

MICROBIOLOGY

A VersaTile-driven platform for rapid hit-to-lead development of engineered lysins

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Health care authorities are calling for new antibacterial therapies to cope with the global emergence of antibiotic-resistant bacteria. Bacteriophage-encoded lysins are a unique class of antibacterials with promising (pre)clinical progress. Custom engineering of lysins allows for the creation of variants against potentially any bacterial pathogen. We here present a high-throughput hit-to-lead development platform for engineered lysins. The platform is driven by VersaTile, a new DNA assembly method for the rapid construction of combinatorial libraries of engineered lysins. We constructed approximately 10,000 lysin variants. Using an iterative screening procedure, we identified a lead variant with high antibacterial activity against *Acinetobacter baumannii* in human serum and an ex vivo pig burn wound model. This generic platform could offer new opportunities to populate the preclinical pipeline with engineered lysins for diverse (therapeutic) applications.

INTRODUCTION

The rapid emergence and spread of multi- and extensively drug-resistant bacteria are a major public health threat that is further exacerbated by the lack of novel antibiotics. Projections made by the World Health Organization indicate that antibiotic-resistant bacteria may be responsible for more than 10 million deaths by 2050, thereby outnumbering cancer-related deaths, if no global action is undertaken (1). The once successful Waksman platform, mining natural antibiotics from soil-derived actinomycetes, heralded the golden era of antibiotic discovery during the 1940s to 1960s. However, the Waksman platform increasingly led to the rediscovery of the same classes of antibiotics (2). The diminishing success spurred the pharmaceutical industry to establish new discovery platforms. With the emergence of genomics in the 1990s, the focus shifted to high-throughput screening campaigns of large libraries of synthetic compounds against essential and conserved bacterial targets. Although lead compounds were identified, they failed under in vivo conditions. One of the main causes was the lack of penetration into bacterial cells (3). This critical shortcoming was most pronounced in the case of Gram-negative pathogens due to their outer membrane (2, 4). This approach resulted in a disappointing and financially unsustainable outcome, contributing to the declining interest in antibiotic discovery by the pharmaceutical industry (4). For more than 50 years, no new antibiotic classes have been introduced.

New discovery platforms are therefore needed to identify lead antibacterials to expand the portfolio of antibiotics. Nonconventional approaches that differ from the classic small-molecule antibiotics are increasingly considered, both as prophylactics and as therapeutics: antibodies, probiotics, natural and engineered bacteriophages, immune stimulation, vaccines, peptides, and lysins. A recent pipeline

portfolio review of antibacterials identified seven (pre)clinical lysin projects and underscored the need for further basic research to expand the therapeutic options of lysins (5). Lysins are peptidoglycan-degrading enzymes produced by (bacterio)phages, including endolysins and virion-associated peptidoglycan hydrolases (VAPGHs). Owing to immense abundance of phage, the reservoir of lysins appears inexhaustible, with candidates against any potential pathogen (6). The first lysins targeting *Staphylococcus aureus* (CF-301, SAL200, P128, and Staphhefekt™) are currently being evaluated in clinical trials (7, 8). Given the typical attrition rates during clinical evaluation and for a guaranteed translation into new therapies against a diversity of pathogens, a higher number of candidates under clinical evaluation are desired (5). Thus, a reliable and robust discovery platform for efficient lysins is needed.

While wild-type lysins are highly promising, protein engineering offers many opportunities to increase this natural diversity. Directed evolution by protein engineering resulted in engineered lysins with improved features toward their specific applications. Besides mutagenesis, recombination of the modular domains of lysins has been, by far, the most popular approach, as recently reviewed by our group (7). Recombination of cell wall binding (CBD) and enzymatic activity domains (EADs) has been used to alter the specificity or to improve the activity, stability, and solubility of lysins. A library of 174 natural, recombined, and truncated variants of lysins and other bacterial peptidoglycan hydrolases was screened for optimal activity against *S. aureus* in milk (9). Another library of 126 combinatorial constructs was used to develop a chimeric lysin with activity against planktonic and biofilm methicillin-resistant *S. aureus* (10). To expand the potential of lysins to include killing of Gram-negative pathogens, specific outer membrane permeabilizing peptides (OMPs) were fused to lysins in a proper way for transfer across the outer membrane, resulting in peptidoglycan degradation and death due to osmotic lysis (11–14). These engineered lysins are also known as Artilysins® (Lysando AG). In a previous study, we created a library of 49 variants using classic restriction-ligation protocols. Each variant was composed of a combination of at least one OMP, a linker, one CBD, and one EAD. We concluded that each component affects the antibacterial activity but that further empirical research is needed to find the best combination for targeting a specific species (12).

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On the basis of the huge diversity of OMPs, linkers, CBDs, and EADs, libraries of much higher complexity targeting any pathogen can be envisioned. Yet, this progress is impeded by the cumbersome, time-consuming, and labor-intensive molecular techniques to create such modular libraries because methods for combinatorial assembly of nonhomologous sequences (such as OMPs, linkers, CBDs, and EADs) within an open reading frame are inexistent (15). To identify the best engineered lysins from the practically infinite sequence space of combinatorially shuffled lysins, we describe here a discovery platform to perform design, build, test, and learn cycles for engineered lysins in a short period of time. VersaTile drives this platform as a new technique to construct a practically unlimited number of shuffled lysin sequences. Because an outrageous number of variants were created, an iterative search strategy to establish a hit-to-lead development process was implemented, similar to the lead development of classic pharmaceutical drugs (16). As a proof of concept, we here show the use of this discovery platform to identify a lead lysin variant with broad activity against multidrug-resistant *Acinetobacter baumannii* strains in human serum. We further evaluated this lead variant in an ex vivo model of burn wound infection to assess its effects on biofilm formation on biological skin-based surfaces. The established platform is generic in nature and represents a discovery platform to identify efficient, engineered lysins targeting a broad diversity of pathogens.

RESULTS

Development of a discovery platform for high-throughput engineering and testing of lysins

The platform basically consists of three steps that can be iteratively repeated (Fig. 1A): (i) designing and building a library of engineered lysins using VersaTile, (ii) expressing engineered lysins in parallel and testing their antimicrobial activities, and (iii) learning from the output and defining structure-activity relationships. These structure-activity relationships can be used to create an enriched library with VersaTile as the input for iterative rounds of screening. To evaluate the performance of this approach, we generated a library of approximately 10,000 shuffled lysins to identify the most promising lysin variant active against the Gram-negative pathogen *A. baumannii* in human serum.

Integrating previous knowledge to design a smart library of engineered lysins

During previous work on lysins targeting Gram-negative bacteria, it was observed that both the composition and the specific order of the combined modules affect the antibacterial activity of engineered lysins. The best results were obtained when using modular lysins that comprise both a CBD and an EAD. In addition, an intervening linker had a positive effect and the most active variants had an N-terminal OMP. Therefore, we designed a library of modular variants with four positions and the following specific configuration: OMP-linker-CBD-EAD. The selected CBD-EAD order resembles the natural configuration that is seen in the few modular lysins of phages that infect Gram-negative bacteria (12). We have selected for each position 38 OMPs, 2 linkers, 6 CBDs, and 21 EADs, respectively (table S1).

