Molecular Dissection of Phage Endolysin

AN INTERDOMAIN INTERACTION CONFERS HOST SPECIFICITY IN LYSIN A OF MYCOBACTERIUM PHAGE D29*

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Background: Bacteriophages produce endolysins to hydrolyze host cell wall for their progeny release.

Results: Mycobacterium phage endolysin is inactive in E. coli but is active in mycobacteria. An in-depth study can help in developing strong anti-tuberculosis agents.

Conclusion: An interdomain interaction makes Lysin A inactive in E. coli.

Significance: A hydrolase with built-in mechanism attains host specificity. An in-depth study can help in developing strong anti-tuberculosis agents.

Mycobacterium tuberculosis has always been recognized as one of the most successful pathogens. Bacteriophages that attack and kill mycobacteria offer an alternate mechanism for the curtailment of this bacterium. Upon infection, mycobacteriophages produce lysins that catalyze cell wall peptidoglycan hydrolysis and mycolic acid layer breakdown of the host resulting in bacterial cell rupture and virus release. The ability to lyse bacterial cells makes lysins extremely significant. We report here a detailed molecular dissection of the function and regulation of mycobacteriophage D29 Lysin A. Several truncated versions of Lysin A were constructed, and their activities were analyzed by zymography and by expressing them in both Escherichia coli and Mycobacterium smegmatis. Our experiments establish that Lysin A harbors two catalytically active domains, both of which show E. coli cell lysis upon their expression exclusively in the periplasmic space. However, the expression of only one of these domains and the full-length Lysin A caused M. smegmatis cell lysis. Interestingly, full-length protein remained inactive in E. coli periplasm. Our data suggest that the inactivity is ensued by a C-terminal domain that interacts with the N-terminal domain. This interaction was affirmed by surface plasmon resonance. Our experiments also demonstrate that the C-terminal domain of Lysin A selectively binds to M. tuberculosis and M. smegmatis peptidoglycans. Our methodology of studying E. coli cell lysis by Lysin A and its truncations after expressing these proteins in the bacterial periplasm with the help of signal peptide paves the way for a large scale identification and analysis of such proteins obtained from other bacteriophages.

Mycobacterium tuberculosis, the causative agent of tuberculosis, poses a great threat to mankind due to the continued emergence of antibiotic resistant strains (1, 2). Thus, the need of the hour is to look for alternative mechanisms to restrict the growth of this organism and cure the disease. Mycobacterium phages, or more commonly termed as mycobacteriophages, in this regard, serve as wonderful option, and a detailed study of the mechanisms employed by these phages to lyse mycobacterial cells will certainly help in developing strong anti-tuberculosis agents. These bacteriophages have also shown their potential in detecting M. tuberculosis (3–5). Bacteriophages employ endolysins encoded in their genome to hydrolyze bacterial cell wall peptidoglycan in order to release the newly assembled phage particles into the environment. The process causes rupturing of the host thus resulting in its death (6). This phenomenon has made bacterial viruses popular for phage-based therapies (7–11).

Bacteriophages infecting Gram-negative bacteria express lysins that are, in general, single-domain structures with a few exceptions (12, 13). However, lysins expressed by the phages of the Gram-positive bacteria have been found to be modular with respect to domain presence and arrangement (14–16). These proteins are typically composed of an N-terminal catalytic domain and a C-terminal cell wall binding domain. Chimeric cell wall hydrolases were constructed by fusing the endolysins of different origins. The fusion protein generated in this way showed cell lysis activity against both Streptococcus agalactiae and Staphylococcus aureus (17). Lysins were believed earlier to contain only one catalytic domain with few exceptions (8). However, several reports are available that show the presence of more than one catalytic domain in endolysins (14, 18, 19).

Mycobacteriophage D29 contains three genes in tandem that together form the lytic cassette. Two of these genes encode Lysin A and Lysin B that sandwich one other gene coding for a membrane pore-forming protein, holin (20). The Lysin A (coded by gp10) is responsible for the hydrolysis of PG, whereas Lysin B (coded by gp12) acts at the junction between mycolic acid and the PG (21, 22). It should be noted here that the mycobacterial cell envelope contains, besides PG, an outer

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3 The abbreviations used are: PG, peptidoglycan; IPTG, isopropyl 1-thio-β-D-galactopyranoside; CTD, C-terminal domain; NTD, N-terminal domain; SPR, surface plasmon resonance; Ni-NTA, nickel-nitrilotriacetic acid; LD, lysozyme-like domain.
molecular Dissection of D29 Lysin A

membrane rich in mycolic acid. Holin, in several bacteriophages, is required for the transfer of lysins from the cytosol (its site of production) to the periplasm (its site of action) (23, 24). However, whether it has similar function in D29 or other mycobacteriophages remains to be elucidated. For example, in Ms6, holin is not essential for the translocation of endolysin from cytoplasm to periplasm (25). The D29 and the Ms6 holin were found to have lethal effects on *Escherichia coli* cells upon expression (25). Very recently, the first transmembrane helix of D29 holin was synthesized and studied by carrying out detailed biophysical analysis (26). The peptide was shown to switch between α-helix and β-sheet conformations by means of a Pro-Gly motif, depending upon the peptide concentration in micelles (26). The structure of Lysin B of mycobacteriophage D29 is available, and detailed experiments have been carried out to understand its activity (21). However, a similar level of characterization of Lysin A from the D29 phage has not yet been attempted.

