

# Holins in Bacteria, Eukaryotes, and Archaea: Multifunctional Xenologues with Potential Biotechnological and Biomedical Applications

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**Holins form pores in the cytoplasmic membranes of bacteria for the primary purpose of releasing endolysins that hydrolyze the cell wall and induce cell death. Holins are encoded within bacteriophage genomes, where they promote cell lysis for virion release, and within bacterial genomes, where they serve a diversity of potential or established functions. These include (i) release of gene transfer agents, (ii) facilitation of programs of differentiation such as those that allow sporulation and spore germination, (iii) contribution to biofilm formation, (iv) promotion of responses to stress conditions, and (v) release of toxins and other proteins. There are currently 58 recognized families of holins and putative holins with members exhibiting between 1 and 4 transmembrane  $\alpha$ -helical spanners, but many more families have yet to be discovered. Programmed cell death in animals involves holin-like proteins such as Bax and Bak that may have evolved from bacterial holins. Holin homologues have also been identified in archaea, suggesting that these proteins are ubiquitous throughout the three domains of life. Phage-mediated cell lysis of dual-membrane Gram-negative bacteria also depends on outer membrane-disrupting “spanins” that function independently of, but in conjunction with, holins and endolysins. In this minireview, we provide an overview of their modes of action and the first comprehensive summary of the many currently recognized and postulated functions and uses of these cell lysis systems. It is anticipated that future studies will result in the elucidation of many more such functions and the development of additional applications.**

**B**acteriophages, such as double-stranded DNA phages, infect their bacterial hosts, have their DNA replicated within the host cells, assemble phage particles, and mediate programmed lysis of the infected bacterium (1, 2, 3). Multiple cell lysis strategies that promote release of the virions from phage-infected bacteria have been described elsewhere (4). In general, the lysis genes are found in cassettes localized together on the phage chromosome, and these include at least two genes, one that encodes a small protein which spans the cytoplasmic membrane from one to four times as transmembrane  $\alpha$ -helical sequences (TMSs) (Fig. 1) (1, 5) and another that has catalytic activity designed to hydrolyze or alter certain bonds in the peptidoglycan cell wall (6). Because these muralytic enzymes are produced within the cell that is targeted for lysis, they are referred to specifically as “endolysins” or sometimes more generally as “autolysins.” Since autolysins may function by several mechanisms, we will use the more restrictive “endolysin” term in this minireview.

Endolysins are genome- or phage-encoded enzymes that can be of at least five different types (4, 7). Many of them are made without targeting signal sequences, sequences that are characteristic of proteins exported via the general secretory pathway, the Sec translocase (8) (see the Transporter Classification Database [TCDB]; [www.tcdb.org](http://www.tcdb.org); TC no. 3.A.5) (9, 10). They must therefore use an alternative method of export (1, 11). These enzymes are produced fully folded in the cell cytoplasm and are exported via small transmembrane proteins called holins or hole formers because of their propensity to form large oligomeric flexible pores in the cytoplasmic membranes of bacteria (12).

The term “holin” was coined to refer to proteins that control the phage infection cycle (12), but subsequent studies, discussed comprehensively in this review for the first time, led to the sugges-

tion that these proteins and their homologues may serve a variety of functions in phage-free prokaryotic cells. Holins allow the endolysins to gain access to the cell wall, where they exert their actions by cleaving various bonds in the peptidoglycan polymer, depending on the type of endolysin (4, 13). Genes encoding holin proteins and their target peptidoglycan hydrolases have been identified in a wide variety of Gram-negative and Gram-positive bacteria and their phages (1, 14–17). While possible homologues are present in archaea (see holin families 1.E.3, 1.E.14, and 1.E.43 in TCDB), the functions of these proteins are not yet defined. It is not always clear whether access of endolysins to the cell wall results from secretion, leakage, or membrane lysis, and this may depend on the type of holin (18). It is also unclear whether chromosomally encoded holins are xenologues of phagic origin or whether phage holins are xenologues of chromosomal origin (1).

Holins play two clear roles in the phage infection cycle. They allow release of the endolysin, their primary function (e.g., their membrane permeation function), and they determine the timing of the end of the infection cycle, their secondary function, a “clock” function. This conclusion was first reached by analyzing

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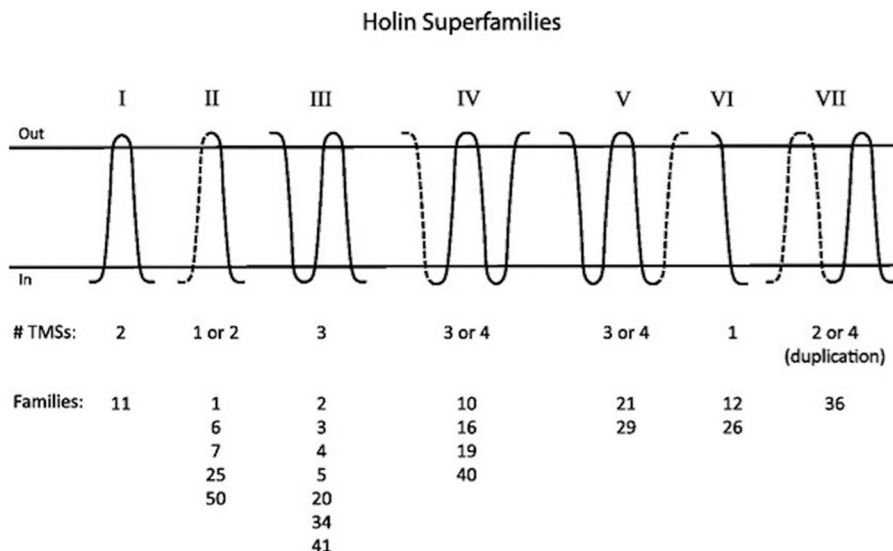
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**FIG 1** Schematic view of the topologies of members of the seven holin superfamilies (I to VII) (Table 1). Solid lines represent TMSs that are present in all known members of the superfamily. Dashed lines represent TMSs present in some but not all members. # TMSs, the number of transmembrane  $\alpha$ -helical segments (TMSs) predicted for the proteins of a superfamily. Family numbers refer to the families in subclass 1.E of the Transporter Classification Database (TCDB; [www.tcdb.org](http://www.tcdb.org)). (Reprinted from the work of Reddy and Saier in *Biochimica et Biophysica Acta* [5].)

