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Deletion of the cyclic di-AMP phosphodiesterase gene (*cnpB*) in *Mycobacterium tuberculosis* leads to reduced virulence in a mouse model of infection

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Summary

Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide. The pathogenesis by the causative agent, *Mycobacterium tuberculosis*, is still not fully understood. We have previously reported that *M. tuberculosis* Rv3586 (*disA*) encodes a diadenylate cyclase, which converts ATP to cyclic di-AMP (c-di-AMP). In this study, we demonstrated that a protein encoded by Rv2837c (*cnpB*) possesses c-di-AMP phosphodiesterase activity and cleaves c-di-AMP exclusively to AMP. Our results showed that in *M. tuberculosis*, deletion of *disA* abolished bacterial c-di-AMP production, whereas deletion of *cnpB* significantly enhanced the bacterial c-di-AMP accumulation and secretion. The c-di-AMP levels in both mutants could be corrected by expressing the respective gene. We also found that macrophages infected with *cnpB* secreted much higher levels of IFN- β than those infected with the wildtype (WT) or the complemented mutant. Interestingly, mice infected with *M. tuberculosis cnpB* displayed significantly reduced inflammation, less bacterial burden in the lungs and spleens, and extended survival compared to those infected with the WT or the complemented mutant. These results indicate that deletion of *cnpB* results in attenuated virulence, which is correlated with elevated c-di-AMP levels.

Keywords

Mycobacterium tuberculosis; c-di-AMP; phosphodiesterase; diadenylate cyclase; IFN- β ; virulence

Introduction

Tuberculosis (TB) is the number one killer worldwide due to a single infectious bacterial agent, and the global burden of TB remains enormous. In 2012, there were an estimated 8.6 million new TB cases and 1.3 million people died from the infection (WHO, Global Tuberculosis Report 2013). Our knowledge about the causative agent, *Mycobacterium*

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tuberculosis, is still limited. Therefore, it is essential to understand the biology of this pathogen in order to eradicate the infection.

Bacterial second messengers are molecules that control important signaling cascades in bacteria as well as the host during infection. For example, cyclic AMP (cAMP) is a ubiquitous second messenger that has been studied for several decades in both eukaryotic and prokaryotic cells. It has been extensively reported to regulate bacterial catabolite repression and microbial virulence (McDonough & Rodriguez, 2011). Signaling by cAMP in *M. tuberculosis* has been well established (Bai *et al.*, 2011a, McDonough & Rodriguez, 2011, Shenoy & Visweswariah, 2006b). Most interestingly, *M. tuberculosis* secretes cAMP directly into the infected macrophages (Agarwal *et al.*, 2009, Bai *et al.*, 2009) and interferes with the signaling pathway of the host.

Recently, cyclic di-AMP (c-di-AMP) was recognized as a new signaling molecule (Witte *et al.*, 2008). It is enzymatically converted from two molecules of ATP by diadenylate cyclase and cleaved by distinct c-di-AMP phosphodiesterases to either phosphoadenylyl adenosine (pApA) (Bai *et al.*, 2013, Corrigan *et al.*, 2011, Rao *et al.*, 2010, Smith *et al.*, 2012, Witte *et al.*, 2013) or AMP (Bai *et al.*, 2013). Most bacteria only have one diadenylate cyclase. However, three diadenylate cyclases have been identified in *Bacillus subtilis* including DisA, CdaA (also referred as YbbP), and CdaS (also referred to as YojJ) (Mehne *et al.*, 2013, Romling, 2008). These cyclases could be individually disrupted from the genome but could not be deleted together except when one of them was ectopically expressed (Mehne *et al.*, 2013). Thus, c-di-AMP is essential for the viability of *B. subtilis*. Additionally, diadenylate cyclase encoding genes in *Staphylococcus aureus*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Mycoplasma genitalium*, and *Mycoplasma pulmonis* are all essential (Corrigan *et al.*, 2011, Woodward *et al.*, 2010, Bai *et al.*, 2013), suggesting that c-di-AMP plays an important role in bacterial biology. A transmembrane c-di-AMP phosphodiesterase, GdpP, has been reported in several bacteria including *B. subtilis*, *S. aureus*, *Lactococcus lactis*, *L. monocytogenes*, and *S. pneumoniae* (Bai *et al.*, 2013, Corrigan *et al.*, 2011, Rao *et al.*, 2010, Smith *et al.*, 2012, Witte *et al.*, 2013). Recently, a cytosolic c-di-AMP phosphodiesterase, Pde2, has been recognized in *S. pneumoniae* (Bai *et al.*, 2013), which possesses DHH and DHHA1 domains, but lacks the transmembrane domain, the atypical GGDEF domain, and the PAS domain that are all present in GdpP-like c-di-AMP phosphodiesterase. Overall, c-di-AMP has been linked to bacterial peptidoglycan homeostasis (Corrigan *et al.*, 2011, Luo & Helmann, 2012), cellular resistance to antibiotics (Banerjee *et al.*, 2010, Corrigan *et al.*, 2011, Griffiths & O'Neill, 2012, Luo & Helmann, 2012, Witte *et al.*, 2013), bacterial size and biofilm formation (Corrigan *et al.*, 2011, Pozzi *et al.*, 2012), chain length of *S. pneumoniae* (Bai *et al.*, 2013), potassium uptake (Bai *et al.*, 2014, Corrigan *et al.*, 2013), bacterial infections (Cron *et al.*, 2011, Pozzi *et al.*, 2012, Witte *et al.*, 2013, Schwartz *et al.*, 2012, Yamamoto *et al.*, 2012), and a class of riboswitch (Nelson *et al.*, 2013). In addition, it has also been shown that c-di-AMP represents a bacterial secondary signaling molecule that triggers a cytosolic pathway of innate immunity (Parvatiyar *et al.*, 2012, Sauer *et al.*, 2011, Schwartz *et al.*, 2012, Woodward *et al.*, 2010, Yamamoto *et al.*, 2012). This response is mediated by STING (stimulator of IFN genes)

(Burdette *et al.*, 2011, Jin *et al.*, 2011, Sauer *et al.*, 2011) and DDX41 proteins (Bowie, 2012, Parvatiyar *et al.*, 2012).