The selected OMPs differ in length (between 9 and 44 amino acids), fold (helical, sheet, and random coil), cationicity, hydrophobicity, and amphipathicity. Some OMPs were designed in silico,

whereas others have a previously demonstrated outer membrane permeabilizing activity. We designed two flexible linkers of different length (6 and 14 amino acids). We selected five different CBDs from previously characterized modular lysins (Φ KZ, EL, OBP, PVP-SE1, and 201phi2-1) (17, 18). These CBDs all directly target peptidoglycan with chemotype A1 γ typical for Gram-negative bacteria. In addition, we included a mutagenized variant of the CBD of the Φ KZ endolysin, which is no longer able to oligomerize through disulfide bridges and which has a higher thermostability (11). We selected 21 different EADs, derived from a diversity of phages infecting Gram-negative species and with diverse biochemical specificities (lysozyme, transglycosylase, amidase, and endopeptidase). We used EADs from both globular and modular endolysins (table S1).

VersaTile is an efficient method for rapid construction of large combinatorial lysin libraries

VersaTile was developed to eliminate the current technical constraints to shuffle the modules of engineered lysins in a high-throughput manner. VersaTile follows a two-step approach. First, a repository of all modules, here called tiles, is constructed. Second, any assembly can be created with these tiles in a directed or random manner (Fig. 1B). Therefore, VersaTile is a Lego-like DNA assembly method specifically for modular proteins such as engineered lysins that do not share DNA homology.

A tile is defined here as a coding sequence for a specific module (e.g., OMP, linker, CBD, or EAD) that is made compatible for use with VersaTile. The coding sequence is therefore flanked by six-nucleotide-long position tags and BsaI recognition sites, cloned in a dedicated entry vector (pVTE; fig. S1). In total, we created for this study a repository with 67 tiles (table S1). Because the lysin design consists of four modules (OMP-linker-CBD-EAD), tiles encoding OMPs are specifically labeled for position 1, tiles encoding linkers for position 2, etc. (table S2). BsaI cleavage within the position tags generates position-specific, single-stranded overhangs that are joined to generate combinatorial libraries (cyclic digestion and ligation using BsaI and T4 DNA ligase, respectively) in a dedicated destination vector (pVTD; fig. S1). A combinatorial library was constructed with a complexity of 9576 ($= 38 \times 2 \times 6 \times 21$) variants by combining all 38 OMP, 2 linker, 6 CBD, and 21 EAD tiles at their respective positions (table S1). In the resulting constructs, adjacent modules are separated by a six-nucleotide linker encoding two freely selected amino acids. In this study, only glycine, alanine, and serine were used (table S2). These flexible amino acids are expected to not interfere with the autonomous folding and functioning of the modules. The expression of the OMP-linker-CBD-EAD sequences in the library is driven by a T7 promoter.

Standard analysis by polymerase chain reaction (PCR) and Sanger sequencing of randomly selected clones indicated a correct and random assembly with ~95% efficiency. For a comprehensive assessment of the representation and randomness of individual tiles at each position, we sequenced 30,435 single variants of the combinatorial library with nanopore technology as long reads that entirely span assembled lysins. We extracted read counts for each individual tile and assessed the order of the specific tiles order by leveraging the syntenic information contained within the reads. This quality control of the library showed that each individual tile is well represented and can be found in its expected position. Variant counts were distributed in a log-normal manner, and only 99 combinations

