Published in final edited form as:

ACS Chem Biol. 2015 April 17; 10(4): 1110–1117. doi:10.1021/cb500976y.

Genetically Enhanced Lysozyme Evades a Pathogen Derived Inhibitory Protein

Sarah M. Dostal^{1,‡}, Yongliang Fang^{1,‡}, Jonathan C. Guerrette¹, Thomas C. Scanlon^{1,†}, and Karl E. Griswold^{1,2,*}

¹Thayer School of Engineering at Dartmouth College, 14 Engineering Drive, Hanover NH 03755, USA

²Program in Molecular and Cellular Biology, Dartmouth College, Hanover NH 03755, USA

Abstract

The accelerating spread of drug-resistant bacteria is creating demand for novel antibiotics. Bactericidal enzymes, such as human lysozyme (hLYZ), are interesting drug candidates due to their inherent catalytic nature and lack of susceptibility to the resistance mechanisms typically directed towards chemotherapeutics. However, natural antibacterial enzymes have their own limitations. For example, hLYZ is susceptible to pathogen derived inhibitory proteins, such as *Escherichia coli* Ivy. Here, we describe proof of concept studies demonstrating that hLYZ can be effectively redesigned to evade this potent lysozyme inhibitor. Large combinatorial libraries of hLYZ were analyzed using an innovative screening platform based on microbial co-culture in hydrogel microdroplets. Isolated hLYZ variants were orders of magnitude less susceptible to *E. coli* Ivy yet retained high catalytic proficiency and inherent antibacterial activity. Interestingly, the engineered escape variants showed a disadvantageous increase in susceptibility to the related Ivy ortholog from *Pseudomonas aeruginosa* as well as an unrelated *E. coli* inhibitory protein, MliC. Thus, while we have achieved our original objective with respect to escaping *E. coli* Ivy, engineering hLYZ for broad-spectrum evasion of proteinaceous inhibitors will require consideration of the complex and varied determinants that underlie molecular recognition by these emerging virulence factors.

Introduction

Drug-resistant bacterial pathogens represent a significant threat to public health, and a complicated assortment of factors has combined to stymie antibiotic development and fuel this growing crisis (1, 2). The current situation has prompted a need for renewed discovery and development of novel anti-bacterials, however experience has shown that conventional chemotherapies are inevitably undermined by rapid evolution of their target organisms (3). Therefore, to more comprehensively address this threat, conventional antibiotic discovery

*karl.e.griswold@dartmouth.edu.

‡These authors contributed equally to this work.

†Present address: Avitide, Inc. 7 Lucent Drive, Lebanon NH 03766, USA.

Supporting Information Available: Supplemental figures and full ConSurf dataset. This material is available free of charge via the Internet at <http://pubs.acs.org>.

and development strategies need to be complemented by searches within previously untapped molecular reservoirs.

There is a growing body of evidence that bacteriolytic enzymes represent a powerful class of novel therapeutic candidates (4–10). While microbial bacteriocins and phage endolysins have dominated early work, antibacterial enzymes of human origin have the advantage of inherent compatibility with the human immune system. Human lysozyme (hLYZ), an important component of innate immunity (11), represents one protein of particular interest. Lysozymes cleave the core β -(1,4) glycosidic bond in bacterial cell wall peptidoglycan, thereby causing bacterial lysis and death. Additionally, hLYZ and other C-type lysozymes manifest non-catalytic modes of action (12, 13), which contribute to their broad spectrum antibacterial activity. The availability of mass produced recombinant hLYZ has spurred interest in prospective medical applications, and early studies in rodent models have been encouraging (14, 15).

Although hLYZ possesses a range of advantageous properties, the wild type protein has inherent limitations that pose potential roadblocks to clinical translation. For example, in pulmonary infections, hLYZ's cationic character is known to drive electrostatic mediated aggregation with and inhibition by negatively charged biopolymers that accumulate in the infected lung (e.g. DNA, F-actin, mucin, and alginate). To address this limitation, hLYZ's electrostatic potential field has been redesigned (16, 17), and the engineered variant has shown improved efficacy in a murine model of *Pseudomonas aeruginosa* lung infection (18, 19). More generally, this successful redesign of hLYZ has led us to conclude that putative limitations of the wild type protein can be addressed through molecular engineering of performance enhanced variants.

Here we extend our analysis of wild type hLYZ limitations beyond the infected lung environment, and we consider the challenge posed by pathogen-derived, lysozyme-specific inhibitors. The bacterial cell wall represents an acute weakness that has been a favorite target of pharmaceutical scientists (20), and likewise the immune systems of higher organisms have produced a variety of peptidoglycan hydrolases evolved to destroy pathogenic invaders (11). Not surprisingly, bacterial evolution has responded in turn by creating panels of proteinaceous lysozyme inhibitors (21). *Escherichia coli* inhibitor of vertebrate lysozyme, or the Ivyc protein, was the first to be discovered (22). The Ivyc homodimer is a potent inhibitor of C-type lysozymes' hydrolytic activity (23), and it has been shown to play a key role in protecting *E. coli* from lysozyme mediated destruction (24, 25). Moreover, Ivyc orthologs have been found in the important pathogens *Burkholderia cepacia* and *P. aeruginosa* (26), suggesting broader human health implications for these proteins.

We speculated that Ivyc and related inhibitory proteins might limit the clinical efficacy of wild type hLYZ therapies, and we contemplated the potential to engineer Ivy-resistant variants. In an initial effort to subvert Ivy-mediated inhibition, we created a large *Saccharomyces cerevisiae* library of mutant hLYZ genes and used a recently developed high throughput antibiotic screen (27) to search for variants able to evade Ivyc. Here, we describe the isolation and characterization of Ivyc-resistant hLYZ variants, and we place these results

in the context of efforts seeking performance enhanced lysozymes able to destroy pathogens that may produce a multitude of redundant inhibitory proteins.

