‘Artilysisation’ of endolysin λSa2lys strongly improves its enzymatic and antibacterial activity against streptococci

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Endolysins constitute a promising class of antibacterials against Gram-positive bacteria. Recently, endolysins have been engineered with selected peptides to obtain a new generation of lytic proteins, Artilysins, with specific activity against Gram-negative bacteria. Here, we demonstrate that artilysisation can also be used to enhance the antibacterial activity of endolysins against Gram-positive bacteria and to reduce the dependence on external conditions. Art-240, a chimeric protein of the anti-streptococcal endolysin λSa2lys and the polycationic peptide PCNP, shows a similar species specificity as the parental endolysin, but the bactericidal activity against streptococci increases and is less affected by elevated NaCl concentrations and pH variations. Time-kill experiments and time-lapse microscopy demonstrate that the killing rate of Art-240 is approximately two-fold higher compared to wildtype endolysin λSa2lys, with a reduction in viable bacteria of 3 log units after 10 min. In addition, lower doses of Art-240 are required to achieve the same bactericidal effect.

Endolysins are bacteriophage-encoded muralytic enzymes. They digest the peptidoglycan at the last stage of the lytic cycle, resulting in the release of newly formed virions from the infected host bacteria. Endolysin efficacy is of crucial importance for a successful bacteriophage multiplication cycle. Therefore, throughout evolution, bacteriophages have acquired highly efficient enzymes featured by high specificity1 and a low probability of developing bacterial resistance2–4. Endolysins are composed by a single or multiple catalytic domains and in many of them a cell wall binding domain is present5. This feature has facilitated endolysin engineering to design new chimeric proteins with desired properties like altered specificity6 and enhanced lytic activity7,8.

The finding that endolysins are able to attack Gram-positive bacteria and lyse them when added exogenously prompted their study as novel antimicrobial agents. Numerous in vitro and in vivo studies have shown the efficacy of endolysins to eliminate pathogenic bacteria from mucosal surfaces and systemic infections9. These characteristics, along with their efficacy against antibiotic-resistant bacteria, lack of toxicity and synergy with antibiotics makes them promising candidates to be exploited in the therapy of infectious diseases. In addition to clinical use, other applications of endolysins include their use in food safety, pathogen detection, disinfection, nanotechnology and vaccine development10.

Recently, endolysins have been engineered to overcome one of the main limitations in their use, i.e., their inefficacy against Gram-negative bacteria due to the presence of the outer membrane. Artilysis are optimized fusions of selected endolysins to specific outer membrane permeabilizing peptides, which promote transfer of the fusion protein across the outer membrane structure11. Upon translocation, the peptidoglycan is degraded, causing a rapid cell death by osmotic lysis12. Artilysis have been developed against Gram-negative pathogens.

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including *Pseudomonas aeruginosa* and *Acinetobacter baumannii in vitro and in vivo*, and have a low probability of resistance development similar to endolysins. They are highly bactericidal against multidrug-resistant isolates and persisters and may therefore be useful in the treatment of human and animal chronic infections.11,13,14

The endolysin λSa2lys from *Streptococcus agalactiae* prophage λSa2 has two catalytic domains showing β-D-glutaminyl-L-lysine endopeptidase and β-D-N-acetylglucosaminidase activity, respectively, and one cell wall binding domain (CBD), which comprises on its turn two Cpl-7 type subdomains.15 This enzyme has lytic activity against *Streptococcus pyogenes*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Streptococcus equi*, Group G *Streptococcus* and Group E *Streptococcus*.16 Chimeric proteins derived from endolysin λSa2lys through fusion of the endopeptidase domain to staphylococcal cell wall binding domains from either lysostaphin or staphylococcal endolysin LysK are highly active against *Streptococcus agalactiae*, *S. uberis* and *Staphylococcus aureus*.17,18

In this study, the ‘artilysation’ of an endolysin, i.e. the modification of an endolysin with a specific peptide with a dedicated function, was explored as a new approach to improve the muration and antibacterial properties of endolysins acting against Gram-positive bacteria. Contrary to Gram-negative targeting Artilyses, which comprise a peptide with an outer membrane permeabilizing function, the peptide in this novel Artilysin targeting a Gram-positive species is selected to improve cell wall affinity under a broader range of environmental conditions such as pH and ionic strength. As such, ‘artilysation’ does not only unlock the antibacterial potential of endolysins against Gram-negative species, but would also strengthen the antibacterial activity against Gram-positive species in a broader scope of applications. Here, we report on Art-240, a chimeric endolysin which is a fusion of the polycationic nonapeptide (PCNP) (composed of a mixture of arginine and lysine residues) to the C-terminus of λSa2lys. Analysis of Art-240 shows that artilysation generally improves both the muralytic and antibacterial properties of λSa2lys under a broad range of conditions.