A Discovery platform for engineered lysins

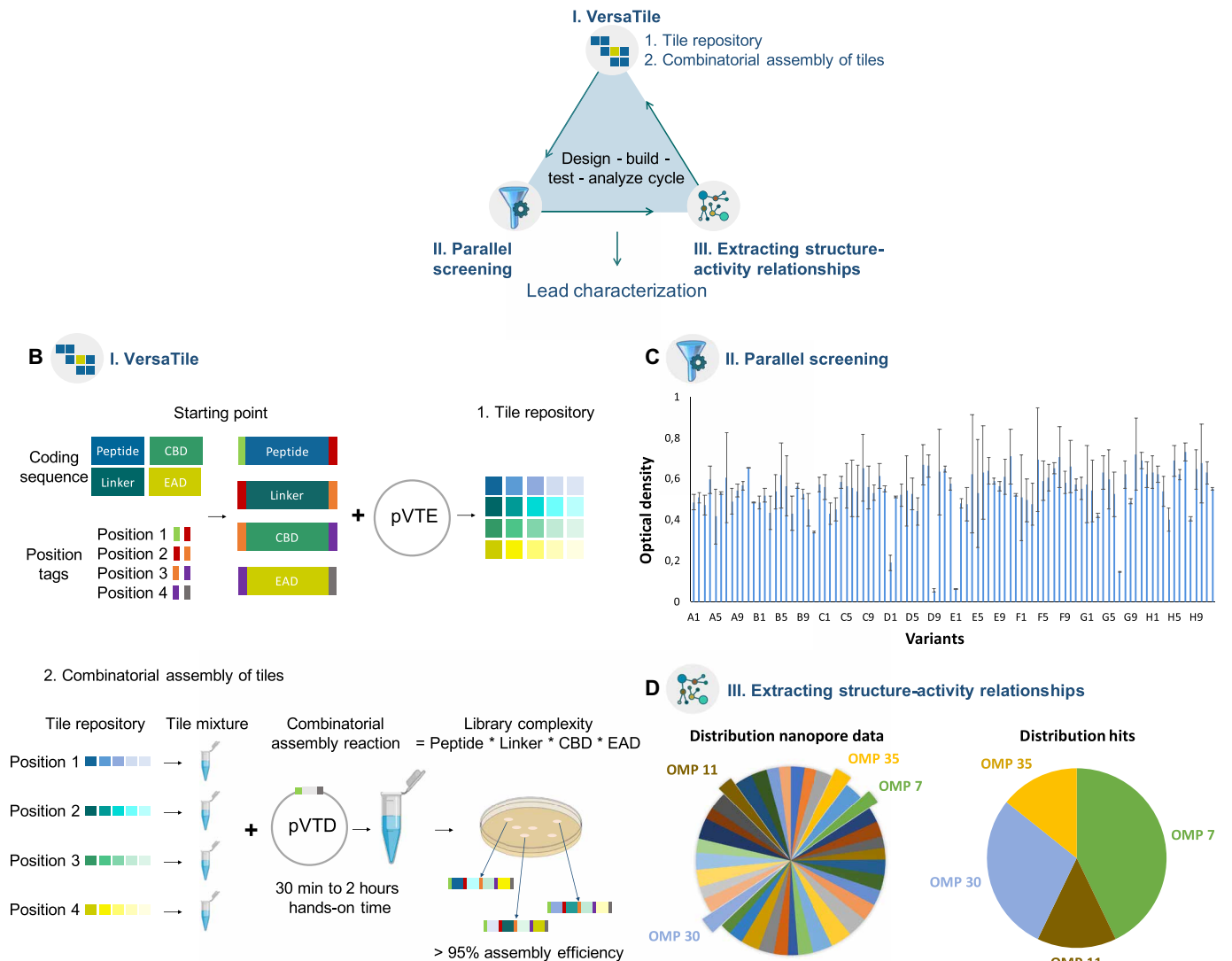


Fig. 1. An iterative three-step approach to design, build, test, and analyze engineered lysins. (A) Overview of the three steps of the discovery platform for engineered lysins. These steps are iteratively repeated to obtain a lead variant that fulfills the selection criteria. (B) Library of shuffled variants is produced with VersaTile. A tile repository of all modules (OMPs, linkers, CBDs, and EADs) is constructed. Each module is cloned into the pVTE vector, flanked with position-specific tags (indicated with different colors) and a BsaI recognition site. The restriction site of BsaI is located in the position tag. All tiles from the repository are then mixed per position and combined with the pVTD vector for a one-tube combinatorial assembly reaction. After transformation, every clone will have a different assembly of tiles. (C) The variants are randomly selected, expressed, and analyzed by a growth inhibitory assay against a panel of four isolates in triplicate. Low optical density indicates a growth inhibitory effect. (D) On the basis of the proportional enrichment of specific tiles in the identified hits compared to the nanopore sequenced library, structure-activity relationships are extracted. These rules serve as input for the next library construction, reusing the tile repository.

were not observed in the sequencing data, without any specific patterns of missingness (fig. S2).

Hits are identified through parallel screening of random variants for growth inhibition

We implemented parallel protein expression and growth inhibitory assessment to screen for hit candidates present within the combinatorial library (Fig. 1C). *Escherichia coli* BL21(DE3)-RIL cells were transformed with the combinatorial library, and 380 clones were randomly picked. The shuffled lysin genes were expressed in parallel in 96-deep-well plates. The use of autoinduction medium ensured

expression induction at the same growth stage of all clones. Lysates of all expressed variants were obtained by exposure of the bacteria to chloroform vapor. Complete lysis of the cells was confirmed by the absence of viable bacteria after spotting the lysates.

We tested these cleared lysates for growth inhibition of four different multidrug-resistant *A. baumannii* strains [10^6 colony-forming units (CFU)/ml]. Seven variants (~2%) showed a complete growth inhibition of at least one strain (Table 1). Specifically, variant 1D9 completely inhibited all *A. baumannii* strains tested, whereas variant 1G7 was highly active against three strains. The other variants completely inhibited two strains (1B11 and 1H4) or

Table 1. Characterization of seven hits selected after the first screening campaign. The modular composition, growth inhibitory activity against four multidrug-resistant *A. baumannii* strains, and muralytic activity are given for the variants that completely inhibited the growth of at least one strain. The variants are ranked according to growth inhibitory effect. A full annotation of the modules is available in table S1. No correlation is observed between growth inhibitory activity and enzymatic activity.

Engineered lysin		Growth inhibitory activity*				Muralytic activity (U/ml)
		NCTC 13423	LUH 5875	RUH 875	RUH 134	
1D9	OMP7-link1-CBD6-EAD8	+++	+++	+++	+++	3333
1G7	OMP7-link2-CBD2-EAD9	+++	+++	+++	++	2533
1B11	OMP35-link2-CBD1-EAD2	+++	++	+++	+	4196
1H4	OMP11-link1-CBD6-EAD18	+++	+++	++	+	1267
1D1	OMP30-link2-CBD2-EAD7	+++	++	+	–	8899
1A10	OMP7-link2-CBD2-EAD10	+++	+	++	–	7366
1H3	OMP30-link2-CBD4-EAD10	+++	–	–	–	2000

*The different categories of growth inhibition after 24 hours of incubation at 30°C were classified as follows: +++, no bacterial growth was observed; ++, bacterial growth with OD_{655nm} between 0 and 0.1; +, bacterial growth with OD_{655nm} between 0.1 and 0.2; –, bacterial growth with OD_{655nm} larger than 0.2. The OD_{655nm} values were corrected for background OD_{655nm} of the medium.

one strain (1D1, 1A10, and 1H3), respectively. *A. baumannii* NCTC 13423 appeared to be the most susceptible strain, whereas RUH 134 was only completely inhibited by one variant. The multidrug-resistant strains thus showed a variable susceptibility. Spotting the cell cultures after 24-hour exposure to these variants confirmed their bactericidal effect. All variants also showed muralytic activity (Table 1), but no correlation was observed between muralytic and growth inhibitory activity.

The active engineered lysins show conserved structure-activity relationships

The tile composition of the seven hits was determined by sequencing to search for structure-activity relationships (Table 1). Some tiles were more prevalent at position 1 (OMPs) and position 3 (CBDs) (Fig. 1D). Specifically, four of seven active variants contain a cecropin-related peptide (3× OMP7; 1× OMP11) at position 1. Cecropin-related peptides contain a conserved α helix interrupted by a hinge (fig. S3) (19), which might be an indicative feature for a positive effect related to this type of peptides. Of six possible CBDs, four were present. CBD2 and CBD6 were present in the two most active hits, respectively, and also most prevalent (threefold and twofold, respectively). In addition, CBD6 is a mutagenized derivative of the CBD1 of lysin KZ144 (18) and was previously shown to improve stability and activity in Art-175 (11). No enrichment of a specific linker or EAD was observed.

An iterative combinatorial library screen results in an increased ratio of engineered lysins with improved and broader efficacy

On the basis of the inferred structure-activity relationships for positions 1 and 3, a second, focused library with a complexity of 336 (4 × 2 × 2 × 21) variants was constructed using VersaTile, reusing

the tiles from the repository (table S3). For position 1, the number of OMPs was reduced from 38 to 4, including only cecropin-related peptides (OMP6, OMP7, OMP10, and OMP11). For position 3, CBD2 and CBD6 were retained. A number of 188 clones were randomly selected, expressed, and analyzed against the same test panel of multidrug-resistant strains. The median growth inhibitory effect was similar for both libraries, but the distribution of the growth inhibitory effects of engineered lysins from the second library was consistently broader compared to variants from the first library, with both more inferior and superior variants (Fig. 2A). Under the same selection criteria as in the first cycle, 40 variants showed full growth inhibitory activity against at least one strain, significantly increasing the hit rate from 2 to 21% (40 of 188) ($P < 0.001$). This obvious increase in the proportion of active engineered lysins is also accompanied by an increase in the number of variants that completely inhibited the four strains at the cost of variants that only completely inhibited a single strain (Fig. 2B and table S4). Both observations support that one or both implemented structure-activity relationships effectively contribute to a higher hit rate.

The composition of all 40 hits was determined (table S4). OMP7, i.e., cecropin A from *Aedes aegypti*, is the most prevalent OMP with 31 of 40 occurrences and exclusively present in the hits with the broadest activity. The remaining hits have OMP11, i.e., sarcotoxin IA from *Sarcophaga peregrina*, at their first position. Two other cecropin-related OMPs that were included in the second library did not appear in the list of hits. Linker 1 (15 of 40) or linker 2 (25 of 40) were both well represented, whereas CBD6 was overrepresented (32 of 40) compared to CBD2 (8 of 40). Sixteen different EADs (of 21 EADs included in the second library) were identified, showing flexibility at this position. Yet, EAD4 was most abundant with 6 of 40 occurrences.

