

The Entner-Doudoroff Pathway Is Obligatory for Gluconate Utilization and Contributes to the Pathogenicity of *Vibrio cholerae*

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The Entner-Doudoroff (ED) pathway has recently been shown to play an important role in sugar catabolism for many organisms although very little information is available on the functionality of this pathway in *Vibrio cholerae*, the causative agent of cholera. In this study, activation of the genes *edd* and *eda*, encoding 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase, was used as a marker of a functional ED pathway in *V. cholerae*. Transcriptional activation analyses and gene silencing experiments with cells grown in sugar-supplemented M9 medium demonstrated that the ED pathway is functional in *V. cholerae* and is obligatory for gluconate catabolism. Importantly, selective activation of the ED pathway led to concurrent elevation of transcripts of prime virulence genes (*ctxA* and *tcpA*) and their regulator (*toxT*). Further, lowering of these transcript levels and cholera toxin production *in vitro* by an ED pathway-defective mutant (strain N16961 with a Δ *edd* mutation [Δ *edd*_{N16961} strain]) suggested the importance of this pathway in regulating *V. cholerae* virulence. The *in vivo* relevance of these data was established as the mutant failed to colonize in suckling mice intestine or to induce fluid accumulation in ligated rabbit ileal loops. Activation of the ED pathway in *V. cholerae* was shown to inhibit biofilm formation *in vitro* that could be reversed in the mutant. As further support for these results, comparative transcriptome analysis with cells grown in the presence of glucose or gluconate revealed that a functional ED pathway led to activation of a subset of previously reported *in vivo* expressed genes. All of these results suggest the importance of the ED pathway in *V. cholerae* pathogenesis.

Carbohydrates play an important role in the assimilation of energy by living cells and act as carbon sources for the synthesis of important metabolites. The breakdown of sugars through different catabolic pathways provides energy to the bacterial cells in the form of ATP and other reducing equivalents. The best-characterized pathways for sugar catabolism in bacteria are the Embden-Meyerhof-Parnas (EMP), the pentose phosphate (PP), and the Entner-Doudoroff (ED) pathways. These pathways are widespread in prokaryotes and are crucial for their physiology and nutrition. The overall schemes of the ED and EMP pathways are quite similar: 6-carbon sugars are primed by phosphorylation and subsequently cleaved by the aldolase enzyme into two 3-carbon intermediates (27). The two key enzymes distinctive to the ED pathway are (i) 6-phosphogluconate dehydratase (Edd) (EC 4.2.1.12), which catalyzes dehydration of 6-phosphogluconate to form 2-keto-3-deoxy-6-phosphogluconate (KDPG), and (ii) KDPG aldolase (Eda) (EC 4.1.2.14), which cleaves KDPG to pyruvate and glyceraldehyde 3-phosphate, the latter being further catabolized through the EMP pathway and tricarboxylic acid (TCA) cycle (6). The ED pathway was discovered in 1952 in *Pseudomonas saccharophila* (9) and several years later was shown to be present in *Escherichia coli* (8). Initially, the ED pathway was considered to be restricted to Gram-negative bacteria, but current studies indicate that it is widely distributed from *Archaea* to *Eukarya* (6). It is now believed that the ED pathway predates the EMP pathway in the evolution of microbes (30).

The enzymes Edd and Eda of the ED pathway play key roles in gluconate (Gnt) catabolism. Gnt, a sugar acid which is available in the intestinal milieu, can be utilized by various microorganisms as an energy source. The organism *E. coli* utilizes Gnt through the ED pathway (27). This bacterium has high-affinity (GntT) and low-affinity (GntU) Gnt transporters and a thermoresistant gluconokinase enzyme (GntK) (EC 2.7.1.12) involved in phosphorylation of Gnt. Catabolism of Gnt via the ED pathway in the

organism is controlled by the repressor protein GntR. The *edd*, *eda*, *gntT*, *gntU*, and *gntK* genes are crucial for functioning of the ED pathway, and their expression is negatively regulated by *gntR*, whereas Gnt acts as a true inducer (26). Recent studies have suggested that the ED pathway may play an important role in the physiology of *E. coli* bacteria toward their adaptation specific for the intestinal milieu (4). Apart from *E. coli*, the ED pathway may be important in the survival of pathogenic organisms like *Salmonella enterica*, *Neisseria gonorrhoeae*, *Klebsiella pneumoniae*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Pasteurella pestis*, *Xanthomonas campestris*, *Pectobacterium carotovorum*, etc. in their respective host systems (2, 3, 10, 15, 20, 21, 23, 24, 31, 33).

The Gram-negative gammaproteobacterium *Vibrio cholerae*, the etiological agent of the diarrheal disease cholera, has a life cycle that includes its existence in intestinal (in humans) as well as extraintestinal (marine aquatic) environments, preferably by forming biofilms (12). Clearly, the adaptation of the organism in such distinct environments would require appropriate metabolic pathways to be functional and efficient to derive energy from diverse nutrient sources. To date, our knowledge is quite limited in understanding the physiological changes that *V. cholerae* adopts during the transition between the two extreme environments and their pathological consequences. An analysis of genomic data of *V. cholerae* has revealed the presence of a Gnt utilization system in-

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cluding the ED pathway genes with a unique arrangement (29). The present study was undertaken with the following objectives: (i) to determine whether the ED pathway is functional in *V. cholerae*, (ii) to determine whether activation of the ED pathway has any role in the expression of virulence genes *in vitro*, (iii) to ascertain the relevance of a functional ED pathway toward the expression of its pathogenicity *in vivo*, and (iv) to evaluate the role of a functional ED pathway in biofilm formation. The significance of our results was analyzed and discussed using a set of comparative microarray-based gene expression data generated with the organism grown separately in the presence of glucose (Glu) or Gnt as the sole carbon source.

MATERIALS AND METHODS

Bacteriology. Strains of *V. cholerae* O1 classical (O395) and El Tor (N16961) biotypes were included in the study. Studies were carried out with *V. cholerae* strains grown in M9 minimal medium supplemented individually with 0.2% concentrations of different sugars such as Glu, *N*-acetylglucosamine (GlcNAc), Gnt, and ribose and incubated at 37°C for 12 h.

