Effect of Trehalose and Trehalose Transport on the Tolerance of *Clostridium perfringens* to Environmental Stress in a Wild Type Strain and Its Fluoroquinolone-Resistant Mutant

Miseon Park,¹ Wilfrid J. Mitchell,² and Fatemeh Rafii¹

¹Division of Microbiology, National Center for Toxicological Research, FDA, Jefferson, AR 72079, USA
²School of Life Sciences, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, UK

Correspondence should be addressed to Fatemeh Rafii; fatemeh.rafii@fda.hhs.gov

Received 1 September 2016; Accepted 10 October 2016

Copyright © 2016 Miseon Park et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Trehalose has been shown to protect bacterial cells from environmental stress. Its uptake and osmoprotective effect in *Clostridium perfringens* were investigated by comparing wild type *C. perfringens* ATCC 13124 with a fluoroquinolone- (gatifloxacin-) resistant mutant. In a chemically defined medium, trehalose and sucrose supported the growth of the wild type but not that of the mutant. Microarray data and qRT-PCR showed that putative genes for the phosphorylation and transport of sucrose and trehalose (via phosphoenolpyruvate-dependent phosphotransferase systems, PTS) and some regulatory genes were downregulated in the mutant. The wild type had greater tolerance than the mutant to salts and low pH; trehalose and sucrose further enhanced the osmotolerance of the wild type to NaCl. Expression of the trehalose-specific PTS was lower in the fluoroquinolone-resistant mutant. Protection of *C. perfringens* from environmental stress could therefore be correlated with the ability to take up trehalose.

1. Introduction

*Clostridium perfringens*, a Gram-positive, spore-forming anaerobic bacterium, produces several different toxins. Although a pathogen, it is a member of the normal gastrointestinal tract microbiota of humans and animals and is found in soil, sewage, and foods [1]. Its ubiquitous distribution indicates that *C. perfringens* can overcome environmental stresses to survive. Forming spores allows *C. perfringens* to cope with harsh environmental conditions [2]. A two-component signal transduction system, VirR and VirS, enables *C. perfringens* to sense environmental changes and regulate the transcription of genes for the expression of the appropriate response [3].

In addition, bacteria may accumulate protective compounds, such as osmolytes, which prevent bacterial damage under stressful conditions by sheltering macromolecules and membranes from an otherwise harmful environment [4–9]. The disaccharides trehalose and sucrose are among these osmolytes that protect cells from environmental stress [5, 6, 10–15]. Increased intracellular trehalose has been reported in bacteria after treatment with NaCl [12, 16]. 1 mM trehalose has been shown to protect bacteria against 0.5 M NaCl [14]. Although the protective role of sucrose has been shown during L-form formation in *C. perfringens* [17], the role of trehalose in the protection of *C. perfringens* from environmental stress is not known. *Escherichia coli* and some other bacteria have enzymes for trehalose biosynthesis [4, 12, 13, 18–20] but no putative gene for trehalose synthesis has been detected in the genomes of strains of *C. perfringens* that have been sequenced.

In clostridia, like other bacteria, sugars may be transported into the cell via the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), which both transports and phosphorylates the substrate [21–23]. Based on the structural similarities to enzymes of the PTS IIBC subunit sugar transport genes, a putative gene for transport and metabolism of trehalose via the PTS has been identified in *C. perfringens* [2, 24]. Three genes (*treB, treC, and treR*) have been annotated as involved in trehalose transport, metabolism, and regulation [2, 24]. As in other bacteria, the
treB gene product is proposed to transport trehalose into the cell as trehalose 6-phosphate, which is then hydrolyzed via the treC gene product α,β-phosphotrehalase (trehalose-6-phosphate hydrolyase), to produce glucose 6-phosphate and glucose [18, 25]. In Bacillus subtilis, both treR and carbon catabolite repression (CCR) regulate the transport and metabolism of trehalose [26]. No trehalose-specific EIIA gene is listed among the genes found in the sequence of C. perfringens ATCC 13124 in GenBank. However, the missing EIIA function may be provided by the EIIA domain of another PTS, as has been demonstrated in the case of the trehalose PTS in B. subtilis [27].

In food and in the gastrointestinal tract, C. perfringens may come in contact with trehalose synthesized by other organisms [9] and also may be exposed to antimicrobial agents used for the treatment of infections [28, 29]. Exposure to antimicrobial agents may result in the development of resistant bacterial strains with changes that affect their metabolic activities [30–32]. We have shown strain-specific changes in fluoroquinolone-resistant strains of C. perfringens generated in the laboratory [28, 32–34]. In gatifloxacin-resistant laboratory mutants of C. perfringens ATCC 13124 [35], the expression of various genes and production of toxins has been altered [28]. In this study, we have investigated other alterations in the mutant by comparing the growth of mutant and wild type C. perfringens under various conditions and the role of trehalose in protecting them from environmental stress. We found that the genes designated as treB and treC are functionally involved in trehalose utilization and that their expression and their role in protection of cells from environmental stress have been altered in one fluoroquinolone-resistant mutant.

