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Bacterial *c*-type cytochromes and pathogenicity

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Abstract: During the course of infection, bacterial pathogens must adjust their metabolism for growth in the host and to counteract host response systems that would otherwise eliminate the pathogen. A group of proteins that can participate in such metabolic shifts are the *c*-type cytochromes, a widely distributed class of extracellular hemoprotiens involved in many types of redox chemistry, such as respiration and H_2O_2 scavenging. The *c*-type cytochromes are characterized by covalently attached heme and are assembled at the cell surface by cytochrome *c* maturation systems. Several studies have provided insight into the functions of bacterial *c*-type cytochromes and cytochrome *c* biogenesis systems in the ability to colonize the host and induce pathogenic damage.

Key words: *c*-type cytochrome; cytochrome *c*, virulence; host-pathogen interaction; biofilm, toxin.

Body

c-type cytochromes are widely distributed hemo-proteins important to electron transfer in aerobic and anae-robic respiration as well as various redox reactions. *c*-type cytochromes differ from other types of cytochromes in that the heme prosthetic group is covalently bound to the thiol groups of the cysteine residues in a characteristic CXXCH motif. *c*-type cytochromes can be found in both mono-he-me and multi-heme forms. Some cytochromes utilize non-heme cofactors, such as the *caa*3 and *cbb*3 terminal oxidases that require copper (1). *c*-type cytochromes are found in prokaryotic, algal, and plant chloroplasts where they are important to photosynthesis (2). In human cells, cytochrome *c* is present in mitochondria where it is asso-ciated with induction of apoptosis (3). In bacterial cells, *c*-type cytochromes are found outside the surface of the cytoplasmic membrane, anchored to the outer surface of the membrane or free in the cytoplasm (1).

Cytochrome *c* maturation (Ccm) is a complex, mul-ti-step process that involves separate export of apo-cyto-chrome *c* and the heme prosthetic group followed by at-tachment of heme at the extracellular surface. Six distinct classes of Ccm systems have been identified (4). System I for cytochrome *c* maturation in *Escherichia coli* and other Gram-negative bacteria comprises the CcmA–H proteins (5). System II in *Bacillus subtilis* and other Gram-posi-tive bacteria contains the ResA-C proteins (6). Animal mitochondria use System III (4). System IV is associated with oxygenic phototrophs (7). System V in euglenozoan protozoa and System VI in *Bacillus* species are predic-ted based upon genomic analysis (4). There is significant overlap in the distribution Ccm systems as some orga-nisms carry multiple Ccm systems of different classes (8).

While modulation of mammalian host mitochondrial cytochrome *c* to control apoptosis is a well studied mecha-nism in a number of species (9-12), bacterially-produced cytochrome *c* can also influence virulence. This review will examine several examples of the *c*-type cytochromes and their maturation systems affecting bacterial pathoge-nesis.

Growth under host conditions

Mycobacterium tuberculosis is an obligate aerobic bacterium responsible for the lung disease tuberculosis. *M. tuberculosis* grows slowly in the alveolar macrophages and can persist within the infected host in a long-term asymptomatic state (13). These features have led to stu-dy of respiration in *M. tuberculosis*. Mutagenesis screens indicate that the *ccm*, *qcrCAB* (cytochrome *bc*1 complex), and *ctaCDE* (*aa*3-type cytochrome *c* oxidase) genes are essential to mycobacterial growth (14, 15). Cytochrome *c* oxidase is most efficient terminal oxidase used during exponential growth of *M. tuberculosis*. Transcript levels of *ctaD* and *qcrC* were reduced as part of an overall shift in respiration in a mouse lung model tuberculosis (16). The Ccm protein CcsX is important to growth *in vitro* and in mouse lung model tuberculosis. Loss of CcsX, which eliminates cytochrome *c* maturation, caused an increase in the less efficient cytochrome *bd* production to compen-sate for loss of the cytochrome *c* oxidase (17). Similarly, treatment of *M. tuberculosis* with cytochrome *c* oxidase inhibitors resulted in increased transcription of the *cyd* operon, encoding cytochrome *bd* (18). The importance of cytochromes to mycobacterial infection has led to the characterization of inhibitors of the cytochrome *bc*1 com-plex as drug candidates in the treatment of tuberculosis (19, 20).