In this manuscript we have performed a detailed examination of the Lysin A of D29 phage. The full-length protein showed lethal effect on bacterial survival, when it was expressed in *Mycobacterium smegmatis*, whereas it was inactive in *E. coli*. We further show that the reason for the inactivation of Lysin A in *E. coli* is an interdomain interaction. Additionally, we have developed a new method for the in vivo activity assay of lysins in *E. coli* by using a signal peptide sequence that makes the Holin requirement for such experiments redundant.

**EXPERIMENTAL PROCEDURES**

- **Reagents, Bacterial Strains, Media, and Growth Conditions—** *E. coli* strain XL1-Blue (Stratagene) was used for all cloning procedures, and BL21(DE3) strain (Lucigen) of this bacterium was used in all experiments involving protein expression. *E. coli* strains were grown in Luria-Bertani medium (HiMedia) having 100 μg/ml ampicillin at 37 °C and with constant shaking at 200 rpm unless specified otherwise. Broth was supplemented with 1.5% agar for solid medium culture. 1.5% agar was included in the medium, whereas Tween 80 was avoided for solid medium culture. 1.5% agar was included in the broth unless specified otherwise. The phage was grown in MiddleBrook 7H9 medium (Difco) containing 2% glucose, 0.05% Tween 80, and 40 μg/ml kanamycin at 37 °C with constant shaking at 200 rpm. 1.5% agar was included in the medium, whereas Tween 80 was avoided for solid medium culture. All the reagents used were of highest quality and were obtained from Sigma unless specified otherwise.

- **Construction of Clones**—The clones used in this study and the oligos utilized to generate these clones have been listed in Table 1. Table 2 lists the oligos along with their sequences. The mycobacteriophage D29 was a kind gift from Graham Hatfull (University of Pittsburgh) and was cultured following the published protocol (22). The genomic DNA of the phage was isolated as described (27). Plasmid purification, restriction digestion, ligation, and transformation were carried out as described (27). Site-directed mutagenesis was carried out following the single primer method (28, 29). The gp10 gene was PCR-amplified using a set of primers and D29 genomic DNA as template. For its expression in *E. coli*, cloning was carried out in pET21b between Nhel and NotI sites to yield a hexahistidine tag at the C-terminus of the expressed protein. For expression of proteins in mycobacteria, *E. coli-Mycobacterium* shuttle vector, pMV261 (kind gift from William R. Jacobs, Howard Hughes Medical Institute) was modified by site-directed mutagenesis to yield an Ndel site at the translation start site of *hsp60*, resulting in pMV261-Ndel. This vector was further modified by replacing *hsp60* promoter cassette with an ~2.6-kb PCR-amplified upstream region of formamidase gene from *M. smegmatis* to yield pMV261Acet vector (kindly provided by Manika I. Singh, IISER Bhopal). This cloning, although followed by the published method (30), will be detailed elsewhere. The vector has been utilized for the expression of all the constructs in *M. smegmatis* upon induction by acetamide; acetamide-mediated induction has been shown to be very useful for the controlled expression of proteins in mycobacteria (31, 32). Cloning in this

**TABLE 1**

**Clones used in the present study and the oligos used to generate these clones**

The vectors derived from the pET backbone carry ampicillin resistance marker, whereas those derived from pMV contain the kanamycin marker. Source: 1, from Novagen; 2, from Dr. S. Kamilla, IISER Bhopal; 3, from Dr. W. R. Jacobs, HHMI; 4, from M. I. Singh, IISER Bhopal; 5, from Dr. M. Reddy, Centre for Cellular and Molecular Biology, Hyderabad. All the remaining vectors were generated in this study.

| Plasmid | Protein | Forward | Reverse | Template |
|---------|---------|---------|---------|----------|
| pET21b  |         |         |         | D29 GENOMIC DNA |
| pETGIP1WT | WT      | FOR_G10 | REV_G10 | D29 GENOMIC DNA |
| pETGIP1LD | LD      | LYSODM-FOR | LYSODM-REV | pETGIP1WT |
| pETGIP1DT | NTD     | PETFOR | GPH10DMDREV | pETGIP1WT |
| pETGIP1CTD | CTD     | GP100D2FOR | PETRECEVOR | pETGIP1WT |
| pETGIP1NCD | NCD     | A | PETFOR | GP10NCDFUS-CDF08 | pETGIP1WT |
| pETGIP1SE WT | rec-WT  | B | GP100NCDFUS-CDF08 | PETRECEVOR |
| pETGIP1SE LD | rec-LD | C | PETFOR | PETRECEVOR |
| pETGIP1SEC-LD | rec-LD | A | PETFOR | PETRECEVOR |
| pETGIP1SEC NTD | rec-NTD | B | GPH10NCDFUS-CDF08 | PETRECEVOR |
| pETGIP1SEC NCD | rec-NCD | C | PETFOR | PETRECEVOR |
| pETGIP1SEC CTD | rec-CTD | A | PETFOR | PETRECEVOR |
| pETGIP1SEC403 | STOP 403 | C | PETFOR | PETRECEVOR |
| pUCGFP |         |         |         |         |
| pETCD-GFP | CTD-GFP | CTR-GFP646Xhol | CTR-GFPPrev-Xhol | pUCGFP |
| pETGIP1SEC rec-WT | rec-WT | CTR-GFP646Xhol | CTR-GFPPrev-Xhol | pUCGFP |
| WTGFP |         |         |         |         |
| pMV261 |         |         |         |         |
| pMV261/NDE1 | NDE1 |         |         | pMV261 |
| pMVACET |         |         |         |         |
| pMVACET WT | WT | G10 WT | PETFOR | PETRECEVOR | pETGIP1WT |
| pMVACET G10F475 | G10 | F475 | Fsmut | PETRECEVOR |
| pMVACETNCD | NCD | PETFOR | PETRECEVOR | pETGIP1WT |
| pMVACETNTD | NTD | PETFOR | PETRECEVOR | pETGIP1WT |
| pMVACETLD | LD | PETFOR | PETRECEVOR | pETGIP1WT |
| pMVACETCTD | CTD | PETFOR | PETRECEVOR | pETGIP1WT |
| pMN86 |         |         |         |         |