2. Materials and Methods

2.1. Growth of Strains. Clostridium perfringens ATCC 13124 (wild type) and its fluoroquinolone-resistant mutant ATCC 13124 CR, which has a stable double mutation in gyrA [35], were used for the experiment. The mutant was developed with exposure to 10 µg/ml of gatifloxacin [35]. The susceptibility of the mutant to different fluoroquinolones, including gatifloxacin, varied 128-fold [35]. The resistance development affected the expression of various genes [28]. The bacteria were grown in an anaerobic glove box (Coy Laboratory Products, Inc.) under 85% N₂, 10% CO₂, and 5% H₂, in brain heart infusion (BHI) medium for all experiments unless otherwise stated.

2.2. Assay for Growth on Different Sugars in Minimal Medium and BHI. The minimal medium, in phosphate buffer, included 19 amino acids, uracil, adenine, vitamins (biotin, calcium D-pantothenate, riboflavin, and pyridoxamine), and salts [36]. The chemically defined media, with and without sugars, were filter-sterilized. To assay strains for the ability to metabolize glucose, fructose, galactose, sucrose, and trehalose, a final concentration of 1% of one of the sugars was added to the minimal medium. The minimal medium without sugar was used for controls. The media were dispensed into triplicate wells in 96-well microtiter plates, inoculated with washed cells of overnight cultures of the wild type and the mutant, and incubated at 37 °C under anaerobic conditions. Growth was measured spectrophotometrically (Biotek Instruments) at 600 nm after 24 h.

The effect of trehalose on growth was also examined in brain-heart infusion (BHI) medium. Heat sterilized BHI with and without trehalose (at concentrations up to 5%) was placed in triplicate wells of microtiter plates and inoculated with the mutant and wild type. Growth was examined after the plates were incubated at 37 °C for 24 h under anaerobic conditions.

2.3. PCR Analysis. The sequence of C. perfringens ATCC 13124 from GenBank (accession number CP000246) was used to design primers for the amplification of genes involved in the metabolism of carbohydrates. The primers were designed to amplify the genes for trehalose utilization, treB, treC, and treR, the flanking sequences upstream and downstream of these genes and some regulatory genes (Table 1); DNA Star software was used to design primers. DNA was extracted according to a method described previously [35]. The 100 µl PCR mixture contained template DNA, 2 mM of dNTP mix, 0.5 µM of each forward and reverse primer, and 2.5 units of Taq polymerase from Roche or Applied Biosystems. The MyCycler thermocycler from Bio–Rad was used to amplify the genes. After initial denaturation at 95 °C for 2 min, the genes were amplified during 30 cycles with the following parameters: 95 °C for 15 sec, 52 °C (or another appropriate temperature for the annealing of primers) for 30 sec, and 72 °C for 90 sec, and final extension for 5 min at 72 °C. The PCR products were purified from agarose gels and sequenced using an ABI Biosystems sequencer and dideoxy terminator.

2.4. Preparation of RNA. Total RNA was isolated from cultures of the wild type and mutant [28]. Briefly, cells were harvested by centrifugation (15,000 × g, 10 min, 4 °C), washed with 10 mM Tris and 1 mM EDTA (pH 8.0), and suspended in buffer containing 1 mg/ml of lysozyme (Sigma) and 2% SDS. RNA Bee (Gentaur, Brussels, Belgium) was added to the mixture and then the RNA was extracted with chloroform. The RNA in the top layer was precipitated with isopropanol by placing the mixture at −70 °C for 30 min. After centrifugation, the pellet was drained and washed twice with 75% ethanol and dissolved in diethylpyrocarbonate- (DEPC-) treated water before treatment with DNase I (Ambion, Santa Clara, CA). Qiagen RNasy columns were used to further purify the RNA according to the manufacturer’s instructions.

2.5. qRT-PCR for Comparison of Relative Gene Expression in the Wild Type and Mutant Strain. qRT-PCR was performed using the SYBR®GreenER™ qPCR SuperMix Universal Two-Step qRT-PCR kit (Invitrogen) according to the manufacturer’s instructions, using the primers listed in Table 2. Primers for 16S rRNA were used as controls. In the first step of qRT-PCR, cDNA was synthesized from RNA using the SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) component of the kit and 25 ng/µl of RNA, in reaction mixtures with and without the RT enzyme. The cDNA was cooled and mixed with E. coli RNase H, provided in the kit, and incubated for 20 min at 37 °C. The cDNA was diluted
### Table 1: Primers designed for the amplification of trehalose operon genes in *C. perfringens* along with the upstream regions.

