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Effects of Iron and Temperature on Shiga-Like Toxin I Production by *Escherichia coli*

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Iron is known to depress Shiga toxin production by *Shigella dysenteriae* 1, and temperature has been shown to regulate several genes required for *Shigella* invasiveness. In this study, the influence of iron and temperature on regulation of a highly related toxin, Shiga-like toxin I (SLT-I) of enterohemorrhagic *Escherichia coli*, was examined in strains lysogenic for the toxin-converting coliphage 933J and in strains carrying the cloned *slt*-I genes on a high-copy-number plasmid vector. For comparison, *S. dysenteriae* 1 was included in these studies. As expected, iron suppressed Shiga toxin synthesis, and reduced growth temperature was also found to decrease Shiga toxin production. Iron also suppressed SLT-I synthesis in *E. coli* lysogenized with phage 933J but did not demonstrably repress toxin synthesis in *E. coli* strains carrying the cloned *slt*-I genes. Temperature had no effect on SLT-I synthesis. Mini-Mu *lac* operon fusions were then isolated in the cloned *slt*-I genes and used to test for regulation of β-galactosidase by iron. Iron did not decrease β-galactosidase production in strains that harbored these operon fusion plasmids. Taken together, these results indicate that iron but not temperature represses SLT-I synthesis when the *slt*-I genes are phage associated but this suppression is not easily demonstrated when the *slt*-I genes are cloned on a high-copy-number plasmid.

Strains of *Escherichia coli* have been previously identified which produce a cytotoxin similar to that produced by *Shigella dysenteriae* 1 (Shiga toxin). This toxin, referred to as Shiga-like toxin I (SLT-I; 24), was first described in 1982 by O'Brien et al. (21) and seems to be the same as the *E. coli* O26 strain H3O Vero cell cytotoxin described by Konowalchuk et al. (11). The biologic properties of SLT-I are comparable to those of Shiga toxin, including enterotoxicity for rabbit ileal segments, paralysis of and lethality for mice, and cytotoxicity for selected cell lines in vitro (20). The toxin is comprised of one copy of an A polypeptide and multiple copies of a B polypeptide (20).

The structural genes for the A and B polypeptides of SLT-I, designated *slt*-I A and *slt*-I B, respectively, have been cloned from toxin-converting coliphages of *E. coli* O26 strain H-19 by Willshaw et al. (26) and Huang et al. (9) and from the toxin-converting coliphage 933J (22) in our laboratory (18). Phage 933J was originally isolated from enterohemorrhagic *E. coli* O157:H7 strain 933 (22). The smallest hybrid plasmid which expressed SLT-I was designated pJN25 and contains an insert of approximately 3.1 kilobases (18).

Recently, the nucleotide sequence of the *slt*-I genes contained in pJN25 was determined (10). A 12-base-pair gap was found to separate *slt*-I A and *slt*-I B, an observation in accordance with the findings of Betley et al. (1). Nucleotide sequences consistent with ribosome-binding sites were identified 5′ to both the *slt*-I A and *slt*-I B genes. Translation of these genes indicated that the A and B polypeptides of SLT-I are synthesized as precursors with signal sequences that are removed by proteolysis to form the mature A and B polypeptides. The calculated molecular weights of the mature A and B polypeptides are 32,211 and 7,690, respectively. In the proposed model for expression of the *slt*-I operon, the *slt*-I A and *slt*-I B genes are transcribed on a single polycistronic mRNA. Translation of the sequence that encodes the B polypeptide is presumed to be more efficient than translation of the A coding sequence, resulting in synthesis of several copies of the B polypeptide for each A polypeptide, as required for assembly of the holotoxin.

