Decoding the Molecular Properties of Mycobacteriophage D29 Holin Provides Insights into Holin Engineering

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ABSTRACT Holins are bacteriophage-encoded small transmembrane proteins that determine the phage infection cycle duration by forming nonspecific holes in the host cell membrane at a specific time postinfection. Thus, holins are also termed “protein clocks.” Holins have one or more transmembrane domains, and a charged C-terminal region, which, although conserved among holins, has not yet been examined in detail. Here, we characterize the molecular properties of the mycobacteriophage D29 holin C-terminal region, and investigate the significance of the charged residues and coiled-coil (CC) domain present therein. We show that the CC domain is indispensable for holin-mediated efficient bacterial cell lysis. We further demonstrate that, out of the positively- and negatively-charged residues present in the C-terminal region, substituting the former, and not the latter, with serine, renders holin nontoxic. Moreover, the basic residues present between the 59th and the 79th amino acids are the most crucial for holin-mediated toxicity. We also constructed an engineered holin, HolHC, by duplicating the C-terminal region. Compared to the wild type, the HolHC protein shows higher toxicity in both *Escherichia coli* and *Mycobacterium smegmatis* and causes rapid killing of both bacteria upon expression. An oligomerization property of HolHC similar to that of the wild-type holin allows us to propose that the C-terminal region of the D29 holin determines the timing, and not the extent, of oligomerization and, thereby, hole formation. Such knowledge-based engineering of mycobacteriophage holin will help in developing novel phage-based therapeutics to kill pathogenic mycobacteria, including *Mycobacterium tuberculosis*.

IMPORTANCE Holins are bacteriophage-encoded small membrane perforators that play an important role in determining the timing of host cell lysis toward the end of the phage infection cycle. The ability of holin to precisely time the hole formation in the cell membrane, ensuring cell lysis, is both interesting and intriguing. Here, we examined the molecular properties of the mycobacteriophage D29 holin C-terminal region, which harbors several polar charged residues and a coiled-coil domain. Our data allowed us to engineer holin with an ability to rapidly kill bacteria and to show higher toxicity than the wild-type protein. Due to their ability to kill host bacteria by membrane disruption, it becomes important to explore the molecular properties of holins that allow them to function in a timely and efficient manner. Understanding these details can help us modulate holin activity and engineer bacteriophages with superior lytic properties to kill pathogenic bacteria, curtail infections, and combat antimicrobial resistance.

KEYWORDS mycobacteriophage, holin, holin engineering, *in vivo* cross-linking, membrane perforation, D29 phage

Bacteriophages utilize a highly sophisticated host cell lysis system in order to release newly formed progeny phages. This is achieved by overcoming host physical barriers viz. plasma membrane, cell wall, and outer membrane. To break down these

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layers, phage genomes possess a “lytic cassette,” which codes for at least a muralytic enzyme termed endolysin that targets peptidoglycan and a membrane-perforating protein, holin (1–4). Phages which infect either Gram-negative bacteria or mycobacteria also encode other accessory proteins, such as spanins or mycoarabinogalactan esterase, respectively, which help them to break down the outer membrane of the host (5, 6). Holins are small, pore-forming, integral membrane proteins encoded by most of the double-stranded DNA bacteriophages. These toxic proteins are transcribed during the late phase of the phage infection (2, 7–9). Upon expression, holin localizes into the host cell membrane without causing any harm and oligomerizes, forming “death raft”-like structures (9, 10). After reaching a critical concentration, it suddenly undergoes a structural change to form holes, which result in an immediate disruption of proton motive force across the plasma membrane (8, 10). The holes formed are nonspecific in nature and consequently allow either the release of fully folded endolysins initially accumulated in the cytoplasm into the periplasmic space or an activation of the endolysins already present in the periplasmic space, leading to an efficient host cell lysis by allowing enzymatic digestion of cell wall peptidoglycan (9, 11–15). Holins, therefore, determine the timing of host cell lysis precisely at a genetically programmed time and thus act as a “molecular clock” for the bacteriophage life cycle (16).

Holins are a diverse group of proteins. They are segregated in seven superfamilies that share no primary sequence similarity, although they harbor the only conserved function of hole formation (17, 18). A generalized structure of holin constitutes a small N-terminal region followed by the transmembrane domains interconnected by loop(s), and a long cytoplasmic (11)—or periplasmic in some cases (19)—C-terminal region, which generally possesses polar charged residues (11). It has been reported that although holins have diverse sequences, the polar charged residues present in the C-terminal region are generally conserved among all holins (11, 16, 20). This is, however, in contrast with the holin homologues such as Bak and Bax expressed by eukaryotic cells, which promote apoptosis by forming pores into the outer membrane of mitochondria (17, 21, 22). In contrast to bacteriophage holins, the C-terminal region of Bax is hydrophobic in nature and is found to be essential for the insertion into the outer mitochondrial membrane to exhibit its proapoptotic activity (17, 22, 23).

