JB Journals.ASM.org

Dynamics of Expression and Maturation of the Type III Secretion System of Enteropathogenic *Escherichia coli*

Gal Yerushalmi, Yael Litvak, Lihi Gur-Arie, Ilan Rosenshine

Department of Microbiology and Molecular Genetics, IMRIC, The Hebrew University of Jerusalem, Faculty of Medicine, Jerusalem, Israel

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of food poisoning, leading to significant morbidity and mortality. EPEC virulence is dependent on a type III secretion system (T3SS), a molecular syringe employed by EPEC to inject effector proteins into host cells. The injected effector proteins subvert host cellular functions to the benefit of the infecting bacteria. The T3SS and related genes reside in several operons clustered in the locus of enterocyte effacement (LEE). We carried out simultaneous analysis of the expression dynamics of all the LEE promoters and the rate of maturation of the T3SS. The results showed that expression of the *LEE1* operon is activated immediately upon shifting the culture to inducing conditions, while expression of other LEE promoters is activated only ~70 min postinduction. Parallel analysis showed that the T3SS becomes functional around 100 min postinduction. The T3SS core proteins EscS, EscT, EscU, and EscR are predicted to be involved in the first step of T3SS assembly and are therefore included among the *LEE1* genes. However, interfering with the temporal regulation of EscS, EscT, EscU, and EscR expression has only a marginal effect on the rate of the T3SS assembly. This study provides a comprehensive description of the transcription dynamics of all the LEE genes and correlates it to that of T3SS biogenesis.

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) represent a major global health problem. EPEC is an important cause of potentially fatal infant diarrhea in developing countries (1). EHEC is an emerging pathogen which causes bloody diarrhea and hemolytic uremic syndrome. The infection by these pathogens is characterized by the formation of "attaching and effacing" (AE) intestinal lesions (1). The AE histopathology is defined by a localized destruction of the brush border microvilli and an assembly of highly organized pedestal-like actin structures in the epithelial cells beneath the attached bacteria (2). The AE histopathology and actin pedestal formation require the activity of a bacterial type III protein secretion system (T3SS), which functions as a molecular syringe to translocate >20 effector proteins from the bacterial cytoplasm directly into the cytoplasm of the host epithelial cells (3). These effectors promote interaction with the intestinal cells, interfere with the host cytoskeleton dynamics (causing the generation of actin pedestals and AE histopathology), and modulate initiation of the immune response to the infecting bacteria (3).

The T3SS organelle consists of a cylindrical basal structure spanning the two bacterial membranes and the peptidoglycan, connected to a hollow needle, followed by a filament. More than 15 proteins are needed to build the T3SS, some of which are highly conserved in all known T3SSs. EscR, EscS, EscT, and EscU are among these conserved proteins; assembly of an inner membrane complex containing these proteins might represent a critical early step in the T3SS biogenesis (4).

The genes encoding T3SS components and related proteins, including regulators, chaperones, and some effectors, are clustered in a 35-kb chromosomal region termed the locus of enterocyte effacement (LEE) (5). The LEE contains 41 genes organized in five major operons (designated *LEE1* to *-5*) and several smaller transcriptional units (6). Expression of the *LEE* genes is thermoregulated. The *LEE* promoters, except the *LEE1* promoter (P_{LEE1}), are silenced by H-NS both at 27°C and at 37°C (7). P_{LEE1} is repressed by H-NS at 27°C, but upon shifting of the culture to 37°C, P_{LEE1} is activated and no longer repressed by H-NS. This *LEE1* activation is mediated by PerC and/or GrlA, which are redundant positive regulators of P_{LEE1} (8, 9). PerC is encoded by a large plasmid, and GrlA is encoded by a bicistronic operon within the LEE (*LEE7*) (10). The first gene in the *LEE1* operon is *ler*, encoding Ler, a positive regulator of all the other LEE promoters. Ler functions as an anti-H-NS, releasing the H-NS-mediated repression (7, 11), and was shown to activate the transcription of the *LEE2*, *LEE3*, *LEE4*, *LEE5*, *espG*, *grlRA*, and *map* genes/operons (6, 12).

Although much is known about the regulation of the LEE genes and the process of T3SS assembly, little is known regarding the temporal dynamics of these processes. The aim of this study was to address this gap in our knowledge by analyzing the temporal order and dynamics of expression of all the LEE promoters and to correlate them to the dynamics of T3SS biogenesis. To this end, we carried out simultaneous real-time analysis of LEE gene transcription (using green fluorescent protein [GFP] gene transcriptional fusions) and T3SS activity (using *blaM* translational fusions). This study provides a comprehensive description of the transcription dynamics of all the LEE genes and correlates them to those of T3SS biogenesis.

MATERIALS AND METHODS

Cells, bacteria, plasmids, and activation conditions. HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Biological Industries) and 100 units/ml penicillin–0.1 mg/ml streptomycin (Pen-Strep; Biological Industries) at 37°C with 5% CO_2. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Strains were grown under repressive conditions in Luria-Bertani (LB) broth supplemented with 20 mM $(NH_4)_2SO_4$ at 27°C or 30°C. To induce *LEE* gene expression, overnight

Received 20 January 2014 Accepted 13 May 2014
Published ahead of print 16 May 2014
Address correspondence to Ilan Rosenshine, ilanr@ekmd.huji.ac.il.

doi:10.1128/JB.00069-14

TABLE 1 Strains used in this study

Strain	Description	Reference or source
E2348/69	EPEC wild type	J. Kaper
DF2	E2348/69 *ler*::*kan*	28
SM10 λpir	*thi-1 thr leu tonA lacY recA supE* (RP4-2 Km^r *tet*::Mu)	Lab collection
MC4100	*araD* Δ*(argF-lac)U169 rpsL relA flbB deoC ptsF rbsR*	Lab collection
MC1061λpir	(λ*pir*) *thi thr-1 leu6 proA2 his-4 argE2 lacY1 galK2 ara14 xyl5 supE44* λ*pir*	Lab collection
GY2455	E2348/69 *LEE1reg-ler*::*gfp*$^+$	29
GY2529	E2348/69 P_{LEE2}::*gfp*$^+$	This study
GY4315	E2348/69 *escRSTU*::*kan*	This study
GY4714	GY4315 carrying *escRSTU* from the P_{LEE6} promoter	This study
GY4455	E2348/69 *escU*::3× FLAG	This study
GY5058	E2348/69 expressing GFP$^+$ from the LEE1 promoter	This study

