Transcriptomic Analysis of Shiga-Toxigenic Bacteriophage Carriage Reveals a Profound Regulatory Effect on Acid Resistance in Escherichia coli

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Shiga-toxigenic bacteria are converting lambdoid phages that impart the ability to produce Shiga toxin to their hosts. Little is known about the function of most of the genes carried by these phages or the impact that lysogeny has on the Escherichia coli host. Here we use next-generation sequencing to compare the transcriptomes of E. coli strains infected with an Stx phage, before and after triggering of the bacterial SOS response that initiates the lytic cycle of the phage. We were able to discriminate between bacteriophage genes expressed in the lysogenic and lytic cycles, and we describe transcriptional changes that occur in the bacterial host as a consequence of Stx phage carriage. Having identified upregulation of the glutamic acid decarboxylase (GAD) operon, confirmed by reverse transcription-quantitative PCR (RT-qPCR), we used phenotypic assays to establish the ability of the Stx prophage to confer a greater acid resistance phenotype on the E. coli host. Known phage regulators were overexpressed in E. coli, and the acid resistance of the recombinant strains was tested. The phage-encoded transcriptional regulator CII was identified as the controller of the acid response in the lysogen. Infection of an E. coli O157 strain, from which integrated Stx prophages were previously removed, showed increased acid resistance following infection with a nontoxigenic phage, φ24n. In addition to demonstrating this link between Stx phage carriage and E. coli acid resistance, with its implications for survival post-ingestion, the data set provides a number of other potential insights into the impact of lambdoid phage carriage on the biology of E. coli.

Bacteriophages have become widely recognized as important drivers of bacterial diversification and evolution (1–4). They play important roles in the adaptation of established pathogens to new metazoan hosts as well as the general emergence of new pathogens (5–7); bacteriophages can transform their commensal bacterial host to a pathogen or simply add to the virulence of pathogenic bacterial hosts (7–9), a process termed “lysogenic conversion.” In the particular case of Shiga-toxigenic Escherichia coli (STEC), especially the subset of enterohemorrhagic E. coli (EHEC), infection with Shiga toxin-encoding bacteriophages (Stx phages) was the key event leading to the emergence of these pathogens as a major health concern following their first association with an outbreak of foodborne disease in 1982 (10–12). EHEC is able to colonize the intestinal tract with great efficiency; the resulting gastrointestinal infection is potentially fatal due to the production and release of Shiga toxin, leading to hemolytic colitis and, in some cases, hemolytic-uremic syndrome (HUS) (12–14).

Stx phages are lambdoid phages because they possess genomes that have an organization similar to that of bacteriophage lambda (λ) (7). A recent genomic comparison of 11 Stx phages and λ revealed a high degree of mosaicism in this group, and all but one, φP27, had a significantly larger genome than that of λ (15). The function of this extra genomic material has yet to be determined. Between 40 and 60% of the genes carried by these 11 sequenced Stx phages are of unknown function, including genes that are highly conserved across this group (15). While Shiga toxin itself has been extensively studied (16–18) and putatively assigned the biological role of protecting a STEC population from predation by grazing protozoa (19, 20), little attention has been paid to the identification of other possible virulence or fitness factors encoded on the genome of Stx phages. The key feature of Shiga toxin production by E. coli lysogens (STEC) is that Shiga toxin gene expression, and, hence, disease, is a direct consequence of the Stx prophage entering the lytic phase when infectious bacteriophage particles are released along with the toxin itself. The lytic replication cycle of Stx prophages, in common with all known lambdoid bacteriophages, is induced by the autocleavage of the CI repressor, which is driven by the activation of RecA and can be achieved in vitro by exposure to UV radiation, mitomycin C, norfloxacin, or other agents that result in DNA damage (21, 22).