Mycobacterium phage D29 is a virulent phage capable of infecting and killing both fast- and slow-growing mycobacteria, including pathogenic *Mycobacterium tuberculosis* (23–30). The lytic cassette of mycobacteriophage D29 possesses the *gp11* gene, which codes for holin and is sandwiched between *gp10*, which codes for LysA, and *gp12*, which produces LysB (7, 12, 31). Computational analysis of D29 holin shows the presence of two transmembrane domains (TMDs) at the N terminus, followed by a long C-terminal region, which also carries a coiled-coil (CC) domain. *In vitro* expression of this ~15-kDa membrane perforating protein has also been shown to be toxic for non-pathogenic *Mycobacterium smegmatis*, as well as for *Escherichia coli*. (31). We have previously shown with the holin knockout variant of the D29 phage that holin is mandatory for the timely and efficient lysis of the host *M. smegmatis*, but not for the phage viability (29). In the absence of holin, the latent period of D29 phage infection cycle is increased because of the delayed cell lysis, and such holin-knockout phage produced smaller plaques with reduced burst size (29), thus demonstrating the significance of holin in D29 phage physiology.

We earlier showed that the length of the C-terminal region of D29 holin is crucial for its activity (31). However, the significance of polar charged residues present in the C-terminal region in protein's activity has not yet been investigated. In the present manuscript, we explore the significance of the polar charged residues, and hence the C-terminal region, in D29 holin's activity. We were able to engineer D29 holin with higher toxicity compared to that of the wild-type holin; the engineered protein is capable of rapidly killing both *E. coli* and *M. smegmatis* upon expression. Considering the growing interest in phage therapy and phage-based therapeutics to curtail bacterial infections and combat antimicrobial resistance (32–46), we believe that our data will

FIG 1 Effect of Hol Δ CC on the growth of *E. coli*. (A) Schematic representation of HolWT and Hol Δ CC; the N and C termini are labeled in HolWT. The two transmembrane domains (labeled 1 and 2) are shown as filled cylinders in the lipid bilayer in each case. The CC domain is shown as a spiral from amino acid 87 to 116 as marked in HolWT only; it is absent in Hol Δ CC, where the 117th amino acid is immediately followed by the 86th residue because of CC domain deletion. (Panel A created using BioRender.) (B) Growth profiles of bacteria expressing HolWT and Hol Δ CC. The growth was monitored by measuring the optical density of the culture at 600 nm (OD₆₀₀) after inducing the culture with isopropyl- β -D-thiogalactopyranoside (IPTG) at time 0. The induced and uninduced cultures are marked "In" and "UI," respectively. The data normalized with the 0-min time point are plotted here. (C) Spot assays carried out with induced cultures of HolWT and Hol Δ CC. Serial dilutions (indicated as triangles above each image) were spotted from the cell suspension of induced cultures collected at different time points, as specified. Zero-minute samples were collected just before the induction. (D) Shows the Sytox assay for the real-time monitoring of the membrane perturbation of cells expressing HolWT and Hol Δ CC. The induced and uninduced cultures are marked "In" and "UI," respectively. (B and D) Experiments were repeated at least thrice, with error bars indicating standard deviation. (C) Experiments were repeated at least thrice; only one representative image is shown.

help in the designing and engineering of phage proteins and the phages harboring them for treating drug-resistant infections.

RESULTS

D29 holin coiled-coil domain is essential for mediating efficient host cell lysis.

Mycobacteriophage D29 holin possesses two transmembrane domains (TMD) at its N-terminal and a long, charged C-terminal region that also harbors a coiled-coil (CC) domain that spans from amino acids 87 to 116 (31). We previously showed that when the two TMDs, TMD-1 ranging from amino acids 8 to 26 and TMD-2 ranging from amino acids 40 to 59 (31) located at the N terminus of the holin are fused with the CC domain, the resulting protein was able to show toxicity upon its expression in *E. coli*, although the cell lysis in this case was delayed significantly (31). To further understand the effect of the CC domain on the functionality of holin, we deleted the CC domain from the full-length holin, thus generating Hol Δ CC (Fig. 1A), and studied its effect on

bacterial growth and survival. Our data show that while the Hol Δ CC expression shows cell growth inhibition in *E. coli* BL21(DE3), the cell lysis usually seen with wild-type holin expression is not observed (Fig. 1B). This is further verified by the spot assay, where Hol Δ CC shows extremely low toxicity compared to that of the wild-type holin (Fig. 1C). We also monitored the cell membrane disruption in real-time by carrying out a Sytox assay (47). Sytox dye binds to the DNA and fluoresces; however, it is membrane impermeable and does not enter live cells with intact membranes (48). Our Sytox dye binding data clearly show delay in the cell lysis upon Hol Δ CC expression compared to that with wild-type holin. Additionally, the Sytox fluorescence intensity in the case of Hol Δ CC does not reach the level of that with wild-type holin (Fig. 1D), which suggests an inefficient disruption of the membrane. Taken together, our data show that the deletion of the CC domain from the holin renders the protein inefficient in its pore-forming ability, resulting in poor cell lysis activity.

