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Involvement of *tonB–exbBD1D2* operon in infection of *Xanthomonas campestris* phage ϕL7

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Abstract

ϕL7 is a lytic bacteriophage infecting *Xanthomonas campestris* pv. campestris, a Gram-negative bacterium producing xanthan gum and causing black rot in crucifers. A mutant resistant to ϕL7 was isolated by Tn5 mutagenesis. Sequence analysis indicated that the gene responsible for the mutation is *tonB* encoding an inner membrane protein previously shown to be required for iron uptake and pathogenesis. This gene is clustered with three other genes, *tonB–exbB–exbD1–exbD2*. Results of insertional mutations, DNA and protein sequence analyses, phage sensitivity tests, transfection tests, complementation tests, and phage adsorption assays together with the cellular location of the proteins indicate that TonB, ExbB, and ExbD1 are essential for penetration of phage ϕL7. The genome organization, structural features of the *tonB–exb* region, and transcriptional analyses including Northern hybridization, reporter assays, and primer extension together indicate that the four genes are organized into an operon.

Keywords: *Xanthomonas*; Bacteriophage; Penetration; *tonB* gene; Transcriptional analysis

The Gram-negative *Xanthomonas campestris* pv. campestris (Xc) is the causative agent of black rot in cruciferous plants [1]. This organism is able to produce great amounts of an exopolysaccharide, xanthan gum, which is involved in pathogenicity and has various applications in oil drilling, cosmetics, food, and agriculture as a thickening, emulsifying, and suspending agent [2,3]. Several bacteriophages specifically infecting *X. campestris* pv. campestris have been reported but not well characterized [4–8].

ϕL7 is a lytic phage isolated in our laboratory from the soil of a local garden, in which cabbage plants had been infected by *X. campestris* pv. campestris and showed symptoms of black rot [7]. To understand the interaction between ϕL7 and its host, we have previously exploited Tn5 transposition to isolate a ϕL7-resistant mutant (CH7LR) from Xc17, which is defective in *xanA* gene coding for phosphoglucomutase/phosphomannomutase required for the synthesis of both lipopolysaccharide (LPS) and xanthan gum [9]. CH7LR and the other *xanA* mutants isolated by marker exchange are still capable of adsorbing ϕL7 at an efficiency which is three orders of magnitude lower than that of the wild-type cells. Depending on this low efficiency of adsorption, only very turbid lysis zones can be formed after dropping ϕL7 suspension on the lawns of CH7LR (spot tests) followed by an incubation of 16 h, comparing to 1.5 h in tests with the Xc17 lawns. While single plagues are visible on the Xc17 lawns at about 4 h post-plating in double-layered assays, none is formed on the lawns of *xanA* mutants after incubation for 24 h [9]. Based on these observations, we suggested the ϕL7 receptor to be a complex structure formed by LPS and other component(s), such as an outer membrane protein [9].

In this study, we isolated a ϕL7-resistant mutant from *X. campestris* pv. campestris P20H by mini-Tn5Tc

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transposition. Sequencing of the DNA region around the Tn insertion site revealed six open reading frames. The last four are the genes analogous to *tonB–exbB–exbD1–exbD2* in *X. campestris* pv. campestris B100 that were previously shown to be required for pathogenicity, iron uptake, and hypersensitive response in non-host plant [10,11]. These four genes are shown to form an operon and, except for *exbD2*, are essential for ϕL7 penetration.

Materials and methods

Bacterial strains, phages, and plasmids. The tadpole-shaped lytic ϕL7 and the filamentous ϕLf were bacteriophages specifically infecting *X. campestris* pv. campestris [7,12]. *X. campestris* pv. campestris P20H was a non-mucoid avirulent strain derived from Xc11, resistant to ampicillin and rifampicin [13]. *Escherichia coli* strains DH5α [14] and S17-1(pUT-miniTn*5*Tc) [15] were the host for gene cloning and the donor cell in conjugation, respectively. pOK12 (2.1 kb, Km^r) [16] was an *E. coli* cloning vector derived from P15A replicon, which cannot be maintained in *X. campestris*. Plasmid pRK415 (10.5 kb, Tc^r) [17], derived from the broad-host-range RK2, was used to clone the desired genes for complementation tests. LB broth and L agar were used as the media for growing *X. campestris* pv. campestris (28 °C) and *E. coli* (37 °C). XOLN was a basal salt medium (with $FeSO_4$) supplemented with 0.0625% tryptone and 0.0625% yeast extract [18]. Antibiotics, gentamycin (15 μg/ml), kanamycin (50 μg/ml), rifampicin (100 μg/ml), and tetracycline (15 μg/ml) were added when necessary.

