(p)ppGpp, a Small Nucleotide Regulator, Directs the Metabolic Fate of Glucose in Vibrio cholerae

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Background: Bacteria respond to nutrient starvation by (p)ppGpp that activates the stringent response. Results: V. cholerae mutants defective in (p)ppGpp production lost their viability during glucose-supplemented growth because of overproduction of organic acids. Conclusion: (p)ppGpp regulates energy metabolism, contributing to the successful proliferation of V. cholerae under glucose-rich environments. Significance: We report a previously unexplored role of (p)ppGpp in V. cholerae glucose metabolism.

When V. cholerae encounters nutritional stress, it activates (p)ppGpp-mediated stringent response. The genes relA and relV are involved in the production of (p)ppGpp, whereas the spoT gene encodes an enzyme that hydrolyzes it. Herein, we show that the bacterial capability to produce (p)ppGpp plays an essential role in glucose metabolism. The V. cholerae mutants defective in (p)ppGpp production (i.e. ΔrelAΔrelV and ΔrelAΔrelVΔspoT mutants) lost their viability because of uncontrolled production of organic acids, when grown with extra glucose. In contrast, the ΔrelAΔspoT mutant, a (p)ppGpp overproducer strain, exhibited better growth in the presence of the same glucose concentration. An RNA sequencing analysis demonstrated that transcriptions of genes consisting of an operon for acetoin biosynthesis were markedly elevated in N16961, a seventh pandemic O1 strain, but not in its (p)ppGpp0 mutant during glucose-stimulated growth. Transposon insertion in acetoin biosynthesis gene cluster resulted in glucose-induced loss of viability of the ΔrelAΔspoT mutant, further suggesting the crucial role of acetoin production in balanced growth under glucose-rich environments. Additional deletion of the aphA gene, encoding a negative regulator for acetoin production, failed to rescue the (p)ppGpp0 mutant from the defective glucose-mediated growth, suggesting that (p)ppGpp-mediated acetoin production occurs independent of the presence of AphA. Overall, our results reveal that (p)ppGpp, in addition to its well known role as a stringent response mediator, positively regulates acetoin production that contributes to the successful glucose metabolism and consequently the proliferation of V. cholerae cells under a glucose-rich environment, a condition that may mimic the human intestine.

The Gram-negative bacterium Vibrio cholerae is the causative agent of the acute diarrheal disease, cholera, and its associated deadly pandemics (1, 2). V. cholerae inhabits aquatic environments, but when ingested by a human host, it is able to colonize the small intestine through expression of toxin-co-regulated pillus (3, 4). The pathogen also produces cholera toxin, which is responsible for massive watery diarrhea (5–7). Until now, oral rehydration solution (ORS),2 which consists of glucose and electrolytes, has been used as the primary treatment for cholera (8, 9). Because cholera patients are treated with high volumes of ORS containing a large amount of glucose to prevent the severe dehydration induced by V. cholerae infection (10), it is speculated that continuous administration of ORS may create glucose-enriched microenvironments in the stomach or intestine. Such conditions subsequently have the potential to influence growth and virulence of V. cholerae, and identification of mutant(s) exhibiting defects during glucose-induced growth would be desirable to propose a better strategy for infection control. The efficacy of ORS is based on the ability of glucose to serve as a cofactor to stimulate ions (Na+, K+, Cl−, and HCO3−) and fluid absorption in the small intestine of the host via a cyclic AMP-independent process (11, 12). The effect of glucose on the bacteriological and virulence properties of V. cholerae, however, has received comparatively little attention.

V. cholerae O1 El Tor biotype strains produce acetoin, a metabolite that provides a growth advantage over the classical strains under glucose-rich growth condition (13). The biological function of acetoin or 2,3-butanediol, which is formed by single step reduction of acetoin, is a known strategy that allows the bacterial cells to avoid fatal acidification of the growth environment (14–16). Moreover, acetoin, when added to the cultured intestinal epithelial cells, suppressed the flagellum-induced production of proinflammatory cytokines through the inhibition of NF-κB pathway (17), demonstrating that the capa

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†‡This article contains supplemental Table S1.