Real-time PCR assay. *V. cholerae* strains were grown in minimal medium supplemented with different sugars. Total RNA was extracted by using the TRIzol method. Reverse transcriptase PCR was performed with 4 µg of total RNA as the template with random hexamers and MultiScribe reverse transcriptase (high-capacity cDNA synthesis reverse transcriptase kit; Applied Biosystems). The cDNA thus synthesized was subsequently used for a SYBR green-based real-time PCR assay performed in an ABI 7900 instrument using gene-specific primers that included genes for metabolic activities, virulence factors, regulatory elements, and certain other genes of interest (see Table S1 in the supplemental material). The transcript levels of *recA* served as an internal control. All of the real-time PCR assays were performed with total RNA (before cDNA synthesis), which showed no amplification, and this was to exclude the possibility of DNA contamination in the total RNA as extracted from the cells.

GM₁ ELISA for CT estimation. The ability of *V. cholerae* strains to express cholera toxin (CT) *in vitro* was assayed by GM₁ enzyme-linked immunosorbent assay (ELISA) using cell-free culture supernatants of cells grown for 12 h (14). For each set of experiments, a known amount of purified CT was used to generate a standard curve showing the interrelationship between the optical density at 492 nm (OD₄₉₂) and the amount of CT. The OD₄₉₂ average obtained from triplicate wells of the experimental sets was considered to estimate the amount of CT present in the culture supernatant using the standard curve (7).

Development of plasmid constructs for antisense-mediated inhibition. An antisense strategy was utilized to inhibit functional activity of genes encoding 6-phosphofructokinase (*pfk*), 6-phosphogluconate dehydratase (*edd*), and 6-phosphogluconate dehydrogenase (*gnd*), key enzymes of the EMP pathway, ED pathway, and PP pathway, respectively. PCR amplicons specific for *pfk*, *edd*, and *gnd* were initially cloned into the vector pCR2.1. Cloned *pfk*, *edd*, and *gnd* were subsequently subcloned into the EcoRI site of a modified pBAD vector to generate plasmid constructs pTPApfk, pTPAgnd, and pTPAedd, respectively. Partial deletion was introduced into the catabolite activator protein (CAP) binding site of pBAD using a QuikChange II site-directed mutagenesis kit (Stratagene) to generate a modified pBAD, which was used for generating antisense constructs. The genes *pfk*, *gnd*, and *edd* present in pTPApfk, pTPAgnd, and pTPAedd, respectively, existed in reverse orientation relative to the arabinose-inducible promoter. These constructs were electroporated into the *V. cholerae* N16961 strain, and the effect of conditional as well as specific gene silencing was evaluated using minimal medium supplemented with or without the inducer arabinose. The enzyme encoded by *edd* converts 6-phosphogluconate to KDPG, which is subsequently converted to pyruvate and glyceraldehyde 3-phosphate by the action of the KDPG aldolase encoded by *eda*. Deactivation of *eda* will therefore lead to accumulation of

KDPG within the cells. It has been reported earlier that accumulated KDPG at higher concentrations resulted in bacteriostasis (11). Thus, we selected *edd* but not *eda* for antisense suppression assays.

Generation of an in-frame deletion mutant of *edd*. Construction of a Δ *edd* *V. cholerae* mutant was generated by an allelic exchange procedure. For this, an internal deletion was introduced in the *edd* gene by a PCR fragment-joining method, and the resulting amplicon was cloned into SacI sites of a suicide vector, pDS132 (chloramphenicol resistant [Cm^r]) (28), to generate the suicidal construct pTPedd, which can replicate only in the presence of *pir* protein. The suicidal construct pTPedd was mobilized into recipient *V. cholerae* strains by conjugal transfer, and the transconjugants were selected on plates containing 7 µg/ml chloramphenicol and 100 U/ml polymyxin B. Selection of these transconjugants on the Cm-containing plates caused integration of pTPedd into the *V. cholerae* genome by homologous recombination in the *edd* locus. These integrants were subsequently passaged on plates containing 10% sucrose to generate an in-frame deletion mutant of *edd* (due to removal of the vector backbone along with a functional copy of *edd*). Successful removal of the integrated vector backbone led to Cm sensitivity in the Δ *edd* mutant of strain N16961 (Δ *edd*_{N16961} strain). In-frame deletion of *edd* was confirmed by nucleotide sequencing. Deactivation of *edd* resulted in a non-functional ED pathway in this mutant. The Δ *edd*_{N16961} mutant was further modified by electroporation with the antisense plasmid construct pTPApfk to create a double mutant with a defective ED pathway and EMP pathway under arabinose-inducible conditions.

Animal experimentations. The potential of the *V. cholerae* strains to colonize the intestine was evaluated using the suckling mouse model. Following 18 h of challenge, animals were sacrificed, and their intestines were removed, washed, and homogenized in normal saline. The challenge dose and the intestinal homogenates were serially diluted and plated on Luria agar plates to determine the number of CFU. The colonization potential was determined as the ratio of the number of CFU recovered to the number of CFU used for oral challenge (5). The overall virulence potential of *V. cholerae* strains was also determined by a rabbit ileal loop (RIL) assay. After 18 h of challenge, rabbits were killed, and the fluid accumulation (FA) ratio was determined by measuring the amount of fluid accumulated per unit length of the loop. Reduction of the FA ratio served as an index of the attenuation of the virulence phenotype.

Biofilm assay. The biofilm-forming capacities of the *V. cholerae* strains were determined by crystal violet staining after 18 h of incubating the culture in borosilicate glass tubes at 37°C under static conditions (5) in minimal medium supplemented with either Glu, Gnt, or GlcNAc. Absorbed dye in the attached cells was solubilized in dimethyl sulfoxide (DMSO) and the OD₅₇₀ was measured spectrophotometrically (18).

Gene expression profiling. The *V. cholerae* O1 El Tor strain N16961 was grown in minimal medium supplemented with either 0.2% Glu or 0.2% Gnt for 12 h, and total RNA was isolated using TRIzol reagent (Invitrogen), treated with RNase-free DNase I (Ambion), and finally purified with the use of an RNeasy kit (Qiagen). The microarray experiment was performed by Genotypic Technology, India. Polyadenylation of the bacterial RNA samples using Epicentre's A-plus Poly(A) polymerase tailing kit was followed by T7 oligo(dT)-based cDNA synthesis and subsequent amplification and labeling by *in vitro* transcription using Cy3-CTP, Agilent's low-input linear amplification kit. The normalization of the signals generated after hybridization was done using GeneSpring GX, version 11.0, software, and final data were obtained after normalization using the signals from cells grown Glu-supplemented medium.