Isolation of mutants by miniTn5-Tc transposition. To isolate mutants by Tn5 mutagenesis, *E. coli* S17-1(pUT-miniTn5-Tc) [15] and P20H were used as the donor and the recipient, respectively, for conjugation following the procedures described previously [9]. Transconjugants were selected on L agar plates containing tetracycline and rifampicin.

Construction of mutants by insertional mutation. The mutants were constructed by insertional mutation, either by marker exchange or interruption by integration of the whole plasmid containing a DNA fragment internal to the target gene. For marker exchange, the gene of interest was cloned into pOK12, and then the GmΩ cartridge form pUCGmΩ [19] was inserted into an appropriate restriction site within the coding region. The resultant plasmid was electroporated into P20H, allowing for integration by double crossover. For the mutants of *orf268*, *orf398*, *tonB*, and *exbB*, the GmΩ cartridge was inserted into the *Hin*dIII site, the *Bgl*II site, the *Eco*RV site, and the *Nae*I site, resulting in mutants CH120(*orf268*::GmΩ), CH220(*orf398*::GmΩ), CH320(*tonB*::GmΩ), and CH420(*exbB*::Gm), respectively. The *exbD1* and *exbD2* mutants were constructed by plasmid integration which caused gene interruption. Plasmids pOD1201 and pUD2190 were derivatives of pOK12 and pUC19G [20] containing a 201-bp PCR fragment (nt 4126-4336 in Fig. 2) and a 190-bp PCR fragment (nt 4516–4705 in Fig. 2) internal to *exbD1* and *exbD2*, respectively. These plasmids were separately electroporated into P20H, allowing for single crossover, resulting in mutants CH520(*exbD1*::pOD1201) and CH620(*exbD2*::pUD2190), respectively.

Southern hybridization was carried out to verify that double crossover had occurred in marker exchange, in which insertion of the cartridge caused fragment size enlargement. For the mutants constructed by plasmid integration, single crossover was verified by the acquisition of Km^r phenotype and by Southern hybridization to detect fragment size enlargement caused by the insertion.

Phage propagation, tittering, and sensitivity tests. Phage ϕL7 was propagated as previously described [9], except that P20H was used as the host. The lysate, after centrifugation (12,000*g*, 15 min), was filtered through a 0.45-μm Millipore membrane filter and then stored at 4 °C until use.

Phage titer was determined by the double-layered agar method [21]. Phage sensitivity was tested by spot tests, which were performed by dropping 5 μl of the phage lysate (ca. 1×10^9 PFU/ml) onto a lawn of *X. campestris* pv. campestris freshly prepared by including ca. 1×10^9 cells in the top agar or spreading the cells in an area of about 2 cm^2 on the surface of an agar plate. The latter method was used in screening a large number of transconjugants. Lysis zone formation indicated a sensitive response.

Phage adsorption assay. Preparation of the cells and the procedures for adsorption assays were the same as described previously [9], except that P20H and its mutants were used. The assay mixtures were incubated on ice for 15 min and the phage particles adsorbed were pelleted together with the cells, followed by measuring the phage titers.

Promoter activity assay. Promoter activity was assayed by using the promoter-less *lacZ* gene as the reporter. The DNA fragment containing putative promoter sequences was cloned in the multiple cloning sites upstream of the *lacZ* gene in the promoter-probing vector pFY13-9 [22]. Nucleotide sequence was determined to ensure that no mutation was caused by PCR amplification and the orientation of the fragment was correct. To measure the β-galactosidase activity, cells from the overnight cultures were inoculated into fresh LB medium and grown until OD_{550} was 1.0. The enzyme activity was expressed as Miller units [23].