* The LD construct that expresses from this plasmid is a truncated version. Due to a frameshift mutation, only the 140-amino acid polypeptide is expressed that lacks a hexahistidine tag.
vector was carried out by PCR amplifying the necessary genes from their pET21b clones using PETFOR and PETREVECORI primers followed by digestion of amplicons with NdeI and EcoRI enzymes and ligation in pMV261Acet vector at NdeI and EcoRI sites (Table 1).

Overlapping PCR was carried out to generate constructs with signal sequence (33). Three sets of primers were utilized that successively added a 60-bp DNA sequence upstream of the gene. Briefly, primers from Set A and Set B were used to PCR-amplify a part of the upstream region of the gene from the vector and the gene, respectively. The amplified regions were successively added a 60-bp DNA sequence upstream of the gene from the vector, and the gene, respectively. The amplified regions were mixed together and used as a template with the primers in Set C to amplify the fusion product (Table 1). Vector, pUCGFP, was kindly provided by Soumya Kamilla, IISER Bhopal. The gene was expressed as described (36) with some modifications. Briefly, primers followed by digestion of amplicons with NdeI and EcoRI enzymes and ligation in pUC18 at SmaI. This vector was further used for the preparation of the final vector, pUCGFP, required. The green fluorescent protein (GFP) was expressed and purified as detailed earlier (34). The plasmid, pMN86 (kindly provided by Manjula Reddy, Centre for Cellular and Molecular Biology, Hyderabad) was used for expressing and purifying C-terminal histidine-tagged YebA (37) and following their expression in E. coli BL21(DE3) after IPTG addition. This was carried out in a 96-well microtiter plate using HiGro plate shaker (Digilab). The cells were used as negative and positive controls, respectively.

| Table 2
| Oligos and their sequence used in the present study |
|---|
| **Label** | **Sequence (5’-3’)** |
| 1-PQN | CGACCCCTTCCGAAATCTAGGGTCGCCGCTAGAG |
| 2-HAR | GAGGATCCGAATGTAAGATCTCTGCCACCTAGCC |
| 3-RAR | GAGAATCCCATATGGCAGACAATTG |
| 4-LAQ | GGTGTCGCGTGAGCATGCTTTAGGCTGCGCTACGGCAG |
| 6-RFI | GGGATCCGGCGCCTTCTCTACGCTAGCGCACGCGCCTGG |
| 7-HTV | GGGATCCGGCGCCTTCTCTACGCTAGCGCACGCGCCTGG |
| Cter-GFPfor-Xhol | GAAGGATCCCTAGGACATGCTTTAGGCTGCGCTACGGCAG |
| Cter-GFPrev-Xhol | GAAGGATCCCTAGGACATGCTTTAGGCTGCGCTACGGCAG |
| FOR_GP10 | CACGAGAAGAGACCATGCTGATTAGAGGATG |
| F-SECR-GP10CD | CGATGGATTATGTCGCGTGTGAGCATGCTTTAGGCTGCGCTACGGCAG |
| GP10DOM1REV | GGGATCCGGCGCCTTCTCTACGCTAGCGCACGCGCCTGG |
| GP10DOM2FOR | CATATGGACGCGACACTACTAGTCTGCTATC |
| GP10NCFDFS-CDFOR | CGCAAGCCGGGCGCAACCTAGCTAGCTG |
| GP10NCFDFS-NDFREV | GTAAGTATGGTGGGGCGGCGGCTTCTCAGAC |
| LYSDOM-FOR | GGATCCGGCGCCTTCTCTACGCTAGCGCACGCGCCTGG |
| LYSDOM-REV | GGATCCGGCGCCTTCTCTACGCTAGCGCACGCGCCTGG |
| MV261NDEI | GAGAATCCCATATGGCAGACAATTG |
| OMPA-STOP | GTGGTGGGCTGAGTAGGATG |
| PETFOR | ATCGAGAATCCTAGCTACGCTAGCGCACGCGCCTGG |
| PETREVECORI | GCTTCTTCTGAAATCTTTAGGACGCGCCTAG |
| PETUPSTFOR | GGGATCCGGCGCCTTCTCTACGCTAGCGCACGCGCCTGG |
| REV_GP10 | GGGATCCGGCGCCTTCTCTACGCTAGCGCACGCGCCTGG |
| R-SECR-GP10 | CACGAGAAGAGACCATGCTGATTAGAGGATG |
| Sig-NTD-For | GTAGGCGCTACTACTAGTCTGCTATC |
| Sig-NTD-NHE | GTGAGAATCCCATATGGCAGACAATTG |
| FSmut | GTGGTGGGCTGAGTAGGATG |

