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NOTE

Roles of the superoxide dismutase SodB and the catalase KatA in the antibiotic resistance of *Campylobacter jejuni*

Sunyoung Hwang[2], Sangryeol Ryu[2] and Byeonghwa Jeon[1]

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Reactive oxygen species (ROS) are inevitable by-products from aerobiosis and give damages to biomolecules.[1] Recently, it was reported that treatment with bactericidal antibiotics enhances the generation of ROS in *Escherichia coli*, and then the increased ROS production affects bacterial lethality under the treatment of bactericidal antibiotics.[2] This is considered a new novel paradigm of bacterial killing by antibiotics but has thus far been examined only in a few bacterial species, including *E. coli, Staphylococcus aureus* and *Enterococcus faecalis*.[2,3] In *Listeria monocytogenes*, however, oxidative stress mutations did not alter the lethal kinetics of bactericidal antibiotics.[4] These previous reports suggest that the ROS-mediated lethality mechanism may have different impacts on antibiotic resistance depending on bacterial species.

Campylobacter species, particularly *Campylobacter jejuni*, are a major bacterial cause of human gastroenteritis, accounting for approximately 400–500 million infection cases in humans worldwide per year.[5] In addition, increasing prevalence of *Campylobacter* resistant to clinically important antibiotics, such as fluoroquinolones and macrolides, emerged as a serious public health concern.[6] *C. jejuni* is a microaerophilic bacterium and has unique oxidative stress defense systems.[7] For example, *C. jejuni* possesses only the sole superoxide dismutase (*sodB*) and catalase (*katA*) gene,[7] whereas *E. coli* carries three superoxide dismutase genes (*sodA*, *sodB* and *sodC*) and two catalase genes (*katG* and *katE*).[1] In this study, we constructed single mutants of *sodB* and *katA*, two key genes of oxidative stress resistance, and a double mutant of *sodB* and *katA*, and investigated the effect of oxidative stress defense systems on antibiotic resistance in *C. jejuni*.

C. jejuni NCTC 11168 and its derivatives were routinely grown at 42 °C on Mueller–Hinton (MH) media (Difco, Franklin Lakes, NJ, USA) under microaerobic conditions generated by MART (Anoxomat, Mart Microbiology B.V., Drachten, the Netherlands). Culture medium was supplemented with kanamycin (50 mg l^{-1}) or chloramphenicol (10 mg l^{-1}), where required. We knocked out the *sodB* and *katA* genes with suicide plasmids as described previously.[8] Briefly, a DNA fragment containing *sodB* and its flanking region was amplified from *C. jejuni* NCTC 11168 using the primers sodB_F: GAC TTA TAT CAA GGC TGG GAT TAT TGA and sodB_R: CAC TCC ATT TCC ACA ACC AAA ATC AAG T, and was ligated to pUC19. The chloramphenicol resistance cassette (*cat*) was PCR-amplified from pRY112,[9] and inserted into a *Bgl*II site of *sodB* in pUC19. To construct a *katA* mutant, the PCR product amplified with the primers (katA_F: TAA CGA TTT TGG ATC CAT TAT AGC and katA_R: AAT TAT TTT TCT AAA GCT TTT TTA ATT CC) was cloned to pUC19. The kanamycin resistance cassette (*aphA*) was generated by PCR from pMW10,[10] and inserted to a *Bgl*II site of *katA* on pUC19. The constructed suicide plasmids were introduced to *C. jejuni* NCTC 11168 by electroporation. The *sodB* and *katA* mutants were selected by growing on MH agar plates supplemented with chloramphenicol (10 mg l^{-1}) and kanamycin (50 mg l^{-1}), respectively. The *sodB* and *katA* double mutant was constructed by transferring the *sodB* mutation to the *katA* mutant by natural transformation.[8] To construct complementation strains, intact copies of *sodB* and *katA* were integrated into the chromosome with a method described elsewhere.[11] Briefly, DNA fragments containing the intact copy of *sodB* and *katA* were amplified with primer pairs (sodB-CF: GAT TAA ATA TCT AGA AAT TTT ACC CAC and sodB-CR: CCC TTT TTG TCT AGA ATG TTG AC and katA-CF: TTT TGA AAT TTA TTA TCT AGA AAA TGC AAT TAT TC and katA-CR: TTA TTA TAA TCT CTA GAT TGC CAC CAA AAG) and cloned into an *Xba*I site of a pUC19 derivative carrying an rRNA gene cluster.[11–13] The plasmids were delivered to the *sodB* and *katA* mutants by electroporation. Allelic

[1]Department of Public Health Sciences, University of Alberta, Edmonton, Alberta, Canada and [2]Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, and Center for Agricultural Biomaterials, Seoul National University, Seoul, Republic of Korea
Correspondence: Dr B Jeon, Department of Public Health Sciences, University of Alberta, Edmonton, Alberta T6G 2G7, Canada.
E-mail: bjeon@ualberta.ca
or Dr S Ryu, Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, and Center for Agricultural Biomaterials, Seoul National University, Seoul 151–921, Republic of Korea.
E-mail: sangryu@snu.ac.kr
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exchanges in the *sodB* and *katA* mutants were confirmed by PCR and sequencing a flanking region of the mutated genes (data not shown). The minimum inhibitory concentrations (MICs) of several antibiotics (ciprofloxacin, erythromycin, cefotaxime, gentamicin, rifampicin, polymyxin B and tetracycline) were measured with the microtitre broth dilution method.[14] Rapid killing of *Campylobacter* by antibiotics was determined as described previously with some minor modifications.[9] Briefly, *C. jejuni* grown overnight on MH agar plates was resuspended in fresh MH broth to an OD at 600 nm of $\sim$0.06 (10^7 CFU ml^{-1}). Aliquots of bacterial suspension were exposed to antibiotics. At every 1 h, samples were serially diluted and applied to MH agar plates for viable counts.

