Chlamydial Type III Secretion System Is Encoded on Ten Operons Preceded by Sigma 70-Like Promoter Elements^{∇†}

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Received 13 July 2006/Accepted 11 October 2006

Many gram-negative bacterial pathogens employ type III secretion systems for infectious processes. Chlamydiae are obligate intracellular bacteria that encode a conserved type III secretion system that is likely requisite for growth. Typically, genes encoding type III secretion systems are located in a single locus; however, for chlamydiae these genes are scattered throughout the genome. Little is known regarding the gene regulatory mechanisms for this essential virulence determinant. To facilitate identification of *cis*-acting transcriptional regulatory elements, the operon structure was determined. This analysis revealed 10 operons that contained 37 genes associated with the type III secretion system. Linkage within these operons suggests a role in type III secretion for each of these genes, including 13 genes encoding proteins with unknown function. The transcriptional start site for each operon was determined. In conjunction with promoter activity assays, this analysis revealed that the type III secretion system operons encode σ^{70} -like promoter elements. Transcriptional initiation by a sigma factor responsible for constitutive gene expression indicates that undefined activators or repressors regulate developmental stage-specific expression of chlamydial type III secretion system genes.

Type III secretion systems are virulence determinants employed by many gram-negative bacterial pathogens to evade and exploit eukaryotic host cells (24). These intricate secretion systems are comprised of multiple structural and chaperone components that deliver bacterial proteins to the surfaces of or into eukaryotic host cells (18). As a result, type III secretion systems facilitate numerous infectious processes, including attachment, uptake, vacuolar escape or survival, and avoidance of host immune responses (17). These secretion system components are tightly regulated to ensure proper expression, assembly, and secretion following detection of diverse environmental stimuli (42).

Chlamydiae are obligate intracellular bacteria that manipulate host cells throughout their developmental cycle. Based on sequence homology, chlamydiae carry an almost-complete repertoire of genes representing a type III secretion system (23, 55). Chlamydiae enter host cells as metabolically inert elementary bodies (EB) that have surface projections reminiscent of a type III secretion apparatus (40). EB invade nonphagocytic cells (5), and entry is associated with a candidate type III secretion effector protein, TARP (7). Once internalized, chlamydiae establish a unique vacuole called the inclusion that avoids fusion with endosomes and lysosomes yet acquires exocytic lipids (20, 51). Chlamydia-encoded proteins decorate the inclusion membrane and have been implicated in intracellular vesicular trafficking (47). Inclusion membrane-localized proteins have also been proposed to be secreted by the type III

secretion system (57). Within the established inclusion, EB convert into the metabolically active and replication-competent reticulate bodies (RB). As RB divide, they are tightly associated with the inclusion membrane and have observable projections protruding through the inclusion membrane that are thought to be type III secretion apparatuses (39). Through unknown signals and ill-defined mechanisms, RB asynchronously convert into EB which are eventually released into the extracellular environment, thus completing the developmental cycle.

Relatively little is known regarding the mechanisms regulating the chlamydial developmental cycle. Global gene expression profiling during development emphasized the governing role of transcriptional regulation. Within 6 to 8 h postinfection (hpi), greater than 75% of predicted *Chlamydia trachomatis* open reading frames are transcribed, and most are constitutively expressed throughout the developmental cycle (2, 43). Chlamydiae encode a primary DNA-dependent RNA polymerase σ factor (σ^{66}) that is homologous to σ^{70} factors responsible for constitutive gene expression (11, 27). While few chlamydial promoters have been experimentally defined, σ^{66} promoter elements precede constitutively expressed genes (21). At approximately 18 to 24 hpi, coinciding with conversion from early RB to EB in *C. trachomatis*, virtually all differentially expressed genes (~20% of the open reading frames) are upregulated (43). Of the differentially expressed genes analyzed, all but one (*hctB*) (65) also have σ^{66} promoter elements (21). These data indicate that transcriptional activators or repressors play a critical role in chlamydial differential gene expression (13). While it is anticipated that the alternative σ factors encoded by chlamydiae, σ^{28} and σ^{54} , are also involved in developmental regulation, only σ^{28} -directed *hctB* expression has been demonstrated (65).

Type III secretion system genes are among those upregulated during RB-to-EB conversion (43); however, the regulatory mechanisms are unknown. Determining chlamydial type

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[†] Supplemental material for this article may be found at <http://j.b.asm.org/>.

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[∇] Published ahead of print on 20 October 2006.

III secretion system regulatory elements involved in gene expression is complicated by the unique genomic structure. Typically, type III secretion system genes are similarly organized and tightly clustered within a single locus located in a chromosomal pathogenicity island or virulence-associated plasmid (24). Among chlamydial species, the type III secretion system genetic content and organization are highly conserved (25). However, chlamydial type III secretion system genes are disordered and chromosomally dispersed within six loci (55). Within these loci are structural components that comprise the needle structure, specific chaperones for secreted proteins (effectors), and two translocator systems that are necessary for secreted proteins to cross the host cell membrane (24). Additionally, genes encoding proteins with unknown function flank many type III secretion system homologs. While transcriptional regulators of type III secretion genes are located predominantly within the same locus in other organisms (24), a homolog has not been identified in the chlamydial loci.