In *M. tuberculosis*, Rv3586 (*disA*, also referred as *dacA*) encodes a diadenylate cyclase (Bai *et al.*, 2012, Witte *et al.*, 2008). This protein forms an octamer in solution, which is similar to *B. subtilis* DisA (Bai *et al.*, 2012). Two motifs, DGA and RHR, are highly conserved in DisA of the two bacterial species. It has been recently reported that synthesis of c-di-AMP by a DisA homolog in *Mycobacterium smegmatis* is inhibited by RadA through a physical interaction with DisA (Zhang & He, 2013). Furthermore, a c-di-AMP binding transcription factor, DarR, was identified in *M. smegmatis*, and this transcription factor represses the expression of several genes associated with fatty acid metabolism and transportation (Zhang *et al.*, 2013). However, a DarR ortholog has not been identified in *M. tuberculosis*. How *M. tuberculosis* maintains c-di-AMP homeostasis and transduces the signal remains unknown. In this study, we identify and characterize a c-di-AMP phosphodiesterase in this important pathogen.

Results

M. tuberculosis Rv2837c encodes a c-di-AMP phosphodiesterase

We have characterized *M. tuberculosis* Rv3586 (DisA) as a diadenylate cyclase (Bai *et al.*, 2012). In this study, we identify a c-di-AMP phosphodiesterase in *M. tuberculosis*. From sequence analysis with the *M. tuberculosis* genome (Cole *et al.*, 1998), we were unable to identify a *gdpP* (or *yybT*) ortholog, which encodes a well-characterized c-di-AMP phosphodiesterase (Rao *et al.*, 2010, Corrigan *et al.*, 2011). However, Rv2837c is an ortholog of *S. pneumoniae* *pde2* (Bai *et al.*, 2013), which contains a DHH domain and a DHHA1 domain (Fig. 1A). The Rv2837c protein shares 22.5% identity in amino acid sequence with Pde2, suggesting that Rv2837c encodes a putative c-di-AMP phosphodiesterase. Thus, we designated Rv2837c *cnpB* and the encoded protein CnpB as the second cyclic nucleotide phosphodiesterase in addition to Rv0805 that has been reported to cleave 2',3'-cAMP and 2', 3'-cGMP in *M. tuberculosis* (Keppetipola & Shuman, 2008, Podobnik *et al.*, 2009).

We overexpressed His-tagged CnpB in *E. coli* and purified the protein to homogeneity. The purified protein exhibited an apparent molecular mass of 34 kDa (Fig. 1B). Gel filtration analysis indicated that this protein forms a stable dimer in solution (Fig. 1C), similar to *S. pneumoniae* Pde2 (Bai *et al.*, 2013). In order to optimize the catalytic activity of CnpB, we first utilized bis-*p*-nitrophenyl phosphate (BNPP), a nonspecific substrate of phosphatases and phosphodiesterases, to determine the enzymatic activities of CnpB under different conditions. Our results showed that CnpB preferred Co^{2+} and Mn^{2+} from the metal cations that we tested (Fig. 1D). In terms of pH, CnpB exhibited the highest enzymatic activity at pH 8.5 (Fig. 1E). These results are consistent with that of *S. pneumoniae* Pde2 (Bai *et al.*, 2013).

We determined the phosphodiesterase activity of CnpB towards cleavage of c-di-AMP and cyclic di-GMP (c-di-GMP). Our result showed that CnpB cleaved c-di-AMP exclusively to AMP (Fig. 2A and B) with a Michaelis-Menten constant (*K_m*) of $11.42 \pm 2.96 \mu\text{M}$.

Additionally, CnpB was also capable of converting pApA to AMP (Fig. 2A). These results are also consistent with the activity of *S. pneumoniae* Pde2 (Bai *et al.*, 2013). Furthermore, CnpB exhibited very low activity in cleavage of c-di-GMP to GMP (Fig. 2B). The preference of CnpB to c-di-AMP over c-di-GMP is similar to the observation with GdpP (Rao *et al.*, 2010). These observations indicate that c-di-AMP and c-di-GMP are cleaved by distinct phosphodiesterases.

Both DHH and DHHA1 motifs of CnpB are essential for c-di-AMP phosphodiesterase activity

GdpP possesses two transmembrane domains, a PAS domain, a modified GGDEF domain, a DHH domain, and a DHHA1 domain (Corrigan *et al.*, 2011, Rao *et al.*, 2010). In contrast, CnpB or *S. pneumoniae* Pde2 only possesses a DHH domain and a DHHA1 domain, which supports the observation that DHH and DHHA1 domains are essential for GdpP's phosphodiesterase activity (Rao *et al.*, 2010). Sequence analysis revealed that CnpB belongs to phosphodiesterase RecJ domain-containing proteins. These proteins possess highly conserved DxD, DHH, and GGGH motifs (Fig. 2C). Structural analysis with C3nD software using *Thermus thermophilus* RecJ (PDB code 1IR6) as a template displayed that both the DxD and DHH motifs coordinate Mn^{2+} (Yamagata *et al.*, 2002), while the GGGH residues are structurally adjacent to the DHH motif although it is located in a different domain (Fig. 2D), implicating that these residues might be essential for the enzymatic activity of CnpB. According to the conservation of amino acids in RecJ-like proteins, we replaced both aspartate residues in the DxD motif with alanine, the DHH motif with AAA, or the GGGH motif with GAAH in CnpB (Fig. 2E), and examined the activity of these variants towards cleavage of c-di-AMP. Our result showed that mutation of either the DxD motif or the DHH motif completely abolished the c-di-AMP phosphodiesterase activity of CnpB, indicating that both motifs are essential for the enzymatic activity (Fig. 2F). Mutation of the GGGH motif also significantly reduced the cleavage of c-di-AMP (Fig. 2F), indicating that the GGGH motif also plays a substantial role in cleavage of c-di-AMP. The enzymatic activity of these CnpB variants is consistent with the implication from the structural modeling.

