Cytochrome c_{551} and the Cytochrome c Maturation Pathway Affect Virulence Gene Expression in *Bacillus cereus* ATCC 14579

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Loss of the cytochrome c maturation system in *Bacillus cereus* results in increased transcription of the major enterotoxin genes *nhe*, *hbl*, and *cytK* and the virulence regulator *plcR*. Increased virulence factor production occurs at 37°C under aerobic conditions, similar to previous findings in *Bacillus anthracis*. Unlike *B. anthracis*, much of the increased virulence gene expression can be attributed to loss of only c_{551} , one of the two small c -type cytochromes. Additional virulence factor expression occurs with loss of *resBC*, encoding cytochrome c maturation proteins, independently of the presence of the c -type cytochrome genes. Hemolytic activity of strains missing either *cccB* or *resBC* is increased relative to that in the parental strain, while sporulation efficiency is unaffected in the mutants. Increased virulence gene expression in the Δ *cccB* and Δ *resBC* mutants occurs only in the presence of an intact *plcR* gene, indicating that this process is PlcR dependent. These findings suggest a new mode of regulation of *B. cereus* virulence and reveal intriguing similarities and differences in virulence regulation between *B. cereus* and *B. anthracis*.

Bacillus cereus is a Gram-positive, spore-forming, soil-dwelling bacterium that can be pathogenic in humans. *B. cereus* is most often observed as a causative agent of food poisoning but is also responsible for a wide range of opportunistic and nosocomial infections (reviewed in reference 1). This species belongs to the *B. cereus* group, which includes the closely related species *Bacillus anthracis* and *Bacillus thuringiensis* (2). Despite a high degree of genetic similarity between *B. cereus* and *B. anthracis*, the molecular mechanisms of pathogenesis are dissimilar. Pathogenesis of *B. cereus* relies on a number of hemolytic and nonhemolytic enterotoxins (discussed below) controlled by the quorum-sensing regulatory system PlcR-PapR, all encoded by genes on the main bacterial chromosome (3–5). Pathogenesis of *B. anthracis* relies on production of anthrax toxin and capsule, expression of which is controlled by the virulence regulatory protein AtxA, and these virulence factors are encoded by genes on extrachromosomal virulence plasmids (6–8). While *B. anthracis* carries the same chromosomally encoded enterotoxin genes as *B. cereus*, they are not expressed due to a truncation mutation in *plcR* (3, 9).

B. cereus produces a number of secreted proteins, including hemolysins, phospholipases, and enterotoxins, that cause host cellular damage. The three most important enterotoxins are nonhemolytic enterotoxin (Nhe), hemolysin BL (Hbl), and cytotoxin K (CytK) (10, 11). Nhe is a tripartite toxin encoded by three contiguous genes that contributes to diarrheal disease through pore formation in host cell membranes (10, 12–14). Hbl is a similar tripartite toxin that also causes pore formation in host cell membranes (10, 15, 16). The similarity in structure and activity of Hbl and Nhe enterotoxin complexes has led to the hypothesis that the *nhe* and *hbl* operons are the result of a gene duplication event in an ancestral species (14). CytK is a β -barrel channel-forming toxin that damages host cells by membrane pore formation and contributes to necrotic enteritis, a potentially fatal outcome of *B. cereus* food poisoning (11).

Production of virulence factors in *B. cereus* is tied to the metabolic state of the cell, and several metabolic regulators have been shown to alter the expression profiles of enterotoxin genes. The catabolite control protein CcpA represses transcription of the Nhe and Hbl operons by binding to putative CRE sites (17). Expression

of the enterotoxin genes is also influenced by the pleiotropic regulator CodY, tying virulence expression to intracellular GTP and branched-chain amino acid levels (18). Enterotoxin production is sensitive to the oxidation-reduction potential through complex interactions between multiple regulators. In *B. cereus* strain F4430/73, Nhe and Hbl expression increases during growth under anaerobic conditions (19, 20) due to changes in the activity of the redox regulator Fnr and the two-component system ResDE in response to changes in oxidoreduction potential (ORP). ResDE and Fnr directly regulate expression of Hbl and Nhe and indirectly regulate all enterotoxin expression through changes to PlcR expression (20–22). An additional two-component system, OhrRA, is also involved in regulating enterotoxin production in response to changes in ORP (23).

It was recently shown that in *B. anthracis*, the c -type cytochromes affect virulence gene expression of the anthrax toxin genes (24). c -type cytochromes contribute but are not essential to aerobic respiration and are distinct from other cytochromes due to their covalently attached heme (25). In *B. subtilis*, five membrane-bound proteins with covalently bound heme have been identified: the 39-kDa subunit II of cytochrome *caa*₃ (CtaC), the 28-kDa cytochrome c subunit of the *bc* complex (QcrC), the 25-kDa cytochrome *b* subunit of the cytochrome *bc* complex (QcrB), the 13-kDa cytochrome c_{550} (CccA), and the 10-kDa cytochrome c_{551} (CccB) (26, 27). The functions of the two small c -type cytochromes, CccA and CccB, remain unknown. Both the *B. cereus* and *B. anthracis* genomes contain c -type cytochrome genes similar

Received 21 July 2014 Accepted 20 November 2014

Accepted manuscript posted online 24 November 2014

Citation Han H, Sullivan T, Wilson AC. 2015. Cytochrome c_{551} and the cytochrome c maturation pathway affect virulence gene expression in *Bacillus cereus* ATCC 14579. *J Bacteriol* 197:626–635. doi:10.1128/JB.02125-14.