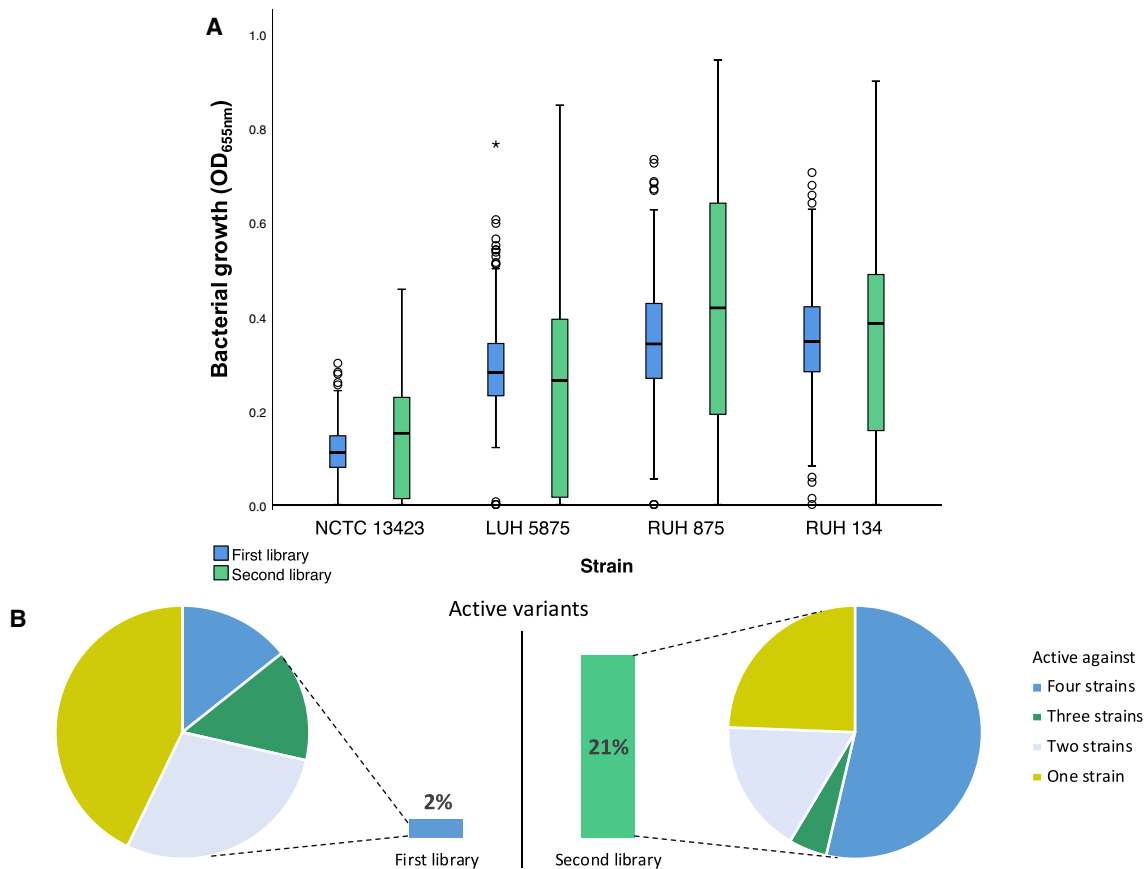


Fig. 2. Growth inhibitory activity of engineered lysins from the first library and the second library. (A) Boxplot distribution of the growth inhibitory activities of 380 variants (first library: blue) and 188 variants (second library: green) against four epidemiological *A. baumannii* strains. Both libraries have a similar median value, but the second library consistently shows a broader distribution with an increased number of both superior and inferior variants. (B) The percentage of hits inhibiting four *A. baumannii* strains increases from 2 to 21%. The hits of the second library show a broader activity against the tested *A. baumannii* strains, with more than half of them inhibiting all strains.

Identification of a lead variant active against *A. baumannii* in human serum

The most active variants (24 of 40) against three or four of the *A. baumannii* strains (table S4) were withheld to test bactericidal activity under more stringent conditions (50% human serum) to better mimic human physiological conditions. The most susceptible and resistant *A. baumannii* strains (NCTC 13423 and RUH 134, respectively) were spotted after 24-hour exposure to the different variants. Five variants (21%) showed a complete absence of surviving cells in human serum, with one of them (1D10) completely killing both strains (table S5). This engineered lysin (OMP7-linker 1-CBD6-EAD20) was therefore finally selected as the lead variant.

Large-scale expression of the lead variant yielded 22 mg per liter expression volume after purification with nickel affinity chromatography. Minimum inhibitory concentration (MIC) values of the lead variant against the four epidemiological *A. baumannii* strains (table S6), a diverse set of 27 pathogenic *A. baumannii* strains and 22 *Acinetobacter* species, including 34 clinical burn wound isolates (table S7) ranged between 4 and 24 $\mu\text{g/ml}$ ($11.8 \pm 5.2 \mu\text{g/ml}$) (20). Addition of 0.2 mM EDTA, which acts as a chelator of divalent cations present in the outer membrane, further decreases the MIC values to 2 to 20 $\mu\text{g/ml}$ ($4.4 \pm 3.6 \mu\text{g/ml}$). The number of bacteria of

the four tested strains was reduced with at least 5 logs (Fig. 3). The lead variant showed a strain-dependent killing in 90% human serum. At a dose of $10\times$ MIC, strain NCTC 13423 was the most susceptible strain, reaching a 4.5-log reduction in cell number after 30 min, whereas the cell number of strains LUH 5875, RUH 875, and RUH 134 was reduced between 2.4 and 2.7 logs, respectively. The inclusion of 0.2 mM EDTA did slightly enhance the cell log reduction to 2.7 to 3.1 logs, respectively ($P < 0.05$) (Fig. 3).

The lead variant slows bacterial growth and biofilm formation in an ex vivo pig skin explant burn wound model

To simulate bacterial growth and biofilm formation on burn wounds, a pig skin explant model was established. A standardized burn wound bed was created on small pieces of pig skin, followed by *A. baumannii* infection. After bacterial adhesion, a wound bed was sprayed with 50 μg of lysin. In general, the lead variant could control bacterial growth and biofilm formation of all tested strains after one single-dose treatment (Fig. 4A). This inhibition resulted in a reduction of 2.4 to 3.8 log units in the burn wound model at 8 hours after treatment compared to the untreated control. This reduction was improved when using consecutive dose treatments (2 and 4 hours after the first treatment). The best outcomes were

