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Bacteriophage control of Shiga toxin 1 production and release by *Escherichia coli*

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Summary

The *stx* genes of many Shiga toxin-encoding *Escherichia coli* (STEC) strains are encoded by prophages of the λ bacteriophage family. In the genome of the Stx1-encoding phage H-19B, the stx_1AB genes are found ≈ 1 kb downstream of the late phage promoter, p_R', but are known to be regulated by the associated iron-regulated promoter, p_{Stx1}. Growth of H-19B lysogens in low iron concentrations or in conditions that induce the prophage results in increased Stx1 production. Although the mechanism by which low iron concentration induces Stx1 production is well understood, the mechanisms by which phage induction enhances toxin production have not been extensively characterized. The studies reported here identify the factors that contribute to Stx1 production after induction of the H-19B prophage. We found that replication of the phage genome, with the associated increase in stx_1AB copy number, is the most quantitatively important mechanism by which H-19B induction increases Stx1 production. Three promoters are shown to be involved in stx_1AB transcription after phage induction, the iron-regulated p_{Stx1} and the phage-regulated p_R and p_R' promoters, the relative importance of which varies with environmental conditions. Late phage transcription initiating at the p_R' promoter, contrary to previous findings in the related Stx2-encoding phage φ361, was found to be unnecessary for high-level Stx1 production after phage induction. Finally, we present evidence that phage-mediated lysis regulates the quantity of Stx1 produced by determining the duration of Stx1 accumulation and provides a mechanism for Stx1 release. By amplifying stx_1AB copy number, regulating stx_1AB transcription and allowing for Stx1 release, the biology of the Stx-encoding phages contributes greatly to the production of Stx, the principal virulence factor of STEC.

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are pathogens responsible for outbreaks and sporadic cases of diarrhoea (Head *et al*., 1988; Al-Jumaili *et al*., 1992; Muhldorfer *et al*., 1996). STEC are distinguished from other types of diarrhoeaogenic *E. coli* by their production of Shiga toxins, which account for the severe clinical manifestations of STEC infection, including haemorrhagic colitis and the haemolytic uraemic syndrome (Griffin, 1995). Two principal types of Stx, Stx1 and Stx2, have been described, each of which is composed of eukaryotic glycolipid-binding B subunits and an enzymatic A subunit that inhibits protein synthesis by cleaving ribosomal RNA (Acheson and Keusch, 1996). In nearly all cases examined, the *stx* genes in STEC are closely associated with bacteriophage sequences (Newland and Neill, 1988; Datz *et al*., 1996; Unkmeir and Schmidt, 2000) and, in many cases, the *stx* genes reside within functional prophages of the λ family (Karch *et al*., 1999; Mizutani *et al*., 1999).

H-19B is an Stx1-encoding bacteriophage isolated from the O26:H11 STEC strain H19, obtained in 1967 from an infant with diarrhoea (Smith and Linggood, 1971). After the cloning of the stx_1 genes from purified H-19B DNA (Huang *et al*., 1986), several groups identified a functional promoter, p_{Stx1}, directly upstream from the stx_1 coding sequence (Calderwood *et al*., 1987; De Grandis *et al*., 1987; Jackson *et al*., 1987). The activity of this promoter is regulated by environmental iron concentration by a mechanism involving the iron-dependent Fur transcriptional repressor, which is thought to bind to a site near p_{Stx1} (Calderwood and Mekalanos, 1987; Calderwood *et al*., 1987; De Grandis, 1987). H-19B is a

λ-like phage, sharing with λ a similar genome organization and many genes and sites that are identical or functionally related (Fig. 1) (Huang *et al*., 1986; 1987). Agents that induce the λ-like Stx-encoding prophages, such as mitomycin C, have been shown to increase toxin production by STEC strains (Head *et al*., 1988; Acheson *et al*., 1989; Hull *et al*., 1991; 1993). Thus, Stx1 production by STEC strains can be induced in two ways: through low-iron induction of p_{Stx1} and through prophage induction.

Induction of λ-like prophages occurs when phage repressor levels fall below the minimal concentration necessary to block transcription initiating at the two promoters for early transcription, p_L and p_R. Induction occurs at a low level spontaneously or at a high level in the presence of agents such as mitomycin C that damage DNA and activate RecA, which in turn facilitates autocleavage of phage repressor (Roberts and Devoret, 1983; Little, 1995). Removal of repression allows for transcription initiation at p_L and p_R. p_L-initiated transcription results in expression of the *N* antiterminator gene, whereas p_R-initiated transcription results in expression of the replication genes, *O* and *P*, which is inefficient because of the terminator, *t*R1, directly downstream of p_R (Friedman and Gottesman, 1983) (Fig. 1). Subsequently, transcription from p_R is modified by the N antiterminator, which acts with a group of host factors to modify RNA polymerase to a form that over-rides termination barriers (Das, 1992; Richardson and Greenblatt, 1996; Friedman and Court, 2001). N-modified transcription from p_R proceeds efficiently through *O* and *P* and into the region encoding a second antiterminator gene, *Q*, near the late phage promoter p_R'. Transcription from p_R' is constitutive but, in the absence of *Q*, terminates at an immediately downstream terminator, t_R' (Roberts *et al*., 1998). *Q* modifies transcription from p_R' such that it proceeds through t_R', allowing efficient transcription of the late phage genes, which include lysis and morphogenesis genes.

The *stx* genes are located between p_R'-like sequences and putative lysis genes in the Stx-encoding phages H-19B (Neely and Friedman, 1998a) and 933 W (Neely and Friedman, 1998b; Plunkett *et al*., 1999). Thus, they could be expressed as late phage genes, dependent upon the *Q* antiterminator and the p_R' promoter for efficient transcription. Consistent with this hypothesis, overexpression of the *Q* gene of H-19B resulted in marked induction of toxin expression and phage-mediated lysis in lysogens of H-19B and the Stx2-encoding phage 933W, which share a nearly identical *Q* gene sequence (Neely and Friedman, 1998b). Moreover, mutants lacking the *Q*-p_R' sequence in an Stx2-encoding STEC isolate were found to be drastically impaired in Stx2 production *in vitro* and in a mouse intestinal model (Wagner *et al*., 2001a), indicating the critical importance of phage late transcription for Stx2 production. The importance of the *Q*-p_R' sequence for Stx1 production has not been tested directly, and was uncertain given the presence of the adjacent, iron-regulated p_{Stx1} promoter.

Besides influencing production of Stx, phage induction could also lead to Stx release, via phage-mediated lysis, given the tight genetic linkage between the p_R', *stx* and lysis gene sequences (Neely and Friedman, 1998a; Plunkett *et al*., 1999). If transcription of stx_1 is initiated from p_R', it would be coupled with transcription of the downstream phage lysis genes, whereas if it is initiated from p_{Stx1}, it would not. This is because *Q*-modified transcription from p_R' would over-ride a terminator, t_{Stx1}, found between stx_1AB and the downstream lysis genes (De Grandis, 1987) and would consequently proceed into the lysis genes, whereas transcription initiating at p_{Stx1} would not be modified by an antiterminator and therefore would terminate at t_{Stx1} (Fig. 1). The contribution of phage-mediated lysis to Stx release has not been tested directly. We set out to determine whether phage-mediated lysis is important in Stx release and to determine what mechanisms account for increased Stx1 production and release by *E. coli* lysogens of the H-19B prophage.

Results

Induction of Stx1 production and release by low iron and mitomycin C

In this study, we used prophage induction with mitomycin C to assess the contribution of phage induction to toxin production, and the low-iron syncase broth to assess the contribution of the iron-regulated p_{Stx1} promoter to toxin production and release. These artificial conditions allowed us to distinguish these stimuli to facilitate the study of the mechanisms involved in their effects. In order to establish a baseline for comparison in our mutational analyses (see below), we confirmed the previously described effects of iron concentration and phage induction on Stx1 production. Low media iron concentration resulted in a fivefold increase in Stx1 production by a K-12 lysogen of H-19B, named MC1000(H-19B) (Wagner *et al*., 1999) relative to growth in iron-rich conditions (Fig. 2), probably reflecting diminished Fur repressor activity at p_{Stx1} (Calderwood, 1987). We next assessed Stx1 production after H-19B prophage induction with mitomycin C, finding an ≈ 70-fold increase in Stx1 production under these conditions. Because this was done in iron-replete media, this increase probably primarily reflects the contribution of phage gene products to Stx1 production, with little contribution from the iron-repressed p_{Stx1} promoter. We also assessed toxin production during growth in conditions in which both stimuli for toxin production were present.