Editor: O. Schneewind

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doi:10.1128/JB.02125-14

to those of *B. subtilis*. In *B. anthracis*, loss of either the *c*-type cytochrome maturation system or simultaneous loss of CccA and CccB results in increased toxin gene production through altered transcription of the *B. anthracis*-specific virulence regulator AtxA. Neither CtaC nor QcrBC contributes to virulence regulation (24). Production of *c*-type cytochromes requires a number of proteins that constitute the cytochrome *c* maturation system. *c*-type cytochromes are initially produced as apo-proteins in the cytoplasm, missing their required cofactor, heme. The apo-cytochrome *c* is secreted across the membrane by the Sec translocation system. At the membrane, the oxidoreductase ResA reduces cysteine residues in the heme binding pocket of apo-cytochrome (28), while ResB and ResC transport and covalently attach heme to the reduced apo-cytochrome to generate the final, active form of cytochrome *c* (29, 30). Loss of ResB and ResC in *B. anthracis* also results in increased toxin gene production through AtxA. Toxin and AtxA gene expression is identical in the Δ cccAB strain and the Δ resBC strain, indicating that the role of ResBC in altered virulence gene expression is the production of active CccA and CccB (24).

Here we report that virulence gene expression in *B. cereus* is also influenced by *c*-type cytochromes and their maturation system. Loss of only the small *c*-type cytochrome CccB increases enterotoxin and *plcR* expression, but deletion of the cytochrome maturation system increases virulence gene expression more strongly than the loss of CccB alone. The increased virulence gene expression is *plcR* dependent. Hemolytic activity in the cytochrome and cytochrome maturation system mutants is also increased. These results reveal a newly identified regulatory network controlling *B. cereus* virulence gene expression and highlight differences in virulence regulation between *B. cereus* and *B. anthracis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. cereus* strain ATCC 14579 and its derivatives were routinely grown in LB, nutrient sporulation medium phosphate (NSMP) (31), or brain heart infusion (BHI) broth supplemented with the appropriate antibiotics at the following concentrations: chloramphenicol (7.5 μ g/ml), erythromycin (5 μ g/ml), lincomycin (25 μ g/ml), and kanamycin (150 μ g/ml). 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (40 μ g/ml) was added to LB agar to monitor β -galactosidase activity as necessary. Competent cells of *B. cereus* were prepared following the method of Koehler et al. (32), and electroporation was performed using the Bio-Rad Gene Pulser according to the instructions of the supplier.

Escherichia coli TG1, C600, and DH5 α competent cells were used for the propagation and isolation of all plasmid constructs. *E. coli* transformation was performed in chemically competent cells as previously described (33). Transformants were selected on LB agar supplemented with ampicillin (100 μ g/ml), chloramphenicol (7.5 μ g/ml), or kanamycin (30 μ g/ml).

Plasmid and strain construction. Strains and plasmids are listed in Table 1. Oligonucleotide primers are listed in Table 2. Markerless gene deletions in *B. cereus* were generated through a modification of the technique of Janes and Stibitz (34), as previously described (35). The β -galactosidase reporter plasmid pAW325 was created by digesting pTCV-lac (36) with SnaBI and PstI, blunt-ending with T4 DNA polymerase, and religating, resulting in a promoterless *lacZ* transcriptional fusion plasmid carrying only a kanamycin resistance cassette.

β -Galactosidase assays. *B. cereus* strains harboring gene promoter fusions on the replicative vector pTCV-lac or pAW325 were grown at 37°C in LB supplemented with the appropriate antibiotics. β -Galactosidase activity was assayed as described previously, and specific activity was expressed in Miller units (37, 38).