Both DisA and CnpB contribute to c-di-AMP homeostasis in *M. tuberculosis*

In order to examine the biological role of c-di-AMP in *M. tuberculosis*, we generated *disA* and *cnpB* mutants in *M. tuberculosis* H37Rv strain by homologous recombination and complemented both mutants with their open reading frames (ORFs) controlled by *M. tuberculosis* Rv0805 or *tuf* promoter (Table 1). Both complemented strains were engineered in a single copy and integrated at an att-int site (Bai *et al.*, 2011b). We prepared bacterial lysates and detected c-di-AMP levels using a competitive enzyme-linked immunosorbent assay (ELISA). Our result showed that deletion of *disA* in *M. tuberculosis* abolished the production of bacterial c-di-AMP (Fig. 3A), indicating that DisA might be the unique diadenylate cyclase in this pathogen. In contrast, deletion of *cnpB* significantly enhanced the levels of c-di-AMP (Fig. 3A). The c-di-AMP levels of both mutants could be corrected by complementation with the respective gene, indicating that the activities of both DisA and CnpB within *M. tuberculosis* are consistent to the *in vitro* analyses, and both enzymes are required for maintaining c-di-AMP homeostasis in *M. tuberculosis*. We also determined the c-di-AMP levels directly in 7-d culture supernatant of each bacterial strain. Our results

showed that c-di-AMP was undetectable in the culture supernatant with our assay for all the strains except the *cnpB* mutant (Fig. 3B). The relatively large amount of c-di-AMP accumulated in *cnpB* and secreted by this strain suggests that the *M. tuberculosis* wildtype (WT) may also secrete c-di-AMP, but at levels that are beyond our detectable limit, which is ~10 nM.

Deletion of *cnpB* reduces bacterial length of *M. tuberculosis*

It has been reported that deletion of *gdpP* in *S. aureus* significantly reduces bacterial size (Corrigan *et al.*, 2011). In this study, we grew *M. tuberculosis* WT, *disA*, *cnpB*, and the complemented mutants in mycomedium and determined the bacterial growth rate by monitoring optical density (OD) at 600 nm (OD₆₀₀). Our result showed that *disA* grew slightly slower than the WT but the defective growth could not be corrected by complementation (Fig. 4A), suggesting that it is likely caused by a polar effect. The growth rate of *cnpB* is indistinguishable from that of the WT (Fig. 4B). For the bacterial size, *disA* is similar to the WT (not shown). Interestingly, the bacterial length of *cnpB* was reduced approximately 30% relative to that of the WT and the complemented mutant analyzed using Image *J* software (Fig. 4C and D), which is consistent with the report of *S. aureus*. Our result suggests that enhanced c-di-AMP in *M. tuberculosis* modulates bacterial size similar to *S. aureus*.

c-di-AMP produced by *M. tuberculosis* induces IFN- β production

It has been reported that c-di-AMP stimulates a host type I IFN response during infection of *L. monocytogenes* or *C. trachomatis* (Barker *et al.*, 2013, Woodward *et al.*, 2010). Additionally, mycobacterial DNA and peptidoglycan have also been shown to stimulate a type I IFN response (Manzanillo *et al.*, 2012, Pandey *et al.*, 2009). To characterize the host type I IFN response to *M. tuberculosis* *disA* and *cnpB*, we first infected bone marrow derived macrophages (BMDMs) isolated from C57BL/6 WT mice with *M. tuberculosis* and its derivatives and examined the IFN- β secretion from the infected cells. Our result showed that by 5 h post-infection, IFN- β secreted by the *disA* infected macrophages was approximately 4-fold less than those infected by the WT. In contrast, the *cnpB* infected macrophages secreted 10-fold more IFN- β than those infected with the WT (Fig. 5A). This result is coincident with the secretion of c-di-AMP by *cnpB* (Fig. 3B). The enhanced IFN- β secretion by macrophages infected with *cnpB* is unlikely contributed by bacterial DNA since similar numbers of viable bacteria were recovered from macrophages infected with WT, *cnpB*, and the complemented mutant, respectively, at 5 h post-infection (Fig. 5B). However, the present results do not fully rule out the possibility that perturbations of cell wall metabolism in *cnpB* are leading to increased release of DNA. Our ongoing studies using cGAS^{-/-} and STING^{-/-} mice will distinguish these two possibilities. Furthermore, it is well known that bacterial peptidoglycan can induce a type I interferon response through a NOD2 signaling pathway. However, BMDMs isolated from WT and NOD2^{-/-} mice secreted similar levels of IFN- β after infection with either *M. tuberculosis* WT or *cnpB* (Fig. 5C), indicating that the enhanced IFN- β response to *cnpB* is not stimulated by bacterial peptidoglycan. Together, our results suggest that c-di-AMP secreted by *M. tuberculosis* stimulates an IFN- β response in infected macrophages.

Deletion of *cnpB* in *M. tuberculosis* attenuates virulence during infection of mice

In order to investigate the role of *cnpB* in virulence, mice were infected with *M. tuberculosis* WT, *cnpB*, and the complemented mutant, respectively, examined for pathology and bacterial burden in the lungs and spleens at 2, 4, 8, 16 weeks post-infection, and monitored for survival rate over time. From 4 weeks post-infection, the lungs and spleens of mice infected with *cnpB* exhibited smaller sizes compared to those infected with the WT or the complemented mutant (Fig. 6A). Meanwhile, the lungs of mice infected with *cnpB* displayed less inflammation compared to those infected with the WT or the complemented mutant (Fig. 6B). These results indicate that deletion of *cnpB* reduced the immunopathology in the infected lungs and spleens. At 1 d post-infection, similar numbers of bacteria were recovered from the lungs of mice infected with the WT, *cnpB*, or the complemented mutant (Fig. 6C), indicating that mice were initially infected with similar numbers of bacteria. From 8 weeks post-infection, the bacterial burden in the lungs of mice infected with *cnpB* was 10 to 50-fold less compared to those infected with the WT or the complemented mutant (Fig. 6C). Meanwhile, the bacterial burden in the spleens of mice infected with *cnpB* was 5 to 10-fold less than those infected with the WT or the complemented mutant (Fig. 6D). These results are in accordance with the observation that mice infected with *M. tuberculosis disA* exhibited exacerbated lung pathology and bacillary load (Dey *et al.*, 2013). For the survival rate, all mice infected with either the WT or the complemented *cnpB* mutant succumbed to the experiment. The median time of survival for mice infected with the WT and the complemented mutant were 208 d and 203 d, respectively. However, all 15 mice infected with *cnpB* survived the experiment and appeared healthy throughout the experiment. The experiment was terminated at 220 d post-infection (Fig. 6E). Taken together, deletion of *cnpB* in *M. tuberculosis* attenuates virulence in the mouse model of infection, which is likely due to the significantly elevated c-di-AMP levels.