| Name      | Gene                 | Sequence                                      | Location          | Size   |
|------------|----------------------|-----------------------------------------------|-------------------|--------|
| CPF_0539F  | Sigma-70 factor      | TGAGTTCCAAAAATAGACAGAT                       | 640794–640815     | 22-mer |
| CPF_0539R  | Sigma-70 factor      | CCCATAAGCCAGGGTGTT                           | 642208–642224     | 17-mer |
| CPF_0540F  | Hypothetical protein | AATTGGATAGGAGGATGAGAT                       | 642361–642394     | 24-mer |
| CPF_0540R  | Hypothetical protein | CTTTTCCAGCTCTATTGTTTTTCA                     | 644393–644592     | 24-mer |
| 1985F      | CPF_0540 upstream    | ACAACCTTATTGAGGAGAT                          | 641983–642004     | 20-mer |
| tre373F    | treB                 | TTGGGACTTTGGATTTGCT                         | 642793–642816     | 24-mer |
| tre1027F   | treB                 | TATGGGCGAATTATAATGTAAG                      | 643447–643470     | 24-mer |
| tre2172R   | treB                 | TCAGTGTTATGATTTGTTTTTCA                     | 644569–644592     | 24-mer |
| tre1920F   | treB                 | TATAGTGGCAGGCTTTACATGCT                    | 643440–643463     | 24-mer |
| tre3777R   | treC                 | CTCTCCCTAGCTCTCAACACATCT                    | 645525–645548     | 24-mer |
| tre3090F   | treC                 | GAGGGGAATGCTGAGGAGT                        | 645510–645529     | 20-mer |
| tre373R    | treC                 | CCCAGTTTAAATCAGCCTGT                        | 646180–646197     | 18-mer |
| tre5283R   | treC                 | CTTTAAATAGCTGGTGATAGTT                     | 646837–646845     | 20-mer |

### Table 2: Comparison of the gene transcription in wild type *C. perfringens* ATCC 13124 and gatifloxacin resistant mutant 13124GR by qRT-PCR.

| Locus          | Gene        | Primer                      | Fold (mutant/wild) |
|----------------|-------------|-----------------------------|--------------------|
| CPF_0541upstream | treB upstream | ACAGAGTATCATAGGGCGAAGAGAGA TTTCAACATCTGCTTTTGCTT | −333.89 / −828.5 / −330 |
| CPF_0541       | treB        | TGGGACCTTTGAGTTTGCT GCCTGCGACATGATGCTCTG  | −93.81 / −227.5 / −22.28 |
| CPF_0542       | treC        | GAGCTTTAAAGGGCGAAGGAA CCCAGTTTAAATCAGGCTGT  | −45.1 / −44.5 / −16.80 |
| CPF_0543       | treR        | GCCTTCGAATACTCAACATTTATG GCCCTGACCTTTTCTAAGAGC  | −1.69 / −1.79 / −1.31 |
| CPF_0539       | RNA polymerase sigma-70 factor | TTGGTGAAGAGATGACACCAGAGGACCCCTCTTTACCCTCA | 2.29 / 3.63 / 2.99 |
| CPF_0069       | Transcription antiterminator | ATTCGGCAAAGAACACAGGA GCAACCTTAAAGGATTTGGGA | −90.74 / −711.9 / −116.6 |
| CPF_0042       | plc         | TGACAGGGGGAATCACAAGGCTGAGATTGCTTGA TCAAATCTGGCAATGCTGA  | −9.58 / −14.20 |
| CPF_0623       | revR        | AGTTCTTAAAGATGATGAGGAGGGCCTGACCTTTATGCTGAG  | −1.92 / −1.73 / −1.51 |
| CPF_0007       | gyrA        | TGCCAGAATAAGTGGGAA GCATGTCAGTCAACAGAC  | 1.08 / 1.27 |
| CPF_1204       | vrr         | CAAAAAGATTAAAAAGCTGCAA TTTGATATGAAAGCAAGAATGACTG  | −596.80 / −133.1 |
| CPF_1956       | topA        | ACCTTGGCCTGACCTTGA CAGACCTCAATCCCCTCTTATC  | −2.38 / −2.42 / −2.07 |
| CPF_1760       | topB        | AACTTGGCCTTATGCGCATT GCTAGACCTCCTTCTTCTCT  | −1.61 |
| CPF_1785       | PTS sucrose-specific IIBC component | GGGGCAATATTGGGAGTAA CGACTCTGCAGGCTTCTTCTTCTT | −92.15 |

*treB and treC are trehalose-specific genes. The transcription of several genes was considerably decreased in the mutant 13124GR in comparison with the wild type ATCC 13124; the data presented represent those obtained in different experiments. The expression of topoisomerase genes (gyr and top) and response regulator gene (revR) was not markedly changed. Pfo, which was also downregulated, was reported previously [28].*
and amplified in the second step of qRT-PCR, using the SYBR®GreenER™ qPCR SuperMix component of the kit. The C1000 thermal cycler CFX96 real-time system from Bio-Rad was used for the synthesis with the following parameters: 50°C for 2 min, 95°C for 8.5 min, and 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The instrument was programmed to measure the melting curve within 65–95°C (1°C/sec). The Ct (cycle threshold) for each of the genes was determined. The fold change in gene expression was calculated for each of the genes in the mutant and wild type [28]. The 2^−ΔΔCT method was used to calculate the relative level of mRNA expression, according to the thermocycler application guide [28].