Iron is known to suppress synthesis of Shiga toxin by *S. dysenteriae* (6, 15, 19, 25), and unpublished data indicating that SLT-I synthesis is also affected by iron were cited in a recent review (1). Other recent studies demonstrated that temperature plays an important role in regulating a set of genes required for invasiveness and virulence in *Shigella* spp. (14). In the present investigation, the effects of iron and temperature on regulation of SLT-I production in *E. coli* were examined both in strains lysogenic for the toxin-converting coliphage 933J and in strains carrying the cloned *slt*-I genes in high-copy-number plasmid vectors. In addition, mini-Mu *lac* operon fusions were constructed and used to confirm the organization of the *slt*-I operon, determine the effects of fusions within the promoter-proximal *slt*-I A gene on production of the *slt*-I B gene product, and test for regulation of β-galactosidase by iron with the *lac* gene under control of the *slt*-I promoter.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. *S. dysenteriae* 1 strain 60R was obtained from S. Formal, Walter Reed Army Institute for Research, Washington, D.C. *E. coli* MC4100 was described by Casadaban (3), and *E. coli* M8820(Mu cts), POI1681TR, and POI1683 were described by Castilho et al. (4). *E. coli* C600 and C600(933J) were described by O'Brien et al. (22). Phage 933J is a toxin-converting coliphage that contains the SLT-I structural genes *slt*-I A and *slt*-I B. Plasmid pJN25 contains *slt*-I A and *slt*-I B on a 3.1-kilobase restriction fragment from phage 933J cloned into the plasmid vector pBR328 (18). Studies using strains transformed with pJN25 were performed under BL3+EK1 physical contain-

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ment conditions using the guidelines of the National Institutes of Health Recombinant DNA Advisory Committee (Fed. Regist. 51:16972, 1986). Plasmid pJN25 was purified from *E. coli* HB101(pJN25) (18) by treatment with sodium dodecyl sulfate-sodium hydroxide followed by cesium chloride gradient purification as described by Maniatis et al. (12) or by the rapid isolation method of Birnboim and Doly (2) and used to transform *E. coli* MC4100, POI1681TR, and POI1683 by published methods (12). Isolation of mini-Mu *lac* fusions with plasmid pJN25 is described below.

Media and culture conditions. For iron and temperature regulation studies, 50-ml cultures of the indicated strains were grown for 24 h at 30 or 37°C in Chelex (Bio-Rad Laboratories, Richmond, Calif.)-treated glucose-syncase medium prepared as previously described (20), with or without the addition of 10 μg of Fe^{3+} per ml as $FeCl_3$. Unless otherwise stated, all strains containing mini-Mu *lac* fusion plasmids were cultured in Luria broth (16) or on MacConkey lactose agar (Difco Laboratories, Detroit, Mich.) for 18 to 24 h at 30°C. When indicated, media were supplemented with ampicillin and kanamycin, each at 50 μg/ml. For assays of Shiga toxin or SLT-I, culture supernatants were prepared and sterilized by filtration and bacterial lysates were prepared by sonication of bacterial suspensions as described previously (13).

Cytotoxicity, toxin neutralization, and colony immunoblot assays. The microcytotoxicity assay of Gentry and Dalrymple (7) was used to test the toxicity for HeLa cells of fourfold serial dilutions of filter-sterilized culture supernatants or sonic lysates. After 48 h the HeLa cells remaining in the microtiter wells were fixed to the plastic with formaldehyde and stained with crystal violet. The plates were then scanned on a Titertek Multiscan MC reader (Flow Laboratories, Inc., McLean, Va.) at 600 nm, and the absorption of toxin-treated wells was compared with that of untreated wells. The last dilution in which greater than or equal to 50% of the HeLa cells detached from the plastic, as assessed by A_{620}, was considered the 50% cytotoxic dose (CD_{50}) per 0.1 ml of sample. Toxin neutralization assays with monoclonal antibody to the B subunit of SLT-I (23) were done as described previously (20). The immunoblot assay to detect production of the B subunit of SLT-I by individual bacterial colonies was performed as described by Strockbine et al. (23), with minor modifications. Briefly, bacteria were grown on glucose-syncase agar for 48 h. The colonies were overlaid with nitrocellulose paper and then lysed with polymyxin B (Sigma Chemical Co., St. Louis, Mo.). After extensive washing of the paper with phosphate-buffered saline and blocking of the unreacted sites with gelatin, the blot was overlaid with monoclonal antibody specific for the SLT-I B subunit. The blot was again washed and then incubated with goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad Laboratories). After a final wash, the blots were developed with the substrate 0.05% (wt/vol) 4-chloro-1-naphthol and 0.015% (vol/vol) hydrogen peroxide for 30 min.