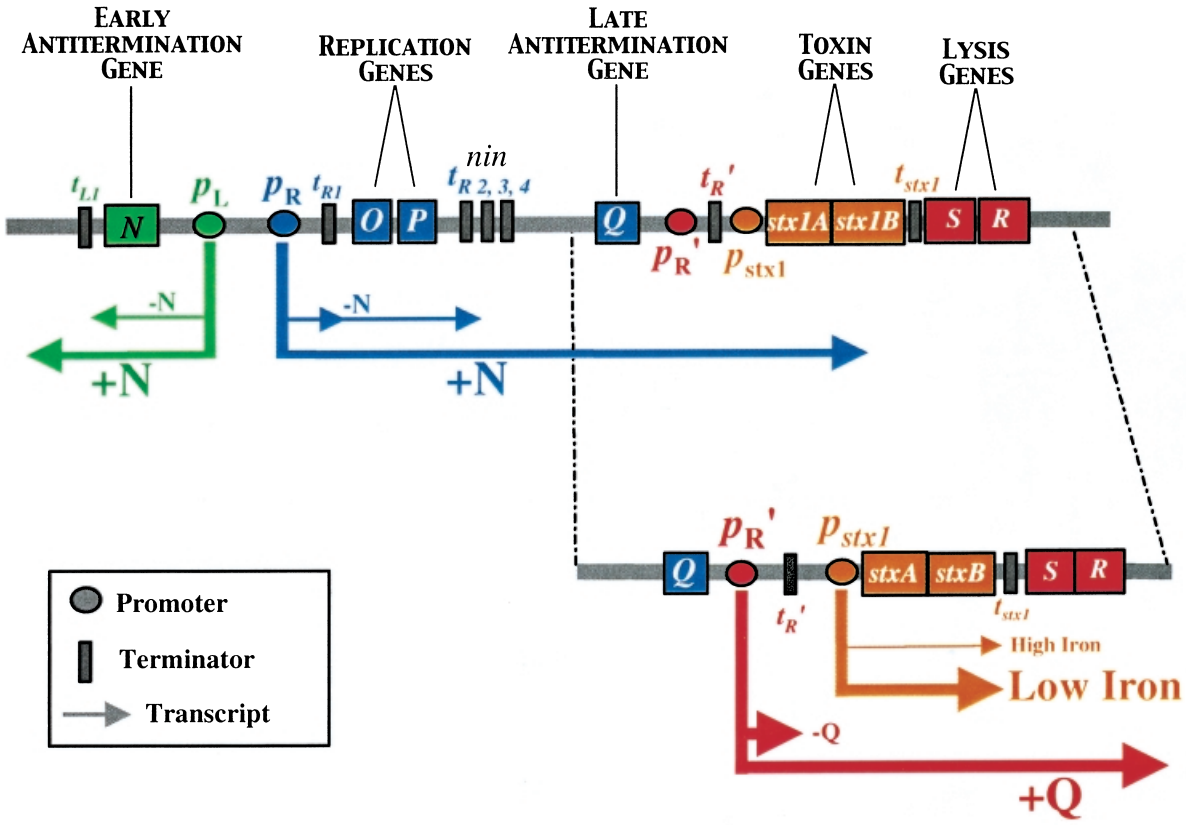

Fig. 1. Map and transcription patterns of the segment of the H-19B genome involved in Stx1 regulation (Neely and Friedman, 1998a). Like phage λ, the H-19B prophage is repressed by the activity of phage repressor at the early control region, containing early promoters p_L and p_R. Induction results from a loss of repressor activity, allowing transcription to initiate at p_L and p_R. The N antiterminator, transcribed from p_L, acts to render transcription complexes initiating at p_L and p_R resistant to termination signals, allowing transcripts from p_R to efficiently readthrough *t*R1, the *O* and *P* replication genes, the *nin* region terminators (*t*R2, *t*R3 and *t*R4), and subsequently transcribe the *Q* late antitermination gene.
Inset. Transcription of the *stx*$_1$ genes from p_{Stx1} or p_R'. Transcription initiating at p_{Stx1} is inducible in low-iron conditions and terminates at t_{Stx1}, located just downstream of *stx*$_1$*B* and upstream of the lysis genes. Transcription initiating at p_R' is constitutive, but terminates immediately downstream at t_R' in the absence of the late antiterminator *Q*. After phage induction, *Q* acts through p_R' to render transcription complexes initiating at p_R' unsusceptible to termination, and *Q*-modified transcription proceeds through t_R', the toxin genes, t_{Stx1} and into the phage lysis genes. As N-modified transcription initiating at p_R is also resistant to termination signals, transcription from p_R can also proceed through t_R' and into the toxin genes, allowing this promoter to contribute to toxin production after prophage induction.

Under these conditions, MC1000(H-19B) made ≈ 100-fold more toxin than during growth in non-inducing conditions (Fig. 2). As it is unlikely that these optimized *in vitro*-inducing conditions resemble those encountered *in situ* by STEC, we cannot assess the relative importance of low iron concentration versus prophage induction as clinically relevant stimuli for Stx production.

Although both stimuli, low iron and mitomycin C, were known to induce toxin production, it was not known whether they lead to toxin release. We studied release of Stx1 from the clinical isolate, H19, from which phage H-19B was isolated. We chose H19 as there could be mechanisms for Stx1 release operative in the clinical isolate H19 but not operative in MC1000(H-19B). Cultures of H19 were grown under low-iron conditions or phage-inducing conditions, and intracellular and extracellular Stx1 concentrations were measured in these cultures at defined intervals. Both conditions strongly induced Stx1 production (Fig. 3). However, we observed a dramatic difference in the localization of Stx1 in these two cultures. Less than 1% of the Stx1 was found in the supernatant of cultures grown in low-iron media, even after overnight growth (Fig. 3A; data not shown). In contrast, after 3 h growth in the presence of mitomycin C, there was an increase in the Stx1 concentration in supernatants of H-19 cultures, and more than 99% of Stx1 was extracellular after overnight growth (Fig. 3B; data not shown). Thus, although both low iron and mitomycin C induced Stx1 production, substantial Stx1 release into the supernatant occurred only under phage-inducing conditions.