### Results

**Art-240 has the same specificity as λSa2lys, but shows increased bactericidal activity.** Both λSa2lys and Art-240 were purified to homogeneity by metal affinity chromatography, resulting in a total yield of 11.5 and 8.36 mg/l of *Escherichia coli* expression culture, respectively. Purity of proteins was evaluated by SDS-PAGE (>95%). Major protein bands with estimated molecular masses of about 50 kDa, correlating well to the predicted molecular masses for λSa2lys and Art-240 (50.8 and 53.4 kDa), were observed (Supplementary File 1A,B). Melting curves of both λSa2lys and Art-240 were determined by monitoring the loss of α-helicity by CD spectroscopy at 222 nm and show that the structural stability of λSa2lys is conserved after artilysation with melting temperatures of 61.1 °C and 63.9 °C, respectively (Supplementary File 1C).

![ enzymatic activity against a broader pH range and a broader range of salt concentration.](https://www.nature.com/scientificreports/images/scientific-reports-9-35382.png)

**Art-240 has increased enzymatic activity over a broader pH range and a broader range of salt concentration.** The muralytic activity of Art-240 against *S. uberis* and *S. agalactiae* was tested at different pH values and compared to the activity of the λSa2lys endolysin. Enzymatic activity of Art-240 against *S. agalactiae* LiCC S461 (Fig. 2) is higher than that of λSa2lys at all assayed pH values (P < 0.01; P < 0.001). Similar to endolysin λSa2lys, Art-240 shows an optimal activity at pH 7–8. When *S. uberis* LiCC S648 is used as substrate (Supplementary File 3), a similar outcome is observed, but with more pronounced differences for alkaline pH values. A 6–fold increase in enzymatic activity is observed for Art-240 at pH 9 (P < 0.001). Art-240 retains some activity at pH 10, contrary to λSa2lys. In summary, artilysation of λSa2lys improves the enzymatic activity in a broad pH range.