DNA techniques. The standard procedures were used for the preparation of chromosome, plasmid, and phage DNA, agarose gel electrophoresis, preparation of ^{32}P-labeled probes, Southern hybridization, and transformation of *E. coli* [24]. Introduction of plasmid (transformation) and phage DNA (transfection) into *X. campestris* pv. campestris was performed by electroporation [25]. Nucleotide sequence was determined by the dideoxy chain termination method of Sanger et al. [26]. The sequence of 4981 bp containing *orf268–orf398–tonB–exbB–exbD1–exbD2* has been deposited in GenBank under Accession No. AF527951.

Results and discussion

CH20R isolated from P20H by Tn5 transposition is a ϕL7-resistant mutant

Xanthomonas campestris pv. campestris P20H is sensitive to ϕL7. To isolate mutants resistant to ϕL7, P20H was mutagenized with mini-Tn*5*Tc [15]. About 3000 tetracycline-resistant (Tc^r) transconjugants were obtained. After testing for phage sensitivity by dropping ϕL7 suspension on the thick spread of the cells from these transconjugants, several of them were found unable to form lysis zones, indicating resistance to ϕL7. One of them was designated as CH20R and further studied.

In spot tests with the cells being included in the top soft agar, lysis zones were visible with the lawns of P20H at 4 h after dropping the phage (Fig. 1A). In contrast, no lysis zones were caused on the lawns of CH20R, even after incubating for 3 days (Fig. 1B).

CH20R is capable of releasing infective phage particles upon transfection with ϕL7 genomic DNA

Introduction of intact phage genomic DNA into a phage-resistant mutant by electroporation (transfection)

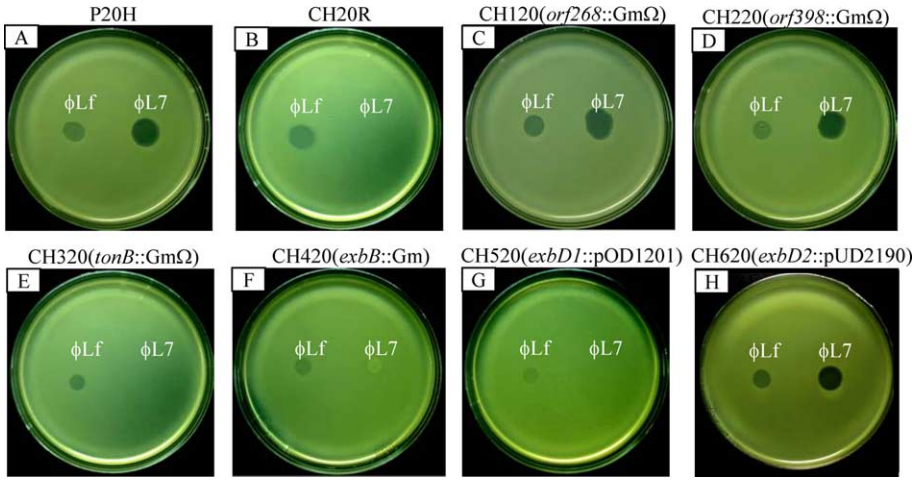

Fig. 1. Lysis zone formation in spot tests. Suspensions of ϕL7 and ϕLf were separately dropped onto the lawns of P20H and its mutants as indicated.

is a useful treatment to skip the early steps of infection, adsorption, and penetration, and to test whether the strain retains the ability to support phage propagation [22]. In this study, the ϕL7 genomic DNA was electroporated into CH20R and P20H, followed by counting the infective centers. The efficiencies of transfection varied from one experiment to another using different batches of ϕL7 DNA. The experiments were repeated three times, each with triplicate samples, and efficiencies ranging from 210 to 3600 PFU/μg DNA were obtained for either of the strains, which were about the same as that obtained in chemical transfection with $CaCl_2$-treated Xc17 cells [7]. These data indicated that CH20R retained the normal ability to support ϕL7 propagation, but had lost the function required for the early steps of ϕL7 infection, i.e., adsorption or penetration or both.

