Functional and structural features of the holin HOL protein of the Lactobacillus plantarum phage Φg1e: analysis in Escherichia coli system

Functional and structural features of the holin HOL protein of the *Lactobacillus plantarum* phage ϕg1e: analysis in *Escherichia coli* system

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Abstract

Lactobacillus plantarum phage ϕg1e has two consecutive cell lysis genes *hol–lys* (Oki et al., 1996b). In the present study, functional and structural properties of the *hol* protein (Hol) were characterized in *Escherichia coli*. Electron microscopic examinations showed that *hol* under p*lac* in *E. coli* XL1-Blue injured the inner membrane to yield empty ghost cells with the bulk of the cell wall undisturbed. Northern blot analysis indicated that *hol–lys* genes under p*lac* were co-transcribed, although the amount of *hol* transcript was larger than that of *lys*, ceasing via an apparently ρ-independent terminator just downstream of *hol*. However, deletion and/or fusion experiments suggested that: (1) the N-terminal half of ϕg1e Hol composed of three putative transmembrane domains may be responsible for interaction with membrane; (2) the N-terminal end (five amino acids) seems nonessential; and (3) the C-terminal half containing charged amino acids appears to be involved in proper *hol* function. These results suggest that ϕg1e Hol is a member of the lambdoid holin family, but divergent in several properties from lambda holin.

Keywords: Bacteriophage ϕg1e; Deletion; Fusion; Holin; Transmembrane

1. Introduction

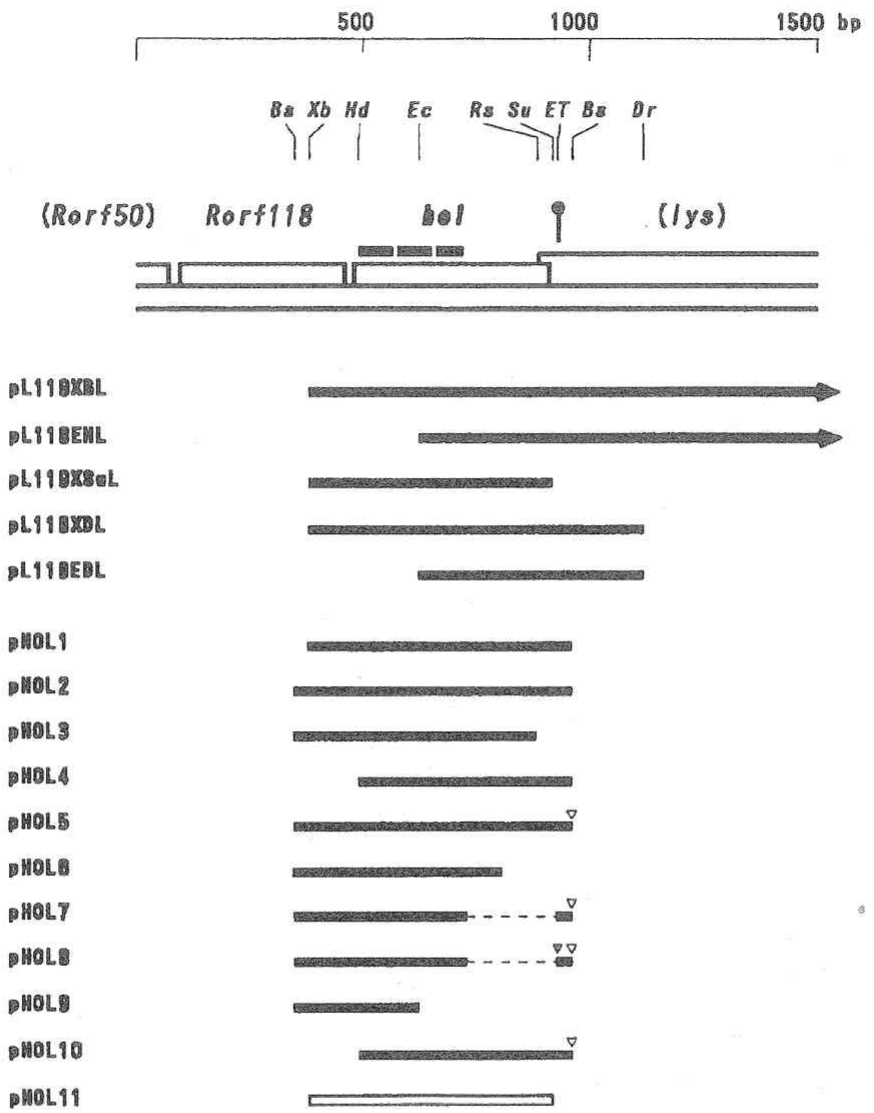
For release of progeny particles from the host cell, phages appear to encode a set of enzymes that degrade the host cell envelope, consisting of several structural components, including peptidoglycan layer and cytoplamic membrane (Young, 1992). In coliphages, such as lambda (Blasi and Young, 1996) and P2 (Ziermann et al., 1994), the cell lysis has been presumed to depend upon phage-encoded proteins: e.g. holin and endolysin. Holins have been thought to form a hole in the cytoplasmic membrane, through which endolysin can attack the peptidoglycan layer.