The effect of artilysation was evaluated with regard to salt tolerance for both *S. agalactiae* LiCC S461 (Fig. 3) and *S. uberis* LiCC S648 (Supplementary File 4). The enzymatic activity of Art-240 is significantly higher than the activity of λSa2lys over a broad range of NaCl concentrations for both *S. agalactiae* LiCC S461 (P < 0.01; P < 0.001) and *S. uberis* LiCC S648 (P < 0.01; P < 0.001). Although the enzymatic activity of Art-240 is also clearly affected by NaCl concentrations above 0.5 M (*S. agalactiae* LiCC S461) or 0.75 M (*S. uberis* LiCC S648), activity...
| Bacteria        | Strain          | LiCC Nr. | λSa2lys 5µM | λSa2lys 2µM | λSa2lys 1µM | Art-240 5µM | Art-240 2µM | Art-240 1µM | PNCP 10µM | Buffer |
|-----------------|-----------------|----------|-------------|-------------|-------------|-------------|-------------|-------------|-----------|--------|
| *Streptococcus* |                 |          |             |             |             |             |             |             |           |        |
| *S. agalactiae* | B12-11.20.0190  | S439     | ++          | +/+         | ++          | +/+         | ++          | +/+         | ++        | —      |
|                 | vaO0413         | S461     | ++          | ++          | +/+         | ++          | ++          | +/+         | ++        | —      |
|                 | va80306         | S1047    | ++          | +/+         | +/+         | +/+         | ++          | +/+         | ++        | —      |
|                 | 1560            | S1046    | +           | +/+         | +/+         | +/+         | +/+         | +/+         | ++        | —      |
| *S. dysgalactiae* | B12-11.14.0245 | S436     | +++         | +++         | +++         | +++         | +++         | +++         | ++        | —      |
| *S. pyogenes*   | DSMZ 11728      | S271     | +++         | +++         | +++         | +++         | +++         | +++         | ++        | —      |
|                 |                 | 3347     | +++         | +++         | +++         | +++         | +++         | +++         | ++        | —      |
|                 | 10651           | S1049    | +++         | +++         | +++         | +++         | +++         | +++         | ++        | —      |
| *S. uberis*     | C2638           | S636     | +           | +           | ++          | +           | ++          | ++          | +        | —      |
|                 | C2673           | S637     | +           | +           | ++          | +           | ++          | ++          | +        | —      |
|                 | C2623           | S639     | +           | +           | ++          | +           | ++          | ++          | +        | —      |
|                 | C2691           | S640     | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | ++        | —      |
|                 | C2697           | S641     | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | ++        | —      |
| *S. suis*       | SN 16770        | S772     | +/+         | —           | —           | +          | +/+         | —           | —        | —      |
|                 | SN 19395        | S774     | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
|                 | SN 19396        | S775     | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
|                 | SN 20014        | S777     | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
|                 | SN 23674        | S778     | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
|                 | SN 26556        | S779     | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
| *S. porcinus*   | 2231            | S1048    | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
|                 | 34/11/13        | S848     | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
| *S. gordonii*   | 6365            | S1050    | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
| *S. sanguinis*  | 10505           | S1053    | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
| *S. viridans*   | 7351            | S1054    | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
| *Staphylococcus*|                 |          |             |             |             |             |             |             |           |        |
| *S. aureus*     | DSMZ 346        | S20      | —           | —           | —           | —           | —           | —           | —         | —      |
|                 | DSMZ 20231      | S21      | —           | —           | —           | —           | —           | —           | —         | —      |
|                 | Sp10            | S165     | —           | —           | —           | —           | —           | —           | —         | —      |
|                 | Sp10res         | S416     | —           | —           | —           | —           | —           | —           | —         | —      |
| *S. epidermidis*| B12-11.20.0190  | S440     | +/+         | +/+         | +/+         | +/+         | +/+         | +/+         | +/+       | —      |
| *Enterococcus*  |                 |          |             |             |             |             |             |             |           |        |
| *E. faecium*    | NCI1MB 11181    | S526     | —           | —           | —           | —           | —           | —           | —         | —      |
| *Pseudomonas*   |                 |          |             |             |             |             |             |             |           |        |
| *P. aeruginosa* | PAO1p           | S52      | —           | —           | —           | —           | —           | —           | —         | —      |
| *Escherichia*   |                 |          |             |             |             |             |             |             |           |        |
| *E. coli*       | 06-08410 (O103:H2) | S68 | —           | —           | —           | —           | —           | —           | —         | —      |
|                 | 03-07953 (O157:H7) | S72 | —           | —           | —           | —           | —           | —           | —         | —      |
| *Salmonella*    |                 |          |             |             |             |             |             |             |           |        |
| *S. Hadar*      | LGL-107 (Group C2-C3) | S796 | —           | —           | —           | —           | —           | —           | —         | —      |
| *S. Typhimurium*| LGL-57 (Group B)  | S808     | —           | —           | —           | —           | —           | —           | —         | —      |

**Table 1.** Spectrum of activity for endolysin λSa2lys and Art-240. Plate lysis assay with thirty-three *Streptococcus* sp., five *Staphylococcus* sp. and one *Enterococcus faecium* bacterial strains as well as three Gram-negative bacterial strains (*E. coli*, *Pseudomonas aeruginosa*, *Salmonella* sp.). Lysis zones were analyzed in a semi-quantitative manner, with +— corresponding to no visible lysis, and +/+—, ++, ++++ corresponding to an increasingly clear lysis determined in pairwise comparisons. Controls: buffer, PNCP peptide (10 µM spotted); Abbreviations: PCNP = polycationic nonapeptide. +++ very good, ++ good, + low, +/+—detectable, — no activity.
could be detected at high salt conditions up to 5 M NaCl. Artilysation with a polycationic peptide leads thus to increased enzymatic activity under a broad range of NaCl concentrations.

**Art-240 is enzymatically active against both exponential and stationary bacterial cells.** To analyze the influence of the growth stage on the susceptibility to Art-240, turbidity reduction assays using 0.1 μM of both proteins (Art-240 and endolysin λSa2lys) have been performed. *S. agalactiae* LiCC S461 cells collected...
during exponential (Fig. 4A) and stationary (Fig. 4B) growth phase are both susceptible to Art-240 with an enzymatic activity of 121 ± 16 and 84 ± 27 units, respectively. Similarly, endolysin λSa2lys is also active against cells in both growth stages but with reduced activities (61 ± 13 and 30 ± 4 units against exponential and stationary cells, respectively). Significant differences could be observed between Art-240 and endolysin λSa2lys activities on exponential cells after 5, 10 and 15 min of treatment (\(P < 0.05\); \(P < 0.001\)). The polycationic nonapeptide (PCNP) alone or the buffer alone do not lyse cells in both phases. Stationary phase cells are thus less susceptible for λSa2lys and Art-240, but the reduced activity of Art-240 against stationary phase cells remains similar compared to exponential phase cells.
to the enzymatic activity of λSa2lys against exponential phase cells. Artilylation of λSa2lys thus compensates the reduction in activity which is intrinsically related to stationary phase cells.