**Protein Expression and Purification—**E. coli BL21(DE3) carrying the desired plasmid was grown at 25°C with constant shaking at 150 rpm until the OD$_{600}$ reached 0.6. The cells were then induced with 0.25 mM IPTG and were allowed to grow further for 16 h after which they were harvested and lysed by sonication in lysis buffer containing 40 mM Tris-Cl, pH 8.0, 500 mM NaCl, 25 mM imidazole, and 5 mM 2-mercaptoethanol. Lysate was cleared by centrifugation at 14,000 rpm for 30 min at 4°C, and the supernatant was incubated with pre-equilibrated Ni-NTA (Qiagen) beads for 3 h. Beads were then washed with wash buffer containing 40 mM Tris-Cl, pH 8.0, 500 mM NaCl, 25 mM imidazole, and 5 mM 2-mercaptoethanol. Elution of proteins was carried out in the elution buffer containing 40 mM Tris-Cl, pH 8.0, 200 mM NaCl, 300 mM imidazole, and 5 mM 2-mercaptoethanol. Elution fractions containing the highest amount of protein (as judged by SDS-PAGE) were pooled together and dialyzed against the storage buffer containing 40 mM Tris-Cl, pH 8.0, 200 mM NaCl, 1 mM dithiothreitol, and 40% glycerol. After three successive changes, the protein was collected, centrifuged at 14,000 rpm for 15 min, and stored at −20°C until required. The green fluorescent protein (GFP) was expressed and purified as detailed earlier (34). The plasmid, pMN86 (kindly provided by Manjula Reddy, Centre for Cellular and Molecular Biology, Hyderabad) was used for expressing and purifying C-terminal histidine-tagged YebA as described (35).

**Zymography—**Zymograms were prepared to test the activity of the purified proteins using 0.2% Micrococcus lysodeikticus cells (Sigma) as substrate in a 12% SDS-polyacrylamide gel. The experiment was carried out as described (36) with some modifications. Briefly, 5 μg of each of the proteins were boiled in SDS sample loading buffer and loaded on to the SDS-polyacrylamide gel containing the substrate. After electrophoresis, the gel was washed twice with renaturation buffer containing 25 mM Tris-Cl, pH 8.0, 1% Triton X-100, and 5 mM EDTA and incubated in the same buffer at 25°C for 12 h. It was then developed essentially as described (36) and photographed. BSA and lysozyme were used as negative and positive controls, respectively.

**Bacterial Survival Assay—**The assays of Lysin A and its variants prepared in the present study were attempted in the bacterial cells by constructing the fusion of Lysin A variants with an OmpA secretory signal sequence (MMKKTAAIAALAVAGFATVAQQ) (37) and following their expression in E. coli BL21(DE3) after IPTG addition. This was carried out in a 96-well microtiter plate using HiGro plate shaker (Digilab).
Molecular Dissection of D29 Lysin A

Bacterial growth was carried out at 25 °C in a 200 μl volume with constant shaking at 450 rpm. The supply of humidified air (zero air) was controlled by a solenoid valve with on and off cycles for 0.5 s and 0.5 min, respectively. After 1 h of growth, cells were induced with 0.25 mM IPTG and were allowed to grow further in HiGro shaker. The optical density at 600 nm (A_{600}) was monitored using SpectraMax M5 plate reader (Molecular Devices) at specific time intervals. Lysin A constructs were expressed in M. smegmatis using a modified pMV261 vector that allowed protein production upon the addition of 0.2% acetamide. Growth was carried out in 5 ml of MB7H9 broth at 37 °C and monitored by measuring A_{600} at various time points using spectrophotometer. All the growth profile experiments were carried out in triplicate, and the data represent the average of them. These experiments were repeated at least three times to look for the consistency in the profile. To observe bacterial survival on solid agar medium, 2 μl of respective dilution culture (as mentioned in each case) were spotted on either LB agar (for E. coli) or MB7H9 agar (for M. smegmatis). The plates were then incubated at 37 °C for 10 h (for E. coli) and 48 h (for M. smegmatis).

**PG Preparation—** M. smegmatis peptidoglycan was prepared using a modified protocol (38). M. smegmatis cells were grown in 2 liters of culture medium until late log phase after which they were harvested and resuspended in 100 ml of breaking buffer containing 20 mM Tris-Cl, pH 8.0, 500 mM NaCl, and 10% glycerol. The cells were lysed by passing the suspension three times through a cell homogenizer (Constant Systems Ltd.) at 30,000 lb/in² followed by sonication with a pulse duration of 15 s on and 15 s off for 10 min. The lysate was then centrifuged at 18,000 rpm for 30 min, and the resultant pellet was resuspended in breaking buffer containing 2% SDS. The resulting suspension was stirred at 45 °C for 3 h. It was then cooled to room temperature and centrifuged at 18,000 rpm for 30 min at 25 °C. The pellet thus obtained was resuspended in 1% SDS. Pronase E (Sigma) at a final concentration of 100 μg/ml was added and stirred slowly at 45 °C for 3 h following by stirring at 80 °C for 1 h. The resulting suspension was then allowed to cool to room temperature and centrifuged at 18,000 rpm for 30 min, and the pellet thus obtained was resuspended in 1% SDS and incubated in a sonicating water bath for complete resuspension. It was then centrifuged, and the collected pellet was extracted with water three times to remove any SDS. The extraction process required resuspension in water using sonication followed by centrifugation at high speed. The final pellet thus obtained was washed with ethanol-diethyl ether (ratio:1 v/v) and lyophilized. The resulting mycolyl-arabinogalactan-peptidoglycan complex was collected and stored in −80 °C for further use. The E. coli peptidoglycan was prepared following published protocol (39).