Discussion

M. tuberculosis CnpB has been shown to dephosphorylate 3'-phosphoadenosine 5'-phosphate (pAp) to AMP (Postic *et al.*, 2012). In this study, we demonstrated that *M. tuberculosis cnpB* encodes a c-di-AMP phosphodiesterase and that deletion of *cnpB* significantly enhanced c-di-AMP accumulation and secretion in *M. tuberculosis*. Furthermore, *M. tuberculosis cnpB* exhibited reduced virulence in the mouse TB model. Interestingly, the attenuation of virulence we observed is consistent with previous reports that deletion of c-di-AMP or c-di-GMP phosphodiesterase inhibits bacterial virulence. For instance, deletion of both *pde1* and *pde2*, which possess c-di-AMP phosphodiesterase activity, significantly attenuated virulence of *S. pneumoniae* (Cron *et al.*, 2011, Bai *et al.*, 2013). Moreover, deletion of *gdpP* in *S. pyogenes* also attenuated virulence in a murine subcutaneous infection model (Cho & Kang, 2013). Additionally, deletion of c-di-GMP phosphodiesterase in several pathogens reduces virulence (Bobrov *et al.*, 2011, Kulasakara *et al.*, 2006, Ryan *et al.*, 2009, Sultan *et al.*, 2010, Tamayo *et al.*, 2008, Tischler & Camilli, 2005), whereas deletion of diguanylate cyclase may result in elevated or no change in virulence (Bobrov *et al.*, 2011, Romling *et al.*, 2013, Tischler & Camilli, 2005). Taken

together, we conclude that the attenuated virulence during infection of mice with *cnpB* is caused by the over production of c-di-AMP.

The mechanism by which c-di-AMP attenuates virulence is unclear. It is likely that c-di-AMP induces a protective immune response and/or regulates expression of bacterial virulence genes. Several studies have demonstrated that c-di-AMP stimulates a STING-dependent type I IFN response during infection with *L. monocytogenes* (Jin *et al.*, 2011, Sauer *et al.*, 2011) and *C. trachomatis* (Barker *et al.*, 2013). Our result also suggests that *M. tuberculosis cnpB* induces stronger type I IFN response than the WT. However, the induction of type I IFN has mostly been associated with pathogenesis of *M. tuberculosis* (Berry *et al.*, 2010, Teles *et al.*, 2013, Stanley *et al.*, 2007), and it has been shown that signaling through type I IFNR is important for full virulence of *M. tuberculosis* (Stanley *et al.*, 2007). On the other hand, a protective role of type I IFN during *M. tuberculosis* may not be completely ruled out, as a recent study established a model in which type I IFN limits the number of cells that *M. tuberculosis* can infect in the lungs (Desvignes *et al.*, 2012). Moreover, it has also been shown that both c-di-AMP and c-di-GMP can activate NLRP3 inflammasome, which leads to production of IL-1 β (Abdul-Sater *et al.*, 2013). Our future study will determine whether it is type I IFN or other c-di-AMP stimulated host response that leads to the attenuated infection of *cnpB*.

It has been noted that DAC domain-containing proteins are most frequently found in Gram-positive bacteria phyla Firmicutes and Actinobacteria, and most bacteria contain only one diadenylate cyclase (Corrigan & Grundling, 2013). In contrast, c-di-GMP producing bacteria usually possess many more c-di-GMP synthesis/cleavage enzymes (Romling *et al.*, 2013). Interestingly, both c-di-AMP and c-di-GMP associated genes have been identified in some bacteria, such as *B. subtilis* (Chen *et al.*, 2012, Gao *et al.*, 2013, Luo & Helmann, 2012, Mehne *et al.*, 2013, Rao *et al.*, 2010, Witte *et al.*, 2008) and *M. tuberculosis* (Bai *et al.*, 2012, Gupta *et al.*, 2010). We have reported that *M. tuberculosis* DisA has a preference for ATP, but not GTP, as a substrate (Bai *et al.*, 2012). In this study, we showed that CnpB specifically cleaves c-di-AMP, rather than c-di-GMP, which is consistent with the activity of GdpP in *B. subtilis* (Rao *et al.*, 2010). Additionally, the *M. tuberculosis* Rv1354c protein converts GTP to c-di-GMP, and both Rv1357c and Rv1354c proteins are able to hydrolyze c-di-GMP (Gupta *et al.*, 2010). Therefore, the enzymes for synthesis and cleavage of both c-di-AMP and c-di-GMP are distinct. In contrast to *B. subtilis*, *M. tuberculosis* has fewer enzymes for both c-di-AMP and c-di-GMP metabolism. Thus, we will explore whether there is any overlapping between the signaling pathways of the two dinucleotides in *M. tuberculosis*.

Little is known about how bacteria secrete c-di-AMP, except that *L. monocytogenes* is capable of secreting c-di-AMP via efflux pumps (Schwartz *et al.*, 2012, Woodward *et al.*, 2010). In this study, a large amount of c-di-AMP was detected in *cnpB* and its culture supernatant, indicating that *M. tuberculosis* may also secrete c-di-AMP. However, we were unable to detect c-di-AMP in the culture supernatant of the *M. tuberculosis* WT, suggesting that the levels of c-di-AMP in the culture supernatant might be less than 10 nM, which is much lower than that of cAMP secreted by *M. tuberculosis* (Agarwal *et al.*, 2009, Bai *et al.*, 2009). This is not a surprise since at least 15 adenylate cyclases have been found in *M.*

tuberculosis (Bai *et al.*, 2011a, McCue *et al.*, 2000, McDonough & Rodriguez, 2011, Shenoy & Visweswariah, 2006a), whereas only one diadenylate cyclase has been identified in this pathogen (Bai *et al.*, 2012).