**Art-240 kills bacteria faster than its parental endolysin λSa2lys.** To quantify the killing rate of Art-240 time-kill experiments were performed using *S. agalactiae* LiCC S461 cultures treated with 0.1 μM of each lytic protein at the optimal pH (pH 7) (Fig. 5). Art-240 acts more rapidly than λSa2lys. Art-240 causes a reduction in cell number of 1.7 ± 0.3 log units after 5 min (*P* < 0.05), whereas λSa2lys needs 30 minutes to cause a similar reduction in cell number (1.8 ± 0.1 log units). The rapid onset of action by Art-240 is also evidenced after 2 min of incubation (*P* < 0.05) with a reduction in the viable bacterial count (one log unit) while no activity of the endolysin λSa2lys is detected after this short exposure time. In addition, the extent of killing is significantly greater in case of Art-240 with a reduction of 3.8 ± 0.2 log units after 30 min. This represents a twofold increase compared to λSa2lys at the same time point (*P* < 0.001). The experiment was repeated under identical conditions, but now time-dependent killing was monitored using time-lapse microscopy (Fig. 6, Movie S1). While both λSa2lys and
Art-240 induce osmotic lysis of bacterial cells, the more rapid killing of Art-240 is clearly visualized and a more complete lysis is achieved in comparison to the λSa2lys endolysin. Controls (PCNP or buffer alone) do not visibly affect the structural integrity.

Lower doses of Art-240 are required to achieve a similar bactericidal effect against *S. agalactiae* LiCC S461 compared to λSa2lys. A dose-response curve from 6.25 nM to 200 nM of Art-240 was determined at pH 7. The bactericidal effect was quantified after 1 h of incubation for the different doses and compared to those obtained after endolysin λSa2lys exposure (Fig. 7). The antibacterial activity of Art-240 outcompetes the λSa2lys antibacterial activity, which is most outspoken at low concentrations (P < 0.05). To achieve the same bactericidal effect, a 4 to 12-fold higher dose of λSa2lys is needed. E.g., a reduction of about 2 log units is observed for 12.5 nM Art-240, while 150 nM λSa2lys is needed to cause the same reduction. Moreover, an increase in λSa2lys concentration over 50 nM does not further improve the antibacterial activity but a higher reduction in bacterial counts (P < 0.01; P < 0.001) is achieved with Art-240 (Fig. 7). In conclusion, Art-240 is more effective and requires smaller doses than the endolysin to achieve a comparable bactericidal effect.

**Discussion**

Artilysins represent a new class of engineered endolysins with high antibacterial activity against Gram-negative bacteria. The design of these enzymes implies the fusion of a selected outer membrane permeabilizing peptide to a specific endolysin. The cationic or amphipathic peptide moiety destabilizes the lipopolysaccharide (LPS) layer of the outer membrane by interfering with the ionic interactions between divalent cations and LPS phosphate groups and the hydrophobic forces between the fatty acids of lipid A that stabilize the outer membrane structure. The peptidoglycan layer becomes accessible to the endolysin moiety, resulting in peptidoglycan degradation and cell death by osmotic lysis. Here, we have shown that the process of artilysation, i.e. modifying the properties of an endolysin by fusion with a specific peptide, represents a novel approach to improve the enzymatic and antibacterial properties of an endolysin active against Gram-positive bacteria. For Art-240 we made use of the polycationic nonapeptide (PCNP) which was previously successfully used in the construction of Artilysins against Gram-negative *P. aeruginosa*. The rationally designed Art-240 outperforms λSa2lys, the endolysin from which it is derived, in (i) an increased enzymatic activity under a broad range of pH and NaCl conditions, (ii) a faster and more complete lysis/killing and (iv) the requirement of reduced doses. At the same time, the good protein stability of λSa2lys, its high specificity for streptococci and activity against stationary cells is conserved after artilysation.