Contrary to the lambdoid system, molecular details on the cell lysis by the temperate phages of lactic acid bacteria are still scanty, although several putative genes for holin and endolysin have been reported from the *Lactobacillus gasseri* phage ϕadh (Henrich et al., 1995), the *Lactobacillus bulgaricus* phage mv1 (Boizet et al., 1990), the *Lactococcus lactis* phage ϕLC3 (Birkeland, 1994), Tuc2009 (Arendt et al., 1994) and ϕvML3 (Shearman et al., 1994), and the *Listeria* monocins (Zink et al., 1995).

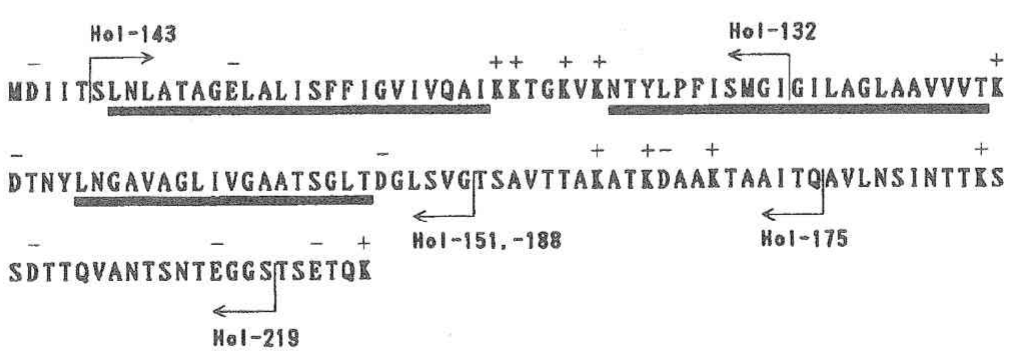
Recently, we isolated a new phage ϕg1e (Kakikawa et al., 1996) from *Lactobacillus* sp., now identified as *L.*

Abbreviations: am, amber; Amp, ampicillin; β-gal, β-galactosidase; bp, base pair; EDTA, ethylenediaminetetra-acetic acid; IPTG, isopropyl-β-D-thiogalacto-pyranoside; *hol*, gene encoding holin (Hol); Hol-143, Hol-219, and Hol-151, Hol derivatives; kDa, kilodalton; LB, Luria–Bertani (medium); *lys*, gene encoding endolysin (Lys); p*I*, isoelectric point; p*lac*, promoter of *lacZ'* gene; r, resistant; *Rorf118*, putative gene encoding 118-amino-acid protein; *Rorf50*, putative gene encoding 50-amino-acid protein; ^{s}, sensitive; SD, Shine Dalgarno; SDS, sodium dodecyl sulfate; T_{hol}, putative terminator downstream of *hol*; T_{lys}, putative terminator downstream of *lys*; vol., volume; $^+$, utilizable.

(A)


500
1000
1500 bp
Ba Xb Hd Ec Rs Su ET Ba Dr
(Rorf50)
Rorf118
Bel
(lys)
pL118XBL
pL118EHL
pL118XSaL
pL118XDL
pL118EBL
pHOL1
pHOL2
pHOL3
pHOL4
pHOL5
pHOL6
pHOL7
pHOL8
pHOL9
pHOL10
pHOL11

(B)


Hol-143
Hol-132
MDIITSLNLATAGELALISFFIGVIVQAIKKTGKVKNTYLPFISMGIGILAGLAAVVVTK
DTNYLNGAVAGLIVGAATSGLTDGLSVGTSAVTTAKATKDAAKTAAITQAVLNSINTTKS
Hol-151, -188
Hol-175
SDTTQVANTSNTEGGSTSETQK
Hol-219

plantarum, and have determined the whole genome sequence of 42 259 bp (Kodaira et al., 1997; EMBL accession number X98106). In a previous study (Oki et al., 1996b), we reported two putative cell lysis genes, *hol–lys*, encoded within an approximately 1500-bp DNA region of the ϕg1e genome. *hol* encodes a basic protein (Hol) of 142 amino acids with an estimated p*I* of 9.45, and *lys* encodes an endolysin-like protein (Lys) of 422 amino acids.

Hol contains potential transmembrane domains separated by a short beta-turn linker in the N-terminal half and charged amino acids in the C-terminal half, structurally resembling presumed holins from other phages from lactic acid bacteria, such as mv1 and ϕadh, as well as coliphage lambda (Blasi and Young, 1996). Otherwise, ϕg1e Hol shares no amino acid homology with other holins. Unlike lambda holin, the N-terminal end of ϕg1e Hol has no basic amino acid, such as Lys residue that is thought to be pivotal in lambda holin. When expressed in *E. coli*, ϕg1e *hol* significantly decreased the cell turbidity, suggesting that Hol is probably involved in cell lysis directly, as a holin.

In the present study, further functional and structural properties of ϕg1e *hol* were characterized in *E. coli*, and showed that Hol probably belongs to the lambdoid holin family, but somewhat deviates from lambda holin.

2. Results and discussion

2.1. *Outlines of the ϕg1e* hol *gene*

Fig. 1 shows an 1500-bp physical map containing ϕg1e *hol* and the predicted amino-acid sequence of the *hol* protein (Hol). As shown in Fig. 2A, *hol* has only one possible start codon ATG, accompanied by two potential SD sequences, 5′-AAGGAGG-3′ and 5′-GGAaGT-3′, and contains no secondary structures overlapping the putative SD sequence, essential for the so-called dual start motif in lambda holin (Young, 1992). In an upstream region of the start codon of *hol*, ϕg1e has three direct repeats with 8-bp consensus sequence 5′-GCATGATA-3′ (Fig. 2A). As to *hol* transcription, two potential termination sites were found in this study just downstream regions of *hol* and *lys* (Fig. 2; for details, see below), but its transcriptional initiation site(s) is still unknown.