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>B. cereus</i> strains		
ATCC 14579	Type strain of <i>B. cereus</i>	Laboratory stock
AW-C006	Markerless deletion of <i>resBC</i>	This study
AW-C007	Markerless deletion of <i>plcR-papR</i>	This study
AW-C008	Markerless deletion of <i>cccA</i>	This study
AW-C009	Markerless deletion of <i>cccB</i>	This study
AW-C011	Markerless deletions of <i>cccA</i> and <i>cccB</i>	This study
AW-C012	Markerless deletions of <i>resBC</i> and <i>plcR-papR</i>	This study
AW-C013	Markerless deletion of <i>ctaC</i>	This study
AW-C017	Markerless deletion of <i>qcrBC</i>	This study
AW-C016	Markerless deletions of <i>cccA</i> , <i>cccB</i> , <i>ctaC</i> , and <i>qcrBC</i>	This study
AW-C019	Markerless deletion of <i>resBC</i>	This study
Plasmids		
pHT315s	Shuttle vector with P _{SPAC} promoter; Amp ^r Erm ^r	45
pORI-Cm-I-SceI	pORI-Cm vector with I-SceI recognition site; Cm ^r	40
pSS4332	I-SceI expression plasmid; Kan ^r	35
pTCV-lac	Promoterless vector for <i>lacZ</i> fusion; Erm ^r Kan ^r	36
pAW250	<i>nheA-lacZ</i> transcriptional fusion in pTCV-lac; Erm ^r Kan ^r	This study
pAW270	<i>cytK-lacZ</i> transcriptional fusion in pTCV-lac; Erm ^r Kan ^r	This study
pAW285	Xylose-inducible expression plasmid; Cm ^r	35
pAW287	<i>hblL2-lacZ</i> transcriptional fusion in pTCV-lac; Erm ^r Kan ^r	This study
pAW288	<i>plcR-lacZ</i> transcriptional fusion in pTCV-lac; Erm ^r Kan ^r	This study
pAW325	Promoterless vector for transcriptional <i>lacZ</i> fusion; Kan ^r	This study
pAW330	Regions flanking BC1474-BC1476 in pORI-Cm-I-SceI; Cm ^r	This study
pAW340	Regions flanking BC5349-BC5350 in pORI-Cm-I-SceI; Cm ^r	This study
pAW349	Regions flanking BC4288 in pORI-Cm-I-SceI; Cm ^r	This study
pAW352	Regions flanking BC5187 in pORI-Cm-I-SceI; Cm ^r	This study
pAW353	<i>cccB</i> expression vector; Cm ^r	This study
pAW368	Regions flanking BC3943 in pORI-Cm-I-SceI; Cm ^r	This study
pAW369	Regions flanking BC1523-BC1524 in pORI-Cm-I-SceI; Cm ^r	This study
pAW395	<i>cytK-lacZ</i> transcriptional fusion in pAW325; Kan ^r	This study
pAW396	pHT315S-based <i>cccB</i> expression vector; Amp ^r Erm ^r	This study
pAW426	pAW285-based <i>resBC</i> expression vector; Cm ^r	This study

TMPD oxidase staining. The presence of active *c*-type cytochrome oxidase was verified by a colorimetric assay using *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as an artificial electron donor that can be oxidized by cytochrome *caa*₃. TMPD oxidase staining was performed on *B. cereus* grown on NSMP as previously described (24, 29).