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2 The abbreviations used are: ORS, oral rehydration solution; RPKM, reads per kilobase per million mapped; SR, stringent response; QS, quorum-sensing; CFU, colony forming unit; Tn, transposon; RNA-sequencing; (p)ppGpp, guanosine pentaphosphate or tetraphosphate.
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bility for acetoin production may also offer V. cholerae cells a survival advantage during the infectious process by down-regulating the host innate immune responses. Acetoin biosynthesis was determined to be regulated by two transcriptional regulators, AphA (quorum-sensing (QS) dependent virulence activator) and AlsR (acetate-responsive LysR-type regulator) (18). This finding suggested that the QS system likely plays a role in controlling glucose metabolism in V. cholerae. Similarly, the level of acetoin fermentation was also regulated by QS in Serratia plymuthica (21). The SR is characterized as a bacterial stress response in response to nutrient starvation (22). It is activated by (p)ppGpp, also known as a stress alarmone, refers to weak synthetase and strong hydrolase activities (24). In Gram-negative proteobacteria, (p)ppGpp biosynthesis is modulated by the strong monofunctional synthetase RelA and by the bifunctional protein SpoT, which exerts weak synthetase and strong hydrolase activities (24). V. cholerae has an additional small (pppGpp synthetase, RelV, which lacks the N-terminal hydrolase domain (25). Because the capability of bacterial pathogens to manage adverse conditions including rapidly changing nutritional availability and host-derived immune stress is required to establish successful infection, the possibility that the stress-induced SR influences bacterial metabolism warrants in-depth investigation. In the current study, we investigated the molecular basis of our unexpected observation that (pppGpp nonproducing V. cholerae mutant strains lost their viability during growth with extra glucose. This report provides strong evidence that (pppGpp, a small nucleotide regulator, modulates changes in glucose metabolism, and this process is critical to the maintenance of growth and viability of V. cholerae under glucose-rich conditions.

Experimental Procedures

Bacterial Strains and Growth Conditions—All of the strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37 °C in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl). A single colony grown in a LB agar plate was picked and inoculated to start a preculture. Precultures grown overnight were then diluted 100-fold in LB or LBG for the main cultures. The antibiotics were used in the following concentrations: streptomycin, 200 μg/ml; kanamycin, 50 μg/ml; and gentamycin, 50 μg/ml. The effect of glucose was assessed using cells grown in LB containing 1% glucose. Bacterial growth was monitored spectrophotometrically by measuring the optimal density at 600 nm. Phenol red (20 mg/liter; Sigma) was added to the agar plates as a pH indicator. The number of colony forming units (CFU) was measured by plating serial dilutions of bacterial cultures.

Construction of Mutants and Promoter-lacZ Fusion Strains—V. cholerae mutants were created by allele replacement, as previously described (26, 27). The 500 bp flanking sequences located at both ends that were used to introduce the mutation were amplified by PCR with the primers listed in Table 2. Construction of a chromosomal P_{VC1589::lacZ} transcriptional fusion was performed as described previously (7).

β-Galactosidase Activity Assay—For β-galactosidase assays, V. cholerae cells were harvested after 4 h of growth in LB or LBG (LB containing 1% glucose). β-Galactosidase activity assays were performed as described previously (7).

Construction of a Random Transposon Insertion Mutant Library—To create a V. cholerae transposon (Tn) insertion mutant library, we performed Tn mutagenesis of ∆relA∆spoT and ∆relAΔrelV∆spoT (i.e. (pppGpp)) mutants using the plasmid, pBTK30, which contains a Gm resistance-marked trans-
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TABLE 2

Primers used in this study

| Gene name | Direction | Primer sequence (5′ → 3′)* | Restriction sites |
|-----------|-----------|-----------------------------|------------------|
| Cloning   |            |                             |                  |
| VCI589-promoter fusion | Forward | CA/TGGAATTTCCTGATCTGGAGATCGGTTTCACTGCA | EcoRI |
|           | Reverse   | TA/ATTCTCACTGTTACGAGGAGAAGTGAAAG | SalI  |
| aphA left | Forward   | CA/TAGACGTTAAGTTTGCTGAAGCTTGAAG | SacI  |
| aphA left | Reverse   | AATT/ATGCCTGTAATAGCAAGTCCTACCTCCA | BamHI |
| aphA right| Forward   | CTT/ATTACGGTCCATAGTACGGTTTCACTGCA | BamHI |
|           | Reverse   | GA/ATTCTCACTGTTACGAGGAGAAGTGAAAG | SacI  |

Arbitrary PCR

| BTK30-Tnp1, 1 round | Forward | CACCCGGTCTGCTGTCACAG |                  |
| BTK30-Tnp2, 2 round | Reverse | CC/TACACGGCACAAGCCTCTGACCTAGTACGGTTTCACTGCA |                  |
| BTK30-Tnp3, 2 round | Forward | GAGAGCTGCTACGCTGTTACGAGGAGAAGTGAAAG |                  |
| BTK30-Tnp3, 2 round | Reverse | CT/TACACGGCACAAGCCTCTGACCTAGTACGGTTTCACTGCA |                  |

* Restriction enzyme recognition sequences are underlined.

posable element and a mariner C9 transposase (28). Suspensions of the Escherichia coli strain, SM10/Apir, which harbors pBTK30, and the V. cholerae recipient strains were mixed at a ratio of 2:1 onto LB plates and incubated for 6 h at 37 °C. Bacteria were then suspended in LB, and dilutions were plated on LB plates containing 50 μg/ml gentamycin and 200 μg/ml streptomycin. Bacterial colonies were then screened for mutants that formed a yellow zone around a single colony in a LBG agar medium supplemented with 20 mg/liter phenol red (Sigma-Aldrich). Mutant candidates that acidified the culture medium were harvested at 3 h postinoculation. To extract the high quality bacterial RNA, an RNeasy Protect kit (Qiagen) was used (13). Where all HPLC analyses were performed. The glucose level remaining in the medium was measured using a glucose assay kit (Sigma). The acetoin level was quantitatively measured using the Voges-Proskauer test as described previously (13).