Results and Discussion

Design and construction of Ivyc escape library

We used a high-resolution crystal structure of Ivyc bound to hen egg white lysozyme (HEWL) to guide our molecular engineering efforts. To facilitate the design of hLYZ variants that evade Ivyc, an inhibitor-bound model was constructed from hLYZ structure 1JWR (28) and the Ivyc-HEWL co-crystal structure 1GPQ (26). The energy-minimized Ivyc-hLYZ model was found to overlay closely with the Ivyc-HEWL structure (1.5 Å RMSD over 2225 matched atoms, Supplemental Figure 1A). The Ivyc-hLYZ binding interface was found to be large and complex, with a contact surface of 965 Å² and a total of 26 hLYZ residues within 4 Å of the Ivyc homodimer. The structural basis of Ivyc inhibition is well defined, involving tight binding and insertion of a “CKPHDC” loop into the lysozyme active site (26). However, there exists little to no information regarding lysozyme residues that might be mutated so as to affect Ivy evasion while still maintaining high inherent antibacterial activity.

To better understand the designability of key residues at the Ivyc binding interface, the evolutionary conservation of hLYZ residues was mapped onto its molecular structure using the Consurf web server (29). Most of the 26 candidate residues were highly conserved among hLYZ homologs (Supplemental Dataset 1), but a subset of six amino acids occupied positions at the binding interface while also exhibiting a low degree of evolutionary conservation (Val2, Lys33, Trp34, Gly37, Arg41, Arg115) (Supplemental Figure 1B). While these sites were putatively important for Ivyc molecular recognition, it was not obvious which specific substitutions or combination of substitutions would produce highly active, Ivyc-evading variants. Therefore, the six target sites were subjected to combinatorial mutagenesis, with the intent of performing high throughput functional screening to identify functionally enhanced enzymes.

So as to maintain a manageable library size, the NDT degenerate codon was incorporated at all six sites by gene reassembly. NDT encodes 12 residues that are broadly representative of the 20 natural amino acids (30), and when incorporated at six positions, it gives a theoretical library size of $\sim 3 \times 10^6$ equiprobable protein variants (as opposed to 6.4×10^7 variants from a full saturation library). The wild type hLYZ residues Lys33 and Trp34 were not encoded by the NDT codon, but the biochemically similar residues His/Arg and His/Tyr/Phe, respectively, are among the twelve NDT encoded amino acids. Following library construction from synthetic oligonucleotides, the mutant genes were cloned by homologous recombination in *S. cerevisiae*, yielding 2×10^7 transformants and 99.9% expected coverage of the 3 million member library (31).

GMD-FACS screen

Functional library screening must tightly couple observable phenotype to the encoding genotype. In this case, the desired phenotype is enzyme-mediated bacterial killing in the presence of Ivyc, and the cognate genotype is encoded by the individual yeast cells from the

library population. While bacterial killing is readily quantified using various viability probes, coupling a bacterium's death to the causative genotype of a separate expression host represents a technically challenging problem in ultra-high throughput formats. To identify hLYZ variants able to kill bacteria in the presence of Ivyc, we modified our previously described gel microdroplet – fluorescence activated cell sorting assay (GMD-FACS, Supplemental Figure 2) (27). Yeast cells that secrete wild type hLYZ efficiently kill adjacent *M. luteus* target bacteria that have been co-encapsulated within micron scale agarose hydrogel droplets (Figure 1A). Addition of 70 nM Ivyc renders the secreted hLYZ largely ineffective, resulting in a concomitant reduction in SYTOX Orange staining (Figure 1B). By producing GMDs of approximately 30–50 μm diameter, large yeast populations can be rapidly and semi-quantitatively analyzed by flow cytometry. Control experiments suggested that the dynamic range of the Ivyc-modified GMD-FACS screen was adequate for hLYZ library enrichment (Figure 1C).

First Library Screen

Two successive library screens were performed: a 'First Screen' and a 'Second Screen' (Supplemental Figure 4). In the First Screen, two parallel Round 1 FACS sorts were performed on the naïve library: a plating sort used a high stringency sort gate to isolate highly active individual clones for analysis, and an outgrowth sort employed a moderately stringent gate to broadly capture an enriched population for subsequent iterative screening (Supplemental Figure 4, left). The plating sort isolated 2500 GMDs having the highest 0.33% SYTOX signal (Figure 1D), and the activity of these clones was verified by plating on indicating agar containing *M. luteus* reporter bacteria and 700 nM Ivyc; active Ivyc escape clones generate halos or zones of clearance (Supplemental Figure 5). In the naïve library, halo forming clones were not detected (<0.2% frequency), whereas 3.1% of the Round 1 clones generated marked zones of clearance (Figure 2A). Sequencing of 21 halo-forming clones from Round 1 yielded 12 distinct genotypes (Figure 2B).

The companion Round 1 outgrowth sort employed a more modest 1.66% SYTOX sort gate to collect 63,000 events, which were amplified by overnight outgrowth in selective liquid medium. The resulting enriched population was subsequently re-encapsulated with *M. luteus* and subjected to a more stringent Round 2 sort (Supplemental Figure 4, left). Similar to the Round 1 clones, 3.4% of the Round 2 clones exhibited a halo-forming phenotype on indicating agar (Figure 2A). Subsequent sequencing of 14 halo-forming clones from Round 2 yielded 13 distinct genotypes, two of which were also found among Round 1 clones (Figure 2B). Thus, while the GMD-FACS screen effectively enriched Ivyc escape variants from the naïve library, it failed to narrow the diversity of functional clones between Rounds 1 and 2. We speculated that, in bulk suspensions of GMDs, trans-killing by prevalent functional clones might explain the lack of Round 2 enrichment. We have previously shown that this problem is readily addressed by simply diluting the GMD suspension in a manner proportional to the fraction of functional clones in the population (27).

