Studies on NonA-dependent Bacteriophage Abortive Infection in *Bacillus subtilis* Marburg Strain

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Introduction.

Bacteriophages (phages) were discovered by Twort in 1915 and have played a key role in genetics and molecular biology. Bacteria and phages exist in almost all environment. Phages outnumber bacteria by approximately 1 to 10-fold and ~ 10^{25} phages initiate infection per second on a global scale. Thus, bacteria have developed several phage resistance systems. These resistance systems can be classified into four groups as follows: preventing phage adsorption, preventing phage DNA entry, cutting phage nucleic acids and abortive infection. Phage adsorption onto host cell is the initial step of infection. Recognition of host-specific cell surface component is required for adsorption to host cell. Bacteria can resist phage adsorption by modulating the availability of, or denying phages access to receptors. After adsorption to the host cell surface, phages inject their DNA into the host cell. Immunity against specific phages is conferred via superinfection-exclusion proteins that block phage DNA injection into host cells. Restriction enzymes or CRISPR/Cas complexes immediately recognize and cleave phage DNA upon its entry into a host cell and thus restrict phage growth. Abortive infection genes affects a crucial step in the phage infection cycle, such as

genomic DNA replication, RNA transcription, translation, or host cell lysis, preventing the spread of phages. In contrast to former three phage resistance systems, mode of action of abortive infection gene has been poorly characterized due to their diversity. Since most abortive infection genes are toxic for the host, these genes should be tightly regulated. However, the regulatory mechanisms for most of them remain unknown. Thus, studies on abortive infection are important to increase understanding of how bacteria resist phage infection.

Bacillus subtilis Marburg strain is resistant to bacteriophage SP10, but strains harboring mutations in both *nonA* and *nonB* genes are susceptible to this phage. The *nonB* mutation is a nonsense mutation in *ydiR*, which is a component of the restriction system. On the other hand, mutational analysis predicted that the *nonA* gene is located on the intergenic region between *bnrdE* and *bnrdF*, which encompasses SPβ prophage region. It is predicted that *nonA* is an abortive infection gene, but its coding region have not yet been determined. Here I identified the locus of the *nonA* gene and analyzed its transcriptional regulation and mode of function.

Results.

To determine the location and direction of *nonA* gene, I carried out Northern blotting with RNA probes and 5'/3' RACE. Total RNAs were prepared from the ASK3000 (Δ*ydiO-ydjA* (*nonB*)) and ASK3002 (ΔSPβ, Δ*ydiO-ydjA*) strains with or without SP10 infection. Northern blotting and RACE analysis revealed that *nonA* was transcribed as 370 nt RNA from *bnrdEF* intergenic region, and its direction was opposite to that of *bnrdEF*. The region located 130 nt from 3' end of *nonA* overlaps with the coding sequence of *bnrdE* gene. I found 72 amino acids ORF in the *nonA* transcriptional region. Mutations were introduced into ribosomal binding site or start codon of the ORF. Strains harboring the mutated *nonA* gene were susceptible to SP10 phage, indicating that the 72 amino acids protein is required for *nonA*-dependent resistance to the SP10 phage in *B. subtilis*.

A blast search of NonA identified no homologs. I predicted domain structure of NonA and found a predicted trans membrane domain in the middle of NonA. Western blotting with cytoplasmic and membrane fractionated lysate from SP10 infected cells was performed to determined localization of NonA. The band corresponding to NonA was detected in membrane fraction, indicating that it localizes

to the membrane.

The *nonA* mRNA was detectable at late stage of SP10 infection but not in the cells without SP10 infection. This suggested that the product(s) of SP10 gene(s) activated the transcription of *nonA*. SP10 phage has three sigma factor homologs, Orf120, Orf183 and Orf199-200. I cloned each gene under the control of the xylose-inducible promoter in pWH1520 and transformed the plasmid into *B. subtilis*. When each sigma factor homologue was expressed in non-infected cells, the *nonA* transcript was detected after the induction of $\sigma^{Orf199\text{-}200}$, indicating that *nonA* transcription is regulated by the sigma factor, Orf199-200.