**Peptidoglycan-Protein Binding Assay**—The protein binding to peptidoglycan was assessed as described earlier (40). Lyophilized peptidoglycan from M. smegmatis, E. coli, Bacillus subtilis (Sigma), and M. tuberculosis (BEI Resources) were resuspended in water to prepare a stock of 5 mg/ml (w/v). 50 μl of the peptidoglycan suspension was added in 300 μl of binding buffer containing 25 mM Tris-Cl, pH 8.0, 200 mM NaCl, 0.05% Triton X-100, and BSA as specified in each case. The BSA was added as a blocking agent to avoid any nonspecific adsorption of protein onto the PG. 2 μg of either CTD-GFP or GFP was added to this mixture and incubated at 37 °C for 1 h with constant mixing. The PG suspension was then collected by centrifugation at 13,000 rpm for 10 min. The pellet was washed 3 times with the binding buffer, resuspended in 50 μl of 8 M urea, electrophoresed on an SDS-polyacrylamide gel, and analyzed by Western blotting using anti-His_{6} tag (Thermo Scientific) and anti-gp10 antibodies. 60 μg of purified YebA was subjected to binding to PG of E. coli, M. smegmatis, and M. tuberculosis following the method described above; the protein was probed with anti-His_{6} tag antibodies. Blots were scanned using Odyssey infrared imaging system (LI-COR Biosciences).

**Surface Plasmon Resonance (SPR) Analysis of Protein Interaction**—The N-terminal domain (NTD)-CTD interaction was monitored by carrying out SPR experiment using T-200 (GE Healthcare). The NTD was immobilized on a CM5 chip using the amine coupling kit. Both the CM5 chip and the coupling kit were purchased from GE Healthcare and were used following the supplier’s instructions. The concentration of NTD was 50 μg/ml, and sufficient protein was immobilized to obtain 800 response units. The CTD was then passed onto it at a flow rate of 30 μl/min with binding and dissociation run for 120 and 150 s, respectively. Running buffer contained 40 mM Tris-Cl, pH 8.0, 100 mM NaCl, 2 mM CaCl_{2}, and 2 mM MgSO_{4}. Data were fitted using Biacore T200 Evaluation software Version 1.0. The 1:1 Langmuir binding model was used for fitting the data and calculating the binding constants.

**Microscopy**—Differential interference contrast and fluorescence imaging were carried out on a Leica DM2500 system equipped with 100X PL APO objective. 4’,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was obtained from Invitrogen and was used following the supplier’s instructions. A confocal laser scanning microscope (LSM 780, Zeiss) was used to visualize sec-WT-GFP expression with DAPI as a counterstain. Z-stacking was carried out to obtain optical sections of the bacteria in confocal microscope. The bacterial cultures, in all of these experiments after induction with IPTG, were mixed with equal volumes of 0.5% agarose and placed on a glass slide for imaging.

**RESULTS**

The Lysin A of Mycobacteriophage D29 Contains Two Catalytic Domains—Lysins of mycobacteriophages have been shown to contain more than one domain that carries out cell wall binding and PG hydrolysis functions (14). Furthermore, these proteins have been predicted to contain either one or two catalytic domains for PG hydrolysis. We carried out a detailed dissection of the Lysin A by making several constructs to examine the presence of the catalytic domain(s) and to arrive at the shortest protein sequence that is sufficient to lyse bacterial cells. The Lysin A of D29 is a 54.8-kDa protein. The protein was scanned for the presence of domains by carrying out a conserved domain database search at the National Center for Biotechnology Information. This resulted in the identification of only the LD domain located in the middle of the protein (data not shown). However, Lysin A of D29 has been predicted to have, additionally, an NTD-carrying peptidase activity and a CTD having the cell wall binding activity; the two sandwich a
Molecular Dissection of D29 Lysin A

glycoside hydrolase domain (14). A series of smaller constructs along with full-length wild-type protein (WT) (as shown in Fig. 1A) was prepared, expressed, and purified on Ni-NTA metal ion affinity chromatography (Fig. 1B). We examined these proteins for their in vitro PG hydrolytic activity using Micrococcus lysodeikticus cells as substrate immobilized in a polycrylamide gel. The procedure, known as zymography, is a well-established method for assaying the cell wall lysis activity of the enzyme in question, and Micrococcus lysodeikticus cells are commonly used as substrate in these experiments (36, 41). The cell lysis activity of the proteins appeared as a clear zone after staining the gel with methylene blue (Fig. 1C). The Lysin A and two of its deleted versions, namely NTD (harboring only the N-terminal region) and LD (containing the lysozyme-like activity domain), showed PG hydrolysis activity; the NCD construct containing the NTD and the CTD regions of the protein has been discussed later separately. The CTD protein (containing only the C-terminal region) did not show any activity (data not shown).

We were surprised to see the inactivity in the Lysin A in this experiment. Endolysins have, however, been suggested to be species-specific, and this property in the protein is attributed by the cell wall binding domain (15, 42). We, therefore, believe that the presence of CTD in Lysin A makes the protein inactive in E. coli only when it is expressed inside the cell (42). It is, however, active on Micrococcus cells when assayed in the zymography experiment (Fig. 1).

Additionally, spot assays were carried out to assess the level of toxicity posed on the cells by NTD and LD proteins. We found the expression of these proteins tagged with secretory signal peptide to be lethal as judged by the reduction in spot density (Fig. 2E). We wish to add here that the sec-LD construct does not express the complete LD domain due to a frameshift mutation at 950th bp (with respect to the full-length wild-type DNA sequence) resulting in a stop codon appearance. The LD domain, thus expressed, is of 140 amino acids instead of the desired 184 amino acids, and we proceeded with this truncated protein for our experiments. It should, however, be noted that all other proteins including LD without the signal peptide have the desired sequence.