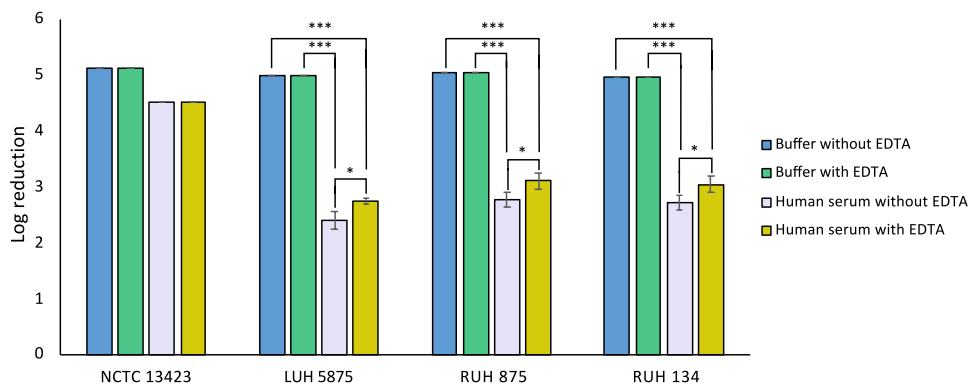


Fig. 3. Time-kill assay with the lead variant against four tested *A. baumannii* strains in buffer and human serum. The used concentration of the lead variant is $1\times$ MIC in buffer and $10\times$ MIC in human serum, in the presence or absence of 0.2 mM EDTA. All experiments in buffer reached the detection limit of the assay, as well as all experiments with *A. baumannii* NCTC 13423. Each bar represents the mean \pm SD of three independent experiments. * $P < 0.05$, Student's *t* test; *** $P < 0.001$, Student's *t* test.

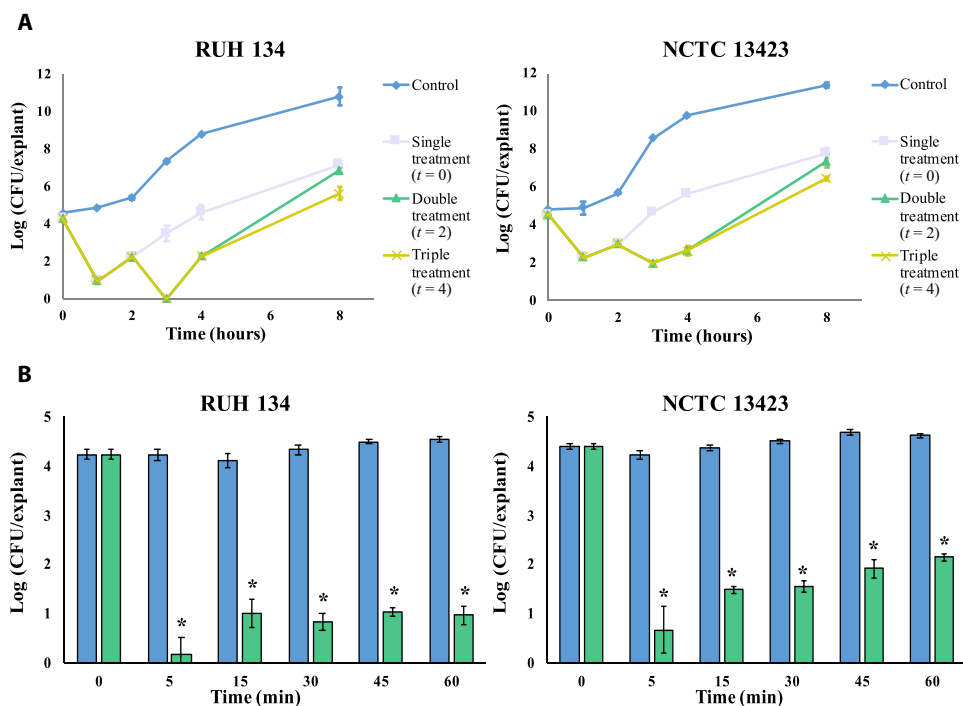


Fig. 4. Activity of the lead variant against *A. baumannii* RUH 134 and NCTC 13423 in an ex vivo pig skin model of burn wound infection. (A) Data represent log of CFU per explant after treatment with either a single dose of the lead variant (50 μ g) at time 0 hour, a double dose of protein (2 hours after the first dose), or a triple dose (2 and 4 hours after the first treatment). The control represents the bacterial number without protein treatment. (B) Bars (blue bar: control, green bar: treated with 1D10) represent the log of CFU per explant after a single dose of protein (50 μ g; time 0 min) during a short period of time (60 min). Values correspond to the mean \pm SD of four replicates. Asterisks indicate statistical differences between the control and treated sample ($P < 0.05$, Student's *t* test). The bacterial detection limit is 5 CFU per explant.

obtained after the application of a triple-dose treatment (reduction of 4.8 to 5.1 log units 8 hours after treatment). The antibacterial activity of the lead variant takes place during the first 5 min after protein application with a reduction of 3 and 2 log units against *A. baumannii* RUH 134 and NCTC 13423, respectively (Fig. 4B). *A. baumannii* RUH 134 became the most sensitive strain in this ex vivo model of infection. Similar results were obtained in the case of a topical wound infection without burning (fig. S4).

DISCUSSION

New antimicrobial discovery platforms are needed to replenish the portfolio of antibiotics in response to the rapid emergence of multi- and pandrug-resistant bacteria. Whereas the traditional Waksman platform got exhausted and suffers from rediscovery, alternative platforms to identify antimicrobial (engineered) peptides or antibiotics from uncultivable organisms have been developed (21–25). Recent clinical progress up to phase 2 trials has demonstrated the

promising prospects of lysins as a broad, novel class of antibacterials. Lysins may be identified against each pathogen. In addition, they are being further engineered to strengthen their properties for their use as antibacterials or to render them active against Gram-negative bacteria (7). Yet, an efficient high-throughput approach to construct and identify the most promising engineered lysin is still lacking. The approach presented here combines high-throughput combinatorial shuffling of lysin modules by VersaTile with parallel and iterative antibacterial screening. This approach establishes a hit-to-lead development process for engineered lysins to saturate the pipeline with new potential candidates for preclinical evaluation.

The development and implementation of VersaTile allows the generation of a large combinatorial libraries of shuffled engineered lysins. In general, a method that allows full combinatorial shuffling in a homology-independent way starting from an unlimited number of parental sequences was nonexistent (15). The method of choice to create engineered lysins has since long been traditional restriction/ligation. This approach is adequate but becomes increasingly cumbersome and laborious when more modules have to be combined or when a large number of combinatorially shuffled variants are needed (15). The largest reported libraries of engineered lysins using these methods count 49, 126, and 174 variants (9, 10, 12). The oldest and most frequently used methods for combinatorial shuffling are based on homologous recombination [e.g., deoxyribonuclease (DNase)-based DNA shuffling] but are not applicable for the recombination of well-defined, nonhomologous building blocks such as the OMPs, linkers, CBDs, and EADs. More recent, homology-independent methods such as ITCHY, SCRATCY, SHIPREC, SCOPE, SISDC, and USERec have been established (26–32) but only partially fill this gap as they all come along with their specific drawbacks (e.g., combination of two modules only, highly laborious due to multiple PCRs, and no high-throughput combinatorial shuffling possible). VersaTile allows us to shuffle nonrelated DNA sequences in a rational or combinatorial manner by exploiting the inherent ability of a type IIS restriction enzyme to directionally cut outside its recognition site with the possibility to freely choose the restriction site, as in other methods such as Golden Gate shuffling (33). With VersaTile, these restriction sites are embedded in six-nucleotide position tags that encode flexible amino acids that intervene two modules and are expected to not interfere with the autonomous folding and functioning of the modules. On the basis of previous lysin engineering work, glycine, serine, and alanine were chosen as small amino acids.