CH20R is mutated in tonB, a gene clustered with exbB, exbD1, and exbD2

To isolate the wild-type gene that could complement the mutation in CH20R, the inserted mini-Tn*5*Tc together with the flanking regions (a 4.3-kb *Bgl*II–*Kpn*I fragment) from the CH20R chromosome was cloned into pOK12, giving pOKTc4.3, and used as the probe for hybridization with a P20H genomic library constructed by cloning the DNA fragments generated by partially cutting with *Sau*3A1 into the cosmid pSupercos (Stratagene). In Southern hybridization, three clones showed positive signals and one of them carrying a 32-kb insert was designated as pP2032. The pP2032 insert was partially digested with *Sau*3A1 and cloned into the broad-host-range vector pRK415. One of the clones (pTON5) with a ca. 5-kb insert was capable of restoring ϕL7 sensitivity to the transformant, CH20R(pTON5), as assayed by spot testing. This result indicated that the pTON5 insert contained the gene responsible for the

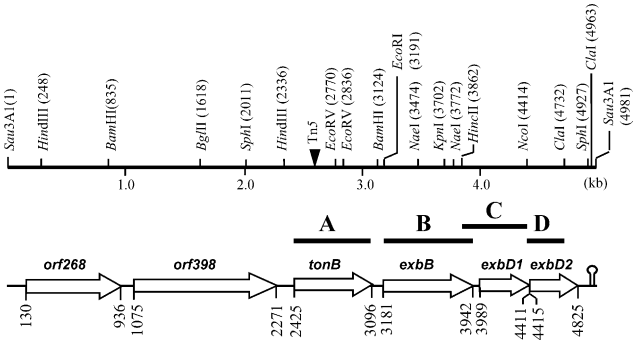

Fig. 2. Restriction map of the 4981-bp fragment containing *orf268*, *orf398*, *tonB*, *exbB*, *exbD1*, and *exbD2*, cloned from *X. campestris* pv. campestris P20H. Important restriction enzyme sites and the positions of the genes are indicated by Arabic numbers. The thick bars represent the position of the probes used in Northern hybridization.

CH20R mutation. In these complementation tests, it was noticed that the lysis zones formed were less clear compared to those formed on the lawns of P20H cells (data not shown).

Sequences in both strands were determined for the pTON5 insert (4981 bp). As shown in Fig. 2, this region contained six open reading frames, *orf268–orf398–tonB–exbB–exbD1–exbD2*. The amino acid sequences deduced from these six genes showed over 98% identities, with a few synonymous changes of amino acid residues, to those of the corresponding proteins from two *X. campestris* pv. campestris strains (B100 and ATCC 33913) and *X. axonopodis* pv. citri 306 [10,27]. In addition, the genome organizations of these genes in the four *Xanthomonas* strains are the same. Using ^{32}P-labeled pTON5 as the probe for Southern hybridization, only one hybridization band was detected in the digests from the P20H and Xc17 chromosomes (data not shown), indicating that a single copy of these genes is present.

The *orfs 268* and *398* encoded uncharacterized hypothetical proteins [27]. The amino acid sequence

deduced from *orf268* shared 64% and 56% identities with the hypothetical proteins XF0006 (306 aa) from *Xyllela fastidiosa* (gi 9104766) and VCA0581 (263 aa) from *Vibrio cholerae* (gi 9657992), respectively. The amino acid sequence deduced from *orf398* only shared significant identity (56%) with the *X. fastidiosa* hypothetical protein XF0007 (397 aa, gi 9104767).

To determine the site of mini-Tn5Tc insertion in CH20R, we also sequenced the downstream border region in the pOKTc4.3 insert. The results indicated that the insertion occurred at nt 155 downstream of the *tonB* initiation codon. In other words, CH20R was a *tonB* mutant of *X. campestris* pv. campestris P20H.