Second Library Screen

To demonstrate that iterative application of the GMD-FACS screen could effectively narrow the diversity of selected Ivyc escape clones, we conducted a Second Screen starting again

with the naïve library population (Supplemental Figure 4, right). In this revised screening effort, we used progressively more dilute GMD suspensions to further increase screening quality. We conducted three serial Rounds of GMD-FACS sorting, progressively enriching the fraction of halo-forming variants in each round (Figure 2C). We sequenced all 19 halo-forming colonies from Round 1 and 25 representative halo formers each from Rounds 2 and 3. Unlike the First Screen, this Second Screen effectively narrowed the diversity of functional clones from 18 distinct sequences in Round 1 to only three genotypes in Round 3 (Figure 2D). One of these Round 3 clones, variant 2A, generated particularly large halos, and the sequencing analysis demonstrated that this specific clone was itself progressively enriched from one round of sorting to the next (Round 1 frequency=1/18; Round 2=12/25; Round 3=16/25). Thus, as we had shown in earlier control studies (27), the quality of the GMD-FACS selection can be tuned so as to control selective pressure and enable isolation of promising candidates, even from substantially enriched library populations.

Preliminary characterization of isolated variants

While the GMD-FACS screen effectively enriched functional clones from a large and diverse library population, it was only semi-quantitative in nature. For example, it did not explicitly account for variable expression levels among different yeast clones. To more directly compare the molecular performance of isolated enzyme variants, 13 of the sequenced constructs (see Supplemental Figure 6 for alignment) were expressed, purified, and their specific lytic activities were quantified in the absence of inhibitor, with 200 nM Ivyc, and with 2000 nM Ivyc. Wild type hLYZ proved to be the single most active enzyme in the absence of inhibitor, but its activity was reduced to background levels at both 200 nM and 2000 nM Ivyc (Figure 3).

In the absence of inhibitor, the engineered variants maintained anywhere from 20% to 90% of hLYZ's inherent activity. There was a similarly large range of activities in the presence of the Ivyc inhibitor. Several of the variants from the First Screen exhibited inhibition kinetics similar to wild type hLYZ (enzymes 1A, 1B, 1C, 1E, 1G, and 1H, Figure 3A). Given the poor overall performance of these variants and the fact that they were isolated during the First Screen, it seems likely that they were carried through the GMD-FACS selection in part due to the trans-killing issue discussed above. In contrast, clones from the more carefully controlled Second Screen all exhibited 5- to 11-fold higher activity in the presence of 200 nM Ivyc (Figure 3B). However, only the dominant variant from the Second Screen, enzyme 2A, exhibited high lytic rates at 2000 nM Ivyc, retaining approximately 60% of its inherent activity. Variants 1D and 1F from the First Screen also retained relatively high activity in the presence of 2000 nM Ivyc (Figure 3A), and these enzymes, along with variant 2A, were selected for more detailed functional analysis.

Detailed analysis of variants 1D, 1F, and 2A

The structural stabilities of the engineered lysozymes were compared to that of wild type hLYZ and HEWL by differential scanning fluorimetry (32). Both hLYZ and HEWL possessed high apparent T_m values of 70 °C, whereas variant 1D and 1F demonstrated 9 °C and 8 °C reductions, respectively (Table 1). Importantly, however, we observed that, like hLYZ and HEWL, both 1F and 1D lost little to no activity after three months at 4 °C (data

not shown), and in general these two engineered enzymes exhibited good stability. On the other hand, variant 2A from the Second Screen exhibited a more substantial 17 °C drop in T_m . This large reduction in stability likely resulted from numerous and complex interactions associated with the variant's five mutations, but it was noteworthy that 2A was the only high functioning variant to encode a cysteine at position 33 (Supplemental Figure 6).

Lysis kinetics were examined more closely via pseudo Michaelis-Menten analysis with the *M. luteus* bacterial substrate (Table 1). The V_{max}/K_m values for wild type hLYZ and HEWL differed by less than 2-fold (HEWL being higher), but their respective efficiencies were driven by a large V_{max} in the case of hLYZ and a low K_m in the case of HEWL. In general, hLYZ is considered the more powerful lytic agent (33), suggesting that a large V_{max} is more important for overall lysozyme activity. Relative to hLYZ, variants 1D and 1F experienced 50% and 60% reductions in V_{max} , respectively, but both remained 2.5 to 3-fold faster than HEWL. The V_{max} of 2A was equivalent to wild type hLYZ, although the variant also possessed the highest K_m among the enzymes tested, requiring 500 µg/ml *M. luteus* to achieve ½ max velocity. Thus, the engineered variants retained high levels of inherent activity, although they all required relatively high bacterial concentrations to achieve maximum reaction velocity.

In presence of Ivyc, the enhanced performance of the engineered variants was striking. Ivyc is known to inhibit C-type lysozymes via a slow-tight binding mechanism, and its K_i for HEWL has been reported previously as 1 nM (22). We observed similar potency towards both HEWL (K_i =10 nM) and hLYZ (K_i =17 nM). Compared to hLYZ, variant 1D exhibited a 15-fold reduction in susceptibility to Ivyc, and 1F was an even more effective escape variant with a 77-fold reduction in K_i (Table 1). Variant 2A retained 60% of its inherent activity at 2000 nM Ivyc, and the enzyme's resistance to Ivyc inhibition was so great that the slow-tight binding model could not be fit to the data (i.e., a K_i could not be calculated). Thus, all three engineered enzymes retained high levels of lytic activity at Ivyc concentrations far beyond those that completely inactivated wild type hLYZ.