Once the repository of tiles was generated, the first library of approximately 10,000 variants could be created in a single day. The initial hit rate of 2% and enriched OMPs and CBDs provided the required input to generate a second library, reusing tiles from the existing tile repository. Screening of this second library showed an increased proportion of active variants up to more than 21%, corroborating the positive contribution (of one) of the implemented design rules. The most active engineered lysins from the second library were selected to test their *in vitro* activity in human serum, mimicking physiological conditions. This led to the identification of 1D10, which decreases the bacterial cell count up to 4.5 log units in 30 min in human serum. The identified variant has a broad inhibitory activity against *Acinetobacter* species and prevents bacterial growth and biofilm development in an *ex vivo* pig skin (burn) wound model with *A. baumannii*.

To date, other (engineered) lysins or peptides derived from lysins have been used against *A. baumannii*, yet they either are not active in wounds or human serum or have not been tested (7, 34–37). Some native endolysins against *Pseudomonas aeruginosa* are active in low serum conditions (38). In addition, lysocin PyS2-GN4, a hybrid of a narrow-spectrum pyocin and a lysin domain, is active in human serum with a 4-log reduction after 12 hours (39). Although variant 1D10 emerged as the lead candidate from a library with clearly defined design rules, the composition of the final lead variant was unexpected. While its OMP was previously demonstrated to be active against *A. baumannii* species (40), its EAD is derived from gp16 of phage BcepC6B. The latter is a VAPGH that locally degrades the peptidoglycan layer during infection and has limited enzymatic activity compared to phage endolysins (41). Nevertheless, the success of a VAPGH can be rationally explained, as a VAPGH acts naturally from without and is often resistant to more harsh external conditions (pH, temperature, presence of salts, or complementary components as in human serum) (42–45).

Because we screened for growth inhibitory activity with cleared lysates, the observed growth inhibitory effects result from the combined effect of intrinsic antibacterial activity and expression yield in the lysate. Because of the substantial sequence variation among the different modular variants, expression yield is variable as well (fig. S5). Variants that perform well for both parameters will emerge at the top of the ranking. Otherwise, this also implies that a variant with low expression yield but high intrinsic activity can score equally well as a variant with high expression yield but low intrinsic activity. The lead variant 1D10 was eventually shown to have a combined high expression yield (22 mg/liter) and high antibacterial activity, including in human serum. Both features are favorable for a pre-clinical analysis and will reduce the eventual production and purification costs. Note that muralytic activity is not an indicator for growth inhibitory activity (Table 1). Whereas the muralytic activity is mainly determined by the CBD-EAD combination, growth inhibitory activity also depends on the OMP efficiency to transfer the lysin moiety through the outer membrane. In addition, the OMP may also partially contribute to the growth inhibitory activity as has been observed before (11).

The presented approach is generic in nature and can be potentially translated to any bacterial target. In addition, each possible configuration can be explored (e.g., doubling of OMPs or EADs, reversed CBD/EAD order, C- instead of N-terminal position of the OMP, and linker variation between CBD and EAD). By extension, VersaTile can be used to create DNA libraries that encode variants of any modular protein, expanding the protein engineering toolbox with many opportunities. Whereas VersaTile has eliminated a major technical hurdle in the engineering of lysins, a next hurdle has emerged in the throughput of downstream assays. Given the lack of methods to analyze all ~10,000 variants, a shortcut trajectory was followed through the sequence space comprising all shuffled variants. The hits from the first screen of randomly selected clones directed us to a subpopulation of the sequence space, where a denser search was performed with the second library to end up as close as possible to the best variant without outrageous efforts for screening. Sequences of the randomly picked clones were only disclosed after hit selection, followed by the extraction of structure-activity relationships between the observed antibacterial activity data and the hit sequences. A different screening strategy can be followed in combination with the VersaTile technology as well. By performing

numerous parallel rational assembly reactions as has been applied in drug discovery with combinatorial chemistry, a combinatorial library with a known systematic variation per position can be created. On the basis of a parallel analysis of the antibacterial activity of these variants as performed in this study, the positive or negative contribution of each tile can be quantitatively calculated based on the known sequences of each variant (46–47). Although this requires more experimental work in library construction and library screening, variants with further improved antibacterial activity may be identified on the basis of those quantitative sequence–function relationships. Eventually, a screening technique with a throughput that can match up with the library complexity created by VersaTile would further leverage the benefits of VersaTile. Much larger libraries could be envisioned. Every additional tile that will be added to the repository can be combined with all existing tiles, e.g., if one linker tile is added, approximately 5000 more engineered lysins can be constructed. Also, other configurations with a different order of the modules can be designed, rapidly increasing the total number of variants.

MATERIALS AND METHODS

Bacterial strains and growth media

All *Acinetobacter* strains used in this study and their source are shown in table S7. The initial set of four strains comprises reference epidemiological clones of the European Union (EU) that have been found worldwide and are also referred to as worldwide clones I to III [I: RUH 875, II: RUH 134, and III: LUH 5875 (20)]. The fourth strain used is the so-called *Iraqibacter* (“T strain”: NCTC 13423, a battlefield isolate from casualties of the Iraq conflict) (48). For plasmid storage and protein expression, *E. coli* TOP10 and BL21(DE3)-RIL (Agilent Technologies, Belgium) were used, respectively. All *Acinetobacter* (30°C) and *E. coli* (37°C) strains were routinely grown in LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) with shaking (200 rpm) or on LB supplemented with 1.5% of agar. For proper selection of the *E. coli* clones, LB was supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml), and 5% (w/v) sucrose.

VersaTile: Preparation of tiles

Depending on the coding sequence length, a tile was constructed either by PCR with primers with extended 5′ sequences or by gene synthesis (>100 nucleotides (nt)) or generated by primer cassette hybridization (<100 nt). Each primer (Integrated DNA Technologies, Leuven, Belgium) contained the following necessary parts (listed starting from the coding sequence to outward): a distinct position tag, a BsaI recognition site (for library construction), and a Sap I restriction and recognition site (for tile construction). All enzymes were from Thermo Fisher Scientific (Belgium), unless stated otherwise. For cassette hybridization, the primers were mixed in equal ratios (5 µM) and incubated at 95°C (5 min), followed by a gradual cooldown to room temperature (20°C). Next, the single-stranded overhangs were filled in with Pfu DNA polymerase (2.5 U, 72°C, 10 min). Sequences (>100 nt) were amplified with Pfu DNA polymerase (2.5 U) following the manufacturer’s instructions with 100 pg of phage genomic DNA as template.

Next, the primer cassettes and amplicons were ligated in the *sapI* site of pVTE using the following protocol: 100 ng of pVTE, 50 ng of the amplicon/primer cassette, 2 µl of T4 DNA ligase buffer, 15 U of T4 DNA ligase, and 10 U of Sap I in a total volume of 20 µl. Chemical

competent *E. coli* TOP10 cells were transformed with the entire ligation mixture and plated on LB 1.5% agar, supplemented with ampicillin (100 µg/ml) and 5% (w/v) sucrose. New tiles were confirmed by Sanger sequencing (LGC Genomics, Germany), and stocks of 50 ng/µl were prepared for each tile.

