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USING SICLOPPS FOR THE DISCOVERY OF NOVEL ANTIMICROBIAL PEPTIDES AND THEIR TARGETS

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Abstract

High throughput screening of SICLOPPS libraries afforded six distinct cyclic peptides that inhibit *Escherichia coli* growth both in liquid and solid media. One of these peptides (LN05) reduced both bacterial growth rate and caused cell aggregation in liquid media. Mutant bacteria immune to LN05 action were obtained at a frequency of 10^{-7} . Over-expression of an *E. coli* genomic library in the presence of LN05 production resulted in enrichment of a single genomic construct, a fragment of the NarZ gene.

Keywords

cyclic peptides; antimicrobial; functional complementation

1. INTRODUCTION

Antimicrobial resistance has proven a formidable scourge that causes more than eleven million people to die every year from preventable or curable diseases [1]. The general response to the spread of antimicrobial resistance has been to modify existing drugs to create generation after generation of antibiotics that inhibit the same cellular targets. As a result, infective microbes have been able to stay one step ahead of medical treatments by neutralizing new drugs with the old resistance mechanisms. This situation has created a paradox where increased antimicrobial resistance is met with fewer new drugs, meanwhile the genomic information of numerous infectious agents remain unexploited for essential metabolic activities [2].

Currently, 170 microbial genomes have been solved. One striking result, repeated across different species, is that 30–50% of all detected ORFs have unknown metabolic functions. The large influx of new gene sequences has left the formidable task of elucidating the biological role of the encoded proteins. In the field of antibiotic research two additional hurdles need to be overcome: First is determining which genes are essential for cell viability; second is the requirement to distill into a single molecular structure, the functional and chemical determinants necessary to make new antibiotics.

To our knowledge, no available method simultaneously identifies new antimicrobial compounds while interrogating complete genomes to detect the affected biological functions. Such a method would require the ability to screen large collections of small molecules for their capacity to change cellular phenotypes.

The natural choice to explore molecular diversity at the cellular level are small, genetically encoded peptides. However, the use of peptides as intracellular probes has been hampered by their instability against cellular catabolism. Cyclization affords a way to stabilize peptides against proteolysis, thus allowing their application in the search for inhibitors of intracellular targets. We have recently [3] demonstrated the feasibility of utilizing the ligase activity of inteins for the *in vivo* backbone cyclization of peptidic chains. This procedure—called SICLOPPS for Split Intein Circular Ligation Of Peptides and ProteinS—has been applied in the creation of large collections of nine and six amino acid cyclic peptides [4]. Libraries of SICLOPPS peptides allow the co-localization of small molecule probes and their targets in a biologically relevant environment, which simplifies the search for new antimicrobial targets while simultaneously providing drug-like compounds to inhibit the detected enzymatic function [5].

In this report we tested SICLOPPS libraries for cyclic peptides that inhibit normal *E. coli* growth. Furthermore, we used functional complementation to detect potential intracellular targets of one of the bacteriostatic peptides found.

2. MATERIALS AND METHODS

a. Reagents, plasmids and bacterial strains:

Enzymes for molecular biology were obtained from Invitrogen Life Technologies, (Carlsbad, CA), Promega Corporation (Madison, WI), and New England Biolabs (Beverly, MA). All other chemicals were of analytical grade or better. *E. coli* strain DH5 α -PRO and plasmid pPROTet.E133 were from Clontech (Palo Alto, CA). *E. coli* strain TUNER(DE3) and plasmid pET24d were from Novagen (Madison, WI). *E. coli* strain XL1-Blue was from Stratagene (La Jolla, CA). Plasmid pMAL-C2X was from New England Biolabs. Plasmid pBAD/HisB was from Invitrogen. The pDIMC7 plasmid was a generous gift from Prof. Stephen Benkovic, the Pennsylvania State University, PA.

b. Construction of genomic library expression vector:

pPROTet.E133 (10 ng/ μ l) was digested with *Bam*HI (0.5 u/ μ l) for 4.5 h at 37 °C. The digested plasmid was subsequently dephosphorylated by adding 1 μ l shrimp alkaline phosphatase directly to the digestion mixture, and further incubated for 1h at 37 °C. Genomic DNA was prepared from *E. coli* XL1-Blue. The genomic DNA was digested with *Sau*3AI to give DNA fragments ranging from 500 bp to 3000 bp.