Basic, and not acidic, residues in the C-terminal region of holin are essential for its cytotoxic activity. Similarly to most other holins, mycobacteriophage D29 holin also harbors a highly charged cytoplasmic C-terminal region that contains both positively (basic) and negatively (acidic) charged amino acids. Even though they are present in abundance, there is no study that shows their importance in holin function. A detailed examination of the D29 holin primary sequence shows that the polar charged residues are distributed in the entire C-terminal region, with a few present within the CC domain (Fig. 2A). In order to understand their contribution to holin function, we designed several different charged residue mutants of holin (Fig. 2A), which are as follows. We substituted either all negative charged or all positive charged residues present within the region from 59 to 133 with serine, thus yielding HolPos (which retains all basic residues) and HolNeg (which retains all acidic residues), respectively. Similarly, to obtain HolNeu, we substituted all the polar charged residues within the region from 59 to 133 with serine. Finally, to obtain HolNeu-CC^{WT}, we substituted all the polar charged residues within the region from 59 to 133 with serine leaving the ones present in the CC domain intact. A multiple-sequence alignment generated using Clustal Omega (49) (Fig. 2B) to depict the various charged amino acid substitutions made in the holin protein.

We next assessed the effect of the expression of each of these constructs on bacterial survival by carrying out both growth curve analysis (Fig. 3A) and Sytox assays (Fig. 3B). Interestingly, we found that only HolPos, which retains all positive charged residues, is able to show toxicity in *E. coli*, although this protein is not as efficient in killing the bacterium as the holin wild type (Fig. 3A and B). Nevertheless, HolPos is found to be more potent than the other constructs in negatively affecting *E. coli* growth. These data immediately suggest that the basic amino acids (positive charged residues) present in the C-terminal region of the holin protein are indispensable in holin-mediated toxicity. These data additionally suggest that the basic amino acids present in at least the CC domain are dispensable, since the HolNeu-CC^{WT} construct, which retains both the acidic and the basic residues in the CC domain, is unable to show toxicity (Fig. 3A and B).

We previously showed that a truncation of holin, Hol116, that lacks the region beyond the CC domain retains toxicity upon its expression in *E. coli* (31). Revisiting these data immediately suggests that the residues beyond the CC domain, and hence the charged residues present therein, are dispensable for holin's activity. It is conceivable, therefore, that the positive charged residues present between the TM2 and the CC domain are important. We therefore attempted to examine if there is a specific basic residue in this region that may impart toxicity in holin by introducing positive charged residues in place of serine at positions 59, 64, 65, and 79 in the HolNeg protein. All of these constructs, when tested for toxicity, were found to be nontoxic upon their expression in *E. coli* (Fig. 3C). Similarly, a dual charge reversal construct, HolNeg SSRK, having serine residues at both 64th and 65th positions restored back to Arg and Lys, respectively, also did not show any toxicity. It is thus plausible that one single residue is not sufficient to impart toxicity in holin, and that multiple residues, maybe all of

FIG 2 Schematic representation of the acidic (negatively charged) and the basic (positively charged) amino acid residue mutants of holin. (A) Schematic representation of the charged amino acid distribution within the C-terminal region of holin and its variants. HolPos and HolNeg proteins retain, within the region 59 to 133, either only basic or only acidic amino acids, respectively; in HolNeu, all of the charged residues are substituted with serine. In HolNeu-CC^{WT}, all of the polar charged residues within the region from 59 to 133 are substituted with serine, leaving only the ones present in the CC domain intact. (Panel A created using BioRender.) (B) Multiple-sequence alignment of the various charged residue mutants for holin generated in this study. The alignment shows the substitutions carried out in the sequence in comparison with the HolWT. The labels of the constructs are as given in panel A. The residue number marked in the protein sequences indicates the position of the amino acid in the primary protein sequence. The residues mutated at a particular position in respective variants are highlighted using black boxes. E142 is present in all the sequences, and is followed by a 6xHis tag. *, conserved residue.

them as in the case of Hol116 (31), are required for holin's activity. Taken together, our data show the importance of charged residues, specifically positive charged residues, in the C-terminal region of holin in yielding protein-mediated toxicity in bacteria.

Duplicating the C-terminal region of holin renders the protein more toxic. Our previous work (31) and the data presented in this study clearly show the significance of the C-terminal region of holin in protein-mediated toxicity upon its expression in bacteria. Taken together, we suggest that an alteration of the C-terminal region alters holin function in terms of timing of cell lysis and/or size of the hole. All of these results tempted us to generate a recombinant holin with higher toxicity. It may be noted that such recombinant phage-inspired proteins are required to develop phage-based

FIG 3 Effect of the mutation of acidic (negatively charged) and basic (positively charged) amino acid residues located in the C-terminal region of Holin on the growth of *Escherichia coli*. (A) Growth profiles of bacteria expressing Holin and its variants. Growth was monitored by measuring the optical density of the culture at 600 nm (OD₆₀₀) after inducing the culture with IPTG at time 0. The data normalized with the 0-min time point are plotted here. (B) Sytox assay for real-time monitoring of the membrane perturbation of cells expressing Holin and its variants. Profiles of uninduced (UI) and induced (In) cultures are shown. (C) Growth profiles of bacteria expressing Holin and its reverse charge mutants. Serine at specific positions in HolNeg protein was substituted with the corresponding basic (positively charged) amino acid present in Holin. "SSRK" represents the substitution of serine at 64th and 65th position with Arg and Lys, respectively. Growth was monitored, and the data were normalized and plotted essentially as described in panel A. In all of the panels, the data represent an average of at least three independent experiments with error bars indicating standard deviation.