As a corollary to our studies, we sought to evaluate the performance of our lysozymes with an Ivyc homolog from the Gram-negative pathogen *P. aeruginosa*. This homolog, Ivyp, has been shown to be a reasonably potent HEWL inhibitor (26). Our own analysis showed that, similar to the literature, Ivyp inhibited HEWL with a K_i =10 nM. We anticipated that Ivyp would likewise prove an effective inhibitor of hLYZ, but surprisingly it had a 24-fold higher K_i value compared to Ivyc (Table 1). Prior analysis demonstrated that although the Ivyc homodimer and Ivyp monomer share only 30% sequence identity, their tertiary structures are strikingly similar (26). Co-crystal structures of both inhibitors with HEWL revealed that each manifests a similar number of intermolecular salt bridges and hydrogen bonds, but each complex leverages interactions between different HEWL residues. In fact, only three strongly interacting HEWL residues are strictly conserved between the Ivyc and Ivyp complexes (26). Thus, while the inhibitory mechanisms of Ivyc and Ivyp are strongly conserved (i.e., insertion of the "CKPHDC" loop into the lysozyme active site), different Ivyc inhibitors appear to leverage distinct molecular determinants when binding to C-type lysozymes. For example, it appears that Ivyc determinants of HEWL and hLYZ inhibition have considerable overlap, leading to similar inhibitory activity. In contrast, the Ivyp

determinants for the same two lysozymes must be substantially different, as they exhibit a nearly 50-fold difference in Ivyp K_i values.

Our results became even more intriguing upon analysis of Ivyp inhibitory activity towards engineered variants 1D, 1F, and 2A. We were surprised to find that, compared to hLYZ, all three variants were significantly more susceptible to Ivyp inhibition (~80, 15, and 7-fold, respectively, Table 1). Among the engineered variants, there appeared to be some correlation between evasion of Ivyc and Ivyp. Higher K_i values for the Ivyc target inhibitor were associated with higher K_i values for the Ivyp homolog. However, even the most effective Ivyc escape variant 2A underperformed with respect to Ivyp inhibition. This unexpected observation underscores the extent of the challenge faced in seeking to design lysozymes that might be broadly evasive towards pathogen derived inhibitors. It is now evident that the solutions to avoiding one inhibitory protein may not necessarily transfer to other inhibitors, irrespective of any structural or mechanistic homologies.

Bactericidal activity towards *E. coli*

We next assessed whether the Ivy-evasive properties of the engineered variants would translate into improved antibacterial activity against *E. coli*. As single agents, lysozymes are poorly active against *E. coli* (13), and we therefore conducted quantitative culture experiments using combinations of our lysozymes and LL37, a membrane disrupting antimicrobial peptide that acts synergistically with lysozyme in time-kill experiments (34). The variants' bactericidal activities towards *E. coli* correlated with their Ivyc evasive capacities, where higher Ivyc K_i values (Table 1) were associated with greater % bacterial killing (Figure 4A, left). Counter to expectations, however, variants 1D and 1F were substantially less bactericidal than wild type hLYZ, and 2A was at best equivalent to wild type hLYZ. To account for the fact that the engineered variants possessed reduced inherent activity compared to hLYZ, we next normalized the cell kill data to the enzymes' relative lytic rates in the absence of inhibitor (Figure 4A, right). In the context of this correction, variant 2A was easily the most effective antibacterial treatment, having 2.5-fold greater normalized activity than hLYZ. However, variants 1D and 1F continued to underperform relative to the wild type enzyme. As a result, and interestingly, it was evident that the antibacterial efficacy of the engineered enzymes was dominated by their Ivyc evasive properties, as opposed to their inherent lytic capacities in non-inhibitory conditions.

Given that 1D and 1F had both inadvertently acquired greater susceptibility to the Ivyp homolog, we contemplated the possibility that they might also have greater sensitivity to MliC, an unrelated membrane-bound lysozyme inhibitor and known *E. coli* virulence factor (35, 36). To test this hypothesis, we purified a soluble form of *E. coli* MliC and tested its inhibitory capacity in microplate kinetic assays (Figure 4B). For wild type hLYZ, MliC was significantly less potent than Ivyc (at 200 nM inhibitor, 86% vs. 6% residual activity, respectively). Conversely, MliC and Ivyc manifested largely equivalent inhibition of 1D (60% vs. 55% residual activity, respectively) and 2A (80% vs. 87% residual activity, respectively). In a complete reversal of the wild type hLYZ trend, 1F was substantially more susceptible to MliC than Ivyc (33% vs. 45% residual activity, respectively). Thus, as was

found with Ivyp, the mutations selected for Ivyc evasion inadvertently increased the variants' sensitivity to the unrelated MliC inhibitor.

Having established that 1D and 1F suffered greater MliC sensitivity than hLYZ, we next examined bactericidal activity in an *E. coli* *mliC* knockout strain. The antibacterial activities of wild type hLYZ and variant 2A were unaffected by the *mliC* genomic modification (compare Figure 4C to 4A), which was consistent with their low degree of sensitivity to MliC inhibition *in vitro* (Figure 4B). On the other hand, both 1D and 1F were significantly more lethal towards the knockout as compared to the parental strain (compare Figure 4C to 4A). Variant 1D killed 18% of the knockout strain compared to 3% of wild type *E. coli*, and 1F showed activity equivalent to wild type hLYZ, killing 25% of the modified bacteria compared to just 10% of wild type *E. coli*. These results are more impressive when placed in the context of the engineered enzymes' reduced inherent activities. Upon normalizing *mliC* *E. coli* killing to the enzymes' respective lytic rates in the absence of any inhibitor, variant 1D was equivalent to hLYZ and 1F exhibited 2-fold enhanced relative killing, approaching the efficacy of variant 2A (Figure 4C, right).

Conclusion

Using structure-guided library design combined with an innovative ultra-high throughput antibiotic screen, we have successfully engineered hLYZ variants that evade the *E. coli* inhibitory protein Ivyc. Surprisingly, however, the top performing engineered enzymes acquired an unexpected sensitivity to the *E. coli* MliC inhibitor, the *P. aeruginosa* Ivyp inhibitor, or both. Efforts to rationalize these observations via molecular modeling of the various enzyme-inhibitor complexes proved inconclusive, and in general these results highlight the complexity of molecular determinants that underlie lysozyme recognition by proteinaceous inhibitors. Thus, it will be a challenge to develop hLYZ variants that are broadly evasive towards different classes of inhibitors or even similar orthologs from different pathogens. However, the diversity of our current Ivyc-resistant variants suggests that there are many alternative solutions to this general molecular engineering problem. Alternative library designs and appropriate application of high throughput screening technologies should enable efficient mining of higher performance enzymes. For example, one reasonable next step would be construction of an error-prone PCR library and more aggressive screening against cocktails of inhibitory proteins. Regardless, the results reported here represent an important proof-of-concept for developing inhibitor-resistant lysozyme variants. We anticipate that future efforts will capitalize on the lessons learned to produce highly active enzymes able to more efficiently attack and kill pathogens that currently subvert nature's repertoire of lysozyme antibiotics.