VersaTile: Preparation of combinatorial libraries

For each position, a tile mixture was made by taking 1 µl of 50 ng/µl of each selected tile. This is repeated for all positions 2, 3, and 4 separately. Subsequently, 1 µl was taken from each tile mixture for a certain position and mixed with 1 µl from the other tile mixtures confining positions 2, 3, and 4. A VersaTile shuffling reaction was set up using the following protocol: 1 µl of pVTD (100 ng/µl), the tile mixture (4 µl), 1 µl of BsaI (10 U/µl), 3 µl of T4 DNA ligase (1 U/µl), and 2 µl of T4 DNA ligation buffer in a total reaction volume of 20 µl. The mixture was incubated in the thermocycler using the following program: (i) 2 min at 37°C and (ii) 3 min at 16°C; steps 1 and 2 are repeated 50 times and followed by 5 min at 50°C and finally 5 min at 80°C. Chemical competent *E. coli* BL21(DE3)-RIL cells were transformed with the entire ligation mixture and plated on LB 1.5% agar, supplemented with kanamycin (50 µg/ml) and 5% (w/v) sucrose.

Nanopore sequencing and sequence analysis of the combinatorial library

This library was sequenced using the MinION sequencer [Oxford Nanopore Technologies (ONT), UK]. Initially, the plasmid DNA preparation of the combinatorial library was linearized with the restriction enzyme Ecl136ii at a site found upstream of the cloning site and absent in all but one of the constructs found in position 4 (hence, no impact on detection capabilities). DNA was then prepared using the one-dimensional (1D) ligation approach of ONT, a PCR-free technique that preserves the integrity of the DNA molecules. The resulting library was sequenced on a MinION sequencer equipped with an R9.4.1 flow cell for 48 hours. Base calling was performed on the data using ONT Albacore v2.3.4, and the resulting reads were filtered stringently to map (BWA-MEM) over their entire length to the original vector pVTSD, except for the region substituted with the VersaTile reaction product (49). Subsequently, we analyzed these substituted regions using local alignments with the EMBOSS needle tool to verify both the order and the type of tiles that made up the constructs (50).

Parallel protein expression and lysate preparation

Overnight cultures were prepared by inoculating single colonies in wells of a 96-deep-well plate, each filled with 500 µl of LB with kanamycin (50 µg/ml). The plate was covered with a tape allowing air exchange (Brand GmbH, Germany) and shaken (900 rpm) at 37°C for 18 hours. A small volume (15 µl) from each well was transferred to a 96-deep-well plate filled with 500 µl of autoinduction medium per well (51). These plates were incubated for 5 hours at 37°C (900 rpm). Then, the cultures were incubated at 16°C for 48 hours. Cells were harvested by centrifugation (3200g, 30 min, 4°C). The pellets containing the cells were further lysed by exposure to chloroform vapor above a chloroform-saturated filter for 1 hour at room temperature. Afterward, the deep-well plate was inverted to evaporate the residual chloroform. Each lysate was suspended in 500 µl of 20 mM HEPES-NaOH, 150 mM NaCl (pH 7.4), and 1 U of DNase I, followed by incubation at 30°C at 100 rpm for 1 hour. Upon complete

resuspension of the pellets, 5 μ l is spotted onto an LB agar plate to confirm complete bacterial lysis. Then, the lysates were cleared by centrifugation (3200g, 45 min, 4°C) and stored at 4°C.

Growth inhibition and muralytic assay

Acinetobacter cultures were grown in Mueller-Hinton (MH) broth (Becton Dickinson, Belgium) for 18 hours at 30°C. Then, the cultures were diluted in 2 \times MH broth to an OD_{625nm} (optical density at 625 nm) = 0.1 ($\sim 5 \times 10^8$ CFU/ml). From this cell suspension, 50 μ l is inoculated to wells of the microtiter plate, as well as 50 μ l of the cleared lysates. Turbidity is measured at 655 nm after 18 and 24 hours. Drops of 5 μ l of the cultures were spotted on LB agar plates to analyze bactericidal activity. To test activity in human serum, the same protocol was used, except that the cultures were diluted in 100% human serum (MP Biomedicals, Belgium). After 24 hours, the suspensions were diluted in 20 mM HEPES-NaOH and 150 mM NaCl (pH 7.4) and dropped on LB agar plates.

The substrate for the muralytic assay was produced as described previously (52). Briefly, *P. aeruginosa* PAO1 is grown overnight at 37°C in an orbital shaker. The next day, fresh LB is inoculated with the overnight culture and the culture is grown up to OD_{600nm} = 0.6. The culture is spun down and resuspended in chloroform-saturated buffer [0.05 M tris-HCl (pH 7.7) and chloroform]. The resuspended solution is gently shaken for exactly 45 min. The solution is washed twice with phosphate-buffered saline (PBS; pH 7.4) (OD_{600nm} = ± 1.5). A volume of 270 μ l of the substrate is mixed with 30 μ l of the cleared lysate. The absorbance (OD_{600nm}) is measured every 30 s for 1 hour at room temperature. Then, the muralytic activity was calculated using a standardized calculation method (52).

Large-scale protein expression and purification

Recombinant expression of the selected hits was performed as previously described (11). Fresh cells [*E. coli* BL21(DE3)-RIL] were inoculated into 0.5 liter of LB medium and grown at 37°C to OD_{600nm} = 0.5 to 0.6. Expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 16°C for 18 hours. *E. coli* cells were lysed in 20 ml of lysis buffer [20 mM NaH₂PO₄/Na₂HPO₄, 0.5 M NaCl, and 50 mM imidazole (pH 7.4)] by freeze-thawing and sonication (Q125, Qsonica) on ice. Lysates were cleared (16,000g; 20 min) and filtered (polyvinylidene difluoride, 0.22- μ m pore size; Novolab, Belgium). Soluble protein was purified with His GraviTrap columns (GE Healthcare, Belgium) according to the manufacturer's instructions. The purified lysin was dialyzed against 20 mM HEPES-NaOH and 500 mM NaCl (pH 7.4) and thereafter against 20 mM HEPES-NaOH and 150 mM NaCl (pH 7.4) using Slide-A-Lyzer MINI Dialysis Devices (Thermo Fisher Scientific, Belgium). The protein concentration was determined spectrophotometrically.

Time-kill assay

A stationary phase culture of the respective strain was diluted (1:10) and incubated at 30°C (200 rpm) until mid-exponential phase (OD_{600nm} = 0.6). The cell suspension was washed three times with 20 mM HEPES-NaOH and 150 mM NaCl (pH 7.4) and diluted 100-fold in 20 mM HEPES-NaOH and 150 mM NaCl (pH 7.4) ($\sim 10^6$ CFU/ml). A cell suspension of 100 μ l was incubated at room temperature with 50 μ l of the dialyzed lysin and 50 μ l of 20 mM HEPES-NaOH and 150 mM NaCl (pH 7.4) with/without 0.2 mM EDTA. The final concentration of the lead variant (1D10) was adjusted for each strain to 1 \times MIC. After 30 min, cell dilutions were

plated in triplicate (30°C). The antibacterial activity was quantified as the relative inactivation level in log units [$\log_{10}(N_0/N_i)$, with N_0 = initial number of untreated cells and N_i = number of residual cells after treatment]. Time-kill assays in serum were performed in a similar way. Volumes were adjusted to achieve 90% human serum, and 10 \times MIC was used instead of 1 \times MIC as the final concentration of the lead variant (1D10).