RNA Sequencing Analysis—Bacterial cultures grown in LB or LBG were harvested at 3 h postinoculation. To extract the high quality bacterial RNA, an RNeasy Protect kit (Qiagen) was used with an RNeasy mini kit (Qiagen) following the manufacturer’s protocol. The quantity and quality of the total RNA were evaluated using RNA electropherograms (Agilent 2100 Bioanalyzer; Agilent Technologies, Waldbronn, Germany)) and by assessing the RNA integrity number. From each sample with a RNA integrity number value greater than 8.0, 8 μg of the total RNA was used as a starting material and treated with the MICROBExpress™ mRNA enrichment kit (Invitrogen). The resulting mRNA samples were processed for the sequencing libraries using the Illumina mRNA-Seq sample preparation kit (Illumina, San Diego, CA) following the manufacturer’s protocols. One lane per sample was used for sequencing by the Illumina Genome Analyzer IIx (Illumina, San Diego, CA) following the manufacturer's protocol. The raw reads were mapped to the reference genome sequences (NCBI BioProject accession number PRJNA57623, identification number 57623) using CLRNASeq version 0.80 (Chunlab, Seoul, Korea). The relative transcript abundance was computed by counting the RPKM (29).

Results

(p)ppGpp Is Essential for Glucose-induced Growth and Survival of V. cholerae—Recently, Raskin and co-workers (30) had constructed mutant strains of V. cholerae producing different levels of (p)ppGpp to characterize the effects of (p)ppGpp on biofilm formation. They determined the role of the mutants in regulating (p)ppGpp production in the exponential and stationary growth phases by thin layer chromatography. It is noteworthy that almost no detectable (p)ppGpp was observed in the ΔrelAΔrelV or the ΔrelAΔrelVΔspoT (termed as (p)ppGpp0 mutant), whereas the ΔrelAΔspoT mutant produced higher levels of (p)ppGpp than the wild type strain. We used these mutant strains in our study. To assess how bacterial growth is affected by (p)ppGpp-dependent glucose metabolism, we explored the growth and viability of the wild type strain N16961 and compared these parameters to those of isogenic mutant strains producing different basal levels of (p)ppGpp in LB or LBG containing 1% glucose (termed LBG). When grown in plain LB, the A600 values were exceeded at 3.0 in all strains except the ΔrelAΔspoT double mutant (A600 value = 1.5) after 16 h of growth (Fig. 1A). Because the initial growth defect caused by accumulation of (p)ppGpp is a hallmark of the SR, the ΔrelAΔspoT double mutant, which is the (p)ppGpp overproducing strain, has been characterized as having slow growth (31). When grown in LBG,
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FIGURE 1. Effects of (p)ppGpp production on growth and viability of V. cholerae strains in glucose-stimulated cultures. A, wild type N16961 and various (p)ppGpp-deficient mutant strains were inoculated in LB or LBG (LB containing 1% glucose), respectively, and grown in aerobic conditions for 16 h. *A600 values were expressed as relative bacterial growth. **p < 0.001 versus A600 values of LB-grown cultures. Three independent experiments were performed, and the means ± S.D. (error bars) are displayed in each bar. B, changes in bacterial cell viability in LB or LBG medium. Aliquots of each culture were sampled at 16 h postinoculation and were 10-fold serially diluted to assess the number of CFU.

The A600 values of N16961, ΔrelA, ΔrelV, and ΔrelAΔspoT strains were slightly increased compared with the respective A600 values in plain LB growth medium. Interestingly, when the ΔrelAΔrelV double mutant and the (p)ppGpp0 mutant were grown in LBG, severe growth defects were observed after 16 h (A600 value = <1.0) (Fig. 1A). Furthermore, loss of viability was observed in these two mutants based on the CFU counting as shown in Fig. 1B. These results strongly suggest that the capability of producing (p)ppGpp confers a growth and survival advantage of V. cholerae in glucose-enriched environments.