TABLE 2 Plasmids used in this study

Plasmid	Description	Reference or source
pIR1	pKK177-3 derivative containing *gfp-mut3*	28
pGY1	pIR1 containing a transcriptional fusion of P_{LEE1}, *ler*, and *gfp-mut3* (P_{LEE1}-*ler-gfp-mut3*)	30
pGY2	pCX391 derivative containing *gfp*$^+$ as transcriptional reporter gene	29
pGY4	pGY2 derivative carrying P_{LEE2}::*gfp*$^+$	This study
pGP704	*pir*-dependent, suicide plasmid	31
pCX391	Derivative of pGP704 with the *blaM* gene was replaced by *tetRA*, contains the T1 terminator of *rrnB* (from pQE30) between *tetR* and the multiple-cloning site	19
pGY7412	pCX391 derivative carrying *rorf1* and *escRSTU*	This study
pGY4701	pSA10 derivative carrying *escRSTU*	This study
pGY3115	pIR1 derivative carrying *gfp-mut3* from the putative P_{rorf1} promoter	This study
pGY3116	pIR1 derivative carrying *gfp-mut3* from the putative P_{LEE6} promoter	This study
pGY3117	pIR1 derivative *gfp-mut3* from the putative P_{LEE1} promoter	This study
pGY3142	pIR1 derivative carrying *gfp-mut3* from the putative P_{rorf3} promoter	This study
pGY3143	pIR1 derivative carrying *gfp-mut3* from the putative P_{LEE7} promoter	This study
pGY3119	pIR1 derivative carrying *gfp-mut3* from the putative P_{grlA} promoter	This study
pGY3120	pIR1 derivative carrying *gfp-mut3* from the putative P_{LEE2} promoter	28
pGY3121	pIR1 derivative carrying *gfp-mut3* from the putative P_{LEE3} promoter	28
pGY3144	pIR1 derivative carrying *gfp-mut3* from the putative P_{cesF} promoter	This study
pGY3145	pIR1 derivative carrying *gfp-mut3* from the putative P_{map} promoter	This study
pGY3122	pIR1 derivative carrying *gfp-mut3* from the putative P_{LEE5} promoter	This study
pGY3123	pIR1 derivative carrying *gfp-mut3* from the putative P_{cesT} promoter	28
pGY3125	pIR1 derivative carrying *gfp-mut3* from the putative P_{escD} promoter	This study
pGY3126	pIR1 derivative *gfp-mut3* from the putative P_{LEE4} promoter	This study
pGY3128	pIR1 derivative carrying *gfp-mut3* from the putative P_{espA} promoter	This study
pGY3129	pIR1 derivative carrying *gfp-mut3* from the putative P_{espF} promoter	This study
pSUB11	3× FLAG tag template	17
pGY5057	Suicide plasmid designed to chromosomally insert *gfp*$^+$ under the transcriptional control of P_{LEE1}	This study

cultures grown under repressive conditions were diluted 1:25 or 1:50 into antibiotic-free DMEM (Gibco) or modified Casamino-DMEM (13) prewarmed to 37°C. Cultures were immediately placed on preseeded HeLa cells and incubated at 37°C. When necessary, media were supplemented with ampicillin (Amp) (Sigma) at 100 μg/ml, streptomycin (Str) (Sigma) at 50 μg/ml, chloramphenicol (Cm) (Sigma) at 25 μg/ml, tetracycline (Tet) (Sigma) at 10 μg/ml, or kanamycin (Kan) (Sigma) at 40 μg/ml.

Strain and plasmid construction. The primers used for plasmid construction are listed in Table 3. PCR was performed using the Phusion or DyNAzyme DNA polymerase (Finnzymes) in the Bioer or Biometra machine. Restriction enzymes were purchased from Fermentas or New England BioLabs. To construct pGY1, a DNA fragment containing P_{LEE1} and *ler* (starting from position 159 upstream to the transcriptional start site) (14) was amplified using the primers specified in Table 3; this amplified fragment was digested with XbaI and BamHI and cloned into pIR1 digested with the same enzymes. To generate the GFP transcriptional fusion to the intergenic regions (IRs) of the *LEE*, each of these regions was PCR amplified using EPEC strain E2348/69 as a template. The fragments were digested with BamHI and/or XbaI and cloned into the pIR1 plasmid, which contains a promoterless GFP-*mut3* gene, digested with the same enzyme(s). The obtained plasmids are specified in Table 3. To generate the EPEC P_{LEE2}::GFP strain, the P_{LEE2} sequence was digested from the PCR product of primers 392 and 393 on wild-type (WT) EPEC as a template with XbaI and BamHI and ligated to pGY2 digested with XbaI and BglII, generating pGY4, which was transferred by conjugation into WT EPEC to generate GY2529. To generate the EPEC P_{LEE6}-*escRSTU* strain, the *rorf1* and *escRSTU* genes were cloned into the suicide plasmid pCX391, generating pGY4712 (*rorf1* was digested from the PCR product of primers 1129 and 1130 on WT EPEC as a template with SmaI and SphI, *escRSTU* was digested from the PCR product of primers 1127 and 1234 on WT EPEC as a template with SphI and XbaI sites, and both fragments were sequentially ligated into pCX391 digested with the same enzymes), which was transferred by conjugation into GY4315 to generate GY4714 (Tet and Strep resistant, as verified by sequencing). To complement the *escRSTU* deletion, *escRSTU* was cloned into the EcoRI and SalI sites of pSA10 (15) to generate pGY4701. To generate the *escRSTU* deletion strain (GY4315), we used the one-step inactivation process, using PCR products as previously described (16). GY4455 is EPEC E2348/69 in which *escU* is tagged with the 3× FLAG tag. The tag was inserted as described previously (17), using primers 1116 and 1162 and plasmid pSUB11 as the template. GY5058 is EPEC E2348/69 with a chromosomal translational fusion of *escU* and the WT GFP gene. The C terminus-encoding region of *escU* and the WT GFP gene (from pZEP08 [18]) were cloned into the BglII and EcoRV restriction sites of pGP704, generating plasmid pGY5057, which was conjugated and integrated into the EPEC E2348/69 chromosome to generate the EscU-GFP fusion.

Measurement of gene expression. Strains containing the plasmids expressing the GFP gene were grown overnight at 27°C in LB medium.

TABLE 3 Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)[a]	Use	Restriction endonuclease site(s)
156	ATGGATCCGTTTATGCAATGAGATCTATC	Cloning of pGY1	BamHI
199	GCTCTAGATGTTAAATATTTTTCAGCGG	Cloning of pGY1	XbaI
1041	GAGAGTCGACGCATTATACGCACCAACTGC	Cloning of pGY4250, pGY4251	SalI
1063	ATGTCTCAATTAATGACCATTGGCTCACAGCCAATATTTT TAATTATTGTAGTGTAGGCTGGAGCTGCTTC	Deletion of *escRSTU*	
1064	TTAATAATCAAGGTCTATCGCAATACGAATCAATTGTGCCA CAGGTTCAAACATATGAATATCCTCCTTAG	Deletion of *escRSTU*	
392	GCTATCTAGATTAGGCATATTTCATCGCTAATCCG	Cloning of P_{LEE2} into pCX391	XbaI
393	GCATGGATCCTCATGATGTCATCCTGCGAACG	Cloning of P_{LEE2} into pCX391	BamHI
1129	TGCCCGGGGCAAACAATTTACAGTAAATAGAGAAAATG	Cloning of *rorf1* into pCX391	SmaI
1130	TAGCATGCGTGACAGACTTACTTATTAGGG	Cloning of *rorf1* into pCX391	SphI
1127	ATGAATTCGCATGCTAAAGAGGAGAAATTAACTATGTC TCAATTAATGACCATTG	Cloning of pGY4712 and pGY4701	SphI, EcoRI
1234	CGGTCGACTCTAGACGATTAATAATCAAGGTCTATCGC	Cloning of pGY4712 and pGY4701	XbaI, SalI
209	GCTCTAGAGTGAGTGTTCTTGAGGTG	Cloning of P_{rorf1}	XbaI
200	CGGGATCCAAGGTATGCCGCATCTG	Cloning of P_{rorf1}	BamHI
14F	GCTCTAGAGGCAACAAGTATCATATAGC	Cloning of P_{LEE6}	XbaI
8R	CGGGATCCGCATGCTTTAATATTTTAAGC	Cloning of P_{LEE6}	BamHI
26F	CGGGATCCAAGCGGATCTGTTTGAGC	Cloning of P_{LEE1}	BamHI
24R	GCTCTAGACCTTCTTCATTGCGGTAG	Cloning of P_{LEE1}	XbaI
503	ATTCTAGAGCACCAACTGCATCCCAT	Cloning of P_{rorf3}	XbaI
211	ATGGATCCAGCACCGAGGGAATTTCG	Cloning of P_{rorf3}	BamHI
210	ATGGATCCGCACCAACTGCATCCCAT	Cloning of P_{LEE7}	BamHI
504	ATTCTAGAAGCACCGAGGGAATTTCG	Cloning of P_{LEE7}	XbaI
205	CGGGATCCACATTGCCATGTAAGAGG	Cloning of P_{grlA}	BamHI
201	GCTCTAGAATATCATTGCGAGAAATCC	Cloning of P_{grlA}	XbaI
505	ATTCTAGATGATCTGGTTCGTTGGTTG	Cloning of P_{cesF}	XbaI
208	ATGGATCCTACGGCCTGTAGTAGCTG	Cloning of P_{cesF}	BamHI
206	ATGGATCCTGATCTGGTTCGTTGGTTG	Cloning of P_{map}	BamHI
506	ATTCTAGATACGGCCTGTAGTAGCTG	Cloning of P_{map}	XbaI
520	CGGGATCCGAGTGGATCCCATTACACG	Cloning of P_{LEE5}	BamHI
521	GCTCTAGAAGAGAAGCGTCTTCTGCTC	Cloning of P_{LEE5}	XbaI
203	ATGGATCCACCTTCCTCACGTATATC	Cloning of P_{escD}	BamHI
204	ATGGATCCAGAAAGCGATGAGCTAGT	Cloning of P_{escD}	BamHI
39F	CGGGATCCATGCCATACCAGAGTG	Cloning of P_{LEE4}	BamHI
49R	GCTCTAGACAGATCGAACGATAGC	Cloning of P_{LEE4}	XbaI
213	ATGGATCCTTCTCGGGTATCGATTGTC	Cloning of P_{espA}	BamHI
513	ATTCTAGAGATCGCTTTGTGCCTGATAC	Cloning of P_{espA}	XbaI
207	CGGGATCCTAGTGGTTGGGTACGAGG	Cloning of P_{espF}	BamHI
202	GCTCTAGAGTAAGACGAACAGCCTG	Cloning of P_{espF}	XbaI
1116	CACCAAATGCAGTAGAACTCAGAAGGCAATACGCAATGA AAATATATAAAACATATGAATATCCTCCTTAG	3× FLAG tagging of *escU*	
1162	TTTTTTGAACCTGTGGCACAATTGATTCGTATTGCGATA GACCTTGATTATGACTACAAAGACCATGACGGT	3× FLAG tagging of *escU*	3× FLAG