the consequences of mutations in the holin of phage lambda which either blocked release of the endolysin or gave rise to early lysis. Both defects resulted in the absence of phage-mediated plaque formation (19, 20). It thus seems that phage holins are highly constrained pore-forming proteins because, in determining the viability of a virus, timing is extremely important. In fact, stochastic holin gene expression has recently been shown to account for lysis time variation in phages such as lambda (21).

In 1995, Young and Blasi (7) grouped holins into 11 families that were suggested to have evolved independently. However, one cannot establish independent origin based solely on a lack of sequence similarity because sequence divergence can mask the common features of homologues that arose from a common ancestry (22–24). In establishing homology, it is imperative to use rigorous statistical methods to identify family and superfamily relationships. This allows one to extrapolate structural, functional, and mechanistic information from one holin to others. Establishment of homology provides the first line of evidence for the functions of thousands of small proteins that resemble the few functionally characterized holins. The inference of common structure, function, and mechanism, based on phylogenetic relationships with known holins, is justified only when homology is rigorously established.

#### CLASSIFICATION AND PHYLOGENETIC CHARACTERIZATION OF HOLINS

Recently, Reddy and Saier (5) identified 52 families of holins, and representative well-characterized members as well as distant homologues of unknown function were entered into TCDB. In these studies, the organismal sources of family members, often derived from many bacterial phyla, were identified. These proteins were analyzed for their topological features, showing that they possess 1 to 4 transmembrane  $\alpha$ -helical sequences (TMSs) (Fig. 1). Some of the 4-TMS holins proved to have arisen by intragenic duplications of primordial 2-TMS-encoding holin genes. Members of a single

family often, but not always, exhibited the same number of TMSs. In general, protein sizes correlated with numbers of TMSs, suggesting that most holins consist primarily of their transmembrane domains. While some families were large, with over 5,000 sequenced members in the NCBI protein database as of January 2012, others proved to be small, with only one or a few recognized members (5).

Using established statistical approaches, 21 of the 52 families could be shown to comprise seven superfamilies as defined by Reddy and Saier (5) (Table 1). For our purposes, a superfamily is operationally defined as a group of protein families in which members are homologous but distantly related. Because of sequence divergence, their homologous relationships cannot usually be established using simple BLAST searches or binary sequence comparisons. Additional methods such as use of the “superfamily principle” (the transitivity rule) must be applied (25). Conserved motifs, a guide to structure and/or function, can sometimes be used to provide additional support for homology, as was possible for many of the holin families and superfamilies. The results were used to expand the “clan” system of Pfam (5).

Superfamily assignment allows extrapolation of structural and functional characteristics from one or a few holins to all other members of the superfamily, with the reliability of such extrapolations being inversely proportional to phylogenetic distance. Phylogenetic trees for the major superfamilies allowed graphical depiction of the relationships of individual proteins as well as the families of a superfamily to each other (5). Since publication of this work, six new holin families and several putative holin families have been identified. A few of these include or consist exclusively of archaeal proteins (see subclasses 1.E and 9.B in TCDB).

#### MECHANISMS OF HOLIN ACTION AND TIMING

Phage lysis of infected Gram-negative bacterial cells is, of necessity, regulated in predetermined temporally programmed processes. Three distinct types of proteins result in destruction of the

TABLE 1 Characteristics of holin superfamilies<sup>a</sup>

Holin superfamily	Superfamily characteristic		Avg size (mean no. of amino acids ± SD)	No. of TMSs
	Family(ies) (TC no.)	Organismal type(s)		
I	1.E.11	<i>Firmicutes, Actinobacteria, Proteobacteria</i>	97 ± 38	2
II	1.E.1, 1.E.6, 1.E.7, 1.E.25, 1.E.50	<i>Proteobacteria</i>	78 ± 14	1/2
III	1.E.2, 1.E.3, 1.E.4, 1.E.5, 1.E.20, 1.E.34, 1.E.41	<i>Proteobacteria, Actinobacteria, Archaea, Synergistetes, Deinococcus/Thermus</i>	114 ± 23	3
IV	1.E.10, 1.E.16, 1.E.19, 1.E.40	<i>Firmicutes, Actinobacteria, Fusobacteria</i>	156 ± 94	3/4
V	1.E.21, 1.E.29	<i>Firmicutes, Chloroflexi, Actinobacteria</i>	98 ± 17	3/4
VI	1.E.12, 1.E.26	<i>Firmicutes, Chloroflexi, Fusobacteria, Tenericutes, Thermotogae, Deinococcus/Thermus</i>	132 ± 21	1
VII	1.E.36	<i>Actinobacteria</i>	105 ± 40	2/4