Presently, only a few studies have explored how the signal of c-di-AMP is transduced inside bacteria. A transcription factor DarR has been reported to bind c-di-AMP and regulate fatty acid metabolism (Zhang *et al.*, 2013). However, the direct role of c-di-AMP in gene regulation was not addressed in that report, and we were unable to identify a homology of DarR in *M. tuberculosis*. Recent reports identified a c-di-AMP binding protein in both *S. aureus* (Corrigan *et al.*, 2013) and *S. pneumoniae* (Bai *et al.*, 2014) that plays a role in potassium uptake. At least in *S. pneumoniae*, c-di-AMP impairs potassium uptake by reducing the interaction between the c-di-AMP binding protein and a potassium transporter (Bai *et al.*, 2014). The biological role of c-di-AMP in *M. tuberculosis* remains largely unexplored, which warrants further investigation.

In summary, our study demonstrated that deletion of c-di-AMP phosphodiesterase *cnpB* in *M. tuberculosis* attenuates virulence in a mouse infection model. The attenuation is likely caused by a protective immune response induced by c-di-AMP and/or by c-di-AMP mediated regulation of virulence gene expression in *M. tuberculosis*. Nonetheless, the protective role of c-di-AMP during infection may provide insights into novel vaccine exploration.

Note

During the revision of this manuscript, a paper presented that *M. tuberculosis* Rv2837c encodes a c-di-AMP phosphodiesterase (Manikandan, *et al.* 2014), which is consistent with our result.

Experimental Procedures

Bacterial strains and growth conditions

M. tuberculosis H37Rv was grown in mycomedium, which is Middlebrook 7H9 medium (BD) supplemented with 0.5% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC), 0.05% Tween-80, as previously described (Florczyk *et al.*, 2003) or on Middlebrook 7H10 agar (BD) supplemented with 10% OADC, and 0.01% cycloheximide. Fresh cultures were inoculated from frozen stocks for every experiment. Bacteria were typically used at late log phase after 7 days of growth. Cultures were grown in tissue culture flasks standing with ambient air. *Escherichia coli* strains were grown in Luria-Bertani broth or on Luria-Bertani agar plates. Kanamycin at 25 µg/ml or hygromycin at 50 µg/ml was added for recombinant strains. All cultures were grown at 37°C, except that *M. smegmatis* was grown at 30°C.

Expression and purification of CnpB

M. tuberculosis *cnpB* ORF was amplified by PCR using primers listed in Table 2 and *M. tuberculosis* genomic DNA as template. The PCR product was cloned into pET28a(+) (Novagen) to generate pGB136. Mutations of DxD, DHH, and GGGH motifs in *cnpB* were generated by overlapping PCR, and the DNA fragments with these mutations were cloned

into pET28a(+) to generate pGB152, pGB153, and pGB156, respectively. The four recombinant plasmids were individually maintained in *E. coli* BL21(DE3). CnpB and its variants were expressed and purified as we reported previously (Bai *et al.*, 2012, El Qaidi *et al.*, 2013). Briefly, Overnight culture was inoculated in 500 ml LB broth containing 25 µg/ml kanamycin and incubated at 37°C with shaking to an OD₆₀₀ of 0.6. The cultures were then cooled down to room temperature followed by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After induction at room temperature for 4 h, the bacteria were harvested by centrifugation at 6,000 rpm for 10 min and the pellets were stored at –80°C until use.

Each bacterial pellet was resuspended in 50 ml of buffer A (50 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 10% glycerol, pH7.5) with 1× protease inhibitor (Roche) and sonicated on ice for 10 min with 5 s pulse and 10 s interval. After centrifugation at 12,000 rpm for 30 min at 4°C, the supernatant was loaded onto a Ni-NTA agarose column pre-equilibrated with buffer A at a flow rate of 0.5 ml/min. The column was subsequently washed with 50 ml of buffer B (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.5) and 50 ml buffer C (50 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, pH7.5) at 1 ml/min. The His-tagged proteins were eluted with 10 ml buffer D (50 mM Tris-HCl, 500 mM NaCl, 300 mM imidazole, pH 7.5) at 0.5 ml/min. All eluted fractions were analyzed by SDS-PAGE, and collections were dialyzed against phosphate buffered saline (PBS) at 4°C. The purified proteins were stored in PBS with 10% glycerol at –80°C until use.

Gel filtration

Size-exclusion chromatography experiment was performed with a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) and connected to a GradiPhrac Automatic Sampler (Amersham Biosciences). The column was equilibrated and eluted at a constant flow rate of 0.5 ml/min with 10% (v/v) glycerol in PBS. Molecular mass of the protein was determined by using a Gel Filtration Standard (Bio-Rad) per the instruction of the Gel Filtration Principles and Methods (GE Healthcare).

Cleavage of BNPP

Reaction (50 µl) mixtures consisted of 50 mM Tris-HCl at specified pH, 10 mM NaCl, 5 µM 2-mercaptoethanol, 0.1 mM of specified metal cation, and 2 mM BNPP. The reaction was initiated by addition of 3 µM purified CnpB and was incubated at room temperature for 10 min. Relative BNPP cleavage was determined by measuring OD at 405 nm (OD₄₀₅) using a microplate reader (Spectrum Max 340PC, Molecular Devices). The assay conditions used for cation screening contained 0.1 mM CaCl₂, CoCl₂, FeCl₂, Fe(NO₃)₃, MgCl₂, MnCl₂, NiSO₄, or ZnSO₄. For pH analysis, reaction mixtures consisted of 0.1 mM MnCl₂ and 50 mM Tris-HCl at pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, or 9.5.

High-performance liquid chromatography (HPLC)

The reaction (10 µl) mixtures to determine the activity of CnpB contained 50 mM Tris-HCl (pH 7.5), 1 mM MnCl₂, and 0.5 mM indicated nucleotide. The reaction was initiated by adding 3 µM CnpB and incubated for 1 h at 37 °C. Subsequently, each reaction was terminated by adding 1 µl of 0.5 M EDTA and diluted 1:5 with water. Finally, 20 µl of each

sample was injected and separated by reverse-phase HPLC with a C-18 column (250×4.6 mm, Vydac) using a Waters 625 LC system equipped with a 996 Photodiode Array Detector and a 717 Autosampler (Waters). Samples were eluted using the same buffers and program as previously reported (Ryjenkov *et al.*, 2005). Nucleotides were monitored at 254 nm.