Generally, endolysins are specific to a species (or genus) of bacteria naturally infected by the phages that encode them. In good agreement with this, λSa2lys shows high activity against different species of *Streptococcus* and low activity against *S. epidermidis*. Table 1 shows a similar specificity for Art-240, demonstrating that the PCNP did not extend the spectrum to other Gram-positive microorganisms, such as *E. faecium*, or Gram-negative bacteria. Indeed, the fusion of PCNP to endolysin λSa2lys may allow transport of Art-240 through the outer membrane of Gram-negative bacteria as has been shown before for other engineered endolysins but if this were to happen, the transfer appears not to be sufficient to lyse them. This was likely because of the different peptidoglycan chemotype between Gram-negative bacteria (A1γ) and streptococci (A3γ). Indeed, the specificity of endolysins is due to the cell wall binding and/or the catalytic domains. λSa2lys harbors apart from two catalytic domains (a glucosaminidase and endopeptidase domain) a cell wall binding domain (CBD) that comprises two Cpl-7 subdomains. Typically, CBDs of endolysins show a high affinity and specificity for their targets with low affinity constants. Changes in the activity spectrum of the endolysin λSa2lys were previously reported as bacterial reduction (log10 units) relative to the untreated control after 1 h of treatment. Data are means ± standard deviations of three replicates. Student t-test was performed to compare the activity of λSa2lys and Art-240. *P < 0.05; **P < 0.01; ***P < 0.001.
### Table 2. Overview of oligonucleotides.

The list of primers for PCR amplification used in this study are summarized. The restriction sites used for cloning are boxed in the respective primer sequence. Nucleotides encoding for the polycationic nonapeptide (PCNP) are indicated in bold. Nucleotides encoding the His6-tag are underlined and in bold.

| Constructs | Oligonucleotides | Primer sequence |
|------------|------------------|-----------------|
| λSa2lys   | λSa2lysNdeIFw    | 5′-GGATTCGATATGAGATTAACACCGAAATCGC-3′ |
| λSa2lys   | λSa2lysXhoIRv    | 5′-GGATTCCACGGGTGTTTTTGGTCAG-3′ |
| Art-240   | λSa2lysNdeIFw    | 5′-GGATTCGATATGAGATTAACCCGAAATCGC-3′ |
| λSa-His6AflI-P-XhoIRv-1 | Sa2lysNdeIFw  | 5′-GGATTCGATATGAGATTAACCCGAAATCGC-3′ |
| λSa-His6AflI-P-XhoIRv-2 | Sa2lysXhoIRv | 5′-GGATTCCACGGGTGTTTTTGGTCAG-3′ |

### Materials and Methods

#### Bacterial strains and growth conditions.

All bacterial strains used in this study were provided by Goldberg-Klinik Kelheim GmbH, Germany, RWTH University Aachen, Germany, Bayerisches Landesamt für Gesundheit u. Lebensmittelsicherheit (LGL), Germany, Universitätsklinikum Regensburg, Germany, DSMZ (German Collection of Microorganisms and Cell Cultures), KU Leuven (Belgium), University of Veterinary Medicine Hanover (TiHs, Germany), or Robert Koch Institute (Wernigerode, Germany), each of which has been assigned with a specific LCC (Lisando Culture Collection) number. All strains were grown in brain heart infusion broth (BHI) (Oxoid Deutschland GmbH), except *E. faecalis, E. coli* and *S. Typhimurium*, which were grown in lysogeny broth (LB; Sambrook *et al.*, 1989) with or without shaking at 37 °C. *E. faecalis, S. suis, S. viridans* group, *S. sanguinis, and S. gordini* were grown under microaerophilic conditions (85% N₂, 10% CO₂, 5% O₂). Likewise, different *E. coli* strains were used in this study: *E. coli* NEB Turbo (New England Biolabs GmbH, Country) for DNA cloning and cell stock storage, and *E. coli* BL21(DE3)pLysS (New England Biolabs) as host strain for protein expression. For proper selection, ampicillin (Roche Diagnostics, Mannheim, Germany) (100 μg/ml) was used.