Construction and characterization of mini-Mu *lac* fusions with pJN25. Fusions derived by insertion of the transposable element mini-Mu *lac* into plasmid pJN25 were prepared by the methods described by Castilho et al. (4). After confirming that strains POI1681TR(pJN25) and POI1683(pJN25) produced SLT-I, the strains were heat induced, and phage lysates were prepared. These lysates were used to infect the recipient strain M8820(Mu *c*ts). Lactose-fermenting and lactose-nonfermenting transductants that contained fusion plasmids were identified as red and white colonies, respectively, on MacConkey lactose agar containing ampicillin and kanamycin.

To identify insertion mutants in which SLT-I synthesis was reduced or ablated, Km^r Ap^r mini-Mu *lac* transductants were grown in 1 ml of Luria broth overnight at 30°C, and filter-sterilized culture supernatants were tested for toxicity. Cultures were grown at 30°C rather than 37°C to prevent thermal induction of Mu *c*ts in the host strain *E. coli* M8820(Mu *c*ts). Both undiluted culture supernatants and supernatants diluted 1:100 were tested in this screening assay to identify transductants that failed to release extracellular toxin or released less than did *E. coli* POI1681TR transformed with pJN25 and cultured under the same conditions. The cell-associated and extracellular cytotoxic activities in 50-ml cultures of mutants so identified were then determined as described by Marques et al. (13). Nontoxinogenic and hypotoxinogenic transductants were also tested in the colony immunoblot assay to determine whether they produced the immunoreactive B subunit of SLT-I. For immunoblot tests, the transductants were grown at 30°C to prevent thermal induction of phage Mu functions. To test quantitatively for β-galactosidase activity, selected transductants were grown in glucose-syncase broth, and cells were lysed by chloroform-sodium dodecyl sulfate treatment. β-Galactosidase assays were performed on the lysates as described by Miller (16). The sites of insertion of mini-Mu into pJN25 in selected fusion plasmids were determined by restriction enzyme analysis of purified plasmid DNA.

RESULTS

The effects of iron and temperature on production of cytotoxin were compared in *S. dysenteriae* 1 and in strains of *E. coli* that had been rendered toxinogenic either by lysogenization with the toxin-converting wild-type phage 933J or by transformation with the multicopy hybrid plasmid pJN25, which carries the cloned *slt*-I structural genes (Table 1). Iron significantly suppressed total toxin synthesis by *S. dysenteriae* 1 strain 60R grown at 37°C, as expected based on previous studies (6, 15, 19, 25). The effect was more dramatic when the total toxin units were divided by bacterial growth (A_{600}) because the 60R cultures without added iron grew less well than the cultures with added iron. Iron also suppressed total toxin production by *E. coli* C600(933J) grown at 37°C but had less influence on the growth of strain C600(933J) than on that of *S. dysenteriae* 1 strain 60R. No statistically significant difference in total toxin was observed when iron was added to cultures of *E. coli* MC4100(pJN25) grown at 37°C nor did the A_{600} differ in the presence or absence of added iron. However, significantly more toxin was produced at 37°C by *E. coli* MC4100(pJN25) than by either *S. dysenteriae* 1 strain 60R or *E. coli* C600(933J).

At 30°C the addition of iron to cultures of *S. dysenteriae* 1 strain 60R and *E. coli* C600(933J) suppressed total toxin synthesis per A_{600} unit (Table 1). For *S. dysenteriae* 1, iron also significantly suppressed total toxin production. In contrast, for *E. coli* C600(933J), MC4100(pJN25), and POI168 1TR(pJN25) at 30°C, iron did not affect total toxin synthesis. *E. coli* C600(933J) and MC4100(pJN25) grew to significantly higher A_{600} values in cultures with added iron, and for these two strains the total toxin per A_{600} unit was significantly less with added iron. For strain POI1681TR(pJN25), the effect of iron on toxin production at 37°C could not be determined because it is lysogenized with a thermally inducible Mu phage (4).