Construction and complementation of mutants

In this study, *M. tuberculosis* *disA* was generated using homologous recombination as previously reported (Bai *et al.*, 2011b). Briefly, upstream and downstream fragments of *disA* were amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as template. The two flanking DNA fragments were sequentially sub-cloned into pMBC851 at NotI and PflMI sites, respectively, and sequencing verified to generate pMBC1194. This plasmid was linearized with PacI and ligated with PacI-digested shuttle phasmid phAE159 (kindly provided by Dr. William Jacobs) (Vilcheze *et al.*, 2008). The ligation mixture was packaged using MaxPlax lambda packaging extracts (Epicentre Biotechnologies) and transduced into *E. coli* HB101. Phasmid DNA (designated pMBC1206) was prepared from hygromycin-resistant colonies and transformed into *M. smegmatis* mc²155. Phages were prepared from transformed *M. smegmatis* similar as earlier reports (Bardarov *et al.*, 2002, Tufariello *et al.*, 2004). *M. tuberculosis* grown to log phase were infected with high titer phages (Bardarov *et al.*, 2002). *disA* candidates were screened by PCR using primers as listed in Table 2.

M. tuberculosis *cnpB* mutant was generated similarly but using a different plasmid. Briefly, an upstream fragment was cloned into pJSC407 (Kindly provided by Dr. Jeffery Cox) (Grundner *et al.*, 2008) between EcoRV and HindIII sites to generate pGB128, and a downstream fragment of *cnpB* was then cloned into pGB128 between XbaI and KpnI sites to generate pGB132. This plasmid was then used to generate phage and delete *cnpB* using the same procedure as that for deletion of *disA*.

To complement the mutants, *M. tuberculosis* *tuf* promoter was amplified by PCR and cloned into pMBC304 between HindIII and KpnI sites to generate pMBC1258, The *disA* ORF was then cloned into this plasmid to generate pGB086, and this plasmid was transformed into *disA* to complement the mutation. Meanwhile, Rv0805 promoter was amplified by PCR and cloned into pMBC304 between HindIII and KpnI sites to generate pMBC1260. The *cnpB* ORF was then cloned into this plasmid to generate pGB120. This plasmid was eventually transformed into *cnpB* to complement the mutation.

Determination of bacterial growth and size

M. tuberculosis WT, *cnpB*, and the complemented mutant were grown in mycomedium until 11 d post inoculation. Bacterial growth was determined on day 1, 3, 5, 7, and 11 by measuring OD₆₀₀. To examine bacterial size, 7-d culture was washed once with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Bacteria were then smeared, stained with acid-fast staining (Bai *et al.*, 2009), and bacterial length of the microscopic images of 100 individual bacteria, which are 10 bacteria randomly selected in each of 10 fields, were analyzed with Image J software (<http://rsbweb.nih.gov/ij/>).

Detection of c-di-AMP

Bacteria were grown in Sauton's broth (Bai *et al.*, 2009) for 7 d and the OD₆₀₀ of each strain was determined. Bacteria were then harvested and the supernatant was taken to determine secreted c-di-AMP. Each bacterial pellet was resuspended in 0.5 ml 50 mM Tris-HCl (pH8.0), heat-killed, and disrupted with 1 mm beads with a bead-beater (BioSpec). The lysate was collected, and the bacterial debris was removed by centrifugation for 10 min at 13,000 rpm. The supernatant was used as bacterial c-di-AMP sample. The concentration of c-di-AMP was then determined using a competitive ELISA as we described previously (Bai *et al.*, 2013, Bai *et al.*, 2014).

Infection of macrophages and detection of IFN- β

Murine BMDMs of C57BL/6 and NOD2^{-/-} mice (The Jackson Laboratory) were generated as previously described (Jin *et al.*, 2011). Bone marrow cells harvested from mouse femurs were cultured in DMEM (Life Technologies) containing 20% FBS (BioSource International), 10% M-CSF-containing media, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M β -mercaptoethanol. The medium was exchanged after 3 d, and cells were stained for the macrophage marker F4/80 at day 7.

Cells were seeded in 6-well or 12-well plates as specified. On the following day, the medium was exchanged with infection media with a multiplicity of infection (MOI) at 2:1, which was verified by plating dilutions on Middlebrook 7H10 plates to numerate colony-forming units (CFU) after 3 weeks of incubation. At 5 h post-infection, the supernatant was collected and filtered to determine IFN- β using the Verikine Mouse IFN- β ELISA kit (PBL Assay Science) per the manufacture's manual. To determine intracellular bacterial CFU, cells were washed with PBS for three times and then lysed in 0.5% Triton and 0.5% sodium deoxycholate (DOC) as previously described (Bai *et al.*, 2011b). Serial dilutions of the lysate were plated onto Middlebrook 7H10 plates and CFU were enumerated after 3 weeks.

Infection of mice

This experiment was performed at Johns Hopkins Center for TB Research through an NIH contract. *M. tuberculosis* H37Rv WT, *cnpB*, and the complemented mutant were grown in mycomedium. Female BALB/c mice at 4- to 5-week old were infected with an aerosol of *M. tuberculosis* culture using Glas-Col aerosol chambers by adjusting the inoculum to implant ~ 10,000 CFU in the lungs. At 0, 2, 4, 8, 12, and 16 weeks post infection, 5 mice infected with each bacterial strain were euthanized at each time point to examine bacterial burden in the lungs and spleens, gross pathology, and tissue histology of the lungs. For bacterial burden, lungs and spleens were individually homogenized in sterile equipment. The homogenates were plated to enumerate CFU after 21 d of incubation. Additionally, 15 mice of each infected group were monitored daily for time-to-mortality until 220 d post-infection.

Sequence analyses, structural modeling, and statistics

Multiple sequence alignment was performed using ClustalW method of MegAlign (Lasergene). Protein structural modeling was performed using C3nD (Wang *et al.*, 2000). A

structural of *T. thermophilus* RecJ (PDB code 1IR6) (Yamagata *et al.*, 2002) was used as template. Data of c-di-AMP levels, bacterial CFU, and IFN- β secretion were analyzed by a two-tailed *t*-test, except specified, using Prism 5 (GraphPad Software). Survival rate of the infected mice was analyzed by Log-rank (Matel-Cox) test using Prism 5. *P* values of < 0.05 were considered to be statistically significant.