Chlamydiae have a significant impact on public health worldwide and are associated with a diverse spectrum of disease manifestations, including blindness, pneumonia, atherosclerosis, and reproductive sterility (49). Despite the medical significance, very little is known regarding the mechanisms of pathogenicity. This study was designed to elucidate regulatory systems for the type III secretion virulence factors in *C. trachomatis*. Due to the genetic complexity of the chlamydial type III secretion system, the operon structure was characterized to facilitate identification of *cis*-acting regulatory elements. Genes contained within a transcriptional unit predominantly encode proteins involved in the same cellular function (29). Therefore, determining the chlamydial type III secretion system operon structure also identified proteins likely involved in type III secretion system-mediated virulence, including candidate regulatory and effector proteins.

MATERIALS AND METHODS

Culture strains. *C. trachomatis* L2/434/Bu was used to infect mouse fibroblast L929 cultures at a multiplicity of infection of 5. RPMI 1640 tissue culture medium (Invitrogen, Carlsbad, CA) was supplemented with 5% fetal bovine serum and 50 µg/ml vancomycin. For isolating *C. trachomatis* RNA, L929 cells were grown in suspension prior to infection. For studies isolating total RNA, L929 cells were grown in monolayers prior to infection. *Escherichia coli* strain TOP10 (Invitrogen) was utilized to cultivate plasmids for cloning and β-galactosidase activity experiments.

Transcriptional linkage analysis. To define operon structure, reverse transcription coupled with PCR (RT-PCR) was utilized to determine if flanking genes are contained on the same transcriptional unit (operon). Chlamydial RNA was isolated at 24 hpi as described previously (43). cDNA was generated from 5 µg of RNA by using random hexamers and Superscript III reverse transcriptase according to the manufacturer's protocol (Invitrogen). PCR was performed using primers designed to generate an approximately 800-bp PCR product (amplicon) representative of a region approximately 400 bp upstream and downstream of the predicted translational start site (see Table S1 in the supplemental material). Amplicons were separated and visualized in ethidium bromide-stained 1% agarose gels.

Quantitative gene expression analysis. Total RNA was isolated directly from *C. trachomatis*-infected L929 cells at 6-h increments postinfection through 36 h postinfection by using Trizol (Invitrogen, Carlsbad, CA). Isolated RNA was treated with RQ1 DNase (Promega, Madison, WI) for 2 h prior to purification according to the manufacturer's protocol (RNeasy Cleanup Column; QIAGEN, Valencia, CA). For each RNA sample, cDNA was generated using random hexamers and Superscript III reverse transcriptase (Invitrogen), following the manufacturer's procedures. Real-time PCR analysis was performed using 16S ribosomal primers, verifying that RNA samples were free of contaminating DNA

and confirming cDNA generation. For each gene, quantitative PCR was performed on each cDNA sample (including an RT-negative sample) and on dilutions of *C. trachomatis* genomic DNA (5×10^{-9} through 6.4×10^{-15} grams), using an Applied Biosystems 7500 real-time PCR detection system (Applied Biosystems, Foster City, CA). Each reaction mixture contained template, 300 nmol of each primer, and 2× SYBR green PCR master mix (Applied Biosystems). Threshold fluorescence was established within the geometric phase of logarithmic amplification for each gene, and the cycle threshold (C_T) was determined. Log genomic DNA versus C_T was plotted to establish standard curves for each gene, and genomic equivalents were determined from each cDNA sample. Genomic equivalents were averaged and standard deviations calculated. For normalization, ratios to the constitutively expressed CT190 (*gyrB*) for each time point were ascertained.

Identification of 5' transcript ends. To identify the transcriptional start sites for each operon, 5' transcript termini were determined following the 5' rapid amplification of cDNA ends (RACE) protocol of Invitrogen, with the following exceptions. cDNA was generated from chlamydial RNA isolated at 24 hpi, using primers listed in Table S1 in the supplemental material and Superscript reverse transcriptase III (Invitrogen) with a 55°C annealing temperature. Terminal deoxytransferase reaction mixtures were incubated at 37°C for 20 min before heat inactivation at 65°C for 10 min. Initial PCRs were performed using 30 cycles of 94°C, 55°C, and 72°C for 30 seconds each cycle with gene-specific RACE nested 3' primers (see Table S1 in the supplemental material). Secondary PCRs (30 µl) were performed using 5 µl of a 1:500 dilution of the primary PCR products with an annealing temperature of 60°C. Amplicons were separated on a 1.8% agarose gel, and bands unique to reactions containing terminal deoxytransferase were excised. DNA was purified from excised bands following the manufacturer's protocol (DNA gel purification kit; QIAGEN), and amplicons were sequenced directly using RACE nested primers (see Table S1 in the supplemental material) (University of California, Berkeley, DNA core sequencing facility and Applied Biosystems 3730). Sequencing trace files were analyzed for the first nucleotide after the incorporation of a string of dCTPs to predict the transcriptional 5' termini.