2.6. Effect of Sodium Chloride on the Bacterial Growth in the Presence and Absence of Trehalose. NaCl concentrations of 0–2% were added to triplicate wells of 96-well microtiter plates containing BHI, with or without trehalose (0.5%). The wells were inoculated with the wild type and mutant. To find out the effect of trehalose in protection of cells from NaCl, the kinetics of the growth of wild type C. perfringens ATCC 13124 in the presence of 1% glucose, sucrose, or trehalose and also combinations of 1% glucose + 1% trehalose and 1% glucose + 1% sucrose were measured. 0.5 ml aliquots of minimal media supplemented with sugars were inoculated with overnight cultures of C. perfringens ATCC 13124, in the presence of 0%, 0.5%, 1%, and 2% NaCl in 48-well microtiter plates. The wells were filled with 0.9 ml of sterile mineral oil to keep the cultures anaerobic. The plates were placed in the Synergy Max Spectrophotometer (Biotek Instruments) and the kinetics module of Gene 5 software (Biotek Instruments) was programmed to measure the A600 every 30 min at 37°C. The kinetics analysis was performed using Microsoft Excel.

2.7. Effect of Sodium Nitrite on Growth in the Presence and Absence of Trehalose. NaN02 concentrations of 0–0.8% were added to triplicate wells of 96-well microtiter plates containing BHI with and without 0.5% trehalose. The wells were inoculated with the wild type or mutant and incubated anaerobically at 37°C. Growth was monitored at 600 nm with a spectrophotometer.

2.8. Effect of pH on the Growth of Wild Type and Mutant in the Presence and Absence of Trehalose. BHI medium, with pH values ranging within 4.8–10, was prepared and dispensed in triplicate wells of two 96-well microtiter plates. In one of the plates, trehalose (0.5%) was added to the wells. The plates were inoculated with overnight cultures of wild type and mutant and incubated anaerobically at 37°C. Growth was measured spectrophotometrically at 600 nm after 24 h.

3. Results

3.1. Growth of C. perfringens on Different Sugars in Minimal Medium and BHI. To find out if there were differences between the mutant and wild type in metabolizing different sugars, bacteria were grown in both minimal and rich media containing various sugars. The wild type ATCC 13124 grew in defined minimal medium containing glucose, sucrose, trehalose, fructose, or galactose (Figure 1). The mutant 13124GR had less growth than the wild type on glucose, fructose, and galactose and did not grow on trehalose or sucrose (Figure 1).

In BHI medium supplemented with sugars, both strains grew (Figure 2). Addition of different concentrations of trehalose to BHI resulted in increased growth of the wild type, but only up to 1.25% trehalose. Addition of trehalose to BHI cultures of the mutant 13124GR had no effect on growth (Figure 2). Gatifloxacin resistance affected the expression of various genes, decreasing the fitness of the 13124GR resistant mutant in comparison to the wild type [28, 32]. Therefore, the resistant mutant had less growth than the wild type, both in BHI medium and in the minimal medium with sugars that supported its growth.

3.2. Sequence Analysis and Expression of Genes Involved in Trehalose Transport and Metabolism and Regulatory Genes. To find out if the lack of ability of the mutant to grow on trehalose was because of mutation in the annotated trehalose transport gene or related PTS regulatory genes, these genes were amplified by PCR and sequenced. The primers used for
the amplification of genes of the trehalose transport operon amplified the same size fragments from \textit{treB}, \textit{treC}, and \textit{treR} in DNA from both mutant and wild type. The sequences of the amplified genes for \textit{treB}, a trehalose-specific IIBC component of the PTS; \textit{treC}, trehalose-6-phosphate hydrolase; \textit{treR}, a transcriptional regulator for these genes; and the flanking regions were identical for the mutant and wild type with one exception: a mutation of $G^{168}T$ was observed in \textit{treB} that resulted in the substitution of Leu 90 to Ser.

Other known PTS and global regulatory genes from \textit{C. perfringens} were also sequenced. The sequences of the HPr kinase/phosphorylase (HPrK, CPF_{1261}), the catabolite control protein A (CcpA, CPF_{2863}), and the sigma factor 70 ($\sigma^{70}$, CPF_{0539}) that are involved in PTS-related regulatory functions were identical for the wild type and the mutant. The sequence of the global regulatory gene \textit{vrr}, which regulates a variety of genes in \textit{C. perfringens}, was also identical in the wild type and mutant. However, a PTS regulatory gene (CPF_{0069}), which codes for a transcription antiterminator, had a single mutation resulting in the conversion of Leu 90 to Ser.

3.3. Comparison of the Transcription of Genes Involved in Trehalose Transport and Regulatory Genes. To find out if there were differences in expression of genes involved in trehalose transport in wild type and the mutant, the results of microarray data and gene transcription, as measured by qRT-PCR, of these genes in the two strains were compared. Microarray analysis data (http://www.ebi.ac.uk/arrayexpress/arrays/A-MEXP-2008/) showed alteration of the transcription of a range of genes in the mutant 13124^{GR} compared to the wild type [28]. Among the downregulated genes were \textit{treB} and \textit{treC}, which are involved in trehalose transport and metabolism. The qRT-PCR experiment, performed to compare the expression of several genes in the wild type and mutant, confirmed the microarray data on the downregulation of expression of \textit{treB} and \textit{treC} in the mutant 13124^{GR}. The transcription of the gene encoding the IIBC component of the putative sucrose PTS was also downregulated (Table 2). In addition, expression of the global regulatory gene \textit{vrr} (CPF_{1204}) and a PTS-associated antiterminator gene (CPF_{0069}) were also decreased substantially in the mutant 13124^{GR}. CPF_{0069} is located upstream of the putative PTS N-acetylglucosamine-specific EIia and EIIBC operon; the expression of those genes was also downregulated.