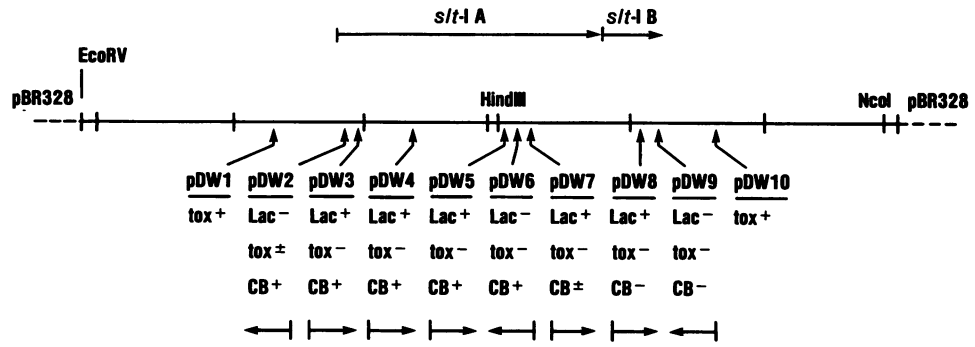

FIG. 1. Locations of mini-Mu insertions into the *Eco*RV-to-*Nco*I insert of pJN25. The locations of the *slt*-I A and *slt*-I B genes and their transcriptional orientation as determined from nucleotide sequence analysis (10) are shown above the map. Increments of 0.5 kilobases are indicated along the map. The vertical arrows below the insert indicate the insertion sites of mini-Mu. The horizontal arrows represent the orientation of the reading frame for the *lacZ* gene. Lac^+ or Lac^-, Ability or inability of the strains carrying the plasmid to ferment lactose; tox^+, tox^-, or $tox^\pm$, toxinogenicity, nontoxinogenicity, or hypotoxinogenicity of strains carrying the plasmid; CB^+, CB^-, or $CB^\pm$, ability, inability, or partial ability of strains carrying the plasmid to produce immunoreactive SLT-I subunit B as assessed by colony blot.

The effects of temperature on toxin production were evaluated by comparing cultures grown in identical media at different temperatures (Table 1). At 37°C without added iron, *S. dysenteriae* 1 strain 60R produced significantly larger amounts of total toxin and toxin per A_{600} unit than at 30°C. In contrast, the effect of temperature on cultures of *S. dysenteriae* 1 with added iron was not statistically significant. Therefore, the effects of higher temperature and iron deprivation on toxin production by *S. dysenteriae* 1 are additive. With *E. coli*, temperature did not significantly affect the total amount of toxin produced per A_{600} unit for any of the strains tested. For *E. coli* C600(933J) grown in medium without added iron, the total amount of toxin produced at 37°C was significantly greater than at 30°C, but this difference was entirely accounted for by the greater growth at 37°C.

The distribution of toxin between extracellular and cell-associated compartments was also determined for each culture (Table 1). Toxin was predominantly cell associated under every set of conditions except for *S. dysenteriae* 1

TABLE 1. Effects of iron and temperature on Shiga toxin and SLT-I production

Organism (n^a)	Added iron	Temp (°C)	A_{600} (mean ± 2 SEM)	Amt of cytotoxin (mean ± 2 SEM)[b]			% Extracellular[c]	Total/A_{600}[d]
				Cell associated	Extracellular	Total		
S. dysenteriae 1 60R (3)	−	37	3.3 ± 0.4	7.8 ± 0.5	8.1 ± 0.8	8.6 ± 0.1	79.5	8.1 ± 0.1
	+	37	6.7 ± 1.6[e]	7.1 ± 0.3	6.5 ± 0.4[e]	7.2 ± 0.2[e]	29.5	6.5 ± 0.2[e]
E. coli C600(933J) (8)	−	37	3.4 ± 0.7	8.2 ± 0.3	6.7 ± 0.3	8.3 ± 0.3	3.0	7.7 ± 0.4
	+	37	4.3 ± 0.8	7.2 ± 0.3[e]	6.6 ± 0.2	7.3 ± 0.2[e]	22.8	6.7 ± 0.3[e]
E. coli MC4100(pJN25) (7)	−	37	4.3 ± 0.3	9.7 ± 0.4	8.5 ± 0.7	9.8 ± 0.4	9.2	9.2 ± 0.4
	+	37	4.3 ± 0.3	9.5 ± 0.2	7.7 ± 0.6	9.5 ± 0.2	2.7	8.9 ± 0.2
S. dysenteriae 1 60R (3)	−	30	3.2 ± 0.3	7.6 ± 0.1	6.7 ± 0.0[f]	7.6 ± 0.1[f]	11.3	7.1 ± 0.1[f]
	+	30	5.9 ± 0.2[e]	6.9 ± 0.4[e]	6.1 ± 0.0[e]	7.3 ± 0.1[e]	7.1	6.5 ± 0.1[e]
E. coli C600(933J) (7)	−	30	1.9 ± 0.3[f]	7.6 ± 0.3[f]	5.5 ± 0.2[f]	7.6 ± 0.3[f]	1.1	7.3 ± 0.2
	+	30	4.0 ± 0.9[e]	7.4 ± 0.3	5.1 ± 0.2[e,f]	7.4 ± 0.3	0.6	6.8 ± 0.2[e]
E. coli MC4100(pJN25) (4)	−	30	3.4 ± 0.3[f]	9.3 ± 0.2	8.7 ± 0.0	9.4 ± 0.1	20.3	8.9 ± 0.2
	+	30	5.8 ± 0.9[e,f]	9.4 ± 0.1	6.5 ± 0.2[e,f]	9.4 ± 0.1	0.2	8.6 ± 0.0[e]
E. coli POI1681TR(pJN25) (5)	−	30	2.3 ± 0.0	8.9 ± 0.2	7.6 ± 0.4	8.9 ± 0.2	5.9	8.5 ± 0.2
	+	30	2.7 ± 0.4	8.7 ± 0.7	7.4 ± 0.5	8.8 ± 0.7	5.6	8.4 ± 0.7