The digested vector and genomic DNA fragments were ligated following standard procedures [6] to yield pPRO(Ecoli_genomic). After ligation, the mixture was digested with *Bam*HI, prior to transformation into electro competent *E. coli* DH5 α -PRO. The transformed population yielded approximately 1.5×10^5 colonies. Plasmid DNA was extracted from individual colonies and analyzed by digestion with *Nco*I. The majority of inserts were between 900 and 1200 bp long. Approximately 20% of the plasmids tested were parental pPROTet.E133. Transformed colonies were pooled and stored as a 20 % glycerol stock at -80 °C.

c. Construction of SICLOPPS libraries:

The I_C fragment of the *DnaE* split intein from *Synechocystis sp. PCC6803* was cloned between the *SacI* and *EcoRI* sites of pMAL-C2X. The I_N fragment was cloned downstream of I_C using the *XbaI* and *HindIII* sites to yield pMAL(SICLOPPS). Two different SICLOPPS libraries were prepared following published procedures [7]. Both peptide libraries have four fixed positions (serine, glycine, proline, and leucine) to improve cyclization efficiency. The 4+5 (cyclo[SGXXXXXPL]) library has five variable positions, while the 4+6 (cyclo[SGxrefPL]) library has six variable positions. Both libraries were cloned into pMAL(SICLOPPS), in the standard SICLOPPS configuration [3] to yield pMAL(4+5) and pMAL(4+6). The libraries were subsequently subcloned into pBAD/HisB to yield pBAD(4+5) and pBAD(4+6), pET24d to yield pET(4+5) and pET(4+6), and pDIMC7 to yield pDIMC(4+5) and pDIMC(4+6).

d. Screening for bacteriostatic peptides:

Tuner(DE3) cells were transformed with pMAL(4+6) or pET(4+6), and plated on LB agar containing the appropriate antibiotic and 1% glucose. Plates were incubated overnight at 37 °C. Colonies appearing on these master plates were replica plated onto LB agar supplemented with 1 mM isopropyl-β-D-1- thiogalactopyranoside (IPTG) (Fig. 1, a). Colonies able to grow on the master plates and unable to grow on the inducing plates were marked as producing toxic peptides. Selected colonies were picked from the master plate and grown overnight liquid LB media. The resulting cultures were diluted to OD₆₀₀ < 0.1 in two different wells containing 1 mM and 0 mM IPTG, respectively (Fig. 1, b). The resulting 96-well plates were incubated at 37 °C and the OD₆₀₀ was monitored continuously. Toxicity of the induced SICLOPPS members were scored as the ratio of growth rate of induced versus uninduced cells. The experiment was repeated at 0.1, 0.2, 0.4 and 0.8 mM IPTG. SICLOPPS constructs that stopped cell growth at lower inducer concentrations were extracted by plasmid purification and their identity determined by DNA sequencing (Fig. 1, c). Plasmids encoding toxic peptides were individually re-transformed into fresh cells (Fig. 1, d). The selection scheme was repeated to confirm peptides' bacteriostatic and bactericidal effect. Screening of the pMAL(4+6) library resulted in the identification of three different SICLOPPS constructs (LN01, LN05, and LN06) that affected cell viability. Screening of the pET(4+6) library afforded three more bacteriotoxic constructs (ML04, ML07, and ML11).

e Transient induction of SICLOPPS peptides:

Cells encoding peptides ML04, ML07, and ML11 were grown in LB media for 2 hours at 37 °C. The resulting cultures were induced with 1 mM IPTG for 0, 4, 6, or 20 hours. Aliquots containing 10³ CFU were individually plated on non-inducing LB agar to determine the actual CFU. All plates were incubated overnight at 37 °C. Survival percentage for each sample was obtained by dividing the CFU of induced cultures by the CFU of non-induced cultures.

f Appearance of cell strains resistant to SICLOPPS peptides:

DH5 α PRO cells transformed with pMAL(LN05) were diluted with LB media and 10^9 CFU were plated on inducing LB agar as above. After 48 hours incubation at 37 °C, plates were scored for normal size colonies (as compared with noninducing plates) above a background of pin-point colonies. Four normal sized and one pin-point colonies were picked and grown in LB media overnight at 37 °C. The resulting cell cultures were diluted to OD₆₀₀ < 0.1. Cells were incubated at 37 °C until OD₆₀₀ = 0.4. Every cell culture was divided into two aliquots. One aliquot was treated with IPTG to a final concentration of 1 mM and the other was treated with equal volume of distilled water. All fractions were further incubated overnight at 37 °C. A 500 μ l aliquot from each culture was concentrated by centrifugation. Cell pellets were diluted with SDS-PAGE loading buffer to a final OD₆₀₀ = 20.0. Cell suspensions were incubated in boiling water for 15 minutes and 5 μ l loaded on a 15% SDS-PAGE gel. The gel was run with Tris-glycine buffer for 30 minutes and stained with Coomassie brilliant blue.

