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Functional and genetic analysis of regulatory regions of coliphage H-19B: location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release

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Summary

Analysis of the DNA sequence of a 17 kb region of the coli lambdoid phage H-19B genome located the genes encoding shiga-like toxin I (Stx-I) downstream of the gene encoding the analogue of the phage λ Q transcription activator with its site of action, *qut* at the associated p_R' late promoter, and upstream of the analogues of λ genes encoding lysis functions. Functional studies, including measurement of the effect of H-19B Q action on levels of Stx expressed from an H-19B prophage, show that the H-19B Q acts as a transcription activator with its associated p_R'*(qut)* by promoting readthrough of transcription terminators. Another toxin-producing phage, 933W, has the identical *Q* gene and p_R'*(qut)* upstream of the *stx-II* genes. The H-19B Q also activates Stx-II expression from a 933W prophage. An ORF in H-19B corresponding to the holin lysis genes of other lambdoid phages differs by having only one instead of the usual two closely spaced translation initiation signals that are thought to contribute to the time of lysis. These observations suggest that *stx-I* expression can be enhanced by transcription from p_R' as well as a model for toxin release through cell lysis mediated by action of phage-encoded lysis functions. Functional studies show that open reading frames (ORFs) and sites in H-19B that resemble components of the N transcription antitermination systems controlling early operons of other lambdoid phages similarly promote antitermination. However, this N-like system differs significantly from those of other lambdoid phages.

Introduction

Phages related to the well-characterized coliphage λ have been identified as carriers of the genes encoding shiga-like toxins (Stxs) (Smith *et al*., 1983; 1984; Scotland *et al*., 1983; O'Brien *et al*., 1984a; Newland *et al*., 1985; Huang *et al*., 1987). Stxs, cytotoxins related to the toxin produced by *Shigella dysenteriae* type 1, are produced in other bacterial genera (O'Brien *et al*., 1984b; 1992; Acheson *et al*., 1991), including several different *Escherichia coli* serotypes. The genes encoding Stxs have been found in resident prophages in most toxigenic *E. coli* (Newland and Neill, 1988). The conversion to toxigenicity by a phage, referred to by the general term lysogenic conversion, has been described for a number of pathogenic bacteria (Bishai and Murphy, 1988). Classical examples of phage-encoded toxins are the diphtheria toxin produced by *Corynebacterium diphtheriae*, enterotoxin A from *Staphylococcus aureus* and neurotoxin from *Clostridium botulinum* (reviewed in Bishai and Murphy, 1988).

Stxs have been implicated as the causative agents of serious diseases such as haemorrhagic colitis, infantile diarrhoea and haemolytic uraemic syndrome. Stxs, like shiga toxin, are composed of the A enzymatic subunit and the B eukaryotic cell membrane-binding subunit (reviewed in O'Brien and Holmes, 1987; 1996). Two different forms of prophage encoding shiga-like toxin in *E. coli* have been identified: Stx-I, differing from shiga toxin by only one amino acid in the A subunit and Stx-II, differing more extensively in both subunits.

H-19B, originally identified as a relative of λ (lambdoid phages) through restriction mapping and hybridization studies with λ DNA (Huang *et al*., 1987), is the focus of this study. H-19B was first isolated from *E. coli* 026 strain H19, which caused an outbreak of infantile diarrhoea in a day care centre in Great Britain (Smith *et al*., 1983). Huang *et al*. (1986) demonstrated that the structural genes encoding shiga-like toxin I subunits were encoded in the H-19B genome. Another lambdoid phage, 933W, carries genes encoding the Stx-II form of shiga-like toxin. The Stx-II expressed from *E. coli* 0157:H7, which carries the 933W prophage, has been demonstrated to be the cause of serious outbreaks of haemorrhagic *E. coli* infections (O'Brien *et al*., 1984a; Knight, 1997). The genome of 933W has been completely sequenced (F. Blattner, personal communication).

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Phages are important vectors for the horizontal transfer of pathogenic elements between bacteria (Cheetham and Katz, 1995). Because H-19B is a member of a well-characterized family of phages, it should be an excellent model system for studying how phages acquire, transfer and possibly contribute to the expression of virulence genes.

We have determined the sequence of an ≈17 kb region of H-19B that enabled us to precisely locate the position of the toxin genes on the phage genome and identify elements and open reading frames (ORFs) resembling those found in other members of the lambdoid family of phages. Sequences have been deposited in GenBank under accession no. AF034975. Based on this comparison and results of functional studies, we have identified what appear to be regulatory regions controlling transcription of delayed early and late genes of H-19B. These studies suggest a role for the regulated expression of phage lysis genes in the release of toxin.

Results and discussion

Regulation of expression of delayed early genes

Expression of delayed early genes in lambdoid phages is regulated temporally through a process of transcription termination and subsequent antitermination (reviewed in Das, 1992; Friedman and Court, 1995; Richardson and Greenblatt, 1996). Antitermination by the λ N protein regulates expression of gene products required early in infection. Immediately after infection or induction, transcription initiating at the early λ promoters, p_L and p_R, is halted at downstream terminators relatively close to the promoters. Consequently, expression of genes downstream of the terminators is either reduced or blocked. Both terminator-proximal transcripts include sites, NUTR and NUTL, that interact with N (Salstrom and Szybalski, 1978) and a number of *E. coli* proteins, termed Nus factors, to alter RNA polymerase so that transcription overrides subsequent termination signals. We use the following convention: sites in the RNA will be capitalized, e.g. NUT, and sites in the DNA will be lower case and italicized, e.g. *nut*.

Comparison of *nutL* and *nutR* regions from a number of lambdoid phages suggested that the NUT site can be divided into two specific components: BOXA, a recognition site for the *E. coli* NusB and possibly NusE (ribosomal protein S10) proteins (Friedman *et al*., 1990; Nodwell and Greenblatt, 1993), and BOXB, a region of stem–loop structure that is recognized by N and possibly the *E. coli* NusA protein (Chattopadhyay *et al*., 1995; Mogridge *et al*., 1995). The *boxA* components from a number of lambdoid phages share significant homology and thus a consensus sequence can be derived (Friedman *et al*., 1990) (Fig. 1). The *boxB* components are identified by a region of hyphenated dyad symmetry in the DNA that can result in the formation of a stem–loop structure in the RNA, although they differ in sequence (Olson *et al*., 1982; Franklin, 1985a).