There is some evidence that Stx phages have a more profound impact on the E. coli host than merely enabling it to produce Shiga toxin. Preliminary data acquired during signature-tagged mutagenesis experiments identified genes implicated in increased gut adhesion of STEC in calves; of the 59 genes identified, 7 were carried by Stx prophages (23). Microarray analysis of the impact of lysogeny with the Stx2 phage φMin27 on the transcriptome of E. coli MG1655 showed upregulation of the expression of 104 genes and downregulation of 62 genes (24). Finally, the lambdoid phage transcriptional regulator CII has been linked to repression of the...
type III secretion system in EHEC (25), the expression of which is necessary for colonization of the mammalian gut (26).

We previously described the identification of Stx phage genes that are expressed in an E. coli lysogen culture and linked the expression of at least two Stx phage genes of unknown function, \( \nu_b \)-\( 24B \)-\( 13c \) and \( \nu_b \)-\( 15 \), to the lysogenic state. In any lysogen culture, there is always a background of spontaneous phage induction, which confounds the ability to link the expression of a specific gene with the lysogenic state, and this is further constrained by the sensitivity of the methods used to identify proteins present in low abundance (27). Here we use transcriptome sequencing (RNA-Seq) to produce preliminary gene expression patterns controlled by, or derived from, Stx prophage carriage and determine the influence of one such upregulated operon, glutamic acid decarboxylase (GAD), on the acid resistance phenotype that has been associated with disease-causing Shiga-toxigenic E. coli.

MATERIALS AND METHODS

**Bacterial strains, growth conditions, and RNA extraction.** E. coli K-12 strain MC1061 was used in all experiments; naive MC1061 refers to cells that have not been infected with bacteriophage \( \phi 24A \)-\( 24B \)-\( Kan \) (28), and MC1061(\( \phi 24B \)) refers to MC1061 lysogens of \( \phi 24A \)-\( 24B \)-\( Kan \) (see Table S1 in the supplemental material). MC1061 and MC1061(\( \phi 24B \)) were propagated overnight in three biological replicates (16 h), subcultured (1:10), and grown to mid-exponential phase (optical density at 600 nm \([OD_{600}]\) of 0.5). Aliquots (1 ml) were harvested, and RNA was extracted by using the RNeasy minikit from Qiagen according to the manufacturer’s instructions. These 6 cultures, 3 MC1061 and 3 MC1061(\( \phi 24B \)) cultures, were then induced with norfloxacin as described previously (27); briefly, cultures were incubated with norfloxacin (1 \( \mu g \cdot ml^{-1} \)) for 1 h at 37°C with shaking at 200 rpm, followed by dilution of the cultures 1:10 in fresh lysogeny broth, and allowed to recover at 37°C for 40 min with shaking at 200 rpm. Cells were then collected by centrifugation, and RNA was immediately extracted as described above.

**Sequencing of the MC1061(\( \phi 24B \)) genome.** Prior to the RNA-Seq analysis of the four transcriptomes generated in the study, the genome of MC1061(\( \phi 24B \)) was sequenced, annotated, and deposited, along with the four transcript libraries, in the European Nucleotide Archive. The genome assembly consists of 5,027,118 bp in 15 scaffolds. The E. coli MC1061(\( \phi 24B \)) genome was obtained through whole-genome shotgun pyrosequencing by generating a standard DNA mate-pair library with an 8-kb insert size, using a 454 preparation kit (Roche Applied Sciences, Indianapolis, IN). The genomic DNA (gDNA) sample was sequenced with a GS-FLX system using Titanium chemistry (454 Life Sciences, Roche Applied Sciences). The 454 reads were assembled with Newbler (August 2010 R&D version of GSAssembler; Roche Applied Sciences). The final assembly was annotated on the RAST (Rapid Annotation Using Subsystem Technology) server (29). To improve the quality of the phage sequence within the lysogen, the 57,677-bp genome of \( \phi 24A \) (GenBank accession number HM208303.1) was used to replace the lower-quality phage sequence residing in the lysogen genome sequence between bp 3129539 and 3186979 (57,440 bp) to form a hybrid genome. The subsequent analyses and RNA-Seq mapping were done by using the hybrid genome. To compensate for the coordinate numbering following the creation of the hybrid sequence, 236 bp (57,677 bp – 57,440 bp – 1 bp = 236 bp) were added at the starting and ending coordinates of each gene from the original assembly after bp 3186979, while 3,129,538 bp (3,129,539 bp – 1 bp = 3,129,538 bp) were added at the starting and ending coordinates of each gene from the \( \phi 24A \) prophage genome.