Computational prediction of σ^{70} promoter elements. To predict the presence of σ^{70} promoter elements, Neural Network Promoter Prediction 2.2 was utilized (4). One-hundred-base-pair regions corresponding to 80 and 20 bp upstream and downstream of experimentally identified transcriptional start sites, respectively, were analyzed. Predicted promoters are provided a score ranging from 0 to 1. Other than for CT663, a threshold of 0.9 was applied. At this threshold, the false-positive rate was 0.3% and the correlation coefficient was 0.71 for *E. coli* σ^{70} promoters. Additionally, approximately 50% of *E. coli* σ^{70} promoters scored higher than 0.9 (M. Reese, personal communication; http://www.fruitfly.org/seq_tools/nnppHelp.html).

Computational transcriptional terminator prediction. The *C. trachomatis* genome has been analyzed for Rho-independent termination sites by using the TransTerm algorithm (12), and results are available at <http://www.cbcb.umd.edu/software/TransTerm/>. For each predicted operon, regions downstream of the stop codon for the 3'-most proximal gene were inspected for Rho-independent transcriptional termination sites. For those genes for which sites were not identified using TransTerm, regions were searched for the presence of inverted palindromes separated by 3 to 10 bp that could form stem-loop structures. Free energies for predicted stem-loops were calculated using Mfold (67).

β-Galactosidase expression analysis. β-Galactosidase activity was used to measure the transcriptional compatibility of type III secretion system promoters in *E. coli*. An expression vector containing a *lacZ* gene lacking a promoter (pAC-lacZ) was generated by cloning the *lacZ* gene into pACYC184. The *lacZ* gene from the pBAD-lacZ vector (Invitrogen) was amplified and cloned using the *EagI* and *HindIII* sites. Upstream regions containing type III secretion system promoter elements were amplified using primers listed in Table S1 in the supplemental material and were cloned into the *EagI* site. All clones were sequenced to confirm insertion and orientation by using *lacZ* 3' screen primer (5'-TTGAGGGGACGACGACAGTATC-3'). For β-galactosidase activity assays, triplicate samples from overnight cultures grown in Luria-Bertani broth and chloramphenicol (50 µg/ml) were diluted 1:100 in fresh broth and grown at 37°C for approximately 2 h (early to mid-log phase) before the optical density at 600 nm (OD_{600}) was measured. β-Galactosidase expression analysis was performed as described previously (41) with the following exceptions to adapt the analysis to 1.8-ml microcentrifuge tubes: cells were diluted in a total volume of 500 µl with Z-buffer; 50 µl of chloroform and 25 µl of 0.1% sodium dodecyl sulfate were added, vortexed for 10 seconds, and incubated at room temperature for 10 min; 100 µl of *ortho*-nitrophenyl-β-galactoside (4 mg/ml) was added; and the time was measured before addition of 250 µl of 1 M Na_2CO_3 to stop reactions. Reaction

FIG. 1. Transcriptional linkage analysis of chlamydial type III secretion system loci. To determine if genes share a common transcript, PCR was performed on 24-hpi cDNA using primers designed to amplify approximately 400 bp on either side of a translational start site for the pairs of genes shown. For each gene pair, the products of four reactions were analyzed: lanes 1, RT positive; lanes 2, RT negative; lanes 3, DNA positive; and lanes 4, DNA negative. Type III secretion homologs are not shaded, and genes are numbered according to the *C. trachomatis* serovar D sequence (55). Above each locus, predicted transcripts are indicated by an arrow.

mixtures were centrifuged for 5 min at $16,000 \times g$, the upper phase was removed, and the OD_{420} and OD_{550} ratio was measured.

In vitro transcription assays. To analyze the transcriptional compatibility of *E. coli* σ^{70} and type III secretion system promoters, in vitro transcription reactions were performed. One and a half picomoles of core RNA polymerase or σ^{70} saturated holoenzyme (Epicenter, Madison, WI) and 40 units of RNasin (Promega) were mixed with transcription buffer (200 mM Tris-HCl [pH 7.5], 750 μ M KCl, 50 mM $MgCl_2$, 0.05% Triton X-100, and 10 mM dithiothreitol) prior to addition of 50 femtomoles of template DNA. Template DNA consisted of the type III secretion system promoter-*lacZ* fusion constructs utilized in β -galactosidase assays. Reactions were incubated at 22°C for 10 min to allow RNAP to bind to DNA before transcription was initiated by addition of 300 μ M nucleoside triphosphates with 0.1 mg/ml heparin. Reaction mixtures were incubated at 30°C for 20 min before treatment with 5 units of RQ1 RNase-free DNase (Promega) for 4 h at 37°C. Transcribed RNA was purified according to the manufacturer's protocol (RNeasy Cleanup; QIAGEN).