[a] Restriction endonuclease sites are underlined.

The cultures were diluted 1:25 in Casamino-DMEM and then grown in 96-well plates in a microplate reader at 37°C (SPECTRAFluor Plus; Tecan). The fluorescence intensity (filter set at 485-nm excitation and 535-nm emission wavelengths) and optical density at 600 nm (OD_{600}) were read and collected using the Magellan version 5.0 software (Tecan). To determine protein levels, strains were grown in DMEM at 37°C up to an OD_{600} of 0.3 to 0.4. When indicated, different concentrations of IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma) were added. The cultures were centrifuged (3 min, 12,100 × *g*) and the bacteria lysed by boiling in SDS loading buffer. Protein concentrations in the samples were adjusted using the bicinchoninic acid (BCA) kit (Sigma) or by measuring the OD_{600} of the bacterial culture that was used to prepare the sample. The sample proteins were separated on polyacrylamide gels, transferred onto polyvinylidene difluoride (PVDF) transfer membranes (Millipore), and subjected to Western blot analysis using anti-Tir, anti-EspB, or anti-Ler antibodies as primary antibodies and anti-rabbit IgG alkaline phosphatase (produced in goat; Sigma) as the secondary antibody. Membranes were developed using 5-bromo-4-chloro-3-indolylphosphate (BCIP)-nitroblue tetrazolium (NBT) color development substrate (Promega).

Translocation assay. Translocation assays were carried out as previously described (19), with minor modifications. Briefly, HeLa cells were grown in a 96-well plate (Greiner Bio-One), washed with Casamino-DMEM, and loaded with CCF2/AM (CCF2/AM loading kit; Invitrogen) (1 μM CCF2/AM, 2.5 mM probenecid, and 62 μM BLIP [final concentrations]). After 75 min, cells were infected with bacteria diluted 1:25 from an overnight culture grown in LB medium at 27°C. To determine the levels of BlaM activity (CCF2/AM hydrolysis) in the infected cells, plates were inserted into a plate reader (SPECTRAFluor Plus; Tecan) and excited

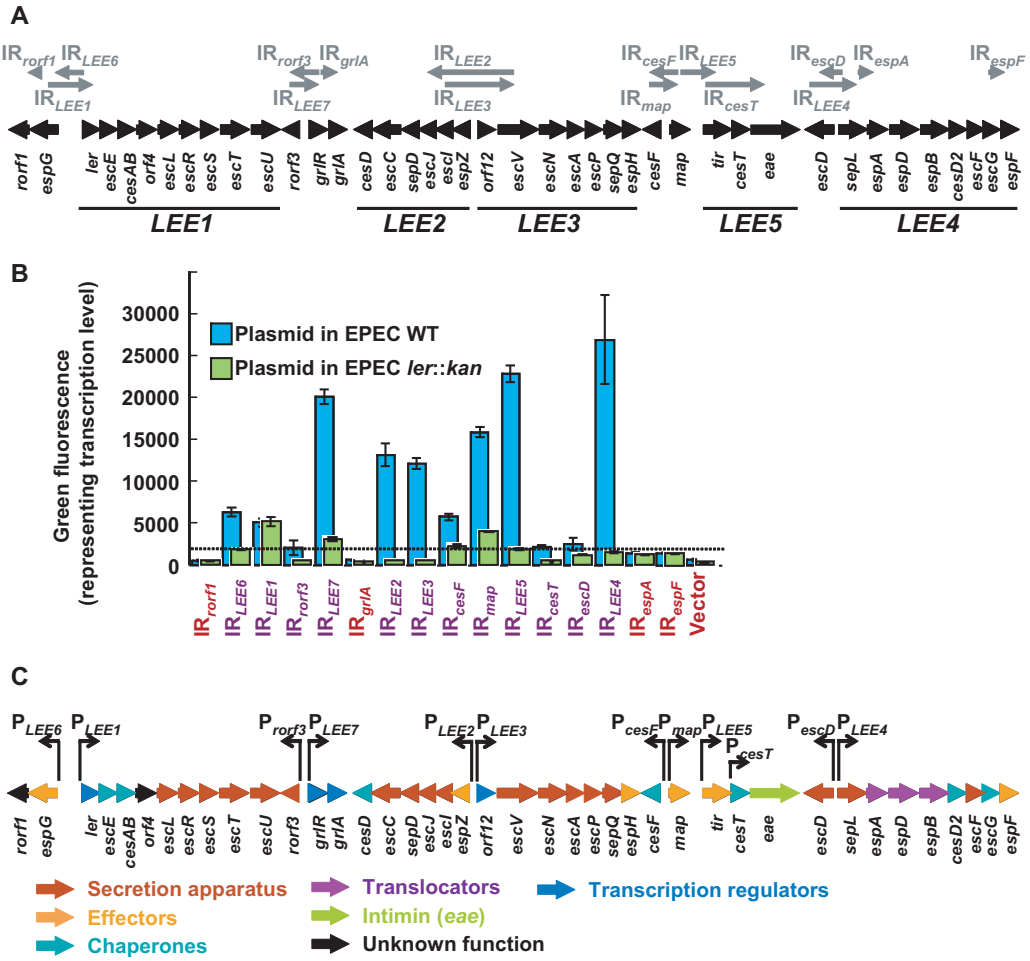

FIG 1 Systematic identification of the *LEE* promoters. (A) LEE intergenic regions (IRs) larger than 40 bp (gray arrows) were cloned as transcriptional fusions upstream of the GFP reporter gene. (B) EPEC (blue) or EPEC *ler*::*kan* (green) harboring the GFP gene fusions were analyzed for green fluorescence, representing promoter activity from the sequence fused to the GFP gene. The sequences IR_{LEE6}, IR_{LEE1}, IR_{rorf3}, IR_{LEE7}, IR_{LEE2}, IR_{LEE3}, IR_{cesF}, IR_{map}, IR_{LEE5}, IR_{cesT}, IR_{escD}, and IR_{LEE4} were found to contain promoters, while the sequences IR_{rorf1}, IR_{grlA}, IR_{espA}, and IR_{espF} exhibited no promoter activity, similarly to the vector. The transcription from all the promoters except P_{LEE1} was found to be activated by Ler. The experiment was performed at least twice for each strain, with similar results. (C) Map of the LEE promoters based on the results in panel B.