METHODS

Strains and Plasmids

The *Saccharomyces cerevisiae* expressing yEGFP previously described (27) was used and *Micrococcus luteus* (ATCC 4698) was obtained from ATCC. The centromeric expression vector p4GM-LYZ was constructed as previously described (37). *E. coli* strains BW25113 (F⁻, DE(araD-araB)567, lacZ4787(del)::rrnB-3, LAM⁻, rph-1, DE(rhaD-rhaB)568,

hsdR514) and JW1631-1 (F-, DE(araD-araB)567, lacZ4787(del)::rrnB-3, LAM-, rph-1, DE(rhaD-rhaB)568, hsdR514, ydhA788::kan) were purchased from The Coli Genetic Stock Center at Yale University, CT, USA. ydhA was previously renamed mliC (38). *E. coli* T7 Shuffle Express was purchased from New England Biolabs.

Model construction and library design

Using YASARA v13.4.21 (39), a single molecule from hLYZ structure 1JWR (28) was superimposed on the Ivyc-HEWL co-crystal structure 1GPQ (26), and the HEWL molecules were then deleted. The resulting hLYZ-Ivyc complex was energy minimized using the AMBER99 forcefield (40). Six residues were selected for mutation based on the modeled interaction between hLYZ and *E. coli* Ivyc. A ConSurf (29) bioinformatics analysis, using default parameters, scored the hLYZ residues at the interface between hLYZ and Ivyc for evolutionary conservation. Poorly conserved residues were then examined for interactions with Ivyc. Residues V2, W34 and G37 have close packed van der Waals contacts at the binding interface. Residues K33, R41 and R115 have extensive hydrogen bonding with Ivyc.

Library construction

A site directed *hlyz* library, using the NDT degenerate codon at target sites (30), was constructed from synthetic oligonucleotides as described in detail elsewhere (37). The external N- and C-terminal primers included overlap with the EcoRI and XhoI restriction sites in the p416-GAL1- α MF vector (41). The library was cloned into the the p416-GAL1- α MF vector by yeast gap repair homologous recombination in the BJ5464::yEGFP *S. cerevisiae* strain (27). The statistics of library coverage were calculated using the GLUE algorithm (31).

Purification of lysozyme inhibitors

Hexa-His tagged Ivyc, Ivyp, and *E. coli* MliC proteins were obtained using similar procedures. Genes coding for Ivyc and *E. coli* MliC were amplified from *E. coli* JM105, and that for Ivyp from *P. aeruginosa* strain PA01. Expression vectors pET26b-Ivyc-his, pET26b-Ivyp-his, and pET26b-MliC-his were constructed and subsequently transformed into Shuffle® T7 Express cells (New England Biolabs, USA) and produced as described previously (42).

GMD production

GMD were produced as described in detail elsewhere (27), with the exception that *M. luteus*, instead of *Staphylococcus aureus*, was employed as the target bacteria. The stirred tank method of GMD production was used here.

First Screen

The First Screen included two sequential Rounds of sorting (see Supplemental Figure 4 for details). The GMDs were grown in induction medium, containing purified Ivyc, at 30°C for 19hrs, after which they were sieved over a 20 μ m mesh and then recovered from the sieve in fresh PBS. SYTOX Orange (Life Technologies, USA) was added to a final concentration of 100 nM just before sorting. GMD were sorted on an iCYT Synergy flow cytometer equipped

with a 126 µm nozzle and laser lines of 488 and 561 nm for excitation of yEGFP and SYTOX Orange, respectively. Individual GMD isolated from high stringency plating sorts were placed on *M. luteus* indicator plates (0.5 mg/ml *M. luteus*, 10 µg/ml *E. coli* Ivy, 0.5% dextrose, 1.5% galactose, 0.71% Difco™ YNB without (NH₄)₂SO₄, 0.25% (NH₄)₂SO₄, 0.077% CSM^{-ura}) and grown four days at 30°C. Prevalent halo-forming colonies were selected for sequencing.

Second Screen

The Second Screen followed the same general methods as the first, with the exception that GMDs were incubated in progressively more dilute concentrations at each round of sorting (Round 1=100,000 GMDs/ml, Round 2=10,000 GMDs/ml and Round 3=1000 GMDs/ml; See Supplemental Figure 4 for details).

Lysozyme Purification

Lysozyme variants were purified as described in detail elsewhere (16, 42).

Protein Characterization

The lytic activities of lysozymes were analyzed by light scattering assays as previously described (43). Commercially sourced human lysozyme (Sigma, USA) and hen egg white lysozyme (Fisher Scientific, USA) were used as controls. Kinetic assays used 300 µg/ml *M. luteus* and 14 nM enzyme in 100 mM KHPO₄ buffer, pH 6.0. The plates were assessed for decrease in absorbance at 450 nm over 30 minutes on a Spectra Max 190 plate reader at 25 °C, and specific rates were calculated from the initial linear portion of the curve. Pseudo Michaelis–Menten kinetics were assessed at four different substrate concentrations (50, 100, 200 and 400 µg/ml *M. luteus*) with 14 nM enzyme in 100 mM KHPO₄, pH 6.0. The parameters K_m, V_{max} and V_{max}/K_m were calculated by non-linear regression using Prism version 5.0 (GraphPad Software Inc., USA). K_i values were accessed by measuring the specific activities (using 300 µg/ml *M. luteus* and 14 nM enzymes in 100 mM KHPO₄, pH 6.0) in the presence of Ivyc, *E. coli* MliC, and *P. aeruginosa* Ivyp at concentrations varying from 0 to 15 µM. K_i was computed by non-linear regression with equation 1 (22):

$$(V_{Ivy}/V_0)^2 + (V_{Ivy}/V_0) * (I_t/E_t + K_i/E_t - 1) - K_i/E_t = 0, \quad (1)$$

where V_{Ivy} is the initial rate in the presence of inhibitor, V₀ is the initial rate in the absence of inhibitor, I_t is the total inhibitor concentration, and E_t is the total lysozyme concentration.