The amount of transcribed RNA in each reaction was determined by quantitative PCR. cDNA was generated from equal volumes of RNA eluted from each reaction, using a 3' *lacZ* primer (GGGCGCATCGTAACCGTGCATCTGC) and Superscript III reverse transcriptase according to the manufacturer's protocol (Invitrogen). Quantitative PCR was performed on 1:500 dilutions of cDNA containing 300 nmol of each primer (*lacZ* RT [5' GCTGGCGTAATAGCGAA GAGG] and 3' TTGAGGGGACGACGACAGTATC) and 2 $\times$ SYBR green PCR master mix (Applied Biosystems). Threshold fluorescence was established within the geometric phase of exponential amplification, and the C_T was determined for each reaction. Fold change in transcript ($E_{\sigma^{70}}$ versus core RNA polymerase) was determined using the $2^{-\Delta\Delta C_T}$ method (32).

RESULTS

Genes are often organized into a coexpressed and coregulated unit termed an operon that shares an RNA transcript (29). Operon expression occurs through regulatory factors which typically exert control through interaction with *cis*-acting elements located near transcriptional initiation sites (33). Characterizing an operon structure indicates the 5'-most proximal gene and facilitates identification of transcriptional start sites. To elucidate the regulatory factors associated with the chlamydial type III secretion system, the operon structure was characterized through two approaches: RT-PCR transcriptional linkage analysis and quantitative gene expression profiling. RT-PCR transcriptional linkage analysis enables the stepwise analysis of whether adjacent genes share a common mRNA transcript. Gene clusters were then analyzed by quantitative gene expression profiling to confirm the RT-PCR linkage and to identify internal promoter activity within operons. Based on predicted operon structure, RACE was applied to the 5'-most proximal gene of each operon to identify transcriptional start sites and *cis*-acting regulatory elements.

Transcriptional linkage analysis reveals seven type III secretion operons. Detection of RT-PCR products by using

primers specific to genes flanking an intergenic region indicates genes that share a common transcript. However, transcripts with extensive 5' and 3' untranslated regions could overlap neighboring genes, causing inaccurate prediction of transcriptional linkage. In *E. coli*, transcriptional initiation and termination sites are approximately 100 bp upstream or downstream of translational start and stop sites, respectively (4). Information regarding the lengths of chlamydial untranslated regions is limited; however, the chlamydial genome is compact, with small intergenic regions (55). Therefore, primers were designed to amplify regions that correlate to approximately 400 bp on either side of a translational start site to diminish potential false positives.

Transcriptional linkage analysis was performed on the six loci that harbor homologs to the type III secretion system (see Fig. 5) (55). This analysis revealed that within these six loci, seven operons are present (Fig. 1). These operons are comprised of 37 genes and include 13 genes encoding proteins of unknown function (CT560, CT577, CT663 to -668, CT670, CT671, CT716, CT718, and CT863) as well as 4 genes identified as homologs to MalQ (CT087), LpdH (CT557), LipA (CT558), and Pkn5 (CT673). Thus, these gene relationships define 7 operons consisting of coordinately regulated genes.

Quantitative gene expression profiling reveals three internal operons. A limitation of transcriptional linkage analysis is the inability to detect operons embedded within an overlapping transcript. Typically, ectopic genes or operons are regulated independently and thus have different expression patterns. To reveal the presence of these in the type III secretion loci, expression of individual genes was quantitated at 6, 12, 18, 24, 30, or 36 hpi using quantitative PCR (Fig. 2).

Three internal operons were suggested by their distinct patterns of gene expression. Within the CT663-CT672 operon, the expression levels of the first two genes in the operon (CT663 and -664) were constant between 6, 12, and 18 hpi (Fig. 2). In contrast, CT665-CT672 displayed a different temporal pattern, with respective genes increasing over eightfold during these time periods, indicating separate regulation. Similarly, within the CT557-CT564 operon, transcript levels for CT559 were approximately two- to eightfold higher more than those for upstream CT557-CT558, also suggesting internal regulation. Additionally, levels and patterns of CT673 and CT674 transcription were different. At 6 hpi, CT674 transcripts were twice as abundant as CT673 transcripts, and this disparity increased to almost 16-fold by 24 hpi. Together, these data indicate that CT665-CT672, CT559-CT564, and CT674 are independently regulated internal operons but are also expressed in the parental operon.

Given the divergent expression patterns of genes bordering the 5' end of internal operons (CT558-CT559, CT664-CT665, and CT673-CT674), RT-PCR linkage analysis was repeated with an added level of specificity to confirm the presence of a shared parental transcript. cDNA was generated using primers specific for downstream genes compared to random hexamers. PCR of the resulting cDNA produced amplicons of the expected size, and sequencing of these products confirmed that RNA transcripts correlating to these regions are indeed present (data not shown).