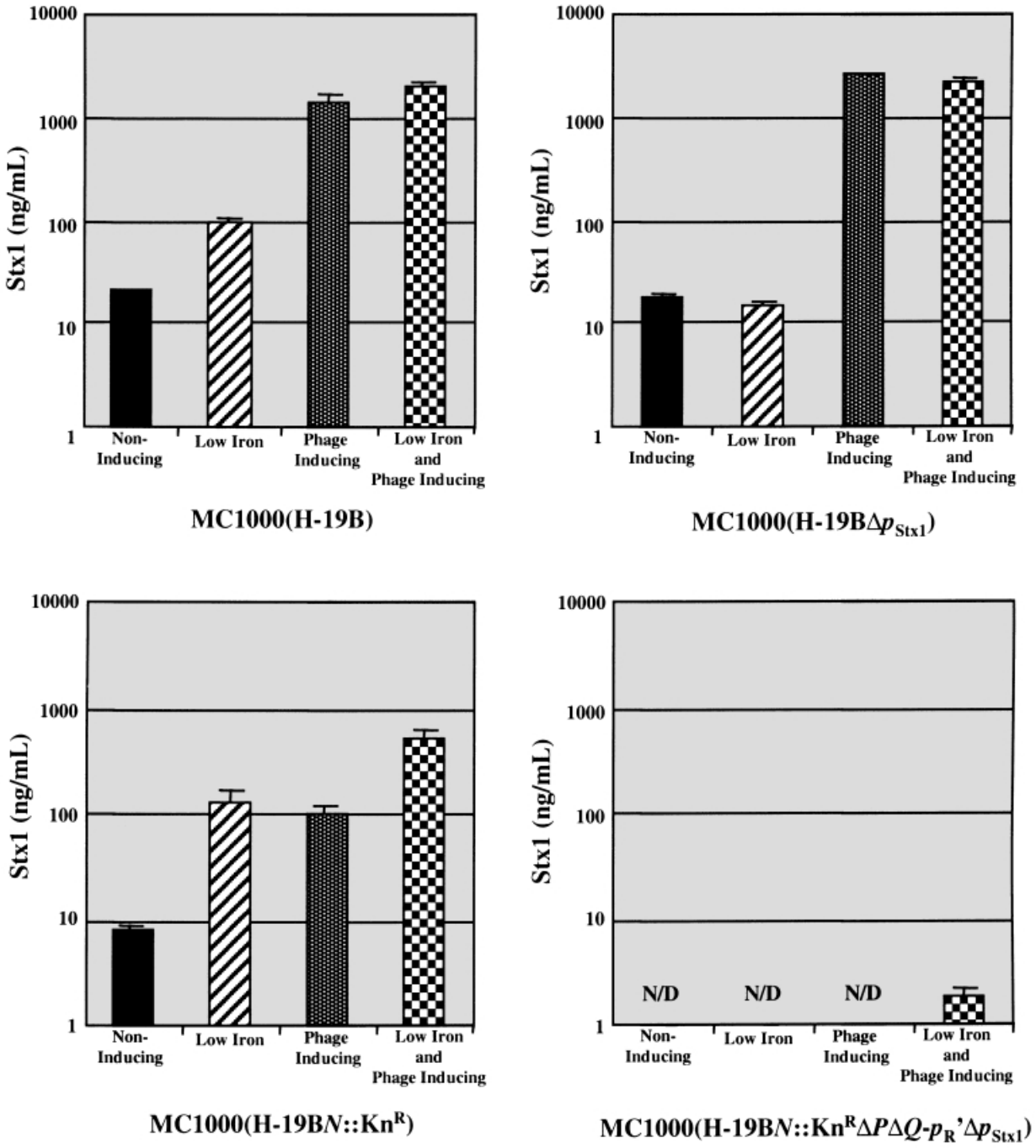

Fig. 2. Stx1 production under various growth conditions by MC1000(H-19B) and isogenic derivatives containing mutations in the H-19B prophage. Mean values derived from three independent cultures are shown along with the standard deviations. ND, not detected (<0.075 ng Stx1 ml^{-1}). Stx1 concentrations were measured after 3 h growth in the indicated media.

Contribution of the N antiterminator to Stx1 production

The N antiterminator, by facilitating efficient transcription from the early promoter p_R, plays an early role in the cascade of regulatory events that results in expression of all phage functions required for lytic growth (Fig. 1). Therefore, deletion of the *N* gene was predicted to result in a defect in phage replication as well as to limit phage transcription, both from the early p_R promoter and from the late phage promoter p_R', which ultimately relies on N-facilitated *Q* transcription. A deletion–substitution of *N* was introduced into the H-19B prophage to assess the importance of *N* in Stx1 production. The resulting lysogen, MC1000(H-19BΔ*N*::Kn^R), made ≈ 14-fold less Stx1 than

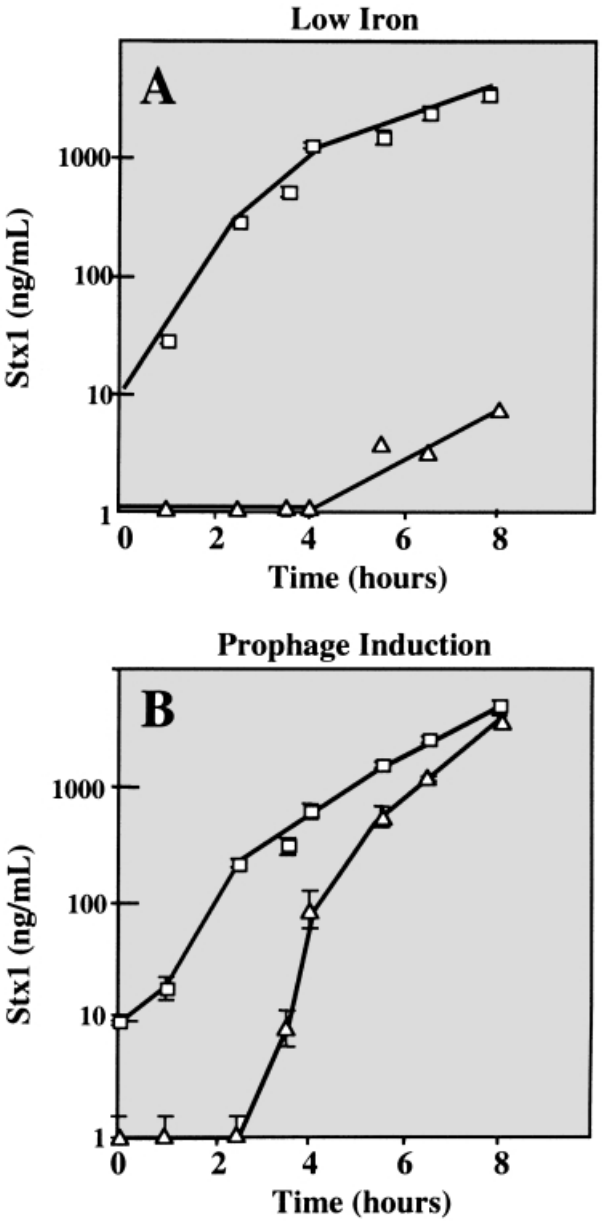

Fig. 3. Kinetics of Stx1 production and release by clinical isolate H19.
A. Total Stx1 (squares) and supernatant Stx1 (triangles) in cultures of H-19 grown in low-iron conditions (syncase broth).
B. Total Stx1 (squares) and supernatant Stx1 (triangles) in cultures of H19 grown in phage-inducing conditions (syncase broth plus iron and mitomycin C).

wild type during growth in mitomycin C, indicating that Stx1 production after phage induction is largely dependent upon the N antiterminator. The residual Stx1 production by MC1000(H-19BΔ*N*::Kn^R) after prophage induction probably reflects (i) the likelihood that some amount of transcription initiating at p_R proceeds through the downstream *t*R1 terminator even in the absence of N (Court *et al.*, 1980), resulting in some prophage replication (Signer, 1969); (ii) the presence of a functional p_{Stx1} allowing phage induction-independent toxin production; and (iii) the fact that MC1000(H-19BΔ*N*::Kn^R) is defective in lysis and therefore survives to accumulate toxin under conditions in which wild-type cells would lyse (see below).

Contribution of H-19B replication to Stx1 production

Because the stx_1 genes are located within the phage genome, replication of the H-19B prophage will result in an increase in stx_1 gene dosage. We assessed the effect of prophage induction on stx_1A copy number in strains H-19 and MC1000(H-19B) using Southern hybridization. We found markedly increased hybridization of an stx_1A probe to DNA prepared from cultures of H-19 and MC1000(H-19B) after growth in mitomycin C (Fig. 4). Confirming that this increase in stx_1AB copy number specifically reflects replication of the phage genome, we observed no increase in hybridization with a probe for the *prfC* gene, located elsewhere on the bacterial chromosome (Fig. 4). As expected, induction of p_{Stx1} activity by growth in low-iron media did not affect stx_1A copy number in H-19 (Fig. 4) or MC1000(H-19B) (data not shown).