**Preparation and sequencing of cDNA libraries.** The quantity and quality of harvested RNA were assessed on an Agilent 2100 bioanalyzer using an RNA 6000 Nano Chip kit from Ambion. Equal amounts of RNA were pooled from each biological triplicate to produce four libraries (naive cultures, naive cultures treated with norfloxacin, lysogen cultures, and lysogen cultures treated with norfloxacin). rRNA was depleted from the total RNA samples by using the MICROBExpress bacterial RNA enrichment kit according to the manufacturer’s instructions, and the resulting mRNA quantity and quality were evaluated on an Agilent 2100 bioanalyzer using an RNA 6000 Nano Chip from Ambion. The RNA was then randomly fragmented by incubation with RNase III (Applied Biosystems) at 37°C for 10 min, recovered by using the RiboMinus concentration module (Invitrogen), and assessed for quantity and size distribution on a bioanalyzer using the RNA 6000 Pico Chip (Agilent). The processed RNA fragments were hybridized and ligated to the adapters from the SOLiD small RNA expression kit (Ambion), and reverse transcription was performed by using ArrayScript reverse transcriptase (Ambion). The resulting cDNA was purified by using the MinElute PCR purification kit (QiaGen). Size selection of cDNA (100 to 200 bp) was performed by using Novex 6% Tris-borate-EDTA (TBE)–urea gels (Invitrogen). The harvested DNA was amplified by using components from the SOLiD small RNA expression kit (Ambion) according to the manufacturer’s instructions, and the products were purified by using the PureLink PCR microkit (Invitrogen).

**Data processing and gene expression analysis.** RNA samples as cDNA were sequenced on an ABI SOLiD sequencing platform using v4 chemistry. More than 8 \( \times 10^8 \) high-quality, single-end, 50-bp color-space reads were generated per sample. The reads were analyzed by using the ABI BioScope v1.3 whole-transcriptome analysis (WTA) pipeline. The WTA pipeline first aligned the reads onto the E. coli lysogen sequence [MC1061(\( \phi 24B \))] and reported the alignments as a BAM file (30). WTA was then used to enumerate reads aligning to each genomic feature in the lysogen genome. Each defined genomic feature was subsequently assigned a tag count, which is the total number of alignments that pass the stringency filters. Finally, the WTA pipeline was used to calculate the normalized level of expression of each genomic feature by using RPKM (reads per kilobase of exon model, per million mapped reads) values (31).

**Statistical analysis** was performed on normalized read counts for all four samples by using the R platform. The DESeq package was used to analyze differential gene expression across the four libraries (32). The script used in the analysis uses the command cds-estimateVariationFunctions(cds, method = “blind”), as recommended in the DESeq manual (32), for processing of samples that are pools and therefore not replicated: table<-read.table(“L10.txt”, header = T), cnds<-factor(c(“L”, “N”)), cds<-newCountDataSet(table, cnds), cnds<-estimateSizeFactors(cds), cds<-estimateVarianceFunctions(cds, method = “blind”), res<-nbinomTest(cds, “L”, “N”), and write.table(res, file = “L10res.txt”, sep = “	”).

Hierarchical cluster analysis was performed on the base 2 logarithm of gene read counts by using the “apeplots” packages in Bioconductor (34).

**Arabinose induction of phage transcriptional regulators.** The \( \phi 24A \) genes encoding the regulators CI, CII, and CIII were amplified from MC1061(\( \phi 24A \)) DNA by using the appropriate PCR primers (see Table S2 in the supplemental material). CI was purified and digested with NcoI and Sall endonucleases. The arabinose-inducible plasmid pBAD/Myc-His (see Table S1 in the supplemental material) was digested with the same enzymes, and both products were cloned to create pB24aCI. A construct bearing both CII and CIII was made by PCR using the primers CI_CIII_phusion and CII_SalI (see Table S2 in the supplemental material), digested with NcoI and Sall, and cloned into pBAD/Myc-His to create pB24acIIICII. MC1061 and MC1061(\( \phi 24A \)) competent cells were transformed with each of the plasmid constructs, and transformants were selected on LB agar (LBA) containing ampicillin (100 \( \mu g \cdot ml^{-1} \)).