g Selection of genomic fragments that counteract peptide action:

DH5 α PRO cells containing pPRO(Ecoli_genomic) were transformed with either pMAL(LN05) or pMAL(SICLOPPS) (Fig. 2, a). Cells were individually plated onto LB agar containing the appropriate antibiotics and 1 % glucose. Both plates were incubated overnight at 37 °C. Approximately 10^5 colonies were obtained in each plate. All colonies in each plate were pooled, and stored at -80 °C as 20% glycerol stocks.

Individual 10 μ l aliquots of frozen cells were grown overnight in 4 ml LB media at 37 °C. 40 μ l aliquots of the overnight cultures were diluted to 4 ml and grown until OD₆₀₀ reached 0.8. Approximately 10^6 CFU of each culture were plated onto LB agar supplemented with the appropriate antibiotics and either 1 % glucose or 1 mM IPTG / 20 mg/l of anhydrotetracycline (aTc). Both plates were incubated overnight at 37 °C. Cells overexpressing the inhibited gene product will produce an excess of the targeted activity, thus overcoming peptide inhibition and rescuing growth (Fig. 2, b & c). DNA from ten individual colonies from the IPTG / aTc plate was isolated and genomic constructs identities determined by DNA sequencing. All colonies from the IPTG / aTc plate were pooled and the plasmid DNA extracted. The resulting plasmid mixture was retransformed in DH5 α PRO cells (Fig. 2, d). The selection scheme was repeated to remove false positives resulting from naturally appearing resistance not associated with genomic construct over-expression.

3. RESULTS AND DISCUSSION

a. Promoter strength and plasmid copy number modulate the toxicity of SICLOPPS peptides:

The 4+5 and 4+6 SICLOPPS libraries were expressed from inducible promoters with varying strength. Expression of the pBAD(4+5) library from the stronger arabinose promoter resulted in the loss of colony forming ability in approximately 80% of transformed cells. Expression of the model peptide, Pseudostellarin F under the same conditions did not reduce CFU [3], demonstrating that loss of viability is not related to synthesis of intein

intermediates, synergy between antibiotic and arabinose, or sequestering of the cellular transcription/translation machinery by the SICLOPPS constructs.

Since SICLOPPS precursor protein is soluble when expressed at 28 °C, but form inclusion bodies at 37 °C, temperature-dependent induction provides for a sensitive test on the toxic effect of soluble SICLOPPS intermediates [4]. Peptide production at 28 °C caused complete growth inhibition by selected pBAD(4+5) constructs, while expression of the same constructs at 37 °C had only a slight effect on growth rate.

Expressing the pET(4+5) or pET(4+6) libraries from a moderately strong T7 promoter ameliorated toxicity, although 30–50 % of the cells lost the ability to produce the precursor protein altogether. This result is probably due to the loss of the integrated DE3 prophage needed for induction from T7-based expression vectors—a typical response to toxic product expression in cells containing an integrated prophage—. The T7 promoter was also induced with lactose to test if toxicity could arise from sugar metabolism. No difference in bacterial growth rate was seen when peptide production was induced with lactose or its non-hydrolysable analog, IPTG.

Both pMAL(4+6) and pDIMC(4+6) were expressed from a weak Tac promoter. However, pMAL(4+6) has a ColE1 origin of replication which allows maintenance of 75–100 plasmid copies per cell. On the other hand, pDIMC(4+6) has a p15A origin of replication that only sustains 15 plasmid copies per cell.

Induction of the pMAL(4+6) library further ameliorated peptide toxicity by reducing the CFGU losses to 1–5%. From this plasmid, the SICLOPPS constructs were translated as a fusion with maltose binding protein (MBP). The solubilization properties of MBP, allows the toxicity of the peptide constructs to be retained both at 28 °C and 37 °C. Induction of pDIMC(4+6) resulted in the complete suppression of peptide antimicrobial activity.

b. Identification of individual toxic peptides:

The pMAL(4+6) and pET(4+6) libraries were plated on LB agar and replica plated onto LB agar plates containing 1 mM IPTG. Approximately 200 colonies unable to grow upon peptide induction were picked from the non-inducing plate and grown on LB media with varying IPTG concentrations. Six peptides were selected that stopped cell at the lowest IPTG concentration.