Loss of Viability in (p)ppGpp-deficient Mutants Is Caused by Medium Acidification through the Accumulation of Organic Acids—When bacterial cells are grown in glucose-containing media, diverse organic acid fermentations occur (32–34). These pathways can be fatal to some bacteria, including V. cholerae, that do not grow well at relatively low pH levels (13, 35). Therefore, we hypothesized that accumulation of organic acids induced by glucose metabolism would lead to loss of viability in (p)ppGpp-deficient mutants. To address this question, we measured diverse growth-associated parameters including A600 values, bacterial cell viability, medium pH, glucose consumption rate, and production rate of acetic and lactic acids under standard and glucose-supplemented conditions. As expected, severe growth defect was observed in the (p)ppGpp0 mutant during growth in LBG. A600 values were not increased, plateauing at ~1.5 after the 4-h growth time point (Fig. 2A). Likewise, bacterial viability was also sharply decreased during growth in LBG (Fig. 2B). Notably, the medium pH of the (p)ppGpp0 mutant culture was reduced to ~5.0 during the first 4 h and maintained at the same value throughout the growth (Fig. 2C). However, the medium pH was increased to ~8.0, after an initial drop to ~5.5, in N16961 or ΔrelAΔspoT mutant (Fig. 2C). The (p)ppGpp0 mutant exhibited defective glucose consumption, whereas the other two strains were capable of complete utilization of glucose (Fig. 2D). Because V. cholerae grows well in media with pH of above ~5.5, acidification of culture medium resulted in loss of viability in the (p)ppGpp-deficient mutants. To determine the source of the medium acidification, we quantitatively monitored acetic and lactic acids, two major end products of glucose fermentation, using HPLC. As shown in Fig. 2 (E and F), both acids accumulated continuously in the (p)ppGpp0 mutant for 10 h. In contrast, the wild type strain and the ΔrelAΔspoT mutant produced a less than 2-fold lower levels of acids than the (p)ppGpp-deficient mutant at 10 h postincubation. Remarkably, the two organic acids accumulated during the early stage of growth in all bacterial strains, whereas they no longer accumulated in N16961 or the ΔrelAΔspoT mutant at the later stage (Fig. 2, E and F). These results suggest the existence of a potential mechanism to regulate changes in glucose metabolism in a ppGpp-dependent manner, and this mechanism may be activated during the later stage of growth. To further confirm whether the loss of viability was due to medium acidification, bacterial cells were inoculated in buffered (pH 7.5) LB media supplemented with or without 1% glucose. As shown in Fig. 2 (G and H), regardless of the presence of glucose in the culture media, there was no growth defect or loss of viability in (p)ppGpp0 mutant.

Genome-wide Expression Monitoring Reveals (p)ppGpp-dependent Expression of Genes Involved in Acetoin Production—To understand the (p)ppGpp-dependent changes in glucose metabolism behind the glucose-induced pH drop, we compared the transcriptional profile of the N16961 to that of the (p)ppGpp0 mutant grown in LB medium with or without glucose using RNA sequencing. Because the rate of organic acid fermentation was decreased after 2 h in N16961, the total RNA was extracted at 3 h postinoculation from cells grown in LB or LBG. From the quantitative RNA analysis, 790 genes (up-regulated genes: 529, down-regulated genes: 261) and 1,843 genes (up-regulated: 1,430, down-regulated: 413) were identified, which expressed differentially more than two times in N16961 versus (p)ppGpp0 mutant, under LB or LBG condition, respectively (Fig. 3A). The Venn diagram in Fig. 3A shows that 322 and 50 genes with expression levels being decreased or increased by the lack of (p)ppGpp production are shared in both growth conditions. The number of differentially expressed genes under glucose-rich condition was 2.3-fold higher than that induced in LB condition, suggesting that (p)ppGpp plays a more important role in regulating gene expression under glucose-enriched condition. Next, the (p)ppGpp-dependent genes during glucose-stimulated growth were classified by function into 23 categories through the DEG browser of the CLRNASeq program as described under “Experimental Procedures” (Fig. 3B). The functional families that contain larger number of (p)ppGpp-regulated genes include (i) functional, not assigned, 14.46% (275 genes); (ii) general function prediction only, 8.78% (167 genes); (iii) amino acid transport and metabolism, 8.25% (157 genes); (iv) function unknown, 7.31% (139 genes); (v) signal transduction mechanisms, 7.20% (137 genes); and (vi) transcription, 6.52% (124 genes) (Fig. 3B). In Fig. 4, we illustrated seven regions showing noticeable differences in gene expression between the wild type and the (p)ppGpp0 strain during glucose-amended growth. The genetic composition and expression levels in each region are shown in Table 3. The expression of various stress-related genes encoding chaperones were significantly increased in (p)ppGpp0 cells compared with wild type cells during glucose-stimulated growth in regions 1, 3, 6, and 7. Such genes include those encoding 16 kDa HspA, GrpE, DnaK, DnaJ, GroEL, and GroES. Additionally, alternative RNA polymerase sigma factors, such as σ22 and σ4 and their regula-
tors (RseA and RseC) were highly expressed in (p)ppGpp⁰ cells during glucose metabolism (Fig. 4 and Table 3, region 2 and 5). Bacterial σ⁸⁷ and σ⁵ are alternative sigma factors that control the extra-cytoplasmic stress responses (24, 36). Together, these results strongly suggest that (p)ppGpp⁰ mutant encounters cellular stress (likely the one induced by pH drop) during glucose metabolism.