TABLE 2 Oligonucleotide primers used in this study

Name	Function	Sequence
BC14745' Bam	Region upstream of BC1474 for amplification of <i>resB</i>	5'-CACCGGATCCTTGCGGTAACCTGAGGTTAGAA
BC14753' Sal	Region downstream of BC1475 for amplification of <i>resB</i>	5'-TGCGTCGACATCGTTTCCCCACCTTAACCT
BcresBU5' Acc	Region upstream of BC1474 for deletion of <i>resBC</i>	5'-CACCGGTACCTGGTGCTGTAGGATTACCC
BcresBU3' Sal	Region upstream of BC1474 for deletion of <i>resBC</i>	5'-TGCGTCGACTTCTAACCTCAAGTTACCGCAAT
BcresCD5' Sal	Region downstream of BC1476 for deletion of <i>resBC</i>	5'-CACCGTCGACAATTATTGCCGGTTTACATTCA
BcresCD3' Pst	Region downstream of BC1476 for deletion of <i>resBC</i>	5'-TGCTGCACTGTCGCTTTCTTCTCACGAA
BcresBUSeq	Check primer upstream of BC1474	5'-GCGAGAGAATTGACACCACA
BC4288U5' Bam	Region upstream of BC4288 for deletion of <i>cccA</i>	5'-CACCGGATCCGGACCATTGCAGCCTATGAAT
BC4288U3' Sal	Region upstream of BC4288 for deletion of <i>cccA</i>	5'-TGCGTCGACTCCCCCTTCTATACCCCTGA
BC4288D5' Sal	Region downstream of BC4288 for deletion of <i>cccA</i>	5'-CACCGTCGACCGCATGACAAGCCTTTTGC
BC4288D3' Pst	Region downstream of BC4288 for deletion of <i>cccA</i>	5'-TGCTGCACTCATGGATAAGCTCCCAACC
BC4288Useq	Check primer upstream of BC4288	5'-TAGGCCGTGGTATGCTTTTC
BC4288DSeq	Check primer downstream of BC4288	5'-AGAGTAAGGCGCTGCAACAT
BC5187U5' Bam	Region upstream of BC5187 for deletion of <i>cccB</i>	5'-CACCGGATCCGGAGAGCAAAAAGAAATCG
BC5187U3' Sal	Region upstream of BC5187 for deletion of <i>cccB</i>	5'-TGCGTCGACACTTCTATCGCTCCCCATT
BC5187D5' Sal	Region downstream of BC5187 for deletion of <i>cccB</i>	5'-CACCGTCGACAAGGTGAAATGGACGAGTTGA
BC5187D3' Pst	Region downstream of BC5187 for deletion of <i>cccB</i>	5'-TGCTGCACTACACGCTCACGAATTGCAT
BC5187E5' Bam	Amplification of <i>cccB</i> ORF	5'-CACCGGATCCGGGGAGCGATAGAAGTGAAA
BC5187E3' Sal	Amplification of <i>cccB</i> ORF	5'-TGCGTCGACTCAACTCGTCCATTTCACCTT
plcRU5' Bam	Region upstream of <i>plcR</i> for deletion of <i>plcR-papR</i>	5'-CACCGGATCCACTAACAGTTGTGATTACTCCATT
plcRU3' Sal	Region upstream of <i>plcR</i> for deletion of <i>plcR-papR</i>	5'-TGCGTCGACGTGCATACTTACTCACCATCC
papRD5' Sal	Region downstream of <i>papR</i> for deletion of <i>plcR-papR</i>	5'-CACCGTCGACCGCTTACTTAGGCGGTTG
papRD3' Pst	Region downstream of <i>papR</i> for deletion of <i>plcR-papR</i>	5'-TGCTGCACTGCGCTGCCCAATTTATCCCTCT
plcR3' promBam	Region upstream of <i>plcR</i> for <i>lacZ</i> fusion	5'-TGCGGATCCTAATTTTTCTGCGTGCAT
plcR5' promXma2	Region upstream of <i>plcR</i> for <i>lacZ</i> fusion	5'-CACCCCGGGTTTGCAAATTCATTTGATACGG
nhe5' promXma	Region upstream of <i>nheA</i> for <i>lacZ</i> fusion	5'-CACCCCGGGCCGCGTTACGGTCGTTATAC
nhe3' promBam	Region upstream of <i>nheA</i> for <i>lacZ</i> fusion	5'-TGCGGATCCGCTTACAGGAATGAAGCAA
cytK5' promEco	Region upstream of <i>cytK</i> for <i>lacZ</i> fusion	5'-CACCGAATTCACAGCCAGCCTCCTTATTGA
cytK3' promBam	Region upstream of <i>cytK</i> for <i>lacZ</i> fusion	5'-TGCGGATCCGACAGCTTTCATACAACTCCTCC
hbl5' promEco2	Region upstream of <i>hbl</i> for <i>lacZ</i> fusion	5'-CACCGAATTCGTTAATAATGATATTAGGATGTTTTGTG
hbl3' promBam2	Region upstream of <i>hbl</i> for <i>lacZ</i> fusion	5'-TGCGGATCCACTCCTTTACCATTGTTTTTATACT
BcctaCU5' Bam	Region upstream of BC3943 for deletion of <i>ctaC</i>	5'-CACCGGATCCTGGGATTATTGGTGCTTTCA
BcctaCU3' Sal	Region upstream of BC3943 for deletion of <i>ctaC</i>	5'-TGCGTCGACAGTCGCCACTGTTCTTCA
BcctaD5' Sal	Region downstream of BC3943 for deletion of <i>ctaC</i>	5'-CACCGTCGACTGACTGGCAAATATGGCAAC
BcctaCD3' Pst	Region downstream of BC3943 for deletion of <i>ctaC</i>	5'-TGCTGCACTGAGATTGCAAGCCGAGTA
BcctaCUSeq	Check primer upstream of BC3943	5'-AAGAACAAGACCCGTCCAA
BcqrBU5' Bam	Region upstream of BC1523 for deletion of <i>qcrB</i>	5'-CACCGGATCCTGACCTGTGCATGAGCGTAT
BcqrBU3' Sal	Region upstream of BC1523 for deletion of <i>qcrB</i>	5'-TGCGTCGACAATCTTTCATCCACCCAATCA
BcqrCD5' Sal	Region downstream of BC1524 for deletion of <i>qcrC</i>	5'-CACCGTCGACGAAGCTTTCGGAATTCGTTG
BcqrCD3' Pst	Region downstream of BC1524 for deletion of <i>qcrC</i>	5'-TGCTGCACTGCTTATGCAACGTCAC
BcqrBUSeq	Check primer upstream of BC1523	5'-TTGGAAGAGAAATGGGGAAA
BcresBCE5' Sal	Amplification of <i>resB</i> and <i>resC</i> ORF	5'-CACCGTCGACTAGATACAGGGGCGGTGAGA
BcresBC53' Pst	Amplification of <i>resB</i> and <i>resC</i> ORF	5'-TGCTGCACTGACATGCACTCCCTATTTT

Sporulation analysis. Sporulation assays were performed using strains grown in NSMP or SM (Schaeffer's sporulation medium) (39) at 37°C for the time indicated for each experiment. Sporulation was initially monitored by visualization of cells on a Zeiss Axio Imager microscope and enumeration of spores and vegetative cells from multiple independent fields. Sporulation efficiency was assayed by chloroform treatment and enumeration of spores and vegetative cells as previously described for *B. anthracis* (40). Sporulation efficiency is presented as percentage of spores relative to total viable cells.