Melting temperatures were measured using differential scanning fluorimetry. Lysozymes and SYPRO Orange (Invitrogen, USA) were prepared in 20 µl 10 mM KHPO₄, pH 6.0 at final concentrations of 100 µg/ml lysozyme and 5X SYPRO Orange. Samples were run on a Q-PCR 7500 (Life Technologies Corporation, USA) and analyzed using Prism and Excel following the protocols provided by Niesen *et al.* (32).

Quantitative Culture

Single colonies of *E. coli* BW25113 and *E. coli* JW1631-1 were inoculated in 5 ml of LB and grown at 37°C overnight. Overnights were sub-cultured 1:100 into 5 ml of fresh LB and

grown at 37°C to mid-log ($OD_{600}=0.6-0.9$). Subsequently, cells were diluted to 0.01 OD_{600} in either 1 ml of control solution (10% LB medium and 100 mM $KHPO_4$, pH 6.0,) or 1 ml of working solution (10% LB medium, 100 mM $KHPO_4$, pH 6.0, 0.5 μ g/ml LL37, and 100 μ g/ml lysozymes), respectively. Cell suspensions were incubated at 37°C for 2 hours, serially diluted, and plated in triplicate on LB agar. Plates were grown at 37°C overnight, and colonies were enumerated the next morning. The bactericidal activities of lysozymes were represented by percentage of killing, equation 2:

$$\%Killing = [1 - (\#colonies \text{ from working solution}) / (\#colonies \text{ from control solution})] * 100 \quad (2)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a pilot grant from the Hitchcock Foundation (to K. Griswold), a pilot grant from the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P30GM106394 (to K. Griswold), and grant R21AI094391 (to K. Griswold) from the NIH National Institute of Allergy and Infectious Disease. J. Guerrette was supported in part by a Renal Function and Disease Training Grant (T32 DK007301) from the NIH. The authors would like to thank D. Osipovitch and Dr. H. Zhao for helpful discussions and assistance with assay development.

References

1. CDC. Antibiotic Resistance Threats in the United States, 2013. Centers for Disease Control; 2013.
2. WHO. Antimicrobial Resistance: Global Report on Surveillance, 2014. Geneva: WHO; 2014.
3. Taubes G. The Bacteria Fight Back. *Science*. 2008; 321:356–361. [PubMed: 18635788]
4. Kokai-Kun JF, Chanturiya T, Mond JJ. Lysostaphin as a treatment for systemic *Staphylococcus aureus* infection in a mouse model. *Journal of Antimicrobial Chemotherapy*. 2007; 60:1051–1059. [PubMed: 17848374]
5. Parisien A, Allain B, Zhang J, Mandeville R, Lan CQ. Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. *Journal of Applied Microbiology*. 2008; 104:1–13. [PubMed: 18171378]
6. Nelson, DC.; Schmelcher, M.; Rodriguez-Rubio, L.; Klumpp, J.; Pritchard, DG.; Dong, SL.; Donovan, DM. Endolysins as Antimicrobials. In: Lobočka, M.; Szybalski, WT., editors. *Advances in Virus Research*, Vol 83: Bacteriophages. 2012. p. 299-365.
7. Pastagia M, Schuch R, Fischetti VA, Huang DB. Lysins: the arrival of pathogen-directed anti-infectives. *Journal of Medical Microbiology*. 2013; 62:1506–1516. [PubMed: 23813275]
8. Rodriguez-Rubio L, Martinez B, Rodriguez A, Donovan DM, Goetz F, Garcia P. The Phage Lytic Proteins from the *Staphylococcus aureus* Bacteriophage ν B_SauS- ϕ IPLA88 Display Multiple Active Catalytic Domains and Do Not Trigger Staphylococcal Resistance. *PLoS One*. 2013; 8
9. Schuch R, Lee HM, Schneider BC, Sauve KL, Law C, Khan BK, Rotolo JA, Horiuchi Y, Couto DE, Raz A, Fischetti VA, Huang DB, Nowinski RC, Wittekind M. Combination Therapy With Lysin CF-301 and Antibiotic Is Superior to Antibiotic Alone for Treating Methicillin-Resistant *Staphylococcus aureus*-Induced Murine Bacteremia. *Journal of Infectious Diseases*. 2014; 209:1469–1478. [PubMed: 24286983]
10. Osipovitch DC, Griswold KE. Fusion with a Cell Wall Binding Domain Renders Autolysin LytM a Potent Anti-*Staphylococcus aureus* Agent. *FEMS Microbiol Lett*. 2014 epub ahead of print. 10.1093/femsle/fnu1035
11. Callewaert L, Michiels CW. Lysozymes in the animal kingdom. *Journal of Biosciences*. 2010; 35:127–160. [PubMed: 20413917]