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Fig. 1. Purification and characterization of CnpB. (A) Domain architecture of CnpB. The numbers indicated under the DHH and DHHA1 domains are amino acid positions in the full-length protein. (B) His-tagged CnpB was expressed and purified from *E. coli*, and was analyzed by SDS-PAGE stained with Coomassie bright blue. Lane M, broad range protein ladder (BioRad). (C) Gel filtration chromatograph of CnpB. The molecular mass (in kDa) and the retention volumes of the standards are indicated. (D and E) Cleavage of BNPP by purified CnpB in the presence of indicated metal cations (D) or at different pH (E). The reaction in “Control” of panel D was not supplemented with any cation. The data shown in panels D and E are the means of three independent experiments. The error bars indicate the standard errors of the means (SEMs).

Fig. 2.

Phosphodiesterase activity of CnpB. (A) CnpB was incubated with c-di-AMP or pApA, as indicated. Each sample was separated by HPLC and monitored at 254 nm. Purified c-di-AMP, pApA, and AMP were used as standards. (B) Kinetics of CnpB in cleavage of c-di-AMP and c-di-GMP. c-di-AMP or c-di-GMP at indicated concentrations was incubated with CnpB for 1 h. The enzymatic activities were determined using HPLC. The data shown are the means of three independent experiments. The error bars indicate the SEMs. (C) Sequence alignment of *M. tuberculosis* CnpB with *S. pneumoniae* Pde1 and Pde2. Identical

residues are highlighted in yellow. Highly conserved amino acids in phosphoesterase RecJ domain-containing proteins are indicated with asterisks. (D) Structural modeling of *T. thermophilus* RecJ (PDB code 1IR6) with Cn3D software. The indicated residues, DAD, DHH, and GG, are shown in yellow. A grey ball indicates Mn^{2+} . DHH domain, DHHA1 domain, and a long helix connecting the two domains are shown in purple, brown, and blue, respectively. (E) Purification of CnpB and CnpB with mutation of DHH, DAD, and GG to AAA, AAA, and AA, respectively. Approximately 2 μ g of each indicated protein was loaded. Lane M, Fisher BioReagents EZ-run prestained Rec protein ladder (Fisher Scientific). (F) Enzymatic activity of CnpB variants relative to native CnpB in cleavage of c-di-AMP to AMP. The same amount of CnpB and the indicated proteins were used to determine the activity. The amount of cleaved c-di-AMP detected in the reaction with protein containing GG to AA mutation was compared to that with CnpB, as shown in percentage.

Fig. 3.

Determination of bacterial (A) and secreted (B) c-di-AMP. Bacteria were grown in Sauton's broth for 7 d and were harvested by centrifugation. The c-di-AMP levels in the supernatant (Secreted) and in the bacterial lysate (Bacterial) were determined using ELISA as described in the Experimental Procedures section. The data shown are the means of three independent experiments. The error bars indicate the SEMs, ***, $P < 0.001$.

Fig. 4.

Growth curve and bacterial size of *M. tuberculosis* and its derivatives. (A and B) Growth curve of *M. tuberculosis* WT, the indicated mutants, and the complemented mutants in mycomedium. The growth was monitored at days 1, 3, 5, 7, and 11. The data shown are the means of three independent experiments. The error bars indicate the SEMs. Note that the growth rate of *disA* is the same as the complemented *disA* (A) and both *cnpB* and the complemented mutant are indistinguishable from the WT (B). (C) Morphology of *cnpB*. *M. tuberculosis* WT, *cnpB*, and the complemented mutant were fixed with 4% paraformaldehyde and stained with Kinyoun acid-fast stain (Kinyoun, 1915). (D) Determination of bacterial size. Bacteria were grown in mycomedium for 7 d and analyzed microscopically. The relative cell length (in arbitrary unit) of 100 bacteria per strain was analyzed with Image *J* software. The data shown are the means of 100 bacteria. The error bars indicate the SEMs. ***, $P < 0.0001$. The graph is a representative of three independent experiments.

Fig. 5.

Analysis of IFN- β secreted by BMDMs. (A) IFN- β secreted by BMDMs of WT mice 5 h post-infection with *M. tuberculosis* and its derivatives. Cells were seeded in 6-well plates at 2×10^6 cells/well. "Control" denotes BMDM cells without infection. The data shown are the means of three independent experiments. The error bars indicate the SEMs. *, $P < 0.05$ in a one-tailed t -test compared to the WT infected macrophages. (B) Bacteria recovered from infected BMDMs in experiments shown in panel A. The data shown are the means of three independent experiments. The error bars indicate the SEMs. (C) IFN- β secreted by BMDMs

of WT and NOD2^{-/-} mice 5 h post-infection with *M. tuberculosis* and its derivatives, respectively. BMDM cells were seeded in 12-well plates at 1×10^6 cells/well in triplicates. The data shown are the means of samples in triplicate. The error bars indicate the SEMs. **, $P < 0.01$, ***, $P < 0.001$.

Fig. 6.

Evaluation of virulence of *cnpB* in a mouse infection model. (A) Gross pathology of lungs and spleens of the mice at 8 weeks post-infection with *M. tuberculosis* WT, *cnpB*, and the complemented mutant. Data shown is a representative of 5 mice for each group. (B) H&E stained lung sections of an uninfected mouse or infected mice at 8 weeks post-infection with *M. tuberculosis* WT, *cnpB*, and the complemented mutant. The data shown is a representative of 5 mice for each group. Note that the lung section of an uninfected BALB/c mouse was stained separately from the infected lungs. (C and D) Bacterial burden in the

lungs (C) and spleens (D) of the infected mice at the indicated time points post-infection with *M. tuberculosis* WT, *cnpB*, and the complemented mutant (n = 5). The data shown are the means of CFU. The error bars indicate the SEMs. **, $P < 0.01$; ***, $P < 0.001$. (E) Survival rate of mice infected with *M. tuberculosis* WT, *cnpB*, and the complemented mutant (n = 15). Mice were infected with approximately 10,000 CFU of each strain to the lungs using an aerosol chamber. ***, $P < 0.001$ analyzed using Log-rank test.