Quantitative hemolysis assay. Overnight cultures of *B. cereus* were diluted to an optical density at 600 nm (OD_{600}) of 0.01 in LB and grown for 7 h at 37°C. Samples were centrifuged for 5 min at $10,000 \times g$, and supernatants were transferred to a new tube. Supernatant samples were diluted in phosphate-buffered saline (PBS). Supernatant samples were then added to 3% washed sheep red blood cells in PBS and loaded onto 96-well microtiter plates. PBS only was added as a 0% hemolysis control, and 1% Triton X-100 was added as a 100% hemolysis control.

Plates were incubated for 30 min at 37°C and then read at OD_{540} . Percentage hemolysis was calculated as $\{1 - [(OD_{\text{sample}} - OD_{100\% \text{ control}}) / (OD_{0\% \text{ control}} - OD_{100\% \text{ control}})]\} \times 100$. Results were plotted, and a 50% hemolysis value was calculated.

RESULTS

***B. cereus* ATCC 14579 carries a single *resB* gene and produces active cytochrome *c* oxidase.** In *B. subtilis* and *B. anthracis*, a single *resB* gene encodes a protein required for covalent attachment of heme to apo-cytochrome *c* to produce active *c*-type cytochromes. According to the published genome sequence of *B. cereus* type strain ATCC 14579, ResB is encoded by two genes, BC1474 and BC1475, predicted to encode the N-terminal and C-terminal portions of ResB, respectively (41). BC1474 (GenBank no. [AAP08454.1](#)) and BC1475 (GenBank no. [AAP08455.1](#)) have 98% amino acid identity relative to ResB of *B. anthracis* (GenBank

TABLE 3 TMPD activity and sporulation efficiency in wild-type and mutant *B. cereus* strains

Strain	Genotype	TMPD activity ^a	% sporulation ^b
ATCC 14579	Parental	+	27.3 ± 2.8
AW-C006	$\Delta resBC$	—	26.7 ± 1.6
AW-C008	$\Delta cccA$	+	ND
AW-C009	$\Delta cccB$	+	27.8 ± 3.3
AW-C011	$\Delta cccAB$	+	27.5 ± 2.7
AW-C013	$\Delta ctaC$	—	ND
AW-C017	$\Delta qcrBC$	+	ND
AW-C016	4 cyt	—	26.2 ± 2.0

^a +, TMPD staining detected; —, no TMPD staining detected.^b Percentage of spores relative to the total number of viable cells. Values are means and standard deviations from the means for three independent assays. ND, not determined.

no. AAT53704.1). To confirm this observation, the region carrying BC1474 and BC1475 was amplified by PCR and sequenced. Complete sequencing of *resBC* region demonstrated that the frameshift mutation indicated in the ATCC 14579 genome sequence is not present in our amplified sequence (data not shown), indicating that ResB is encoded by a single, full-length gene highly similar to *B. anthracis resB*. *B. cereus* ATCC 14579 is positive for TMPD oxidase activity, confirming cytochrome *c* maturation activity of the strain (Table 3).

Loss of *resBC* results in increased expression of virulence genes. In *B. anthracis*, loss of the cytochrome *c* maturation pathway results in increased transcription of the anthrax toxin genes (24, 35). In order to investigate the role of cytochrome *c* maturation pathways in *B. cereus* virulence gene regulation, a strain containing a markerless deletion of *resBC* was created (AW-C006). Loss of *resBC* resulted in complete abolition of cytochrome *c* oxidase activity as indicated by TMPD oxidase staining (Table 3).

Loss of *resBC* did not significantly affect growth in LB (Fig. 1A) or BHI (data not shown). To assay the role of ResBC in virulence gene expression, the previously mapped promoter regions of the major enterotoxin genes *cytK* (42), *hbl* (43), and *nhe* (44), as well as the virulence regulator *plcR* (3, 5), were fused to a promoterless *lacZ* gene, and promoter activity was monitored by β -galactosidase analysis. As shown in Fig. 1B, promoter activity of all three enterotoxin genes increased relative to that in the parental strain as the cultures entered stationary phase. In *B. anthracis*, increased toxin gene expression was associated with an increase in transcription of the virulence regulatory gene *atxA* during exponential growth (24). In *B. cereus*, an increase in *plcR* promoter activity was observed during stationary phase relative to that in the parental strain, similar to the profiles of the enterotoxin genes (Fig. 1B, inset). A second, independent *resBC* deletion strain was generated (AW-C019), and the phenotypes associated with this strain are the same as those of the first $\Delta resBC$ strain (data not shown).

To confirm of the role of *resBC* in virulence gene expression, a region containing *resBC* was cloned into plasmid pAW285 (35), placing *resBC* transcription under the control of an exogenous promoter. Figure 2 presents virulence gene expression at 7 h post-inoculation, during stationary phase, when maximum differences in promoter activity were seen in the $\Delta resBC$ strain. Addition of *resBC* to the parental strain ATCC 14579 had no effect on *cytK* promoter activity relative to that in the parental strain transformed with empty vector. Complementation of *resBC* in *trans* expressed from the *B. subtilis xylA* promoter reduced *cytK* expression to parental levels in the $\Delta resBC$ strain, confirming that the increased virulence gene expression in these mutants is due to the loss of *resBC*.