3.4. Growth of the Mutant and Wild Type with Sodium Chloride. To find out if there were differences between the NaCl tolerance of mutant and wild type and whether trehalose protects \textit{C. perfringens} from the effect of high salt concentration, the strains were grown with different concentrations of NaCl, with or without trehalose. In BHI medium, the growth of the mutant 13124^{GR} was less than that of the wild type (Figure 3). Addition of up to 0.25% NaCl did not affect the growth of either strain substantially, but unlike the wild type ATCC 13124 the mutant 13124^{GR} could not grow with added 0.5% NaCl. BHI medium, in addition to proteinaceous ingredients, contains NaCl and other sodium salts. It is more viscous than the minimal medium and the actual concentration of salt in this medium is higher than 0.5%. Addition of trehalose increased the tolerance of the wild type to NaCl but did not affect the tolerance of the mutant (Figure 3).

To find out if there were differences between \textit{C. perfringens} against NaCl, the kinetics of growth of ATCC 13124 in the minimal medium with glucose, sucrose, and trehalose were measured, separately and in combination, in the presence of 0, 0.5, 1, and 2% NaCl (Figure 4). The growth rates of the wild type were the same for all sugars in the absence and the presence of 0.5% NaCl (Figure 4). In the presence of 1% NaCl, the rate of growth in the culture containing glucose had decreased in comparison with the growth in cultures with trehalose or sucrose by 42% and 38%, respectively. Cultures containing trehalose had the highest rate of growth, indicating a trehalose osmoprotective effect; sucrose was also osmoprotective but to a lesser extent. The osmoprotective effect of trehalose was also greater than sucrose in cultures containing 2% NaCl, in which, after an extended lag phase of 8 h, bacteria grew in cultures containing trehalose or sucrose. The combination of trehalose with glucose or sucrose did not support growth of the wild type in the presence of 2% NaCl. Glucose may have had a repressive effect on the uptake of protective trehalose and sucrose.

3.5. Effect of Sodium Nitrite on the Wild Type and Mutant, with and without Trehalose. Since NaNO$_2$ is used as a preservative in food, its effects on the growth of the wild type and the mutant were also examined in the presence and absence of trehalose. The concentration of NaNO$_2$ that inhibited the growth of the wild type ATCC 13124 was twofold higher than that for the mutant 13124^{GR} (Figure 5). Trehalose did not appear to protect either strain against NaNO$_2$, but it enhanced the growth of the wild type in both the presence and absence of NaNO$_2$ (Figure 5).
Figure 4: Growth rate of wild type C. perfringens ATCC 13124 with different concentrations of NaCl in the presence and absence of trehalose and sucrose in the minimal medium. Without NaCl (a), 0.5% NaCl (b), 1% NaCl (c), and 2% NaCl (d). G = glucose, T = trehalose, S = sucrose, GS = glucose + sucrose, and GT = glucose + trehalose.

3.6. Effect of pH on the Growth of Wild Type ATCC 13124 and Mutant 13124GR with and without Trehalose. Since C. perfringens has potential to survive in different environmental conditions, the survival of the strains in the media with different pH was also compared in the presence and absence of trehalose. The wild type ATCC 13124 grew better than the mutant 13124GR in BHl medium at all pH values tested (4.8–10.2) (Figure 6). The lowest pH supporting the growth of the mutant was 5.2, but the wild type could grow in cultures with pH as low as 4.8, both in the presence and in absence of
The mutant could not grow with sucrose or trehalose in minimal medium and the genes for the transport of these carbohydrates and two regulatory genes, the transcription antiterminator CPF_0069 and vrr, were substantially downregulated in the mutant. The mutant was also less tolerant to acidic pH and NaCl. In the BHI medium, trehalose stimulated the growth of the wild type and increased its tolerance to low pH and high salt concentration. In the minimal medium, trehalose had an osmoprotective effect and enabled the bacteria to grow at concentrations up to 2% NaCl. Trehalose is therefore implicated in the protection of the bacteria from environmental stress; and the lack of growth of the mutant 13124GR on trehalose and sucrose in the minimal medium was consistent with inability to transport the sugars into the cell. Although the sequences of treB, treC, and treR, and the region upstream from these genes, which includes the sigma factor $\sigma^70$, were identical except for the mutation in treB that resulted in conversion of $\text{T}^\text{GR}\text{G}$, the transcription of treB and treC was substantially downregulated, as indicated by both the microarray data and qRT-PCR. Surprisingly, the trehalose transport regulatory gene treR (CPF_0543) was not markedly downregulated in the mutant (Table 2). treR belongs to the large GntR family of transcriptional regulators; the members of this family regulate varied biological processes.