Analysis of the H-19B DNA sequence suggests a transcription antitermination system resembling the N-mediated systems of most other lambdoid phages (Franklin, 1985a,b). We identified a sequence resembling *nut* sequences between the putative H-19B *cro* and *c*II genes, the analogous position of *nutR* in λ, HK97, another recently identified and sequenced lambdoid phage (R. Hendrix, personal communication), contains motifs almost identical to the H-19B putative *nut* sequences, suggesting that the two phages share the same *N* gene. We exploited this similarity to identify and sequence the putative H-19B *N* gene (see *Experimental procedures*). The putative H-19B *N* gene, located at a position on the H-19B genome analogous to the positions of the *N* genes of other lambdoid phages, encodes a 127-amino-acid protein that contains an arginine-rich motif like those found to be important for function of the N proteins of other lambdoid phages (Lazinski *et al*., 1989; Franklin, 1985b). The derived amino acid sequence

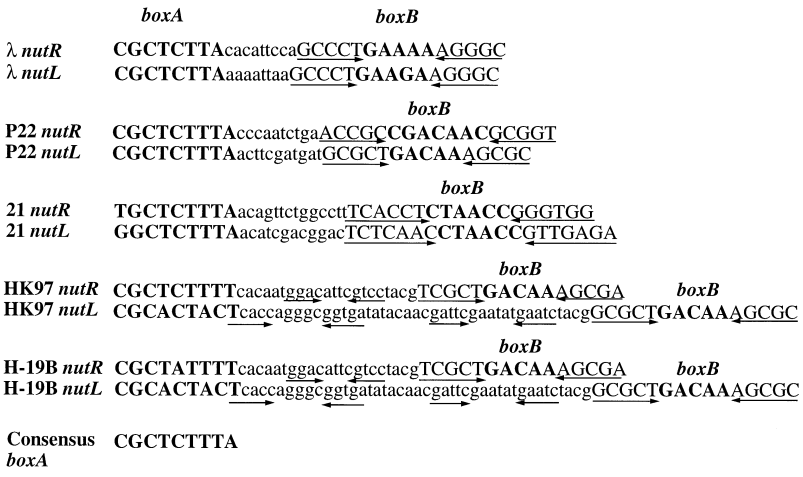

Fig. 1. Alignment of the *nut* regions of H-19B with those of other lambdoid phages. The *boxA* and *boxB* sequences are shown in bold type. Underlined sequences are regions of possible secondary structure in the RNA. The consensus *boxA* sequence was derived from comparison of the *boxA* sequences of P22, P21, λ and that found in the leader region of the *E. coli rrn* operons (Olson *et al*., 1982).

for this N protein varies from that of HK97 by only three amino acids.

Both putative *nut* regions in H-19B have significant differences from those of other lambdoid phages (see Fig. 1). First, there are variations from the *boxA* consensus, the H-19B *nutR* sequence has a C→A change at the highly conserved fifth position in *boxA*. The A at this position is functionally significant as N-mediated antitermination is reduced when the λ *nutR boxA* sequence has this change (Robledo *et al*., 1990; Patterson *et al*., 1994). The H-19B *nutL boxA* sequence differences are even more pronounced. Not only does this *boxA* sequence have less homology to the consensus *boxA* sequence, but it also deviates from the *boxA* sequence in its own *nutR*. The H-19B *nutL boxA* is missing the run of Ts shown in the consensus *boxA* sequence, which has been demonstrated to be important for N-mediated antitermination in λ (Friedman *et al*., 1990).

A second difference found in the H-19B *nut* regions is the unusually long spacing between the *boxA* and *boxB* sequences compared with that seen with *nut* regions from other phages. These sequences appear to be functionally relevant as changes in the spacer sequence of λ *nut* regions influence N-mediated antitermination (Peltz *et al*., 1985; Zuber *et al*., 1987; Doelling and Franklin, 1989). The spacer sequence in lambdoid phages varies from the eight nucleotides seen in λ *nutL* to the 14 nucleotides in phage 21 *nutR* (Friedman and Gottesman, 1983). The spacers in H-19B are significantly larger, 22 nucleotides in *nutR* and 42 nucleotides in *nutL*. Moreover, unlike other *nut* regions, the spacer regions of both the *nutR* and *nutL* of H-19B have regions of hyphenated dyad symmetry between *boxA* and *boxB* that potentially could result in NUT RNAs that form additional stem–loop structures.

Lambdoid phage HK022 has an early transcription antitermination mechanism that varies significantly from that described above for other lambdoid phages (Gottesman and Weisberg, 1995). Instead of a *nut* site, HK022 has a *put* site that is an extensive region of RNA secondary structure having no detectable homology to *nut* sites (Oberto *et al*., 1993). Remarkably, antitermination is mediated entirely by the PUT RNA, no proteins, phage or bacterial, appear to be involved in altering the RNA polymerase to a terminator-resistant form (King *et al*., 1996). Thus, the antitermination complex in this phage appears to consist entirely of two RNA stem–loop structures modifying RNA polymerase. The three significant deviations in the H-19B *nut* region, the degenerate *boxA*, the large spacer region and the potential additional stem–loop structure, suggest that H-19B may also have an alternative mode of antitermination; one that, like λ, requires some host proteins (this laboratory, unpublished), and, like HK022, has added structure in the RNA recognition element.

To test for function of this putative early regulatory system in H-19B, we constructed a two-plasmid system in which one plasmid supplies N and the other has a reporter system with the putative target site for N (Fig. 2). The reporter system, based on a standard procedure used in the *in vivo* analyses of other lambdoid phage antitermination systems (De Crombrugghe *et al*., 1979), has a promoter fused to a reporter gene with intervening inserts that can contain various combinations of transcription termination signals and *nut* sites. In our experiments, which are based on the system used by Patterson *et al*. (1994), the essential elements of the construct were ordered as follows, the *plac* promoter, the putative *nutR* site from H-19B, a terminator cassette with three terminators followed by the *lacZ* reporter gene. The plasmids supplying N have the *N* gene cloned downstream of *plac*. Because both components are expressed from *plac*, the system is under the control of Lac repressor and can be regulated using IPTG. As a control, we used a similar two-plasmid system with the *N* gene of λ on one plasmid and the reporter construct with *nutR* from λ on the other. The basic strategy is presented in Fig. 2.