therapeutics to combat bacterial infections, especially those caused by drug-resistant bacteria (32, 38, 50). We generated a recombinant holin by duplicating its C-terminal region in the wild-type protein, yielding HolHC; the duplication was carried out by installing the 69th to 141st residue segment of the protein at the C terminus of the wild-type protein (Fig. 4A). We next performed the growth kinetics of *E. coli* BL21(DE3) expressing HolHC protein. Very interestingly, *E. coli* cells expressing HolHC also show a drastic decrease in optical density of the culture at 600 nm (OD₆₀₀) (Fig. 4B to E). However, upon changing the isopropyl- β -D-thiogalactopyranoside (IPTG) concentrations and/or temperature of growth, the profiles start to differ. The HolHC construct shows higher toxicity in *E. coli* compared to that of Holin at low IPTG concentrations (Fig. 4B to E), and this is more apparent at lower temperatures (Fig. 4C to E). In other words, the difference in the toxicity of the two proteins could be readily observed by either reducing the IPTG concentrations or by lowering the temperature. The data thus clearly show that HolHC is more toxic to *E. coli* than the wild-type holin. Furthermore, we also monitored the loss of membrane integrity due to hole formation for both Holin- and HolHC-expressing *E. coli* cells by Sytox assay. The early increase in the fluorescence intensity of the cultures expressing HolHC compared to Holin is clearly

FIG 4 Engineering of holin for higher toxicity. (A) Schematic representation of HolWT and HolHC constructs. The two transmembrane domains are shown as filled cylinders in the lipid bilayer in each case. The CC domain is shown as spiral in both the proteins; in HolHC, two such domains are shown. (Panel A created using BioRender.) (B, C, D, and E) Growth kinetics of *E. coli* cells expressing HolWT and HolHC after induction with various concentrations of IPTG and at different temperatures, as mentioned in each plot. The growth was monitored by measuring the optical density of the culture at 600 nm (OD₆₀₀) at specified time points. The data were normalized with respect to time zero (induction time) and plotted. The uninduced culture is indicated as "UI"; induced cultures are marked with their respective IPTG concentrations for both HolWT and HolHC. For all of the growth curves, experiments were repeated at least thrice, with error bars indicating standard deviation.

observed at different temperatures, which suggests an early perturbation in membrane integrity due to hole formation (Fig. 5A to D). These data also point to the fact that HolHC forms pores sooner than HolWT. The higher cell lytic ability of HolHC is further confirmed by our β -galactosidase assay, which showed a higher β -galactosidase

FIG 5 The engineered holin causes membrane perturbation earlier than the wild-type protein. (A, B, C, and D) Show Sytox assay-based fluorescence data for the real-time monitoring of membrane perturbation of *E. coli* upon protein expression. The fluorescence was recorded for the uninduced (UI) and induced (In) cultures expressing either HolHC or HolWT at different temperatures, as mentioned in each panel. Induction was carried out with 0.3 mM IPTG in each case. (E) β -Galactosidase activity monitored as absorbance at 420 nm from *E. coli* culture supernatant collected from respective induced cultures only at 0- and 15-min time points. Cultures were induced with 1 mM IPTG at 37°C. In all of the panels, the experiments were repeated at least thrice, with error bars indicating standard deviation.

activity in supernatant of the culture expressing HolHC as early as 15 min (Fig. 5E). Taken together, our data support the interpretation that the HolHC protein engineered here is more toxic to *E. coli* than the wild-type protein, and that the extended C-terminal region is responsible for the enhanced toxicity and early cell lysis.

The engineered HolHC protein causes early cell lysis of the host *M. smegmatis*.

Since mycobacteria are the natural hosts for mycobacteriophage D29, we tested the effect of HolHC expression on the nonpathogenic host *M. smegmatis*. The HolHC protein was expressed from an acetamide-inducible promoter (31). The log-phase cultures were induced with acetamide, and the survival of mycobacterial cells was monitored by spot assay. Our data show that, although the expression of both HolHC and HolWT

FIG 6 Effect of HolHC on *Mycobacterium smegmatis* survival. (A) Spot assays carried out with induced *M. smegmatis* cultures of HolWT and HolHC. Serial dilutions (indicated by triangles above each image) were spotted from the cell suspension of acetamide-induced cultures collected at different time points as specified. Zero-hour samples were collected just before the induction. The experiment was repeated at least thrice; only one representative image is shown. (B) Luminescence as an indicative of release of ATP into the culture supernatant upon *M. smegmatis* cell membrane disruption by either HolWT or HolHC at various time points, as specified. The cultures were induced (In) with 2% acetamide, and the luminescence was compared with that of uninduced (UI) cultures. The experiments were carried out at least thrice; error bars indicate standard deviation.

is toxic to the host cells, bacteria expressing HolHC exhibit an early decrease in bacterial spot density compared to that of the wild-type holin (Fig. 6A). This immediately indicates an early host cell lysis. We wish to add here that these experiments were carried out at 37°C and, unlike in *E. coli*, we are able to see distinct difference between HolWT- and HolHC-mediated toxicity in *M. smegmatis* at this temperature. In addition, we monitored the ATP released into the culture medium as a measure of cell lysis (7, 29). The culture supernatant was collected at various time points after centrifugation, and the amount of ATP present in it was measured using the BacTiter-Glo microbial cell viability assay kit (Promega). We observe larger amounts of ATP released at the 1-h time point from the bacteria expressing HolHC compared to that expressing HolWT; furthermore, the level of released ATP increases with time (Fig. 6B).

