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LETTER TO THE EDITOR

Persister mechanisms in *Borrelia burgdorferi*: implications for improved intervention

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Dear Editor,

Lyme disease caused by *Borrelia burgdorferi* is the most common vector borne disease in the United States and Europe.[1,2] The current treatment for Lyme disease is a 2-4 week antibiotic monotherapy with doxycycline, amoxicillin or cefuroxime.[3] While this treatment is effective for the majority of Lyme disease patients, about 10%-20% of patients still have persisting symptoms such as fatigue, muscular pain, and neurological impairment even six months after the treatment,[1] a collection of symptoms called Post Treatment Lyme Disease Syndrome (PTLDS).[4] While the cause of PTLDS remains unclear and controversial, several hypotheses have been proposed to explain PTLDS, including host response to continued presence of bacterial debris,[5] autoimmunity,[6] co-infections,[7] and presence of bacterial persisters not killed by the current Lyme antibiotics.[7] Consistent with the persisting organisms not killed by current antibiotics, experiments in various animal models such as mice, dogs and monkeys have shown *B. burgdorferi* could still be detected after treatment with different Lyme antibiotics though viable organisms could not be cultured.[8-10] *In vitro* studies also demonstrated that *B. burgdorferi* could develop antibiotic tolerant persisters.[11] Although persister mechanisms have been reported in the model organism *E. coli*,[12] the mechanisms of *B. burgdorferi* persisters remain unknown.

Here we performed RNA Sequencing (RNA-Seq) analysis to determine the gene expression profile of *B. burgdorferi* persisters that survived antibiotic treatment to shed light on the mechanisms of *B. burgdorferi* persistence. *B. burgdorferi* B31 cultures were grown for five days to 1×10^7 spirochetes/mL in Barbour-Stoenner-Kelly (BSK) BSK-H medium followed by treatment with 50 μg/mL doxycycline or 50 μg/mL amoxicillin for six days. Triplicate biological samples were used for each group. The total RNA was extracted and ribosomal RNA was removed prior to preparation of cDNA (complementary DNA) libraries for Illumina sequencing. We analyzed the up-regulated and down-regulated genes in the amoxicillin and doxycycline treated *B. burgdorferi* persisters compared with drug free control (Figure 1). The RNA-Seq data was analyzed by BLAST analysis and mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Real-time quantitative reverse transcription PCR (RT-PCR) was performed for selected candidate genes that were upregulated (*bmpD* and *clpP*) or down-regulated (gene of membrane protein BB_0428 and *rpmE*) to validate the RNA-Seq data (see below).

For doxycycline treated *B. burgdorferi* persisters, a total of 675 genes were differentially expressed between doxycycline tolerant persisters and drug free group, with 335 genes upregulated and 340 genes down-regulated in doxycycline treated group. A total of 35 genes were identified by more than two-fold changes among up-regulated genes (Figure 1A), including five transporter genes (BB_0164, BB_0116, BB_0637, BB_0729 and BB_B29), four bacterial envelope protein coding genes (BB_0158, BB_A05, BB_0385 and BB_0201), three DNA repair related genes (BB_G32, BB_0552, BB_0830), two bacterial chemotaxis genes (BB_0114 and BB_0515), one bacterial secretion gene *secD* (BB_0652), and the *clpP* (BB_0757) gene encoding Clp protease. On the other hand, the majority of down-regulated (by more than two-fold) genes (33 genes) are associated with genes encoding outer membrane proteins and ribosome proteins.

clpP encoding ATP-dependent Clp protease proteolytic subunit was the most highly upregulated (30 fold) in doxycycline tolerant *B. burgdorferi* persisters. ClpP protease is an intracellular protease which could recognize and degrade misfolded proteins with the aid of ClpX, C or A subunits. Doxycycline could disturb bacterial protein synthesis by binding to the 30S ribosomal subunit that might lead to misfolded proteins. The up-regulation of *clpP* could be a response to this situation. Meanwhile we also found some genes encoding heat shock proteins (HSP) were upregulated. The HSP molecular chaperones could stabilize proteins to ensure correct folding and help to refold damaged proteins under stress, which could be important for persister survival.

Five up-regulated transporter genes (encoding phosphotransferase system, maltose and glucose uptake transporter, Na^+/Ca^{2+} exchanger, dicarboxylate/amino acid uptake transporter and Na/H antiporter) could facilitate uptake of nutrients (carbohydrates and amino acid) and regulate intracellular ion concentration under the pressure of doxycycline. We also found some up-regulated genes are associated with DNA repair, which may help to maintain stability of DNA under the doxycycline stress.