<sup>a</sup> See TCDB and reference 5 for family descriptions and primary reference sources. (Modified from the work of Reddy and Saier in *Biochimica et Biophysica Acta* [5].)

three layers of the cell envelope: the inner membrane, the peptidoglycan cell wall, and the outer membrane (8, 26). A typical phage-encoded holin may accumulate harmlessly in the cytoplasmic membrane until triggered at a specific time to form huge pores, allowing an endolysin to reach the periplasm to degrade the peptidoglycan cell wall (27). In a parallel pathway, an entirely different type of holin, a “pinholin,” can form small channels, sometimes heptameric channels, that depolarize the membrane. Pinholins are associated with endolysins that accumulate in the periplasm as inactive membrane-tethered enzymes. A pinholin collapses the proton motive force (PMF), allowing the endolysin to fold to an active form and hydrolyze bonds in the peptidoglycan cell wall (12). In a third step, a spanin complex, consisting of a small outer membrane lipoprotein and an integral cytoplasmic membrane protein (o-spanin and i-spanin, respectively), disrupts the outer membrane. Single-component spanins, called u-spanins, consist of an N-terminal outer membrane lipoprotein domain and a C-terminal inner membrane domain connected by a linker that spans the periplasm (see Fig. 2) (28). Possibly, spanins function to disrupt the outer membrane by fusion of the inner and outer membranes (29, 30).

As noted above and visualized in Fig. 2, two primary functions of most phage-encoded holins are to (i) dissipate the PMF (accomplished by small-pore-forming pinholins) and (ii) allow release of endolysins (accomplished by large-pore-forming holins). In general, the holin accumulates in the cytoplasmic membrane until a signal triggers oligomerization and consequent pore formation, allowing endolysin release (13). In some cases, including the well-studied phage lambda and phage 21 lysis systems, antiholin proteins, homologous to and nearly identical to the target holin, control lysis timing. In the case of some well-characterized holin-antiholin pairs, the dominant activity of the antiholin is due to an N-terminal positively charged residue that confers a PMF-dependent block to the holin topological alteration that is required for lysis (31). The presence of homologous holin-antiholin pairs that may or may not be derived from a single gene is reminiscent of apoptosis in animal cells (32). This topic will be considered below.

#### OUTER MEMBRANE LYSIS MEDIATED BY SPANINS

In addition to holins and endolysins, which disrupt the inner membrane and the cell wall, respectively, disruption of the Gram-negative bacterial outer membrane depends on a third class of lysis

proteins that comprise a “spanin” complex. This complex spans the two membranes of the bacterial cell envelope as well as the periplasmic space between these two membranes (8, 28, 33). Under normal aerobic laboratory growth conditions with agitation, lysis by bacteriophage lambda requires only the holin and endolysin genes but not the spanin (Rz and Rz1) genes of the lysis cassette. Under these laboratory conditions, defects in either Rz or Rz1 block lysis only in the presence of high concentrations of divalent cations, not in their absence. Presumably, divalent cations stabilize the outer lipopolysaccharide membrane against the shearing forces that result from rotary shaking of the cultures (30). The divalent cations may create salt bridges, neutralizing the neg-

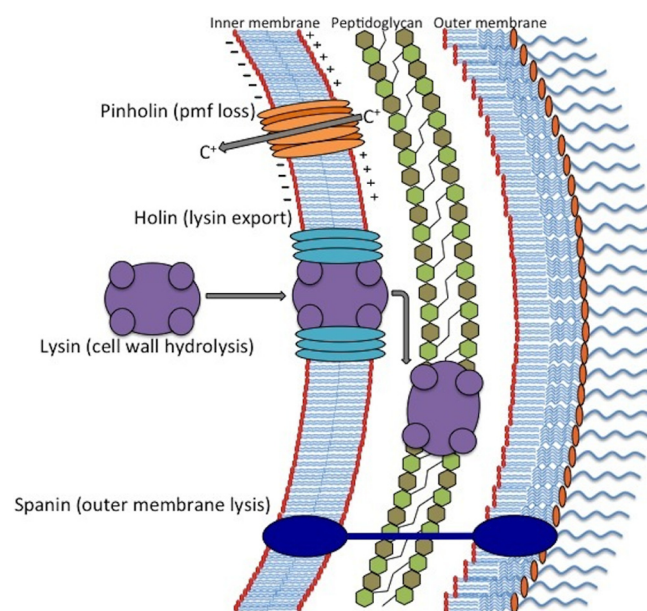


FIG 2 Schematic depiction of the proteins involved in Gram-negative bacterial cell envelope disruption by holin-type lysis systems. Pinholins form small heptameric pores that collapse the membrane potential (the PMF) across the inner membrane, while the more conventional holins form large multisubunit pores of variable sizes that allow release of fully folded endolysins from the cytoplasm, which in the periplasm hydrolyze specific bonds in the peptidoglycan cell wall, depending on the lysin type. One- or two-component spanins disrupt the outer membrane by an unknown mechanism, possibly involving fusion of the outer membrane with the inner membrane. C<sup>+</sup>, a cation.

ative charges on the lipid and polysaccharide constituents, thereby minimizing electrostatic repulsion. The two lysis proteins, Rz and Rz1, act cooperatively, interacting as covalent, disulfide-bonded, intermolecular links in homodimeric structures that are required for function (34).