We next asked if the higher toxicity demonstrated by HolHC is due to the larger number of higher-order aggregates formed by this protein compared to HolWT. To investigate this, we carried out *in vivo* cross-linking of both HolWT and HolHC proteins using dithiobis-succinimidylpropionate (DSP) after expressing them in *M. smegmatis*. We observed specific size bands in both proteins corresponding to various oligomeric species on the Western blot. Briefly, we are able to observe cross-linked species corresponding to monomer, dimer, trimer, and tetramer in both the proteins. All of the cross-linked products were able to convert into monomer upon treatment with reducing agent dithiothreitol (DTT), suggesting that the higher-molecular-weight bands indeed correspond to the cross-linked proteins (Fig. 7). Our data thus show that both HolWT and HolHC are able to assemble as higher-order aggregates in *M. smegmatis*, as has been suggested for holin proteins in other bacteria (51, 52). Taken together, our data unambiguously establish that HolHC is more toxic and leads to early cell lysis of *M. smegmatis*, and that this property of HolHC is likely due to its rapid assembly into oligomer in the bacterial membrane, which is contributed by the extended C-terminal region. It would be interesting to discover how this protein functions in *M. tuberculosis*.

DISCUSSION

Bacteriophages are present almost everywhere in the world and outnumber their host bacteria by over 10-fold (53). They are very specific for a given host and infect it in order to propagate. At the end of their life cycle inside the bacterial host, most double-stranded DNA (dsDNA) bacteriophages lyse their host to release their progeny. To achieve this, they overcome the biophysical barriers of their host cell such as cell membrane and cell wall by producing two proteins, holin and endolysin. Endolysin acts on

FIG 7 Multimerization of HolHC in *M. smegmatis*. *In vivo* cross-linking of HolWT or HolHC in *M. smegmatis* was carried out with 0.6 mM dithiobis-succinimidylpropionate (DSP). Cross-linked HolWT (lanes 1 and 2) or HolHC (lanes 3 and 4) were separated by SDS-PAGE and were probed with anti-His antibodies on a Western blot. Cross-linking was carried out for either 3 h or 5 h, as indicated. Dithiothreitol (DTT; 50 mM) was used for reversing the cross-linking (lanes 5 and 6) for the 3 h samples in both cases. Lane L represents the molecular weight marker. The various cross-linked species obtained on the blot are marked as follow: m, monomer; *, dimer; \$, trimer; #, tetramer. The monomeric molecular weight of HolWT and HolHC are mentioned.

the peptidoglycan of the host bacterium and hydrolyses it (7, 12, 54). Holin, on the other hand, accumulates nonspecifically in the plasma membrane and forms holes that, depending upon the phage, can either be small (pinholin) or large (canonical holin), leading to either the disruption of proton motive force or the passage of endolysin to the periplasm, respectively (8–10, 55). In addition to these proteins, the lytic cassette of some phages also encodes other accessory proteins, such as spanins and esterase, in order to overcome outer cell membrane of Gram-negative bacteria and the mycolic acid layer of mycobacteria, respectively (5, 6).

Holins have been shown to kill bacterial cells upon their expression, and this is irrespective of their origin (10, 11, 16, 17, 56, 57). This property of holin is not only essential for a phage to efficiently complete its life cycle in a time-dependent manner, but it also makes holin a potent antimicrobial agent. The cytotoxicity of holin is not only limited to prokaryotic cells; it has also been shown to reduce eukaryotic tumor cell viability *in vitro* and reduce tumor size up to 98% *in vivo*, which suggests that holin can also be developed as an anticancer agent (57). Hence, it becomes important to understand the molecular factors that regulate holin activity, which will further help to reprogram holin function in order to use it for various biotechnological applications and therapeutic purposes (17).

Mycobacteriophage D29 has the ability to infect and kill mycobacteria, including the pathogenic *M. tuberculosis* (30, 58). We have previously shown that holin is essential for the D29 phage to carry out timely and efficient host cell lysis (29). In several cases, the timing of hole formation by holin is controlled by the presence of its counter protein called antiholin, which inhibits early hole formation by holin (17, 59). This includes phages such as lambda, P22, PS3, and 21, which possess holin with a dual start motif (16). However, to date, no anti-Holin-like molecule has been reported from mycobacteriophage D29. Additionally, its lytic cassette does not encode a holin with a dual start motif (31). Conceivably, therefore, the control of keeping the clock function of D29 holin solely lies in its intrinsic molecular properties.

Holin of the D29 phage harbors two transmembrane domains that are involved

in hole formation (31, 60–62). The C-terminal region of D29 holin possesses a coiled-coil (CC) domain and carries a large number of polar charged residues (31). Here, we carried out a detailed examination of the C-terminal region of D29 holin, and were able to successfully engineer a recombinant holin with higher toxicity. The dramatic reduction in the toxicity exhibited by the holin mutant Hol Δ CC on the growth of *E. coli* due to deletion of the CC domain clearly highlights its significance in D29 holin.