Interestingly, the expression of genes in region 4, including α-acetolactate decarboxylase (alsD), acetolactate synthase (alsS), and short chain dehydrogenase (alsO), were dramatically increased in N16961 during growth with glucose. Of note is that such a great increase was not observed in the (p)ppGpp⁰ mutant. These genes were acting as an operon to encode the acetoin biosynthetic gene cluster. Furthermore, the LysR family transcriptional regulator, alsR, a known regulator of acetoin biosynthesis, shares the promoter with alsD acetolactate decarboxylase, and its expression was also repressed in the (p)ppGpp⁰ mutant during growth in LBG (Table 3). These results suggest that acetoin biosynthesis is likely inhibited in ppGpp⁰ mutants during glucose metabolism. Because the role
FIGURE 3. Transcriptome analysis of (p)ppGpp-dependent genes during growth under LB or LBG. A, Venn diagrams showing the number of genes that are up-regulated and down-regulated in a (p)ppGpp-dependent manner under LB (green) or LBG (pink) growth conditions. B, functional categories of genes differentially expressed in the (p)ppGpp<sup>0</sup> mutant compared with the wild type N16961 strain under glucose-supplemented growth. The number of genes whose expression is differentially expressed in (p)ppGpp<sup>0</sup> mutants compared with wild type cells are presented according to the functions assigned by the DEG (differentially expressed genes) browser of the CLRNA-Seq data analysis software. Red and blue represent the number of genes whose expression is repressed and induced in the (p)ppGpp<sup>0</sup> mutant, respectively.

FIGURE 4. RNA sequencing analysis. A, linear view of the changes in transcription at the whole genome scale of *V. cholerae* using the CLRNA-Seq mapping browser. Transcripts were extracted at 3 h from bacterial cells grown under LB or LBG. Direction of the pick represents the direction of gene transcription. B, each region of magnified images are indicated by an arrow. Each pick represents the RPKM value of the transcript.
of the acetoin biosynthetic pathway in avoiding lethal acid stress during glucose metabolism is well established (13), our data suggest a clear link between the inhibition of acetoin biosynthesis and the up-regulation of stress-related genes encoding molecular chaperones and stress-responsive sigma factors in the (p)ppGpp0 mutant.

To provide additional evidence supporting the role of (p)ppGpp as a regulatory determinant in acetoin biosynthesis, we measured the promoter activity of alsD (VC1598) by constructing a lacZ reporter gene fusion in wild type N16961, ΔrelAΔspoT double mutant, and (p)ppGpp0 mutant. The promoter activity measured in LB with or without supplemented glucose were monitored via β-galactosidase activity. As shown in Fig. 5A, the alsD promoter activity of the wild type strain was increased more than 8-fold, when grown with glucose versus in LB. The (p)ppGpp0 mutant, however, showed little promoter activity in both growth conditions. Notably, the level of glucose-induced increase in the alsD promoter activity was ~1.5-fold higher in the (p)ppGpp accumulating ΔrelAΔspoT double mutant compared with N16961. We next quantified the levels of acetoin detected in bacterial culture supernatants during growth in LBG. As expected, no acetoin was produced in the (p)ppGpp0 mutant, whereas the acetoin production was high as 10 and 14 mM, respectively (Fig. 6B). These data correlate with RNA sequencing analysis data, which showed decreased transcripts of a gene cluster encoding acetoin biosynthesis in the (p)ppGpp0 mutant grown in LBG. Together, these results clearly demonstrate that acetoin biosynthesis in glucose-supplemented growth conditions, phenol red, a pH-dependent colorimetric indicator that turns yellow or pink under acidic or basic conditions, respectively, was used as the primary screening tool. Only two Tn-inserted mutants that exhibited yellow color and a small sized colony were selected from ~3,000 colonies and were confirmed by single colony streaking on agar plates containing both glucose and phenol red, with N16961 and the ΔrelAΔspoT mutant as controls (Fig. 6A). The transposon insertion sites in ΔrelAΔspoT-Tn-1 and ΔrelAΔspoT-Tn-2 mutants were determined to be in the promoter region of VC1589 (alsD), encoding acetalactate decarboxylase and inside the coding region of VC1590 (alsS), encoding acetalactate synthase, respectively (Fig. 6B). Interestingly, two Tn-inserted mutant strains showed a phenotype identical to the (p)ppGpp0 mutant in growth and medium acidification during glucose-supplemented growth (Fig. 6, C–E). Next, we investigated acetoin biosynthesis in two Tn-inserted mutant strains and in the (p)ppGpp0 mutant strain. It is noteworthy that the production of acetoin is dramatically decreased in the two Tn-inserted mutants and in the (p)ppGpp0 mutant, whereas wild types and the ΔrelAΔspoT mutants could produce levels of acetoin as high as 10 and 14 mM, respectively (Fig. 6F). These data correlate with RNA sequencing analysis data, which showed decreased transcripts of a gene cluster encoding acetoin biosynthesis in the (p)ppGpp0 mutant grown in LBG. Together, these results clearly demonstrate that acetoin biosynthesis is promoted by (p)ppGpp during glucose-supplemented growth, and such a promotion is essential for the maintenance of cell growth and viability in V. cholerae.