Amazingly, the Rz1 gene, encoding the outer membrane lipoprotein constituent of the complex, is completely embedded within the Rz gene, which encodes the cytoplasmic membrane constituent. Although genes embedded in genes are known in bacteria (35), this is the only known example where a single DNA sequence encodes two distinct proteins that mediate a single function (30). Interestingly, this is not always the case; in other phages, the genes for the Rz1 and Rz proteins are nonoverlapping.

While Rz and Rz1 homologues have been identified in some phages, such as *Escherichia coli* P2 and T7, many other phages, including P1, T1, T4, Mu, and SP6, lack recognizably homologous Rz/Rz1 equivalents (28). It now seems clear that other nonhomologous but functionally analogous proteins provide this disruptive function.

Summer et al. (28) reported that a search strategy based primarily on gene arrangement and membrane localization signals, rather than sequence similarity, revealed that Rz/Rz1 equivalents are common among phages of Gram-negative bacteria. In fact, 120 of 137 phages were found to possess genes that fit these search criteria (28). In the case of T4 phage, deletion of a nonoverlapping gene pair, termed *pseT.2* and *pseT.3*, considered to be Rz/Rz1 equivalents, resulted in the same divalent cation-dependent lysis phenotype for aerobic shake flask-grown cells cited above for mutants of phage lambda and T7. Interestingly, in phage T1 and each of six other phages studied, Rz/Rz1 pairs were not found, but a single gene encoding an N-terminal outer membrane lipoprotein with a C-terminal inner membrane transmembrane domain was identified (28). These proteins were named u-spanins since they were predicted to span the cell envelope, providing a physical bridge between the two membranes.

The T1 u-spanin proved to complement the lambda Rz/Rz1 lysis defect, furnishing strong evidence that these one-component spanins function as Rz/Rz1 equivalents. The widespread occurrence of spanin equivalents in most examined Gram-negative bacterial phages suggests that they provide a selective advantage to the phage. Their role in the natural environments of these phages is now known to be much more important than inferred from the divalent cation dependency observed under normal laboratory conditions (28, 30).

#### HOLIN-LIKE ANIMAL APOPTOSIS MEDIATORS Bax AND Bak

Animal apoptosis-promoting proteins Bax and Bak appear to be functional holins that may or may not have been derived from bacterial holins. In response to apoptotic signals, mitochondria of animal cells undergo outer membrane permeabilization. This allows the release of cytochrome *c* and other mitochondrial proteins into the cytosol to initiate a series of proteolytic events, the “caspase cascade” (36–38). Mitochondrial permeabilization is controlled by Bcl-2 family proteins, which include antiapoptotic proteins such as Bcl-2 and Bcl-x<sub>L</sub> as well as proapoptotic proteins, including Bax and Bak. The antiapoptotic proteins apparently inhibit permeabilization by suppressing the functions of proapoptotic Bcl-2 family proteins. Thus, antiapoptotic proteins functionally resemble antiholins while proapoptotic proteins resemble holins.

Other proteins involved in animal apoptosis function to neutralize the antiapoptotic proteins or to trigger Bax/Bak activation (39–43). Genetic analyses of mice deficient for both Bax and Bak have established an essential role of these proteins in apoptosis (44).

During apoptosis, Bax or Bak becomes activated to form homo-oligomers in the outer mitochondrial membranes (45–47), and these then cause pore formation. In fact, they can permeabilize liposomes and outer membrane vesicles in artificial *in vitro* systems, providing confirmation that this is their primary function in apoptosis (43, 48). The molecular compositions of the pores have been unclear until recently (49, 50).

Free-living bacteria share many similarities with mitochondria. This is reasonable since alphaproteobacteria gave rise to mitochondria in an endosymbiotic evolutionary process (51). Similarities between the processes involving mitochondrial and bacterial cell permeabilization during mammalian cell apoptosis and holin-mediated bacterial cell death have been noted (52).

Pang et al. (32) explored the functional link between active Bax/Bak and holins. They characterized Bax/Bak-promoted lesions in bacterial membranes and investigated the involvement of Bax helices in the control of pore formation. In these studies, they expressed active Bax/Bak in bacteria and examined their functional similarities to lambda phage holin. They showed that active Bax or Bak, but not one of the antiapoptotic Bcl-2 family proteins, displays holin-like behavior, causing bacterial lysis by releasing endolysins in a process dependent on Bax or Bak oligomerization. Replacing the native holin gene with active alleles of Bax or Bak resulted in plaque-forming phage. Moreover, active Bax was shown to produce large membrane holes, the sizes of which were controlled by the inherent features of Bax. Lysis by Bax was inhibited by Bcl-x<sub>L</sub>, an apoptotic inhibitor, and the lytic activity of the wild-type Bax was stimulated by a BH3-only protein which normally promotes apoptosis in the animal cell. These observations clearly imply that active Bax and Bak are functional holins and that pore formation in the mitochondrial membrane parallels that during bacterial phage release.

A surprising difference is that holins and Bax/Bak, when expressed in bacteria, permeabilize the inner membrane, but that Bax and Bak, when expressed in animal cells, permeabilize the mitochondrial outer membrane. How this distinction arose presumably depends on the protein targeting signals and the cellular apparatuses that recognize them. However, while these proteins are made inside the bacterium, they are made outside mitochondria, because they are encoded in the nucleus of a eukaryotic cell. Thus, in targeting to mitochondria, Bax and Bak may associate with the first membrane that they encounter, the outer membrane, in a process mediated by the TOM complex (see the work of Rapaport [53] for further consideration of this problem).