The holin truncation mutants from our previous study (31), along with the charged residue mutants examined here, together allow us to conclude that the basic or positively charged amino acids present between the 59th and 79th positions in the holin are critical for protein's activity. Furthermore, our *in silico* analysis of all of the holin mutants showed no change in the holin topology with respect to the wild-type protein (data not shown). The significance of these charged residues is further supported by the fact that our HolPos construct that possesses a +6 net positive charge in the C-terminal region shows delayed cell lysis, which is similar to what has been shown previously for a lambda holin mutant that carried a net +4 charge (20). It may be noted here that the C-terminal region of holin S from lambda is found to be nonessential for holin oligomerization and does not participate in the hole structure formation, although it has a role in regulating the timing of cell lysis (20, 63). Taken together, our observations strongly indicate the significance of the molecular factors present in the C-terminal region of D29 holin that contribute to and determine the timing and efficiency of the host cell lysis.

The presence of holins is not limited to bacteriophages alone; these proteins are also present in prokaryotes, as well as in eukaryotes, in the form of homologues or orthologues. Being cell membrane-perturbing agents, holins are not only found to be involved in various physiological processes, such as sporulation, germination, biofilm formation, horizontal gene transfer, and programmed cell death (17, 64–66), but they have also inspired many biotechnological applications, including therapeutics in medicine, biocontrol agents in food safety, and the production of various materials of biological significance (17, 67–70). One such application includes the redesigning of the first transmembrane domain into a nanopore ion channel (71). After delineating the importance of the C-terminal region, we also attempted to engineer D29 holin for higher toxicity. Our HolHC construct, which was generated by duplicating the C-terminal region of wild-type holin, is convincingly more toxic than HolWT and leads to rapid cell lysis upon expression in both *E. coli* and *M. smegmatis*. Since our *in vivo* cross-linking data show similar levels of oligomerization in both engineered and wild-type holins, we believe that rapid lysis caused by the engineered holin is due to the faster assembly of aggregates in the membrane, which is aided by the extended C-terminal region. Such engineered holins may certainly find use in various therapeutic applications. It would be interesting to discover if a mycobacteriophage carrying such engineered holins becomes more potent (lowered burst time and/or increased burst size) in killing mycobacteria, including the pathogenic *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* XL1-Blue strain was used for all cloning and mutagenesis experiments, whereas the BL21(DE3) strain was used for all the protein expression studies. *E. coli* carrying the plasmid was grown in LB broth (Difco) supplemented with either ampicillin or kanamycin, as required, at a final concentration of 100 or 50 μ g/ml, respectively. *M. smegmatis* mc²155 transformed with plasmid was grown in Middlebrook 7H9 broth (Difco) supplemented with 2% (wt/vol) glucose (Hi-Media), 0.05% (vol/vol) Tween 80 (Difco); kanamycin was added to a final concentration of 50 μ g/ml (7, 31, 72). All liquid cultures were grown at 37°C with constant shaking at 200 rpm, unless otherwise specified. For bacterial culture on solid medium, broth was supplemented with 1.5% agar. Plasmids used in this study are mentioned in Table 1.

Construction of holin variants. All of the required genes were cloned in pET21b between the NdeI and XhoI sites (unless mentioned otherwise) in order to add a 6 $\times$ His tag at the C terminus of the protein. pSK-HolFL carries the D29 phage *gp11* gene and codes for the wild-type full-length holin (31), which has been labeled HolWT throughout this article. This plasmid was used as a template to prepare various

TABLE 1 List of plasmids used in the present study

Plasmid	Protein product	Antibiotic selection marker ^a	Source of the plasmid (reference)
pET21b		Amp	Novagen
pMVAcet-NdeI		Kan	Laboratory plasmid (12)
pSK-HolFL ^b	HolWT	Amp	Laboratory plasmid (31)
pVB-HolΔCC	HolΔCC	Amp	Present study
pVB-HolPos	HolPos	Amp	Present study
pVB-HolNeg	HolNeg	Amp	Present study
pVB-HolNeu	HolNeu	Amp	Present study
pVB-HolNeu-CC ^{WT}	HolNeu-CC ^{WT}	Amp	Present study
pVB-HolNeg S59K	HolNeg S59K	Amp	Present study
pVB-HolNeg S64R	HolNeg S64R	Amp	Present study
pVB-HolNeg S65K	HolNeg S65K	Amp	Present study
pVB-HolNeg SSRK	HolNeg SSRK	Amp	Present study
pVB-HolNeg S79R	HolNeg S79R	Amp	Present study
pVB-HolHC	HolHC	Amp	Present study
pSKMs-HolFL ^b	HolWT	Kan	Laboratory plasmid (31)
pVBMs-HolHC	HolHC	Kan	Present study

^aAmp, ampicillin; Kan, kanamycin.^bPlasmid carries the gene *gp11* that codes for full-length wild-type holin (HolFL), which in this study has been labeled HolWT.