To assess the role of phage replication in Stx production, we constructed an H-19B prophage derivative defective for replication by deleting the prophage *P* gene, which belongs to the H-19B replication cassette that is nearly identical to that of λ (Neely and Friedman, 1998a). As the *P* gene is necessary for λ replication (Furth and Wickner, 1983), we predicted that, in the absence of *P*, there should be a failure in H-19B replication and therefore no increase in stx_1A copy number after mitomycin C induction. Southern analysis proved this to be the case; there was no detectable amplification of the stx_1A gene in mitomycin C-treated cultures of MC1000(H-19BΔ*P*) (Fig. 4, lanes 5–8). In terms of Stx1 production, induction of the mutant prophage H-19BΔ*P* with mitomycin C resulted in significantly less Stx1 production relative to the wild-type H-19B prophage, ≈ 13-fold less in low-iron media with mitomycin C (Fig. 5). Thus, the *P* gene product, necessary for stx_1A amplification after phage induction, contributes significantly to high-level, phage-related Stx1 production. In contrast, eliminating phage replication had little effect on Stx1 production under conditions in which the prophage is not induced or p_{Stx1} is induced by a low environmental iron concentration (data not shown).

stx_1 *transcription: the* p_{Stx1} *promoter*

To assess the importance of p_{Stx1} for Stx1 production, we constructed and characterized a deletion mutant, designated MC1000(H-19BΔp_{Stx1}), that lacks p_{Stx1}. A Northern blot was used to assess stx_1 transcription in this mutant by comparing hybridization of an stx_1A probe with RNA

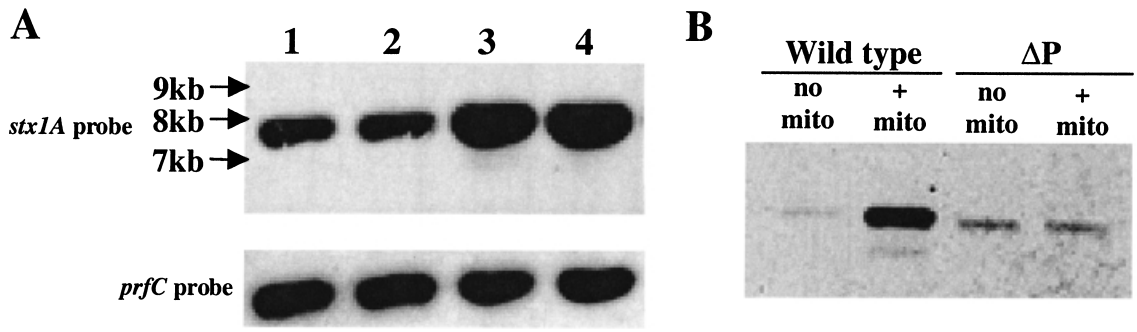

Fig. 4. A. Southern hybridization with probes *stx*$_1$*A* (top) and *prfC* (bottom) to DNA purified from cultures: 1, clinical isolate H19 grown in syncase + iron; 2, H19 in syncase (low-iron); 3, H19 in syncase + iron + mitomycin C (phage-inducing); 4, H19 in syncase + mitomycin C (low-iron and phage-inducing).
B. Southern hybridization with *stx*$_1$*A* probe in cultures of MC1000(H-19B) or MC1000(H-19BΔ*P*) grown in the presence or absence of mitomycin C as indicated.

purified from cultures of MC1000(H-19B) and MC1000(H-19BΔp_{Stx1}). As a prominent band, labelled B in Fig. 6, was present in RNA from MC1000(H-19B) (lane I) but not in RNA from MC1000(H-19BΔp_{Stx1}) (lane III), band B probably represents transcription from p_{Stx1} that was eliminated by the Δp_{Stx1} mutation. Another prominent band, labelled A in Fig. 3, was replaced by a shorter band, labelled A′, in RNA from MC1000(H-19BΔp_{Stx1}) (lane III). Band A probably represents transcription from an upstream promoter that is shortened to a smaller band, A′, because of the 150 bp Δp_{Stx1} deletion in strain MC1000(H-19BΔp_{Stx1}) (lane III). Using an enzyme-linked immunosorbent assay (ELISA), we found that this mutation affected Stx1 production by MC1000(H-19BΔp_{Stx1}), which was not enhanced during growth in low-iron media, but was increased during growth in phage-inducing conditions (Fig. 2). Taken together, these results indicate that the p_{Stx1} sequence is critical for induction of Stx1 production during growth in low iron, but that this promoter is not essential for phage-related induction of Stx1 production.

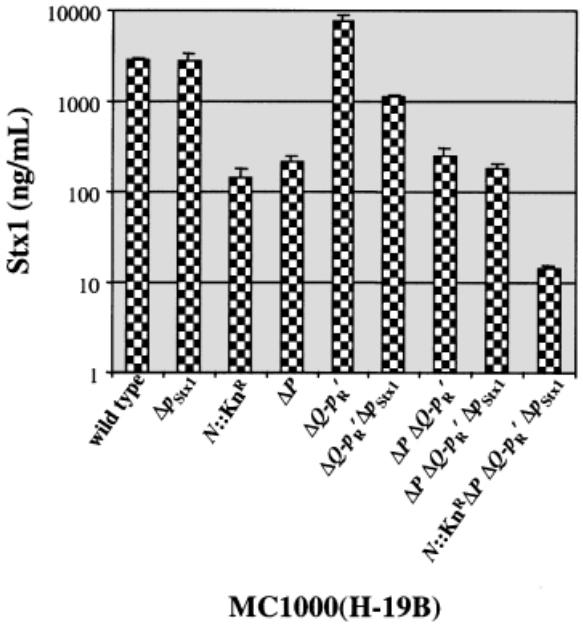

Fig. 5. Stx1 production by MC1000(H-19B) and isogenic derivatives containing the indicated mutations in the H-19B prophage during growth in syncase + mitomycin C (low-iron and phage-inducing) media. Mean values derived from three independent cultures are shown along with standard deviations. Stx1 concentrations were measured after 3 h growth.

stx$_1$ *transcription: the* p$_R$′ *promoter*

As the mutant lacking p_{Stx1} remained inducible by mitomycin C, we hypothesized that a phage-regulated promoter(s) located upstream of p_{Stx1} might also contribute to

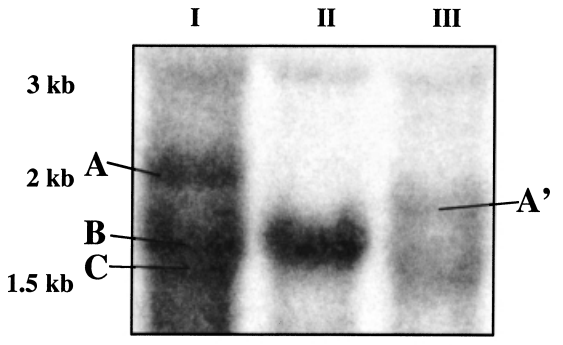

Fig. 6. Northern hybridization of *stx*$_1$*A* probe to total RNA prepared from (I) MC1000(H-19B); (II) MC1000(H-19BΔ*Q*-p_R′); (III) MC1000(H-19BΔp_{Stx1}). A, band present in wild-type (I) and in the Δp_{Stx1} (III) lanes, but shorter in lane III (A′) by the length of the deletion. B, band present in wild-type (I) and Δ*Q*-p_R′ (II) lanes but not present in the Δp_{Stx1} lane (III). C, band present in the wild-type (I) and Δp_{Stx1} (III) lanes, but not in the Δ*Q*-p_R′ lane (II).

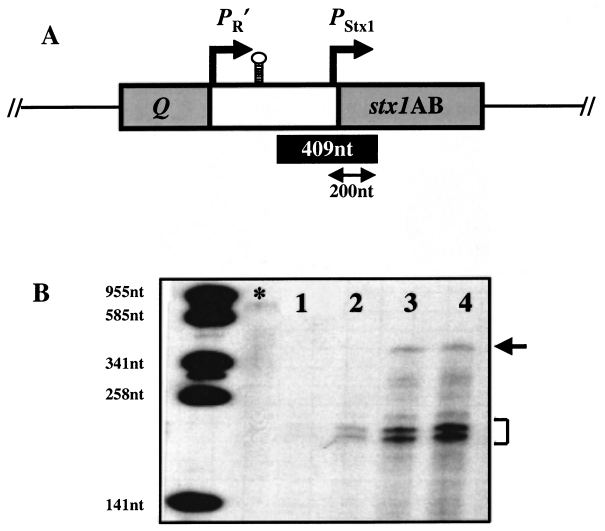

Fig. 7. Detection of stx_1 transcription initiating 5′ of p_{Stx1} by ribonuclease protection assay.
A. Map (not to scale) of *Q-stx*$_1$*AB* region of phage H-19B showing promoters p_R' and p_{Stx1}, the region used to construct the 409 nt probe (dark rectangle) and the region of 200 nt expected to be protected by messages initiating at p_{Stx1}.
B. Polyacrylamide gel of RPA products with RNA ladder and probe (*) unexposed to ribonuclease. RNA was prepared from MC1000(H-19) grown in: 1, syncase + iron; 2, syncase (low-iron); 3, syncase + iron + mitomycin C (phage-inducing); or 4, syncase + mitomycin C (low-iron and phage-inducing.) The arrow indicates a protected band consistent in size with a transcript initiating at p_R', and the bracket indicates protected bands consistent in size with transcripts initiating near p_{Stx1}. Size markers are shown on the left.