MC1061 and MC1061(\( \phi 24A \)) cultures bearing pB24acCI or pB24acIIICII were grown at 37°C to an OD \( 600 \) of \(< 0.4\), when expression of the cloned phage genes was stimulated by the addition of arabinose (0.01% [wt/vol]), and the cultures were further incubated for 1 h. Production of CI, CII, and CIII was confirmed by SDS-PAGE and Western blot analyses (data not shown).
Acid resistance assay. Eight independent cultures of MC1061, MC1061(\(\phi\)24B), MC1061/p\(\phi\)24B-cIIcIII, MC1061(\(\phi\)24B)/p\(\phi\)24B-cIIcIII, and MC1061(\(\phi\)24B)/p\(\phi\)24B-cIIcIII were grown at 37°C with shaking to an OD\(_{600}\) of 0.4. The expression of CI, CII, and CIII was induced with arabinose as described above. After 1 h of incubation with arabinose, each culture was diluted 1:10 in fresh LB at pH 2.5 and pH 7.5 and incubated again at 37°C for 2 h. Aliquots of 50 μl of each culture were harvested in triplicate before and after incubation at pH 2.5 and pH 7.5 and serially diluted, and colony counts on LBA were determined. Survival curves were produced for each biological replicate, and statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test; P values of <0.05 were considered to be statistically significant.

Relative RT-qPCR. RNA samples for reverse transcription-quantitative PCR (RT-qPCR) were prepared, independently, as described above, from three biological replicates of MC1061 and MC1061(\(\phi\)24B) cultures. Three biological replicates of MC1061/p\(\phi\)24B-cIIcIII, MC1061(\(\phi\)24B)/p\(\phi\)24B-cIIcIII, and MC1061(\(\phi\)24B)/p\(\phi\)24B-cIIcIII cultures were also propagated overnight (16 h), subcultured (1:10), and grown until they reached an OD\(_{600}\) of 0.4; arabinose (0.01% [wt/vol]) was added to stimulate protein production; and cultures were grown for a further hour. RNA was treated with Turbo DNase (Ambion, TX, USA) according to the manufacturer’s instructions. The absence of DNA was verified by qPCR, and the amount of RNA was quantified by using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Each RNA sample (1,000 ng) was reverse transcribed by using random hexamers (Bioline, London, United Kingdom) and the cDNA synthesis kit from Bioline (London, United Kingdom).

RT-qPCR was performed by using a StepOnePlus real-time PCR system (Applied Biosystems); each reaction mixture consisted of 100 ng of cDNA, a 1× SensiFAST SYBR Hi-ROX kit (Bioline, London, United Kingdom), and 200 nM specific primers in a 20-μl reaction mix. The amplification cycling conditions were as follows: an initial denaturation step at 95°C for 2 min, followed by 39 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 10 s, and extension at 72°C for 5 s. A melting curve analysis was performed after each amplification reaction, with a temperature gradient of 0.1°C from 55°C to 95°C. No-template controls were included in every experiment. The 2\(^{-\Delta\Delta Ct}\) method (35) was used to calculate relative gene expression levels by using the \(\Delta\Delta CT\) method (36) for the PCR amplicons of each target gene that had been cloned into the PCR-Blunt vector (Invitrogen, Paisley, United Kingdom) and linearized with NcoI (NEB, Herts, United Kingdom) and the cDNA synthesis kit from Bioline (London, United Kingdom). Technical replicates were run for every sample, and means were calculated. Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA) using an ANOVA followed by Tukey’s post hoc test. P values of <0.05 were considered to be statistically significant.