constructs mentioned in Table 1, following methods described previously (31). To generate HolΔCC, the segment of the gene corresponding to the CC domain ranging from amino acids 87 to 116 was deleted from *gp11* by overlapping PCR as described earlier (54) using primers P1, P2, P5, and P6 (Table 2). For the preparation of C-terminal charge residue mutants (HolPos, HolNeg, HolNeu, and HolNeu-CC^{WT}), required DNA fragments (Table 3) coding for the C-terminal region of holin (59th to 133rd amino acids) and carrying various mutations as needed were procured from Genewiz (New Jersey, USA). These fragments were further used to generate full-length holin mutants by fusing them with rest of the N-terminal holin-coding DNA segment by means of overlapping PCR. Briefly, all of the mutated versions were PCR amplified using the following primers: HolPos (P3, P4, P7, and P8); HolNeg (P3, P4, P9, and P10); HolNeu and HolNeu-CC^{WT} (P3, P4, P11, and P12); HolNeg_S59K (P3, P4, P13, and P14); HolNeg_S64R (P3, P4, P15, and P16); HolNeg_S65K (P3, P4, P17, and P18); HolNeg_SSRK (P3, P4, P19, and P20);

TABLE 2 Primers used in this study for the preparation of plasmid constructs

Primer name	Sequence (5'–3') ^a
P1	ATCGAGATCTCGATCCCGGAAATTAATACG
P2	CCTTCGGGCTTTGTTAGCAGC
P3	ATCGGAATGGAGCC CATATG AGCCCCAAGATC
P4	ATCCAGGGCTTG CCTCGAG GTTCAGGGCTGC
P5	CGTCGAGCAGGTGCTCCTGGCCAGCCAGATCCTCAACG
P6	CTGGCTGGCCAGGAGCACCTGCTCGACGCCCTG
P7	CACGGCCGCTGTCAAGGTCAACCAGCAGCGCAAG
P8	GGTTGACCTTGACAGCGGCCGTGGCCGGTGC
P9	CACGGCCGCTGTCTGTCAACCAGCAGTCTCTG
P10	GGTTGACAGAGACAGCGGCCGTGGCCGGTGC
P11	CACGGCCGCTGTCTGTCAACCAGCAGTCTCTCAG
P12	CTGGTTGACAGAGACAGCGGCCGTGGCCGGTGC
P13	GCTGTCAAGGTCAACCAGCAGTCTCTC
P14	CTGCTGGTTGACCTTGACAGCGGCCGTG
P15	CAACCAGCAGCGCTCTGATGGCACGCTG
P16	CATCAGAGCGCTGCTGGTTGACAGAGACAGC
P17	GCAGTCCAAGGATGGCAGCTGACCACTC
P18	GTGCCATCCTTGACTGCTGGTTGACAGAGAC
P19	GTCAACCAGCAGCGCAAGGATGGCAGCTGACCACTC
P20	CGTGCCATCCTTGCGCTGCTGGTTGACAGAGAC
P21	CAGGTCAACAGGGGCGTCGAGCAGGTGCTC
P22	CTCGACGCCCTGGTGACCTGATCCAC
P23	AAGGAT CCTCGAG CTGACCACTCCCCGGTG
P24	GGTGGGGAT CTAGA AGTGACGCGGTCTCAAGC
P25	GAAACTACCTCG CATATG TGGACTCCCTTCTC

^aRestriction enzyme sites, wherever present, are boldfaced.

TABLE 3 Sequence of synthetically generated DNA fragments for the preparation of holin charge residue mutants

Construct	Sequence of DNA fragment (5'–3') ^a
HolPos	AAGGTCAACAGCAGCGCAAGTCTGGCAGCTGACCACCTCCCGGTGTCTCAGGTACACAGGGCGCTCTCCAGGTGCTCGCGGCCAAGCA GAACGCTTCCGCTTCTGTCTCCCGCTCAAGCAGGCTCTGTCTCCGCTGTCAACGGCGCGGTCCCCAGCTCGGCCCGTGGCCAGCCAGA TCCTCAACGGCATCAACCGGCCTACAGCCAGCCGTTCTCCCGCACACGAGCCCTGGAAC CTCGAG
HolNeg	TCTGTCAACAGCAGTCTCTGATGGCAGCTGACCACCTCCCGGTGGATCAGGTACCTCCGCGCTCGAGCAGGTGCTCGCGGCTCCAG AACGCTGAGGCTGAGGTGAGTCTGTCTCCAGGCTCTGGAGTCCGCTGTCAACGGCGCGGTCCCCAGCTCGGCCCGTGGCCAGCCAGA TCCTCAACGGCATCAACCGGCCTACAGCCAGCCGTTCTCGACCCGACACGAGCCCTGGAAC CTCGAG
HolNeu	TCTGTCAACAGCAGTCTCTTTCAGGCAGCTGACCACCTCCCGGTGTCTCAGGTACCTCCGCGCTCTCTCAGGTGCTCGCGGCTCCAG ACGCTTCTGCTTCCGCTCTTCCGCTCTCAGGCTCTGCTCCGCTGTCAACGGCGCGGTCCCCAGCTCGGCCCGTGGCCAGCCAGATCC TCAACGGCATCAACCGGCCTACAGCCAGCCGTTCTCTCCGCACACGAGCCCTGGAAC CTCGAG
HolNeu-CC ^{WT}	TCTGTCAACAGCAGTCTCTTTCAGGCAGCTGACCACCTCCCGGTGTCTCAGGTACCTCCGCGCTCTCTCAGGTGCTCGCGGCAAGCAG AACGCTGAGGCTGAGGTGAGCGCGTCAAGCAGGCTCTGGAGTCCGCTGTCAACGGCGCGGTCCCCAGCTCGGCCCGTGGCCAGCC AGATCTCAACGGCATCAACCGGCCTACAGCCAGCCGTTCTCTCCGCACACGAGCCCTGGAAC CTCGAG

^aXhoI restriction enzyme site present in each DNA sequence is boldfaced.