The results of the experiments with these constructs are shown in Fig. 3. We look first at the control λ system. With induction, the reporter construct in the absence of N expresses low levels of *lacZ* (column 2). However, in the presence of λ N, high levels of β-galactosidase are synthesized (column 3). Without induction, expression of *lacZ* is very low (data not shown). We look next at the constructs containing components from H-19B. The results with H-19B components were analogous to those found with the λ factors; expression of the putative H-19B *N* product allowed NUT-dependent readthrough of the terminators (column 8). The results in Fig. 3 also demonstrate that the N of H-19B, as is the case for N proteins of other lambdoid phages (Franklin, 1985b), is specific for its cognate NUT site. Neither the λ nor H-19B N protein mediates antitermination with the heterologous NUT site (columns 4 and 7). Thus, delayed early transcription of H-19B is regulated by an N-NUT system analogous to that of other lambdoid phages. However, the differences in the arrangement of the *nut* regions of H-19B from those of other lambdoid phages suggests that the components required for the N-mediated H-19B antitermination may differ significantly from those of other lambdoid phages. Experiments that will be reported elsewhere support this inference (M. Neely and D. Friedman, in preparation).

Genes involved in late gene regulation and toxin production

The *Q* gene of λ encodes a delayed early function that is located downstream of the *nin* region (reviewed in Roberts, 1992; Friedman and Court, 1995). The *Q* gene product

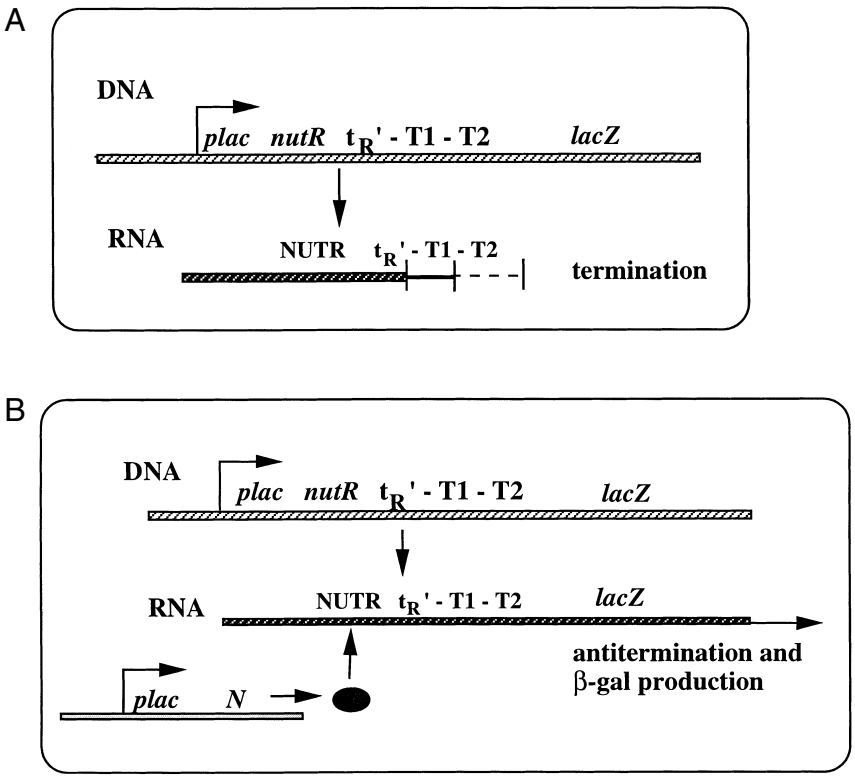

Fig. 2. Vector system for testing N-mediated antitermination. Antitermination is assessed by the level of expression of *lacZ* from a plasmid construct with the *plac* promoter, a *nut* site, a terminator cassette and a *lacZ* reporter gene constructed using pTL61T.
A. In the absence of N, termination will occur within the terminator cassette resulting in low levels of β-galactosidase.
B. In the presence of N, expressed from the second plasmid, constructed using pGB2–plac, there will be readthrough of the terminator cassette resulting in high levels of β-galactosidase.

functions as a second transcription antiterminator that regulates expression of late phage genes by modifying transcription complexes initiating at the late promoter, p_R'. The 207-amino-acid λ Q protein acts at the *qut* site that includes sequences in the p_R' promoter. Other lambdoid phages express Q functions that are specific for their own *qut* site. Unlike NUT, which is an RNA site, *qut* is a DNA site.

Examination of the H-19B sequence reveals, at the position expected for a *Q* gene, an ORF of 144 amino acids with sequence homology to the bacteriophage 21 Q protein (41% identity) that encodes a 162 amino acid protein

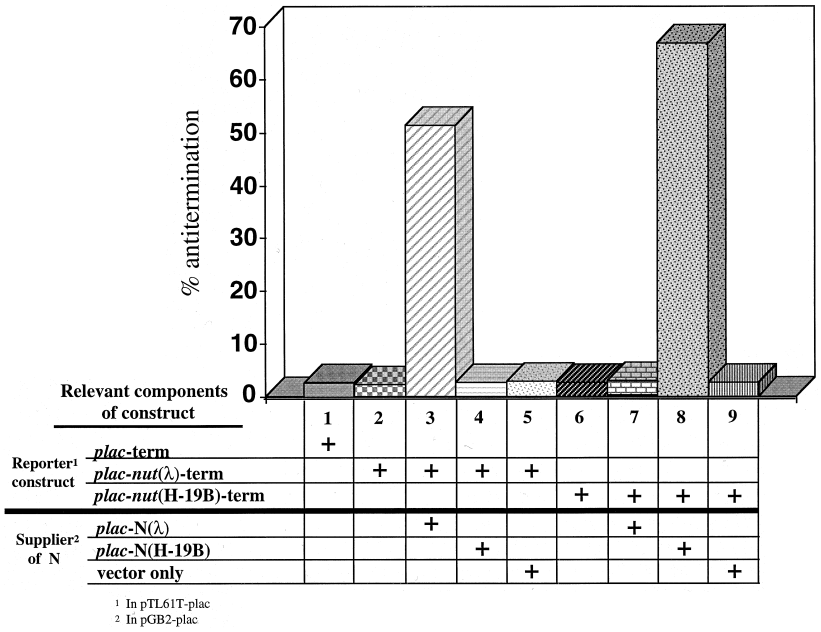

Fig. 3. Role of λ and H-19B N and NUT sites in mediating transcription antitermination. The basic elements of the two plasmid system used in this assay are those described in Fig. 2. The extent of antitermination, as shown by the bar graphs, was determined from the levels of β-galactosidase synthesized by the various combinations of plasmid constructs. The indicated values were all normalized to the level of β-galactosidase synthesized from a *plac–nut–lacZ* construct, without terminators, which was set at 100% antitermination (≈10 000 Miller units). The chart under the bar graph identifies the relevant features of the plasmids contained in the strain being analysed. Term refers to the DNA fragment containing the three terminators, t_R'-T1-T2, referred to in the text. Values shown are from one experiment, but are representative of the values obtained from at least six independent experiments. See *Experimental procedures* for details.