Loss of only *cccB* results in increased expression of virulence genes. In *B. anthracis*, the increased virulence gene expression

FIG 1 Growth and virulence gene expression in parental and $\Delta resBC$ mutant strain. (A) Cell growth of parental and mutant strains grown in LB at 37°C. ■, ATCC 14579; □, AW-C006 ($\Delta resBC$). (B) β -Galactosidase activity in virulence gene reporter strains grown in LB supplemented with kanamycin at 37°C. ■, ATCC 14579 *nhe-lacZ*; ◆, ATCC 14579 *cytK-lacZ*; ▲, ATCC 14579 *hbl-lacZ*; ●, ATCC 14579 *plcR-lacZ*; □, AW-C006 *nhe-lacZ*; ◇, AW-C006 *cytK-lacZ*; △, AW-C006 *hbl-lacZ*; ○, AW-C006 *plcR-lacZ*. (Inset) Magnification of the results from *plcR-lacZ* reporters.

FIG 2 Complementation analysis in $\Delta resBC$ mutant strain. The β -galactosidase activity of ATCC 14579 and AW-C006 ($\Delta resBC$) strains carrying the *cytK-lacZ* reporter plasmid and either empty complementation plasmid (pAW285) or *resBC* complementation plasmid (pAW426), as indicated, and grown in LB supplemented with kanamycin and chloramphenicol at 37°C for 7 h postinoculation was measured. Data were obtained from 3 independent cultures, and error bars represent standard deviations from the means. A two-tailed unpaired *t* test indicated a *P* value of <0.0001 between empty vector and complementation plasmid in the $\Delta resBC$ strain.

associated with loss of cytochrome *c* maturation is the result of deficiency in both the two small *c*-type cytochromes encoded by *cccA* and *cccB*. Loss of other *c*-type cytochromes has no effect on virulence gene expression (24). Deletions of all four *c*-type cytochromes (*cccA*, *cccB*, *ctaC*, and *qcrBC*) singly and in combinations were made. Cytochrome *c* activity was assayed in the mutants by TMPD oxidase analysis (Table 3). As expected, strains lacking *ctaC*, encoding a subunit of cytochrome *c* oxidase, were TMPD negative. All other cytochrome mutants were TMPD positive, showing that loss of these genes has no effect on the activity of cytochrome *c* oxidase. None of the cytochrome *c* mutants displayed a growth defect in LB when grown under aerobic conditions. To analyze gene expression, β -galactosidase analysis was performed using the four virulence gene reporters. Figure 3 represents promoter activity at 7 h postinoculation, when maximum differences in promoter activity were seen in the $\Delta resBC$ strain. The relative gene expression profiles of all four virulence reporters are similar across the strains tested. Loss of *cccA* does not alter promoter activity, while loss of *cccB* increases promoter activity, though not as strongly as in the $\Delta resBC$ strain. A strain missing both *cccA* and *cccB* was similar to the $\Delta cccB$ strain. This is different from what was seen in *B. anthracis*, where loss of either small *c*-type cytochrome had no effect individually and loss of both *cccA* and *cccB* completely replicated the virulence gene overexpression

phenotype of the $\Delta resBC$ mutant (24). Single deletions of *ctaC* and *qcrBC* had no effect on promoter activity (data not shown). Promoter activity in a strain carrying deletions of all *c*-type cytochrome genes was similar to that in the $\Delta cccB$ single deletion mutant but still below the levels in the $\Delta resBC$ mutant.

The *cccB* coding region was cloned into plasmid pHT315S (45), placing *cccB* transcription under the control of a constitutively active, exogenous promoter. Addition of *cccB* to the parental strain ATCC 14579 had no effect on *cytK* promoter activity relative to the parental strain transformed with empty vector (Fig. 4). Complementation of *cccB* in *trans* expressed from a constitutively active, artificial SPAC promoter reduced *cytK* promoter activity to parental levels in the $\Delta cccB$ and $\Delta cccAB$ strains. Similar reductions in promoter activity were seen in the $\Delta cccB$ and $\Delta cccAB$ strains carrying other virulence gene reporters (data not shown), further confirming that the increased promoter activity in these mutants is due to the loss of *cccB*.

Hemolytic activity is increased in cytochrome mutants. *B. cereus* is a beta-hemolytic bacterium due to the production of hemolysins, including hemolysin BL, encoded by the *hbl* gene used in our promoter analysis. In support of the gene expression analysis, quantitative hemolysis assays were performed on the cytochrome mutants. As shown in Fig. 5, hemolysis in the $\Delta cccA$ mutant was similar to that in the parental strain. Strains that

FIG 3 Virulence gene expression in parental and mutant strains. The β -galactosidase activity of parental and mutant strains carrying reporters, as indicated, and grown in LB supplemented with kanamycin at 37°C for 7 h postinoculation was measured. Data were obtained from 3 independent cultures, and error bars represent standard deviations from the means. (A) *nhe-lacZ* strain; (B) *cytK-lacZ* strain; (C) *hbl-lacZ* strain; (D) *plrC-lacZ* strain. Two-tailed unpaired *t* tests indicated *P* values of <0.0001 between the $\Delta resBC$ and parental strains and <0.005 between the $\Delta resBC$ and $\Delta cccB$ mutants for all reporters.

lacked *cccB*, *cccAB*, or all four *c*-type cytochrome genes had hemolysis that was increased to similarly high levels relative to that in the parental strain. Deletion of *resBC* further increased hemolysis beyond the levels measured in the $\Delta cccB$ mutant. These results are consistent with the β -galactosidase data and provide functional confirmation of gene expression analysis.