MIC assay

The bacterium of interest was grown overnight in MH broth (30°C, 200 rpm). The overnight culture was then diluted 1:10 and grown to OD_{600nm} = 0.6, followed by a 5000-fold dilution in MH broth ($\sim 2 \times 10^5$ CFU/ml). The dialyzed lysin [20 mM HEPES-NaOH and 150 mM NaCl (pH 7.4)] was added to the cell suspension with a final concentration ranging between 0 and 20 μ g/ml in 2 μ g/ml steps, with and without 0.2 mM EDTA. Blank and controls included the bacterium of interest without protein and without EDTA and uninoculated broth. The plate was incubated for 18 hours at 30°C, and the MIC was determined as the lowest concentration that gave complete growth inhibition.

Pig skin explant model of (burn) wound infection

To test the capacity of the lead variant (1D10) to reduce bacterial biofilm development, we used a model of wound infection previously described with some modifications (53). Briefly, pig skin was obtained from Minimally Invasive Surgery Center Jesús Usón (Cáceres, Spain). Skin was shaved carefully with scissors and razors. Moreover, the skin was further cleaned with 70% isopropyl alcohol and allowed to dry at room temperature for 30 min. Then, the skin explants were obtained by cutting 1 cm \times 1 cm. The wound bed was created as previously described (51) using a handheld high-speed drill with a 4.8-mm high-speed, round, cutter bit (Dremel #192). This consistently forms a central "wound bed" of 4.8 mm diameter and 1 mm depth. On the other hand, to create the burn wound, a stainless-steel tool with a flat end of 4.8 mm diameter was made. To create the "burn wound bed," the end of the tool was burned for 30 s and then stamped onto the skin explant for 2 s. Immediately, all the skin explants with a wound or a burn wound were sterilized by immersion in 70% ethanol, followed by washing in physiological saline solution (0.9% NaCl, pH 5.5), and finally ultraviolet sterilization for 10 min. Sterile explants were then transferred to 24-well plates filled with 1 ml of physiological saline solution (0.9% NaCl, pH 5.5) supplemented with 0.5% of agar to mimic human body conditions.

To prepare the bacterial inoculum, *A. baumannii* strains were grown in shaking LB broth at 30°C. Next, bacteria were diluted to a final concentration of 10⁶ CFU/ml in 20 mM HEPES-NaOH and 150 mM NaCl (pH 7.4). Then, 10 μ l of this dilution was added to the wound or the burn wound bed ($\sim 10^4$ CFU per explant) in duplicate (four explants per strain per sampling point). Bacteria were then let to adhere by incubating 15 min at room temperature. Last, 100 μ l of dialyzed lysin was sprayed onto the skin (final concentration of 50 μ g per explant). Samples corresponding to time point 0 were immediately processed after spraying. The rest of the samples were incubated at 37°C with 0.5% CO₂. In addition, some samples were treated with the protein twice (double-dose treatment, 2 hours after the first treatment) or three times (4 hours after the first treatment). HEPES-NaOH (20 mM) and NaCl (150 mM, pH 7.4) were used as a control of nontreated infection. At each time point, samples

were taken and 1.5 μ l of proteinase K was added to inactivate the protein. Each explant was aseptically transferred to a stomacher bag (BagPage, BagSystem, Interscience, St-Nom-la-Breteche, France) containing 5 ml of PBS (pH 7.4) and homogenized for 90 s in a stomacher (model 80, Seward Medical, London, UK). After homogenization, dilutions were plated onto LB agar plates. Plates were incubated at 30°C for 16 hours. Every time point consists of two biological replicates and two technical replicates using two different skin explants.

Homology-based modeling of OMPs present in the active engineered lysins in the first screening round

To predict the 3D structure of the OMPs that were present in the active engineered lysins after the first screening round, we used Iterative Threading ASSEmbly Refinement with standard parameters (19).

Statistical analysis

The data obtained from the in vitro activity tests (time-kill assays and MIC assays) were expressed as the mean \pm SD of three replicates. For the ex vivo model of wound and burn wound infection, data represent the mean \pm SD of four replicates. In all cases, the Student's *t* test was used to compare the differences between the treated and untreated bacterial cultures at a level of significance $P < 0.05$ (SPSS-PC+11.0 software, Chicago, IL, USA). The Pearson chi-square with the Fisher's exact test was used to compare the difference in hit rate between the first and iterated combinatorial library at a level of significance $P < 0.001$ (SPSS-PC+11.0 software, Chicago, IL, USA). The empirical distribution of tiles obtained with nanopore sequencing was analyzed with the package `fitdistrplus` (X) (54), using the `chisq.test` function available in the R statistical environment (55) to compare it to a simulated log normal distribution ($P > 0.05$).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/23/eaaz1136/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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Acknowledgments: We thank J.-P. Pirnay and L. V. Peixe for providing the *Acinetobacter* strains. We thank I. Staes and J. Verwaeren for their assistance with the design of the figures and statistics, respectively. **Funding:** This work was supported by the Research Foundation–Flanders (FWO) under the scope of the strategic funding of an SB scholarship (1532217N and 1564718N) and “Krediet aan Navorsers” (FWOKAN2015002001). **Author contributions:** H.G., J.L., Y.B., and R.L. designed the experiments. H.G., D.Gr., R.L., and Y.B. developed VersaTile. H.G. and D.Gr. generated all tiles. H.G. did both screenings and characterization of the lead variant. D.Gu. and A.R. did ex vivo pig skin experiments. C.L., R.L., and V.-v.N. performed the nanopore sequencing and analysis. H.G., D.Gu., and Y.B. wrote the paper. All other authors provided feedback on the manuscript. **Competing interests:** Y.B. acted as a scientific advisor of Lisando GmbH. H.G., D.Gr., R.L., and Y.B. are inventors on a patent application related to this work filed by Ghent University and the University of Leuven (no. WO/2018/114980, filed on 19 February 2017, published on 28 June 2018). The authors declare that they have no other competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the corresponding author. The VersaTile vectors can be provided by Y.B. for academic research pending scientific review and a completed material transfer agreement. Requests for the VersaTile vectors should be submitted to: yves.briers@ugent.be.

Submitted 30 September 2019

Accepted 24 March 2020

Published 3 June 2020

10.1126/sciadv.aaz1136

Citation: H. Gerstmans, D. Grimon, D. Gutiérrez, C. Lood, A. Rodríguez, V. van Noort, J. Lammertyn, R. Lavigne, Y. Briers, A VersaTile-driven platform for rapid hit-to-lead development of engineered lysins. *Sci. Adv.* **6**, eaaz1136 (2020).