Brucella suis, a zoonotic pathogen that can cause brucellosis in humans, also replicates within macrophages during infection. *B. suis* carries two high affinity termi-nal oxidases, cytochrome *cbb*3 and cytochrome *bd*. When grown under microaerobic conditions, the genes enco-

Table 1. Examples of cytochrome *c* contributing to bacterial virulence.

Organism	Cytochrome	Effect	Citation
Bacillus anthracis	^{c}550, ^{c}551	Toxin expression	56-57
Bacillus cereus	Ccm, c_{551}	Toxin expression	60
Brucella suis	cbb_3	Intracellular growth	22
Campylobacter jejuni	multiple	Intracellular growth	24-32
		Intracellular growth	
Legionella pneumophila	Ccm, c_1, c_4, c_5	Iron acquisition	41-43,47
Mycobacterium tuberculosis	Ccm, bc_1, caa_3	Intracellular growth	14-18
Neisseria gonorrhoeae	cbb_3	Biofilm formation	54
Pseudomonas aeruginosa	cbb_3	Biofilm formation	49-53

ding cytochrome *cbb*3 are strongly induced. A *bd* mutant was attenuated relative to wild type or a *cbb*3 mutant in a THP-1 cell culture model of infection, suggesting *bd* is the primary terminal oxidase when growing intracellular-ly (21). In contrast, when the mutant strains were tested in a mouse model of infection, the *cbb*3 mutant was essential for chronic infection. Attenuation of the *cbb*3 mutant was more pronounced in the lower-oxygen liver in compari-son to the more highly oxygenated spleen. Expression of only the genes encoding the *cbb*3 complex was induced when examined in the THP-1 cell culture model under microaerobic conditions (22). These findings emphasize that a cell culture model of infection may not adequately replicate conditions within an animal host. Both *M. tuber-culosis* and *B. suis* differentially utilize terminal oxidases, preferentially using a *c*-type cytochrome complex during intracellular infection.

Campylobacter jejuni, a Gram-negative microaero-phile, is a zoonotic pathogen often spread to humans by contaminated poultry products (23). The *C. jejuni* respi-ratory electron chain is comprised of two terminal oxi-dases: a cyanide-insensitive oxidase with low oxygen affinity and a *cbb*3-type cytochrome *c* oxidase with high oxygen affinity (24). The *cbb*3-type oxidase is required for microaerophillic growth and colonization of the chicken cecum (24, 25). When grown for an extended time in mammalian cells, expression of the *cbb*3-type cytochrome *c* oxidase and the ubiquinol-cytochrome *c* reductase were strongly reduced as part of an adaptation to the intracel-lular low-oxygen environment (26). *C. jejuni* carries a sulfite:cytochrome *c* oxidoreductase system, which al-lows sulphite respiration system and the detoxification of sulfite (27). This system has been shown to modulate motility, host cell adherence and invasion (28). *C. jejuni* also carries two cytochrome *c* peroxidases. Cytochrome *c* peroxidases typically protect cells by reducing hydrogen peroxide to water (29). Neither cytochrome c peroxidase contributes to hydrogen peroxide resistance *in vitro* but are instead required for host colonization by other mecha-nisms (30). Other cytochrome *c* containing complexes may also contribute to pathogenesis as genomic analyses indicate that additional predicted *c*-type cytochromes are found in strains that have high colonization potentials (31) or are highly pathogenic (32). *C. jejuni* utilizes a number of *c*-type cytochromes in adaptation to conditions in the external environment, the poultry host, and mammalian cells.