Sporulation is unaffected in cytochrome mutants. A previous report suggested that loss of the small *c*-type cytochrome *c*₅₅₀, encoded by *cccA*, alters sporulation initiation in *Bacillus subtilis* (46). The effect of loss of *c*-type cytochromes or *c*-type cytochrome maturation on sporulation in *B. cereus* was measured by a liquid sporulation assay. As shown in Table 3, sporulation efficiency was similar to that in the parental strain in all mutants tested, including mutants missing *cccA*, indicating that loss of neither the *c*-type cytochromes nor the *c*-type cytochrome maturation system affects sporulation in *B. cereus*.

Toxin expression in *resBC* mutant is PlcR dependent. In *B. anthracis*, increased toxin gene expression was the result of increased *atxA* transcription (24). *plrC* transcription was elevated in the $\Delta resBC$ mutant and in the mutants containing a deletion of

cccB. To further investigate the role of *plrC*, markerless deletion strains missing *plrC-papR* and both *resBC* and *plrC-papR* were constructed. As shown in Fig. 6, toxin gene and *plrC* expression levels were very low in the $\Delta plrC-papR$ mutant, as expected based on previous findings (3, 5), but expression levels were above those in strains harboring promoterless pTCV-lac. Promoter activities in the $\Delta resBC \Delta plrC-papR$ strain were similar to those in the $\Delta plrC-papR$ deletion strain, suggesting that loss of *resBC* has no effect on virulence gene expression in the absence of *plrC-papR*.

DISCUSSION

This study reveals a role for the *c*-type cytochromes and the *c*-type cytochrome maturation pathway in the regulation of virulence gene expression in *B. cereus*. Loss of the cytochrome *c* maturation genes *resB* and *resC* results in increased expression of the major enterotoxins and the virulence regulator *plrC*. This effect can be partially, but not completely, attributed to loss of *cccB*, encoding cytochrome CccB (*c*₅₅₁). These findings are similar to a previous report that the two small *c*-type cytochromes, CccA and CccB, control virulence gene expression in *B. anthracis*; however, there

FIG 4 Complementation analysis in $\Delta cccB$ mutant strains. The β -galactosidase activity of ATCC 14579, AW-C009 ($\Delta cccB$), and AW-C011 ($\Delta cccAB$) carrying the *cytK-lacZ* reporter plasmid and either empty complementation plasmid (pHT315S) or *cccB* complementation plasmid (pAW396), as indicated, and grown in LB supplemented with kanamycin, erythromycin, and lincomycin at 37°C for 7 h postinoculation was measured. Data were obtained from 3 independent cultures, and error bars represent standard deviations from the means. Two-tailed unpaired *t* tests indicated *P* values of <0.0001 between empty vector and complementation plasmid in both $\Delta cccB$ and $\Delta cccAB$ strains.

appear to be significant differences in the molecular mechanisms of regulation. Unlike *B. anthracis*, where loss of both *cccA* and *cccB* is required for increased anthrax toxin expression, loss of only *cccB* in *B. cereus* results in increased virulence gene expression. In *B.*

anthracis, the presence of CccA can complement the loss of CccB, but in *B. cereus*, CccA does not play a role in this regulatory pathway. CccA and CccB are similar in the amino acid sequences of their extracellular heme-binding domains but differ in their mem-

FIG 5 Hemolytic activity of parental and mutant strains. Cell supernatants of the indicated strains grown in LB at 37°C for 7 h were diluted as indicated. (A) Hemolytic activity, presented as percentage of red blood cell lysis relative to 0% hemolysis and 100% hemolysis controls. Data are averages for three independent cultures. ■, ATCC 14579; ◆, AW-C006 ($\Delta resBC$); ▲, AW-C008 ($\Delta cccA$); ●, AW-C009 ($\Delta cccB$); □, AW-C011 ($\Delta cccAB$); ◇, AW-C016 (4cyt). (B) Mean dilution value at which 50% hemolysis occurs, determined as the mean for three independent cultures $\pm$ standard deviation of the mean.