#### Construction of plasmids.

Inducible plasmid constructs for expression of either λSa2lys (NP_688827) or Art-240 were created using the pET21b backbone. Briefly, the sequence encoding λSa2lys was obtained as a synthetic construct from Thermo Fisher Scientific (Germany). The λSa2lys open reading frame was amplified with PCR primers (Table 2) comprising either a terminal NdeI or XhoI site to introduce appropriate restriction enzymes. The specificity of the endolysin λSa2lys for *Sa2lys* changes slightly by substituting the Cpl-7 cell wall binding domain for the staphylococcal specific SH3b domain, rendering the protein active against both staphylococci and streptococci. We used both a plate lysis and killing assay and noted a different susceptibility for some strains, especially when the susceptibility is close to the detection limit. Specifically, *S. aureus* showed susceptibility in the killing assay, whereas no inhibition was observed in the plate lysis assay. This is consistent with previous reports stating that results from different assays are not always comparable. Here, we believe that diffusion in the agar compromises the sensitivity of the plate lysis assay compared to the killing assay which was performed in suspension. Overall, our results demonstrate that *Sa2lys* contains a C-terminal PCNP increases both muralytic and antibacterial activity of λSa2lys. Based on our current knowledge, these improvements can be best explained by the positive charges of PCNP, which are expected to strengthen the interactions of Art-240 with the polyamionic cell surface (phosphate groups in teichoic acids) and consequently increases the local concentration at the site of action. The peptide PCNP (pI 12.3) solely contains positively charged arginines and lysines at pH 7.4. This increased contact of the catalytic domains of the endolysin with peptidoglycan may accelerate hydrolysis and subsequent osmotic lysis of the bacterial cell. In this regard, one may argue that the increased enzymatic activity of Art-240 over a broad pH range is also due to the highly positive charge of PCNP, because Art-240 (pI 8.8) is expected to interact with the bacterial surface in a broader pH range with a higher affinity compared to endolysin λSa2lys (pI 6.3) alone. A positive correlation between the positive charge of catalytic domains and bacteriolytic activity was obtained by the inversion of the net charge (from negative to positive) of the cell wall binding domain of the Cpl-7 endolysin. The hypothesis that electrostatic interactions constitute the basis for the enhanced antibacterial and muralytic activity of Art-240 compared to λSa2lys, is also supported by the ability of Art-240 to degrade peptidoglycan more efficiently than λSa2lys in a wide range of NaCl concentrations (0–5 M). The influence of ionic strength on the lytic activity of endolysins has been described previously and for most endolysins optimal activity was obtained at lower salt concentrations. In the case of Art-240, the strong positive charge of the cationic peptide enhances the ability to remain bound to the cell wall in the presence of a high concentration of salt ions, which generally compete with electrostatic interactions.

In summary, this study demonstrates that artilysation is a promising strategy for the development of improved endolysin-based antimicrobials against Gram-positive bacteria. A more in-depth knowledge about the interactions between the endolysin and the peptide moieties of the Artilysin, and the peptide with the cell surface will help in the design of novel endolysins with enhanced antibacterial properties against any possible pathogen.
enzyme sites for sub-cloning into pET21b. The PCR product was gel purified, digested with NdeI and XhoI, purified using QIAquick PCR Purification Kit (QIAGEN GmbH, Germany) and introduced into similarly digested, dephosphorylated, and gel-purified pET21b via conventional means. Expression from the resultant pET21b/λSa2lys construct results in the addition of eight amino acid residues to the C-terminus of λSa2lys (corresponding to the XhoI site (Leu-Glu) followed by six His residues). The coding sequence for Art-240 was constructed by fusing the sequence encoding the polycationic nonapeptide (PCNP)\(^\text{11}\) to the 3’ end of the open reading frame of λSa2lys endolysin using a two-step standard PCR cloning method with PCR primer pairs: Sa2lysNdeIFw and Sa-His6AflII-P-XhoIRv-1 in a first PCR and Sa2lysNdeIFw and HissAflIII-P-XhoIRv-2 in a second PCR using the product of the first PCR as a template (Table 2). The PCR amplified fragment (encoding Art-240) comprises subsequently the coding sequence for λSa2lys, a His\(_{6}\)-tag for affinity purification, a unique AflII restriction site for flexible construction of different PNCP-based constructs and the PCNP-tag. The resulting λSa2lys-His\(_{6}\)-AflII-PNCP encoding cassette was then introduced to modified pET21b, in which the C-terminal Hiss\(_{6}\)-tag was removed, via Quick Change mutagenesis (QS site-directed mutagenesis kit; New England BioLabs, GmbH) by introduction of two stop codons after the Xhol restriction endonuclease site. An internal Hiss\(_{6}\)-tag was preferred to ensure a free terminal PCNP.