Statistical analysis. Comparative real-time PCR analysis of the transcript levels of various genes was analyzed by the INSTAT2 program, and *P* values were calculated using a paired *t* test.

RESULTS

Catabolic pathway preferences for the utilization of Gnt and GlcNAc by *V. cholerae*. Relative transcript levels of *pfk*, *gnd*, *eda*, and *edd* were evaluated by real-time PCR assays using cells grown

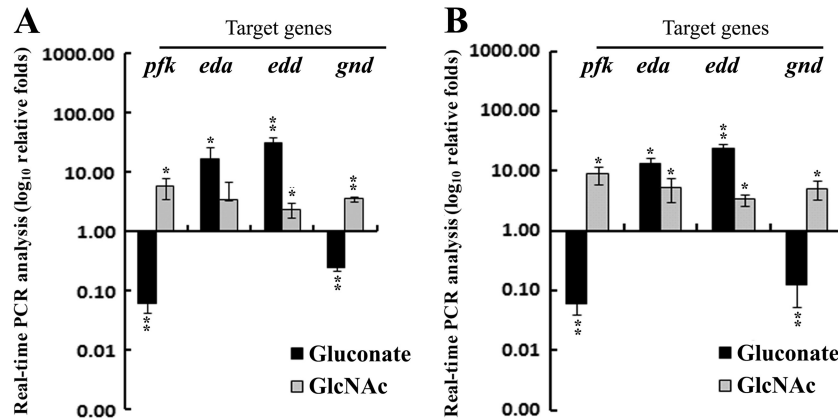


FIG 1 Relative transcript levels were measured by real-time PCR assay in *V. cholerae* strains of El Tor (N16961) (A) and classical (O395) (B) biotypes grown separately in M9 minimal medium supplemented with a 0.2% concentration of either glucose (Glu) or gluconate (Gnt) or *N*-acetylglucosamine (GlcNAc) as a sole carbon source. Normalization of the transcript levels was made against cells grown with Glu as a reference. Error bars show standard deviations for triplicate samples. *pfk*, 6-phosphofructokinase; *eda*, 2-keto-3-deoxy-6-phosphogluconate aldolase; *edd*, 6-phosphogluconate dehydratase; *gnd*, 6-phosphogluconate dehydrogenase. *, $0.005 > P < 0.05$; **, $P \leq 0.005$. Activation of *pfk*, *eda* and *edd*, and *gnd* has been considered an index for functional activity of the EMP, the ED, and the PP pathways, respectively.

in M9 minimal medium supplemented individually with either Glu or Gnt or GlcNAc, which served as an indicator for involvement of the EMP, PP, or ED pathway, respectively. The real-time PCR results were normalized to the transcript level of *recA*, and the respective transcript levels in cells grown in the M9 medium with Glu supplementation (M9-Glu) served as a reference (Fig. 1). Data generated with strains belonging to El Tor (N16961) and classical (O395) biotypes showed elevated levels of both the *eda* and *edd* marker genes for the ED pathway in cells grown in M9 medium with Gnt supplementation (M9-Gnt) and lower transcript levels of the *pfk* and *gnd* genes, markers for the EMP and PP pathways, respectively, compared to cells grown in M9-Glu medium. Supplementation in M9 medium with GlcNAc (M9-GlcNAc) caused elevation of *pfk*, *gnd*, *eda*, and *edd* transcript levels compared to cells grown in M9-Glu medium.

Antisense-mediated suppression of pathway-specific marker genes and its effect on *V. cholerae* grown in sugar-supplemented medium *in vitro*. Antisense-mediated suppression of pathway-specific marker genes (*pfk*, *edd*, and *gnd*) was performed by the introduction of the appropriate construct (pTPApfk, pTAAedd, and pTPAgnd) into *V. cholerae* El Tor strain N16961. Expression of antisense RNA was controlled by an arabinose-inducible promoter for all of these constructs. As a negative control, *V. cholerae* N16961 carrying only a modified pBAD vector was also included in this assay. Figure 2A shows that vector alone did not confer any effect on the growth characteristics of a *V. cholerae* strain grown in minimal medium supplemented with different sugars and in the presence or absence of the inducer arabinose. *V. cholerae* strains carrying an antisense construct were allowed to grow in the M9 minimal medium with or without the inducer arabinose to evaluate functional involvement of different pathways, if any. A *V. cholerae* strain carrying pTPAedd, antisense to *edd*, failed to grow in M9-Gnt medium in the presence of the inducer arabinose (Fig. 2C, tube 4) (final OD₆₀₀ of 0.01 from an OD₆₀₀ of 0.01 due to the addition of inoculum). Arabinose-induced antisense pTPAedd caused no growth in M9-Gnt medium but failed to show any growth-inhibitory effect in either M9-Glu or M9-GlcNAc medium. Similarly, a strain carrying pTPApfk, antisense to *pfk*,

showed no growth in M9-Glu medium with the inducer arabinose (Fig. 2D, tube 2) (final OD₆₀₀ of 0.02 from an OD₆₀₀ of 0.01). Arabinose-induced pTPApfk-mediated growth inhibition was observable in M9-GlcNAc medium (Fig. 2D, tube 6) (final OD₆₀₀ of 0.27 from an OD₆₀₀ of 0.02), but no such effect was noted in medium supplemented with Gnt. The construct pTPAgnd failed to show any growth-inhibitory effect in minimal medium supplemented with Glu or Gnt or GlcNAc, even in the presence of the inducer arabinose (Fig. 2B). The above findings established involvement of the ED and EMP pathways in *V. cholerae* for Gnt and Glu utilization, respectively. Assessment of the involvement of the PP pathway was further addressed through growth experiments using a Δedd_{N16961} mutant carrying pTPApfk (a double mutant due to a defective ED pathway and an arabinose-inducible defective EMP pathway) in M9 medium supplemented individually with all of the above sugars as well as with ribose, a pentose sugar. Results are presented in Fig. 3. As expected no growth was observed in M9 medium supplemented with Gnt (Fig. 3A, tubes 3 and 4). A conditional double mutant showed good growth in M9 medium supplemented with either Glu or GlcNAc or ribose in the absence of the arabinose inducer (Fig. 3A, tubes 1, 5, and 7). Induction of *pfk* antisense caused no growth in M9 medium supplemented with Glu (Fig. 3A, tube 2) (final OD₆₀₀ of 0.01 from an OD₆₀₀ of 0.01). The observed absence of growth in either Gnt-supplemented medium or under arabinose-induced conditions for Glu-supplemented medium confirmed nonfunctional ED and EMP pathways under arabinose-induced conditions. This mutant grew very well in M9-GlcNAc medium (Fig. 3A, tube 5) but showed growth inhibition under arabinose-inducible conditions (Fig. 3A, tube 6) (final OD₆₀₀ of 0.12 from an OD₆₀₀ of 0.02). These data indicated that a conditional double mutant can utilize GlcNAc through the PP pathway with growth suppression. Studies with ribose as a supplement in M9 medium further confirmed that the PP pathway is operative in *V. cholerae* as the conditional double mutant showed good growth in M9-ribose medium with or without the inducer arabinose (Fig. 3A, tubes 7 and 8).