stx_1 expression. To assess directly whether such initiation sites for stx_1 transcription exist, we used a ribonuclease protection assay to detect stx_1A transcripts initiating 5′ of p_{Stx1}. The labelled RNA probe in this assay contained a 409 nucleotide (nt) region complementary to sequences surrounding p_{Stx1}, including 209 nt 5′ to the previously mapped stx_1A transcription initiation site (Fig. 7). Transcripts initiating at the p_{Stx1} promoter would be expected to protect a 200 nt segment of the labelled probe, whereas longer protected species, up to 409 nt, would be expected if upstream promoters are involved in stx_1AB transcription. For these assays (Fig. 7), total RNA was prepared from cultures of H-19 grown under non-inducing conditions for the prophage or p_{Stx1} (lane 1), low-iron conditions (lane 2), phage-inducing conditions (lane 3) or low-iron and phage-inducing conditions (lane 4). An ≈ 200 nt doublet, consistent in length with a p_{Stx1}-initiated transcript, was observed in all lanes (Fig. 7, brackets). The presence of a doublet is consistent with a previous report that two transcription initiation sites exist for stx_1A in the vicinity of p_{Stx1} (Kozlov *et al*., 1988). These two bands were more intense in low-iron cultures, probably reflecting reduced Fur repressor activity. They were also more intense in mitomycin C-treated cultures, presumably reflecting an increased stx_1 template copy number.

In mitomycin C-treated cultures, a larger protected species also became apparent (lanes 3 and 4, arrow). The presence of this mitomycin C-inducible band, consistent in size with the length of the homologous region in the probe (409 nt), indicates that an stx_1AB transcriptional start site(s) located 5′ of p_{Stx1} becomes active after phage induction. Based on the results of previous studies with H-19B (Neely and Friedman, 1998b), ϕ361 (Wagner *et al*., 2001a) and other lambdoid phages (Roberts *et al*., 1998), we predicted that p_R' would be the primary phage promoter involved in *stx* expression. To assess the role of p_R' in Stx1 synthesis, we constructed a derivative of an H-19B prophage lacking p_R' and a portion of the *Q* gene, a mutation identical to the ΔQ-p_R' deletion we studied previously in ϕ361(Wagner *et al*., 2001a). Northern hybridization was used to assess stx_1 transcription in this mutant, which was found to lack bands detected in RNA prepared from the wild-type lysogen (Fig. 6). Specifically, the RNA from MC1000(H-19BΔQ-p_R') (lane II) lacked the bands designated A and C, which are present in RNA derived from wild-type cultures (lane I). Band C probably represents a processing product of the p_R'-initiated transcript (Daniels *et al*., 1988). Moreover, the bands absent in MC1000(H-19BΔQ-p_R') were different from those bands eliminated in the MC1000(H-19BΔp_{Stx1}) mutant (lane III), suggesting that these two mutations interfere with different sites of transcription initiation.

Deletion of the *Q*-p_R' sequence of the H-19B prophage, in marked contrast to our previous results with a similarly deleted ϕ361 prophage, did not result in reduced toxin production during growth in mitomycin C. In contrast, MC1000(H-19BΔQ-p_R'), produced 1.5-fold as much Stx1 relative to an isogenic lysogen with a wild-type H-19B prophage after 3 h growth in mitomycin C (data not shown). As expected, toxin production by MC1000(H-19BΔQ-p_R') was induced by growth in low-iron media (data not shown), confirming the activity of p_{Stx1} in this mutant. Thus, the p_R' promoter is not essential for stx_1 transcription after phage induction.

The increased Stx1 production by a mutant lacking *Q*-p_R' could be explained by the role of *Q* and p_R' in transcription of the phage lysis genes. By eliminating transcription of lysis genes and thus cell lysis, the ΔQ-p_R' mutation could result in the induced lysogen accumulating Stx1-encoding phage genomes that could serve as templates for toxin transcription. Accumulation of Stx under these conditions would require the action of a promoter such as p_{Stx1} to initiate stx_1 transcription. Consistent with this explanation, the majority of Stx1 produced by MC1000(H-19BΔQ-p_R') remained intracellular (data not shown). If delayed lysis accounts for the increase in Stx1 production by MC1000(H-19BΔQ-p_R'), an increase in the

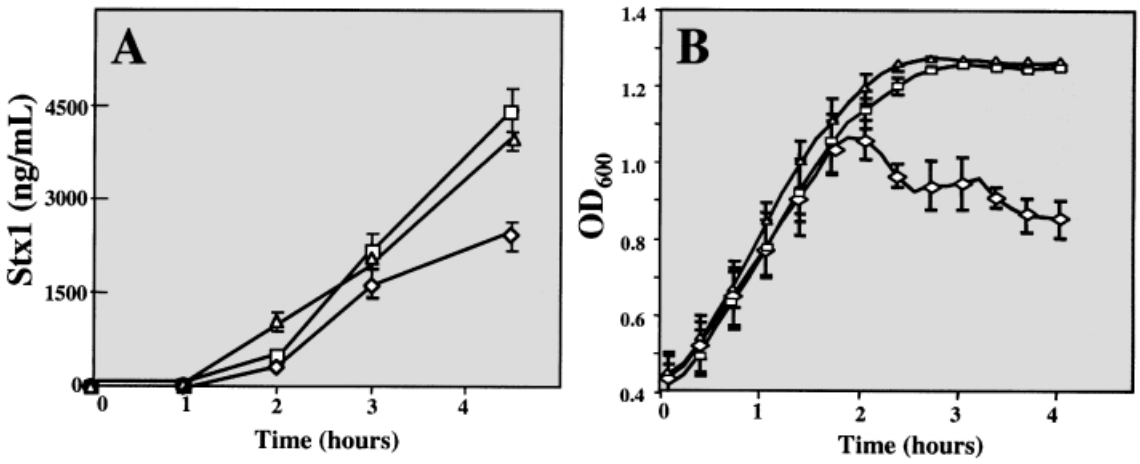

Fig. 8. Kinetics of Stx1 production (A) and culture turbidity (B) of MC1000(H-19B) (diamonds), MC1000(H-19BΔ*Q*-p_R') (squares) and MC1000(H-19BΔ*S*) (triangles) during growth in syncase + iron + mitomcyin C.

duration but not in the rate of toxin production by this mutant might be expected. We confirmed this prediction by comparing Stx1 production by MC1000(H-19B) and MC1000(H-19BΔ*Q*-p_R') during growth in phage-inducing conditions (Fig. 8A). These cultures produced comparable amounts of toxin until ≈ 3 h after induction (Fig. 8A), after which time only MC1000(H-19BΔ*Q*-p_R') continued to accumulate Stx1. Optical density readings of these cultures showed that the strain containing the wild-type H-19B prophage began to lyse, whereas the strain with the H-19BΔ*Q*-p_R' prophage did not. This failure in lysis was associated with prolonged accumulation of Stx1 to a level eightfold greater than wild type after overnight growth (not shown). Hence, Stx1 production by H-19B lysogens does not require the *Q*-p_R' sequence, and removal of this sequence allows for prolonged Stx1 accumulation.

stx$_1$ *transcription: the* p$_R$ *promoter*

As neither the Δp_{Stx1} mutation nor the Δ*Q*-p_R' mutation prevented Stx1 production after phage induction, we hypothesized that each promoter could initiate *stx*$_1$ transcription and could therefore compensate for the other's absence in the single deletion mutants described above. We therefore produced a double mutant lacking both promoters, yielding a strain designated MC1000(H-19BΔ*Q*-p_R'Δp_{Stx1}). Under phage-inducing conditions, strain MC1000(H-19BΔ*Q*-p_R'Δp_{Stx1}) made significantly less Stx1 than wild type (Fig. 5). As neither single promoter deletion mutant was impaired for Stx1 production but the double mutant was impaired, these data provide evidence for the activity of both promoters, p_{Stx1} and p_R', in toxin production after phage induction.