Trehalose is implicated in the protection of several bacteria from environmental stress [4, 5, 8, 9, 14] and we have shown its osmoprotective effect in C. perfringens by demonstrating that addition of trehalose to cultures of ATCC 13124 improved the tolerance of the cultures to increased salt concentration. By comparing the expression of genes proposed to be involved in the transport and metabolism of sucrose and trehalose [2, 24], in the mutant that could not grow on sucrose or trehalose in minimal medium, we have shown that treB and treC are involved in trehalose transport and metabolism. The reason for their transcription downregulation in the mutant is not known.

In addition to downregulation of the transcription of treB and treC, the qRT-PCR results confirmed microarray data indicating that the expression of the transcriptional antiterminator CPF_0069 was substantially downregulated in the mutant 13124GR in comparison with the wild type. CPF_0069 codes for an RNA-binding protein similar to the BglG-like transcriptional antiterminators found in other bacteria [22, 37, 38]. CPF_0069 is located upstream of CPF_0070 and CPF_0071, which, respectively, encode the IIA and IICB components of a putative N-acetylglucosamine PTS. Microarray data showed that CPF_0070 and CPF_0071 were also downregulated in the mutant and most likely are regulated by the associated antiterminator. The mutant therefore shows several defects in expression of phosphotransferases involved in uptake and phosphorylation of sugar substrates. However, the role that mutation in CPF_0069, or its downregulation, may have played in altering the expression of other genes in the resistant mutant is not known and merits further investigation.

Another PTS-associated mechanism of controlling the transcription of genes involves the catabolite control protein A (CcpA), a master transcription factor that regulates
global gene transcription by binding to a catabolite response element (cre) when a readily-metabolizable carbohydrate is present [39]. This form of regulation is mediated through phosphorylation of the PTS phosphocarrier protein HPr at amino acid Ser46 by the metabolite-activated protein kinase, HPrK. HPr(ser)P then forms a complex with CcpA, resulting in stimulation of DNA binding. The coding regions of these proteins were identical in the wild type ATCC 13124 and mutant 13124GR, and there was less than a twofold difference between the levels of transcription of HPrK (CPF_2621) and CcpA (CPF_2683) in the wild type and mutant (data not shown). The role of these proteins, if any, in trehalose transport in the mutant is not known at this time.

In addition to the regulatory genes associated with the PTS, there are other regulatory genes in C. perfringens, notably the two-component transduction (sensor/regulator) system VirR/VirS that regulates a variety of genes, including the genes for regulatory RNA (vrr) and several toxins [40, 41]. vrr is a global response regulatory RNA (VR-RNA), transcription of which is regulated by VirR/VirS through a complex regulatory network via a VirR/VirS-VR-RNA cascade, and its transcript VR-RNA, rather than a protein, has regulatory activity [40, 41]. vrr was substantially down-regulated in the mutant. Using next-generation sequencing, a mutation was found in virS of C. perfringens strain 13124GR that resulted in introduction of a stop codon terminating protein synthesis after 18 amino acids. This change, previously undetected [28], was confirmed by PCR and sequencing and may have resulted in changes in transcription of various genes, including the downregulation of vrr. Ohtani et al. [41] found downregulation of genes, including PTS genes (CPE1463–1466), the toxin gene plc (CPE0036) and one antiterminator gene (CPE2553), in a vrr deletion mutant of C. perfringens strain 13. Although the downregulated PTS genes and antiterminator gene in their study were different from those we found in the fluoroquinolone-resistant mutant 13124GR, it indicates that downregulation of vrr may affect the PTS in C. perfringens. The effect of downregulation of vrr observed in this study on disaccharide transport needs further investigation.

Addition of trehalose to cultures of C. perfringens ATCC 13124 improved the tolerance of the cultures to increased salt concentration. The growth rate of the wild type was similar in minimal medium supplemented with different sugars, with or without 0.5% NaCl. When the salt concentration was increased to 1%, the growth rate of cells in cultures containing glucose was 0.076 h⁻¹, much lower than the growth rate in cultures containing either sucrose (0.1235 h⁻¹) or trehalose (0.1335 h⁻¹). In cultures containing 2% NaCl, growth was observed only in cultures containing trehalose or sucrose. This indicates that sucrose and trehalose protected the cells from NaCl and trehalose was more protective. Sucrose is known to protect C. perfringens during L-form formation [17], but this is the first time that the effect of trehalose on the protection of C. perfringens from NaCl has been shown.

In conclusion, the study shows that trehalose can protect C. perfringens from environmental stress. Furthermore, CPF_0541, annotated as treB, is implicated in trehalose transport, and its expression is altered in the gatifloxacin-resistant mutant 13124GR.

Disclosure

The views presented in this manuscript do not necessarily reflect those of the U.S. Food and Drug Administration.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors thank Dr. Robert D. Wagner and Dr. John B. Sutherland for reviewing the manuscript and Dr. Carl E. Cerniglia for research support.