2.2. hol-*mediated morphological changes in* E. coli

As mentioned above, expression of *hol* and/or *lys* in *E. coli* XL1-blue decreased turbidity of the cell culture. The cell lesions induced by the *hol–lys* system were morphologically analyzed by electron microscopy. Upon induction with IPTG, XL1-Blue carrying pL119XSuL encoding *hol* (see Fig. 1A) yielded translucent (empty ghost) cells (Fig. 3A). The cytoplasmic membranes of these ghost cells were disrupted locally and severely, whereas the cell walls seemed to remain almost normal. Contrary to *hol*, *lys* (XL1-Blue/pL118EHL) alone caused curling up of the cell wall on the bulge (Fig. 3B), presumably via an *E. coli* autolytic process as predicted by Young (1992). However, co-expression of *hol–lys* in XL1-Blue/pL119XBL led to substantial changes in both cytoplasmic membrane and cell wall (Fig. 3C). Recently, similar cellular morphological changes have been reported in the *hol–lys* system of pneumococcal phage EJ-1 (Diaz et al., 1996).

2.3. *Transcription of hol in* E. coli

To analyze transcripts of *hol*, the total content of RNA was extracted from *E. coli* XL1-Blue carrying a pL-plasmid, and subjected to Northern blot hybridization (Fig. 4). ϕg1e-specific RNA species were detected with a 300-bp *Eco*RI/*Sau*3AI fragment from the *hol* region as a probe (see Fig. 1A): pL119XBL induced three main RNAs of about 2840, 1860, and 600 bases; pL118EHL, two main RNAs (2240 and 1600 bases) as well as one minor RNA (1000 bases); pL119XSuL, one minor RNA (1040 bases); pL118XDL, two RNAs of 1240 (major) and 600 (minor) bases; and pL118EDL, broad RNA bands centered at about 980 bases. In the three plasmids, pL118XDL, pL119XSuL and

Fig. 1. Construction of the ϕg1e *hol* mutants. (A) At the top, a physical map of 1500-bp *hol* region is shown. The ϕg1e DNA is shown by two thick lines. The four putative genes *Rorf50–Rorf118–hol–lys* (Oki et al., 1996b) are indicated by open boxes. A ρ-independent-like terminator (see Fig. 4) was represented by a closed circle with a bar. Three possible transmembrane domains in the *hol* protein (Hol) are indicated by small closed boxes. Restriction enzyme sites used in this study are also shown: Bs, *Bsm*I; Ec, *Eco*RI; ET, *Eco*T14I; Dr, *Dra*I; Hd, *Hin*dIII; Su, *Sau*3AI; Xb, *Xba*I. The five pL-plasmids were from Oki et al. (1996b): pL119XBL and pL118EHL contained *lys* where indicated by arrows. The pHOL-plasmids were constructed in the present study (see Table 1). pHOL1 and pHOL2, see text; for pHOL3, a 560-bp *Pst*I/*Rsa*I fragment from pHOL2 was cloned into pUC119 using its *Pst*I and *Eco*RI (blunted) sites; pHOL4 (or pHOL10) was derived from pHOL2 (pHOL5), using a *Hin*dIII site; in pHOL5, one 3′-nucleotide (G) of the *Bsm*I/*Bsm*I fragment was deleted by nuclease method (Oki et al., 1996b); pHOL6 and pHOL7 were deletion derivatives from pHOL5 (with the *Eco*T14I site); for pHOL8, a 638-bp *Pst*I/*Rsa*I fragment of pHOL7 was recloned into *Pst*I/*Sma*I sites of pUC119; pHOL9 was a deletion derivative from pHOL5 (with the *Eco*RI site); an open box indicated pHOL11, in which a 530-bp *Xba*I/*Sau*3AI fragment was cloned into pUC118, to make *hol* free from *plac*. Dotted line, deleted regions; ▽, one nucleotide deletion; ▼, four nucleotides insertion. (B) Amino acid sequence of Hol. Amino acid sequence of Hol predicted by DNA sequence was from Oki et al. (1996b). The potential transmembrane domains are underlined. +, basic residues; −, acidic residues. Arrows indicate the start points of Hol mutants confirmed by DNA sequencing (see also Table 2).