The Expression of sec-WT Changes Cellular Morphology—To ascertain that the Lysin A was translocated to the periplasmic space of E. coli, we carried out confocal microscopy of E. coli expressing sec-WT fused with GFP at its C terminus (sec-WT-GFP). The distinct accumulation of sec-WT-GFP at the periphery of the cells confirmed its translocation to the periplasmic space (Fig. 3A). However, an interesting aspect that did not escape our notice was that all the cells showed spherical appearance as opposed to the rod-shape E. coli. We, therefore, carried out differential interference contrast imaging of E. coli coupled with DAPI fluorescence to explore it further. We observed that the sec-WT expression rendered the bacteria spheroid, whereas the protein without secretory signal did not cause such change in cell morphology (Fig. 3B). We, therefore, speculate that the sec-WT, although is incapable of killing E. coli, does contain residual lysozyme-like activity that is sufficient to cause PG hydrolysis leading to cell shape remodeling (43).

It is worth mentioning here that the GFP used in these experiments is a superfolder version of GFP that shows enhanced brightness and does not dimerize. Furthermore, it also functions in the periplasm of bacteria (44–46).
The Full-length Lysin A as Well as the N-terminal Domain Are Functional in Mycobacteria—Because mycobacteria are the natural host for D29 phage, we proceeded with the expression of these proteins in M. smegmatis. All the constructs were cloned in the E. coli-Mycobacterium shuttle vector under acetamide-inducible promoter (pMV261Acet) without OmpA signal sequence, and the resulting plasmids were electroporated in M. smegmatis mc² 155. The full-length protein showed cell lysis upon induction by acetamide (Fig. 4A). This was as expected; Payne and Hatfull (14) have presented a similar observation earlier. Interestingly NTD, but not LD, showed cell lysis activity similar to the WT protein, whereas CTD was found not to have any catalytic activity in M. smegmatis (Fig. 4A). The observed lesser growth of cells carrying WT and NTD constructs even in the absence of acetamide was primarily because of the leaky expression from the acetamide inducible promoter, which was confirmed by Western blotting experiment (data not shown). Additionally, a spot assay of M. smegmatis expressing WT or NTD confirmed the toxic effect of these proteins on the bacterium (Fig. 4B). The empty vector did not show any negative effect on mycobacterial growth (data not shown). These experiments further show the ability of the WT and the NTD proteins to lyse mycobacterial cells.

FIGURE 2. Growth analysis of E. coli upon expression of Lysin A and its variants. The growth curve of E. coli BL21(DE3) was monitored following optical density of the culture at 600 nm (A₆₀₀) after the cultures were induced with IPTG at time 0. The expression of sec-LD (A) and sec-NTD (B) shows reduction in growth, whereas all others including sec-WT (C) and sec-CTD (D) do not significantly affect the growth. The data presented here are an average of the measurements carried out in triplicate; error bars represent the standard error. Spot assays (E) carried out on LB agar show diminished growth. Only 0- and 4-h data have been shown. Dilution factor has been mentioned on the top.

FIGURE 3. Microscopic analysis of the expression of sec-WT in E. coli. A, the sec-WT tagged with GFP at its C terminus (sec-WT-GFP) shows accumulation at the cell periphery upon expression. The images were captured by confocal microscope after inducing the cultures with IPTG. The panels show only GFP (green), only DAPI (blue), and an overlay of these fluorophores. B, the sec-WT, upon expression, alters cell morphology. The WT and sec-WT were expressed in E. coli by IPTG induction and DAPI, and differential interference contrast (DIC) images of the same area on the slides were captured.

The Full-length Lysin A as Well as the N-terminal Domain Are Functional in Mycobacteria—Because mycobacteria are the natural host for D29 phage, we proceeded with the expression of these proteins in M. smegmatis. All the constructs were cloned in the E. coli-Mycobacterium shuttle vector under acetamide-inducible promoter (pMV261Acet) without OmpA signal sequence, and the resulting plasmids were electroporated in M. smegmatis mc² 155. The full-length protein showed cell lysis upon induction by acetamide (Fig. 4A). This was as expected; Payne and Hatfull (14) have presented a similar observation earlier. Interestingly NTD, but not LD, showed cell lysis activity similar to the WT protein, whereas CTD was found not to have any catalytic activity in M. smegmatis (Fig. 4A). The observed lesser growth of cells carrying WT and NTD constructs even in the absence of acetamide was primarily because of the leaky expression from the acetamide inducible promoter, which was confirmed by Western blotting experiment (data not shown). Additionally, a spot assay of M. smegmatis expressing WT or NTD confirmed the toxic effect of these proteins on the bacterium (Fig. 4B). The empty vector did not show any negative effect on mycobacterial growth (data not shown). These experiments further show the ability of the WT and the NTD proteins to lyse mycobacterial cells.
The C-terminal Region of Lysin A Is Required to Inactivate the Protein in E. coli—The full-length protein in our experiments did not show cell lysis ability when it was expressed in the periplasmic space of E. coli although it contains two catalytically active domains. It did, however, demonstrate cell lysis in mycobacteria. Endolysins are considered to be cell-specific, and this property is contributed by a PG binding domain located in the C-terminal of the protein (15, 42). We, therefore, attempted to understand the role of C terminus in the inactivation of the wild-type protein. Several mutants were prepared by introducing stop codons in the C-terminal region of the sec-WT (Fig. 5A) to identify the segment that is sufficient to kill E. coli. It was interesting to note that the presence of stop codon close to the lysozyme-like domain (that essentially removed the CTD) restored the protein lytic activity in E. coli (Fig. 5B). We thus conclude that the C-terminal domain of Lysin A is responsible for the inactivation of both NTD and LD domains in E. coli.