**Recombinant expression and purification.** The recombinant expression of both λSa2lys and Art-240 was performed in 2 liters lysogeny broth (LB) in *E. coli* BL21(DE3)pLysS and *E. coli* BL21(DE3) cells, respectively. Briefly, a preculture (50 ml LB in 100 ml flask inoculated from fresh plate) was grown overnight at 37°C. The cells were inoculated into fresh medium at an optical density of OD\(_{600}=0.1\) and grown at 37°C to an optical density of OD\(_{600}=0.45–0.55\). Next, the expression of the proteins was induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) at 16°C for 18 h. *E. coli* cells were harvested by centrifugation (6,000 × g for 5 min at 4°C), and the obtained cell pellet was resuspended in 40 ml of lysis buffer (20 mM HEPES, 1 M NaCl, 20 mM imidazole and 1 mM MgCl\(_2\); [pH 7.4] supplemented with 25 μg/ml DNaseI). Cell disruption was done by sonication (Bandelin Sonopuls HD3200) under the following conditions: 45% maximum amplitude (51 watts) for 6 min with 10-s pulse/20-s break steps on ice. Cell debris was separated by centrifugation at 15,000 × g for 20 min, and the supernatant was then filtered (Filtropur S0.2, PES-membrane, 0.2 μm pore size; Sarstedt AG & Co., Germany) before application to the column. Purification of the His\(_{6}\)-tagged fusion proteins was performed on an AKTA fast protein liquid chromatography (FPLC) system (GE Healthcare, Little Chalfont, United Kingdom) controlled by UNICORN 5.1 software with Ni\(^{2+}\)-charged immobilized-metal affinity chromatography (IMAC) columns (1 ml HiPrep IMAC FF; GE Healthcare). Bound proteins were washed using buffer A (20 mM HEPES, 1 M NaCl, 20 mM imidazole [pH 7.4]) and eluted with a linear gradient to 100% buffer B (20 mM HEPES, 500 mM NaCl, 500 mM imidazole [pH 7.4]). The fractions containing the purified λSa2lys endolysin or Art-240 were pooled and dialysed against buffer C (20 mM HEPES, 500 mM NaCl [pH 7.4]). After dialysis, the protein concentration was determined spectrophotometrically in silica cuvettes at a wavelength of 280 nm (Jasco V-650; Jasco Corporation, Tokyo, Japan).

**CD spectroscopy.** To measure the melting temperature (Tm), the ellipticity of the proteins was recorded at 220 nm in a Jasco model J-815 circular dichroism (CD) spectrometer (Jasco Corporation). The protein melting temperatures were determined with a heating rate of 1°C/min, an incubation time of 3 s, and a volume of 410 μl in a 1-mm light path Hellma quartz cuvette. All measurements were performed in 50 mM sodium phosphate buffer with 300 mM NaCl at pH 7.4. The midpoint of the unfolding transition was determined by fitting to a sigmoid unfolding model using JASCO analysis software. The proteins were measured at concentrations of 6.03 μM (λSa2lys endolysin) and 6.00 μM (Art-240).

**Muralytic assay.** A standardized turbidity assay modified from Donovan et al.\(^\text{6}\) with *S. agalactiae* LiCC S461 as substrate was used. Briefly, *S. agalactiae* LiCC S461 cells in the mid-exponential phase (OD\(_{600}=0.6\) and stationary phase (OD\(_{600}=1.2\)) were harvested by centrifugation (3,900 × g, 10 min, 4°C) and stored on ice for ≤4 h until just before the assay. The cell pellets were suspended in 20 mM HEPES, 150 mM NaCl (pH 7.4) to an OD\(_{600}=~1.2\). In the experiment to study the effect of different pH values on the enzymatic activity of λSa2lys and Art-240, a universal buffer (150 mM KCl, 10 mM KH\(_2\)PO\(_4\), 10 mM sodium citrate, 10 mM H\(_2\)BO\(_4\)) with different pH values between 4 and 10 was used in order to exclude any possible effect of the used buffer compound. Upon addition of 30 μl of muralytic enzymes (1 μM) to 270 μl of bacterial cells (final protein concentration 0.1 μM), the optical density was measured spectrophotometrically over time at 655 nm using a Microplate Reader 680 system (Bio-Rad). The standardized calculation method previously described\(^\text{5}\) was used to quantify the muralytic activity of the enzymes with one unit corresponding to a decrease in optical density of 0.001/min.