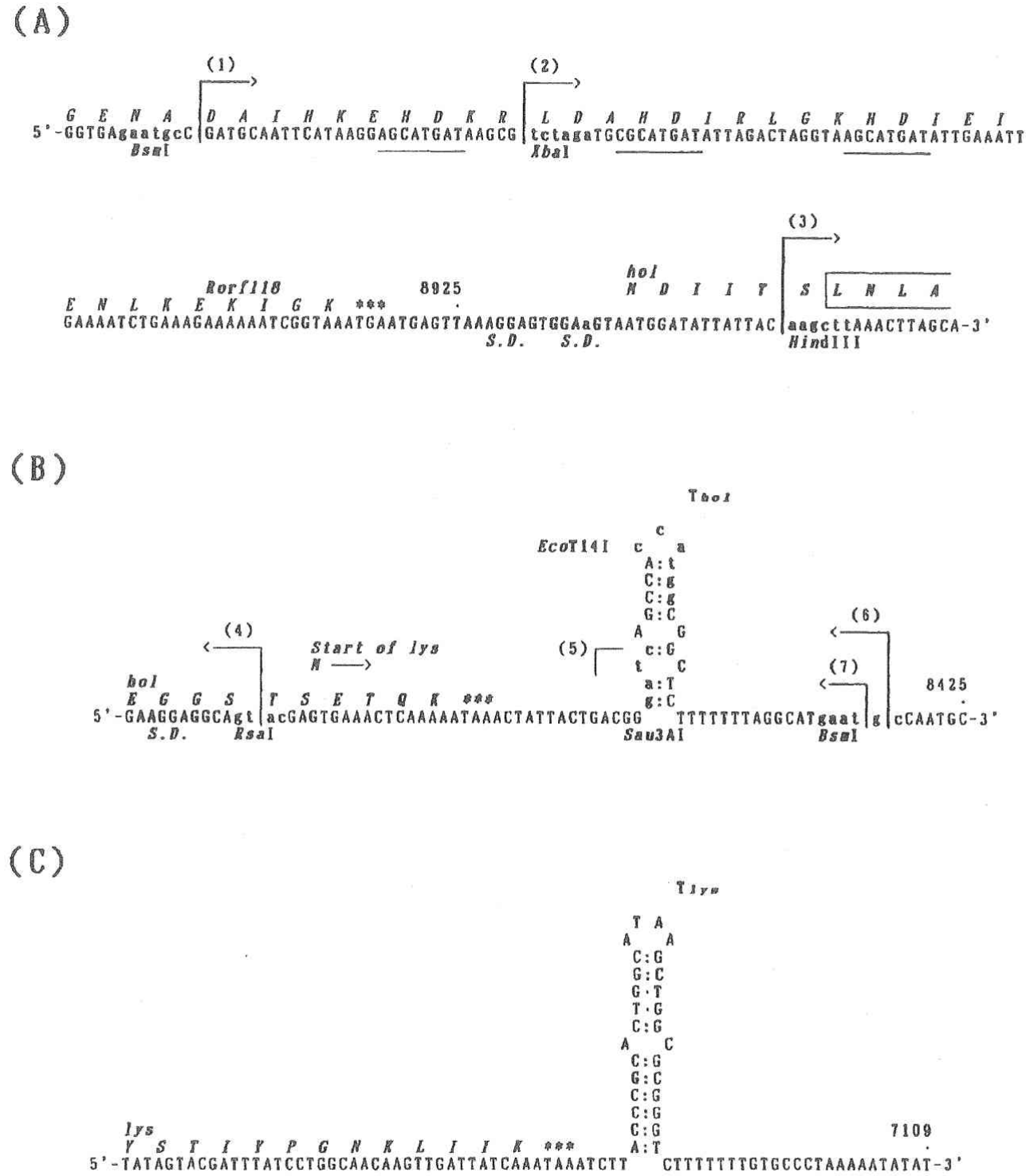


Fig. 2. DNA sequences surrounding *hol*. The nucleotide numbers were from Kodaira et al. (1997): EMBL accession number X98106. The four putative genes *Rorf50–Rorf118–hol–lys* and their predicted amino acids were from Oki et al. (1996b). Restriction sites used in this study are denoted by bold italic lower-case letters. The two possible Shine Dalgarno sequences (SD) are represented by bold letters. Asterisk, stop codon. (A) The upstream region of *hol*. The 8-bp direct repeats are underlined. An open box showed one of the putative transmembrane domains (see Fig. 1B). Arrows (1–3) indicated the cloning points confirmed by DNA sequencing: (1), pHOL2, pHOL3, pHOL5, pHOL6 and pHOL7; (2), pHOL1 and pHOL11; (3) pHOL4 and pHOL10. (B) Secondary structure of a potential terminator (T_{hol}) downstream of *hol*. The amino acid sequence of *lys* was omitted for clarity. Arrows (4–7) indicated the cloning points confirmed by DNA sequencing (Kodaira et al., 1996): (4), pHOL3; (5), pHOL11; (6), pHOL2 and pHOL4; (7), pHOL5 and pHOL10. (C) Secondary structure of a potential terminator (T_{lys}) downstream of *lys*.