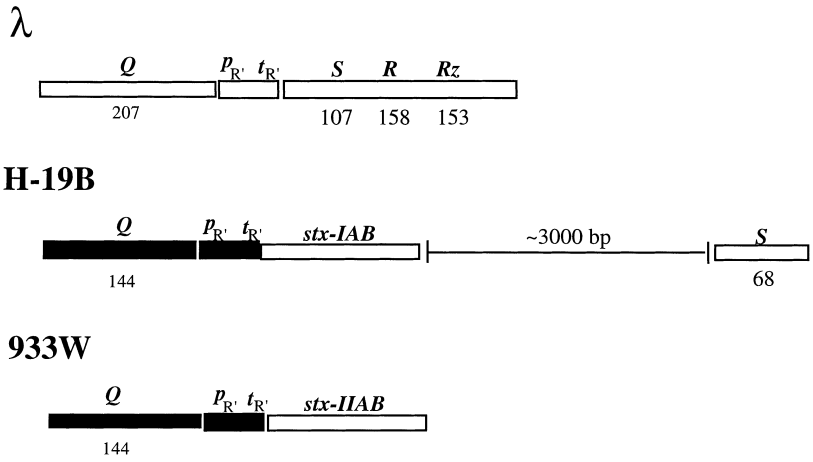

Fig. 4. Map showing DNA sequence homologies between H-19B, 933W and λ in the *Q* through *S* region. Dark boxes are regions of high homology (>90%), whereas open boxes or lines are regions of no homology. Numbers below the maps are the predicted amino acid size of the open reading frames.

(Guo *et al.*, 1991). In λ, the p_R' promoter and associated *qut* site with the t_R' terminator is located directly upstream of the coding sequences for the lysis proteins. H-19B has a similar arrangement downstream of the sequence encoding a putative *Q* gene with sequences conforming to p_R', *qut* and t_R' sites of other lambdoid phages. However, H-19B differs from λ at sequences downstream of the position of the putative t_R'; here it has sequences encoding the shiga-like toxin I A and B subunits (*stx-I*) (Fig. 4) aligned so that they are in the same transcriptional orientation as the surrounding phage genes. This arrangement places the genes encoding Stx-I directly downstream of the strong p_R' promoter (see below). This placement of toxin genes is more precise than that derived in a previous study that used Southern hybridization to compare λ and H-19B sequences and located the toxin genes in the upstream (*nin*) region (Huang *et al.*, 1987).

We used a second two-plasmid system to test whether the H-19B putative Q function activates late gene expression by transcription antitermination. The reporter construct has a fragment of H-19B DNA containing the sequences for p_R', *qut* and t_R' placed directly upstream of the *lacZ* gene in pTI61T (see *Experimental procedures*). The second construct, in the compatible plasmid pGB2-plac, has the H-19B Q analogue cloned downstream of *plac*. Thus, if the putative p_R'*(qut)* in the first plasmid acts with Q as an antiterminator, then increased levels of β-galactosidase should be synthesized when Q is induced from the second plasmid by addition of IPTG. Table 1 shows the results of experiments with this two-plasmid system to assess the H-19B Q-mediated readthrough of two terminator combinations. The high levels of β-galactosidase expressed by the p_R'*(qut)*$\Delta t_R'$ construct in the absence of Q provides a measure of the unterminated transcription initiating at the H-19B p_R' promoter.

We look first at Q-mediated readthrough of the H-19B

Table 1. Action of the H-19B Q protein.

Reporter construct	Q protein supplied by second plasmid: None	*plac*-Q(H-19B)	*plac*-Q(λ)	Per cent antitermination[a] with H-19B Q
	Units of β-galactosidase			
p_R'*(qut)*$\Delta t_R'$–*lacZ*	33 347	–	–	–
p_R'*(qut)*t_R'–*lacZ*	7 350	28 492	4304	81
p_R'*(qut)*t_R'-term[b]–*lacZ*	195	24 985	ND	75

Values shown are from one experiment but are representative of the values obtained from at least six independent experiments.

a. Percentage antitermination was calculated from determination of three values as shown below: (*A*) the β-galactosidase levels produced by the construct with the terminator(s) in the presence of Q; (*B*) the β-galactosidase levels produced by a construct isogenic for all components except for the terminator(s); and (*C*) the β-galactosidase levels produced by the construct with the terminator(s) in the absence of Q.

$$\% \text{ antitermination} = \frac{(A)-(C)}{(B)-(C)} \times 100$$

b. Term refers to a DNA fragment containing the three terminators (t_R'-T1-T2) referred to in the text.

ND, not done.

t_R' terminator region. The low, but significant, level of β-galactosidase expressed by the $p_R'(qut)t_R'$ construct in the absence of Q provides a measure of the basal level of readthrough. The higher levels of β-galactosidase expressed by the $p_R'(qut)t_R'$ construct in the presence of the H-19B Q shows that this Q product effectively supports readthrough of the t_R' terminator.

Because the basal level of readthrough of the H-19B t_R' terminator region was relatively high, it was not possible to assess the strength of the antitermination mediated by the H-19B Q analogue. Therefore, we measured antitermination activity with a construct in which the multiple terminator cassette (t_R'-T1-T2) described above was cloned downstream of the t_R' terminator region and upstream of the *lacZ* reporter gene. In the absence of its cognate Q, this construct expressed extremely low levels of β-galactosidase (195 units), whereas in the presence of the H-19B Q it expressed high levels of β-galactosidase (24 985 units). The H-19B Q stimulated a greater than 100-fold readthrough of the terminators.

The specificity of H-19B $p_R'(qut)$ action was tested by using a third plasmid construct that expresses the Q protein of λ. In the presence of λ Q, the reporter construct again expressed only low levels of *lacZ*, indicating minimal readthrough.

To confirm that our construct with λ *Q* expressed active Q protein, we tested whether the induced plasmid could complement growth of a λ derivative with two amber mutations in the *Q* gene (λ$Qam_{73}am_{501}$) (Herskowitz and Signer, 1970) in a Sup^o host. As shown in Table 2, complementation was observed and, further, complementation was not observed in the presence of the plasmid expressing the Q of H-19B. These results are consistent with our contention that the H-19B Q is specific for the H-19B *qut* site (see below).