SP10 gene was transcribed in $nonA^{+}$ strain as well as in *nonA* deleted strain. This suggests that NonA does not affect transcription of SP10 gene. I analysed whether or not NonA affects SP10 protein synthesis. Cell lysates were prepared from TAY3000 (Δ*ydiO-ydjA*, *amyE*::*kan*), TAY3200 (ΔSPβ, Δ*ydiO-ydjA*, *amyE*::*kan*) and TAY3201 (ΔSPβ, Δ*ydiO-ydjA*, *amyE*::*nonA*, *kan*) infected with SP10, and proteins were resolved by SDS-PAGE and visualized by CBB staining. SP10 capsid and sheath proteins were accumulated in TAY3200 but not in TAY3000 and TAY3201. These proteins are

consistent of phage particle. I used transmission electron microscopy to determine whether or not SP10 particles are formed in the cells. SP10 particles were found in TAY3200 but not in TAY3000 and TAY3201. Taken together, these results suggest that NonA inhibits the accumulation of SP10 particle proteins and diminishes SP10 particle formation.

The NonA was expressed only during SP10 infection. I constructed IPTG-inducible NonA expression *B. subtilis* and *Escherichia coli* to analysing the effect of NonA expression in non-infected cells. The cell growth was halted immediately after NonA expression in both *B. subtilis* and *E. coli*. The efficiency of colony formation was also reduced when NonA protein was expressed. These results show that NonA is harmful to these bacteria. NonA is a membrane-localized protein. I examined whether NonA expressin results in membrane damage that is involved in the inhibition of growth and colony formation. NonA expressed cells of *B. subtilis* was treated with propidium iodide (PI) and observed by fluorescence microscopy. PI can only enter cells with injured membrane. NonA expressed cells were hardly stained by PI, suggesting that expression of NonA does not seriously damage the membrane. I then treated NonA

expressed cells with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). Since the reduced form of CTC emits red fluorescence, CTC specifically stains cells with respiratory activity. CTC did not stain NonA expressed cells. Taken together, these results suggest that NonA expression reduces cellular respiratory activity and inhibit cell growth.

Discussion.

In this study, I found that *nonA* encodes 72-amino acids membrane protein. The NonA has no known motif and domain, and blast search did not find any protein similar to NonA. Complementation analysis showed that NonA protein confers a non-permissive phenotype upon SP10. SP10 gene transcription was not repressed in *nonA*$^{+}$ cells, but accumulation of SP10 capsid and sheath proteins and particle formation were inhibited. It is therefore concluded that the *nonA* is a novel abortive infection gene.

Transcription of *nonA* gene was regulated by SP10 sigma factor, Orf199-200. Thus, *B. subtilis* uses the SP10 phage sigma factor to express NonA in a timely manner to abort SP10 infection. Most abortive infection genes are constitutively expressed, but expression or activation of some of them are regulated by the producs of phage gene. For example, translation of *Lactococcus lactis abiD1* is repressed under normal growth

condition, but bIL66 phage Orf1 protein activates *abiD1* translation. T4 Gol and Stp regulate activities of *E. coli* abortive infection protein Lit and PrrC, respectively. In contrast to these genes, *nonA* was regulated at the transcriptional level. To the best of my knowledge, this is the first demonstration of a sigma factor from a virulent phage regulating the transcription of a phage resistance gene in a host genome.

The amount of SP10 early, middle and late genes transcripts did not differ between $nonA^+$ and *nonA* deficient strains. However, SP10 capsid and sheath protein, which are expressed at late stage of SP10 infection, did not accumulate in $nonA^+$ strain. Middle and late genes transcriptions are regulated by SP10 sigma factor that are expressed at early and middle stage, respectively, suggesting that they are translated in $nonA^+$ cells. Thus, NonA might inhibit SP10 gene expression at post-transcriptional level. Artificial expression of NonA in non-infected cell inhibited cell growth and respiratory activity. The NonA expression level was almost same between NonA induced cell and SP10 infected cell. Based on these data, I speculated that NonA reduces the respiration activity of infected cells, which results in the inhibition of translation and in abortive infection, but not directly inhbit SP10 gene translation.

In this study, I identified a novel abortive infection gene, *nonA*, and revealed the mechanism of its transcriptional regulation. Furthermore, I found that NonA inhibits expression of the structure proteins of SP10 virion particle. Although abortive infection genes have been found in many bacterial species, the mechanisms remain largely unknown. I believe that these findings will increase understanding of the mechanism of abortive infection in bacteria.