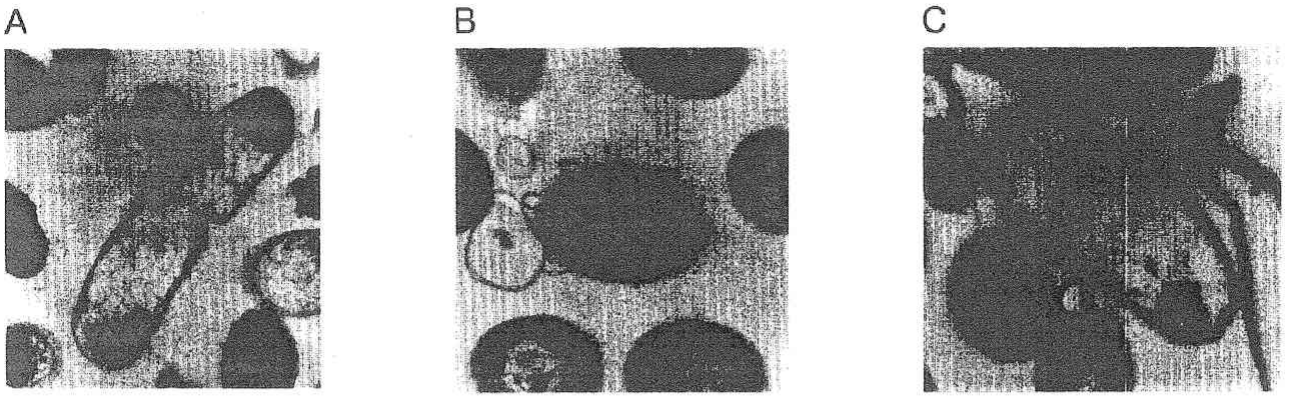


Fig. 3. Morphological changes in *E. coli*. *E. coli* XL1-blue carrying the plasmid pL119XSuL (*hol*), pL118EHL (*lys*) or pL119XBL (*hol–lys*) was grown at 25°C in LB broth containing ampicillin (60 μg/ml). When the A_{660} of the culture had reached 0.3, the growth temperature was shifted to 37°C with concomitant addition of IPTG (1 mM). After 30 min of induction, morphological changes in the cell were examined with electron microscopy (Kakikawa et al., 1996). (**A**) XL1-blue/pL119XSuL (×19 500); (**B**) XL1-blue/pL118EHL (×26 000); (**C**) XL1-blue/pL119XBL (×26 000).

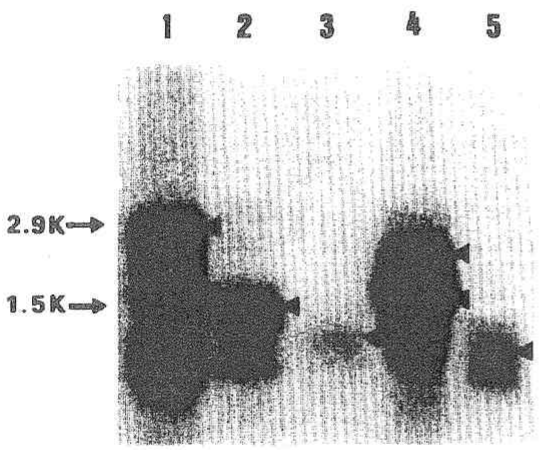


Fig. 4. Transcripts of *hol*. *E. coli* XL1-Blue carrying the plasmid pL119XBL (lane 1), pL118XDL (lane 2), pL119XSuL (lane 3), pL118EHL (lane 4) or pL119EDL (lane 5) was induced by IPTG (1 mM) at 37°C. After 30 min of induction, the total content of RNA was extracted, and subjected to Northern blot analysis (Oki et al., 1996a), using a ^{32}P-labeled 300-bp *Eco*RI/*Sau*3AI fragment as a probe (Fig. 1A). The main RNA bands (see text) are indicated by triangles. The arrows indicate the positions of *E. coli* ribosomal RNAs, 23s and 16S (2.9 and 1.5 kb, respectively).

pL119EDL, the amount of transcripts was significantly smaller than that in p119XBL or pL118EHL.

Using other probes from the *hol–lys* region as well (data not shown), these φgle transcripts were judged to start from p*lac*, and terminate at three different points. Among these termination sites, the two (termed T_{hol} and T_{lys}) were mapped in downstream regions of *hol* and *lys*, respectively (Fig. 2), whereas the third was in the vector. Both T_{hol} and T_{lys} contained a potential secondary structure ending in a run of T residues, which may act as a ρ-independent-like terminator. However, the termination ability of T_{hol} or T_{lys} appeared to be partial (Fig. 4). As expected, pL119XSuL lacking in T_{hol} did not induce such a predicted RNA-600 spanning from p*lac* to T_{hol}. Regardless of the presence of T_{hol}, neither pL118EHL nor pL119EDL induced a detectable amount of a predicted RNA-350 ranging from p*lac* to T_{hol} (Fig. 4). The reason for the absence of RNA-350 is presently unknown.

2.4. *Construction of* hol *mutants*

In the present study, we constructed several *hol* mutants containing *hol–lacZ′* fusions (Table 1 and Fig. 1). First, two restriction fragments containing SD_{hol}–*hol*–T_{hol}, *Xba*I/*Bsm*I and *Bsm*I/*Bsm*I, were cloned into pUC119 to place *hol* under p*lac* control, yielding pHOL1 and pHOL2, respectively. pHOL2 contains three 8-bp consensus (see Section 2.1). The two plasmids carry a *lys*(23 codons)–*lacZ′* fusion. From this *lys–laZ′* hybrid, one 3′-nucleotide (G) of the *Bsm*I/*Bsm*I fragment was deleted, resulting in pHOL5.