Expression of virulence factors in *V. cholerae* cells grown under different culture conditions. Real-time PCR-based analy-

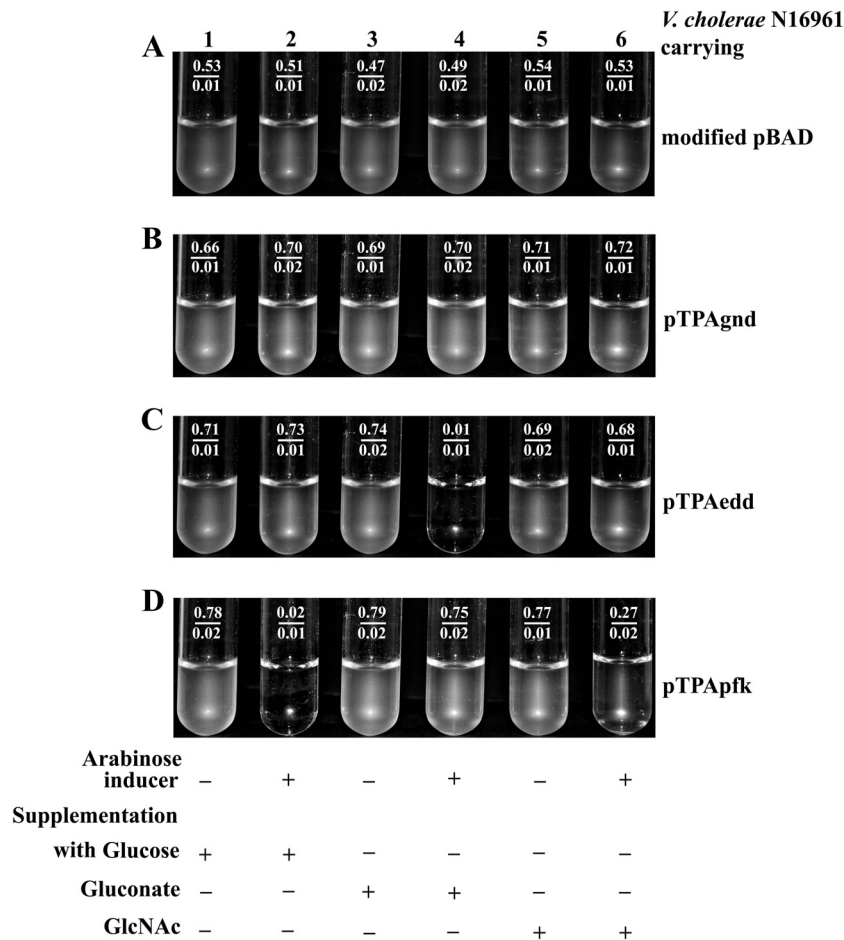


FIG 2 Growth characteristics in medium supplemented with Glu (tubes 1 and 2), Gnt (tubes 3 and 4), and GlcNac (tubes 5 and 6) for *V. cholerae* strain N16961 carrying a modified pBAD vector or carrying the antisense construct pTPAgnd, pTPAedd, or pTPApfk to suppress *gnd*, *edd*, or *pfk*, respectively, under arabinose-inducible conditions (even-numbered tubes). Growth of *V. cholerae* is represented by OD₆₀₀ values, as shown in the numerator in each of the tubes. OD₆₀₀ values in the denominator represent baseline values due to the addition of inoculum. The presence (+) and absence (-) of either inducer or sugar supplements are indicated.

sis was made on relative expression of transcripts of *ctxA*, *tcpA*, and their regulatory element *toxT* in *V. cholerae* strains grown for 12 h at 37°C in M9 medium supplemented with either Glu or Gnt or GlcNac. Elevation in the transcripts of the virulence genes (*ctxA* and *tcpA*) and of their regulator *toxT* was evident in cells grown in M9-Gnt and M9-GlcNac media compared to cells grown in M9-Glu medium (Fig. 4). Similar data were also generated with *V. cholerae* cells grown in Luria broth (LB), pH 6.5, at 30°C or in AKI medium (0.5% NaCl, 0.4% yeast extract, 1.5% Bacto peptone, and 0.3% NaHCO₃) at 37°C for a comparison. As expected, elevation of *ctxA* transcripts was observed in AKI medium but not in LB for El Tor strain N16961 (Fig. 4A) while classical strain O395 showed elevation of *ctxA* transcripts in LB but not in AKI medium (Fig. 4B). The CT production abilities of the *V. cholerae* strains were evaluated in cell-free culture supernatants by growing the cells for 12 h under various culture conditions. The El Tor biotype strain N16961 produced more CT in AKI medium at 37°C than in LB, pH 6.5, at 30°C, and this correlated well with relative transcript levels of *ctxA* under the respective culture conditions (Table 1 and Fig. 4A). The classical biotype strain O395 produced the largest amount of CT with growth in LB (pH 6.5) at

30°C, a condition which also produced a significant increase in *ctxA* transcripts (Table 1 and Fig. 4B). Strains of both of the biotypes produced a good amount of CT in M9 medium supplemented with Gnt but not with Glu or GlcNac (Table 1). However, detection of comparative levels of *ctxA* transcripts was evident when cells were grown in M9 medium supplemented with either Gnt or GlcNac (Fig. 4). Apart from the effect of medium and the supplements, the major reason for discrepancies between *ctxA* transcript levels and CT estimation may be due to certain inherent basic differences between these two assays. The real-time PCR-based detection of *ctxA* transcripts reflected relative amounts of the transcripts present at the 12-h growth time point as mRNAs are known to be very unstable in nature. On the other hand, cell-free CT estimation in the spent medium reflected the cumulative effect of CT production over a 12-h growth period.