Despite the absence of two promoters presumed to be involved in toxin transcription, MC1000(H-19BΔ*Q*-p_R'Δp_{Stx1}) produced a substantial amount of toxin during growth in low-iron, phage-inducing conditions. Based on the λ paradigm (Court *et al*., 1980), we hypothesized that mitomycin C-inducible, N-modified transcription from the early phage promoter p_R (located 5′ of and in the same orientation as *stx*$_1$) could contribute directly to *stx* transcription. To assess this possibility, we constructed two additional mutants, a triple mutant, MC1000(H-19BΔ*P*Δ*Q*-p_R'Δp_{Stx1}), lacking the p_R' and p_{Stx1} promoters and the *P* replication gene, and a quadruple mutant lysogen, MC1000(H-19BΔ*N*::Kn^{R}Δ*P*Δ*Q*-p_R'Δp_{Stx1}) that additionally has a deletion–substitution of the *N* gene. As the N antiterminator is required for efficient p_R-initiated transcription, any contribution of p_R to *stx*$_1$*AB* transcription should be intact in the triple mutant but diminished in the quadruple mutant. In fact, the quadruple mutant made ≈ 10-fold less Stx1 than the triple mutant during growth in mitomycin C and >200-fold less Stx1 than wild-type MC1000(H-19B) (Fig. 5). The results of this comparison show that, when transcription from p_R' and p_{Stx1} is eliminated, essentially all the remaining production of Stx results from N-modified transcription, which most probably initiates at p_R.

If this interpretation is correct, it should be possible to detect N-dependent *stx*$_1$*AB* transcripts encoding sequences upstream of p_R'. We used reverse transcriptase–polymerase chain reaction (RT–PCR) to detect such transcripts (Fig. 9). cDNA was synthesized from cultures of MC1000(H-19B) grown in low-iron, phage-inducing conditions using an antisense *stx*$_1$*A* primer. Subsequent PCR with primers designed to anneal within the *Q* gene (5′ relative to p_R') and the *stx*$_1$*A* gene yielded a band consistent with the expected size of 989 bp (not shown), indicating that some fraction of *stx*$_1$*A* transcripts in wild-type strains contains sequences upstream of p_R'. These *stx*$_1$*A* transcripts were found to depend on the N antiterminator in experiments comparing RT–PCR products obtained from lysogens of the triple and quadruple mutant H-19B

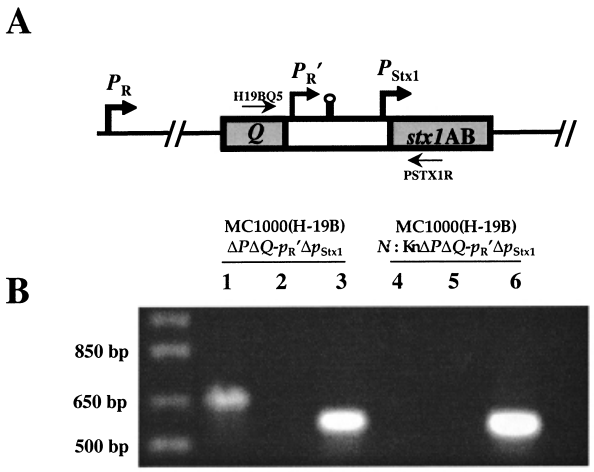

Fig. 9. *N*-dependent stx_1A transcription initiating 5′ of p_R' detected using reverse transcriptase (RT)–polymerase chain reaction.
A. Map of H-19B (not to scale) showing p_R, p_R' and p_{Stx1}, as well as the stx_1A antisense primer (PSTX1R) used for cDNA synthesis and the *Q* primer (H19BQ5) used with the stx_1A primer for PCR.
B. Agarose gel of PCR products with *Q* and stx_1A primers using, as template cDNA derived from MC1000(H-19BΔ*P*Δ*Q*-p_R'Δp_{Stx1}) RNA (lanes 1–3) or MC1000(H-19BΔ*N*::Kn^RΔ*P*Δ*Q*-p_R'Δp_{Stx1}) RNA (lanes 4–6). Lanes 2 and 5 are negative controls to which no RT was added. Lanes 3 and 6 are *rpoB* control RT–PCRs using cDNA derived from MC1000(H-19BΔ*N*::Kn^RΔ*P*Δ*Q*-p_R'Δp_{Stx1}) and MC1000(H-19BΔ*N*::Kn^RΔ*P*Δ*Q*-p_R'Δp_{Stx1}) respectively.

prophages. This comparison revealed a prominent band for the triple mutant, MC1000(H-19BΔ*P*Δ*Q*-p_R'Δp_{Stx1}) and only a very weak band for the quadruple mutant MC1000(H-19BΔ*N*::Kn^RΔ*P*Δ*Q*-p_R'Δp_{Stx1}) (Fig. 9, lane 1 versus lane 4). This contrasts with the comparable levels of PCR product obtained from control reactions amplifying the *rpoB* housekeeping gene (Fig. 9, lane 3 versus lane 6),and indicates that stx_1 transcription initiating 5′ of p_R' is heavily dependent on N.

Role of H-19B-mediated lysis in Stx1 production and release

Deletion of the late phage transcriptional control element, *Q*-p_R', enhanced Stx1 production (see above), most probably as a result of defects in phage-mediated lysis, which requires *Q*-modified transcription initiating at the p_R' promoter and extending through the *S* and *R* lysis genes. The timing of cell lysis by lambdoid phages is determined by the *S* gene product, which acts to permeabilize the bacterial cell inner membrane at a genetically programmed interval after induction. This allows the *R* endolysin gene product to enter the periplasm and degrade the cell wall. The *S* gene product also regulates the number of phage particles released, as phage titre depends upon the amount of time available for intracellular particle assembly before lysis (Young *et al*., 2000). To assess the effect of host cell lysis on toxin production, we constructed a lysogen carrying an H-19B prophage with an in frame deletion of the *S* gene, MC1000(H-19BΔ*S*). We compared the growth of this strain with the growth of wild-type MC1000(H-19B) after phage induction with mitomycin C (Fig. 8B). MC1000(H-19BΔ*S*) cultures continued to grow after wild-type cultures began to decrease in optical density, and we found that, although MC1000(H-19BΔ*S*) initially produced toxin at a rate comparable with that of the wild-type strain, MC1000(H-19BΔ*S*) continued to accumulate toxin at this rate longer than the wild-type strain (Fig. 8A). After overnight growth, the strain defective in phage-mediated lysis contained sixfold more toxin than the wild-type strain (data not shown). These results provide evidence that the timing of phage-mediated lysis, encoded by the *S* gene, is a determinant of the quantity of Stx1 produced by lysogens of H-19B.

Discussion

Virulence genes are frequently encoded in mobile genetic elements, including bacteriophages, and are acquired by pathogenic bacteria via lateral transfer of these elements (Ochman *et al*., 2000; Davis and Waldor, 2002). Expression of these acquired genes may be regulated by factors encoded within the mobile element itself, by factors encoded by the ancestral bacterial chromosome, or both. Stx1 provides an example of a virulence factor whose production is regulated both by the life cycle of the encoding phage, H-19B, and by the iron-sensing Fur regulator encoded by the *E. coli* ancestral chromosome. That prophage induction may play a critical role in the pathogenesis of STEC is suggested by the observations that phage-inducing antibiotics have been associated with increased severity of STEC infection (Butler *et al*., 1987; Carter *et al*., 1987; Pavia *et al*., 1990; Wong *et al*., 2000), and that endogenous agents such as H_2O_2 and neutrophils are capable of inducing Stx2 production in a manner dependent upon prophage induction (Wagner *et al*., 2001b). Although the mechanism by which Fur regulates Stx1 production has been well characterized, the mechanisms by which prophage induction regulates Stx1 production are not and, in this study, we used lysogens containing mutant H-19B prophages to define the mechanisms by which prophage induction enhances Stx1 production and release.