References

[1] J. K. Cheung, J. A. Wisniewski, V. M. Adams, N. S. Quinsey, and J. I. Rood, “Analysis of the virulence-associated RevSR two-component signal transduction system of Clostridium perfringens,” International Journal of Medical Microbiology, vol. 306, no. 6, pp. 429–442, 2016.
[2] G. S. A. Myers, D. A. Rasko, J. K. Cheung et al., “Skewed genomic variability in strains of the toxigenic bacterial pathogen, Clostridium perfringens,” Genome Research, vol. 16, no. 8, pp. 1031–1040, 2006.
[3] K. Ohtani and T. Shimizu, “Regulation of toxin gene expression in Clostridium perfringens,” Research in Microbiology, vol. 166, no. 4, pp. 280–289, 2015.
[4] A. R. Strom and I. Kaasen, “Trehalose metabolism in Escherichia coli: stress protection and stress regulation of gene expression,” Molecular Microbiology, vol. 8, no. 2, pp. 205–210, 1993.
[5] S. B. Leslie, E. Israeli, B. Lighthart, J. H. Crowe, and L. M. Crowe, “Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying,” Applied and Environmental Microbiology, vol. 61, no. 10, pp. 3592–3597, 1995.
[6] B. Kempf and E. Bremer, “Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments,” Archives of Microbiology, vol. 170, no. 5, pp. 319–330, 1998.
[7] R. D. Sleator and C. Hill, “Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence,” FEMS Microbiology Reviews, vol. 26, no. 1, pp. 49–71, 2002.
[8] A. D. Elbein, Y. T. Pan, I. Pastuszak, and D. Carroll, “New insights on trehalose: a multifunctional molecule,” Glycobiology, vol. 13, no. 4, pp. 17R–27R, 2003.
[9] F. S. Cardoso, R. F. Castro, N. Borges, and H. Santos, “Biochemical and genetic characterization of the pathways for trehalose metabolism in Propionibacterium freudenreichii, and their role in stress response,” Microbiology, vol. 153, no. 1, pp. 270–280, 2007.
[10] H. M. Giaever, O. B. Styrvold, I. Kaasen, and A. R. Strom, “Biochemical and genetic characterization of osmoregulatory trehalose synthesis in Escherichia coli,” Journal of Bacteriology, vol. 170, no. 6, pp. 2841–2849, 1988.
[11] S. Mikkat, U. Effnert, and M. Hagemann, “Uptake and use of the osmoprotective compounds trehalose, glucosylglycerol, and...
sucrose by the *Cyanobacterium synechocystis* sp. PCC6803,” *Archives of Microbiology*, vol. 167, no. 2-3, pp. 112–118, 1997.

[12] F. Makihara, M. Tsuzuki, K. Sato et al., “Role of trehalose synthesis pathways in salt tolerance mechanism of *Rhodobacter sphaeroides* T. sp. *denitrificans* LL106,” *Archives of Microbiology*, vol. 184, no. 1, pp. 56–65, 2005.

[13] J. E. Purvis, L. P. Yomano, and L. O. Ingram, “Enhanced trehalose production improves growth of *Escherichia coli* under osmotic stress,” *Applied and Environmental Microbiology*, vol. 71, no. 7, pp. 3761–3769, 2005.

[14] L. Barra, N. Pica, K. Gouffi, G. C. Walker, C. Blanco, and A. Trautwetter, “Glucose 6-phosphate dehydrogenase is required for sucrose and trehalose to be efficient osmoprotectants in *Sinorhizobium mellotii*,” *FEMS Microbiology Letters*, vol. 229, no. 2, pp. 183–188, 2003.

[15] N. K. Jain and I. Roy, “Effect of trehalose on protein structure,” *Protein Science*, vol. 18, no. 1, pp. 24–36, 2009.

[16] P. Zhao, Z. Zhou, W. Zhang, M. Lin, M. Chen, and G. Wei, “Global transcriptional analysis of *Escherichia coli* expressing IrpE, a regulator from *Deinococcus radiodurans*, in response to NaCl shock,” *Molecular BioSystems*, vol. 11, no. 4, pp. 1165–1171, 2015.

[17] D. E. Mahony, J. A. Mader, and J. R. Dubel, “Transformation of *Clostridium perfringens* I forms with shuttle plasmid DNA,” *Applied and Environmental Microbiology*, vol. 54, no. 1, pp. 264–267, 1988.

[18] M. Rimmle and W. Boos, “Trehalose-6-phosphate hydrolase of *Escherichia coli*,” *Journal of Bacteriology*, vol. 176, no. 18, pp. 5654–5664, 1994.

[19] A. L. Carvalho, F. S. Cardoso, A. Bohn, A. R. Neves, and H. Santos, “Engineering trehalose synthesis in *Lactococcus lactis* for improved stress tolerance,” *Applied and Environmental Microbiology*, vol. 77, no. 12, pp. 4189–4199, 2011.

[20] Y. Gao, Y. Xi, X.-L. Lu et al., “Cloning, expression and functional characterization of a novel trehalose synthase from marine *Pseudomonas* sp. F8005,” *World Journal of Microbiology & Biotechnoloy*, vol. 29, no. 11, pp. 2195–2206, 2013.

[21] L. R. Maréchal, “Transport and metabolism of trehalose in *Escherichia coli* and *Salmonella typhimurium*,” *Archives of Microbiology*, vol. 137, no. 1, pp. 70–73, 1984.