at 405 nm, and emission at 465 nm and 535 nm was recorded at 3-min intervals. Data were collected with Magellan5 software (Tecan). To calculate translocation levels, the emission records at 465 nm (product values) and 535 nm (substrate values) were collected from five different duplicate wells for each strain. The initial product value ($t = 0$) was subtracted from the product records, and the result was divided by the initial substrate record for each well. These values were plotted against time, and the average slope of the linear regression lines was calculated as the mean translocation level.

Determination of the rate of pedestal assembly. HeLa cells were seeded and grown overnight on glass coverslips in 24-well plates. Bacteria were grown in LB medium at 27°C overnight. Infection at a multiplicity of infection (MOI) of 1:100 was carried out in DMEM for 3 h and stopped at different time points by fixation with phosphate-buffered saline (PBS) containing 3.7% paraformaldehyde for 10 min. The fixed cells were washed with PBS, permeabilized for 10 min with 0.25% Triton X-100 in PBS, and washed as before. The actin filaments were stained by overlaying the coverslips with 0.1 μg/ml phalloidin-rhodamine (Sigma) in Tris-buffered saline (TBS). After 1 h of incubation, the samples were washed, mounted on glass slides (using Immumount [Thermo Scientific]), and analyzed. Pedestal formation was quantified by counting cells exhibiting at least 5 clear pedestals out of least 100 cells per slide, in duplicates.

Determination of NleD activity (JNK cleavage). Adherent HeLa cells were washed two times with PBS and infected with wild-type EPEC or the P_{LEE6}-*escRSTU* strain. At different time points postinfection, cells were washed again with PBS, scraped from the plate, centrifuged, and lysed in PBS supplemented with 0.5% Triton X-100, 1 mM dithiothreitol (DTT), and complete protease inhibitor (Roche Diagnostics). The cleared lysate was subjected to Western blot analysis using antibody against Jun N-terminal protein kinase (JNK) (554285; BD Pharmingen).

RESULTS

Defining the LEE promoters. We first aimed to systematically define all the promoter regions within the LEE. To this end, we cloned all the LEE intergenic regions (IRs) larger than 40 bp into a plasmid containing a GFP transcriptional reporter gene. This approach is based on two assumptions: (i) most promoters are located in intergenic regions, and (ii) a DNA region of >40 bp is sufficient to accommodate the binding of RNA polymerase, and thus each of the cloned intergenic regions might contain a promoter. The cloned regions and their orientations with respect to the GFP gene are shown in Fig. 1A. We next tested for the capacity of each of the cloned IRs to promote GFP gene expression and for

a possible role for Ler in the activity of each of the putative promoters. Plasmids carrying the IR-GFP gene fusions were introduced into wild-type and *ler*::*kan* EPEC strains. The resulting strains harboring the transcriptional fusions were grown overnight in LB broth at 27°C, which are conditions known to repress the expression of most LEE operons. Next, the cultures were used for infection of HeLa cells under conditions known to activate the expression of the *LEE* genes. After 250 min of infection, we determined the expression levels by measuring the fluorescence intensity at 485-nm excitation and 535-nm emission wavelengths. All the cultures exhibited similar growth rates regardless of the specific plasmid they carried (data not shown). The analysis revealed 12 intergenic regions containing active promoters within the LEE (Fig. 1B). These regions are P_{LEE6} (the *espG-rorf1* operon), P_{LEE1}, P_{rorf3}, P_{LEE7} (the *grlRA* operon), P_{LEE2}, P_{LEE3}, P_{cesF}, P_{map}, P_{LEE5}, P_{cesT}, P_{escD}, and P_{LEE4}. Our analysis further revealed that all the identified *LEE* promoters are upregulated in the presence of Ler (Fig. 1B), with the exception of the *ler* promoter, P_{LEE1}. Expression of GFP from the intergenic regions IR_{rorf1}, IR_{grlA}, IR_{espA}, and IR_{espF} was similar to that of the background level of EPEC containing the vector with a promoterless GFP gene (Fig. 1B), suggesting that there is no promoter immediately upstream of *rorf1*, *grlA*, *espA*, and *espF*. In conclusion, our results confirmed and extended previous reports of the transcriptional structure of the LEE region (Fig. 1C) (7, 8, 10, 11, 13, 14, 20–22). Furthermore, the results highlight the notion that different promoters exhibit marked differences in expression levels.

Transcription dynamics of the LEE-carried genes and T3SS maturation. We next carried out simultaneous analysis of the activity of the entire cohort of LEE promoters as well as the activity of the T3SS itself. To do so, EPEC cultures containing the IR-GFP gene fusions were grown overnight in LB broth at 27°C and diluted 1:25 in Casamino-DMEM prewarmed to 37°C. Infection of HeLa cells was carried out in a 96-well plate in a plate reader preset to 37°C. The activity of each of the LEE promoters was measured by following the changes in fluorescence intensity throughout the infection process at 5-min intervals. The translocation dynamics of the EPEC effector Tir were recorded simultaneously in the same experiment in the same 96-well plate. In this experiment, Tir translocation was used as a readout for completion of assembly of a fully functional T3SS. To follow T3SS activity, HeLa cells in some of the wells were preloaded with CCF2, a caged β-lactamase substrate composed of two fluorophores linked by a β-lactamic ring. The cells in these wells were infected with an EPEC strain that contains a chromosomal copy of *tir* translationally fused to the β-lactamase gene (*blaM*), producing Tir-BlaM. Upon translocation, Tir-BlaM catalyzes CCF2 hydrolysis, thus disrupting Förster (fluorescence) resonance energy transfer (FRET) between the two fluorophores, generating a dynamic fluorescence shift that was traced by the fluorometer plate reader, as described previously (19).

The results showed that LEE promoters exhibit differences in their basal activity when grown under repressing conditions (LB, 27°C). The *LEE7* (*grlRA*) and *map* promoters exhibited a relatively high basal activity (Fig. 2A) (time = 0 min.). Within 10 min after shifting the culture to inducing conditions (infection of HeLa cells in DMEM at 37°C), transcription via the *LEE1* promoter was evident, while all other LEE promoters remained silent. These silent LEE promoters (at 10 min) were activated approximately at the same time, about 70 min postinfection. The *LEE7* and *map* promoters exhibited similar kinetic and expression patterns, except that their basal-level activity was higher than that of the other promoters (Fig. 2A). Under the same conditions, initiation of Tir translocation was detected at ~100 min postinfection (Fig. 2A, lower panel). These results indicate that the assembly of the T3SS is completed around 100 min after shifting the culture to inducing conditions. We also followed the growth of EPEC during the infection process by measuring the OD_{600} of the culture. The EPEC generation time during infection was about 50 min, suggesting that from the time of *LEE1* activation, two generation times are required for the assembly of a functional T3SS (Fig. 2B).