#### INVOLVEMENT OF HOLINS IN BACTERIAL GENE TRANSFER

Lang and Beatty first described a novel mechanism for the transfer of genetic material from one bacterium (the donor bearing a “gene transfer agent” [GTA]) to another, the recipient. Their analyses revealed that GTA had some key features of bacteriophage, even though GTA appeared to lack some of the typical phage genes (54). GTA proved to be a phage-like particle, generated in the cytoplasm of the alphaproteobacterium *Rhodobacter capsulatus*. In fact, GTA looks like a short-tailed phage, although it could not form plaques on a lawn of bacteria because of its lack of several



genes essential for normal viral function. Consequently, GTA can function only to transfer genes.

*R. capsulatus* GTA can incorporate about 4.5 kb of linear double-stranded DNA (chromosomal or plasmid), and incorporation appears to be random; that is, it is not selective for a particular DNA sequence. The GTA structural and regulatory genes were cloned following Tn5 transposon mutagenesis that resulted in the loss of GTA activity (55). All mutants mapped to a 30,000-bp region of the chromosome, encoding at least 19 proteins then recognized. These genes proved to encode (i) phage-like capsid proteins, (ii) a head, and (iii) tail fibers. Their expression seemed to be regulated by a pair of cellular proteins, CckA, a sensor kinase, and CtrA, a response regulator. Together, these two proteins appeared to comprise the signaling pathway for regulated expression of the GTA structural genes. They also proved to regulate expression of many other genes, including those involved in motility (56).

GTA synthesis is growth phase dependent, increasing in stationary phase when the cell density is high. Apparently, GTA evolved in the chromosome under the control of the cell cycle and growth phase (57). Similar genetic elements are widespread in bacteria and possibly archaea. An interesting question is whether GTA is an evolutionary precursor of a phage or whether it is a defective phage (58).

Matson et al. (59) identified genes of a spirochete phage-like gene transfer agent that resemble phage lysis cassettes. VSH-1 is a mitomycin C-inducible prophage-like element on the chromosome of the anaerobic spirochete *Brachyspira hyodysenteriae*. Like the *R. capsulatus* GTA, purified VSH-1 particles incorporate random fragments of the bacterial genome, in this case about 7.5 kb, but these particles are noninfectious. Thus, analogously to *R. capsulatus* GTA, these particles solely mediate generalized gene transfer between *B. hyodysenteriae* cells.

Spirochete GTA particles were purified and analyzed (59). The VSH-1 genes span 16.3 kb of the *B. hyodysenteriae* chromosome and are flanked by typical bacterial genes. VSH-1 genes are consecutively organized in (a) head (seven recognized genes), (b) tail (seven recognized genes), and (c) lysis (four recognized genes) clusters, all in the same direction of transcription. The lysis genes encode an endolysin and a holin-like protein, and the endolysin was shown to hydrolyze bonds in the peptidoglycan cell wall of *B. hyodysenteriae* cells. The types of genes present explained the noninfectious nature of VSH-1 virions and confirmed their resemblance to known prophage-like GTAs of other bacteria (54).

As noted above, GTA of *R. capsulatus* is a genetic element with the sole known function of horizontal gene transfer. Westbye et al. (60) identified the promoter of the first gene in this GTA gene cluster and showed that gene transfer frequencies depend on the growth medium. Surprisingly, millimolar concentrations of phosphate posttranslationally inhibited the lysis-dependent release of GTA from the cells. Cell lysis required two genes, rcc00555 and rcc00556, which, when expressed in *E. coli*, proved to encode an endolysin and a holin, respectively. Transcription of these lysis genes was shown to be regulated by the histidine sensor kinase CckA. Evidently, a holin-endolysin system, not recognized in the early studies by Lang and Beatty, mediates GTA release from this alphaproteobacterium (60). It is likely that this mechanism generally provides the means for GTA release.

## INVOLVEMENT OF HOLINS IN BACTERIAL BIOFILM FORMATION

Bacteria can exist in a motile, free-swimming, planktonic state or a sessile communal state, generally referred to as a biofilm (61). Several reports have led to the conclusion that holins probably play important roles in biofilm formation. For example, the *Staphylococcus aureus* *cid* and *lrg* operons are known to be involved in biofilm formation by controlling cell lysis and the release of genomic DNA, which ultimately becomes a structural component of the biofilm matrix (62, 63). Evidence has been presented suggesting that the *cidA* and *lrgA* genes encode holin- and antiholin-like proteins which function to regulate processes similarly to bacteriophage-induced death and lysis. Ranjit et al. (64) focused on the biochemical and molecular characterization of CidA (TC no. 1.E.14.1.2) and LrgA (TC no. 1.E.14.1.1) with the goal of testing the holin model. Membrane fractionation and fluorescent protein fusion studies revealed, first, that CidA and LrgA are membrane-associated proteins and, second, that, similarly to holins, CidA and LrgA oligomerize into high-molecular-mass complexes in a process dependent on disulfide bridge formation. An *S. aureus* mutant, in which the wild-type *cidA* or *lrgA* gene was replaced with a cysteine-to-serine mutant allele, exhibited altered cell lysis during stationary phase. Moreover, during early biofilm development, altered cell adhesion was observed, and dead cells accumulated during biofilm maturation. Thus, it appeared that CidA, probably in conjunction with LrgA, functions to temporally control the timing of cell lysis and DNA release during biofilm development. It should be noted, however, that although the available evidence favors the proposed holin and antiholin functions of CidA and LrgA, respectively, their functions have not been rigorously established.