Gene expression profiling indicates early type III secretion system expression. EB are prearmed with type III secretion

FIG. 2. Quantitative gene expression analysis of chlamydial type III secretion system loci. Total RNA was isolated at 6, 12, 18, 24, 30, and 36 hpi. For each gene at each time point, the amount of cDNA was determined by quantitative PCR. Transcript quantity was transformed to a log₂ ratio of constitutively expressed CT190 (*gyrB*) (43). Two genes, CT510 (*secY*) and CT046 (*hctB*), were analyzed as constitutive and RB-to-EB conversion associated controls, respectively (43). Genes CT577, -578, -089, -090, and -671 were also analyzed, and expression patterns and levels similar to those for flanking genes were observed. These were not included to conserve figure space. Error bars indicate standard errors of the means.

machinery (40) that is likely utilized for host cell entry (7). It has been proposed that other early infectious processes that require type III secretion, such as secretion of inclusion proteins, also utilize preformed type III secretion machinery (16).

FIG. 3. Transcriptional start sites for chlamydial type III secretion system operons. The 5' ends of gene transcripts were determined by RACE. (A) DNA sequence upstream of transcriptional start sites for the 5'-most proximal gene of each operon. The transcriptional start site is indicated in boldface, and -35 and -10 regions are labeled above. The distances between transcriptional and translational start sites are also indicated. Computational promoter prediction scores are indicated in parentheses. (B to D) Weblogo (8) graphical nucleotide frequency depiction of chlamydial type III secretion (B), previously predicted chlamydial σ^{70} -like promoters ($n = 9$) (21) (C), or combined (type III secretion promoter and previously identified) -35 and -10 hexamer sequences (D) illustrate the conservation at each nucleotide position.

Global gene expression analyses reported that genes encoding type III secretion structural components are upregulated during the middle to late stages of the developmental cycle as RB-to-EB conversion occurs (2, 16, 43). This supports late-stage gene expression and prearming of EB with functional type III secretion system machinery; however, early expression (6 to 8 hpi) of some structural type III secretion components was also detected. Gene expression profiling of individual type III secretion genes confirmed upregulation of structural components as the developmental cycle progresses (Fig. 2). Additionally, while many expression levels were lower than that of the constitutively expressed reference *gyrB*, RNAs from all type III secretion machinery genes were detected at 6 hpi (Fig. 2). The presence of transcripts at this time indicates that de novo type III secretion apparatus is synthesized and likely functioning, thereby contributing to early stages of infection.

Chlamydial translocases exhibit independent expression patterns. Translocases are proteins secreted via the type III secretion system that form pores in membranes required for effector molecule delivery (24). Chlamydiae have two loci that encode predicted translocase systems (CT576-CT579 and CT863-CT860). Unlike the shared transcriptional profile for the genes that encode structural components of the type III secretion system, distinct patterns of gene expression were observed for the two translocase loci. Quantitative gene expression analysis indicated that CT863-CT860 transcripts were present at 6 hpi, and levels remained static throughout the developmental cycle. In contrast, very low transcription levels were detected for CT576-CT579 during early time points (6 and 12 hpi), but these increased over 64-fold by 24 hpi, accounting for the largest increase in transcription for all genes analyzed in this study. Similar expression patterns for *C. pneumoniae* orthologs were reported by Ouellette et al. (44). These expression patterns suggest that each translocase system is utilized at different stages in the developmental cycle.

Type III secretion system operons contain σ^{70} -like promoter elements. Sigma factors recognize and bind to DNA-encoded promoter elements located immediately upstream of transcriptional initiation sites. Thus, determination of the 5' end of an mRNA is necessary and sufficient to interrogate the nearby

DNA sequence for promoter elements. Moreover, the spacing and sequence of promoter elements can directly implicate a particular σ factor as being involved in its gene transcription (19). Chlamydiae encode three σ factors, σ^{66} , σ^{54} , and σ^{28} , that recognize distinct promoter elements (55). To identify the chlamydial σ factor likely associated with type III secretion system expression, 5' transcript ends of operons were determined and upstream regions analyzed for the presence of promoter motifs.

Based upon operon predictions for the seven major operons and three internal operons, an unambiguous 5' terminus was determined for all except the previously identified CT673-CT674 operon (38) (Fig. 3A). The upstream regions were analyzed for the presence of specific σ factor promoter elements. To test whether σ^{70} -like promoter elements were present, a neural network algorithm trained with experimentally defined *E. coli* σ^{70} promoter elements (4) was applied to regions surrounding transcriptional start sites of each operon. With the exception of CT663, *E. coli* σ^{70} -like promoters were predicted computationally for each operon. The scores associated with promoter predictions were high (>0.93 on a scale of 0 to 1) (Fig. 3A). Moreover, these elements correlated with the experimentally defined 5' termini.

