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A Genetic Screen to Identify Bacteriophage Lysins

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Abstract

Lysins are phage-encoded, peptidoglycan (cell wall) hydrolases that accumulate in the bacterial cytoplasm during a lytic infection cycle. Late during infection, the lysins undergo holin-mediated translocation across the inner membrane into the peptidoglycan matrix where they cleave cell wall covalent bonds required for wall stability and allow bacterial lysis and progeny phage release. This potent hydrolytic activity is now the foundation of a powerful genetic-based screening process for the identification and analysis of phage lysin proteins. Here, we describe a method for identifying a lysin, PlyG, from a bacteriophage that specifically infects the Gram-positive organism *Bacillus anthracis*, however, the techniques described can be adapted to clone, express and analyze lysins from any phage infecting Gram-positive bacteria or possibly even Gram-negative bacteria.

Keywords

lysin; hydrolase; cell wall; peptidoglycan; lysozyme; Gram-positive; antimicrobial; diagnostic; expression library

1. Introduction

A lysin-based lytic mechanism (1) is widespread amongst phages infecting both Gram-positive and Gram-negative bacteria. Indeed, the lysins likely represent one of the most successful bacteriolytic agents used in nature (2). A major distinguishing feature of the lysin family of proteins concerns their modular design, which can vary dramatically depending on whether a lysin targets the thick, surface exposed cell wall of Gram-positive bacteria, or the very thin peptidoglycan of Gram-negative organisms that lies subjacent to and is protected by the external outer membrane. As such, lysins active against Gram-negative bacteria usually consist of a single 15 to 20 kDa catalytic domain, while those active against Gram-positive bacteria are 25 to 100 kDa and possess one or more distinct catalytic domains (multi-domain protein) fused to a cell wall-binding domain (CBD). The CBD's of Gram-positive lysins are believed to recognize strain- or species-specific carbohydrate structures that decorate the surface of the Gram-positive cell wall (3), thus exerting a level of specificity over the binding of such lysins. A potent lytic activity, combined with an often extreme binding specificity, distinguish the lysins of Gram-positive microorganisms.

Unlike with Gram-negative peptidoglycan, the surface-exposed nature of Gram-positive peptidoglycan renders it vulnerable to hydrolysis by exogenously applied lysins. We have now demonstrated this “lysis from without” using an array of different lysins specifically active against a broad range of Gram-positive bacteria (4). Based on these findings, our laboratory and others are actively pursuing development of lysins as novel therapeutic agents (or alternatives to conventional antibiotics) active against Gram-positive pathogens. Additionally, we are seeking to exploit the binding capacity of these enzymes as the basis for sensitive diagnostic methods. The genetic and biochemical methods developed for these studies are presented.

2. Materials

2.1 Phage, plasmids, and bacteria

1. The γ phage, pBAD24 (5), and *Bacillus cereus* strain RSVF1 are part of The Rockefeller University Collection.
2. *E. coli* XL2-Blue ultracompetant cells (Stratagene; La Jolla, CA; <http://www.stratagene.com/>)

2.2 Equipment

1. Run One Electrophoresis Multicasting System (EmbiTec; San Diego, CA; <http://www.embitec.com/>)
2. Model C24 incubator shaker (New Brunswick Scientific; Edison, NJ.; <http://www.nbsc.com/Main.asp>)
3. SpectraMax Plus spectrophotometer (Molecular Devices; Sunnyvale, CA; <http://www.moleculardevices.com/>)
4. Model 5810R tabletop centrifuge (Eppendorf)
5. Orbital Shaker (Bellco Biotechnology Inc.; Vineland, NJ; <http://www.bellcoglass.com/>)
6. EchoTherm Model IC20 (Torrey Pines Scientific; San Marcos, CA; <http://www.torreypinesscientific.com/>) for incubations at 16°C and 65°C.
7. UV transilluminator (Alpha Innotech Corp.; San Leandro, CA; <http://www.alphainnotech.com/>)
8. Dual chamber water bath (Precision)