The six peptides identified have an amphiphilic character but share no obvious sequence homology (Table 1). All of them, however, were able to stop cell growth upon induction. Furthermore, cells expressing these constructs showed no propidium iodide uptake (data not shown). Thus, these peptides did not target cellular membranes and may stop cell growth by inhibiting different essential enzymatic pathways. One of these constructs, LN05, not only inhibited cell growth, but caused extensive bacterial aggregation when grown in liquid media. Similar phenotypes have been seen when cell structural proteins have been deleted from the have been seen when cell structural proteins have been deleted from the bacterial genome [8]. Thus, we expect that the LN05 peptide is inhibiting the function of proteins necessary to maintain cell integrity or other membrane-bound proteins.

c. Transient expression of SICLOPPS peptides is sufficient for bacteriostatic effect:

ML04, ML07, and ML11 peptides were induced for 4, 6 or 20 hours and then plated on non-inducing LB agar to test for cell viability. After transiently inducing for 4 hours, viability of cells expressing ML07 was reduced more than 90% (Fig. 3). Cells expressing ML04 showed a four fold decrease in CFU, while cells expressing ML11 were basically unaffected. After a 6 hours induction period, survival of ML11 expressing cells decreased to the same level as ML07, while ML04 expressing cells remains the same. Thus, although ML04 had a faster effect on cell viability, the toxicity is not as pronounced as in cells expressing ML07 and ML11. After 20 hours induction, cells expressing ML07 and ML11 showed a slight increase in CFU, probably due to the appearance of resistant clones. Cells expressing ML04, on the other hand, showed a continuous decrease in cell viability.

d. Bacteria develop resistance against the LN05 peptide:

LN05 production in solid media completely inhibited cell growth at low plating density (10^3 CFU/plate) but not at high plating density (10^9 CFU/plate). After 48 hours incubation, normal-sized colonies appeared at a frequency of 10^{-7} over a background of pin-point colonies. Four normal sized colonies showed to be resistant to LN05 peptide action by monitoring growth inhibition during peptide induction. Cells derived from a pin-point colony were still sensitive. SDS-PAGE of induced resistant cells, showed that two transformants (Fig 4, LN05c and LN05d) had lost the ability to produce LN05 peptide. The other two, however, (Fig 4, LN05a and LN05b) still produced SICLOPPSLN05 precursor protein at levels comparable to sensitive cells (Fig 4, LN05e). Thus, these cells have probably mutated the target inhibited by LN05, such that the peptide cannot affect it anymore. The appearance of resistant colonies is being used to determine genomic mutation that caused resistance to LN05 peptide

e. Functional complementation identify genomic constructs that counteract LN05 peptide effect:

A genomic expression system was created by transforming cells with pPRO(Ecoli_genomic). DNA sequencing of 12 random colonies showed that there was no bias towards any given sequence. When the library was plated in the presence on agar media containing 0 or 0.1 $\mu\text{g/ml}$ aTc, no difference in CFU was observed between induced and noninduced plates. Colonies from the noninduced plate were harvested and the resulting cell population was transformed with either pMAL(LN05) or pMAL(SICLOPPS). Co-expression of LN05 peptide and the genomic library resulted in growth of approximately 100 colonies. Concomitantly, co-expression of pMAL(SICLOPPS) and the genomic library resulted in growth of approximately 30 colonies. As expected, cells expressing pMAL(LN05) alone failed to form any colonies. DNA sequencing of ten pMAL(LN05)/pPRO(Ecoli_genomic) doubly transformed colonies showed different genomic constructs in each one. However, two of the constructs sequenced corresponded to fragments of the NarZ and NarG genes. These two genes encode for the same physiological function, namely, both take part in the reduction of nitrates to nitrites. Ten constructs sequenced from pMAL(SICLOPPS)/pPRO(Ecoli_genomic) transformed cells showed totally different genomic sequences. All colonies from the pMAL(LN05)/pPRO(Ecoli_genomic) plate were

harvested, DNA was extracted and used to transform fresh DH5 α -PRO cells. The resulting transformed cells were selected as above. DNA from ten colonies was sequenced. Three clones corresponded to the NarZ gene previously detected. Under these conditions, the probability of randomly picking out the same genomic construct three times out of ten is 10^{-6} .