Iron acquisition and intracellular growth

Legionella pneumophila infects macrophages during human disease, but survives in aquatic environments as intracellular parasites of free-living protozoa (33). In a signature-tagged mutagenesis screen for genes important in infection of amoebas, loss of the Ccm gene *ccmF* de-creased attachment and replication. This *ccmF* mutant did not demonstrate altered ability to infect human macro-phages (34). In Ccm System I, heme is transported across the cytoplasmic membrane by CcmC where it is accep-ted by the heme chaperone CcmE in a process facilitated by CcmD and the ABC transporter composed of CcmAB (35-38). CcmE then shuttles heme to CcmF which, in as-sociation with CcmG and CcmH, acts as a heme lyase to transfer of heme to the cysteine residues of the apocyto-chrome (39, 40). Disruption of *ccmC* reduced the ability of *L. pneumophila* to grow under low-iron conditions and infect human macrophages and amoeba (41, 42). Additio-nal *L. pneumophila* transposon mutants in *ccmB*, *ccmC*, and *ccmF* showed a growth defect on iron-limiting me-dia and were unable to grown in human macrophages or amoeba (43). Ccm proteins contribute to iron acquisition in several environmental species (44-46). Treatment of *L. pneumophila* with an iron chelator reduced macrophage infectivity while supplementation with iron increased macrophage infectivity, though not to infectivity levels of the wild type strain (42, 43). Significantly, the mutants were also growth deficient in the lungs of mice, a model mimicking the pneumonia associated with Legionnaires' disease (43). As the best characterized function of the Ccm system is in the production of active cytochrome *c*, activi-ties of specific *L. pneumophila* *c*-type cytochromes were characterized. Cytochrome *c*4 was required for the produc-tion of the Fe3+ uptake siderophore legiobactin; however, loss of *c*4 did not alter growth on low-iron media or macro-phage infectivity. In contrast, loss of cytochromes *c*1 and *c*5 decreased infection of human macrophages, though le-giobactin production was unaffected (47). That the loss of any single cytochrome *c* cannot replicate the macrophage growth defect of the *ccm* mutant suggests either functional redundancy among cytochromes or additional activities of the Ccm.

Biofilm formation

Production of biofilms is essential to the survival and pathogenesis of many bacteria. The development of bio-films induces changes to the local environment, such as

reduced nutrient and oxygen availability. *Pseudomonas aeruginosa* is an avid biofilm former, and its biofilms contribute to pathogenicity, especially in the lungs of cys-tic fibrosis patients (48). In studying the temporal patterns of protein expression in *P. aeruginosa* biofilm develop-ment, two *c*-type cytochromes were found to be expressed in different patterns. NirN, part of a denitrification sys-tem, was produced in early stages of biofilm production. In later-stage biofilms, a cytochrome *c* oxidase (part of the *cbb*3-1 complex) was preferentially produced. Mutant strains missing *ccoO1*, the gene encoding cytochrome *c* oxidase, could not form mature biofilms, and *ccoO1* trans-cript was only detectible in cells growing in a biofilm (49). *P. aeruginosa* carries two operons encoding *cbb*3 oxidases. *cbb*3-1 is expressed highly and contributes to growth un-der aerobic conditions while *cbb*3-2 expression increases under oxygen limitation (50). Under microaerobic condi-tions, loss of either *cbb*3-1 or *cbb*3-2 did not affect growth, but loss of both simultaneously reduced growth. Similarly, loss of either *cbb*3-1 or *cbb*3-2 did not affect biofilm for-mation, but loss of both simultaneously reduced biofilm formation (51). Beyond the role of the two *cbb*3 in respi-ration under low oxygen conditions, they also participate in denitrification. The *cbb*3 complexes lead to the accu-mulation of NO, resulting in cell elongation (52). This cell elongation promotes formation of robust biofilms in anoxic biofilms (52, 53). The two *cbb*3 oxidases of *P. aeru-ginosa* have multiple, condition-dependent effects on the formation of robust biofilms.