*Staphylococcus epidermidis* is a commensal bacterium that can colonize the hospital environment due to its ability to form biofilms, favoring adhesion to host tissues and medical devices with increased resistance to antibiotics. In this context, the use of phage to destroy biofilms is an interesting and potentially useful possibility. The genomes of two *S. epidermidis* phages, vB\_SepiS-phiIPLA5 and vB\_SepiS-phiIPLA7, each encode lysis components, including a holin/endolysin system (62, 65). Analyses reported by Gutierrez et al. (65) suggested that these phages have antibiofilm activities that have the potential of being exploited for medical purposes.

Holin-like proteins in the dental plaque-forming *Firmicutes* member *Streptococcus mutans* have been shown to affect both biofilm formation and oxidative stress responses (66). These proteins, CidA (TC no. 1.E.14.1.10) and LrgA (TC no. 1.E.14.1.15), homologues of the *Staphylococcus aureus* proteins of the same names, appear to play analogous roles in biofilm formation on the tooth surface. The *cidAB* and *lrgAB* operons in *S. mutans* are regulated in a reciprocal fashion: the *lrg* operon is expressed maximally in the late exponential growth phase while the *cid* operon is expressed maximally during early exponential growth, both being regulated in response to oxygen and glucose. This regulation influences biofilm formation, providing another example where knowledge of holin action may have applications for the control of bacterial biofilm formation, relevant to human health. Further examples of potential medical importance will be presented below.

### CidAB/LrgAB HOMOLOGUES IN PLANT CHLOROPLASTS

In *Staphylococcus* strains, the CidA and LrgA proteins are encoded within the *cidAB* and *lrgAB* operons, respectively, which are expressed in a reciprocal fashion as noted above (67). CidB and LrgB are homologous multispinning membrane proteins of unknown function. The *cidB* and *lrgB* genes are translationally coupled to the *cidA* and *lrgA* genes, respectively, and are therefore likely to be functionally related (67, 68).

Yang et al. (69) reported the presence of an LrgB-containing domain protein in the plant *Arabidopsis thaliana* and showed that it is localized to the leaf chloroplast envelope, where it plays roles in leaf development, carbon partitioning, and leaf senescence. It appears to be equivalent to a fusion of LrgA (5 TMSs) and LrgB (7 TMSs) of *S. aureus*. Subsequently, these investigators provided evidence that this protein is a plastidic glycolate/glycerate transporter (PLGG1), involved in photorespiration (70). However, the mechanism of transport (channel or carrier) was not defined.

In a recent article, Wang and Bayles (71) suggested that the plant LrgAB protein plays a prominent role in programmed cell death, possibly functioning as a holin, replacing the Bcl-2 family proteins that function in apoptosis in animals. They showed that the *A. thaliana* LrgAB protein can augment nystatin-induced cell permeability when produced in yeast (69). This and other observations led another research group to suggest a role in the induction of programmed cell death of chlorotic cells (72). They noted, however, that evidence for the proposed function of this chloroplastic LrgAB protein could be a secondary consequence of an actual primary role in carbohydrate transport and metabolism (71). Interestingly, homologues are found in cell-walled fungi and stramenopiles as well as plants, revealing that if the biochemical functions of these proteins are the same in these eukaryotes, they cannot be restricted to a role in photosynthesis or chloroplast function. The mechanisms of action of these eukaryotic LrgAB proteins and their relationships to their prokaryotic counterparts remain an interesting problem for future study.

### USE OF HOLINS FOR THERAPEUTIC PURPOSES IN MEDICINE

Phage holin/endolysin systems are currently being pursued intensively as novel potential therapeutic agents in the current age of increasing occurrences of bacterial resistance to antibiotics (73). Lytic systems also have the potential to function as delivery systems, introducing drugs, nucleic acids, and proteins into eukaryotic cells (74). Another possibility involves the development of diagnostic methods using genetically engineered phage (73). In this section, we discuss some recent medical applications that depend on holins.

The use of lytic systems to prevent bacterial infections has the advantage over antibiotics and chemical bacteriostatic or bactericidal agents of their high specificity for their respective organisms. These systems, for example, can be used without affecting the beneficial intestinal microflora, and their use is less likely to result in the development of resistance (75, 76). This may prove to be of particular value in treating infections by drug-resistant pathogenic *Firmicutes* such as streptococci, staphylococci, enterococci, and bacilli, which cannot be treated by traditional methods. These *Firmicutes* were the first to develop drug resistance, and thus, there is a need for alternative treatment methods (77, 78).

Phages and their lytic products have been suggested to be potential agents for combating infectious bacteria, particularly in view of the increasing prevalence of multidrug resistance (MDR)

as noted above (79). In view of this suggestion, Shi et al. (80) have considered the development of antibacterial phage as a means to control infections, particularly by multidrug-resistant (MDR) *Streptococcus suis*, a zoonotic pathogen that causes septic arthritis (a toxic shock-like syndrome) and meningitis in humans and farm animals (81–83). These investigators used exogenous holin (TC no. 1.E.10.1.3) and an endolysin from the phage SMP to lyse *S. suis* and other pathogens, including *S. aureus*. Their surprising results led these authors to the conclusion that a holin, used in conjunction with a lysin, can function as an antibacterial agent to help solve the drug resistance problem arising in these and other Gram-positive bacterial pathogens (14, 80).