Corroboration of the expression dynamics of the LEE promoters and T3SS assembly. To corroborate the notion of rapid activation of *LEE1* expression and delayed expression of the other LEE promoters, we examined the dynamics of expression of the GFP gene from two representative chromosomally located promoters. To this end, we constructed two EPEC strains containing GFP transcriptional fusions under the regulation of the native chromosomal promoters of the *LEE1* and *LEE2* operons. To generate these strains, we cloned the *LEE1* or *LEE2* promoters upstream of the GFP-encoding gene on a suicide plasmid (pGY2439 and pGY2527, respectively) and introduced each suicide plasmid into wild-type EPEC. Each of the two resulting strains expressed GFP from either the *LEE1* promoter (strain GY2455) or the *LEE2* promoter (strain GY2529). These strains were used for expression analysis employing a protocol similar to that described for Fig. 2A. The results showed that expression of *LEE1* is activated at around 30 min postinfection, while expression of *LEE2* is initiated only around 75 min postinfection (Fig. 3A). The response rate in this experiment was somewhat slower than the response seen in Fig. 2, probably reflecting differences in detection sensitivity between a single copy (in Fig. 3A) and about 20 copies (in Fig. 2A) of the reporter GFP gene. Nevertheless, these results support the notion of rapid activation of the *LEE1* promoter and delayed activation of the other *LEE* promoters.

A second approach used to confirm the results shown in Fig. 2 was to follow the levels of EspB in the bacteria and EspB secretion. EspB is encoded by the *LEE4* operon, and its levels in the bacteria report on the activation of the *LEE4* promoter. In addition, EspB functions as a translocon component and as such is secreted into the medium shortly upon completion of T3SS assembly. Therefore, secreted EspB was used as a marker for completion of T3SS assembly. In this experiment, we used infection of HeLa cells with wild-type EPEC using the same infection protocol described for the assay shown in Fig. 2A. At different time points postinfection, we sampled the infecting bacteria or the cleared medium. EspB levels in the bacterial extract (Fig. 3B) or medium (Fig. 3C) were detected by Western blotting using anti-EspB antibodies. The results showed initial detection of EspB in the bacteria at 60 min postinfection and initial detection of EspB in the medium at 100 min postinfection (Fig. 3B). The minor differences in initial detection of *LEE4* expression by Western blotting (60 min) (Fig. 3B) and the GFP reporter (70 min) (Fig. 2A) probably reflect the higher sensitivity of Western blot analysis. Nevertheless, in agreement with the results shown in Fig. 2A, the results in Fig. 3B show a delay of around 40 min from the initial expression of *LEE4* to the completion of assembly of a functional T3SS. In agreement with the Tir translocation results (Fig. 2A, lower panel), extracellular EspB, indicating the completion of T3SS assembly, was detected at ~100 min postactivation. Taken together, these results confirmed the data shown in Fig. 2.

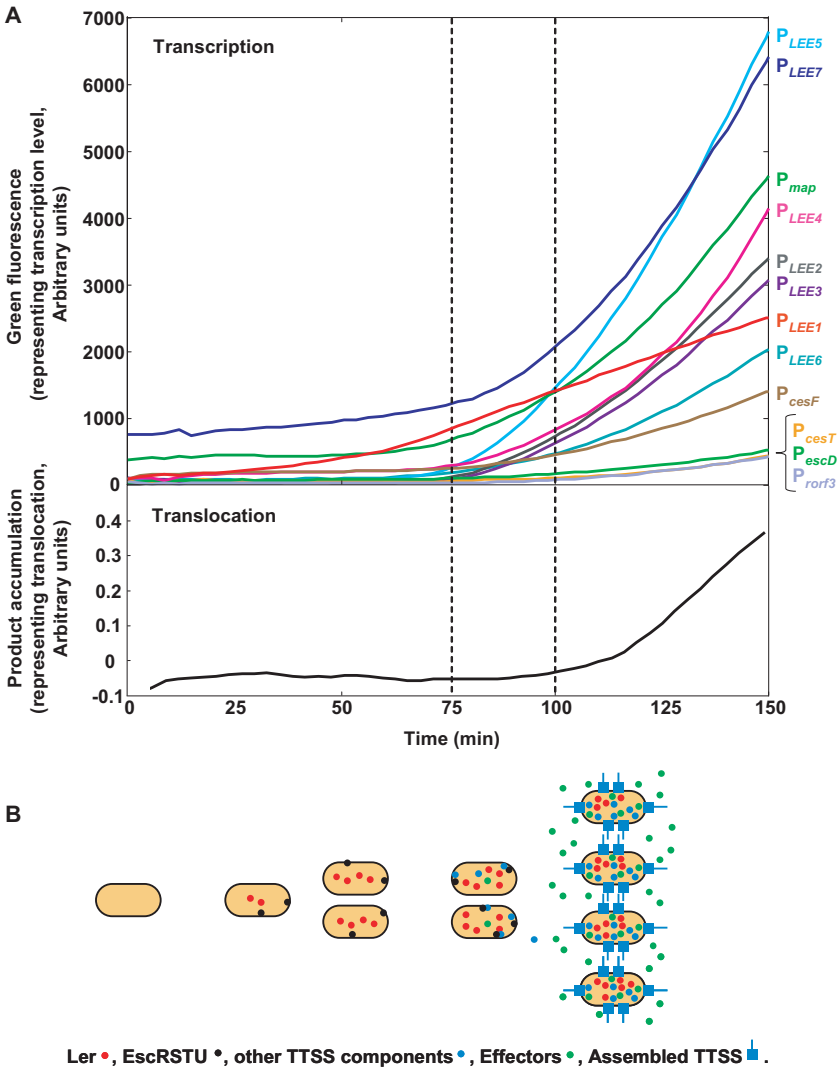

FIG 2 Dynamics of transcription of the *LEE* genes and of T3SS activity. (A) Upper panel, HeLa cells were infected with EPEC harboring the GFP gene fusion, each representing one of the *LEE* promoters. The level of green fluorescence was measured every 5 min, with $t = 0$ the time of shifting the culture to inducing conditions and initiation of infection. Lower panel, T3SS activity was measured simultaneously using HeLa cells loaded with CCF2/AM and infected with bacteria expressing a Tir-BlaM fusion. CCF2/AM hydrolysis by translocated Tir-BlaM was measured over time. Dashed vertical lines represent the time of initiation of transcription of all the *LEE* promoters (except P_{LEE1}) and the time of initiation of translocation. The experiment was performed twice for each strain with similar results. (B) Schematic representation of EPEC growth, expression of LEE proteins, assembly of the T3SS, and secretion of effector proteins.

Is early expression of EscRSTU required for optimal T3SS assembly? The *LEE1* operon comprises nine genes, including *escRSTU*, encoding EscR, EscS, EscT, and EscU, which form a conserved core of the T3SS. Previous reports indicated that in the case of the *Salmonella* SPI1-T3SS, the homologues of EscRSTU (i.e., SpaSPQR) form an initial structure that nucleates the assembly of the entire T3SS (4). Our results suggest that early expression of the *LEE1* operon, including EscRSTU, allows preassembly of the EscRSTU structure. It is possible that this preassembly of the EscRSTU structure optimizes the process of T3SS biogenesis. Furthermore, preassembly of the EscRSTU structure might be required for expression of the other LEE operons. We examined these two hypotheses, as described below.