Nucleotide sequence accession numbers. The genome sequences of MC1061(\(\phi\)24B) along with the four transcript libraries were deposited in the European Nucleotide Archive, and the accession numbers can be found under BioProject record number PRJEB9491.
There is always a basal level of expression of bacteriophage genes associated with the lytic phage replication cycle in any lysogen culture due to some proportion of that culture undergoing spontaneous induction (27). In order to establish whether a phage gene is truly expressed during lysogeny or as part of the lytic cycle, it is necessary to examine the expression of the gene upon induction. Here the expression levels of 68 of the 93 \( \phi 24_B \) genes increased >20-fold upon prophage induction into the lytic cycle, although for 17 of these genes, DESeq analysis did not classify the upregulation as significant (see Table S4 in the supplemental material). The expression levels of 14 genes were unaltered, and these genes include \( cI \), responsible for the maintenance of the lysogenic state; \( P \), the phage integrase which has been shown to be uncoupled from the phage regulatory network in \( \phi 24_B \) (37); \( bor \), characteristic in bacteriophage lambda (38); \( stk \), a serine-threonine kinase; and 10 genes of unknown function (see Table S4 in the supplemental material), 2 of which, \( vb_24B/13c \) and \( res \), were previously shown to be expressed in \( E. coli \) \( \phi 24_B \) lysogens (27).

Four phage genes \( vb_24B/19c, vb_24B/25, vb_24B/28 \), and \( vb_24B/30 \) were selected to validate the RNA-Seq data. The regulatory genes \( cl \) and \( cro \) were included as controls, and relative RT-qPCR was performed as described in Materials and Methods. These data are presented in Fig. 1A, and in accordance with data from the transcriptomic analysis, expression levels of \( cl \), \( vb_24B/19c \), \( vb_24B/28 \), and \( vb_24B/30 \) did not change significantly, while the expression level of \( vb_24B/25 \) showed a significant 10-fold increase upon induction \( P \) value of <0.05. The latter expression level is far short of the increase of >60-fold \( P \) value of <0.005 for \( cro \), the control for gene expression linked to bacteriophage induction. This RT-qPCR-derived value for \( cro \) is in agreement with the 45-fold increase observed in the RNA-Seq data (see Table S4 in the supplemental material).

**Gene expression changes associated with Stx prophage carriage.** Comparison of the MC1061 and MC1061\((\phi 24_B)\) transcriptomes revealed that the majority of bacterial expression changes detected are likely to be simply a consequence of the SOS response occurring in the MC1061\((\phi 24_B)\) sample (see Table S3 in the supplemental material). In most cases, the decreased gene expression in the lysogen can be ascribed to spontaneous bacteriophage induction. However, there were several genes that appear to be truly downregulated in the lysogen, i.e., due to phage carriage. \( mgo \) encodes a malate:quinone oxidoreductase that was downregulated 5-fold in the lysogen sample but showed slightly increased expression levels in both lysogen and naive samples treated with norfloxacin (Table 2). Similarly, the expression levels of \( aceEF \) were significantly decreased (Table 2); \( aceEF \) encode two of the subunits that form pyruvate dehydrogenase, the enzyme that provides acetyl coenzyme A (acetyl-CoA) for the tricarboxylic acid cycle (39, 40). The expression level of the gene encoding the third subunit of pyruvate dehydrogenase, \( aceK \), was reduced 1.7-fold. The expression level of the \( cyoABCD \) operon was also found to be reduced in the lysogen; these genes encode the subunits of the cytochrome \( b \) oxidase, one of the three major terminal oxidases in the aerobic respiratory chain of \( E. coli \). These observations based on RNA-Seq analysis of pooled samples require independent verification, and this is supported by the RT-qPCR data for \( aceK \), \( mgo \), and \( cyoA \) (see Fig. S2 in the supplemental material), which support the RNA-Seq observations.

Among the lysogen-upregulated genes were operons encoding type I fimbriae and the GAD acid stress island (Table 2). The \( fim \) operon comprises 9 genes, 7 of which \( (fimACDEFGI) \) showed significantly increased expression in the lysogen. The genes comprising the GAD acid stress island control the glutamate-dependent acid resistance mechanism in \( E. coli \) (41–43). The \( gadAB \) genes encode two glutamate decarboxylases that, together with \( gadC \), comprise the structural genes of the GAD operon. Expression of the \( gadABC \) genes is under the control of the global regulator \( gadE \) and two additional regulators, \( gadX \) and \( gadW \). All of these genes were significantly upregulated in the lysogen (Table 2).