[a] Number of samples.
[b] Cell associated, $\log_{10}$ CD_{50} per pellet; extracellular, $\log_{10}$ CD_{50}/50 ml of supernatant; total, $\log_{10}$ cell-associated CD_{50} plus $\log_{10}$ extracellular CD_{50}.
[c] Mean of individual values.
[d] Mean of individual values ± 2 standard errors of the mean.
[e] Significantly different ($P < 0.05$) by Student's unpaired *t* test from value for the same strain grown at the same temperature without added iron.
[f] Significantly different ($P < 0.05$) by Student's unpaired *t* test from value for the same strain grown at 37°C under the same conditions of added iron.

TABLE 2. Production of β-galactosidase by mini-Mu *lac* transductants

Fusion strain	Insertion location (subunit)	Lactose fermentation on MacConkey agar	Added iron	Amt of β-galactosidase (avg ± 2 SEM)[a]
M8820(Mu *cts*)		−	−	20 ± 22
M8820(Mu *cts*) harboring plasmid:				
pJN25		−	−	13 ± 24
pDW2	A	−	−	24 ± 40
pDW3	A	+	−	3,697 ± 1,322[b]
pDW4	A	+	−	1,282 ± 485[b]
pDW5	A	+	−	3,036 ± 1,510[b]
pDW6	A	−	−	55 ± 53
pDW7	A	+	−	1,685 ± 704[b]
pDW8	B	+	−	3,179 ± 1,628[b]
pDW9	B	−	−	120 ± 79
pDW3	A	+	−	4,570 ± 1,564[b]
pDW3	A	+	+	3,438 ± 1,280[b]

[a] One unit of enzyme produces 1 nmol of *o*-nitrophenol per min (8). The results are based on data from five or six experiments.

[b] Statistically different from results for M8820(Mu *cts*) ($P < 0.05$).

strain 60R grown at 37°C in iron-deprived medium. For *S. dysenteriae* 1, the percentage of extracellular toxin was greater in iron-deprived medium than in medium with added iron and was greater at 37 than at 30°C. The results with *E. coli* varied by strain, but in all instances at least 75% of the total toxin was cell associated.

In an independent group of experiments we constructed a set of mini-Mu *lac* operon fusions with plasmid pJN25 and used them for additional studies on the organization and expression of the *slt*-I operon. A total of eight nontoxinogenic or hypotoxinogenic mini-Mu transductants were isolated, and the mini-Mu insertion sites were mapped (Fig. 1). Seven of these transductants produced no detectable SLT-I, and one (pDW2) produced at least 100-fold less SLT-I than did the parent, both in supernatants and sonic lysates. Several additional toxinogenic mini-Mu transductants were mapped within the cloned insert, and the two located closest to the *slt*-I genes (pDW1 and pDW10) are shown in Fig. 1.