Surface Plasmon Resonance Experiment Shows an Interaction between N- and the C-terminal Domains—Next, we attempted to find out if an interaction exists between the CTD and the NTD of the protein by a performing SPR experiment, which directly measures the protein-protein interaction. The NTD was immobilized on a CM5 chip, and its interaction with CTD was probed by allowing the increasing concentrations of the latter to pass on it. BSA was used as a negative control (data not shown). The recorded sensograms were used to estimate the dissociation constant (Fig. 6). The $K_D$ was determined to be 14.4 μM. The SPR data hence demonstrates the existence of specific interaction between NTD and CTD. Thus, the data obtained from the mutagenesis experiments, protein expression assays in E. coli and the SPR together, indicate that an interaction between NTD and CTD makes the protein inactive.

The C-terminal Region of Lysin A Binds Selectively to the Mycobacterial Peptidoglycan—We attempted to probe a direct binding of C-terminal domain of Lysin A to the peptidoglycan of M. smegmatis. We prepared a CTD-GFP fusion protein to demonstrate the binding of CTD to the PG present in the intact mycobacterial cells. However, the outcome of such an experiment was inconclusive possibly because of the large abundance of mycolic acid outside the cell wall, present in mycobacteria (data not shown). Cells treated with chloroform to remove mycolic acid also gave no results because of the large amount of cell aggregation (data not shown). We, therefore, purified the PG from M. smegmatis (PG$_{Msm}$), and the CTD-GFP was incubated with this preparation in the presence and absence of large amounts of BSA. The purified GFP was used as a negative control. Western blotting experiment carried out with anti-His$_6$ tag antibodies, which detects both GFP and CTD-GFP, showed a specific binding of CTD-GFP to PG$_{Msm}$ (Fig. 7A). Any nonspecific binding of only GFP to PG remained undetected in this experiment. We wish to emphasize here that the binding of

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**FIGURE 4.** Growth analysis of M. smegmatis upon expression of Lysin A and its variants. The growth curve of M. smegmatis (A) was recorded after inducing the expression of WT, LD, NTD, and CTD proteins using 0.2% acetamide (+acet); −acet corresponds to the absence of the inducer in the medium. Time 0 represents the time of induction. The WT and NTD show reduction in $A_{600}$ value. The growth was monitored for 24 h. Data represent the average of measurements done in triplicate; error bars represent the S.D. The spot assays (B) were carried out for WT and NTD at time 0 and after 8 h. The presence and absence of inducer has been marked by + and −, respectively. Dilution factor has been mentioned on the top.

**FIGURE 5.** The C-terminal truncation of sec-WT leads to the lysis of E. coli. Stop codon mutagenesis was carried out in the sec-WT construct as shown diagrammatically in panel A. Each construct was expressed in E. coli BL21(DE3). The graph shows both uninduced (−IPTG) and induced (+IPTG) cultures at time 0 and after 12 h of growth (B). The labels on the x axis represent the introduction of stop codon with respect to WT protein. The reduction in $A_{600}$ is suggestive of cell death due to the gain of lytic activity by the protein. Data represent the average of growth measurements carried out in triplicate; error bars represent S.D.

**FIGURE 6.** SPR kinetics of NTD and CTD interaction. The sensogram profiles were obtained after immobilizing NTD on a CM5 chip and allowing various concentrations of CTD to flow on it. The 3.25 μM concentration was repeated twice. Time 0 represents the time of injection of the analyte (CTD). The inset shows the data for the constants obtained after carrying out the SPR experiment. The $k_{on}$ and $k_{off}$ represent the association and dissociation rate constant respectively. $K_D$ is the ratio of $k_{off}$ and $k_{on}$, and termed as the equilibrium dissociation constant. $\chi^2$ represents the goodness of fit.
Molecular Dissection of D29 Lysin A

CTD-GFP to PG was observed in the presence of a high amount of BSA that rules out any nonspecific adsorption. We further assessed the binding of CTD-GFP to the PG obtained from three other organisms namely *E. coli* (PG*Eco*), *B. subtilis* (PG*Bst*), and *M. tuberculosis* (PG*Mtu*). Although the *E. coli* PG was purified in the laboratory, PG*Bst* and PG*Mtb* were obtained from Sigma and BEI Resources, respectively. Our Western data, probed with anti-gp10 antibodies, clearly indicates that the CTD-GFP binds selectively to PG*Msm* and PG*Mtb* (Fig. 7B).

We used YebA as a positive control and probed its binding to PG*Eco*, PG*Msm*, and PG*Mtb*. The YebA of *E. coli* is a murein hydrolase that has metal-dependent endopeptidase activity (35) and contains a LysM domain that is known to bind peptidoglycan (47, 48). The purified preparation of YebA showed binding to PG of *E. coli*, *M. smegmatis*, and *M. tuberculosis* when probed with anti-His6 tag antibodies. These experiments thus establish beyond doubt that the CTD binds specifically to the mycobacterial peptidoglycan.