**Antibacterial activity.** Bacterial cells at the mid-exponential growth phase (OD\(_{600}=0.6\)) were harvested by centrifugation (16,000 × g for 5 min), washed, and diluted 100-fold in 20 mM HEPES 150 mM NaCl (pH 7.4) to a final density of ±10\(^{6}\) CFU/ml. Fifty μl of a protein solution containing 0.2 μM λSa2lys endolysin/Art-240 (or to the indicated concentration) in 20 mM HEPES, 150 mM NaCl (pH 7.4) was added to 50 μl of the bacterial cell dilution and further incubated at 25°C, with shaking. After 60-min incubation, the reaction was stopped by addition of 0.15 μg Proteinase K and the appropriate dilutions of the cell suspensions were plated on BHl or LB agar in triplicate. Colonies were counted after overnight incubation at 37°C. The antibacterial activity was quantified as the relative inactivation in log units (log\(_{10}\)[N\(_0\)/N\(_f\)] with N\(_0\) as the initial number of untreated cells and N\(_f\) as the number of residual cells counted after treatment).

**Time–kill curve.** Mid-exponentially growing cells (OD\(_{600}=0.6\)) of *S. agalactiae* LiCC S461 in BHl medium were harvested by centrifugation (16,000 × g for 5 min), washed twice with 1 ml 20 mM HEPES, 150 mM NaCl...
were incubated at 25 °C in a shaker for the times indicated. At the respective time points, 50 μl of the reaction was stopped with 0.15 μl of proteinase K, and appropriate dilutions of the cell suspensions were plated on BHI agar in triplicate. Colonies were counted after overnight incubation at 37 °C. The antibacterial activity was quantified as indicated above.

**Plate lysis assay.** Purified λSa2lys/Art-240 were diluted in sterile 20 mM HEPES, 500 mM NaCl (pH 7.4) buffer, and 5 μl from a stock solution of 5 μM (1.25 μg), 2 μM (0.5 μg) and 1 μM (0.25 μg) was spotted onto BHI or LB agar plate overlaid with 3 ml 0.75% BHI or LB soft agar containing 100 μl of the target bacteria from an overnight culture. The spotted plates were air dried for 10 min under a laminar flow hood and incubated overnight in a 37°C environment. Semi-quantitative scoring of the cleared spots occurred after 24 h of plating the cells.

**Time-lapse microscopy.** Exponentially growing *S. uberis* LiCC S648 cells (37°C, BHI, OD600nm = 0.6) were washed three times in 20 mM HEPES, 500 mM NaCl pH 7.4 and concentrated five times in the same buffer. Equimolar amounts of Art-240, λSa2lys and the PCNP (0.2 μM) in 20 mM HEPES, 500 mM NaCl pH 7.4 were mixed in a 1:1 ratio with the cell suspension (final concentration of compounds is 0.1 μM), and the mixture was immediately transferred to an agarose pad (2% dissolved in 20 mM HEPES, 500 mM NaCl [pH 7.4]) for imaging. A buffer control (20 mM HEPES, 500 mM NaCl [pH 7.4]) was included. Time-lapse microscopy experiments were performed with a temperature controlled (Okolab, Ottoviano, Italy) Eclipse Ti-E inverted microscope (Nikon, Champigny-sur-Marne, France) equipped with a Ti-CT-E motorized condenser and a CoolSNAP HQ2 FireWire CCD camera, as described previously. Images were acquired using NIS-Elements (Nikon), and the resulting pictures were further handled with open-source software, ImageJ (http://rsbweb.nih.gov/ij/).

**Statistical analyses.** The one-tailed Student t-test was used to compare the antibacterial activity of λSa2lys and Art-240 (expressed as log viable reduction) against a panel of strains. The same test was used to compare the effect of pH and NaCl on the enzymatic activity of both proteins against exponential cultures of *S. agalactiae* LiCC S461 and *S. uberis* LiCC S648, as well as their activity against exponential and stationary cultures of *S. agalactiae* LiCC S461 (expressed as log viable reduction) against a panel of strains. The same test was used to compare the effect of the enzymes over time (0–30 min) and at different concentrations (6.25–200 nM) against *S. agalactiae* LiCC S461 suspensions at pH 7.4 were also compared (expressed as log viable reduction). Results are shown as means ± standard deviations. A P value below 0.05 was considered statistically significant.

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Author Contributions
Y.B., R.L. and M.B. conceived and designed the experiments. W.-L.C., L.R.-R., D.G., S.K.G. and A.A. performed the experiments. C.H., B.M., Y.B. and P.G. analysed the data. P.G., A.R., L.R.-R. and Y.B. wrote the paper.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: R.L. and Y.B. act as scientific advisors of Lisando GmbH. W.-L.C., C.H. and M.B. are employees of Lisando GmbH. Artilysin is a registered trademark in the European Union, United States, and other countries.

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