Based on pHOL2 and pHOL5, *hol* mutants with deletion and/or fusion (pHOL3, 4, 6, 7, 8, 9 and 10) were constructed (for details, see Fig. 1). The mutated Hol of all the mutants except pHOL9 retained the three putative transmembrane domains (see Fig. 1B and Table 2); pHOL9 contained the first (N-terminal) transmembrane domain and a truncated second domain. The four plasmids, pHOL3, pHOL6, pHOL8, and pHOL9, carry a *hol–LacZ′* fusion; pHOL3 and pHOL8 gave blue colonies on a X-gal plate, whereas the coloration of pHOL9 was light blue. However, pHOL6 formed white colonies, because its fused LacZ′ is probably too short to yield activity. In pHOL4 or pHOL10, N-terminal 5-amino acids of Hol (MDIIT) were replaced with N-terminal 6-amino acids of LacZ′ (MTMITP), and they were lacking in the three 8-bp direct repeats (see above). The mutated Hol proteins encoded by these

Table 1
Bacteria, plasmids, and phages

Strain	Genotype or construct	Source or reference
E. coli		
W3110	Wild-type	Lab. stock[a]
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	H. Inokuchi
594	*sup°*	H. Inokuchi
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac⁻ F'[proAB⁺ lacI^q lacZ ΔM15 Tn10(tet^r)]*	Lab. stock
Plasmids		
pUC118	*Ap^r*, p*lac*, *lacZ'*, M13 origin	Lab. stock
pUC119	*Ap^r*, p*lac*, *lacZ'*, M13 origin	Lab. stock
pL119PPR	pUC119::2963-bp PstI fragment from ϕg1e DNA (*Rorf50–Rorf118–hol–lys* under p*lac*)	Oki et al. (1996b)
pL119XBL	pUC119::2338-bp *Xba*I–*Bam*HI fragment from pL119PPR (*hol* and *lys* under p*lac*)	Oki et al. (1996b)
pL118EHL	pUC118::1760-bp *Eco*RI–*Hin*dIII fragment from pL119PPR (*lys* under p*lac*)	Oki et al. (1996b)
pL119XSuL	pUC119::530-bp *Xba*I–*Sau*3AI fragment from pL119PPR (*hol* under p*lac*)	Oki et al. (1996b)
pL118XDL	pUC118::732-bp *Xba*I–*Dra*I fragment from pL119PPR (*hol* and *Δlys–ΔlacZ'* under p*lac*)	Oki et al. (1996b)
pL118EDL	pUC118::502-bp *Eco*RI–*Dra*I fragment from pL118XDL (*Δlys–ΔlacZ'* under p*lac*)	Oki et al. (1996b)
pHOL1	pUC119::580-bp *Xba*I–*Bsm*I fragment from pL119PPR (*hol* and *Δlys–ΔlacZ'* under p*lac*)	This study
pHOL2	pUC119::609-bp *Bsm*I fragment from pL119PPR (*hol* and *Δlys–ΔlacZ'* under p*lac*)	This study
pHOL3	Deletion derivative of pHOL2 (*Δhol–ΔlacZ'* under p*lac*)	This study
pHOL4	Derivative of pHOL2 (*ΔlacZ'–Δhol* under p*lac*)	This study
pHOL5	pUC119::608-bp[b] from pL119PPR (*hol* under p*lac*)	This study
pHOL6	Deletion derivative of pHOL5 (*Δhol–ΔlacZ'* under p*lac*)	This study
pHOL7	Deletion derivative of pHOL5 (*Δhol* under p*lac*)	This study
pHOL8	Insertion derivative of pHOL7 (*Δhol–ΔlacZ'* under p*lac*)	This study
pHOL9	Deletion derivative of pHOL5 (*Δhol–ΔlacZ'* under p*lac*)	This study
pHOL10	Derivative of pHOL5 (*ΔlacZ'–Δhol* under p*lac*)	This study
pHOL11	pUC118::530-bp *Xba*I–*Sau*3AI fragment from pL119PPR (*hol* free from p*lac*)	This study
Phages		
λ	Wild-type	Lab. stock
λ *S*am7	Amber mutant of λ *S* gene	H. Inokuchi
λ *R*am216	Amber mutant of λ *R* gene	H. Inokuchi

[a]See Kodaira et al. (1995a,b) and Oki et al. (1996a).
[b]Lacking in one 3'-nucleotide (G) of the 609-bp *Bsm*I fragment (see text).

pHOL-plasmids were summarized in Table 2 (see also Fig. 1B): Hol-219 (encoded by pHOL3), Hol-143 (pHOL4 or pHOL10), Hol-175 (pHOL6), Hol-151 (pHOL7), Hol-188 (pHOL8) and Hol-132 (pHOL9).

In addition, a 530-bp *Xba*I/*Sau*3AI fragment was cloned into pUC118 resulting in pHOL11, whose *hol* was free from p*lac* control. All the pHOL-plasmids thus constructed were retained stably at 25°C in *E. coli*.

2.5. *Cell-lysis properties of* hol *mutants*

To estimate cell-lytic ability, changes in turbidity of *E. coli* XL1-Blue carrying each of the pHOL-plasmids were monitored at different temperatures (Fig. 5). The seven plasmids, pHOL 1–6 and 10, induced a decrease in turbidity of the culture, about 150 min after a temperature shift from 25° to 37°C, although rates of cell lysis varied among the plasmids. This turbidity-decrease at 37°C without IPTG induction has been thought to be due to a leakiness of *lacI^q* control and/or a high copy number of the recombinant plasmid (Oki et al., 1996b). The four plasmids pHOL-7, -8, -9, and -11 did not cause the turbidity decrease.