To determine the direction of transcription of the *slt*-I genes, the lactose fermentation phenotypes of the eight nontoxinogenic or hypotoxinogenic mini-Mu insertions were noted on MacConkey lactose agar and compared with the orientation of the inserts (Fig. 1). The lactose phenotypes were confirmed by performing β-galactosidase assays with cell extracts (Table 2). Five of the transductants were lactose fermenting, and the inserts in all five were oriented so that the *lacZ* gene was transcribed from left to right on the physical map shown in Fig. 1. The other three transductants were lactose nonfermenting, and their inserts were oriented in the opposite direction.

Next, the transductants were tested in the immunoblot assay for immunoreactive B subunit determinants of SLT-I. The five transductants which mapped to the left of pDW7 made easily detectable amounts of the SLT-I B subunit in this assay and were presumed to be located within the *slt*-I A gene (Fig. 1 and 2). The two transductants which mapped to the right of pDW7 did not make detectable SLT-I B subunit and were therefore located within *slt*-I B. The strain containing the pDW7 plasmid made very small amounts of immunoreactive B subunit. These findings located the *slt*-I A gene between the sites of the mini-Mu insertions in pDW1 and pDW8 and the *slt*-I B gene between the insertions in pDW7 and pDW10. These sites are consistent with the positions of the genes inferred from DNA sequence analysis. The observation that insertions of the mini-Mu *lac* element into the *slt*-I A gene eliminated toxicity but permitted expression of immunoreactive B subunit regardless of the orientation of mini-Mu indicated that the SLT-I B subunit can be produced independently of the SLT-I A subunit. These results suggest that a promoter 5′ to *slt*-I B is in the distal portion of *slt*-I A. The possibility that an independent

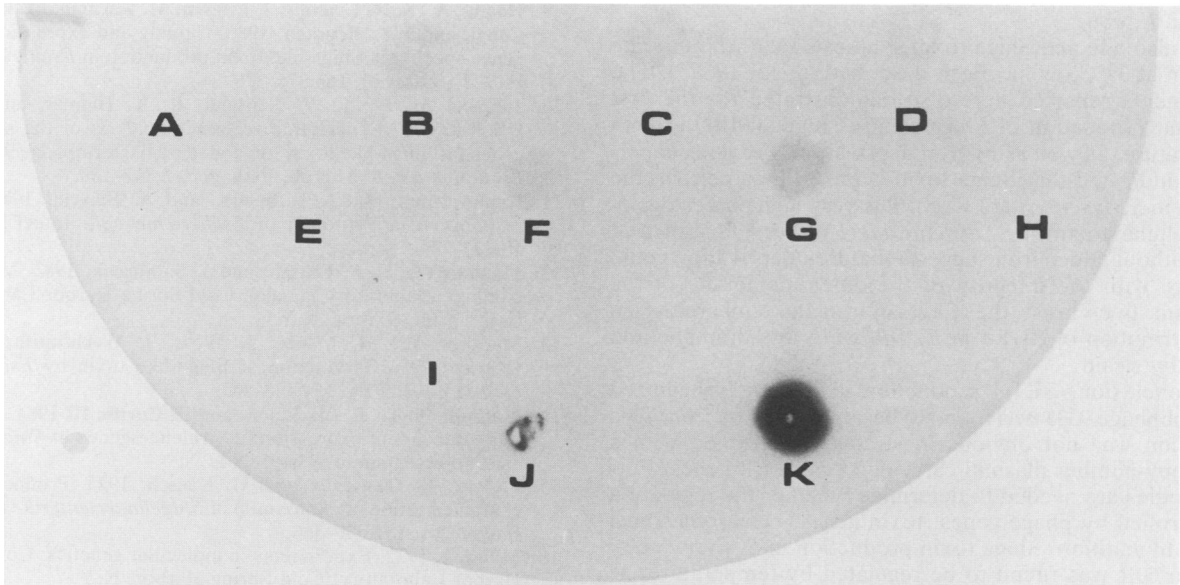

FIG. 2. Colony immunoblot assay (23) for detection of SLT-I B subunit in nontoxinogenic and hypotoxinogenic mini-Mu transductants. Blots for *E. coli* M8820(Mu *cts*) harboring the following plasmids: A, pDW7; B, pDW2; C, pDW4; D, pDW5; E, pDW9; F, pDW6; G, pDW3; H, pDW8; I, pJN28 (18), produces SLT-I A subunit only; J, culture supernatant from pJN25; K, pJN26 (18), produces SLT-I B subunit only.

promoter for *slt*-I B consistent with the findings of Newland et al.(18) and Huang et al. (9), who showed by minicell analysis and in vitro transcription analysis, respectively, that clones lacking the *slt*-I A promoter could express the B subunit of SLT-I.