As discussed above, the genes for shiga-like toxin II (*stx-II*) are found on the genome of another phage, 933W (Smith *et al*., 1984; O'Brien *et al*., 1989). Mühldorfer *et al*. (1996) concluded that a phage-encoded factor must exert a positive regulatory role on the expression of *stx-II* based on experiments with a plasmid carrying a translational fusion between the *stx-IIA* gene and a reporter gene (*phoA*). This plasmid-based fusion has the proximal portion of *stx-IIA* from phage 933W, with the associated promoter and some additional upstream region fused to the reporter gene. In their work, it was shown that mitomycin C induction of either a 933W or an H-19B prophage in a lysogen carrying this reporter construct resulted in significantly higher levels of expression from the reporter construct. By damaging DNA, mitomycin C treatment results in prophage induction that leads to expression of phage functions and ultimately lytic growth (al-Jumaili *et al*., 1992). Thus, there should be an increase in Stx-II toxin expression because of increased copies of the phage genome resulting from phage replication. What seemed remarkable was the increased expression of the reporter gene from the plasmid. Mühldorfer *et al*. (1996) concluded that, 'a phage-encoded factor, which is also dependent on phage induction, has a positive regulatory impact on the *stx-II* operon'.

Table 2. Specificity of action of Q proteins of λ and H-19B.[a]

Plasmids	Relevant genotype	EOP[b] λ	EOP[b] λ Qam_{73}-am_{501}
–	Sup^o	1.6	< 0.0001
plac-Q(λ)	λ *Q* gene	1	1.8
plac-Q(H-19B)	H-19B *Q* gene	0.8	< 0.0001

a. The bacteria used were K3093 (Sup^o) or derivatives with the indicated plasmid.
b. Efficiency of plating (EOP) was determined by comparing plaque formation on the indicated host lawn with that observed on a lawn of an *E. coli SupF* strain (K60).

The position of the *stx-I* gene in the H-19B genome suggested the Q protein as a likely candidate for the predicted phage-encoded regulatory function that turns on the *stx-II* gene and also suggested that the *stx* genes of 933W and H-19B occupy analogous positions in their genomes. Moreover, if the positive activator of transcription from the 933W *stx-IIA* fragment is the Q of H-19B, then H-19B and 933W would probably share the same *Q* gene. To test this idea, we sequenced the *Q-stx-II* region of 933W using primers designed from the sequence of the H-19B *Q* gene and the published sequence of *stx-II* (GenBank no. X07865). The results of this DNA sequencing showed that 933W has the same *Q*, p_R', *qut* and t_R' as H-19B located upstream of the *stx-II* genes (Fig. 4).

Collectively, we think this is strong evidence that the positive regulatory functions expressed by the induced lysogens enhancing *stx-II–phoA* expression from the reporter gene construct are the *Q* gene products of phages H-19B and 933W. As the *stx-II* gene in 933W is located at the same position as *stx-I* in H-19B, the phage DNA fragment in the reporter gene construct, which includes ≈2.4 kb of phage DNA upstream of the *stx-II* gene, should have the p_R' promoter region of 933W. Transcription initiating at p_R' would be expected to terminate at t_R' unless there is a mechanism of antitermination modifying transcription from p_R'. Accordingly, after phage induction, the Q protein would bind at the *qut* site at p_R' and modify RNA polymerase, resulting in readthrough of terminators and enhanced expression of toxin and other downstream genes. The *stx-I* and *stx-II* promoters are different (Calderwood and Mekalanos, 1987; de Grandis *et al*., 1987; Sung *et al*., 1990), yet induction of both an H-19B and a 933W prophage induced transcription of the 933W *stx-IIA–phoA* gene fusion (Mühldorfer *et al*., 1996). Therefore, the two activators must recognize a site associated with another promoter, most

likely p_R'. Consistent with this argument was the observation of Mühldorfer *et al*. (1996) that mitomycin C induction of a λ lysogen did not induce expression of the plasmid-based gene fusion. As we have shown, the Q protein of λ does not activate expression from the p_R' of H-19B. In accord with the argument presented above, it is unlikely to do so with the p_R' of 933W.

We used an enzyme immunoassay (EIA) using a polyclonal antisera raised against both Stx-I and Stx-II to directly determine if Stx expression can be enhanced by Q-mediated antitermination of transcription initiating at the p_R' promoter. Lysogens carrying either an H-19B or 933W prophage provided natural constructs of the p_R'*qut–stx* arrangements and, when required, Q was supplied from a plasmid, *plac-Q*(H-19B). As the prophages were never induced, neither phage encoded regulatory proteins nor gene copy number are factors that need be considered in assessing Stx levels.

Figure 5 shows the results of experiments in which we assessed the effect of Q on Stx expression from resident prophages. Although the *Q* gene on the plasmid was obtained from H-19B, as stated above, the *Q* gene of 933W has an identical DNA sequence. Expression of this shared Q protein significantly enhances expression of the *stx* genes from both 933W and H-19B prophages; the former by 17-fold (compare columns 1 and 2) and the latter by fourfold (compare columns 3 and 4). These results are consistent with those of Mühldorfer *et al*. (1996) discussed above and therefore support our contention that the *trans*-acting phage-encoded factors that turn on the *stx-II–phoA* fusion are the Q proteins of the two phages. The differences in the levels of Stx expressed by the H-19B and 933W prophages in the presence of Q could reflect one or more of a number of factors: the differences in the promoters for the *stx-I* and *stx-II* genes (Calderwood and Mekalanos, 1987; de Grandis *et al*., 1987; Sung *et al*., 1990); significant differences in the two genomes between the p_R'*(qut)*t_R' region and the *stx* genes; and possible differences in downstream terminators. Currently, we are unable to distinguish between these alternatives.

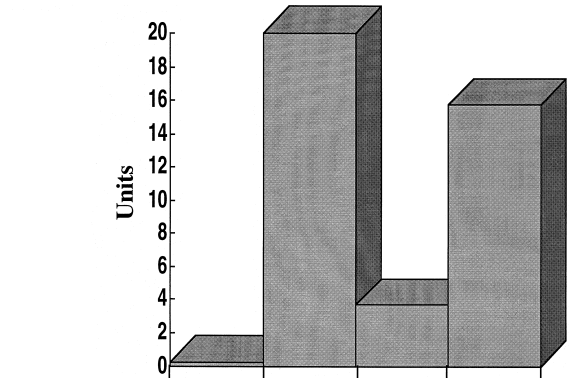

Fig. 5. Measurement of Stx produced from H-19B and 933W lysogens and lysogen derivatives carrying plasmids with an inducible Q protein. The non-specific background was determined by measuring antibody-binding activity in extracts from the C600 parent strain. The toxin units shown in the chart were calculated by subtracting the background level from the actual measured levels of antibody binding activity and are represented as units over background. Values shown are representative of the values obtained from at least six independent experiments.