In an earlier study, Agu et al. (84) had shown that the cytotoxic activity of phage lambda holin reduced tumor growth rates in mammary cancer cell xenograft nodules. This work aimed to use holins in a therapeutic approach for cancer treatment because of their ability to disturb membrane integrity and inhibit protein synthesis. Stably transfected human cell lines expressed the lambda holin gene *in vivo*, first in a human breast cancer cell tumor xenograft and second in a mammary adenocarcinoma mouse model system. When the holin was inducibly expressed, a reduction in tumor cell viability by up to 98% resulted. Thus, lambda holin proved to be cytotoxic to tumor cells *in vitro* and *in vivo*, leading to its potential for use for cancer gene therapy (84). Logical extrapolation suggests that numerous holin/lysin systems could substitute for the lambda system and that each such system will have its own specific applications and disadvantages.

In a follow up study, these same investigators demonstrated that phage lambda holin can cause caspase-independent, nonapoptotic mammalian cell death (85). They showed that in this case, the holin localized to the endoplasmic reticular and mitochondrial membranes. The latter association resulted in loss of the mitochondrial membrane potential. Vacuolization also occurred, indicative of necrosis. These findings provided mechanistic information underlying the potential therapeutic use of holins to control cancer. Toward this end, the use of retroviral vectors for cancer gene therapy has proven effective (86). In this case, a cytotoxic gene was stably expressed, resulting in death to the targeted cell.

In another study, Kuo et al. (87) used a stably engineered suicidal strain of *Listeria monocytogenes* to deliver proteins or nucleic acids to fully differentiated human intestinal epithelial cells. These investigators used a holin/endolysin gene pair from *Listeria*-specific phage A118 and expressed it in a strain of *L. monocytogenes* under the control of a *Listeria* promoter. Upon induction, the bacteria lysed inside the mammalian intestinal cells, releasing their contents. Engineered shuttle vectors were designed to facilitate expression of transgenes in the attenuated bacteria, releasing the synthesized protein into the cell cytoplasm. This *Listeria* delivery system has potential use for oral vaccination using antigens synthesized by a bacterium (87). The possibilities of using holins for medical purposes will require creative efforts before their use can be realized in practice.

Phages and their bacteriolytic gene cassettes can serve as biocontrol agents for food safety (88). Examples abound. *Shewanella putrefaciens*, *Photobacterium phosphoreum*, and *Pseudomonas* spp. are common contaminants of harvested fish and can drastically decrease the shelf life of chilled fish (89). In fresh fish, *S. putrefaciens* can account for as much as 75% of the bacterial spoilage organisms. Bacterial lytic systems, involving endolysins released by holins from the bacterial cytoplasm during phage infection, are

TABLE 2 Holins involved in physiological processes other than phage-promoted lysis

Application	Organism(s)	Holin	TC no.	Reference
Spore morphogenesis and germination	<i>Bacillus subtilis</i>	YwcE	1.E.23.1.1	105
Biofilm formation and DNA release	<i>Staphylococcus aureus</i>	CidA	1.E.14.1.2	99
		LrgA	1.E.14.1.1	
Programmed cell death and acetate metabolism	<i>Staphylococcus aureus</i>	CidA	1.E.14.1.2	106
		LrgA	1.E.14.1.1	
Biofilm formation and oxidative stress adaptation	<i>Streptococcus mutans</i>	LrgA	1.E.14.1.10	102
Gene transfer	<i>Rhodococcus capsulatus</i>	GTA holin	1.E.54.1.1	58
Gene transfer	<i>Brachyspira hyodysenteriae</i>	GTA holin	1.E.55.1.1	59
Programmed cell death	<i>Homo sapiens</i> and other mammals	Bak	1.A.21.1.2	32
		Bax	1.A.21.1.3	

effective food preservatives (90). These systems have been used for pathogen control in human food sources, including fish, milk, cheese, eggs, and poultry (91–93). They similarly should be useful antibacterial agents in animal feed stocks. It is possible that the use of phage endolysins will prove to be especially applicable to low-G+C-content Gram-positive bacteria which lack an outer membrane.

#### USE OF HOLINS FOR BIOTECHNOLOGICAL MATERIAL PRODUCTION

In the previous section, several potential medical applications of holins were discussed, and some of these approaches can potentially be used to bring about technological advances as well. For example, microbes are used to produce all sorts of compounds, from drugs to biofuels. In the absence of an efficient active efflux system, these compounds can be sequestered inside the cell where they may exert deleterious or even toxic effects, inhibiting growth and substrate production. The use of holins may obviate the former problem by promoting release of the desired products by controlled pore formation. In other cases, holin-mediated cell lysis provides an effective release mechanism.

An example was provided by Gao et al. (94). They noted that product release from microbial cells is often the rate-limiting step for industrial-scale production of biotechnology-based chemicals, and the nature of cell lysis via mechanical disruption or solvent extraction can contribute substantially to the total cost of production. They further pointed out that holins provide a cheap and efficient means of product release. An advantage is that holin production can be controlled by conditionally inducible regulatory signals and the judicious use of effective expression promoters. These approaches have been applied, for example, to the production of fatty acids and polyhydroxyalkanoates. Gao et al. (94) argued that inducible cell lysis systems can be both economically feasible and subject to precise controlled timing.