The effects of high and low iron concentrations on the β-galactosidase activity in the lactose-fermenting mini-Mu operon fusions were also examined. The results of several experiments with pDW3, the lactose-fermenting insert closest to the promoter 5′ of *slt*-I A (Fig. 1), are given in Table 2. No significant differences were noted in the levels of β-galactosidase produced by pDW3 under high- or low-iron conditions. Similar results were found with the other lactose-fermenting insertion mutants (data not shown).

DISCUSSION

Iron suppressed cytotoxin synthesis by *E. coli* C600(933J) cultured at 30 or 37°C. In contrast, iron had no demonstrable effect on β-galactosidase activity in *slt*-I operon fusions (Table 2) nor did it alter cytotoxin production by *E. coli* strains transformed with pJN25 and cultured at 37°C. Several explanations can be proposed to resolve these discrepant findings. One possibility is that regulation of SLT-I synthesis by iron in *E. coli* C600(933J) is controlled by phage genes that are not present in the plasmid clone pJN25 or the mini-Mu *lac* fusions derived from pJN25. A second possibility is that the high copy number of pJN25 or the operon fusion plasmids and the resulting increase in the number of *slt* gene copies per cell are responsible for the failure to observe regulation by iron.

Other phage-encoded toxin genes are known to be regulated by iron. For example, expression of the diphtheria toxin operon of the β phage of *Corynebacterium diphtheriae* has been postulated to be negatively regulated by iron functioning as a corepressor in conjunction with a corynebacterial regulatory protein (17). In a recent review, Betley et al. (1) have suggested a similar mechanism for the iron regulation of SLT-I synthesis involving the *fur* gene, which mediates the iron-dependent regulation of a diverse family of genes in *E. coli*.

Iron also affected Shiga toxin synthesis by *S. dysenteriae* 1 grown at 37°C, as has been described (6, 15, 19, 25). The experiments reported here also demonstrated for the first time that production of Shiga toxin is regulated by growth temperature, as well as by iron deprivation. Previous experiments indicated that Shiga toxin is most like a periplasmic protein in *S. dysenteriae* 1 (5, 8). The very high percentage of extracellular toxin in *S. dysenteriae* 1 strain 60R grown at 37°C without added iron suggests that iron deprivation could interfere with the integrity of the outer membrane of the organism. In contrast, the effects of iron and temperature on the distribution of SLT-I in *E. coli* were less dramatic and varied by strain.

In conclusion, SLT-I production in *E. coli* lysogenized with coliphage 933J was found to be suppressed by iron. This regulation was not obvious in strains transformed with a high-copy-number plasmid carrying cloned *slt*-I genes. Further studies are needed to determine whether the regulation is controlled by phage genes, toxin genes, or chromosomal genes. In addition, Shiga toxin production in *S. dysenteriae* 1 strain 60R was found to be regulated by temperature, as well as by iron. Finally, analysis of the mini-Mu *lac* operon fusions confirmed the location of the *slt*-I B and *slt*-I B genes, the direction of their transcription, and the existence of a second promoter for the *slt*-I A gene distal to the site of insertion in plasmid pDW8.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Two recent reports that were published after the acceptance of this paper present evidence that the *slt*-I promoter is controlled by the *fur* gene product. (S. DeGrandis, J. Ginsberg, M. Toone, S. Climie, J. Friesen, and J. Brunton, J. Bacteriol. **169**:4313-4319, 1987; S. Calderwood, and J. Mekalanos, J. Bacteriol. **169**:4759–4764, 1987).