Regulation of lysis

The lysis genes of lambdoid phages typically are located immediately downstream of their respective *Q* genes (reviewed in Blasi and Young, 1996). There are two well-characterized genes that are involved in lysis; in λ these are the *S* and *R* genes and each has homologues in other lambdoid phages. The *R* gene encodes an endolysin that degrades the peptidoglycan of the cell wall. The R protein lacks a signal sequence, yet it functions in the periplasm. Transport of R across the cytoplasmic membrane is provided by the *S* gene product, which creates membrane lesions and thus is called a holin. The *S* genes are well conserved and share an unusual feature: translation can initiate at either of two closely spaced methionine codons. One model of lysis regulation has proposed that the shorter protein forms the holin ring in the membrane, whereas the longer S protein inhibits ring formation. According to this model, the relative concentration of the two *S* gene products during lytic phage growth would determine the time of lysis.

Based on the map positions of the *S* and *R* genes of other lambdoid phages, we predicted that the lysis genes of H-19B would be located immediately downstream of the toxin genes. However, ≈3 kb of DNA separates the toxin genes from the sequence our analysis predicted to be the putative H-19B *S* gene (Fig. 4). Although the H-19B putative *S* gene shows a high degree of homology to the *S* gene of bacteriophage 21 (76% identity), surprisingly, we find that the derived amino acid sequence, unlike all other identified *S* gene homologues, does not have the highly conserved closely spaced dual translation start sites (Fig. 6). If the two start sites indeed play a role in regulation of the time of lysis of other lambdoid phages, H-19B presumably modulates action of the *S* gene product by a different mechanism.

We propose that the location of the *stx-I* genes downstream of p_R' and upstream of the lysis genes facilitates synthesis as well as release of Stx from the bacteria. Stxs are secreted into the periplasm without an identifiable

A

λ	MetLysMetProGluLys (105, 107)
P22	MetLysLysMetProGluLys (105, 107)
HK97	MetLysMetProGluLys (104, 106)
HK022	MetLysMetProGluLys (105, 107)
21	MetLysSerMetAspLysIle (68, 71)
phi 80	MetTyrArgMetAspLysLeu (70, 73)
933W	MetTyrGlnMetGluLysIleSerTyr (68, 71)
H-19B	MetGluLysIleTyrTyr (68)

B

```
TCGGCGGTGACGGAGACATACATCAGATGGAAAAAATCACAACAGGTGTG
                          M  E  K  I  T  T  G  V

TCATACACCACGTCAGCGGTAGGGACGGGATACTGGTTACTGCAACTGCTG
 S  Y  T  T  S  A  V  G  T  G  Y  W  L  L  Q  L  L

GACAAAGTCTCTCCGTCCCAGTGGGTGGCAATAGGTGTACTGGGGAGTCTG
 D  K  V  S  P  S  Q  W  V  A  I  G  V  L  G  S  L

CTGTTTGGCCTGCTGACGTATCTGACTAACCTGTATTTCAAAATCAGAGAG
 L  F  G  L  L  T  Y  L  T  N  L  Y  F  K  I  R  E

GACCGGCGTAAGGCGGTGCGGGGAGAGTAA
 D  R  R  K  A  V  R  G  E
```

Fig. 6. Comparison of H-19B and other Lambdoid phage holin proteins.
A. Alignment of the amino-terminal portion of lambdoid holin gene products showing dual start motifs. Numbers in parentheses represent amino acid size of the two predicted gene products. Underlined Met indicates start of predicted active holin protein. Sequence data, other than H-19B, from Blasi and Young (1996).
B. Nucleotide and predicted amino acid sequence of H-19B holin gene.

mechanism for transport outside the outer membrane (O'Brien and Holmes, 1987). It is still unclear how the toxin is released from the bacterium to cause disease, although it has been proposed that death of the bacteria with associated autolysis must occur for release of toxin (O'Brien and Holmes, 1987). Specifically, we propose that expression of the phage lysis genes may be an important factor in release of phage-encoded Stxs. One obvious mechanism would be Q-stimulated readthrough from p_R' if the prophage is induced. Alternatively, the placement of the *stx* genes makes it possible that readthrough of transcription initiating at the *stx* promoter could result in sufficient expression of the downstream phage lysis genes to cause cell lysis and release of toxin. This means that transcription would have to traverse the ≈3 kb of DNA between the 3′ end of the toxin genes and the 5′ end of the *S* homologue. This could occur providing that either there were no significant termination signals in the intervening 3 kb or that transcription from the toxin promoter is capable of antitermination. The antitermination of Q-modified transcription from p_R' would allow readthrough of terminators and activate both toxin and lysis gene expression. A functional role for the phage lysis genes in toxin release is consistent with the finding that a different regulatory mechanism for the H-19B *S*-like holin gene product has evolved. Perhaps, prophage induction and consequently cell lysis and toxin release is stimulated by growth in the intestine.

We used the previously described lysogens that carry either an H-19B or a 933W prophage and the Q-expressing plasmid, *plac-Q*(H-19B), to determine if Q-mediated antitermination can lead to expression of phage-encoded lysis function (data not shown). When Q expression was induced from *plac-Q*(H-19B) in lysogens that contained either an H-19B or 933W prophage, lysis occurred within 3 h. However, if Q expression was not induced in these lysogens, lysis did not occur. The most plausible explanation for these results is that Q, acting at the prophage *qut* sites, results in transcription from the p_R' promoters reading through to the lysis genes and beyond. This, of course, leads to synthesis of the phage lysis proteins and cell lysis. The seemingly long delay before lysis is probably the result of the low copy number because the repressed prophages do not replicate.

Recently, prophage induction during growth in the mouse intestine was demonstrated with a filamentous phage, CTXΦ, the phage that carries the cholera toxin genes in *Vibrio cholerae* (Waldorf and Mekalanos, 1996). We propose that this mechanism might also occur with *E. coli* carrying the H-19B (or 933W) prophage, thereby inducing cell lysis and allowing release of the toxin into the intestinal environment.

During phage lytic growth, transcription of lysis genes precedes production of phage particles. The time of lysis of lambdoid phages is apparently regulated, at least in part, by competition between two translation products of the *S* gene. As the H-19B *S* homologue apparently encodes only the active holin, this phage may use another mechanism to prevent premature lysis, one that allows lysis when *stx* is expressed.

An example of lysis functions controlling toxin release has been observed in *E. coli* carrying colicin plasmids,

which produce colicin proteins that are lethal to other *E. coli* and related bacteria (Riley, 1993). Lysis results from expression of a colicin-encoded lysis gene allowing the release of the colicin proteins into the environment. Only a few of the colicin-bearing population lyse, whereas the majority of these *E. coli* survive.