12. Ibrahim HR, Matsuzaki T, Aoki T. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. *Febs Letters*. 2001; 506:27–32. [PubMed: 11591365]
13. Masschalck B, Michiels CW. Antimicrobial Properties of Lysozyme in Relation to Foodborne Vegetative Bacteria. *Critical Reviews in Microbiology*. 2003; 29:191–214. [PubMed: 14582617]
14. Bhavsar T, Liu M, Hardej D, Liu XJ, Cantor J. Aerosolized recombinant human lysozyme ameliorates pseudomonas aeruginosa-induced pneumonia in hamsters. *Experimental Lung Research*. 2010; 36:94–100. [PubMed: 20205599]
15. Bhavsar T, Liu M, Liu XJ, Cantor J. Aerosolized recombinant human lysozyme enhances the bactericidal effect of tobramycin in a hamster model of pseudomonas aeruginosa-induced pneumonia. *Experimental Lung Research*. 2011; 37:536–541. [PubMed: 21967196]
16. Scanlon T, Jewell H, Gray E, Griswold K. The monoclonal myth. *Bioengineered Bugs*. 2010; 1:1–4.
17. Gill A, Scanlon TC, Osipovitch DC, Madden DR, Griswold KE. Crystal Structure of a Charge Engineered Human Lysozyme Having Enhanced Bactericidal Activity. *PLoS One*. 2011; 6:e16788. [PubMed: 21408218]
18. Teneback CC, Scanlon TC, Wargo MJ, Bement J, Griswold KE, Leclair LW. Bioengineered lysozyme reduces bacterial burden and inflammation in a murine model of mucoid Pseudomonas aeruginosa lung infection. *Antimicrobial Agents and Chemotherapy*. 2013; 57:5559–5564. [PubMed: 23979752]
19. Griswold KE, Bement JL, Teneback CC, Scanlon TC, Wargo MJ, Leclair LW. Bioengineered lysozyme in combination therapies for Pseudomonas aeruginosa lung infections. *Bioengineered*. 2014; 5:31–30.
20. Koch AL. Bacterial Wall as Target for Attack: Past, Present, and Future Research. *Clinical Microbiology Reviews*. 2003; 16:673–687. [PubMed: 14557293]
21. Callewaert L, Van Herreweghe JM, Vanderkelen L, Leysen S, Voet A, Michiels CW. Guards of the great wall: bacterial lysozyme inhibitors. *Trends in Microbiology*. 2012; 20:501–510. [PubMed: 22840966]
22. Monchois V, Abergel C, Sturgis J, Jeudy S, Claverie J-M. Escherichia coli ykfE ORF gene Encodes a Potent Inhibitor of C-type Lysozyme. *Journal of Biological Chemistry*. 2001; 276:18437–18441. [PubMed: 11278658]
23. Callewaert L, Masschalck B, Deckers D, Nakimbugwe D, Atanassova M, Aertsen A, Michiels CW. Purification of Ivy, a lysozyme inhibitor from Escherichia coli, and characterisation of its specificity for various lysozymes. *Enzyme and Microbial Technology*. 2005; 37:205–211.
24. Deckers D, Masschalck B, Aertsen A, Callewaert L, Van Tiggelen CGM, Atanassova M, Michiels CW. Periplasmic lysozyme inhibitor contributes to lysozyme resistance in Escherichia coli. *Cellular and Molecular Life Sciences (CMLS)*. 2004; 61:1229–1237. [PubMed: 15141308]
25. Deckers D, Vanlint D, Callewaert L, Aertsen A, Michiels CW. Role of the Lysozyme Inhibitor Ivy in Growth or Survival of Escherichia coli and Pseudomonas aeruginosa Bacteria in Hen Egg White and in Human Saliva and Breast Milk. *Applied and Environmental Microbiology*. 2008; 74:4434–4439. [PubMed: 18515493]
26. Abergel C, Monchois V, Byrne D, Chenivresse S, Lembo F, Lazzaroni J-C, Claverie J-M. Structure and evolution of the Ivy protein family, unexpected lysozyme inhibitors in Gram-negative bacteria. *Proceedings of the National Academy of Sciences, USA*. 2007; 104:6394–6399.
27. Scanlon TC, Dostal SM, Griswold KE. A high-throughput screen for antibiotic drug discovery. *Biotechnology and Bioengineering*. 2014; 111:232–243. [PubMed: 23955804]
28. Higo J, Nakasako M. Hydration structure of human lysozyme investigated by molecular dynamics simulation and cryogenic X-ray crystal structure analyses: On the correlation between crystal water sites, solvent density, and solvent dipole. *Journal of Computational Chemistry*. 2002; 23:1323–1336. [PubMed: 12214315]
29. Landau M, Mayrose I, Rosenberg Y, Glaser F, Martz E, Pupko T, Ben-Tal N. ConSurf 2005: the projection of evolutionary conservation scores of residues on protein structures. *Nucleic Acids Research*. 2005; 33:W299–302. [PubMed: 15980475]
30. Reetz MT, Kahakeaw D, Lohmer R. Addressing the Numbers Problem in Directed Evolution. *ChemBioChem*. 2008; 9:1797–1804. [PubMed: 18567049]

31. Patrick WM, Firth AE, Blackburn JM. User-friendly algorithms for estimating completeness and diversity in randomized protein-encoding libraries. 2003;451–457.
32. Niesen FH, Berglund H, Vedadi M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat Protocols*. 2007; 2:2212–2221.
33. Imoto T. Engineering of lysozyme. *EXS*. 1996; 75:163–181. [PubMed: 8765300]
34. Singh PK, Tack BF, McCray PB Jr, Welsh MJ. Synergistic and additive killing by antimicrobial factors found in human airway surface liquid. *Am J Physiol Lung Cell Mol Physiol*. 2000; 279:L799–805. [PubMed: 11053013]
35. Callewaert L, Aertsen A, Deckers D, Vanoirbeek KGA, Vanderkelen L, Van Herreweghe JM, Masschalck B, Nakimbugwe D, Robben J, Michiels CW. A new family of lysozyme inhibitors contributing to lysozyme tolerance in gram-negative bacteria. *Plos Pathogens*. 2008; 4:9.
36. Vanderkelen L, Ons E, Van Herreweghe JM, Callewaert L, Goddeeris BM, Michiels CW. Role of Lysozyme Inhibitors in the Virulence of Avian Pathogenic *Escherichia coli*. *PLoS One*. 2012; 7
37. Scanlon TC, Gray EC, Griswold KE. Quantifying and resolving multiple vector transformants in *S. cerevisiae* plasmid libraries. *BMC Biotechnology*. 2009; 9:95. [PubMed: 19930565]
38. Callewaert L, Aertsen A, Deckers D, Vanoirbeek KGA, Vanderkelen L, Van Herreweghe JM, Masschalck B, Nakimbugwe D, Robben J, Michiels CW. A new family of lysozyme inhibitors contributing to lysozyme tolerance in gram-negative bacteria. *PLoS Pathog*. 2008; 4:e1000019. [PubMed: 18369469]
39. Krieger E, Koraimann G, Vriend G. Increasing the precision of comparative models with YASARA NOVA—a self-parameterizing force field. *Proteins: Structure, Function, and Bioinformatics*. 2002; 47:393–402.
40. Wang J, Cieplak P, Kollman PA. How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? *Journal of Computational Chemistry*. 2000; 21:1049–1074.
41. Mumberg D, Muller R, Funk M. Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucl Acids Res*. 1994; 22:5767–5768. [PubMed: 7838736]
42. Lamppa JW, Tanyos SA, Griswold KE. Engineering *Escherichia coli* for soluble expression and single step purification of active human lysozyme. *Journal of Biotechnology*. 2013; 164:1–8. [PubMed: 23220215]
43. Lee YC, Yang D. Determination of lysozyme activities in a microplate format. *Anal Biochem*. 2002; 310:223–224. [PubMed: 12423643]