**Increased acid resistance of MC1061\((\phi 24_B)\).** Among the data in Table 2, the 3- to 5-fold-increased expression levels of components of the GAD system, which controls acid resistance in \( E. coli \), are particularly striking. Acid resistance is a well-studied feature of the biology of \( E. coli \) O157 (43), and the low infective dose characteristic of this pathogenic serotype is thought to be related to its ability to survive passage through the stomach. To assess whether \( \phi 24_B \) imparts increased acid resistance to the lysogen via upregulation of the GAD system, both MC1061 and MC1061\((\phi 24_B)\) were incubated at \( pH \) 2.5 for a period of 2 h at \( 37^\circ C \), and survival was determined (Fig. 2). The rate of survival of the lysogen MC1061\((\phi 24_B)\) under acidic conditions was reproducibly ~3-fold higher than that of the naive strain, MC1061 \( (P \) value of <0.05). In order to determine if phage regulator proteins that control phage gene transcription (\( CI \), \( CII \), and \( CIII \)) were involved in activating the GAD system, the genes for these proteins were cloned into MC1061, which was then subjected to a 2-h \( pH \) 2.5 challenge after induction of gene expression. The cloning vector was an arabinose-inducible expression plasmid modified to harbor either the \( cl \) gene or both the \( clI \) and \( clII \) genes, \( pb24B-cl \) or \( pb24B-clCIII \), respectively. \( CI \) is the transcriptional repressor that maintains lysogeny (44). CI is a transcriptional regulator crucial to the lysis-lysogeny decision during the early stage of the phage life cycle, and it is rapidly degraded by the bacterial FtsH protease (44–46). The CIII protein acts by inhibiting FtsH, indirectly protecting CI from degradation (45).

MC1061 harboring \( pb24B-cl \) possessed the same acid survival phenotype as that of naive cells (Fig. 2). In contrast, the MC1061 clone expressing both CI and CIII was more acid resistant than the lysogen (Fig. 2). In order to determine the impact of CII-CIII expression on \( gadE \), \( gadX \), and \( gadC \), the regulators of the acid resistance GAD system in \( E. coli \), RT-qPCR data were generated by using MC1061 and MC1061\((\phi 24_B)\) mid-exponential-phase cultures. The relative expression levels of \( gadE \), \( gadX \), and \( gadC \) were calculated by using the naive MC1061 sample as the calibrator (Fig. 1B). The \( gadX \) expression level was significantly higher in the MC1061\((\phi 24_B)\) samples than in the MC1061 samples \( (P \) value of <0.05), but this gene exhibited a very similar relative expression profile in all other constructs and strains (Fig. 1B). The expression of \( gadE \) was also increased in MC1061\((\phi 24_B)\) samples with respect to MC1061; however, there was no significant effect on \( gadE \) expression due to the presence of \( CI \) or \( CII-CIII \) in MC1061. There was a reduction in \( gadE \) expression in MC1061\((\phi 24_B)\)/\( pb24B-cl \) and an increase in expression in MC1061\((\phi 24_B)\)/\( pb24B-clCIII \) compared to MC1061\((\phi 24_B)\) (Fig. 1B). Interestingly, the most pronounced effect was observed for \( gadC \) expression. There was a very significant difference between the MC1061\((\phi 24_B)\) and MC1061 samples \( (P \) value of <0.01), but expression was significantly reduced by the production of \( CI \) in MC1061\((\phi 24_B)\), while the production of \( CII-CIII \) maintained high levels of \( gadC \) expression (Fig. 1B). GadC is one of the proteins directly involved in acid
resistance (membrane transporter of glutamate) rather than functioning as a regulator.