The N antiterminator, by facilitating readthrough of terminators downstream of the early promoter p_R, plays an essential early role in the expression of all phage functions required for lytic growth (Friedman, 1983). An *E. coli* lysogen of the H-19B prophage lacking the *N* gene was severely impaired in Stx1 production, indicating that N plays a central role in phage-regulated Stx1 production. The residual inducibility of toxin production by a strain

lacking N most probably reflects the fact that, even in the absence of N, there is some transcription of replication genes that should lead to an increase in *stx* copy number. Replication of the phage genome is a significant contributor to Stx1 production, as a deletion of the *P* gene diminished toxin production almost as much as disruption of *N*. Thus, by virtue of being phage encoded, the *stx* genes can be regulated by an increase in their copy number in response to specific environmental stimuli encountered by host bacteria. Whether regulated gene amplification contributes to the expression of other phage-encoded virulence factors remains to be explored; however, this mechanism may account for a similar phenomenon described for the genes encoding cholera toxin (*ctxAB*) (Mekalanos, 1983) before the realization that *ctxAB* are phage encoded.

Our experiments demonstrated that three promoters contribute to stx_1AB expression and that, under some conditions, these contributions are redundant. The contribution of p_{Stx1}, as well as a role for phage promoters upstream of p_{Stx1}, was suggested by toxin assays from mutant lysogens, RPAs, Northern analyses and RT–PCR assays. When lysogens were grown under low-iron, phage-inducing conditions, deletion of any single promoter did not impair toxin production, although a deletion mutant lacking both p_R' and p_{Stx1} was greatly impaired under these conditions. The residual amount of toxin produced by this double mutant was essentially eliminated in strain MC1000(H-19BΔN::$Kn^R\Delta P\Delta Q$-$p_R'\Delta p_{Stx1}$), which, in addition to lacking p_R' and p_{Stx1}, lacked effective p_R-initiated stx_1 transcription on account of the *N* deletion. In summary, we were able to account for all stx_1 transcription initiation through three promoters – p_R, p_R' and p_{Stx1} – the relative importance of which varies with environmental conditions.

Our studies of Stx1 production and release by cultures of H19 are consistent with a requirement for phage-mediated lysis in Stx1 release. Growth in low-iron media, which stimulates toxin expression but not lysis, primarily resulted in the accumulation of intracellular, and not extracellular, Stx1, whereas growth in phage-inducing media resulted in toxin release into the supernatant. Furthermore, mutations that interfered with phage-mediated lysis resulted in a prolonged accumulation of Stx1 relative to wild type, indicating that the timing of lysis is one determinant of the quantity of Stx1 produced. This idea was suggested more than 40 years ago in studies on the regulation of the phage-encoded diphtheria toxin (DT) by Barksdale *et al*. (1960), who noted that a delay in lysis of *Corynebacterium diphtheriae* by the DT-encoding phage resulted in a concomitant increase in toxin production. Although our results strongly suggest that phage-mediated lysis and toxin release may represent the same event, at this point we cannot exclude the possibility that unknown phage-independent mechanisms contribute to Stx1 release *in vivo*. For example, genes with homology to a type II secretion system are present on a plasmid found in many enterohaemorrhagic *E. coli* (EHEC) strains (Makino *et al*., 1998), and it is possible that such a system contributes to the release of Stx. Another possibility is that destruction of bacterial cells by the human immune system leads to release of Stx in the absence of phage-mediated lysis.

The apparent redundancy of promoters able to direct stx_1AB transcription highlights interesting differences in the regulation of expression of phage-encoded toxins. Stx1, Stx2 and DT are all phage-encoded toxins produced at higher levels under phage-inducing conditions. Although Stx1 and DT production is influenced by iron concentration, no environmental condition other than phage induction is known to regulate Stx2 production (Muhldorfer *et al*., 1996). A distinction may be drawn between Stx1 and DT in that phage induction of DT production only occurs in low-iron conditions (Barksdale *et al*., 1960), whereas we have shown that phage induction of Stx1 production can occur in high-iron concentrations or in mutants lacking the iron-repressible p_{Stx1} promoter altogether. These differences can be explained based on prevailing notions of phage genome evolution, in which genetic modules are acquired as intact, self-regulated elements that eventually lose autonomy and become integrated into the regulatory scheme of the encoding phage (Campbell and Botstein, 1983; Hendrix *et al*., 2000). Accordingly, the DT genes have remained an intact, iron-repressible operon, largely unaffected by phage transcriptional control elements, whereas the stx_2AB genes have been fully assimilated into the late regulatory scheme of ϕ361, being expressed solely during the late portion of that phage's life cycle. This would imply that the p_{Stx2} promoter (Sung *et al*., 1990) of ϕ361 is a vestigial promoter-like sequence that was acquired with stx_2AB but no longer contributes significantly to stx_2 expression. stx_1AB could represent an intermediate form between the DT and stx_2AB genes, retaining its iron-repressible promoter while at the same time being incorporated into the transcriptional control scheme of the phage.

Whether the assimilation of *stx* expression into the phage life cycle holds an advantage for these phages or their host strains remains unknown. On the other hand, p_{Stx1}-initiated stx_1AB transcription avoids obligate lysis and may represent a capacity for non-lethal Stx production by lysogens of H-19B, which seems to be lacking in lysogens of ϕ361. However, it remains unclear how Stx1 produced under these circumstances would be released from the cell in the absence of phage-mediated lysis. It is possible that the redundant transcriptional control over stx_1AB reflects adaptation to multiple stimuli in which both the

iron-related and the phage-related mechanisms serve to optimize Stx1 production, perhaps at different times or in different environmental locations. We are limited by our lack of knowledge regarding the free iron concentration or the presence and identity of agents – endogenous (e.g. H_2O_2, neutrophils) or exogenous (e.g. antibiotics) – responsible for phage induction in the intestine. However, our work defining the mechanisms of Stx1 induction should help to identify intestinal parameters that contribute to toxin production and, thereby, to design reagents to prevent the severe consequences of STEC infection.

Experimental procedures

Bacterial strains and strain construction

H-19 is an O26 STEC isolate (Smith and Linggood, 1971) kindly provided by A. O'Brien. MC1000(H-19B) is a previously described *E. coli* K-12 lysogen with a single plaque-purified H-19B prophage (Wagner *et al*., 1999). Strain MC1000(H-19BΔ*P*) is a derivative of MC1000(H-19B) produced using a λ Red-based recombination system (Datsenko and Wanner, 2000). Briefly, PCR primers PKOKN1 (5′-CCTGACAAACA CAGACTGGATTTACGGGGTGGATCTGTGTAGGCTGGAG CTGCTTCG-3′) and PKOKN2 (5′-GCGTCCCCAGGTAATG AATAATTGCCTCTTTGCCCGCATATGAATATCCTCCTTA-3′), containing 36 bp immediately upstream and downstream, respectively, of the *P* gene of H-19B were used to amplify the FRT (FLP recognition target)-flanked kanamycin resistance (Kn^R) gene found in plasmid pKD4. The resulting linear PCR product (designated *P*::Kn^R) was electroporated into MC1000(H-19B) previously transformed with the λ Red-encoding plasmid pKD46. Recombinants containing Kn^R in place of the *P* locus were selected on Kn plates and confirmed by PCR. Deletion of the Kn^R element was accomplished by transformation with the FLP recombinase-encoding plasmid pCP20, resulting in kanamycin-sensitive MC1000(H-19BΔ*P*) mutants that were verified by PCR. Plasmids pKD46 and pCP20 (Datsenko and Wanner, 2000) are temperature sensitive and were cured by overnight growth at 42°C.