Figure 1.

GMD micrographs and FACS data. (a) A fluorescent micrograph of a GMD showing that *M. luteus* bacteria are efficiently killed and stained with SYTOX Orange when co-encapsulated with recombinant yeast secreting wild type hLYZ. (b) Purified Ivyc protein added to GMD induction medium inhibits wild type hLYZ and minimizes bacterial staining with SYTOX. In both panels, yeast colonies expressing yEGFP are seen in green and the grape-like microclusters are bacterial colonies. The white scale bar is 20 μm. The brightness, contrast, and color of both panels were enhanced uniformly. The original images are available as Supplemental Figure 3. (c) Overlaid flow cytometry histograms of GMDs incubated with or without 70 nM Ivyc (blue-left and yellow-right, respectively). X-axis is SYTOX fluorescence intensity. (d) Flow cytometry dot plot of pre-sort GMD library population in the presence of 70 nM Ivyc. X-axis is yEGFP fluorescence intensity and Y-axis is SYTOX fluorescence intensity. The lower right population in circular gate R1 is individual free yeast that outgrew their encapsulating GMDs. The upper left population in rectangular gate R2 is GMD containing bacteria but no yeast. The upper right population is GMD containing both bacteria and yeast. Rectangular gate R3 is a representative sorting gate from library screens.

Figure 2.

Phenotype and genotype analysis during library screening. (a) 500–2500 colonies from each round of Screen 1 sorting were plated on indicating agar containing 700 nM Ivyc. The frequency of halo-forming clones is plotted. (b) Twenty to twenty-five halo-forming colonies from each Screen 1 sort were sequenced, and the total number of distinct genotypes is plotted. Hatched bars represent unique sequences not observed in any previous round, and white bars represent genotypes carried through from previous rounds. (c) The frequency of Ivyc evading clones observed during Screen 2 sorting, analogous to panel a. (d) The number of distinct halo-forming genotypes observed during each round of Screen 2 sorting, analogous to panel b.

Figure 3. Specific lytic activities of thirteen selected enzymes. The kinetics of *M. luteus* lysis were measured for purified enzymes in the presence of 0, 200, and 2000 nM Ivyc. (a) Variants isolated during Screen 1. (b) Variants isolated during Screen 2. Units refer to the decrease in absorbance at 450 nm per minute. Error bars represent standard error of the mean from triplicate measurements. Variants selected for detailed characterization are marked with an asterisk.

Figure 4.

Inherent and relative antibacterial activity towards *E. coli*. The effectiveness of wild type hLYZ (black), 1D (grey), 1F (white), and 2A (hatched) were analyzed by quantitative culture. (a) Compared to no treatment, the percentage of killing is reported for a wild type *E. coli* strain. Values are provided both as raw % and normalized to the enzymes' relative lytic activity in the absence of inhibitor, i.e. normalizing for the reduced activity of the variants. (b) Specific lytic activities of each enzyme with *M. luteus* are reported in the absence of inhibitor, with 200 nM Ivyc, and with 200 nM MliC. (c) Compared to no treatment, the percentage of killing is reported for a *mliC* *E. coli* knockout strain. Values are provided both as raw % and normalized to the enzymes' relative lytic activity in the absence of inhibitor. Error bars represent standard deviation from triplicate measurements made in biological duplicate.

Table 1

Activity and stability of lysozymes

Protein	K_m^* ($\mu\text{g}\cdot\text{mL}^{-1}$)	$V_{\max}^*$ ($\text{units}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	$V_{\max}/K_m$	K_i Iyve (nM)	K_i Iyyp (nM)	T_m ($^{\circ}\text{C}$)
hLYZ	160 $\pm$ 20	930 $\pm$ 60	5.7 $\pm$ 0.7	17 $\pm$ 2	410 $\pm$ 40	70.2 $\pm$ 0.1
HEWL	17 $\pm$ 3	155 $\pm$ 3	9 $\pm$ 2	9.9 $\pm$ 0.5	10 $\pm$ 1	70.1 $\pm$ 0.1
ID	210 $\pm$ 10	460 $\pm$ 10	2.2 $\pm$ 0.1	260 $\pm$ 10	5.2 $\pm$ 0.3	60.9 $\pm$ 0.1
IF	440 $\pm$ 60	390 $\pm$ 30	0.9 $\pm$ 0.1	1310 $\pm$ 60	26.6 $\pm$ 0.8	61.8 $\pm$ 0.1
2A	500 $\pm$ 100	900 $\pm$ 100	1.8 $\pm$ 0.4	N.D.	60 $\pm$ 3	52.9 $\pm$ 0.2

* Apparent values from pseudo Michaelis-Menten analysis using whole cell substrate

N.D. – not determined due to hyper-resistance and lack of slow-tight binding model fit