Effect of *edd* deletion (defective ED pathway) on *V. cholerae* pathogenesis. Genetic manipulation studies were carried out to generate an in-frame *edd* deletion (ED pathway-defective) mutant of *V. cholerae* El Tor biotype strain N16961. An in-frame *edd* deletion mutant (Δedd_{N16961} strain) failed to grow in M9-Gnt medium but grew well in M9-Glu and M9-GlcNac media. This evi-

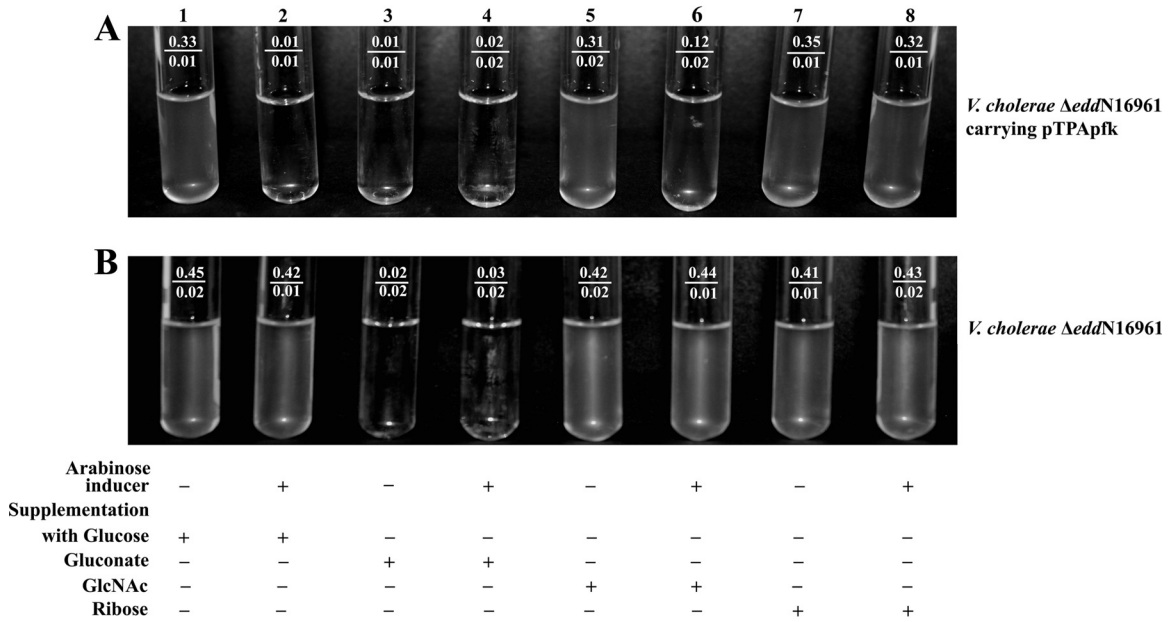


FIG 3 Growth characteristics in medium supplemented with Glu (tubes 1 and 2), Gnt (tubes 3 and 4), GlcNAc (tubes 5 and 6), and ribose (tubes 7 and 8) for an isogenic in-frame *edd* deletion mutant of N16961 (Δedd_{N16961}) carrying pTPApfk (A) and the Δedd_{N16961} strain (B). Antisense construct pTPApfk suppresses *pfk* under arabinose-inducible conditions (even-numbered tubes). Growth of *V. cholerae* is represented by OD₆₀₀ values shown in the numerator in each of the tubes. OD₆₀₀ values in denominator represent baseline values due to the addition of inoculum. The presence (+) and absence (-) of either inducer or sugar supplements are indicated.

dence confirmed the obligatory involvement of the ED pathway in Gnt utilization by *V. cholerae*. Transcriptional differences of the virulence genes such as *ctxA*, *tcpA*, and their regulatory element *toxT* were estimated between the wild-type and the mutant strains grown in AKI medium at 37°C. Significantly decreased levels of the transcripts for *ctxA*, *tcpA*, and *toxT* were evident in the Δedd mutant strain compared to wild-type levels (Fig. 5). These data corroborated the observed decrease in CT production by the Δedd_{N16961} strain compared to the wild type (Table 2). The *in vivo* experiments with the Δedd_{N16961} mutant showed severe attenuation of fluid accumulation in the ligated RIL compared to its isogenic wild-type strain (Table 2). Animal experiments also showed that the mutant failed to colonize in the suckling mouse intestine.

Role of the ED pathway in biofilm formation.

The culture condition-dependent biofilm-forming ability of *V. cholerae* strains was assayed by growing the cells in minimal medium supplemented with either Glu or Gnt or GlcNAc at 37°C under static conditions. The dye retention capacity of the adhered cells served as an index for the extent of biofilm formation, which was measured in DMSO-solubilized cells at 570 nm. OD₅₇₀ values obtained under different culture conditions are presented in Fig. 6. *V. cholerae* strains produced biofilms, but the extent of biofilm formation varied with the culture conditions, as evident by the various OD₅₇₀ values. The OD₅₇₀ for El Tor biotype strain N16961 was higher than that of the classical biotype strain O395 (Fig. 6), which is in accordance with the reported high biofilm formation ability of El Tor strains compared to classical strains. Biofilms formed by the strains grown in M9-Gnt and M9-GlcNAc supple-

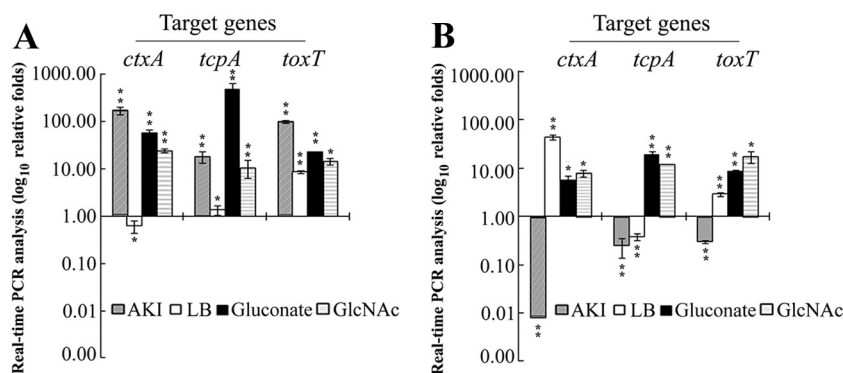


FIG 4 Relative transcripts levels of virulence genes were measured by real-time PCR assay in *V. cholerae* strains of El Tor (N16961) (A) and classical (O395) (B) biotypes grown under different culture conditions for 12 h. Media and conditions used are as follows: AKI medium, pH 7.4 at 37°C; LB, pH 6.5 at 30°C; M9 medium supplemented with a 0.2% concentration of either Glu or Gnt or GlcNAc at 37°C. Transcript levels were normalized to those of cells grown in M9-Glu medium. Error bars show standard deviations for triplicate assays. *, 0.005 > *P* < 0.05; **, *P* ≤ 0.005.