Next, we sought to explore whether a (p)ppGpp-dependent transcriptional regulator, which modulates the expression of acetoin biosynthetic genes, exists. Under the screening condition described in Fig. 6, we failed to identify a new regulator involved in the transcriptional activation of acetoin biosynthetic genes. As an alternative approach, we constructed a Tn mutant library derived from (p)ppGpp0 mutant and screened for mutants displaying a normal growth phenotype in LBG. Our initial purpose was to determine whether there would be a

### Table 3

| Region | Gene no. | Fold change vs. N16961/LB* | (p)ppGpp0/LB | (p)ppGpp0/LBG |
|--------|---------|---------------------------|------------|-------------|
| Region 1 | VC0018 | 1 | 2.1 | 3.3 | 71.8 |
| Region 2 | VC0150 | 1 | 1.7 | 1.1 | 9.6 |
| Region 3 | VC0855 | 1 | 1.2 | 0.7 | 16.8 |
| Region 4 | VC0856 | 2.1 | 2.4 | 92.2 |
| Region 5 | VC1590 | 1 | 2.6 | 6.4 | 200.6 |
| Region 6 | VC1591 | 1 | 524.3 | 0.93 | 24.2 |
| Region 7 | VC2466 | 1 | 1.7 | 2.4 | 5.3 |
| Region 8 | VC2467 | 1 | 1.7 | 1.7 | 4.9 |
| Region 9 | VC2468 | 1 | 3 | 2.6 | 16.9 |
| Region 10 | VC2469 | 1 | 3.3 | 2.2 | 14.6 |
| Region 11 | VC2470 | 1 | 1 | 0.57 | 2.63 |
| Region 12 | VC2664 | 1.83 | 11.9 | 158.3 |
| Region 13 | VC2665 | 1.5 | 6 | 80.3 |
| Region 14 | VC2666 | 1 | 28.3 | 365.3 |
| Region 15 | VC2667 | 1 | 35.2 | 402.8 |
| Region 16 | VC2668 | 1 | 1.1 | 0.7 | 16.8 |
| Region 17 | VC2669 | 1 | 1.7 | 1.1 | 9.6 |
| Region 18 | VC2670 | 1 | 2.1 | 3.3 | 71.8 |
| Region 19 | VC2671 | 1 | 524.3 | 0.93 | 24.2 |

* Transcript levels of the tested genes (indicated in the second column) were normalized with that of N16961/LB sample. Expression of each gene was assessed by measuring RPKM, as described under “Experimental Procedures.” RPKM values of entire genes are presented in supplemental Table S1.
(p)ppGp-dependent regulator that negatively regulates the transcription of acetoin biosynthetic genes. Two Tn-inserted mutants that did not acidify the culture media and showed no growth defects were selected of several thousand colonies under glucose-supplemented growth (Fig. 7, A and B). However, neither of the mutants produced acetoin (Fig. 7C). The genes carried by (p)ppGpp⁰::Tn4–56 and (p)ppGpp⁰::Tn5–12 that were disrupted by Tn insertion were identified as VC0964 (Phosphotransfer system, glucose-specific transporter subunit) and its promoter region, respectively (Fig. 7D). As expected,
glucose consumption was not observed in these mutants (data not shown), suggesting that the lack of medium acidification of these two mutants was caused by the absence of glucose uptake. Further, these results likely suggest that the (p)ppGpp-dependent acetoin fermentation pathway may be controlled by a direct mechanism that modulates the binding affinity between RNA polymerase and the promoter of \textit{alsD} by (p)ppGpp (37).