2.3 Supplies

1. Lambda Maxi Kit (Qiagen Inc.; Valencia, CA; <http://www1.qiagen.com/>)
2. All DNA modifying enzymes and corresponding buffers were purchased from New England Biolabs (Ipswich, MA; <http://www.neb.com/nebecomm/default.asp>).
3. 1.5 ml Eppendorf tubes
4. Phenol, chloroform, NH₄OAc, ethanol, ethidium bromide, NaOH, Tris, L-arabinose, phosphate buffered saline (PBS), LB media, and ampicillin were all from Sigma-Aldrich (St. Louis, MO; <http://www.sigmaaldrich.com/>).
5. 1 kb DNA ladder (Invitrogen Corp.; Carlsbad, CA; <http://www.invitrogen.com/>)
6. TAE buffer
7. Agarose (Cambrex Corp.; <http://www.cambrex.com/default.asp>)

8. NucleoSpin DNA Extraction Columns and NucleoSpin Plasmid Kit (BD Biosciences; San Jose, CA; <http://www.bdbiosciences.com/>)
9. 150 × 15 mm polystyrene and 150 mm glass Petri dish (Fisher, Inc.)
10. Replica transfer apparatus and velvet squares (Cat. No. 11DOTM001; MP Biochemicals; Solon, OH; <http://www.mpbio.com/>)
11. 10 ml round bottom culture tube (Sarstedt)
12. Brain Heart Infusion media (Difco)
13. 96-well microtiter plate (Costar)
14. 125 ml and 2 L Erlenmeyer flasks (VWR)
15. NucleoSpin DNA Extraction Columns (Clontech – a Takara Bio Company; Mountain View, CA; <http://www.clontech.com/clontech/>)

3. Methods

The potent bacteriolytic activity of phage lysins provides the basis by which their coding sequences may be identified (6). Toward this end, we have developed an efficient means to detect lysin activity by screening induced plasmid expression libraries for agents that lyse live bacterial cells. We describe here one such activity screen for identification of a lysin, PlyG, from the purified bacteriophage, γ . The γ phage is a diagnostic tool used in clinical laboratories to identify the Gram-positive pathogen *Bacillus anthracis*, although it also infects some highly related *B. cereus* isolates like RSVF1 (7).

Following our description of lysin identification, we detail the processes by which we define and quantify lysin activity. Owing to the conserved, repeating structure of bacterial peptidoglycan, lysin catalytic domains are limited to one of three enzymatic activities: N-acetylglucosaminidase (lysozyme), N-acetylmuramoyl-L-alanine amidase, or endopeptidase. Unfortunately, because the catalytic domains from Gram positive phage lysins are not enzymatically active *in vitro* there is no assay involving simple chromogenic or fluorogenic substrates that mimic target cell wall bonds and allow kinetic measurements or even simple activity quantitation. A higher order structure, perhaps one containing the CBD carbohydrate binding epitope in association with peptidoglycan, is apparently necessary for activity. Thus, the lysins require either purified cell walls, or whole cells as substrates for *in vitro* analyses. As result, we have adapted a turbidimetric assay (8) to titer lysin activity and define a standard unit. This method is based on observing zones of clearing on lawns of bacterial cells and produces results which are reproducible and allow direct comparison of activity between lysins active on different species.

It is important to note that the details of lysin identification, purification, and analysis will require slight, yet significant, alterations based on variables like the intended lysin source (i.e., is it encoded within a lytic phage, bacterial genome (prophage), or complex environmental sample) and activity target (i.e., Gram-positive vs. Gram-negative organism, fast or slow growing, etc.). Variations that may be incorporated into a lysin screen are described in the **Notes**. Otherwise, the methods described here were specifically developed to identify the PlyG lysin encoded within the purified genomic DNA of γ phage, although this method has also identified lysins active against *Bacillus cereus*, *B. thuringiensis*, *Enterococcus faecalis*, *E. faecium*, *Listeria monocytogenes*, *Streptococcus pyogenes*, *S. pneumoniae*, and *S. agalactiae* (7, 9–13).