Cells expressing LN05 peptide alone, LN05 peptide together with the empty pPROTet.E133 vector, and LN05 peptide together with the NarZ fragment were individually plated onto LB agar supplemented with 0.1 μ g/ml aTc and either 0 or 1 mM IPTG. As before, LN05 peptide production prevented colony formation when expressed alone (Fig. 5, top panels). Co-expression of the pPROTet vector partially ameliorates the bacteriostatic properties of the LN05 peptide (Fig. 5, middle panels). Colonies from co-expressed cells grow slightly better than colonies expressing cyclic peptide alone, indicating that the expression level of the cyclic peptide is at the minimal inhibitory concentration. SDS-PAGE analysis of expression shows that co-expression of LN05 peptide with pPROTet vector lowered the expression level of the cyclic peptide slightly (data not shown). However, when the NarZ fragment was co-expressed with the LN05 peptide, CFU number and colony growth rate was similar for the IPTG induced and non-induced plates (Fig. 5, bottom panels). Co-expression of genomic constructs other than NarZ or NarG failed to rescue cell viability (data not shown).

There are three nitrate reductases in *E. coli*, one periplasmic, Nap (encoded by the napFDAGHBC operon), and two membrane bound, Nitrate Reductase A (NRA, encoded by the narGHJI operon) and Nitrate Reductase Z (NRZ, encoded by the narZYWV operon). Double and triple knockout mutants has been made where either both membrane-bound or all three nitrate reductases were compromised. These knockout clones grow under aerobic, suggesting that nitrate reductases are not essential under aerobic conditions [9]. Since two apparently redundant nitrate reductase enzymes are maintained, the membrane bound reductases may have different physiological functions. These nitrate reductases seem to have evolved by gene duplication because the transcription unit is homologous with 73% identity [10]. Interestingly, they are regulated very differently: NRA is regulated by oxygen and nitrate levels and therefore expressed only during anaerobic conditions. NRZ is constitutively expressed at low levels during exponential growth, and induced by RpoS during stationary phase under aerobic conditions [11].

The fact that NarZ is not essential under aerobic conditions is consistent with our results. The LN05 peptide slows down bacterial growth rate, but does not cause bacterial death. Furthermore, immediately after plating, cells will be in the exponential phase where expression of the NRZ operon is at its lowest levels. If the LN05 peptide inhibits NarZ, cell growth would be slowed (as seen in our results). Thus, no colony formation would be detected. Over time, plated cells would enter into stationery phase with concomitant increased expression of NRZ operon. The increased NarZ concentration, would reduce the inhibitory effect of LN05, allowing the formation of visible colonies. This is, again, consistent with our observations that pin-point colony formation can be seen at very high plate density (where there is strong competition for available IPTG) or after long incubation times (where most cells will have entered into stationary growth phase).

4. SUMMARY

We have developed a novel genetic method to interrogate complete genomes for gene product function. Using plate replication as a selection tool, we identified six different peptides that stop bacterial growth. One of these peptides (LN05) also caused bacterial aggregation upon expression. Functional complementation detected a NarZ gene fragment that counteracted LN05 peptide action. Further characterizations of NarZ-LN05 interaction, as well as identification of metabolic functions inhibited by the other peptides are under way.

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Fig. 1. Selection scheme to detect bactericidal and bacteriostatic peptides. Colonies from a master plate are replica plated on inducing media (a). Colonies unable to grow in the inducing plate are grown with increasing concentrations of inducer (b). Constructs that inhibit cell growth are purified (c). The selected plasmid population is re-transformed into fresh cells (d).

Fig. 2. Selection of genomic constructs that rescue bacteria from SICLOPPS peptide inhibition. Cells are co-transformed with a toxic peptide and a genomic library, so that each cell expresses the same peptide and a unique ORF (a). Expression of targeted functions allows host cells to resume normal growth (b). Surviving cells are able to form colonies (c). Plasmids from selected colonies are re-transformed onto fresh cells (d)

Fig. 3.
SICLOPPS peptides show different kinetics of cell survival after transient induction of peptide production

Fig. 4.
Four different resistant and one sensitive colonies were tested for their ability to produce LN05 construct.

Fig. 5.

LN05 peptide induction eliminates colony formation (Top panels). Coexpression with pPROTET vector partially ameliorates LN05 action (middle panels). NarZ and LN05 co-expression completely eliminated LN05 toxicity.

Table 1.

Bacteriostatic/bactericidal peptide sequences

Name	Peptide sequence
LN01	c[SGYHSVVGPL]
LN05	c[SGWRMWVYPL]
LN06	c[SGMFLWEVPL]
ML04	c[SGSIIVGGPL]
ML07	c[SGLWVLSSPL]
ML11	c[SGPGESWLPL]