In bacteriophage lambda, CII binds to a well-defined sequence, a tetranucleotide repeat (TTGCCN_TTGC) flanking the −35 region of the promoters for P_RE, P_r, and P_Q, controlling the ability of lambda to establish CI production and to express integrase and the anti-Q transcript, respectively (47–49). In φ24B, there is a single nucleotide change in this repeat in the same locations, but the GAD operon had no matches to either of the CII binding sequences, suggesting that the substantial effect of CII on acid resistance is pleiotropic.

The confounding issue with CII increasing acid resistance is that CI (repressor) should turn off all CII expression in the lysogen. We determined the levels of the cII transcript in MC1061(φ24B) cultures by RT-qPCR and demonstrated that measurable numbers of transcripts were present, corroborating our RNA-Seq data (Fig. 1C). We then measured the levels of the cII transcript in MC1061(φ24B)/φφ24N-cI cultures grown in the presence of 0.01% arabinose by RT-qPCR and demonstrated a 3-fold decrease in cII transcript levels (Fig. 1C). These data, combined with the observations that the CI expression level is very low (Fig. 1A) in the lysogen and that it is known that the operator binding sites in φ24B and related phages like 933W are missing or reside in the cl open reading frame (50, 51), support our contention that CII is present and active in the lysogen. Furthermore, we were able to reestablish acid sensitivity in the φ24B lysogen by overexpressing cl (Fig. 2); overexpression of cl has also been shown to abolish CII downregulation of a type III secretion system (52). We made a φ24B lysogen of strain TUV93-0 (EDL933 with both Shiga-toxigenic phages removed) and compared the acid resistances of the naive and lysogen strains. We were able to replicate our observations with MC1061; i.e., the levels of acid resistance were 4-fold higher in the lysogen and >4-fold higher in a TUV93 lysogen (data not shown).

DISCUSSION

Sequencing of the transcriptomes of E. coli MC1061 and MC1061(φ24B) is an invaluable general tool to discern the impact that a bacteriophage can have on its host transcriptome as well as to determine which bacteriophage genes are expressed during the lysogenic state.

Shiga toxin-encoding lambdoid phages were discovered in 1983 (52), and while their contribution to the pathogenic profile of Shiga-toxigenic E. coli has been well documented, their impact on the biology of the E. coli host has not been fully characterized. A microarray-based comparison of gene expression levels in a naive strain and a strain harboring a Shiga-toxigenic E. coli lysogen (EDL933W) (25). We made a φ24B lysogen of strain TUV93-0 (EDL933 with both Shiga-toxigenic phages removed) and compared the acid resistances of the naive and lysogen strains. We were able to replicate our observations with MC1061; i.e., the levels of acid resistance were 4-fold higher in the lysogen and >4-fold higher in a TUV93 lysogen (data not shown).
and anaerobic respiration (see Table S3 in the supplemental material) that are in this category, although further analysis is required to confirm their role in the SOS response.

Bacteriophage lambda carries lom and bor, two genes that are expressed in E. coli and enhance the lysogen’s adhesion to epithelial cells and resistance to immune cell attack (38, 53). These genes are also carried by ph24a, and expression by the lysogen was previously described as being constitutive and uncoupled from the phage regulatory network (27). The RNA-Seq data here fully support that conclusion. The phenomenon represented by prophage-controlled acid resistance, i.e., the control of host cell genes by phage regulators resulting in the alteration of a fitness/virulence trait, was described previously (24), but the mechanism by which the prophage effects this change is addressed here. Our data (from RNA-Seq, RT-qPCR, and acid resistance assays) have demonstrated that the phage-encoded CII transcriptional activator controls the acid response in the lysogen. There are four distinct acid resistance mechanisms described for E. coli (54, 55), and the glutamate-dependent acid resistance system is the most efficient of the four. GAD has long been recognized as a virulence factor in EHEC that contributes to its low infective dose by helping it survive exposure to pHs as low as 1.5 (56, 57).

CII from 933W has also been assigned a role in controlling, specifically decreasing, the expression of a type III secretion system, another virulence factor of EHEC (25), while prophages that encode regulators that upregulate type III secretion have also been found (58). The CII protein of ph24a shares significant homology with the CII protein of 933W. However, these two proteins differ at their carboxyl termini (Fig. 2).