3.1 Construction of a plasmid expression library encoding the partially digested γ phage genome

1. Prepare total genomic DNA from 250 ml of high-titer ($\sim 1 \times 10^9$ PFU ml⁻¹) γ phage lysate using the Qiagen Lambda Maxi Kit (*see* Note 1). Resuspend purified DNA in distilled water at a final concentration of 500 ng μ l⁻¹.
2. Prepare five 1.5-ml Eppendorf tubes, each containing 5 μ g of phage DNA in 50 μ l 1X New England Biolabs (NEB) Buffer 2. Add the NEB restriction endonuclease *Tsp509I* (*see* Note 2) to final enzyme unit amounts of 10, 5, 2.5, 1.0 and 0.5, respectively. Gently suspended the enzyme and incubate tubes for 5 min at 65°C.
3. Since *Tsp509I* cannot be heat inactivated, perform the following: Immediately add 50 μ l phenol:chloroform (1:1 mixture) to each tube, vortex for 10 sec, and centrifuge for 5 min at 4°C in a table-top microcentrifuge (maximum speed). Recover the upper DNA phase, add 50 μ l chloroform, and again vortex and centrifuge. To the resultant upper phase, add 50 μ l 5M NH₄OAc, mix by inversion, add 250 μ l 100% ethanol, and mix again. Centrifuge for 15 min at 4°C, wash each pellet with 70% ethanol, and resuspend in 15 μ l dH₂O.
4. Prepare a 0.4 mm thick, 1% agarose gel. Add gel-loading buffer to each tube and load each (as well as a lane for 1 Kb DNA ladder) into gel with 1X TAE running buffer. The bromphenol blue dye front should be run to the bottom of the gel.
5. Stain gel for 15 min in 1X TAE containing 0.5 μ g ml⁻¹ ethidium bromide and observe on a UV transilluminator, keeping exposure time to a bare minimum. With a clean razor blade, carefully excise gel segments containing partially digested DNA fragments in only the 500 to 2,000 bp range (*see* Note 3).
6. Recover DNA from agarose slices using 4–8 NucleoSpin DNA Extraction Columns and elute each in a final volume of 50 μ l distilled water (dH₂O). Pool samples and run 10 μ l on a 1% agarose gel for quantitation of DNA recovery - a DNA smear should be seen in the 500–2000 bp range.
7. Set up three 15 μ l ligations in 0.2 ml tubes containing 1X ligation buffer, 10 units of T4 DNA ligase and ~ 10 ng of plasmid pBAD24 (previously linearized with *EcoRI* and dephosphorylated with antarctic phosphatase) and either 50, 100, or 200 ng, respectively, of *Tsp509I*-digested phage DNA. Set up a fourth, ligation, in which all phage DNA is omitted, is established as a self-ligation control.
8. After overnight incubation at 16°C, transform 2 μ l of each ligation into 100 μ l XL2-Blue ultracompetant *E. coli* following the manufacturer's protocol (Stratagene, Inc.).
9. Plate cells on LB agar supplemented with 100 μ g ml⁻¹ ampicillin, at a density yielding ~ 300 distinct colonies per 150 $\times$ 15 mm polystyrene Petri dish after

¹For sufficient DNA yield, starting phage titers must be $>1 \times 10^8$ PFU ml⁻¹. Additionally, the Lambda Maxi Kit is effective only for long-tailed phage of the families *Siphoviridae* and *Myoviridae*. For *Podoviridae* (short tails) and *Tectiviridae* (no tails), we incorporated a higher *g*-force spin (ultracentrifugation at 35,000 rpm for 3 h) after the PEG precipitation of phage or purification by CsCl gradient. See **Chapter 17** for additional protocols of phage purification and **Chapter 23** for protocols on DNA extraction.

²*Tsp509I* was chosen for its ability to completely degrade the γ genome (no obvious fragments >500 bp) after overnight digestion. Additionally, it generates cohesive ends (AATT), compatible with *EcoRI* in the pBAD24 MCS. We generally use *Tsp509I* for phage of Gram-positive bacteria. For phage of Gram-negative bacteria, *HaeIII* (GG/CC) is often appropriate for cloning into the *SmaI* site of pBAD24.