Upon induction by IPTG at 37°C (Fig. 5), three plasmids containing wild-type *hol*, pHOL1, 2, and 5, led to a faster onset of turbidity-decrease than that without IPTG treatment. In the *hol* mutants, five plasmids, pHOL3, 4, 6, 8, and 10 also decreased the culture turbidity, accompanied by formation of empty cells (data not shown), although their abilities of cell lysis appeared to be low, when compared with the wild-type.

In other plasmids, pHOL7, lacking in the Hol

Table 2
Amino acid sequences of ϕgle Hol and its deletion and/or fusion derivatives

Putative protein	AA	Cell lysis ability	Sequence
			<——————> turn <——————>turn <——————>
Hol	142	+++++	MDIITS LNLATAGELALISFFIGVIVQAI KKTGKVK NTYLPFISMGIGILAGLAAVVVT KDTNY LNGAVAGLIVGAATSGLT DGLSVGTSAVTTAKATKDAAKTAAITQAVLNS
Hol-143	143	++++	mtm:tp: :::::::::::::::::::::::: ::::::: :::::::::::::::::::::::: ::::: :::::::::::::::::: ::::::::::::::::::::::::::::::::
Hol-219	219	++	:::::: :::::::::::::::::::::::: ::::::: :::::::::::::::::::::::: ::::: :::::::::::::::::: ::::::::::::::::::::::::::::::::
Hol-175	175	++	:::::: :::::::::::::::::::::::: ::::::: :::::::::::::::::::::::: ::::: :::::::::::::::::: ::::::::::::::::::::::::::::::-----
Hol-188	188	++	:::::: :::::::::::::::::::::::: ::::::: :::::::::::::::::::::::: ::::: :::::::::::::::::: ::::::----------------------------
Hol-151	151	-	:::::: :::::::::::::::::::::::: ::::::: :::::::::::::::::::::::: ::::: :::::::::::::::::: ::::::----------------------------
Hol-132	132	-	:::::: :::::::::::::::::::::::: ::::::: ::::::::::::---------- ----- ---------------------- ------------------------------
Hol			INTTKSSDTTQVANTSNTEGGSTSETQK
Hol-143			:::::::::::::::::::::::::::::
Hol-219			:::::::::::::::::::::::::---------- lavvlqrrdwenpgvtqlnrlaahppfaswrnseeartdrpsqqlrslngewrlmryfllthlcgishrirqsnhstrpvaah
Hol-175			---------- lnrlaahppfaswrnseeartdrpsqqlrslngewrlmryfllthlcgishrirqsnhstrpvaah
Hol-188			----------maalflgmnw vpssnslavvlqrrdwenpgvtqlnrlaahppfaswrnseeartdrpsqqlrslngewrlmryfllthlcgishrirqsnhstrpvaah
Hol-151			----------maalflgmngyrarihwpsfynvvtgktlalpnlialqhiplspagviakrpapialpnscaa
Hol-132			---------- nslavvlqrrdwenpgvtqlnrlaahppfaswrnseeartdrpsqqlrslngewrlmryfllthlcgishrirqsnhstrpvaah

Amino acid sequence of Hol was from Oki et al. (1996b). The putative Hol derivatives Hol-143, Hol-219, Hol-175, Hol-188, Hol-151 and Hol-132 are from pHOL4 (or pHOL10), pHOL3, pHOL6, pHOL8, pHOL7 and pHOL9, respectively (see text). AA, amino acid residues; +, lysis ability estimated in *E. coli* (see text); :, amino acids identical to that of Hol; −, deleted amino acids. The possible transmembrane regions were boxed with arrows. turn, possible beta-turn. Hol-143 has the N-terminal end of LacZ′ (boxed lower-case letters). The four derivatives, Hol-219, Hol-175, Hol-188 and Hol-132 contained the C-terminus of LacZ′ (boxed lower-case letters). The C-terminus of Hol-188 contained nine amino acids of Lys (maalflgmn, underlined). The C-terminus of Hol-151 (lower-case letters) was composed of nine amino acids of Lys (maalflgmn, underlined), and 54 amino acids from a different frame of *lacZ′*