(p)ppGpp-mediated Acetoin Production Occurs Independently of the Presence of AphA—It was reported that acetoin biosynthesis is repressed by AphA, a LysR-type transcription factor involved in QS-dependent virulence regulation in \textit{V. cholerae} (18, 38). To determine the relative importance of (p)ppGpp-dependent or AphA-dependent regulation in maintaining the cell viability, we constructed two \textit{aphA} deletion mutant strains, derived from the wild type N16961 and the (p)ppGpp\textsuperscript{0} mutant. As shown in Fig. 8A, the acetoin production was increased \textasciitilde\textasciitilde 1.4-fold in the \textit{ΔaphA} single mutant compared with that of N16961, and this result further confirmed the negative regulation of acetoin biosynthesis by AphA. Acetoin production was also increased by the deletion of \textit{aphA} gene in the (p)ppGpp\textsuperscript{0} mutant (Fig. 8A). However, the level of acetoin produced in the (p)ppGpp\textsuperscript{0}Δ\textit{aphA} quadruple mutant was only \textasciitilde\textasciitilde 20\% of that in \textit{ΔaphA} single mutant. Although the quadruple mutant was capable of producing acetoin, this mutant could not recover the loss of cell viability and did not increase the pH of the culture media (Fig. 8, B and C). These results suggest that (p)ppGpp-mediated acetoin biosynthetic regulation is more critical to maintain cell viability than QS-dependent regulation, which is controlled in an AphA-dependent manner.

Discussion

Enteric pathogens face a number of challenges during the infectious process, including colonization resistance conferred by commensal microbes and immune-mediated stress responses (39). Thus, for pathogens to successfully survive in a host, they must possess extensive regulatory networks with which to orchestrate the expression of virulence factors and metabolic competence in response to environmental changes (40–44). Indeed, studies have shown that the virulence of enteric pathogens is associated with their ability to alter metabolic substrates in accordance with the intestinal environment of the host, which is constantly changing (45, 46). Consistent with this notion, cholera toxin production was greatly induced when \textit{V. cholerae} was grown by anaerobic TMAO respiration, a potential mode of growth that may occur inside the host intestine (31).

In the current study, we demonstrated that the ability of \textit{V. cholerae} to produce (p)ppGpp plays a key role in ensuring its viability in the presence of glucose. In our experiments, bacterial strains were grown in LB containing 1\% glucose, considered to be a nutrient-rich medium. Because SR is induced upon nutrient starvation, active accumulation of (p)ppGpp was not observed in these mutants (data not shown), suggesting that the lack of medium acidification of these two mutants was caused by the absence of glucose uptake. Further, these results likely suggest that the (p)ppGpp-dependent acetoin fermentation pathway may be controlled by a direct mechanism that modulates the binding affinity between RNA polymerase and the promoter of \textit{alsD} by (p)ppGpp (37).
expected to occur in LBG-grown N16961 cells. Our findings therefore suggest that (p)ppGpp, beyond its role as a SR effector, participates in regulating glucose metabolism by V. cholerae. In a recent study by Gaca et al. (20), basal levels of (p)ppGpp produced during nutrient-balanced growth were found to regulate the fermentative processes of E. faecalis, a Gram-positive bacterial species. Interestingly, however, acetoin production increased in a (p)ppGpp mutant of E. faecalis, whereas the production of lactate decreased in the same mutant grown in glucose media. These results suggest that (i) (p)ppGpp production may provide an unfavorable condition for E. faecalis survival during growth in the presence of glucose, and (ii) basal levels of (p)ppGpp likely mediate the regulation of metabolic processes in a species-specific manner. Given that E. faecalis is a core member of the commensal gut microbiota and SR is a conserved regulatory system in the bacterial kingdom, investigating (i) how SR is activated in each species found inside the human intestine and (ii) how V. cholerae regulates (p)ppGpp-dependent energy metabolism in association with other commensal microbes would be interesting, albeit difficult.

Studies suggest that (p)ppGpp production may induce phenotype changes. Recently, researchers reported that glucose exhaustion stimulated persister formation in E. coli, in which (p)ppGpp played a critical role (47, 48). In another study, growth of the wild type strain N16961 in LBG led to acidification of the media during early states of growth, after which increases in pH were observed; meanwhile, during the same stage of growth, glucose was preferentially consumed (13). These results demonstrate that acetoin production may arise as a consequence of bacterial responses to glucose consumption, as was the case in the present study. Interestingly, in a previous study, intracellular levels of both (p)ppGpp and cAMP accumulated upon nutrient limitation in E. coli (49). Also, transcription of the relA gene, which encodes for a (p)ppGpp synthetase, was also found to be activated by cAMP receptor protein in E. coli (50). These results, although observed in E. coli, led us to postulate that glucose consumption induces the intracellular accumulation of cAMP, which in turn activates a metabolic switch toward the production of acetoin in V. cholerae. Consistent with this hypothesis, a V. cholerae mutant lacking the ability to produce cAMP receptor protein also failed to produce acetoin and thus were unable to survive in the presence of glucose (data not shown). Notwithstanding, we suggest that further studies are needed to outline in detail the mechanisms by which cAMP receptor protein and (p)ppGpp induce the transcription of acetoin biosynthetic genes.