**FIGURE 7.** C-terminal domain of Lysin A binds to *M. smegmatis* peptidoglycan. The Western blotting was done with the CTD-GFP to peptidoglycan. The blotting membrane was probed with either anti-His6 tag (A) or anti-gp10 antibodies (B). Panel A shows the binding of CTD-GFP (lanes 1–3) and CTD-GFP (lanes 4–6) to PG*Msm*. Amounts of BSA used in this experiment were 0 μg (lanes 1 and 4), 10 μg (lanes 2 and 5), and 20 μg (lanes 3 and 6). Only CTD-GFP shows binding to PG*Msm*. Panel B shows the binding of Lysin A to peptidoglycan obtained from *M. smegmatis* (lane 1), *B. subtilis* (lane 2), *E. coli* (lane 3), and *M. tuberculosis* (lane 4) in the presence of 20 μg of BSA. Only lanes 1 and 4 show the presence of CTD-GFP, suggesting that CTD-GFP binds selectively to the PG*Msm* and PG*Mtb*. C, the binding of YebA to PG*Msm* (lane 1), PG*Eco* (lane 2), and PG*Mtb* (lane 3) was probed with anti-His6 antibodies. The binding of YebA is seen with all the peptidoglycans.

**FIGURE 8.** The NCD construct is inactive in *E. coli* but is capable of killing *M. smegmatis*. A, growth of *E. coli* was monitored by measuring A600 at various time points. The sec-NCD does not show a lethal effect on *E. coli* upon induction by IPTG. NCD without the sec- signal sequence acted as negative control. B, the expression of NCD in *M. smegmatis* was found to be toxic as judged by the growth profile upon acetamide addition (+acet). The growth was monitored for 24 h. C, the spot assays performed with *M. smegmatis* further showed the negative effect of NCD expression on cell growth. Only 0- and 8-h data have been shown. Dilution factor for each spot has been mentioned on the top. Time 0 in all the panels represents the time of induction. All the data in the graphs represent the average of growth measurements carried out in triplicate; error bars represent the S.D.
expression of NTD domain, but not the LD domain, to be lethal. However, under our experimental conditions we found the CTD results in the inactivation of the Lysin A in late that an intramolecular crosstalk between the NTD and the LD domain also contains the PG hydrolysis activity, and therefore, this may also contribute to the cell wall hydrolysis. In heterologous hosts (such as E. coli), the interaction between NTD and CTD is not disrupted, and therefore, the protein remains inactive.

**DISCUSSION**

Lysins of mycobacteriophages represent a very interesting class of proteins because of their ability to lyse one of the most pathogenic bacteria known to humans. These proteins are composed of various domains required for different functions. In this manuscript we report a detailed molecular dissection of the Lysin A of D29 mycobacteriophage by splitting the protein into individual domains and studying them in isolation and in combination.

The zymography experiments carried out with the purified protein and the growth profiles of E. coli recorded after protein expression provide compelling evidence that the Lysin A of D29 contains two catalytically active domains viz. NTD and LD. However, under our experimental conditions we found the expression of NTD domain, but not the LD domain, to be lethal to M. smegmatis. Interestingly, the full-length protein was inactive in E. coli, whereas it was functional in M. smegmatis. We suggest that the inactivation of wild-type protein in E. coli is due to its C-terminal domain. This is supported by the fact that Lysin A truncation from the C-terminal leads to restoration of cell lysis ability in the C-terminal-truncated Lysin A that an interdomain interaction renders the protein inactive. At this juncture we are tempted to postulate that an intramolecular crosstalk between the NTD and the CTD results in the inactivation of the Lysin A in E. coli periplasm (Fig. 10). Moreover, this interaction is abrogated in mycobacteria, most likely by peptidoglycan that competes out its C-terminal domain. This is supported by the fact that M. smegmatis carrying the mutant construct (gp10FSmut).

The restoration of cell lysis ability in the C-terminal-truncated Lysin A indicates that an interdomain interaction renders the protein inactive. At this juncture we are tempted to postulate that an intramolecular crosstalk between the NTD and the CTD results in the inactivation of the Lysin A in E. coli periplasm (Fig. 10). Moreover, this interaction is abrogated in mycobacteria, most likely by peptidoglycan that competes out NTD in order to bind CTD, resulting in the activation of the Lysin A in mycobacterial cells. Intramolecular interaction as the cause of inactivation of endolysin in Bacillus anthracis phage was suggested earlier also (42). However, after performing differential scanning calorimetry experiment that showed no interaction between the catalytic domain and the cell wall binding domain, the hypothesis was discarded (49). Here, we demonstrate by SPR that such an interaction does exist between NTD and CTD of D29 Lysin A. Post-translational modifications have also been suggested to activate lysins in other bacteriophages such as E. coli T4 tail lysozyme, P1 Lyz, and streptococcal bacteriophage C1 PlyC (50–52). Nonetheless, the idea of Lysin inactivation and activation still remain open for exploration by other experiments.

It was not surprising that the CTD did not differentiate between the PG


gene, thereby strongly indicating that the protein coded by the gp10 of D29 is the only endolysin present.
more experiments are required to understand the exact mechanism of translocation of Lysin A in mycobacterial cells in a Holin-independent manner.

We further wish to add that our experiment with endolysin and its variants fused with the secretory signal for their expression in E. coli periplasm initiates a novel methodology for analyzing endolysin family of proteins. Earlier studies with lysins have utilized chloroform to make the plasma membrane permeable that then allows the diffusion of lysins to the cell wall peptidoglycan and its digestion (53–55). Several other studies have followed the lysin effect after its co-expression with Holin (25, 56). Our methodology developed here will not only help in studying the activity of these proteins in detail but will also aid in identifying new proteins from other bacteriophages when this method of expression is followed in a suitable host.

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