Blue-green bacteria (cyanobacteria) are being developed for lipid/biofuel production (95). These organisms capture CO<sub>2</sub> via photosynthesis and can be grown in aqueous environments on nonarable land. Again, a major problem deals with procedures for extraction. Liu and Curtiss (96) incorporated phage-derived lysis genes into the chromosome of one such production organism, *Synechocystis* sp. strain PCC6803, downstream of a nickel-inducible signal transduction system. Among the systems developed were phage holin/endolysin cassettes. Addition of Ni<sup>2+</sup> to the culture medium resulted in lysis, eliminating the need for mechanical or chemical extraction (96). For similar purposes, Miyake et al.

used a green light-inducible lytic system in the same organism (97).

Park et al. (74) noted that bacterial minicells are sometimes used to deliver drugs and genes to target cells. They developed a strategy, based on a holin/lysin system, to provide minicells in greater purity and yield. They also used a readily controlled induction system to promote autolysis of actively metabolizing cells. In this case, autolysis followed by centrifugation resulted in both increased yields and enhanced degrees of purity in less time and with less expense than conventional multistep procedures (74). It is not surprising that holins are now being considered for many biotechnological applications.

#### PERSPECTIVES AND CONCLUSIONS: A PLETHORA OF HOLIN FUNCTIONS AND USES

In this minireview, we have noted that holins, thought initially to exert their primary effects by allowing phage particle release from virus-infected bacteria, also play roles in and are important for numerous physiological processes in virus-free bacteria (Table 2). These proteins are constituents of chromosomally encoded gene transfer agents (GTAs) (98), which resemble incomplete prophages that lack the ability to infect cells. GTAs function exclusively in horizontal gene transfer between microbes (54).

Holins have also been shown to influence the communal life of bacteria during biofilm formation (65, 99). Phage lytic systems can reduce biofilm formation in some instances (100), while conversely, biofilms may exhibit differential resistance to the lytic activities of phage (101). Holins also play roles in stress responses (102), virulence (66), release of toxins and other proteins (103, 104), and bacterial sporulation and spore germination (105). They may play widespread roles in protein trafficking and secretion to the extracellular environment (18). Holins can function as antimicrobial agents (77) and are useful for biotechnological material production schemes (Table 3) (94). We can anticipate that these chromosomally encoded gene cassettes will prove to have many functions useful to the bacterium as well as humankind.

An intriguing observation concerns the mechanism of apoptosis in mammalian cells. It is well recognized that apoptosis involves mitochondria which play an initializing role by releasing mitochondrial proteins such as cytochrome *c* to the cell cytoplasm. Proteins long known to play important roles in this process are the many members of the Bcl-2 family, including Bax and Bak. The discovery that these Bcl-2 proteins function like holins in promoting apoptosis (32) leads to the obvious suggestion that these animal proteins had their evolutionary origins in bacterial ho-



TABLE 3 Practical applications of holin/lysin systems

Application	Advantages	Reference(s)
Therapeutic control of bacterial or viral infections	Specificity with minimal perturbation of intestinal flora; less opportunity for resistance	73, 80
Delivery of drugs, nucleic acids, and proteins to animal/plant cells	Specificity for target cells or a specific tissue; novel approaches; control of timing	74, 87
Use as anticancer agents	Specificity to disrupt tumor cell membranes; reducing membrane potential; causing apoptosis	84, 85, 86
Oral vaccination	Specificity for antigen delivery; control of timing	87
Food preservation	Specificity for certain bacteria; ease of application	89, 90
Biofuel/product release from cells	Controlled biofuel/product release or controlled cell lysis; cost-effectiveness	94, 95, 96, 97
Diagnostic tool development	Specificity in the use of genetically engineered phage; high sensitivity	73

lins. Since chromosomally encoded holins can mediate programmed cell death in bacteria (106), this possibility seems eminently plausible (107). Of course, in the absence of definitive evidence, convergent functional evolution from dissimilar primordial sources is also possible.

We have identified putative archaeal holin homologues belonging to several recognized families, but none of these proteins has been functionally characterized. What role could they be playing in archaeal physiology? Do they mediate archaeal phage particle release? Do they function in programmed cell death? Do they facilitate biofilm formation as in bacteria? Recent reports suggest that archaeal cell walls consist in part of pseudomurein (108). Moreover, cell wall binding domains, such as the archaeal pseudomurein binding domain, play essential roles in the noncovalent attachment of functional proteins to the cell walls of these organisms, just as LysM domains influence many extracellular functions in bacteria (108). Archaeal holin research represents a new frontier in the study of cell physiology in this “third domain” of living organisms.

All of the observations regarding the diverse functions of holins and their homologous antiholins can be put into an evolutionary framework only if these proteins are classified within families, superfamilies, and subfamilies based on sequence comparisons. This goal has recently been accomplished with bioinformatic analyses that have grouped a substantial number of the currently recognized holin families into seven superfamilies of related proteins (5). Members of a family or superfamily often, but not always, exhibit similar topologies and presumably similar mechanisms of action. However, high-resolution 3-dimensional structural data for these proteins are still lacking. These will greatly facilitate the assignment of distant homologues to the major holin superfamilies and should clarify their mechanisms of action. It seems clear that any systematic structure/function analyses of holins will depend on the phylogenetic characteristics of these small pore-forming proteins.

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