³With an effective partial digestion, the highest and lowest enzyme unit lanes should be completely degraded (a DNA smear smaller than 500 bp) and uncut (a single high-molecular weight band), respectively. With intermediate concentrations, a smear of progressively lower-molecular weight should be observed with increasing enzyme concentration. The lane/s in which a majority of products are between 500 and 2000 bp is appropriate for excision. It may be necessary to repeat the reactions, modifying only the amount of enzyme, in order to get a proper partial digestion pattern.

overnight incubation at 37°C. Ultimately, 10 plates, each containing ~300 colonies, should be obtained (*see* Note 4).

10. The quality of the γ phage expression library, with respect to insert size and diversity, must be assessed. First, isolate total DNA from 25 random transformants by suspending each colony in 25 μ l 0.5 M NaOH, and adding 25 μ l 1 M Tris pH 8.0, 450 μ l dH₂O, and mixing vigorously. Next, use 1.0 μ l DNA samples as template in PCR reactions with primers that flank the pBAD24 MCS (*see* Note 5). Run 7.5 μ l of each reaction on a 1% agarose gel to assess the range of insert sizes. We observed that 24 of 25 transformants had γ phage inserts (from 0.3 to 2.5 kb in size) with no bias toward any particular size as expected for a truly random library (*see* Note 6).

3.2 Screening the γ phage library for lysin activity

1. The γ phage expression library consists of ~3000 *E. coli* transformants on ten 150 $\times$ 15 mm LB plates containing ampicillin.
2. Using 150 mm velvet squares and a replica transfer apparatus, replica plate library onto ten 150 mm glass Petri dishes, each consisting of 90 ml LB agar with 100 μ g μ l⁻¹ ampicillin and 0.2% L-arabinose (*see* Note 7).
3. Mark the master and replica plates with an alcohol-resistant marker at such a position to allow accurate alignment of plate pairs when recovering positive clones.
4. Incubate plates overnight at 37°C for the induced library to grow in.
5. On the day prior to the activity screen, set up a 5 ml LB liquid culture with *B. cereus* strain RSVF1 (*see* Note 8) and incubate overnight at 30°C shaking at 150 rpm.
6. On the day of the activity screen, have available: 55°C LB soft agar (0.75% agar) and a set of ten 10 ml round-bottom culture tubes each containing 100 μ l of *B. cereus* RSVF1 overnight culture and kept at 37°C.
7. Permeabilize induced *E. coli* expression clones with chloroform vapors as follows: in a chemical fume hood, add 10 ml chloroform to the inverted lid of each glass plate, then invert *E. coli* clones over the chloroform and incubate for 10 min, then place the clones face up for 10 min to allow chloroform evaporation (*see* Note 9).

⁴To obtain 300 colonies per plate, trials may be required in which the amount of final transformation mix plated is varied. We first plate 50 and 500 μ l of the 1 ml mixture and then, based on resulting colony counts, we repeat the transformation and plate appropriately on 10 plates.

⁵For sequence analysis of pBAD24 inserts, we use the following primers: BAD1, 5'-CTACTGTTTCTCCATACC-3', and BAD3, 5'-GCAGTTCCTACTCTCGC-3'. PCR reactions use the following conditions: 30 cycles of 95°C 30 s, 50°C 30 s, and 72°C 2 min.

⁶For less efficient libraries, we simply screen a greater number of transformants. The number of insert-bearing transformants that must be screened to identify a lysin gene is predicted with the following equation: $1/(\text{size of the coding region of interest}/[\text{size of phage genome} \times 2])$, where the size of the coding region is 700 bp (average size for *Bacillus* spp. phage lysins), the size of the genome is estimated at 40,000 bp (roughly average size for *Siphovidae* phage of *Bacillus* spp.). The factor 2 accounts for two possible insert orientations in the vector. This equation predicts that we must screen 114 transformants to identify *plyG* (we screened 3000 to be cautious and found 52 *plyG* positive clones).