HolNeg_S79R (P3, P4, P21, and P22). In each of these cases, the third and the fourth primer allowed the overlapping PCR. All of the amplified PCR products were digested with NdeI and XhoI and cloned in pET21b, which was previously digested with the same enzymes. For the preparation of engineered holin constructs, the 3' region of *gp11* corresponding to the C-terminal region of holin (69th to 141st amino acids) was PCR amplified using primers P2 and P23, digested with XhoI, and was ligated in pSK-HolFL and pSKMs-HolFL (predigested with XhoI), to give rise to pVB-HolHC and pVBMs-HolHC, respectively. Both pSKMs-HolFL and pVBMs-HolHC are *E. coli*-mycobacterium shuttle plasmids that carry an acetamide-inducible promoter and a kanamycin antibiotic selection marker (12, 31, 73). Both of these plasmids are based on the pMVAcet vector that was prepared by replacing the *hsp60* promoter region in pMV261-NdeI (12) with an acetamide-inducible promoter region between XbaI and NdeI restriction enzyme sites. The acetamide-inducible promoter region (74) was amplified from the *M. smegmatis* genome using primers P24 and P25 as mentioned in Table 2. The final product, HolHC, possesses an extra C-terminal region at the end of wild-type full-length holin (HolWT). All of the clones generated here were confirmed by DNA sequencing.

Measuring the effect of protein expression on bacterial growth. *E. coli* or *M. smegmatis* cells were transformed with respective plasmids. While in *E. coli*, protein expression was induced with the addition of 1 mM or specified concentration of IPTG, for *M. smegmatis*, 2% acetamide was used for protein production. The growth of the culture was monitored as described previously (31). The uninduced culture acted as a negative control. Spot assays for both *E. coli* and *M. smegmatis* were performed by preparing serial dilutions of the induced cultures at different time intervals and spotted on agar plates containing appropriate antibiotic as described previously (31, 72). Plates were then incubated at 37°C up to appearance of visible growth. For growth kinetics measurements, *E. coli* was grown in a 96-well plate at specified temperature with constant shaking at 800 rpm on a thermomixer (Eppendorf), and the optical density of the culture was measured at 600 nm on a plate reader (Molecular Devices) as described previously (31).

β -Galactosidase activity assay. Culture supernatants were used to measure β -galactosidase activity from the induced cultures of *E. coli* containing respective plasmids. The assay was performed as described previously (31, 75).

Real-time Sytox green assay. The bacterial membrane integrity was monitored in real-time after holin and its variants were expressed in *E. coli*, essentially as described previously (47). The fluorescence was monitored with excitation/emission wavelengths set at 488 and 523 nm, respectively, on a SpectraMax M5 plate reader (Molecular Devices). Fluorescence was monitored every minute for 90 min. The plate was allowed to shake for at least 3 s immediately before the measurement in order to mix the culture in the well.

ATP release assay. *M. smegmatis* cultures harboring either pSKMs-HolFL (31) or pVBMs-HolHC were grown until the OD₆₀₀ reached 0.6, following which the cultures were induced with 2% acetamide. At specified time points, the cultures were collected and immediately centrifuged at 13,000 rpm at 4°C. ATP was estimated in the 100 μ l of the supernatant from each sample (7, 29) by using a BacTiter-Glo microbial Cell Viability assay kit (Promega) in a 96-well white opaque plate (Nunc) following manufacturer's instructions. The luminescence was measured on a SpectraMax M5 plate reader (Molecular Devices). Uninduced cultures were used as a negative control.

In vivo cross-linking and immunoblotting. Dithiobis-succinimidylpropionate (DSP; Thermo Scientific), a homobifunctional cleavable reagent, was used to carry out *in vivo* cross-linking, and was used per the manufacturer's instructions. *M. smegmatis* cells expressing HolWT and HolHC were harvested at 3 h and 5 h postinduction. The cells were immediately harvested, washed by resuspension with 1 $\times$ phosphate-buffered saline with Tween 20 (PBST; 137 mM NaCl, 2.7 mM KCl, 10.0 mM Na₂HPO₄, 1.8 mM KH₂PO₄, and 0.05% Tween 80) at 4°C, and finally resuspended in the same buffer. DSP was then added into the resuspended cells to a final concentration of 0.6 mM. Cross-linking was allowed to occur for 2 h at room temperature. The reaction was then stopped by adding a 1/10th volume of 1 M Tris-Cl (pH 8.0) into the cell suspension. Cells were then harvested and resuspended in lysis buffer containing

8 M urea and 1% Triton X-100, and were lysed by sonication. The cross-linked products of the protein constructs were concentrated on Ni-nitrilotriacetic acid (NTA) agarose beads (Qiagen) and the obtained proteins in the elution were separated on SDS-PAGE followed by Western blotting using anti-6×His tag antibodies as described previously (12, 31). The eluted samples were also treated with 50 mM DTT to observe the reverse cross-linking, and were processed by SDS-PAGE followed by Western blotting as described above.

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We declare that we have no conflicts of interest.

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