TABLE 1 Ability of *V. cholerae* O1 strains of classical (O395) and El Tor (N16961) biotypes to produce CT under various cultural conditions and assayed using cell-free culture supernatant by GM₁ ELISA method

| <i>V. cholerae</i> strain | CT amt (ng ml ⁻¹ OD ₆₀₀ unit ⁻¹) by culture condition ^a | | | | |
|---------------------------|--|--------------------------|---------------------|---------------------|------------------------|
| | LB, pH 6.5, 30°C | AKI medium, pH 7.4, 37°C | M9-Glu medium, 37°C | M9-Gnt medium, 37°C | M9-GlcNAc medium, 37°C |
| O395 | 835 ± 27** | 93 ± 24** | 32 ± 7 | 150 ± 40* | 39 ± 1* |
| N16961 | 22 ± 3* | 569 ± 26** | 13 ± 1 | 163 ± 19** | 26 ± 3** |

^a Values are means ± standard deviations. *, 0.005 > *P* < 0.05; **, *P* < 0.005.

mented media were compared with cells grown in M9-Glu supplemented medium as a reference. Comparative analysis showed that GlcNAc supplementation caused significant induction of biofilm formation in the case of strain N16961 (1.33-fold, *P* < 0.05), while strain O395 showed a 1.18-fold elevation in biofilm formation compared to Glu supplementation. Gnt-supplemented medium, on the other hand, caused a significant (*P* < 0.05) reduction of biofilm formation (1.67-fold and 2.25-fold lower for N16961 and O395, respectively). Deactivation of the ED pathway by the deletion of *edd* resulted in an increased ability of the mutant to produce biofilm (Table 2) (*P* ≤ 0.005), which substantiated the negative impact of a functional ED pathway on biofilm formation by *V. cholerae*.

Comparative transcriptome profiles of *V. cholerae* cells grown in M9-Gnt versus M9-Glu medium. Genome-wide transcriptional profiles of *V. cholerae* N16961 were determined by growing the cells in M9-Gnt and M9-Glu media. Data generated through microarray-based hybridization analysis revealed that 2,284 genes were differentially regulated while the transcript levels of 1,625 genes remained unchanged. Among the differentially regulated genes, 1,285 and 999 genes were upregulated and downregulated, respectively, in cells grown in M9-Gnt medium compared to growth in M9-Glu medium. A comparative analysis of selected genes is presented in Table S2 in the supplemental mate-

rial along with expression profiles adopted from the Gene Expression Omnibus (GEO) database (accession number GSE24405-07) showing *V. cholerae* comparative *in vivo* expression profiles. A fold change (increase or decrease) of 0.6 or more (log₂ expression ratio) were considered significant. Among 115 selected genes, 69 and 18 genes showed significant upregulation and downregulation, respectively (see Table S2). Real-time PCR assays were also carried out on a subset of 42 genes for validation, which showed that the trend as detected in the microarray experiments remains valid in real-time PCR assays (see Table S2).

Cells grown in M9-Gnt medium showed higher expression levels of the genes involved in Gnt utilization, including *gntP*, *gntK*, *edd*, *eda*, and *gntR*. However, the genes involved in the PP pathway, such as *tkl*, *tka*, *gnd*, and *zwf*, and the genes *pgi* and *pfk* involved in the upper part of the EMP pathway were downregulated. Remarkably, the transcription levels of genes like *glgA* and *glgX* and debranching enzymes participating in glycogen synthesis for energy storage were also upregulated in cells grown in M9-Gnt medium (see Table S2 in the supplemental material). These trends were further validated through real-time PCR assays carried out using primers specific to pathway-specific key enzymes (see Table S2).

Transcript levels of genes that encode the major toxigenic factors of *V. cholerae* including *ctxB*, *zot*, and *rtxA* were upregulated in M9-Gnt medium compared to growth in M9-Glu medium. There was a clear trend suggesting that genes responsible for intestinal colonization, like *tcpA*, *mshABCD*, *acf*, and *cep*, were strongly expressed. Higher transcription levels of the regulatory elements, such as *tcpPH*, *toxR*, *toxT*, *tcpT*, *tcpS*, *toxS*, and *aphAB*, were evident in cells with a functionally active ED pathway, i.e., in cells grown in M9-Gnt medium compared to those grown in M9-Glu medium. Another virulence factor, *ompT*, was also transcribed well under M9-Gnt growth conditions. In addition to upregulation of the virulence factors and their regulatory elements, elevated transcript levels were also apparent for genes involved in motility, chemotaxis, and acquisition of iron uptake. These include a set of genes predicted to be involved in chemotaxis, including *cheA*, *cheB*, *cheW*, *cheR*, and several other methyl-accepting chemotaxis protein-encoding genes. Further, transcript levels of genes such as *flgA*, *fliA*, and *flaC* participating in bacterial motility were upregulated with Gnt supplementation and not with Glu supplementation. The genes participating in iron acquisition, vibriobactin synthesis, iron(II) transport (*feoAB*), and hemin ABC transporter complex were also upregulated. In addition, other functional genes (*cadA*, *tprG*, *acnA*, *gltB1*, *ppc*, *thiF*, *pqiA*, *sdaC2*, Na⁺ symporter, histidine sensor kinase, etc.) that were reported to be expressed during *in vivo* growth were also active *in vitro* under M9-Gnt growth conditions (see Table S2 in the supplemental material). Microarray data on key virulence genes and meta-