[22] W. J. Mitchell, “The phosphotransferase system in solventogenic clostridia,” *Journal of Molecular Microbiology and Biotechnology*, vol. 25, no. 2-3, pp. 129–142, 2015.

[23] W. J. Mitchell, “Sugar uptake by the solventogenic clostridia,” *World Journal of Microbiology & Biotechnoloy*, vol. 32, no. 2, article 32, 2016.

[24] T. Shimizu, K. Ohtani, H. Hirakawa et al., “Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 2, pp. 996–1001, 2002.

[25] A. Bhumiratana, R. L. Anderson, and R. N. Costilow, “Trehalose metabolism by *Bacillus popilliae*,” *Journal of Bacteriology*, vol. 119, no. 2, pp. 484–493, 1974.

[26] L. Bürklen, F. Schöck, and M. K. Dahl, “Molecular analysis of the interaction between the *Bacillus subtilis* trehalose repressor TreR and the tre operon,” *Molecular & General Genetics*, vol. 260, no. 1, pp. 48–55, 1998.

[27] M. K. Dahl, “Enzyme II” contributes to trehalose metabolism in *Bacillus subtilis*, *FEMS Microbiology Letters*, vol. 148, no. 2, pp. 233–238, 1997.

[28] S. Park, M. Park, and F. Rafii, “Comparative transcription analysis and toxin production of two fluoroquinolone-resistant mutants of *Clostridium perfringens*,” *BMC Microbiology*, vol. 13, article 50, 2013.

[29] M. Rashid, A. Weintraub, and C. E. Nord, “Comparative effects of the immediate and the extended release formulations of ciprofloxacin on normal human intestinal microflora,” *Journal of Chemotherapy*, vol. 23, no. 3, pp. 145–149, 2011.

[30] P. Komp Lindgren, L. L. Marcussen, D. Sandvang, N. Frimodt-Møller, and D. Hughes, “Biological cost of single and multiple norfloxacin resistance mutations in *Escherichia coli* implicated in urinary tract infections,” *Antimicrobial Agents and Chemotherapy*, vol. 49, no. 6, pp. 2343–2351, 2005.

[31] E. Kugelberg, S. Löfmark, B. Wretlind, and D. I. Andersson, “Reduction of the fitness burden of quinoline resistance in *Pseudomonas aeruginosa*,” *Journal of Antimicrobial Chemotherapy*, vol. 55, no. 1, pp. 22–30, 2005.

[32] M. Park, J. B. Sutherland, J. N. Kim, and F. Rafii, “Effect of fluoroquinolone resistance selection on the fitness of three strains of *Clostridium perfringens*,” *Microbial Drug Resistance*, vol. 19, no. 6, pp. 421–427, 2013.

[33] F. Rafii, M. Park, G. Gamboa da Costa, and L. Camacho, “Comparison of the metabolic activities of four wild-type *Clostridium perfringens* strains with their gatifloxacin-selected resistant mutants,” *Archives of Microbiology*, vol. 191, no. 12, pp. 895–902, 2009.

[34] M. Park and F. Rafii, “Global phenotypic characterization of effects of fluoroquinolone resistance selection on the metabolic activities and drug susceptibilities of *Clostridium perfringens* strains,” *International Journal of Microbiology*, vol. 2014, Article ID 456979, 8 pages, 2014.

[35] F. Rafii, M. Park, and J. S. Novak, “Alterations in DNA gyrase and topoisomerase IV in resistant mutants of *Clostridium perfringens* found after in vitro treatment with fluoroquinolones,” *Antimicrobial Agents and Chemotherapy*, vol. 49, no. 2, pp. 488–492, 2005.

[36] M. Sebold and R. N. Costilow, “Minimal growth requirement for *Clostridium perfringens* and isolation of auxotrophic mutants,” *Applied Microbiology*, vol. 29, no. 1, pp. 1–6, 1975.

[37] M. Tangney and W. J. Mitchell, “Characterisation of a glucose phosphotransferase system in *Clostridium acetobutylicum* ATCC 824,” *Applied Microbiology and Biotechnology*, vol. 74, no. 2, pp. 398–405, 2007.

[38] N. H. Al Makishah and W. J. Mitchell, “Dual substrate specificity of an N-acetylglucosamine phosphotransferase system in *Clostridium beijerinckii*,” *Applied and Environmental Microbiology*, vol. 79, no. 21, pp. 6712–6718, 2013.

[39] J. Deutscher, C. Francke, and P. W. Postma, “How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria,” *Microbiology and Molecular Biology Reviews*, vol. 70, no. 4, pp. 939–1031, 2006.

[40] K. Okumura, K. Ohtani, H. Hayashi, and T. Shimizu, “Characterization of genes regulated directly by the VirR/VirS system in *Clostridium perfringens*,” *Journal of Bacteriology*, vol. 190, no. 23, pp. 7719–7727, 2008.

[41] K. Ohtani, H. Hirakawa, K. Tashiro, S. Yoshizawa, S. Kuhara, and T. Shimizu, “Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*,” *Anaerobe*, vol. 16, no. 3, pp. 258–264, 2010.