Bacterial SR, as a cellular adaptation and survival system, is activated in response to a number of stresses, including amino acid starvation (51, 52), fatty acid starvation (53), carbon source starvation (54), phosphorous limitation (55, 56), iron limitation (57), hyperosmotic shock (58), oxidative stress (59), and diauxic shifts (60, 61). Additionally, bacterial SR has been shown to be important to the persistent survival (62, 63) and virulence (64, 65) of bacterial pathogens. Likewise, a V. cholerae (p)ppGpp mutant showed defective intestinal colonization and cholera toxin production, compared with its parental strain (31). The production of virulence factors has been shown to increase when bacterial cells encounter extracellular stress, often resulting in retarded growth (7, 66–68). Therefore, because V. cholerae must compete with resident gut microbes for nutrients upon entering the human intestine, strains that are capable of mounting SR would be more successful in both proliferating via glucose metabolism and potentiating their virulence inside the stressful microenvironment of the host. Taken together, our results support a close association between bacterial responses to glucose metabolism and virulence potential.

RNA sequencing analysis provides a high throughput platform with which to examine the genome-wide transcriptional profiles of bacterial cells (69, 70). Our results in Fig. 4 show that (i) the expression levels of V. cholerae genes are continuously distributed across the genome, (ii) subsets of genes are actively transcribed in cells under differing conditions, and (iii) RNA-seq analysis offers superior detection sensitivity to that of hybridization-based techniques, such as microarray. In the present study, mRNA corresponding to relA, relV, and spoT were detected only at minimal levels in the (p)ppGpp mutant, compared with those in the N16961 cells (supplemental Table S1), and these results validated the quality of our transcriptome data. Also, a definite role of (p)ppGpp in acetoin biosynthesis was determined by sequential and complementary experiments. A striking increase in VC1590 and VC1591 transcripts was identified by RNA-seq analysis, and this finding was later confirmed by genetic screening and phenotype assays.
Role of (p)pGpp in Glucose Metabolism of V. cholerae

FIGURE 9. Summary of (p)pGpp-mediated regulation of glucose metabolism in V. cholerae. Glucose metabolism is controlled by the bacterial capability to produce a small nucleotide regulator, (p)pGpp, in V. cholerae. During the early stage of the growth, intracellular (p)pGpp levels are low in V. cholerae (30), and glucose metabolism mainly occurs by mixed acid fermentation. As bacterial growth proceeds, (p)pGpp levels are accumulated, and acetoin fermentation pathway is activated. As a result, the medium pH increases and bacterial cells avoid lethal acidification. In the (p)pGpp- mutant, active acetoin production is not induced and mutant cells experience premature cell death because of pH drop. Our results propose a new role of (p)pGpp as a key determinant for the luxurious growth of V. cholerae under glucose-enriched conditions.

Epidemiologically, the V. cholerae O1 classical biotype strains that had caused the first six pandemics therefrom have been replaced by the 7th pandemic O1 El Tor biotype strains (71). In a previous study, researchers suggested that the 7th pandemic El Tor biotype strains exhibit advantages in glucose-mediated growth through acetoin fermentation not observable in the classical biotype strains (13). Additionally, El Tor strains have also been shown to be significantly more competent in biofilm formation than classical strains in the presence of maltose-sensitive hemagglutinin, a monomeric component of chitin, which is one of the most abundant carbohydrate polymers in nature (13). These findings demonstrate that the ability of El Tor strains to metabolize carbohydrates to acetoin has conferred them survival fitness inside the host and the natural environment. Although these results suggest a potential answer to the long lasting question of why classical biotype strains may depend on the ability of the V. cholerae to activate SR under harsh environments inside the human intestine. This implicates SR as a promising drug target, inhibition of which may possibly down-regulate V. cholerae virulence and survival fitness.

In conclusion, we showed that the small nucleotide regulator (p)pGpp induces acetoin fermentation in V. cholerae and that (p)pGpp production is important to maintain the viability of V. cholerae under glucose-rich environments. Thus, for the first time, we propose that (p)pGpp acts as a metabolic switch to globally regulate the ability of bacteria to metabolize glucose (summarized in Fig. 9). Additionally, we showed that the infectious potential of V. cholerae may depend on the ability of the pathogen to activate SR under harsh environments inside the human intestine. This implicates SR as a promising drug target, inhibition of which may possibly down-regulate V. cholerae virulence and survival fitness. Further investigations into (p)pGpp-regulated carbohydrate metabolism by V. cholerae and its impact on the in vivo pathogenicity of V. cholerae are warranted to help eradicate this important human pathogen.

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