The placement of the H-19B *stx-I* gene raises the question of whether there has been selective pressure for genetic linkage of the toxin to the lysis cassette of lambdoid genes. Campbell (1988) has proposed that the lambdoid family of phages, because they commonly recombine with each other to generate variants, are like 'members of a sexually reproducing species, drawing on a common gene pool'. Based on this concept, one can imagine that a cassette of lysis genes associated with an *stx* gene could be a member of this exchangeable gene pool. By having the associated lysis genes, the *stx* genes would bring along the information necessary for their release from the bacterial cell.

Experimental procedures

Media

LB, TB and top agar media used in these studies have been described previously (Miller and Friedman, 1980). EMBO plates are described in Gottesman and Yarmolinsky (1968). M56 media (Monod *et al*., 1951), used for β-galactosidase assays, was supplemented with 0.2% casamino acids and either 0.02% fructose or 0.2% fructose (see below). IPTG was added at a final concentration of 1 mM. The antibiotics spectinomycin and ampicillin were used at 150 $\mu g\,ml^{-1}$ and 100 $\mu g\,ml^{-1}$ respectively.

Bacteria, phage and plasmids

Bacterial strains are listed in Table 3. H-19B, a shiga-like toxin I converting bacteriophage, was originally isolated from *E. coli* 026:H11 strain H19 by H. W. Smith (Smith *et al*., 1983). *E. coli* K-12 strains C600(H-19B) and C600(933W) were provided by Alison D. O'Brien, Uniformed Services University of the Health Sciences, Bethesda, MD, USA. The H-19B phage was isolated from the C600(H-19B) lysogen. The λ phages used in the complementation studies are *cI857* (Sussman and Jacob, 1962) derivatives and were originally obtained from the NIH collection. λQam_{73}-am_{501} also has two amber mutations in the *Q* gene (Vogel and Jensen, 1995). Plasmid pBR325 (Bolivar, 1978) was used for cloning phage DNA for sequencing. Plasmid pTL61T (Linn and St.Pierre, 1990) was used for cloning fragments containing the H-19B *nutR* and the p_R'*(qut)*t_R' sites. pTL61T contains a promoterless *lacZ* gene downstream of an RNase III processing site and has the origin of replication from pBR322. Plasmid pGB2-plac is a derivative of the low-copy-number plasmid pGB2 (Churchward *et al*., 1984), which is compatible with pBR322 and confers spectinomycin resistance. The pGB2-plac derivative was

Table 3. Bacterial strains.

Strain	Relevant genotype	Source
K37	*Sup°*	This laboratory
K60	*SupF*	This laboratory
K99	C600	NIH Collection
K3093	K37 Tn*5*::*lacZ*–$lacI_{Q1}$	This laboratory
K4419	DH5α Δ(*lacIZYA*–*argF*)	This laboratory
K6123	K37 + *plac-N*(λ)	This laboratory
K7523	C600(H-19B)	A. O'Brien
K8948	C600(H-19B) + *plac-Q*(H-19B) + pMMB66HE	This laboratory
K8443	C600(933W)	A. O'Brien
K8952	C600(933W) + *plac-Q*(H-19B) + pMMB66HE	This laboratory
K8210	K37 + pGB2–*plac*	This laboratory
K8234	K37 + p*imm*–H-19B	This laboratory
K8602	K37 + plac-N(H-19B)	This laboratory
K8625	K3093 + pTL61T–*plac*	This laboratory
K8632	K3093 + pTL61T–*plac*–term	This laboratory
K8641	K3093 + pTL61T–*plac*–*nut* (H-19B)-term	This laboratory
K8643	K3093 + pTL61T–*plac*–*nut*(H-19B)-term + *plac-N*(H-19B)	This laboratory
K8654	K3093 + pTL61T–*plac*–*nut*(H-19B)-term + *plac-N*(λ)	This laboratory
K8644	K3093 + pTL61T–*plac*–*nut*(H-19B)-term + pGB2–*plac*	This laboratory
K8642	K3093 + pTL61T–*plac*–*nut*(λ)-term	This laboratory
K8645	K3093 + pTL61T–*plac*–*nut*(λ)-term + *plac-N*(λ)	This laboratory
K8646	K3093 + pTL61T–*plac*–*nut*(λ)-term + pGB2–*plac*	This laboratory
K8656	K3093 + pTL61T–*plac*–*nut*(λ)-term + *plac-N*(H-19B)	This laboratory
K8820	K3093 + *plac-Q*(H-19B)	This laboratory
K8873	K3093 + *plac-Q*(λ)	This laboratory
K8897	DH5α + pTL61T–p_R'*(qut)*Δt_R'	This laboratory
K8900	DH5α + pTL61T–p_R'*(qut)*t_R'	This laboratory
K8901	DH5α + pTL61T–p_R'*(qut)*t_R' + *plac-Q*(H-19B)	This laboratory
K8919	DH5α + pTL61T–p_R'*(qut)*t_R' + *plac-Q*(λ)	This laboratory
K8915	DH5α + pTL61T–p_R'*(qut)*t_R'-term	This laboratory
K8917	DH5α+ pTL61T–p_R'*(qut)*t_R'-term + *plac-Q*(H-19B)	This laboratory

constructed by placing the *plac* promoter fragment from pSRplac (S. Rockenbach and S. Tomich, unpublished) into the *Eco*RI–*Bam*HI site of pGB2 (M. Barber, unpublished from this laboratory). Plasmid pMMB66HE expresses *lacI*^{q} and confers ampicillin resistance (Morales *et al*., 1991).

Plasmid construction

H-19B immunity region plasmid. *Hin*dIII-digested H-19B DNA was cloned into pBR325 using standard methods of cloning (Sambrook *et al*., 1989) to construct p*imm*–H-19B. A clone containing the immunity region of H-19B was identified by streaking transformants against an H-19B phage lysate on EMBO plates and screening for bacteria that were not lysed by the phage. A derivative of strain K37 containing a plasmid with a ≈4.6 kb *Hin*dIII fragment of H-19B DNA was found by this screen. This fragment also contained a portion of the delayed early region and was used for sequencing the *nutR* region.

Plasmids for testing N–Nut interaction. Plasmids containing the *nutR* sites of λ and H-19B were constructed using the pTL61T vector (Linn and St.Pierre, 1990) and maintained by selection against ampicillin. Using the plasmid pSRplac as the template, PCR was used to synthesize a fragment containing the *plac* promoter. This fragment was cloned into pTL61T, generating pTL61T–plac. An ≈400 bp fragment of H-19B including the 3′ portion of the *cro* gene, *nutR*, t_{R1} and the 5′ portion of the *c*II gene was then cloned into plasmid pTL61T-plac behind *plac* creating pTL61T–*plac*–*nut*(H-19B). A ≈600 bp fragment containing the λ t_R' and *rrn* T1-T2 Rho-independent terminators (King *et al*., 1996) was then cloned downstream of the *nutR* fragment resulting in the placement of *plac*–*nutR*–t_R'-T1-T2 directly upstream of an RNase III processing site followed by the *lacZ* gene in the pTL61T vector, creating pTL61T–*plac*–*nut*(H-19B)-term. The same strategy was used in constructing an analogous derivative of pTL61T with the λ *nutR* site, creating pTL61T–*plac*–*nut*(λ)-term.