TonB, ExbB, ExbD1, but not ExbD2, of P20H are essential for ϕL7 infection

To confirm that *tonB* indeed plays a role in ϕL7 infection and to test whether the other five genes are also required for the function, we mutated each of them separately by insertional mutation as described in Materials and methods. The resultant mutants were designated as CH120(*orf268*::GmΩ), CH220(*orf398*::GmΩ), CH320(*tonB*::GmΩ), CH420(*exbB*::Gm), CH520(*exbD1*::pOD1201), and CH620(*exbD2*::pUD2190), with the respective mutated gene bearing the inserted element in parentheses. In spot tests, no lysis zones were formed on the lawns of CH320(*tonB*::GmΩ), CH420(*exbB*::Gm), or CH520(*exbD1*::pOD1201) even after incubating for three days (Figs. 1E, F and G), indicating a complete resistance. These results indicated that *exbB* and *exbD1*, in addition to *tonB*, are essential for the early steps of ϕL7 infection. On the other hand, the remaining three mutants, CH120(*orf268*::GmΩ), CH220(*orf398* ::GmΩ), and CH620(*exbD2*::pUD2190), retained sensitivity to ϕL7 (Figs. 1C, D, and H). Since the mutants of *orf268* and *orf398* were generated by GmΩ insertion, retention of phage sensitivity indicates that no polar effects on the *tonB–exb* functions were caused, which in turn suggests that they are not contained in the same operon with the *tonB–exb* genes. In electroporation experiments, the efficiencies of transfection exhibited by the *tonB*, *exbB*, and *exbD1* mutants were within the same range exhibited by P20H and CH20R (280–4000 PFU/μg DNA), indicating that the ability to support ϕL7 propagation was retained.

Notably, the phenomenon that a mutation in *tonB*, *exbB*, or *exbD1*causes complete resistance to ϕL7 is different from the case of the mutation of *xanA*, causing a defect in LPS biosynthesis, in which a low efficiency of adsorption *xanA* mutant can still support the formation of very turbid lysis zones in spot tests after a prolonged incubation [9].

Previously, *tonB*, *exbB*, *exbD1*, and *exbD2* in *X. campestris* pv. campestris B100 have been shown to be required for hypersensitive response in non-host plant and except for *exbD2* are also required for pathogenicity and iron uptake [10,11]. In this study, we have also tested pathogenicity of the *tonB, exbB, exbD1,* and *exbD2* mutants and found that these mutants, except for *exbD2* mutant, had lost virulence (data not shown). Therefore, taking these data together with the Southern hybridization data showing that only one copy of the genes was present, it is apparent that the *tonB–exb* genes studied here are analogous to the B100 *tonB–exb* genes.

A mutation in tonB, exbB, exbD1, or exbD2 does not affect the ability of ϕL7 adsorption

To determine which part of the function required for the early steps of ϕL7 infection had been affected by the mutation in the *tonB*/*exb* genes, we performed adsorption assays with P20H, CH20R, and the three mutants, CH320(*tonB*::GmΩ), CH420(*exbB*::Gm), and CH520(*exbD1*::pOD1201). Using an MOI of 0.1 (5.0×10^7 PFU for 5.0×10^8 cells in a reaction mixture of 0.5 ml, on ice) for assay, all the strains exhibited similar ability to adsorb ϕL7. In three experiments with triplicate samples, $2.6–3.2 \times 10^7$ PFU of the phage was found to be adsorbed, indicating that each of the mutants retained the normal capability for ϕL7 adsorption. Since normal capabilities in phage adsorption and phage propagation were retained by our mutants, we conclude that TonB, ExbB, and ExbD1 are essential for ϕL7 penetration.

The *E. coli* TonB, an inner membrane protein that spans the periplasmic space, serves to transduce inner membrane-derived energy (proton motive force) to the outer membrane receptor during active transport of iron-siderophore complex and vitamin B12 across the outer membrane [28–32]. In addition, the *E. coli* TonB is also required for the early steps of infection by phages including T1 and ϕ80 [33–35]. The observations that the *X. campestris* pv. campestris *tonB*, *exbB*, and *exbD1* are required for iron uptake [10] and phage penetration suggest similar functions for these homologues and similar patterns of protein–protein interactions in this bacterium.