FIG 6 Virulence gene expression in $\Delta plcR$ -*papR* and $\Delta plcR$ -*papR* $\Delta resBC$ mutant strains. β -Galactosidase activity was measured in virulence gene reporter strains grown in LB supplemented with kanamycin at 37°C. Filled symbols and solid lines represent AW-C007 ($\Delta plcR$ -*papR*), while empty symbols and dashed lines represent AW-C012 ($\Delta plcR$ -*papR* $\Delta resBC$). ■, *nhe-lacZ*; ◆, *cytK-lacZ*; ▲, *hbl-lacZ*; ●, *plcR-lacZ*; ×, promoterless *lacZ*.

brane-anchoring systems: CccA has a predicted α -helical transmembrane polypeptide membrane anchor, while CccB is membrane attached by a diacylglycerol membrane anchor (27). Although CccA and CccB in *B. cereus* may have distinct activities, these functions remain unclear. As in *B. subtilis* and *B. anthracis*, the functions of CccA and CccB are most likely redox related and associated with electron transfer (26), but they are not required for aerobic respiration, as growth is not affected. None of the remaining *c*-type cytochromes (CtaC and QcrBC) are involved in the regulation of virulence gene expression, indicating a specific participation of CccB in enterotoxin gene expression.

Previous studies have examined the role of ORP in enterotoxin production in *B. cereus* F4430/73. Anaerobic conditions lead to increased production of *nhe* and *hbl* (19, 47), while *cytK* and *plcR* transcription is largely unchanged (48). In this study, using *B. cereus* strain ATCC 14579, we demonstrated increased *plcR*, *nhe*, *hbl*, and *cytK* transcription under aerobic conditions in response to the loss of cytochrome *c* maturation or CccB. As control of enterotoxin production occurs at both the transcriptional and posttranscriptional levels (20, 47), increased hemolysis in the $\Delta resBC$ and $\Delta cccB$ functionally demonstrates increased enterotoxin production. These findings may reflect the previously noted differences in response to redox conditions between strains of *B. cereus* (49). The function of CccB remains unclear, as loss of CccB has no effect on growth or sporulation in *B. anthracis*, *B. subtilis*, or *B. cereus* (24, 27). In some thermophilic bacilli, *c*₅₅₁ plays a significant role in oxidation of the terminal oxidase in the respi-

ratory chain and is synthesized under air-limited conditions (50, 51), but the oxidase genes in this pathway are not present in *B. cereus*. It is possible that CccB plays an unappreciated role in respiratory control through an uncharacterized pathway.

These findings are unexpected in light of the differences in virulence gene regulation between *B. cereus* and *B. anthracis*. In *B. anthracis*, loss of cytochrome maturation activity results in increased toxin gene expression through increased *atxA* transcription (24). An *atxA*-like transcriptional regulator is not present in the *B. cereus* genome; instead, transcription of the gene encoding the major virulence regulator in *B. cereus*, *plcR*, is increased in the absence of *c*-type cytochrome maturation. Presence of PlcR is required for the cytochrome-dependent increase in virulence gene expression, arguing against a regulatory mechanism independent of PlcR. There is little similarity in the structure, regulation, or known activities of AtxA and PlcR, which makes a common mechanism acting upon both regulatory proteins unusual. The similarity in phenotype, with loss of cytochrome *c* maturation despite differences in regulatory networks, raises the possibility of a conserved, previously unidentified regulatory pathway at work in both organisms.

The increased virulence gene expression and hemolysis in the $\Delta resBC$ strain relative to the strain missing all known *c*-type cytochromes was surprising given that the only known functions of ResBC are related to heme delivery to *c*-type cytochromes (29, 30). Several possibilities may explain this discrepancy. The ResDE two-component system, known to regulate some enterotoxin gene ex-

pression, is encoded by genes downstream of *resBC*. In *B. cereus* and *B. subtilis*, *resDE* is transcribed both from a promoter upstream of *resA*, producing a *resABCDE* transcript, and from a promoter upstream of *resD*, producing a *resDE* transcript (20, 52). A polar effect on expression of *resDE* is unlikely due to the markerless nature of the *resBC* deletion and the fact that addition of *resBC* in *trans* to the Δ *resBC* mutant can complement the overexpression phenotype. Alternatively, ResBC could transfer heme to a *c*-type cytochrome not found in *B. anthracis* or *B. subtilis*. This is unlikely given the highly similar gene content relative to *B. anthracis*, the lack of additional predicted *c*-type cytochromes in the *B. cereus* ATCC 14579 genome, and the lack of additional predicted transmembrane proteins containing the characteristic CXXCH heme binding motif found in *c*-type cytochromes. Finally, ResBC could have additional activities in *B. cereus* that are not present in or have not been observed in either *B. anthracis* or *B. subtilis*. The role of ResBC in *B. cereus* and related organisms requires further investigation.

Despite the high degree of chromosomal synteny between *B. cereus* and *B. anthracis*, the chromosomally encoded virulence regulatory networks are significantly different. The specificity of virulence control is of interest in light of fatal diseases caused by strains of *B. cereus* that carry virulence genes of both *B. cereus* and *B. anthracis* (53, 54). Insight into the mechanistic differences in the control of virulence factor expression would be helpful in understanding and tracking these dangerous pathogenic strains.

ACKNOWLEDGMENTS

We thank Jenny Kim and Liudmyla Iakovenko for critically reading the manuscript.

This work was supported by Cleon C. Arrington Research Initiation grant UR00010105 from the Georgia State University Research Foundation.

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