Plasmids containing *N* genes were constructed using pGB2-plac and maintained by selection against spectinomycin. *plac-N*(H-19B) and *plac-N*(λ) were constructed by placing, respectively, the H-19B *N* and λ *N* genes downstream of the *plac* promoter of pGB2-plac.

Plasmids for testing Q–p_R'(qut) *interaction*. A plasmid containing a 352 bp fragment of H-19B DNA, with the putative p_R'*(qut)*t_R' region, was constructed using the pTL61T vector (Linn and St.Pierre, 1990), creating pTL61T–p_R'*(qut)*t_R', and was maintained by selection against ampicillin. A second plasmid, pTL61T–p_R'*(qut)*t_R'-term, was constructed by cloning the terminator cassette (λt_R'-T1-T2) at a *Bam*HI site between t_R' and the *lacZ* gene. Plasmid pTL61T–p_R'*(qut)*Δt_R' was constructed by cloning the putative p_R'*(qut)* region without the t_R' terminator.

The *Q* genes from λ and H-19B were individually cloned into the pGB2–plac vector, creating *plac-Q*(λ) and *plac-Q*(H-19B), directly downstream of the *plac* promoter and maintained by selection against spectinomycin.

For the EIA experiments the *plac-Q*(H-19B) plasmid was transformed into C600 lysogens of H-19B and 933W, along with a second, compatible plasmid, pMMB66HE, which expresses *lacI*^{q}, to ensure repression of the *plac*-Q construct until the addition of IPTG.

β-galactosidase assays

Strains were grown overnight in M56 media supplemented with 0.2% casamino acids and 0.02% fructose. Cultures were then diluted 1:20 into fresh M56 media supplemented with 0.2% casamino acids and 0.2% fructose and grown at 37°C for 60 min. At that time, IPTG was added to a final concentration of 1 mM and cultures were grown for another 60 min to an OD_{600} of 0.100–0.200. Appropriate antibiotics were added to maintain plasmids. β-Galactosidase assays were performed and values calculated according to Miller (1992).

Measurement of transcription antitermination

Readthrough of terminators, which was used to assess levels of transcription antitermination, was quantitatively determined by measuring β-galactosidase levels expressed from a downstream *lacZ* reporter gene. These results were converted to percentage antitermination using as the 100% level the units of β-galactosidase synthesized from plasmids with either the *plac*–*nut*–*lacZ* construct (Fig. 3) or the p_R'*(qut)*–*lacZ* (Table 1) construct.

Phage complementation studies

Dilutions of phage were titred on bacterial lawns formed by pouring 2.5 ml of TB top agar containing 0.3 ml of an overnight bacterial culture grown in LB broth onto TB agar plates that were then incubated overnight at 37°C. For those strains carrying a plasmid that expresses *Q*, 150 μg ml^{-1} spectinomycin was added to the overnight cultures and spectinomycin plus 1 mM IPTG were added to the top agar. Phage growth is reported as efficiency of plating (EOP) as described previously (Bear *et al*., 1984).

Phage DNA isolation

Phage lysates were obtained by culturing C600(H-19B) in LB to an OD_{420} of ≈0.700 followed by the addition 5 μg ml^{-1} of mitomycin C. The culture was protected from light and aerated at 37°C until lysis occurred. Phage DNA was isolated from phage lysates using the Qiagen Lambda kit (Qiagen).

DNA sequencing

The immunity region sequence of H-19B was determined from p*imm*–H-19B using primers that anneal to the vector on either side of the cloning site. The template for sequencing of all other regions of the phage genome was purified phage DNAs isolated from phage lysates. Sequences were determined using the Sequenase 2.1 kit or the Thermo Sequenase kit (Amersham) with double-stranded plasmid DNA or phage DNA. Computer analysis of DNA sequences was performed using the Genetics Computer Group (GCG) program (Devereux *et al*., 1984). The DNA sequences of the portion of the

H-19B genome we have determined has been deposited in GenBank and given the accession number AF034975.

Sequencing of the H-19B N *gene*. As N proteins recognize BOXB sequences (Lazinski *et al*., 1989; Chattopadhyay *et al*., 1995; Mogridge *et al*., 1995) and HK97 and H-19B have the same *boxB* in both *nutR* and *nutL* (Fig. 1), we assumed that the two phages have similar *N* sequences. Based on this assumption, we used primers to the HK97 *N* sequence (R. Hendrix, personal communication) to obtain the sequence of an ORF in H-19B that by position and sequence suggested an *N* gene.

Enzyme immunoassays for shiga-like toxin

Overnight cultures were grown in LB or LB + 150 μg ml^{-1} spectinomycin and 100 μg ml^{-1} ampicillin and then subsequently diluted 1:100 into fresh LB (plus antibiotic in strains carrying plasmids) and allowed to grow to a cell density of $OD_{420} = \approx 0.500$. IPTG was added at this point to a final concentration of 1 mM and growth was monitored for 1 h, diluting cultures back with fresh LB (+ supplements when required) periodically so as to keep cultures in the exponential phase of growth (between $OD_{420} = 0.300$ and 0.900). At 1 h after IPTG addition, a final OD_{420} was measured and cultures were placed on ice. Cultures were sonicated on ice for 15 s pulses using a Virsonic sonicator (VirTis,). Total protein was measured using the Bio-Rad protein assay (Bio-Rad Laboratories) according to the manufacturer's directions.

EIA assays were performed using an enzyme immunoassay kit for measuring Stx-I and -II production purchased from Meridian Diagnostics. The kit provided microwells coated with monoclonal antibodies specific for Stx-I and -II, a polyclonal detection antibody specific for Stx-I and -II, a goat anti-rabbit antibody conjugated to horseradish peroxidase and a urea peroxide substrate. The manufacturer's directions were followed exactly, with the exception that cell lysates were isolated from exponential phase of growth (instead of overnight cultures).

The final reaction of the assay was measured at OD_{450}. C600 was used as the negative control as all strain derivatives are in this background. The OD_{450} read for C600 (background) was subtracted from all other reactions and individual results were calculated as units above background based on the OD_{450} for each reaction.

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