Cell structure proteins (envelope proteins) were biggest part of the total protein content, and inhibition of protein synthesis might cause cell envelope defect. We found most lipoproteins and outer membrane proteins were down-regulated, while the S1 and S2 antigens and some inner membrane proteins were up-regulated. The down-regulation of outer membrane lipoproteins could reduce a large demand for protein synthesis that would help *B. burgdorferi* persist under doxycycline stress. On the other hand, defect of outer membrane may allow strengthening of inner membrane structure by up-regulating membrane proteins. Ribosome is the target of doxycycline which inhibits

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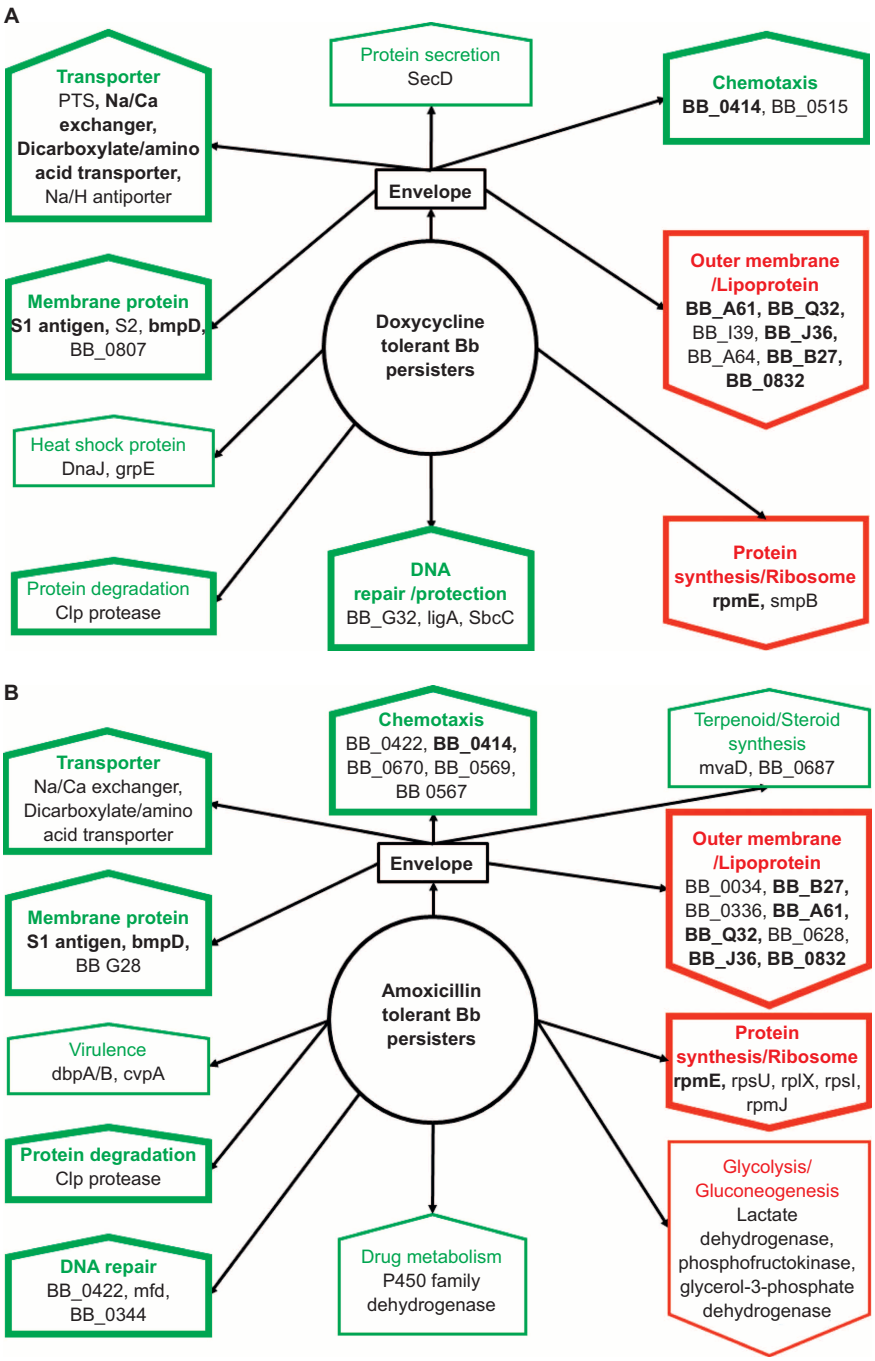