Deletion of *escRSTU* does not affect transcription of *LEE* genes. To examine whether the expression of *escRSTU* has an effect on the expression patterns of the other *LEE* operons, we generated an EPEC strain with the *escRSTU* genes deleted. As expected, the *escRSTU* deletion strain was impaired in T3SS-mediated secretion (Fig. 4A). We next introduced into this strain plasmids containing the promoters of *LEE1*, *LEE2*, *LEE3*, *LEE5*, and *LEE7* fused to the GFP gene and compared the transcription dynamics of these promoters in wild-type EPEC to those exhibited by the EPEC Δ*escRSTU*::*kan* mutant. No significant differences were found in the expression dynamics of the tested promoters of the wild type and the Δ*escRSTU* mutant (Fig. 4B). In conclusion, our results demonstrate that the activity of the *LEE* promoters is not influenced by expression of EscRSTU via the *LEE1* promoter.

Early expression of *escRSTU* is not required for efficient T3SS assembly. Next, we tested whether early expression of EscRSTU is important for rapid T3SS assembly. We constructed an EPEC strain that expresses *escRSTU* at only 70 min postinfection at levels similar to those seen upon expression from the *LEE1*

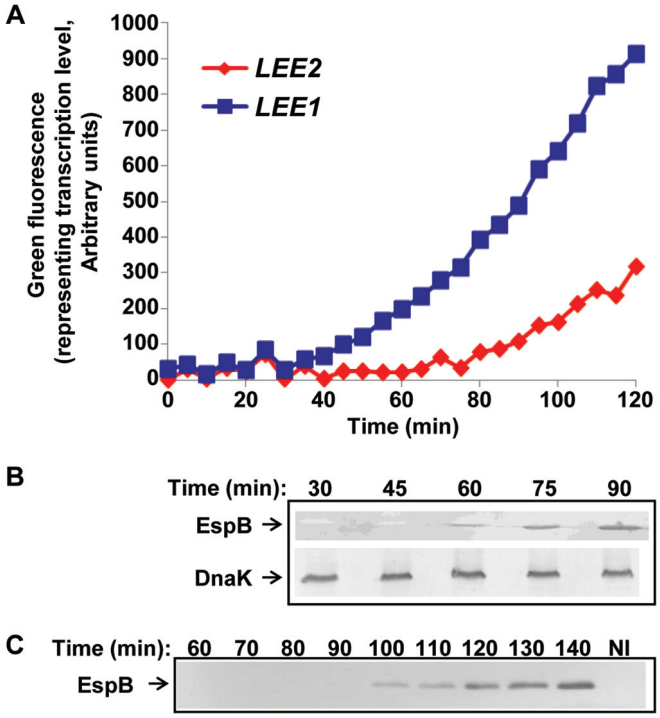

FIG 3 Dynamics of *LEE1*, *LEE2*, and LEE4 expression and T3SS activity. To corroborate the results shown in Fig. 2, EPEC cultures were shifted from repressive to inducing conditions and different parameters were determined. (A) Transcription from the *LEE1* and *LEE2* promoters was monitored using chromosomal transcriptional fusions of the GFP reporter gene to P_{LEE1} (strain GY2455) and P_{LEE2} (strain GY2529). (B) Samples of the activated bacteria were taken at the stated times postactivation and subjected to immunoblotting with anti-EspB, representing *LEE4* expression, and anti-DnaK (loading control). (C) Secretion of EspB by the activated EPEC, used as readout for T3SS activity, was monitored by sampling the supernatant at the stated times postinduction and immunoblot analysis with anti-EspB. The experiment was performed twice for each strain with similar results.

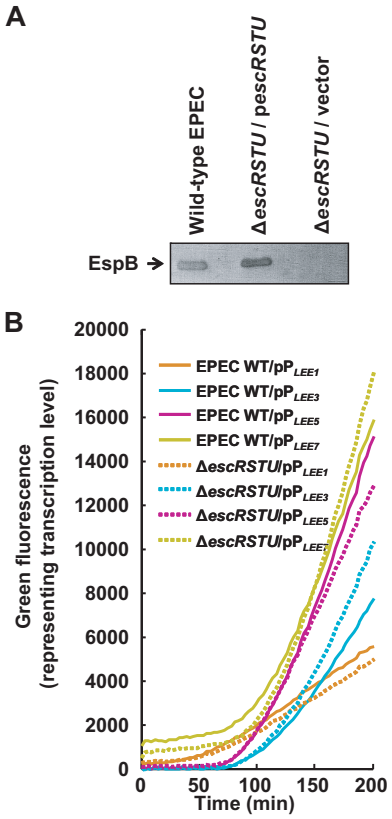

FIG 4 Deletion of *escRSTU* does not affect the kinetics of transcription of the *LEE* genes. (A) An EPEC *escRSTU*::*kan* strain was generated and was found to be unable to secrete EspB. The deletion was complemented by a plasmid encoding *escRSTU* (pGY4701) but not by the empty vector pSA10. (B) HeLa cells were infected with EPEC or EPEC *escRSTU*::*kan* harboring the GFP gene fusions with P_{LEE1}, P_{LEE3}, P_{LEE5}, and P_{LEE7}. The level of green fluorescence was measured every 5 min, with $t = 0$ the time of shifting the culture to inducing conditions and initiation of infection. The kinetics of the *LEE* gene transcription in the *escRSTU*::*kan* strain is similar to that in wild-type EPEC (WT).

promoter. This was done by relocation of the *escRSTU* genes downstream of the P_{LEE6} promoter (Fig. 5A). The P_{LEE6} promoter was chosen for controlling the *escRSTU* expression since it is activated ~70 min postinfection together with all the other *LEE* promoters, and in addition, its activity level is similar to that of the *LEE1* promoter (Fig. 2A). Wild-type EPEC and the newly generated EPEC strain containing P_{LEE6}-*escRSTU* were used to infect HeLa cells. T3SS activity was monitored by three alternative methods: (i) secretion of EspB to the medium; (ii) formation of actin pedestals, which requires translocation of Tir (2); and (iii) cleavage of JNK in the host cell, which requires the translocation of NleD (23). Secretion of EspB, formation of actin pedestals, and JNK cleavage were determined at different time points postinfection. The kinetics of secretion of EspB by the P_{LEE6}-*escRSTU* strain showed no significant difference from that exhibited by wild-type EPEC (compare Fig. 5B and 3C). Formation of actin pedestals and JNK cleavage, induced by the P_{LEE6}-*escRSTU* strain, showed an ~5-min delay compared to those in wild-type EPEC (Fig. 5C and D). Taken together, these results indicate that the T3SS is assembled with efficiency close to that of the wild-type strain even upon a delay in *escRSTU* expression.

A possible explanation for the above result is delayed translation of EscRSTU. To address this possibility, we attempted to evaluate the translation of the last gene in the operon, *escU*, as an indicator for EscRSTU production. We fused the three-FLAG tag or GFP to the C terminus of the chromosomal EscU and tried to use these tags to detect EscU production by Western blotting and microscopy. Unfortunately, we could not detect EscU by either of these methods even after 180 min in DMEM, a time point at which most of the bacteria express active T3SS.