Regions upstream of transcriptional start sites were inspected for the presence of alternative σ factor promoter elements. Neither σ^{28} nor σ^{54} promoter motifs were apparent in any of the adjacent upstream regions. Consistent with computational analysis, σ^{70} -like elements were evident for all but CT663. Similar to *E. coli* canonical σ^{70} motifs, 4 to 7 bases upstream of predicted transcriptional start sites, -10 and -35 hexamers separated by 17 to 19 nucleotides were identified (Fig. 3A). These data indicate that σ^{66} directs type III secretion expression, with perhaps the exception of CT663. The mechanism of CT663 initiation was not apparent from this analysis based on the identified 5' terminus. However, computational analysis did predict a transcriptional start site and σ^{70} -like promoter 19 bp upstream of the 5' terminus. This may indicate that the 5' end of this transcript is particularly labile and that transcription begins at this site.

Within canonical *E. coli* σ^{70} promoter motifs, nucleotide

variation occurs in -10 and -35 hexamers, although certain bases are present at higher frequencies than others (31). For the -10 region, the first two bases (TA) and the last base (T) occur at the highest frequency in *E. coli*. Similarly, the first two and last nucleotides are predominantly TA and T in chlamydial -10 promoter regions. The first three bases (TTG) of the -35 region are prevalent in *E. coli* promoters, and all predicted chlamydial type III secretion system -35 hexamers start with TTG. Thus, the chlamydial -10 and -35 promoter element motifs determined for these operons exhibited nucleotide utilization frequencies similar to those of *E. coli* σ^{70} promoters (Fig. 3B). Conservation of high-frequency nucleotides within these promoter elements suggests that native σ^{66} promoter requirements may be similar to those of *E. coli* σ^{70} (50).

***E. coli* σ^{70} directs transcription of seven chlamydial type III secretion system promoters.** Due to the current lack of a system for genetic exchange in chlamydiae, we analyzed the functionality of the type III secretion promoters within *E. coli* by using in vitro transcription assays. Based on the similarity of the chlamydial promoters to canonical σ^{70} , we tested whether these promoters would direct transcription by *E. coli* σ^{70} . Prior data demonstrated that both *E. coli* σ^{70} and *C. trachomatis* σ^{66} initiate transcription from the chlamydial *dnaK* promoter, which has nearly an exact canonical σ^{70} promoter motif (50). However, a chlamydial promoter with less similarity to σ^{70} was recognized only by *C. trachomatis* σ^{66} (10). Two approaches were employed to test recognition of chlamydial σ^{70} -like promoter elements: determination of promoter-directed β -galactosidase activity in *E. coli* and transcription activity in vitro using purified *E. coli* σ^{70} holoenzyme.

For determination of promoter-directed β -galactosidase activity, chlamydial promoter elements were placed upstream of the *lacZ* gene and β -galactosidase activity was measured during mid-logarithmic growth, which correlates to highest σ^{70} activity (22). Type III secretion promoters resulted in expression of β -galactosidase activity for most promoters (Fig. 4A). Surprisingly, CT559 and -674, which exhibit close similarity to canonical σ^{70} motifs, promoted activity close to vector control levels. Despite this, these data show that six chlamydial type III secretion system promoters (CT091, -557, -576, -663, -665, and -863) are functional and direct transcription in *E. coli*. The lack of CT559-, CT674-, and CT719-directed β -galactosidase activity could be due to many issues, including inhibitory trans-acting factors within *E. coli*. To diminish potential confounding factors and demonstrate specific recognition of σ^{70} to these promoters, in vitro transcription was performed using promoter-*lacZ* fusion vectors. Transcription by *E. coli* core RNA polymerase, free of detectable σ^{70} , was compared to that by σ^{70} -saturated RNA polymerase. Similar to the results of β -galactosidase analysis, CT557, CT665, and CT863 were the most active type III secretion system promoters (Fig. 4B). CT674 produced little β -galactosidase but resulted in σ^{70} -directed transcription. In contrast, CT091 exhibited relatively high β -galactosidase activity but promoted low σ^{70} -specific transcription in vitro. These data show that *E. coli* σ^{70} directed in vitro transcription of five type III secretion system promoters (CT091, -557, -665, -674, and -863).

β -Galactosidase activity and in vitro transcription demonstrate that seven of the nine promoters for chlamydial type III secretion operons are functionally active within a σ^{70} system.

FIG. 4. Functional analysis of chlamydial type III secretion promoters. Putative operon promoters were placed upstream of a promoterless *lacZ* gene and tested for β -galactosidase activity and for transcription in vitro. As a control, a vector containing the chlamydial *dnaK* promoter upstream of *lacZ* was also analyzed. (A) β -Galactosidase activity for chlamydial promoters was assayed in *E. coli* grown to mid-logarithmic phase. (B) Ratio of *lacZ* transcripts produced from in vitro transcriptions using *E. coli* core RNA polymerase or core RNA polymerase saturated with σ^{70} . *, $P < 0.01$ by Student's *t* test with respect to *lacZ* (vector) lacking a promoter. Error bars indicate standard errors of the means.

These data, combined with identification of 5' termini, support that these promoter elements are utilized in vivo.