⁷After pouring, let glass plates dry completely at room temperature prior to replica plating. Once poured, these may be stored at 4°C for up to no more than one month.

⁸RSVF1 is *B. cereus* strain ATCC 4342.

⁹While the library is being permeabilized, chloroform has a tendency to pool at plate edges, or be drawn up between the lid and base. To avoid this, occasionally agitate plates to redistribute chloroform over the surface of the inverted lid. During the chloroform evaporation step, residual chloroform from each lid is discarded, and the lids are washed quickly with soap and water, rinsed and dried.

8. Add 7.5 ml LB soft agar to an RSVF1-containing tube and pour contents over the clones on the glass plate, rapidly rotating to allow overlay of the entire surface (*see* Note 10). Repeat for each glass plate.
9. Incubate plates at room temperature for 4 hours (the RSVF1 lawn may become visible as a very faint haze at this point). Place at 4°C overnight and return to room temperature the following day to allow the RSVF1 to grow in.
10. Lysin-encoding clones are distinguished by the appearance of distinct RSVF1 clearing zones in the overlay surrounding such clones (*see* Figure 1). If no zones become apparent after incubation at room temperature, incubate overnight at 4°C again, then return to room temperature and continue search for clearing zones (*see* Note 11 for alternative methods to screen for lysins). We detected 52 positive clones in our screen (1.7% of the library). In similar screens for other *Siphoviridae* lysins, we observed rates between 0.1 and 2%.
11. Recover master *E. coli* colonies corresponding to positive clones by aligning glass plate-master polystyrene plate pairs. Streak to single colonies on LB agar plates containing 100 µg µl⁻¹ ampicillin and incubate overnight at 37°C.
12. Restreak each clone on LB agar with ampicillin and inoculate 5 ml LB liquid with ampicillin and incubate overnight at 37°C shaking at 200 rpm.
13. Use the LB agar plate cultures to generate frozen stocks (*see* Note 12).
14. Use the LB liquid cultures to prepare plasmid using the NucleoSpin Plasmid Kit. Sequence resulting plasmids with both BAD1 and BAD3 primers (*see* Note 5).
15. Assemble DNA sequences and examine *in silico* to determine whether a lysin has been identified (*see* Note 13).

3.3 Expression of the PlyG lysin

1. To create PlyG expression strain, clone the entire 702 bp *plyG* ORF (include no flanking γ phage sequence) into plasmid expression vector pBAD24 and transform *E. coli* strain XL1-Blue (*see* Note 14).
2. Inoculate XL1-Blue/pBAD24::*plyG* into 15 ml LB liquid culture with 100 µg ml⁻¹ ampicillin (in a 125 ml Erlenmeyer flask) and shake at 30°C overnight at 150 rpm.

¹⁰It is extremely difficult to distribute agar evenly over the entire surface if plates are not at room temperature. To ensure that the overlay process occurs rapidly, we perform this step next to a 55°C water bath containing the LB soft agar and a 37°C water bath with the RSVF1 tubes.

¹¹Although we describe a lysin activity screen, several alternate methods for lysin identification have been developed and used in our lab. One method is the holin activity screen (7): perform this exactly as described for the lysin screen, with the exception that the clones which grow well in non-induced conditions (i.e., master plates) but poorly in induced conditions (i.e., glass plates) can be used to identify a holin gene, which is usually encoded immediately upstream of a lysin. Additional methods involve, **a**) using known lysin DNA sequences in BLAST or PCR-based screens (using primers directed against well-conserved catalytic domains) of bacterial genomes, **b**) direct purification of lysins from phage lysates followed by protein sequence analysis, and **c**) SDS-PAGE separation of phage proteins followed by gel overlays containing dense bacterial cell wall material; subsequent clearing zones identify the phage lysin bands that may then be sequenced, and **d**) total phage genome sequencing.

¹²To make a frozen cell stock, freshly plated bacteria are scraped up from the plate surface and suspended in 1.5 ml cryovials with 1 ml of LB with 15% glycerol. After 15 min incubation, the tubes are placed in the -70°C freezer.