DISCUSSION

Timely and accurate expression of the virulence machinery is essential for successful host colonization by pathogens. Expression of the virulence mechanism of EPEC is subjected to tight and complex regulation by positive and negative regulators. Ler is a master regulator encoded by the *LEE1* operon and positively regulates the expression of other *LEE* operons and other virulence genes (6, 12). In this work we systematically identified all the promoters within the LEE region (Fig. 1C) and showed that all of them except *LEE1* were activated by Ler. Analysis of the temporal dynamics of LEE gene expression showed that the *LEE1* operon, including *ler*, is transcribed immediately upon shifting the culture from repressive to inducing conditions. This is followed by accumulation of Ler, which subsequently displaces H-NS from specific LEE regions (7, 11), leading to activa-

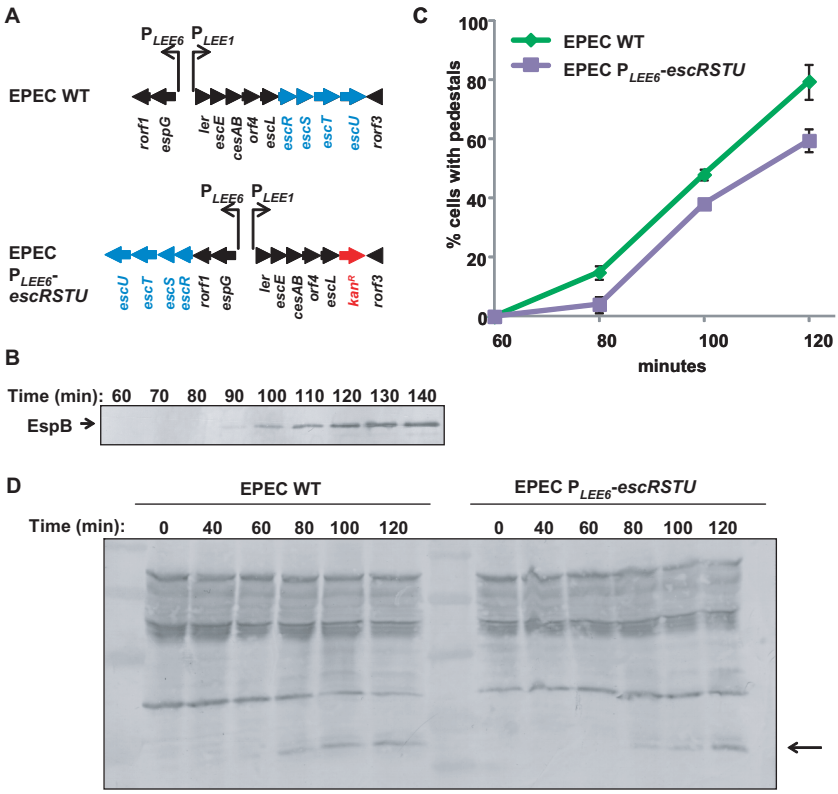

FIG 5 Influence of delayed expression of *escRSTU* on T3SS activity. (A) Schematic presentation of the *LEE1* and *LEE6* operons of wild-type EPEC or the EPEC GY4714 strain (EPEC P_{LEE6}-*escRSTU*). In GY4714, the *escRSTU* genes were deleted from the *LEE1*, replaced by a Kan^r cassette, and placed in *LEE6* under the regulation of the *LEE6* promoter. (B to D) Cultures of wild-type EPEC and the GY4714 strain were grown under repressive conditions and shifted to inducing conditions. Three readouts for T3SS activity were compared between the two cultures: secretion of EspB (B), formation of actin pedestals (C), and cleavage of JNK (D). (B) Secretion of EspB was measured by sampling of the supernatant at the stated times postinduction and analysis by Western blotting using anti-EspB antibody. The experiment was performed twice with similar results. (C) The level of pedestal formation by infected HeLa cells was determined at different time points postinfection. Bars represent standard deviations. Results from one experiment out of two are shown. (D) Levels of JNK cleavage were determined at different time points postinfection. The arrow indicates the JNK cleavage product. The results of one experiment out of three with similar results are shown.

tion of transcription of the other *LEE* promoters. The delay of 70 min between initial Ler expression and initial expression of the other LEE genes is unusually long. The exact mechanism and the biological significance of this delay remain unknown. A possible explanation for this long delay is that H-NS hinders the binding of Ler to the DNA and replication is required to clear H-NS from the DNA, allowing Ler binding and thus preventing the reassembly of stable H-NS–DNA complexes.

Our results show that P_{LEE7} exhibits relatively high basal activity. The bicistronic *LEE7* operon carries *grlRA*. GrlA is a positive regulator of *LEE1*, and GrlR binds directly to GrlA to block its activity (24). Under inducing conditions, the GrlR repression is removed, likely by its targeting to degradation by ClpXP (25), allowing the establishment of a positive feedback loop between GrlA and Ler (9, 10). This mechanism provides a rationale for the relatively high level of basal expression of *LEE7* (Fig. 2A). Constitutive and relatively high levels of the GrlR-GrlA complex in the EPEC cytoplasm should allow rapid establishment of the GrlA-Ler loop upon induction, resulting in GrlR degradation. A second promoter that exhibits unusually high levels of basal expression is the *map* promoter, but the rationale for this behavior is not clear.

The *LEE1* operon carries *ler* and six additional genes, including *escRSTU*, which encode elements of the T3SS basal body. Like *ler*, these genes are transcribed immediately postinfection. We speculated as to whether there is biological significance to the early expression of the *escRSTU* genes, and we raised two hypotheses. The first hypothesis is that the EscRSTU proteins are required for the subsequent transcription of the other *LEE* genes, in analogy to the control of σ^{28} by assembly of the flagellar basal body and secretion of the anti-σ^{28} (FlgM) (26). Our results indicate that T3SS assembles at an efficiency close to that of the wild-type strain, even upon a delay in *escRSTU* transcription. Unfortunately we could not confirm or exclude EscU production by tagging it with 3× FLAG or GFP. The inability to detect the tagged EscU might be related to very low expression levels, to its membrane localization, or to the fact that it undergoes rapid autocleavage upon insertion into the membrane (27).

In conclusion, this study provides a detailed description of the program and timeline of the expression activity of the entire cohort of LEE promoters and of the duration of assembly of active T3SS. The entire process, from the moment of culture shifting to inducing conditions until assembly of the functional T3SS, spans two generation times.

ACKNOWLEDGMENTS

This work was supported by a grant from the Israel Science Foundation funded by The Israel Academy of Science and Humanities. I.R. is an Etta Rosensohn Professor of Bacteriology.

We thank Kobi Baruch for his help with the translocation experiments.