Transcriptional terminators predicted at 3' ends of operons. Identification of transcriptional termination sites, in conjunction with identification of initiation sites and transcriptional linkage analysis, provides support for operon definition. Transcription terminates through Rho-dependent and -independent mechanisms. While Rho-dependent terminators are not identifiable through consensus sequences or structures (46), Rho-independent terminators have been effectively predicted computationally for numerous bacteria (12). This is accomplished through identification of an inverted repeat which forms a stem-loop structure in the mRNA followed by short stretch of uracil residues. In *E. coli* and *Bacillus subtilis*, termination structures are frequently located downstream close to the stop codon, usually within 100 bp (9, 30). To ascertain whether Rho-independent terminators were present downstream of predicted type III secretion operons, a computational approach was employed.

Of the 10 predicted transcripts, transcriptional linkage and expression analysis support that three (CT557-CT564 and CT559-CT564, CT663-CT672 and CT665-CT672, and CT673-CT674 and CT674) share a 3' terminus (Fig. 1 and 2). This indicates that seven genes (CT087, -564, -579, -672, -674, -716, and -860) should have a transcriptional termination site downstream of the stop codon. A computational algorithm (Trans-

TABLE 1. Predicted Rho-independent transcriptional termination sites

3' gene ^a	STS ^b	5' stem	Loop	3' stem (stem -ΔG) ^c	3' tail
CT564 ^d	11	GAAGTCCCTC	CAAAAAA	GAGGGAGTTC (10.7)	TTCTTCACACCACGT
CT579 ^d	42	CCGTAGGCATCCT	AAA	AGGGCCCTACGG (15.1)	TTTTTCTTTACTCTG
CT672	7	AGTGATAGAG	AATC	CTCTGTCATT (10.9)	CTACGTGAGAATTGT
CT674 ^d	76	GTCAAGATGGACTGGAGTTA	GCCG	TATCTCCGGTTCTCTTGGC (21.1)	TTTTTATGGAGTTAG
CT716	92	TGGCCTTCGTT	ATCCCCTC	AACAAAGGCTA (9.0)	ATCGCTCAAATCGAT
CT860 ^d	18	GTCAGATGAG	ATAAA	CTCATCTGAC (14.4)	TTCTCTAATTATAAT

^a 3' genes of predicted operons.^b STS, distance (bases) from stop codon to stem.^c -dG was determined from stem-loop sequences by using Mfold.^d Termination elements were predicted using TransTerm (12).

Term) was designed and applied to numerous bacteria, including *Chlamydia* spp (12). This analysis identified Rho-independent terminators downstream of stop codons for CT564, -579, -674, and -860 (Table 1). One of the components for this analysis relies on the free energy (ΔG) for stem-loop formation for accurate prediction. However, it has been reported that the required ΔG for stem-loop formation can vary in different bacteria, including *Chlamydia* (60). In *E. coli*, the ΔG for non-stem-loop formation is approximately -11.5 and drops to -16 where termination stem-loops have been discovered. In contrast, this variance is approximately -8 to -12 for the same regions in *Chlamydia*. This indicates that the ΔG requirement may be less for functional stem-loop formation in *Chlamydia*. Therefore, we analyzed the downstream regions for the remaining three genes for inverted repeats that could form potential stem-loop structures. Inverted repeats were identified for CT672 and -716, with stem-loop ΔG values of -10.9 and -9 , respectively, although the 3' tails had few uracils. No stem-loop structures with ΔG values less than 9 could be predicted for CT860, which may indicate the Rho-dependent termination.

DISCUSSION

Operons are the primary organized transcriptional unit in prokaryotes, allowing coordinated regulation of groups of genes that predominantly encode functionally linked proteins (45, 48). While relatively few operons have been previously determined experimentally in *Chlamydia* (*trp* [63], *groEL* [59], *incD-G* [52], *incB-C* [1], *pyrG* [64], ribosomal [26], and *omcAB* [61]), all but one (ribosomal/RNA polymerase alpha) encode proteins with shared cellular function. Chlamydial type III secretion system operons included 37 genes, with 13 genes encoding proteins of unknown function (Fig. 5). Due to the propensity of genes within operons to share physiologic function, it is anticipated that many of these genes encoding proteins with unknown function are associated with type III secretion. Supporting this conclusion is the type III-dependent secretion of *C. pneumoniae* orthologs CT671, -718, and -863 in *Shigella* and detection of CT671 in the cytosol of *Chlamydia*-infected cells (56). Additionally, orthologs of CT668 and CT670 are localized to *C. pneumoniae* inclusion membranes, suggesting secretion by a type III mechanism (54). Each of these genes was determined to reside within type III secretion operons.

The global organization of type III secretion system genes in

chlamydiae is fragmented and rearranged relative to that in other bacteria; nevertheless, this organization is highly conserved among diverse members of the *Chlamydiaceae*. The type III secretion machineries are multicomponent structures requiring various molar ratios of each protein. Therefore, gene retention within operons should be advantageous, which suggests that the operon organization for the chlamydial type III secretion system facilitates the expression required for developmental stage-specific morphogenesis of chlamydial organisms. This conclusion is consistent with global gene expression patterns indicating that type III secretion system components are differentially regulated during early stages of development and as RB-to-EB conversion occurs.