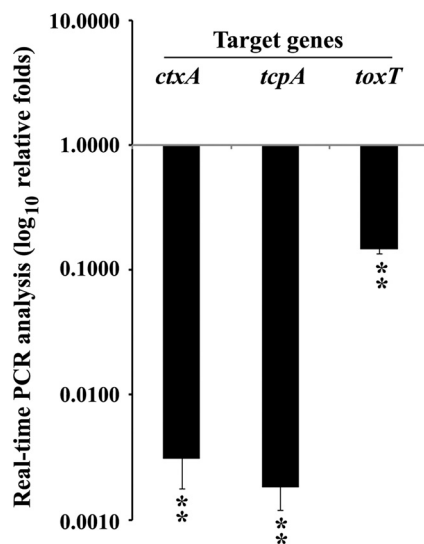


FIG 5 Real-Time PCR-based assay for a comparative analysis of the transcript levels between O1 El Tor wild-type *V. cholerae* strain N16961 and an isogenic in-frame *edd* deletion mutant (Δ *edd*_{N16961} strain) when grown in AKI medium, pH 7.4, at 37°C. Transcript levels were normalized to strain N16961 grown under identical conditions. Error bars show standard deviations for triplicate assays. **, *P* ≤ 0.005.

TABLE 2 Comparison of functional activities between a wild-type (N16961) strain and its isogenic in-frame *edd* deletion mutant (Δedd_{N16961} strain)

| <i>V. cholerae</i> strain | Growth in M9-Gnt medium ^a | Phenotype ^b | | |
|------------------------------|--------------------------------------|---|--|--|
| | | CT amt (ng ml ⁻¹ OD ₆₀₀ unit ⁻¹) ^c | RIL fluid accumulation (ml of fluid cm ⁻¹ of IL) ^d | Biofilm production (OD ₅₇₀) ^e |
| N16961 | + | 569 ± 26 | 1.46 ± 0.12 | 0.51 ± 0.30 |
| Δedd_{N16961} strain | - | 118 ± 37** | 0.07 ± 0.02* | 0.96 ± 0.24** |

^a +, positive growth; -, no growth.

^b Values are means ± standard deviations. *, 0.005 > *P* < 0.05; **, *P* < 0.005.

^c *V. cholerae* strains were grown in AKI medium, pH 7.4, at 37°C for 12 h. The amount of CT produced by the strains was measured by GM₁ ELISA.

^d Fluid accumulation was measured in the RIL assay. IL, intestinal loop.

^e Retention of crystal violet dye by *V. cholerae* strains that formed biofilm on an abiotic surface was estimated at 570 nm.

bolic genes relative to real-time PCR data are presented in Fig. 7 for comparison as well as for validation.

DISCUSSION

Analysis carried out in this study showed that genes for Gnt utilization and the ED pathway were located at different loci in the chromosomes of most of the pathogenic bacteria. The observed ubiquitous presence of the ED pathway in these organisms is an indication of its importance in energy metabolism, along with the involvement of the more efficient EMP pathway. *V. cholerae* whole-genome analysis revealed that the genes involved in the oxidative phase of the PP pathway are localized in a single operon, but the organization of genes involved in the nonoxidative phase and the EMP pathway is scattered within the two chromosomes. Further analysis revealed variations in the average GC content of the EMP and the PP pathways relative to predicted Gnt utilization systems, including ED pathway genes. The average GC content of most of the genes involved in the EMP and PP pathways is about 50%. On the other hand, the average GC content of the predicted Gnt utilization system including the ED pathway genes is 54%, much higher than that of the genome of *V. cholerae*, which is 47% (13). The predicted Gnt utilization system in *V. cholerae* is comprised of two operons, *gntP-eda* and *gntK-edd*, which are located in a single cluster adjacent to *gntR*. The unique organization and

monocistronic arrangement of the genes of the ED pathway and Gnt utilization system in the *V. cholerae* genome indicate that *V. cholerae* might activate the ED pathway, at least in growth medium with Gnt supplementation.

Results presented in this study establish that the ED pathway is functional in *V. cholerae* and that utilization of Gnt is mediated through this pathway (Table 2 and Fig. 2). Growth experiments in M9 medium supplemented with sugars also confirmed that Glu is utilized primarily through the EMP pathway (Fig. 2). Growth experiments with a conditional double mutant (Δedd_{N16961} strain carrying pTPp_{fk}) further show the existence of a functional PP pathway in *V. cholerae*, at least with pentose sugar ribose supplementation (Fig. 3A, tubes 7 and 8). Growth experiments with the conditional double mutant showed that deactivation of both the ED and EMP pathways caused growth suppression in GlcNAc-supplemented M9 medium (Fig. 3A, tube 6), but no such effect was apparent in case of deactivation of only the ED pathway. It is evident that GlcNAc utilization can occur through the PP pathway but with less efficiency. All results generated through growth experiments point out the fact that catabolism of Glu and Gnt occurs through the EMP and ED pathways, respectively; *V. cholerae* utilizes GlcNAc by using all or at least two of the three pathways examined. This is not surprising since this sugar is available in abundance as the breakdown product of the chitinous surface of copepods and other crustacean exoskeletons to which *V. cholerae* attaches itself for survival in the environment (34). The mucous-rich environment of the intestinal milieu, where pathogenic *V. cholerae* survives to cause infection in humans (1), is also a rich source of GlcNAc.

Biofilm formation on an abiotic surface is a multistep process and is believed to be essential for the survival of organisms in the environment. Increased levels of biofilm formation were noted in *V. cholerae* cells grown in the presence of GlcNAc compared to Glu-supplemented medium. A decreased ability to form biofilm was also noted in medium supplemented with Gnt compared to Glu supplementation (Fig. 6). These results suggest that activation of the ED pathway may not be conducive to biofilm formation. Further support for this conclusion is provided by data generated with an *edd* deletion mutant, where deactivation of the ED pathway led to an increase in the biofilm formation ability of the mutant compared to its wild-type control (Table 2). It may be noted, however, that though biofilm formation aids in the survival of *V. cholerae* in the environment, this may not be true in the intestine. The biofilm structure may enhance survival of *V. cholerae* during passage through the gastric acid barrier, but after the bacteria reach the intestine, dispersal of the individual cells from the in-

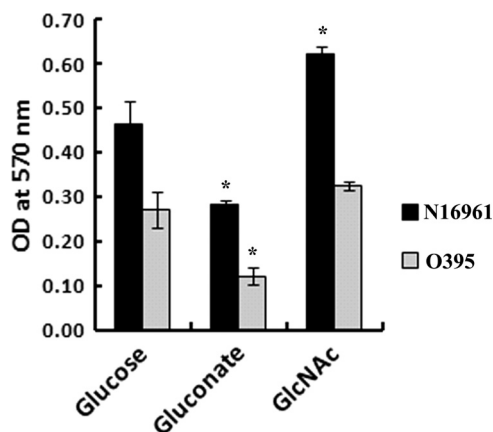


FIG 6 Biofilm formation ability of *V. cholerae* strains N16961 and O395 when grown in M9 medium supplemented with either Glu or Gnt or GlcNAc at 37°C for 18 h under static conditions. The extent of dye retention capacity (OD₅₇₀) of the adhered cells served as an index for the extent of biofilm formation by the cells. Error bars show standard deviations for triplicate assays. *, 0.005 > *P* < 0.05, when estimated against values obtained with cells grown in Glu.