Table 1

Plasmids used in this study

Strain or plasmid	Description ^a	Source
pMBC851	<i>M. tuberculosis</i> knockout plasmid; Hyg ^r	(Bai <i>et al.</i> , 2011b)
phAE159	Knockout phasmid; Carb ^r	(Vilcheze <i>et al.</i> , 2008)
pMBC1193	pMBC851 carrying <i>disA</i> downstream fragment; Hyg ^r	This study
pMBC1194	pMBC1193 carrying <i>disA</i> upstream fragment; Hyg ^r	This study
pMBC1206	phAE159 carrying pMBC1194 plasmid DNA; Hyg ^r	This study
pJSC407	<i>M. tuberculosis</i> knockout plasmid; Hyg ^r	(Grundner <i>et al.</i> , 2008)
pGB128	pJSC407 carrying <i>cnpB</i> upstream fragment; Hyg ^r	This study
pGB132	pGB128 carrying <i>cnpB</i> downstream fragment; Hyg ^r	This study
pGB139	phAE159 carrying pGB132 plasmid DNA; Hyg ^r	This study
pET28a(+)	Expression plasmid; Kan ^r	Novagen
pGB136	pET28a(+) carrying <i>cnpB</i> ORF; Kan ^r	This study
pGB152	pET28a(+) carrying <i>cnpB</i> ORF with DAD to AAA mutation; Kan ^r	This study
pGB153	pET28a(+) carrying <i>cnpB</i> ORF with DHH to AAA mutation; Kan ^r	This study
pGB156	pET28a(+) carrying <i>cnpB</i> ORF with GG to AA mutation; Kan ^r	This study
pMBC304	pCRII carrying <i>M. tuberculosis</i> att-int sequence; Kan ^r ; Ap ^r	This study
pMBC1258	pMBC304 carrying <i>M. tuberculosis</i> <i>tuf</i> promoter; Kan ^r ; Ap ^r	This study
pMBC1260	pMBC304 carrying Rv0805 promoter; Kan ^r ; Ap ^r	This study
pGB086	pMBC1258 carrying <i>disA</i> ORF; Kan ^r ; Ap ^r	This study
pGB120	pMBC1260 carrying <i>cnpB</i> ORF; Kan ^r ; Ap ^r	This study

^a Hyg^r, hygromycin resistance; Kan^r, kanamycin resistance; Carb^r, carbenicillin resistance; Ap^r, ampicillin resistance.

Table 2

Primers used in this study

Primer	Oligo sequence (5' to 3') ^a	Purpose ^b
Mutagenesis		
KM2889	<u>g</u> cgggccggtgcagaccatgtccacc	<i>disA</i> upstream; For
KM2890	<u>g</u> cgggccgacagcctcacgcagggtcg	<i>disA</i> upstream; Rev
KM2891	<u>c</u> catagattgcggaatcgaccatcagc	<i>disA</i> downstream; For
KM2892	<u>c</u> catcttttggacaggccaccgttaacacg	<i>disA</i> downstream; Rev
KM2893	cgtgcacaccgtgctcgacc	Verify <i>disA</i> upstream crossover; For
KM2084	cggccgcataatagactca	Verify <i>disA</i> upstream crossover; Rev
KM2894	gcgaggaggtctgagttacc	Verify <i>disA</i> downstream crossover; For
KM2585	aacggcaggtatgtgatgg	Verify <i>disA</i> downstream crossover; Rev
KM2917	tctgcgatggtggtctctcc	<i>disA</i> internal; For
KM2918	cggtaatacgtgacgttcc	<i>disA</i> internal; Rev
JY207	tttgatactactactcggtcatctacc	<i>cnpB</i> upstream; For
JY208	tttaagcttgcgaccagctcactccttgg	<i>cnpB</i> upstream; Rev
JY209	ttttctagatggccgggtatagcacc	<i>cnpB</i> downstream; For
JY210	tttggtacctcgctagcgaatcaagaac	<i>cnpB</i> downstream; Rev
JY211	tgtgcgcgccgaaggcaagg	Verify <i>cnpB</i> upstream crossover; For
JY002	tcgacgacctgcaggcatgc	Verify <i>cnpB</i> upstream crossover; Rev
JY003	actggcgagttcctctgagg	Verify <i>cnpB</i> downstream crossover; For
JY212	cagcgaaacgccgtcaccc	Verify <i>cnpB</i> downstream crossover; Rev
JY234	tggcgcatctaactgattcc	<i>cnpB</i> internal; For
JY235	catcaaggtcctgctgacgg	<i>cnpB</i> internal; Rev
Complementation		
KM3070	tttaagcttacaccgaggactacatggg	<i>tuf</i> promoter; For
KM3071	tttggtaccgccaattttgtctctcc	<i>tuf</i> promoter; Rev
KM3072	tttaagcttgatgctgctcgtgagttgc	Rv0805 promoter; For
KM3073	tttggtaccacccgaggtgccaaccctc	Rv0805 promoter; Rev
KM3098	tgatcatatgcacgctgtgactgtcc	<i>disA</i> ORF; For
KM3099	tgatcaaagcggataattattgatgc	<i>disA</i> ORF; Rev
JY213	tttggtaccgtgacgacgatcgaccaagg	<i>cnpB</i> ORF; For
JY214	tttgatccgccagctgcgcgatctgacg	<i>cnpB</i> ORF; Rev
Overexpression		
JY217	cggcatatggtgacgacgatcgaccaag	<i>cnpB</i> ORF; For
JY214	tttgatccgccagctgcgcgatctgacg	<i>cnpB</i> ORF; Rev
JY224	gtctgccacgtccaccccgagccgcaaccatcgccgaggattg	CnpB DAD to AAA mutation; For
JY225	caatccggcgccgatggttgcgctgcgggtggacgtggcagac	CnpB DAD to AAA mutation; Rev
JY226	cgggagctccttggaatcgacgagccgctccaacgacctgttc	CnpB DHH to AAA mutation; For
JY227	gaacaggtcgttgaggcggctgctcgattaccaggagctcccg	CnpB DHH to AAA mutation; Rev
JY232	gttgccctctgggttcggtgccgtggtcaccgctgcccgcg	CnpB GG to AA mutation; For
JY233	cgcggccagccggtgaccagcggcaccgaaccagaggcaac	CnpB GG to AA mutation; Rev

^aThe restriction site is underlined if presents in an oligo.

^bFor, forward primer; Rev, reverse primer.