REFERENCES

1. **Nataro JP, Kaper JB.** 1998. Diarrheagenic Escherichia coli. Clin. Microbiol. Rev. **11:**142–201.
2. **Lai Y, Rosenshine I, Leong JM, Frankel G.** 2013. Intimate host attachment: enteropathogenic and enterohaemorrhagic Escherichia coli. Cell. Microbiol. **15:**1796–1808.
3. **Wong AR, Pearson JS, Bright MD, Munera D, Robinson KS, Lee SF, Frankel G, Hartland EL.** 2011. Enteropathogenic and enterohaemorrhagic Escherichia coli: even more subversive elements. Mol. Microbiol. **80:**1420–1438. http://dx.doi.org/10.1111/j.1365-2958.2011.07661.x.
4. **Wagner S, Konigsmaier L, Lara-Tejero M, Lefebre M, Marlovits TC, Galan JE.** 2010. Organization and coordinated assembly of the type III secretion export apparatus. Proc. Natl. Acad. Sci. U. S. A. **107:**17745–17750. http://dx.doi.org/10.1073/pnas.1008053107.
5. **McDaniel TK, Kaper JB.** 1997. A cloned pathogenicity island from enteropathogenic Escherichia coli confers the attaching and effacing phenotype on E. coli K-12. Mol. Microbiol. **23:**399–407. http://dx.doi.org/10.1046/j.1365-2958.1997.2311591.x.
6. **Mellies JL, Barron AM, Carmona AM.** 2007. Enteropathogenic and enterohemorrhagic Escherichia coli virulence gene regulation. Infect. Immun. **75:**4199–4210. http://dx.doi.org/10.1128/IAI.01927-06.
7. **Umanski T, Rosenshine I, Friedberg D.** 2002. Thermoregulated expression of virulence genes in enteropathogenic Escherichia coli. Microbiology **148:**2735–2744.
8. **Bustamante VH, Villalba MI, Garcia-Angulo VA, Vazquez A, Martinez LC, Jimenez R, Puente JL.** 2011. PerC and GrlA independently regulate Ler expression in enteropathogenic Escherichia coli. Mol. Microbiol. **82:**398–415. http://dx.doi.org/10.1111/j.1365-2958.2011.07819.x.
9. **Islam MS, Bingle LE, Pallen MJ, Busby SJ.** 2011. Organization of the LEE1 operon regulatory region of enterohaemorrhagic Escherichia coli O157:H7 and activation by GrlA. Mol. Microbiol. **79:**468–483. http://dx.doi.org/10.1111/j.1365-2958.2010.07460.x.
10. **Jimenez R, Cruz-Migoni SB, Huerta-Saquero A, Bustamante VH, Puente JL.** 2010. Molecular characterization of GrlA, a specific positive regulator of ler expression in enteropathogenic Escherichia coli. J. Bacteriol. **192:**4627–4642. http://dx.doi.org/10.1128/JB.00307-10.
11. **Bustamante VH, Santana FJ, Calva E, Puente JL.** 2001. Transcriptional regulation of type III secretion genes in enteropathogenic Escherichia coli: Ler antagonizes H-NS-dependent repression. Mol. Microbiol. **39:**664–678. http://dx.doi.org/10.1046/j.1365-2958.2001.02209.x.
12. **Bingle LE, Constantinidou C, Shaw RK, Islam MS, Patel M, Snyder LA, Lee DJ, Penn CW, Busby SJ, Pallen MJ.** 2014. Microarray analysis of the Ler regulon in enteropathogenic and enterohaemorrhagic Escherichia coli strains. PLoS One **9:**e80160. http://dx.doi.org/10.1371/journal.pone.0080160.
13. **Berdichevsky T, Friedberg D, Nadler C, Rokney A, Oppenheim A, Rosenshine I.** 2005. Ler is a negative autoregulator of the LEE1 operon in enteropathogenic Escherichia coli. J. Bacteriol. **187:**349–357. http://dx.doi.org/10.1128/JB.187.1.349-357.2005.
14. **Mellies JL, Elliott SJ, Sperandio V, Donnenberg MS, Kaper JB.** 1999. The Per regulon of enteropathogenic Escherichia coli: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). Mol. Microbiol. **33:**296–306. http://dx.doi.org/10.1046/j.1365-2958.1999.01473.x.
15. **Schlosser-Silverman E, Elgrably-Weiss M, Rosenshine I, Kohen R, Altuvia S.** 2000. Characterization of Escherichia coli DNA lesions generated within J774 macrophages. J. Bacteriol. **182:**5225–5230. http://dx.doi.org/10.1128/JB.182.18.5225-5230.2000.
16. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
17. **Uzzau S, Figueroa-Bossi N, Rubino S, Bossi L.** 2001. Epitope tagging of chromosomal genes in Salmonella. Proc. Natl. Acad. Sci. U. S. A. **98:**15264–15269. http://dx.doi.org/10.1073/pnas.261348198.
18. **Hautefort I, Proenca MJ, Hinton JC.** 2003. Single-copy green fluorescent protein gene fusions allow accurate measurement of Salmonella gene expression in vitro and during infection of mammalian cells. Appl. Environ. Microbiol. **69:**7480–7491. http://dx.doi.org/10.1128/AEM.69.12.7480-7491.2003.
19. **Mills E, Baruch K, Charpentier X, Kobi S, Rosenshine I.** 2008. Real-time analysis of effector translocation by the type III secretion system of enteropathogenic Escherichia coli. Cell Host Microbe **3:**104–113. http://dx.doi.org/10.1016/j.chom.2007.11.007.
20. **Haack KR, Robinson CL, Miller KJ, Fowlkes JW, Mellies JL.** 2003. Interaction of Ler at the LEE5 (tir) operon of enteropathogenic Escherichia coli. Infect. Immun. **71:**384–392. http://dx.doi.org/10.1128/IAI.71.1.384-392.2003.
21. **Sanchez-SanMartin C, Bustamante VH, Calva E, Puente JL.** 2001. Transcriptional regulation of the orf19 gene and the tir-cesT-eae operon of enteropathogenic Escherichia coli. J. Bacteriol. **183:**2823–2833. http://dx.doi.org/10.1128/JB.183.9.2823-2833.2001.
22. **Sperandio V, Mellies JL, Delahay RM, Frankel G, Crawford JA, Nguyen W, Kaper JB.** 2000. Activation of enteropathogenic Escherichia coli (EPEC) LEE2 and LEE3 operons by Ler. Mol. Microbiol. **38:**781–793. http://dx.doi.org/10.1046/j.1365-2958.2000.02168.x.
23. **Baruch K, Gur-Arie L, Nadler C, Koby S, Yerushalmi G, Ben-Neriah Y, Yogev O, Shaulian E, Guttman C, Zarivach R, Rosenshine I.** 2011. Metalloprotease type III effectors that specifically cleave JNK and NF-kappaB. EMBO J. **30:**221–231. http://dx.doi.org/10.1038/emboj.2010.297.
24. **Padavannil A, Jobichen C, Mills E, Velazquez-Campoy A, Li M, Leung KY, Mok YK, Rosenshine I, Sivaraman J.** 2013. Structure of GrlR-GrlA complex that prevents GrlA activation of virulence genes. Nat. Commun. **4:**2546. http://dx.doi.org/10.1038/ncomms3546.
25. **Iyoda S, Watanabe H.** 2005. ClpXP protease controls expression of the type III protein secretion system through regulation of RpoS and GrlR levels in enterohemorrhagic Escherichia coli. J. Bacteriol. **187:**4086–4094. http://dx.doi.org/10.1128/JB.187.12.4086-4094.2005.
26. **Karlinsey JE, Tanaka S, Bettenworth V, Yamaguchi S, Boos W, Aizawa SI, Hughes KT.** 2000. Completion of the hook-basal body complex of the Salmonella typhimurium flagellum is coupled to FlgM secretion and fliC transcription. Mol. Microbiol. **37:**1220–1231. http://dx.doi.org/10.1046/j.1365-2958.2000.02081.x.
27. **Zarivach R, Deng W, Vuckovic M, Felise HB, Nguyen HV, Miller SI, Finlay BB, Strynadka NC.** 2008. Structural analysis of the essential self-cleaving type III secretion proteins EscU and SpaS. Nature **453:**124–127. http://dx.doi.org/10.1038/nature06832.
28. **Friedberg D, Umanski T, Fang Y, Rosenshine I.** 1999. Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic Escherichia coli. Mol. Microbiol. **34:**941–952. http://dx.doi.org/10.1046/j.1365-2958.1999.01655.x.
29. **Jobichen C, Li M, Yerushalmi G, Tan YW, Mok YK, Rosenshine I, Leung KY, Sivaraman J.** 2007. Structure of GrlR and the implication of its EDED motif in mediating the regulation of type III secretion system in EHEC. PLoS Pathog. **3:**e69. http://dx.doi.org/10.1371/journal.ppat.0030069.
30. **Yerushalmi G, Nadler C, Berdichevski T, Rosenshine I.** 2008. Mutational analysis of the locus of enterocyte effacement-encoded regulator (Ler) of enteropathogenic Escherichia coli. J. Bacteriol. **190:**7808–7818. http://dx.doi.org/10.1128/JB.00663-08.
31. **Miller VL, Mekalanos JJ.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. J. Bacteriol. **170:**2575–2583.