Expression patterns indicate that in addition to requiring preformed type III secretion machinery for early infection stages, such as entry and inhibition of lysosomal fusion, de novo machinery and associated components are also required. Furthermore, data suggest a functional separation during the developmental cycle for each translocase system. Chlamydiae encounter at least two diverse membranes during the developmental cycle: the host cell plasma and inclusion membranes.

FIG. 5. Genetic organization and operon prediction for six genomic loci containing homologs of type III secretion system genes. Gene orientations and locations are shown by block arrows. Above the genes, line arrows indicate directions and open reading frame inclusions for predicted operons. Proposed functions of type III secretion homologs are indicated by shading. The open reading frame number (above) and annotation (below) are assigned based upon the *C. trachomatis* serovar D annotated nomenclature (55). Chaperone and translocator nomenclature has incorporated the amendments of Fields et al. (14, 15).

Translocases insert into membranes and are required for delivery of effector molecules. Based on expression patterns, we speculate that proteins encoded within CT863-CT860 facilitate delivery of molecules through the inclusion membrane that are necessary to maintain intracellular growth. In contrast, CT576-CT579 translocator expression patterns indicate late developmental stage function interacting with a modified inclusion membrane and/or being packaged into EB for host cell plasma membrane translocation during entry.

Transcriptional initiation is the primary stage for controlling prokaryotic gene expression (3). Sigma factors provide specificity and direct RNA polymerases to initiate transcription at promoter elements located upstream of transcriptional start sites. This study revealed that eight σ^{70} -like promoters precede type III secretion operons and, with one exception (CT863-CT860), are differentially expressed during the developmental cycle. Relatively few promoters have been experimentally defined in *Chlamydia*. Of these, all but three (*hctB* [65] and CT652.1 and CT683 [38]) most closely resemble σ^{70} -like promoters (21). Most of these promoters precede differentially expressed genes, supporting the conclusion that additional regulatory *trans*-acting factors are required. To date, four *trans*-acting transcriptional regulators of differentially expressed genes have been described; however, relatively few gene targets are affected (6, 28, 62, 66). With approximately 20% of chlamydial open reading frames differentially expressed during the developmental cycle (43), and with the paucity of alternative sigma factors and apparent gene targets, it is anticipated that global gene regulatory factors for key stages in the developmental cycle remain to be identified.

The functional differences between chlamydial σ^{66} and *E. coli* σ^{70} have been somewhat enigmatic. This is mainly due to the high conservation of σ^{66} regions that interact with DNA promoter elements (regions 4.2 and 2.4) (27), the presence of σ^{70} -like promoter elements in chlamydiae, and the relatively high transcriptional activity exhibited by σ^{66} in vitro to an almost exact σ^{70} consensus promoter (50). Despite the inherent ability, if not preference, for σ^{66} to initiate transcription from σ^{70} -like promoters, several chlamydial σ^{70} -like promoters are not compatible with *E. coli* σ^{70} (10, 53). Furthermore, chlamydial σ^{66} has less restriction to promoter sequence structure compared to other σ^{70} vegetative sigma factors, which may contribute to this difference (36, 58). Likely overriding issues are that σ^{66} responds optimally to a promoter structure affected by additional amino- and carboxyl-terminal amino acids (37). These extensions are conserved among chlamydial species (data not shown), and among the abundant σ^{70} protein sequences, only one other has been reported in bacteria (34). Moreover, 50% of *E. coli* genes require at least one other factor in addition to σ for appropriate transcriptional initiation, compensating for promoter degeneration (35). It is expected that chlamydial promoters, especially those of differentially expressed genes, may also require additional *trans*-acting factors. In *E. coli*, these factors are likely not compatible with *cis*-acting elements surrounding chlamydial promoters. These observations emphasize the need for experimental systems that will allow for accurate analysis of promoter elements and *trans*-acting regulatory factors.

Mechanisms for chlamydial developmental differentiation are poorly understood. Of the identified 10 operons harboring

37 genes associated with type III secretion, 8 were preceded by σ^{70} -like promoter elements that were confirmed to be transcriptionally active in a σ^{70} -based system. This supports the prediction of functional promoters for type III secretion operons that are regulated by the primary σ factor in *Chlamydia*. Based upon the frequency of chlamydial genes preceded by σ^{66} promoter elements that have expression patterns similar to those of type III secretion genes, *trans*-acting regulators are likely shared. These observations highlight the potentially critical role of unidentified regulators involved in developmental cycle regulation.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI042156 from the National Institute of Allergy and Infectious Diseases and by National Research Service Award AI058490 (to P.S.H.).

We thank K. Hybiske and S. Dela Cuesta for critical review of the manuscript.

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