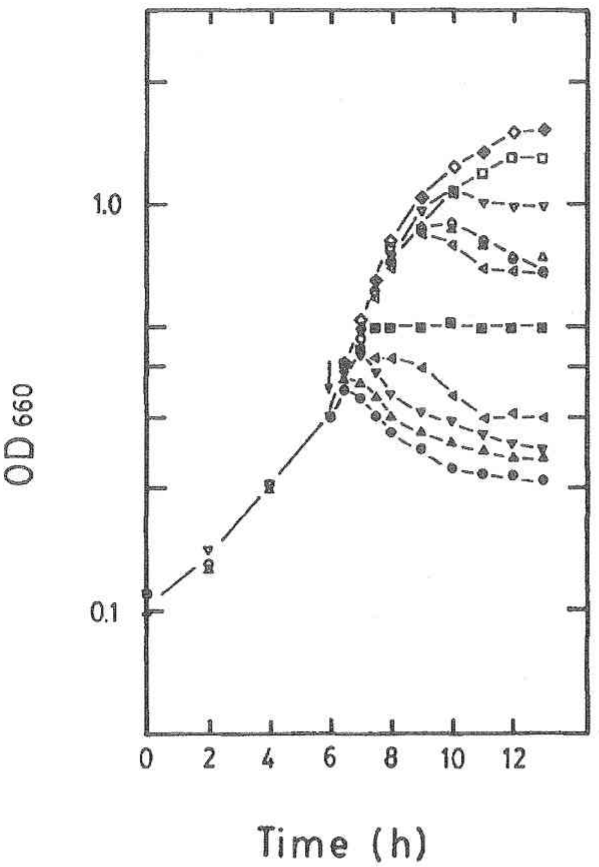


Fig. 5. Profiles of cell lysis. Cell lysis mediated by the pHOL-plasmid. *E. coli* XL1-Blue carrying the pHOL-plasmid was grown at 25°C in LB broth (Sambrook et al., 1989) with ampicillin (60 μg/ml), and increased to 37°C with or without IPTG (1 mM) at $A_{660}=0.3$. Growth of the cells was monitored by measuring the A_{660}. Open symbols, without IPTG; filled symbols, with IPTG. pHOL1, pHOL2, and pHOL5: (▲, △); pHOL4 and pHOL10: (▽, ▼); pHOL3 and pHOL6: (◁, ◀); pHOL7: (□, ■); pHOL8: (◇, ▼); pL118XDL: (○, ●); pHOL11 and pUC119: (◇, ◆). In pHOL9, ◇ (without IPTG); △ (with IPTG).

C-terminal half (Hol-151), inhibited growth of the host cell significantly, and did not decrease the cell turbidity. The cells expressing Hol-151 appeared to be unlysed microscopically (data not shown), but lost their colony-forming ability: the survival at 3 h after induction was about 10%. pHOL9, retaining only the first transmembrane domain (Hol-132), slightly inhibited the cell growth; Hol-132 seems to be somewhat toxic, but incapable of distinct cell lysis. The two plasmids, pHOL4 and pHOL10, lacking in the three 8-bp direct repeats upstream of SD_{hol} (see above) exhibited cell lysis.

2.6. *Localization of the Hol protein*

As mentioned above, the three plasmids, pHOL3 (encoding Hol-219), pHOL8 (Hol-188), and pHOL9 (Hol-132) were accompanied with β-gal activity. Taking advantage of these fusions, localization of the Hol protein in *E. coli* was investigated. β-gal activities associated with these plasmids were detected in a membrane fraction rather than cytoplasm, though the ratios (membrane/cytoplasm) of pHOL8 and pHOL9 were lower than that of pHOL3 (Table 3). These results indicate that the Hol N-terminal half composed of three transmembrane domains may be directly responsible for interaction with membrane, and the C-terminal half appears important in proper *hol* function.

2.7. *Other features of the Hol protein*

The plasmid pHOL11 did not cause a decrease in the cell turbidity at 37°C (Fig. 5). In lambda, holin and endolysin are encoded by *S* and *R* genes, respectively (Blasi and Young, 1996). Therefore, *E. coli* 594 *sup*^{c}/pHOL11 was tested for plaque forming ability of lambda mutants, λ *S*am7 and *R*am216). λ *S*am7 (but *R*am216) yielded substantial number of plaques on 594/pHOL11 (data not shown), although the plating efficiency decreased by one order compared to that on LE392 *sup*$^+$, suggesting that pHOL11 may express Hol, albeit at a low level, being substitutive for lambda holin.

Interestingly, almost all of XL1-Blue cells harboring the pHOL-plasmids spontaneously yielded *hol*-insensitive survivors about 16 h after induction, which retained the original plasmids (data not shown). Characterization of these clones are presently under investigation.

3. Conclusion

The results in the present study suggest that (1) Hol is directly involved in cell lysis (probably acting on cytoplasmic membrane) via its N- and C-terminal

Table 3
Detection of β-Gal activity

Host	Plasmid	Hybrid protein[a]	Ratio of β-gal activity[b] (precipitate/supernatant)
XL1-Blue	pUC119	LacZ′	0.5
XL1-Blue	pHOL3	Hol-219	10.1
XL1-Blue	pHOL8	Hol-188	5.3
XL1-Blue	pHOL9	Hol-132	2.6

E. coli strains and plasmids used were as indicated in Table 1. *E. coli* cells carrying a plasmid were grown in LB broth at 25°C containing 60 μg/ml of ampicillin. When the A_{660} had reached 0.3, the culture was shifted up to 37°C with concomitant addition of IPTG (1 mM). After induction (at 1 h in Hol-219 and Hol-188, or 2 h in Hol-132 and LacZ′), the cells were treated with lysozyme (100 μg/ml) and EDTA (2 mM) in 20% sucrose–0.1 M Tris–HCl (pH 8.0) at 0°C for 10 min. The spheroplasts formed were lysed by the addition of 15 vol. of H_2O, and centrifuged at 18 000 rpm for 10 min. The precipitate and supernatant were subjected to β-gal assay (Oki et al., 1996b).

[a]See Table 2.

[b]The average of three to five experiments.

regions, as proposed in lambda holin (Blasi and Young, 1996), but (2) unlike lambda holin, the N-terminal end of ϕgle Hol appears to be non-essential (at least in *E. coli*), although whether or not ϕgle Hol is regulated by a dual start motif functioning in lambda holin expression is presently uncertain. Further studies on structure, expression, and function of ϕgle holin are now in progress.

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