¹³Raw insert sequence is subjected to BLASTX or ORF Finder (NCBI) analysis. A lysin insert should be similar to other lysins in the protein sequence database. Sequence alignments can be used to assess degrees of relatedness. Alternately, in lieu of significant primary sequence homology, searching the Pfam (protein families) database (<http://www.sanger.ac.uk/Software/Pfam>) is useful to identify conserved domains common to some lysins, such as the recently discovered cysteine/histidine aminopeptidase (CHAP) domain (14). Generally, lysins of Gram-positive bacteria have hallmark structure: a well conserved N-terminal catalytic domain and a poorly conserved C-terminal cell wall binding domain.

¹⁴For cloning of the *plyG* (accession number AF536823), primers including both the first 20 (PLYG1) and last 20 (PLYG2) bases of the ORF were used, with *EcoRI* and *HindIII* sites incorporated into their ends, respectively. The resulting PCR product was cloned into the *EcoRI*-*HindIII* sites of plasmid pBAD24.

3. Dilute overnight culture 1:100 into 1 liter of LB liquid with ampicillin (in a 2 liter Erlenmeyer flask) and shake at 225 rpm for 3 h at 37°C.
4. Add the L-arabinose inducer to a final concentration of 0.2% and continue growth at 30°C overnight (~12 h) (*see* Note 15).
5. Wash culture by spinning down cells at 4000 rpm in an Eppendorf tabletop centrifuge and then adding a 1X volume of 1X PBS to the pellet, remove the supernatant and finally resuspend in 50 ml 1X PBS.
6. For bacterial lysis, add chloroform to a final concentration of 20%, vortex for 1 min, gently agitate in an orbital shaker for 1 hour at 4°C, and vortex again for 1 min (*see* Note 16).
7. Pellet bacterial debris for 15 min at 4°C and 4000 rpm in an Eppendorf tabletop centrifuge and carefully remove supernatant.
8. The supernatant contains crude PlyG, which can be assayed for activity according to section 3.4 below. (*see* Note 17).

3.4 Quantifying lysin activity

1. Grow *B. cereus* strain RSVF1 overnight in 10 ml Brain Heart Infusion (BHI) broth at 30°C shaking at 150 rpm.
2. The next morning, start a 50 ml culture of RSVF1 from a 1:100 dilution of the overnight in fresh BHI media and incubate as above.
3. At exactly 3 hours of incubation for this culture (*see* Note 18), harvest the cells by centrifugation in 50 ml Falcon tubes for 15 min at 4°C and 4000 rpm in an Eppendorf tabletop centrifuge.
4. Resuspend the pellet and wash 1X in 10 mls of phosphate buffered saline (PBS) (*see* Note 19).
5. Resuspend the pellet and adjust the final OD₆₀₀ to 1.0 by the addition of PBS.
6. Prepare PlyG for assay by diluting PlyG 1:10 in PBS and making additional 2 fold dilutions in PBS (i.e. final dilutions will be 1:10, 1:20, 1:40, 1:80, etc.).
7. Pre-warm a 96-well microtiter plate and Molecular Devices spectrophotometer to 37°C (*see* Note 20).
8. Add 100 µl of the PlyG dilutions in duplicate to the 96-well plate. Include a “no PlyG” control with 100 µl PBS only.

¹⁵Expression will have to be optimized for each lysin. We have found that induction in 0.2% arabinose at 30°C overnight is optimum for PlyG. However, some lysins are degraded by *E. coli* proteases with long inductions.

¹⁶Any method of cell disruption is suitable (French press, homogenizer, etc.). Chloroform is quick, inexpensive, gives adequate yields, and does not appear to harm lysins. However, chloroform is not practical for large, scale-up preparations (>10 L cultures).