It has been proposed that proper stoichiometry of TonB, ExbB, and ExbD proteins is important for operation of the system, and an imbalance in production of the components may interfere with their proper incorporation into the inner membrane [36,37]. This may explain our findings in complementation tests that turbid lysis zones were formed on the lawns of the mutant cells carrying the cloned *tonB–exb* genes, because the affected genes would always have lower copy numbers.

tonB, exbB, exbD1, and exbD2 are co-transcribed

The intergenic region between *orf398* and *tonB* was long (155 bp), suggesting that they might be transcribed independently. Furthermore, there was an inverted

repeat (5′-AACGCCACCGgaaaCGGTGGCGTT-3′, followed by a string of T) lying 41 nt downstream of the *exbD2* stop codon, which has the potential to form a stem–loop structure resembling the transcription termination signal. No other sequences having the potential to form such a structure were found in the other three intergenic regions. These together suggested that the *tonB–exb* cluster genes might form an operon, being transcribed as a single unit. To determine the size of the transcript of the *tonB–exb* cluster genes, Northern hybridization was carried out using the total RNA prepared from an overnight culture of P20H. The probes used are shown in Fig. 2. They were two PCR fragments, containing *tonB* (669-nt probe A, nt 2425–3093) and *exbB* (759-nt probe B, nt 3181–3939), the *Hin*cII–*Nco*I fragment containing *exbD1* (553-nt probe C, nt 3862–4414), and the *Nco*I–*Cla*I fragment containing *exbD2* (319-nt probe D, nt 4414–4732,). Probes B and C overlapped by 128 bp, whereas probes C and D were linked restriction fragments. Using the probes with overlapping region, at least two transcripts would be detected if the adjacent genes are transcribed independently. The RNA was separated in agarose gel electrophoresis, followed by blotting onto a nylon membrane. The membrane was cut into four strips, each containing one lane of RNA, and hybridized separately with the labeled probes. The hybridization signals were each associated with a band corresponding to 2.4 kb and no smaller transcripts were detected (Fig. 3). This size was about the same as the total length of the region encompassing *tonB–exb* cluster genes (Fig. 2). Because all the four probes hybridized to mRNA with the same size, these data suggested that the *tonB–exb* genes were contained within the same transcript. This finding is different from the report of Wiggerich et al. [10] whose data of mutational analysis suggested that *exbD1* and *exbD2* genes may also be transcribed independently from *tonB* and *exbB* genes.

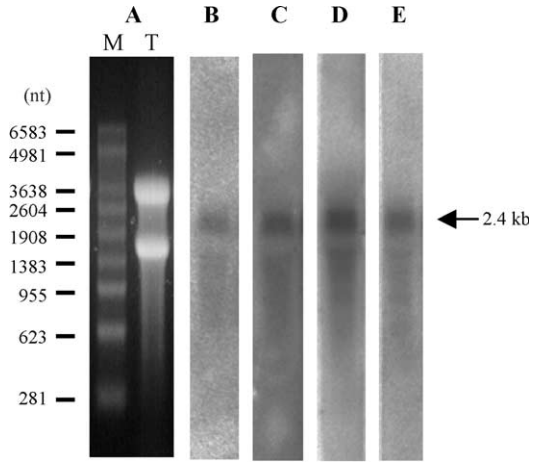

Fig. 3. Determination of transcripts by Northern hybridization. Panel A contained ethidium bromide-stained RNA molecular weight markers (M) and total RNA from P20H (T) after agarose gel electrophoresis. Panels B–E are membranes hybridized with probe A containing *tonB*, probe B containing *exbB*, probe C containing *exbD1*, and probe D containing *exbD2*, respectively.

One promoter is present upstream of tonB

The transcription initiation site was determined by primer extension using the same mRNA samples prepared for Northern hybridization as the template. The 23-mer complementary to nt 82–104 from the *tonB* start codon was used as the primer (Fig. 4B). For comparison, a sequencing reaction was performed with the same primer and plasmid pSE760 containing the 760-bp *Sph*I–*Eco*RV fragment (containing the *tonB* upstream region, nt 2011–2770 in Fig. 2) as the template (Fig. 4A). One primer extension product initiating with a T at −79 nt counting from the *tonB* translation initiation codon was detected (Figs. 4A and B).