Neisseria gonorrhoeae, the causative agent of go-norrhea, can also form biofilms *in vitro* and *in vivo*. Like in *P. aeruginosa*, the cytochrome *c* oxidase subunit of a *cbb*3 complex is induced in bacteria from biofilms relative to free-living bacteria. Several other *c*-type cytochrome proteins were induced under biofilm conditions, including cytochrome *c* peroxidase and a ubiquinol-cytochrome *c* reductase cytochrome *c*1 subunit (54). *c*-type cytochromes are important to adaptation to local environmental change in maturing biofilms.

Toxin expression

Production of anthrax toxin is essential to virulence of *Bacillus anthracis*, the etiological agent of anthrax (55). In a mutagenesis screen for altered toxin expression, loss of the Ccm gene *resB* resulted in increased toxin expres-sion (56). Like other Gram-positive bacteria, *B. anthracis* utilizes the System II Ccm system. ResB and ResC to-gether appear to act as a heme lysase, attaching extracellu-lar heme to apo-cytochrome *c*, similar to CcmF in *E. coli* (6). Systematic inactivation of the *c*-type cytochromes of *B. anthracis* revealed that loss of both *c*550 and *c*551 was required for increased toxin expression. The functions of c_{550} and c_{551} are unknown in *B. anthracis* or *B. subtilis*, but, because loss of either singly did not increase toxin expres-sion, they likely have similar, partially redundant func-tions. The increase in toxin production is due to increased expression of the master virulence regulator AtxA, but the mechanism by which loss of *c*550 and *c*551 causes increased *atxA* transcription remains unknown (56). Loss of the two CcdA proteins, thiol-disulfide oxidoreductases in the Ccm pathway, also abolished *c*-type cytochrome expression and increased toxin expression (57).

Bacillus cereus is a highly genetically similar pa-thogenic species that also relies on toxin expression for induction of pathogenic damage. *B. cereus* does not pro-duce anthrax toxin but, instead, can release a suite of se-veral cell-damaging toxins (58). Loss of the ResBC Ccm proteins caused an increase in toxin gene expression in *B. cereus* strain ATCC14579. Through systematic inacti-vation of the predicted c-type cytochromes, it was shown that only *c*551 contributes to toxin overexpression. Toxin expression in the ResBC mutant was higher than either the *c*551 mutant or a strain missing all four predicted *c*-type cytochromes in *B. cereus*, suggesting additional activities of ResBC proteins that contribute to regulation of toxin gene expression. *B. cereus* does not carry AtxA, the re-gulator required for increased toxin expression in *B. an-thracis* (56). Instead, toxin gene expression is regulated by the PlcR-PapR quorum sensing system (59). Expression of plcR increased in the ResBC mutant, again suggesting a different mode regulation relative to *B. anthracis* (60). Despite the high degree of similarity between *B. anthracis* and *B. cereus*, the mechanisms by which the *c*-type cyto-chromes and the Ccm machinery affect toxin expression appear to differ significantly.

Conclusions and perspectives

Understanding of the impact of *c*-type cytochromes and Ccm systems on bacterial pathogenesis is expanding. Several pathogens require *c*-type cytochromes for respi-ration under external or host environments, as expected based on their long established role as electron transpor-ters and terminal oxidases. These proteins also contri-bute to virulence through less familiar mechanisms, such as iron acquisition or cell elongation in response to NO. *c*-type cytochromes and Ccm systems have diverse other functions, though these have yet to be shown to direct-ly affect virulence. Important pathogenic processes, such as heme synthesis, oxidative stress response, nitrosative stress response, and siderophore production, can be in-fluenced by the *c*-type cytochromes. Continued analysis of the roles of *c*-type cytochromes and Ccm systems will lead to fundamental insights into bacterial physiology and pathogenesis.

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