¹⁷We are not detailing the purification of PlyG, simply because the purification scheme for any given lysin is specific for that protein and is not necessarily applicable as a protocol for other lysins. This being said, most lysins are fairly stable and purify easily with anion exchange or cation exchange column chromatography depending on the isoelectric point of the protein. Tags or fusion proteins can be added to lysin genes in order to simplify purification (i.e. His tagging the protein and purifying on a nickel column). However, in our experience, tags are potentially deleterious to the enzymatic activity of lysins so our personal preference is to express untagged lysins and elucidate a custom purification scheme for each lysin.

¹⁸The time of growth for the day culture is very important. Since lysins are cell wall hydrolases, their actions are more pronounced on mid-log cells than stationary cells, which are more highly crosslinked. To standardize this assay, we always determine the units of lysin activity on mid-log phase cells. For *B. cereus* strain RSVF1, we have found that a 3-hour culture taken from a 1:100 dilution of an overnight is appropriate. This timing may be different for other bacterial species.

¹⁹All assays to titer PlyG and determine units of activity take place in physiological phosphate buffered saline (PBS). While PBS may not offer the exact optimal pH or salt concentration for maximum PlyG activity, PBS is nonetheless chosen as the standard to titer all lysins so a direct comparison can be made between lysins.

9. At time zero, add 100 μ l aliquots of the *B. cereus* strain RSVF1 to each well using a 12-channel micropipette.
10. Agitate for 5 sec and take an OD₆₀₀ endpoint reading. Since the starting OD₆₀₀ was adjusted to 1.0, and these cells were mixed 1:1 with PlyG/PBS, all wells should now have a reading of ~0.5.
11. Allow the door of the spectrophotometer to close, incubate at 37°C with continual agitation, and take another OD₆₀₀ endpoint reading at time=15 min.
12. Measurement of lysin activity is based upon turbidimetric determination of cell lysis. 15 min endpoint readings should form a range from 0.5 (for controls or very dilute PlyG wells) to 0.1 for concentrated PlyG wells. Thus, we *define* the standard lysin “unit” as the highest dilution that produces a half-drop in OD in the 15 min assay. For example, if an 80-fold dilution of PlyG produced a drop in OD₆₀₀ of *B. cereus* strain RSVF1 from 0.5 to 0.25 in 15 min, then we say that PlyG has a titer (or activity) of 80 U/ml for this strain (*see* Note 21).
13. It is our experience that most lysins, when purified to homogeneity, will have a specific activity of ~ 1 U per μ g protein, although some very active lysins may have a specific activity 100 or 1000 times higher.

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²⁰For automation and simplicity, we utilize a 96-well plate spectrophotometer (SpectraMax Plus). This device allows us to monitor the drop in OD in real time (kinetic read), perform 96 simultaneous assays with reference subtraction, incubate samples at 37°C, and continually agitate the 96-well plate so any observed drop in OD is not due to settling of bacterial cells. In practice, only time zero and 15 min endpoint readings are needed to calculate the titer. Therefore, any spectrophotometer will work and assays can be carried out in cuvettes or test tubes as long as the proper proportions and dilutions are maintained and incubations are kept at 37°C with a water bath or heated spectrophotometer.

²¹Units of activity for a particular lysin will vary on different strains and/or species. Similar to the way a host range is determined by titrating a phage on different bacterial species, lysin host ranges can be determined by using identical amounts of lysin on various species and measuring the activity in terms of units. In some cases, the lysin host range and the corresponding phage host range will be identical, as is the case with PlyG and the γ phage. In other instances, the lysin will have a dramatically different host range. For example, the C₁ bacteriophage only forms plaques on Group C streptococci. However, its corresponding lysin is active against Groups A, C, and E streptococci.

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Fig. 1.

Lytic activity screen for the identification of γ phage lysin, PlyG. The induced and permeabilized *E. coli* expression library is overlaid with agar containing *B. cereus* strain RSVF1. Owing to the presence of ampicillin in the LB agar, the ampicillin-sensitive RSVF1 grows only near *E. coli* colonies expressing plasmid-encoded β -lactamase. A clearing zone in the RSVF1 overlay, surrounding one of the library clones (indicated by an arrow), identifies the presence of a cloned lysin.