![FIG 2 Survival of MC1061, MC1061/pH927824-cl, MC1061/pH927824-clCIII, MC1061(d24cl), MC1061(d24cl)pH927824-cl, and MC1061(d24cl)pH927824-clCIII after incubation in LB at pH 2.5. Error bars represent standard errors of the means (n = 8). * represents statistically significant values compared to MC1061, as determined by one-way ANOVA with a post hoc Tukey test (P value of <0.05).](http://aem.asm.org/)

**TABLE 2** Bacterial genes differentially expressed between MC1061 and MC1061(ϕ24a) before norfloxacin treatment

| Gene       | Function                                      | Fold change | P value |
|------------|-----------------------------------------------|-------------|---------|
| **Genes downregulated in** MC1061(ϕ24a) |                  |             |         |
| cyoB       | Cytochrome O ubiquinol oxidase               | 3.41        | 0.001   |
| cyoA       | Cytochrome O ubiquinol oxidase               | 3.78        | 0.000   |
| cyoC       | Cytochrome O ubiquinol oxidase               | 3.12        | 0.001   |
| cyoD       | Cytochrome O ubiquinol oxidase               | 3.12        | 0.002   |
| cypE       | Heme O synthase, protoheme IX farnesyltransferase | 2.1         | 0.022   |
| aceE       | Pyruvate dehydrogenase E1 component         | 3.24        | 0.001   |
| aceF       | Pyruvate dehydrogenase E2 component         | 2.40        | 0.011   |
| **Genes upregulated in** MC1061(ϕ24a) |                  |             |         |
| hdeA       | Chaperone HdeA                               | 3.004       | 0.038   |
| hdeD       | Membrane transporter, H-NS repressed         | 3.127       | 0.044   |
| rpoS       | Sigma transcription factor controlling a regulon of genes required for protection against external stresses | 3.427       | 0.021   |
| gadA       | Glutamate decarboxylase                      | 3.558       | 0.043   |
| gadB       | Glutamate decarboxylase                      | 3.133       | 0.030   |
| gadC       | Probable glutamate/gamma-aminobutyrate antiporter | 3.414       | 0.021   |
| gadE       | Transcriptional activator                    | 5.989       | 0.032   |
| gadW       | HTH-type transcriptional regulator           | 5.196       | 0.000   |
| gadX       | HTH-type transcriptional regulator           | 3.115       | 0.004   |
| fimF       | Minor component of type 1 fimbriae           | 6.543       | 0.028   |
| fimA       | Major subunit of type 1 subunit fimbriae     | 19.451      | 0.000   |
| fimC       | Required for biogenesis of type 1 fimbriae   | 17.396      | 0.000   |
| fimD       | Type 1 fimbria anchoring protein involved in export and assembly of fimA fimbrial subunits across the outer membrane | 11.850      | 0.000   |
| fimE       | Type 1 fimbria regulatory protein            | 21.938      | 0.022   |
| fimG       | Type 1 fimbria adapter subunit               | 9.526       | 0.014   |
| fimI       | Type 1 fimbria protein                       | 20.345      | 0.000   |

*a* Excludes ϕ24a genes that are presented in Table S4 in the supplemental material.

*b* HTH, helix-turn-helix.
CII proteins of φ244r and 933W would be expected to share similar transcriptional regulatory functions without sharing susceptibility to FtsH destruction, while the CII protein encoded by lambda should not have the same phenotypic effects.

The impact of CII expression and its control of the survival of Stx phage lysogens in transit through the gastric system still need to be addressed through animal experimentation, but our data suggest that CII will directly improve the survival of E. coli in the acidic environment of the stomach. Significantly, we were able to introduce φ244r into an E. coli O157-derived strain and demonstrate increased acid resistance in the lysogen. Our data point to the fact that the Stx phages themselves directly contribute to the induction of various lambdoid prophages responsible for production of CII proteins of λ and 933W would be expected to share similar transcriptional regulatory functions without sharing susceptibility to FtsH destruction, while the CII protein encoded by lambda should not have the same phenotypic effects.

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29. Aziz Ando

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Xu Schwartz

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Naylor

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