), and evidence is emerging that the sessile cells in biofilms live in an altered metabolic state (3, 27). Furthermore, the diffusion velocity of antibiotics is limited within biofilms. A reduced rate of antibiotic penetration leads to a gradually increasing concentration of the antibiotic in the deeper layers of a biofilm, which permits adaptation of the biofilm cells to the antibiotic by stress-induced metabolic and transcriptional changes (17). The ϕ11 endolysin most probably destabilizes the biofilm structure by fast lysis of sessile cells, which are embedded in the extracellular matrix or at the interface of the matrix and surface, whereupon the biofilm is dissolved. Another possibility could be an influence of the intrinsic nature of the biofilm. Neither sodium metaperiodate nor proteinase K treatment could completely eradicate biofilms of *S. aureus* NCTC8325 grown in tryptic soy broth (data not shown), which suggests that an elevated portion of proteinogenous biofilm is present along with a biofilm mediated by the characteristic polysaccharide (PIA). PIA consists of β-1,6-linked *N*-acetylglucosamine residues (11) and is not the target of the ϕ11 murein hydrolase. In contrast to *S. aureus* NCTC8325, *S. epidermidis* O-47 biofilms are exclusively polysaccharide mediated. Therefore, different natures of the biofilm matrices tested here could be an explanation for the varying efficacy of the ϕ11 endolysin.

Considering the huge clinical relevance of staphylococcal biofilms in terms of human diseases, the ϕ11 endolysin may constitute a novel strategy to combat *S. aureus* nosocomial infections that are mediated by biofilm formation on medical devices.

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