LITERATURE CITED

1. **Betley, M. J., V. V. Miller, and J. J. Mekalanos.** 1986. Genetics of bacterial enterotoxins. Annu. Rev. Microbiol. **40**:577–605.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104**:541–555.
4. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J. Bacteriol. **158**:488–495.
5. **Donohue-Rolfe, A., and G. T. Keusch.** 1983. *Shigella dysenteriae* 1 cytotoxin: periplasmic protein releasable by polymyxin B and osmotic shock. Infect. Immun. **39**:270–274.
6. **Dubos, R. J., and J. W. Geiger.** 1946. Preparation and properties of Shiga toxin and toxoid. J. Exp. Med. **84**:143–156.
7. **Gentry, M., and J. Dalrymple.** 1980. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. J. Clin. Microbiol. **12**: 361–366.
8. **Griffin, D. E., and P. Gemski.** 1983. Release of Shiga toxin from *Shigella dysenteriae* 1 by polymyxin B. Infect. Immun. **40**: 425–428.
9. **Huang, A., S. De Grandis, J. Friesen, M. Karmali, M. Petric, R. Congi, and J. L. Brunton.** 1986. Cloning and expression of the genes specifying Shiga-like toxin production in *Escherichia coli* H19. J. Bacteriol. **166**:375–379.
10. **Jackson, M. P., J. W. Newland, R. K. Holmes, and A. D. O'Brien.** 1987. Nucleotide sequence analysis of the structural genes for Shiga-like toxin 1 encoded by bacteriophage 933J from *Escherichia coli*. Microb. Pathogen. **2**:147–153.
11. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18**:775–779.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Marques, L., M. Moore, J. Wells, I. Wachsmuth, and A. O'Brien.** 1986. Production of Shiga-like toxin by *Escherichia coli*. J. Infect. Dis. **154**:338–341.
14. **Maurelli, A. T., B. Blackmon, and R. Curtiss III** 1984. Temperature-dependent expression of virulence genes in *Shigella* species. Infect. Immun. **43**:195–201.
15. **McIver, J., G. Grady, and G. Keusch.** 1975. Production and characterization of exotoxin(s) of *Shigella dysenteriae* type 1. J. Infect. Dis. **131**:559–566.
16. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Murphy, J. R., J. Skiver, and G. McBride.** 1976. Isolation and partial characterization of a corynebacteriophage β, *tox* operator constitutive-like mutant lysogen of *Corynebacterium diph-*

theriae. J. Virol. **18**:235–244.

18. **Newland, J. W., N. A. Strockbine, S. F. Miller, A. D. O'Brien, and R. K. Holmes.** 1985. Cloning of Shiga-like toxin structural genes from a toxin converting phage of *Escherichia coli*. Science **230**:179–181.
19. **O'Brien, A. D., and G. D. LaVeck.** 1982. Immunochemical and cytotoxic activities of *Shigella dysenteriae* 1 (Shiga) and Shiga-like toxins. Infect. Immun. **35**:1151–1154.
20. **O'Brien, A. D., and G. D. LaVeck.** 1983. Purification and characterization of a *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. Infect. Immun. **40**:675–683.
21. **O'Brien, A., G. LaVeck, M. Thompson, and S. Formal.** 1982. *Shigella dysenteriae* 1 (Shiga)-like cytotoxin produced by *Escherichia coli*. J. Infect. Dis. **146**:763–769.
22. **O'Brien, A. D., J. W. Newland, F. S. Miller, R. K. Holmes, H. W. Smith, and S. F. Formal.** 1984. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhea. Science **226**:694–696.
23. **Strockbine, N. A., L. R. M. Marques, R. K. Holmes, and A. D. O'Brien.** 1985. Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. Infect. Immun. **50**:695–700.
24. **Strockbine, N. A., L. R. M. Marques, J. W. Newland, H. W. Smith, R. K. Holmes, and A. D. O'Brien.** 1986. Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. Infect. Immun. **53**:135–140.
25. **van Heyningen, W. E., and G. P. Gladstone.** 1953. The neurotoxin of *Shigella shigae*. 1. Production, purification, and properties of the toxin. Br. J. Exp. Pathol. **34**:202–216.
26. **Willshaw, G. A., H. R. Smith, S. M. Scotland, and B. Rowe.** 1985. Cloning of genes determining the production of Vero cytotoxin by *Escherichia coli*. J. Gen. Microbiol. **131**: 3047–3053.