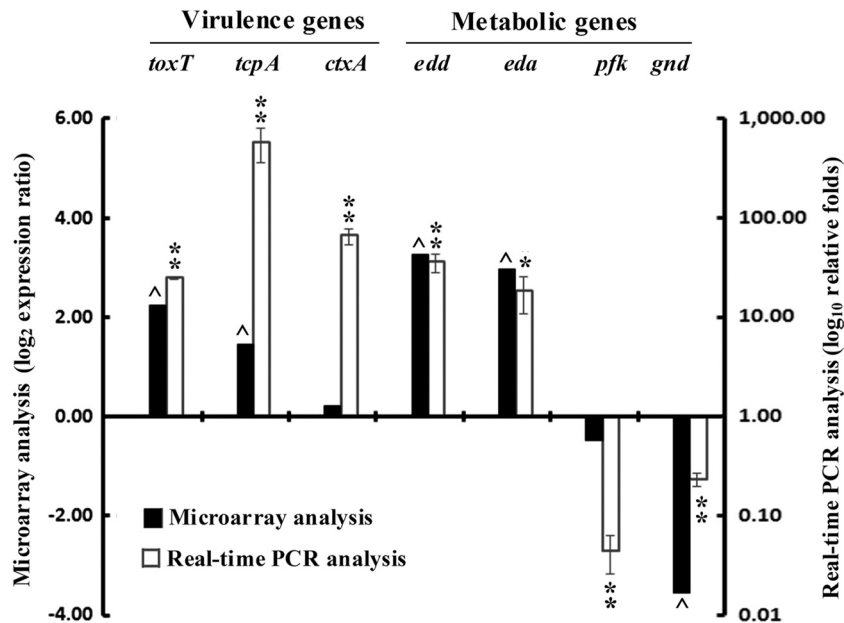


FIG 7 Comparative microarray analysis of gene expression (key virulence and metabolic genes) of *V. cholerae* N16961 grown in M9-Gnt medium compared to growth in M9-Glu medium. Relative expression levels of the genes as determined in microarray experiments are represented by log₂ expression ratio. Microarray data have been compared to real-time PCR analysis determined as log₁₀ relative fold. Data were normalized to cells grown with Glu supplementation. Error bars show standard deviations for triplicate real-time assays. *, 0.005 > *P* < 0.05; **, *P* ≤ 0.005. A change of gene expression level by a factor of 0.6 or more in microarray analysis is indicated by a caret.

gested biofilm clumps is critical to facilitate the colonization process and to establish infection in the gut (36). This may be achieved through the activation of the ED pathway in the intestine, where Gnt is reported to be available from degraded food material, epithelial cells, etc. (27).

A major finding of this study is that the activation of the ED pathway leads to upregulation of the expression of the virulence genes (*toxT*, *tcpA*, and *ctxA*) of *V. cholerae* that are responsible for its colonization and toxin production (Fig. 4). Interestingly, upregulation of these virulence-associated genes in *V. cholerae* grown in the presence of Gnt was found to be independent of its biotype (Fig. 4). It may be mentioned here that *V. cholerae* strains belonging to different biotypes exhibit considerable dependence on their culture conditions *in vitro* for the expression of CT (16, 32). Although we have not been able to explain the basis of the observed upregulation of CT production by the ED pathway, it would be prudent to assume that the regulation of the activation of virulence genes in *V. cholerae* may be quite different in complex enriched medium from that in simple defined medium, as used in this study. The above considerations raise an important question regarding the relevance of functional ED pathway data obtained *in vitro* to the pathogenesis of *V. cholerae* *in vivo*. To address this issue, we generated an ED pathway-defective mutant (Δ *edd*_{N16961} strain) which resulted in marked attenuation of its ability to express these virulence-associated genes *in vitro* (Fig. 5). *In vivo* studies demonstrated that the mutant failed to establish colonization in the suckling mouse model. Additionally, it also failed to cause CT-induced fluid accumulation in the RIL model (Table 2). These results suggest that a functional ED pathway is apparently essential for the expression of the pathogenic potential of *V. cholerae* strains *in vivo*.

Further support for the conclusions drawn above was obtained

from comparative microarray-based gene expression studies. Whole-genome transcriptome profile analysis showed that most of the virulence genes, their regulatory genes, and the virulence-associated genes (like those involved in flagellar movement, chemotaxis, and iron acquisition, etc.) were upregulated in cells grown in M9-Gnt medium compared to cells grown in M9-Glu medium. Other studies have shown that anaerobic conditions exist within the RIL or in the human intestine and that this might drive *V. cholerae* to express increased levels of genes involved during anaerobic respiration and in pathogenesis (35). To survive under poorly oxygenated conditions, *V. cholerae* may drive increased expression levels of the genes that are involved in anaerobic metabolism or respiration. Such an anaerobic condition may give a crucial signal in expressing the virulence factors (17, 22). Studies with *V. cholerae* grown under *in vitro* anaerobic conditions showed a reduction in CT production, and this can be attributed to the effect of repression by the H-NS protein (19). But when the pathogenic *V. cholerae* resides inside the intestine under anaerobic conditions, it produces CT. Our expression profile shows the elevated level of *ctxA* transcription followed by the downregulation of the gene encoding the regulatory H-NS protein. A subset of genes known to be expressed only under *in vivo* conditions (25) was transcribed *in vitro* when cells were grown in M9-Gnt medium. Considering the transcriptional preferences of virulence gene expression, it can be considered that there must be a significant role for the operative ED pathway during the pathogenic cycle of *V. cholerae*.

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