Using BDGP program (http://www.fruitfly.org/seq_tools/promoter.html) for promoter prediction, sequences consensus to the −35 sequence (5′-GTTACA-3′) and the −10 sequence (5′-TATAAG-3′) were found at nt −9 and −29 relative to the *tonB* transcription start site determined here (Fig. 4B). To detect promoter activity, the 371-bp PCR fragment containing the *tonB* upstream region (−268 to +103 relative to the *tonB* translation initiation codon) was amplified using the forward primer F1 (5′-CAA

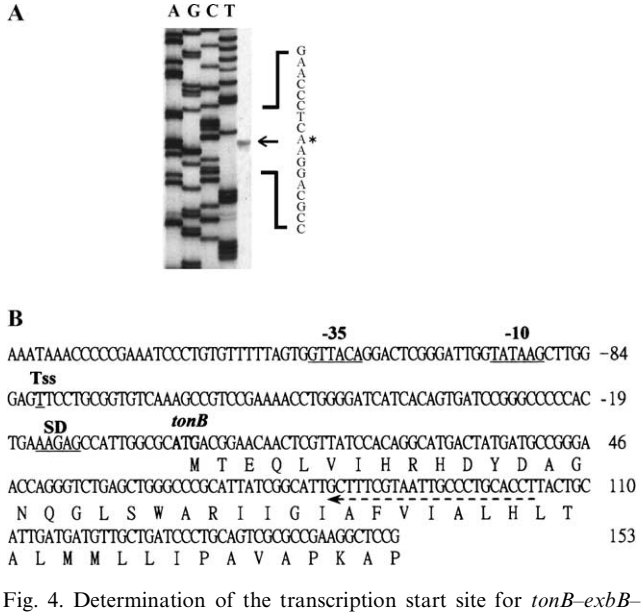

Fig. 4. Determination of the transcription start site for *tonB–exbB–exbD1–exbD2* operon by primer extension. (A) Gel electrophoresis of the primer extension product (indicated by an arrow). The sequence right to the gel is complementary to the sequence displayed in panel B. Asterisk represents the base complementary to the 5′ end of the transcript. (B) Sequence of the *tonB* upstream region. Shown are the −35 and −10 sequences of predicted promoter, transcription start site (Tss, bold-faced T), Shine–Dalgarno sequence (SD), and position of the primer used for primer extension (dashed arrow).

GGTGCTGCATTCGGA-3′, with a *Pst*I site) and the reverse primer R1 (5′-AGGTGCAGGGCAATTACGA AAGC-3′, with an *Xba*I site). The fragment was cloned into the promoter-probing vector pFY13-9 [22], generating pFY223P which was then electroporated into P20H. About 230 U/ml of β-galactosidase activity was measured in P20H containing pFY233P, compared to about 15 U/ml in the same cells containing the vector only. These data indicated that the 371-bp fragment indeed possessed the *tonB* promoter.

The 356-bp *Eco*RV–*Eco*RI fragment containing the *exbB* upstream region was also cloned into pFY13-9 forming plasmid pFY253P. However, when P20H(pFY 253P) was subjected to β-galactosidase assays, no significant enzyme activity was detectable. These data indicated that the *exbB* upstream region did not contain promoter sequences.

Conclusion

Results obtained in this study together with the predicted membrane association of the deduced proteins indicate that TonB, ExbB, and ExbD1 are essential for penetration of phage ϕL7. The structural features of the *tonB–exb* region and data of transcriptional analyses indicate that *tonB*, *exbB*, *exbD1*, and *exbD2* are organized to form an operon. The features and data include (1) presence of the long *orf398 tonB* intergenic region (155 bp), indicating that the two genes are unlikely co-transcribed, (2) mutation in *orf268* or *orf398* by GmΩ insertion does not cause polar effects on *tonB*/*exb* functions, (3) presence of an inverted repeat, resembling a transcription termination signal, downstream of the *exbD2* stop codon, (4) detection of the transcript with a size similar to the total length of the four genes, (5) determination of a transcription start site located upstream of *tonB*, and (6) presence of a promoter upstream of *tonB* but not in other regions.

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