Figure 1 Differentially expressed genes in doxycycline (**A**) and amoxicillin (**B**) tolerant *B. burgdorferi* persisters grouped into pathways. The green upward pentagons indicate upregulated genes, and the red downward pentagons indicate downregulated genes. Pathways in bold frame are differentially expressed in both doxycycline and amoxicillin tolerant persisters. Genes in bold type indicate genes that are shared in both doxycycline and amoxicillin tolerant persisters. Bb: *B. burgdorferi*.

protein synthesis. One persistence strategy of *B. burgdorferi* might be reducing the availability of drug targets. We found five ribosomal genes were down-regulated in the doxycycline treated *B. burgdorferi* persisters. Down-regulation of ribosome proteins may also reduce the metabolism and the demand for nutrients and energy, allowing cells to transition to persistence.

For amoxicillin tolerant *B. burgdorferi* peristers, a total of 516 genes were differentially expressed compared with drug free control, with 342 genes being upregulated and 174 genes down-regulated. A total of 41 up-regulated genes and 45 down-regulated genes by more than two fold change were identified (Figure 1B). The up-regulated genes could be classified into membrane protein (BB_A05, BB_A36, BB_0385, BB_0767, BB_0844), bacterial chemotaxis (BB_0414, BB_0670, BB_0567), DNA repair (BB_0422, BB_0623 or *mfd*, BB_0344), energy production (BB_0782 or *nadD*), transporter (BB_0164, BB_0729), terpenoid/steroid synthesis (BB_0686, BB_0687), virulence (BB_A24, BB_A25 and BB_0766), Clp protease (BB_0757), and P450 family dehydrogenase (BB_G17). On the other hand, the major-

ity of the down-regulated genes belong to outer membrane proteins (most are lipoproteins), ribosome proteins and glycolysis/gluconeogenesis.

Comparison of the pathways of the doxycycline persisters and amoxicillin persisters revealed that they share several common features. For example, transporter (BB_0164, BB_0729), membrane protein (BB_A05, BB_0385), chemotaxis (BB_0414), ClpP protease (BB_0757), and DNA repair genes are up-regulated, while genes for outer membrane proteins/lipoproteins (BB_A61, BB_I39, BB_Q32) and ribosomal proteins (BB_0229) involved in protein synthesis were down-regulated. Besides these common pathways, genes for heat shock proteins and protein secretion protein were upregulated in doxycycline persisters, while amoxicillin persisters had virulence genes (*dbpAB*), terpenoid synthesis genes, and drug metabolism P450 family dehydrogenase gene uprgulated and glycolysis/gluconeogenesis genes down-regulated. These gene expression changes may play important roles in facilitating survival of *B. burgdorferi* persisters under antibiotic stress.

Amoxicillin inhibits the synthesis of bacterial cell wall and induces *B. burgdorferi* into round body form.[13] In the amoxicillin tolerant persisters we also found many down-regulated outer membrane lipoprotein genes, which may provide a survival strategy for persisters. In addition, we found two terpenoid/steroid synthesis genes were up-regulated in amoxicillin tolerant presisters. *B. burgdorferi* is an unusual prokaryote that possesses sterols in its membranes.[14] Up-regulation of terpenoid/steroid synthesis genes in amoxicillin tolerant persisters may increase the content of sterols, and change the cell membrane component to allow survival under amoxicillin treatment. *B. burgdorferi* decorin-binding proteins (Dbp) A (BB_A24) and B (BB_A25) are mainly expressed during mammalian infection to mediate bacterial attachment to the proteoglycan decorin in decorin-expressing mammal cells.[15] The elevated *dbpAB* expression in *B. burgdorferi* persisters without mammalian infection by amoxicillin treatment may enhance interaction with host cell and promote pathogen persistence.

Our findings not only shed new light on the mechanisms of *B. burgdorferi* persisters but also have practical applications. For example, the upregulated genes identified in *B. burgdorferi* persisters may not only serve as targets for developing new drugs for more effective treatment but also antigens for developing diagnostic tests for persistent Lyme disease, and finally for developing therapeutic vaccines for improved treatment. Future studies are needed to address these possibilities for more effective control of Lyme disease.

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