Strain MC1000(H-19BΔ*Q*-p_R') was derived from MC1000(H-19B) using the allele exchange vector, pΔ*Q*-p_R', as described previously (Wagner *et al*., 2001a). PCR with primers annealing outside the 900 bp region of homology in pΔ*Q*-p_R' was used to confirm that MC1000(H-19BΔ*Q*-p_R') contained the intended deletion. Strains MC1000(H-19BΔp_{Stx1}) and MC1000(H-19BΔ*Q*-p_R'Δp_{Stx1}) were derived from MC1000(H-19B) and MC1000(H-19BΔ*Q*-p_R'), respectively, using an allele exchange vector, pPW101. pPW101 is a derivative of pCVD442 (Donnenberg and Kaper, 1991) containing a 920 bp DNA fragment from the p_{Stx1} region of H-19B with a 154 bp deletion of non-coding DNA that includes p_{Stx1} and the associated Fur-binding operator. This deletion was constructed by ligating PCR products that flank the 154 bp region. pPW101 was introduced into MC1000(H-19B) and MC1000(H-19BΔ*Q*-p_R') by conjugation from *E. coli* SM10λ*pir*, and deletion candidates were selected as Sm^R and Ap^R colonies and subsequently screened for resistance to sucrose as described previously (Donnenberg and Kaper, 1991). The presence of the Δp_{Stx1} mutation in sucrose-resistant colonies was confirmed with PCR primers that anneal outside the 920 bp region used to construct pPW101.

MC1000(H-19BΔ*N*::Kn^R) was constructed by replacing the *N* gene in H-19B with the Kn^R gene using the λ Red-based system described by Yu *et al*. (2000). Briefly, the Kn^R gene and its promoter were amplified by PCR with primers carrying 40 bp of homology to regions flanking the *N* gene. This PCR product was purified and electroporated into strain DY329 (from D. Court) carrying a wild-type H-19B prophage. Strain DY329 has a defective λ lysogen that expresses the lambda recombination functions at high temperature. *N*::Kn^R recombinants were selected as Kn^R colonies, and the construction was verified by PCR. The recombinant prophage was transferred to MC1000 by P1 transduction. Transductants were selected as Kn^R colonies and verified by PCR.

Strains MC1000(H-19BΔ*P*Δ*Q*-p_R'), MC1000(H-19BΔ*P*Δp_{Stx1}) and MC1000(H-19BΔ*P*Δ*Q*-p_R'Δp_{Stx1}) were derived from strains MC1000(H-19BΔ*Q*-p_R'), MC1000(H-19BΔp_{Stx1}) and MC1000(H-19BΔ*Q*-p_R'Δp_{Stx1}) respectively. Each parent strain was transformed with plasmid pKD46 and subsequently with the PCR product *P*::Kn^R, with selection and manipulation of recombinants as described above for the production of MC1000(H-19BΔ*P*). Strain MC1000(H-19BΔ*N*::Kn^RΔ*P*Δ*Q*-p_R'Δp_{Stx1}) was constructed by P1 transduction of Kn^R from MC1000(H-19BΔ*N*::Kn^R) to MC1000(H-19BΔ*P*Δ*Q*-p_R'Δp_{Stx1}). Kanamycin-resistant transductants were checked by PCR to confirm the presence of the desired mutations.

Strain MC1000(H-19BΔ*S*) was derived from MC1000(H-19B) using an allele exchange vector, pPW70. pPW70 is a derivative of pCVD442 containing an 828 bp DNA fragment from the *S* region of H-19B with a 150 bp deletion of the coding sequence of the putative H-19B *S* gene, constructed by ligating PCR products that flank the 150 bp region. pPW70 was introduced into MC1000(H-19B) by conjugation from *E. coli* SM10λ*pir*, and deletion candidates were selected as described above. The presence of the Δ*S* mutation in sucrose-resistant colonies was confirmed with PCR primers that anneal outside the 828 bp region used to construct pPW70.

Growth media and Stx1 measurement

Cultures were grown in syncase broth(Donohue-Rolfe *et al*., 1984) supplemented with $FeCl_3$ (Sigma) to 10 μg ml^{-1}, mitomycin C (Sigma) to 0.5 μg ml^{-1}, both $FeCl_3$ and mitomycin C or neither. Fresh overnight cultures were washed several times with fresh media, then diluted to an OD_{550} of 0.1 into 5 ml of the appropriate media before growth at 37°C on a shaker at 250 r.p.m. Determination of Stx1 concentrations was carried out using a previously described Stx1 ELISA (Donohue-Rolfe *et al*., 1989). To liberate intracellular toxin, cultures were diluted into polymixin B (final concentration 2 mg ml^{-1}). Stx1 concentrations thus obtained were reported as the total Stx1 concentration in the culture, i.e. incorporating both intracellular and extracellular Stx1 fractions. In the experiments depicted in Fig. 3, supernatant toxin concentration was determined independently by removing bacterial cells via centrifugation.

Lysis experiments

Cultures were grown in syncase broth and, when required, supplemented with $FeCl_3$ and/or mitomycin C (Sigma). Overnight cultures grown in syncase broth $\pm FeCl_3$ were diluted 1:40 in 2 ml of syncase broth $\pm FeCl_3$, grown for 2.5 h at 37°C, then diluted 1:1 in 100 µl of syncase $\pm FeCl_3$ ± mitomycin C in a 96-well plate. Then, the optical density at 420 nm was determined every 10 min in a SpectraMax 250 plate reader (Molecular Devices). Cultures were grown in duplicate, and experiments were repeated to give four sets of readings. These sets were averaged, and the standard error was determined as ±the standard deviation of each time point divided by 3. High- or low-iron conditions were maintained throughout the experiment including growth of the overnight culture.

Molecular biology techniques

For Southern blots, chromosomal DNA was prepared from cultures of H-19, MC1000(H-19B) or MC1000(H-19BΔ*P*) grown in various conditions, using the Gnome DNA kit (BIO 101). DNA (1 µg) isolated from each culture was digested with *Cla*I (NEB), then separated by electrophoresis in a 1% agarose gel. A 369 bp *stx*$_1$*A*-specific probe was constructed using PCR with primers DA22 (5′-AAATCGCCATTCG TTGACTACTTCT-3′) and DA23 (5′-TGCCATTCTGGCAACT CGCGATGCA-3′), with chromosomal DNA of MC1000(H-19B) serving as the template. The probe was labelled and hybridization detected with the ECL system (Amersham) according to the manufacturer's protocol, using X-Omat Blue XB-1 film (Kodak). The 366 bp *prfC* control probe was prepared using PCR with primers kindly provided by Dr B. Hochhut (Hochhut and Waldor, 1999).

For ribonuclease protection assays, total RNA was prepared from cultures of H-19 grown for 3 h in various conditions with the RNeasy kit (Qiagen). To construct a riboprobe, PCR was initially used to amplify a 409 bp fragment that began 209 bp 5′ of the previously mapped *stx*$_1$*A* transcription initiation site. Primers for this reaction were PSTX1F (5′-AGGATGACCCTGTAACGAAGTTTG-3′) and PSTX1R (5′-CGTCTTTGCAGTCGAGAAGTCTAAG-3′), with MC1000(H-19B) chromosomal DNA serving as template. The 409 bp product was cloned into pCRII-TOPO (Invitrogen), yielding the plasmid pPW158. A single-stranded, antisense RNA probe was prepared by an *in vitro* transcription reaction using SP6 polymerase (Amersham) and ^{32}P-labelled UTP (Amersham), with linearized pPW158 serving as template. Labelled probe was purified on a 5% denaturing polyacrylamide gel, then added to 5 µg of each total RNA preparation and allowed to hybridize overnight at 42°C. Each sample, except for a single control reaction containing only labelled probe, was treated with RNase A/T1 (Amersham) and subsequently electrophoresed on a 5% denaturing polyacrylamide gel.

Northern blotting was performed using standard procedures (Ausubel *et al*., 1999), with total RNA prepared as above. A ^{32}P-labelled *stx*$_1$*A* probe was synthesized using SP6 polymerase (Amersham), with plasmid pPW158 serving as template.

For RT–PCR, RNA was purified from mid-log cultures as above and treated twice with RNase-free DNase I (Qiagen and Ambion). cDNA synthesis was carried out using heat-stable reverse transcriptase (Life Technologies) and either primer PSTX1R (Fig. 9) or a control primer complementary to the coding sequence of the *rpoB* gene of *E. coli*. For both *stx*$_1$*A* and *rpoB* RT–PCR assays, control reactions were carried out without reverse transcriptase. PCR was performed with primers H19BQ5 and PSTX1R (Fig. 9) or with *rpoB* primers.

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