The *katA* mutation decreased the MICs of ciprofloxacin and rifampicin by two-fold compared with the wild-type. The MIC reduction in ciprofloxacin was restored by complementation, while that of rifampicin was not complemented (data not shown). Although the *sodB* single mutation did not make any differences in the MICs of the tested antibiotics, the *sodB* and *katA* double mutation further decreased the MICs of rifampicin and cefotaxime by four- and two-fold, respectively (data not shown). In *E. coli*, similarly, single mutations of *kat* and *sod* genes did not affect MIC levels but a *katG* and *katE* double mutation reduced the MIC of norfloxacin by two-fold.[9] To examine lethality kinetics, *C. jejuni* was exposed to 10 times the MICs of ciprofloxacin and rifampicin, and bacterial viability was determined. Interestingly, the mutants in the oxidative stress defense system demonstrated significantly reduced viability compared with the wild-type (Figure 1). Overall, the viability reduction was more significant in the *katA* mutant compared with the *sodB* mutant, and bacterial killing was synergistically enhanced by the *sodB* and *katA* double mutation particularly under rifampicin treatment (Figure 1b). Similarly, it was reported that a *sodA* mutation in *E. faecalis* resulted in 3.9log$_{10}$ decrease in viable counts by exposure to 20 times the MIC of vancomycin.[3] The treatment of a *sodA* and *sodB* double mutant with 10 times the MIC of norfloxacin for 2 h reduced viable counts by 10-fold compared with the wild-type *E. coli*.[9] Previously, Kohanski *et al.* demonstrated that increased production of ROS by antibiotics leads to the destabilization of iron–sulfur clusters and the released Fe^{2+} produces hydroxyl radicals via the Fenton reaction.[2] Hydroxyl radicals have a critical role in bacterial killing by antibiotics, and the production of hydroxyl radicals is mediated by a metabolic response under antibiotic treatment, which involves the tricarboxylic acid (TCA) pathway and subsequent depletion of NADH.[2] The importance of the TCA cycle in ROS-mediated killing by antibiotics was reassured in *L. monocytogenes*.[4] Unlike the TCA cycle of *E. coli*, *L. monocytogenes* has noncyclic TCA pathway lacking α-ketoglutarate dehydrogenase, oxidative stress mutants, including Δ*sod* and Δ*fri* mutants, did not affect lethal kinetics by bactericidal antibiotics presumably due to the different TCA cycle.[4] As the genome sequence of *C. jejuni* demonstrates the presence of all genes responsible for the TCA cycle,[15] *C. jejuni* would adopt the ROS-mediated killing mechanism similar to *E. coli*. However, the presence of different ROS-detoxifying enzymes and regulatory mechanisms of oxidative stress resistance still propose *C. jejuni* may have a unique mechanism for ROS-mediated killing by antibiotics.

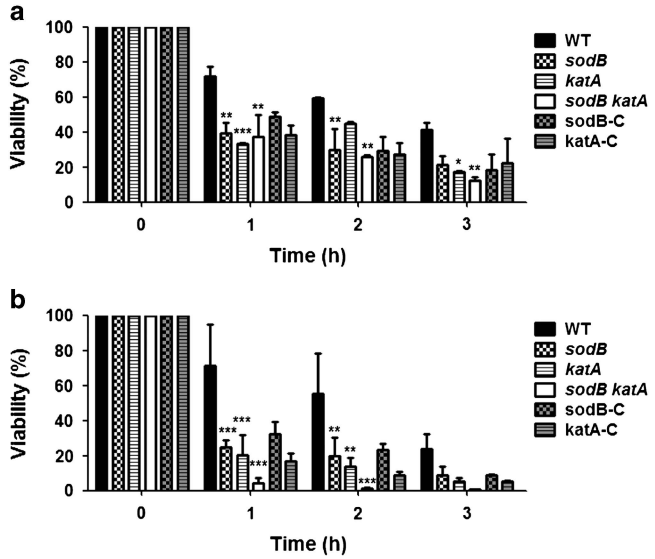

Figure 1 Viability of the wild-type (WT; *C. jejuni* NCTC 11168), *sodB* mutant (*sodB*), *katA* mutant (*katA*), *sodB* and *katA* double mutant (*sodB katA*), the *sodB* complementation strain (sodB-C) and the *katA* complementation strain (katA-C) after exposure to 10 times the MICs of ciprofloxacin (**a**) and rifampicin (**b**). The viability was calculated as CFU after antibiotic treatment was divided by CFU before the treatment. The results show the means and s.d. of three independent experiments. The statistical significance between the WT and the mutants was determined by two-way analysis of variance with Bonferroni's post-test using Prism software (version 5.01; Graphpad software Inc., La Jolla, CA, USA). $*P<0.05$, $**P<0.01$, $***P<0.001$.

On the basis of our findings and previous reports, the effect of oxidative stress defense on antibiotic lethality may vary in different bacterial species. As the sole catalase and superoxide dismutase in *C. jejuni*, KatA and SodB contribute to *C. jejuni*'s resistance to ciprofloxacin and rifampicin. To the best of our knowledge, this is the first report demonstrating the implication of oxidative stress defense in the antibiotic resistance of *Campylobacter*. At this stage, the elucidation of molecular details associated with the ROS-mediated lethal mechanism in *Campylobacter* still awaits future studies.

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