Article

Transcriptomic Changes and satP Gene Function Analysis in Pasteurella multocida with Different Levels of Resistance to Fluoroquinolones

Guanyi Xu[1], HAQMAL MOHAMMAD AMAN[1], Yuxuan Xu[1], Junze Xue[1], Inam Muhammad[1], Haipeng Zhang[2,3], Xuesong Li[1], Yu Jia[2], Yiming Wang[1], Chengguang He[2], Lingcong Kong[1,2*], Hongxia Ma[1,2,3*]

1. College of Animal Medicine, Jilin Agricultural University; Xincheng Street No. 2888, Changchun 130118, China. xuguanyi@mails.jlau.edu.cn(G.X.) ; 1029686675@qq.com(Y.X.) ; Lxsong1207@126.com(X.L.) ;wym27149@jlau.edu.cn(Y.W.);xjz13904404234@163.com(J.X.) dr.inam@sbbu.edu.pk (I.M.);
2 The Key Laboratory of New Veterinary Drug Research and Development of Jilin Province, Jilin Agricultural University; Xincheng Street No. 2888, Changchun 130118, China. hechengguang@163.com; 527115664@qq.com(H.Z.); 66045944@qq.com(Y,J).
3 The Engineering Research Center of Bioreactor and Drug Development, Ministry of Education, Jilin Agricultural University; Xincheng Street No. 2888, Changchun 130118, China. 527115664@qq.com(H.Z.)
* Correspondence: congwbs@126.com(L.K); mahongxia@jlau.edu.cn (H.M.)

Abstract: Under the pressure of fluoroquinolones, *Pasteurella multocida (PM)* can easily develop resistance to fluoroquinolones mediated by QRDR target mutation. It is imperative to find new drug resistance inhibitor targets to combat the rapid development of drug resistance. In order to overcome these problems, we sequenced the transcriptome of *PM* with different levels of resistance to ENR(0.03 μg/mL; 8 μg/mL; 32μg/Ml, Enrofloxacin). The results showed that with the increase of resistance to fluoroquinolones, the expression of *satP* gene was significantly up-regulated. The *satP* gene deletion strain and replenishment strain were constructed, and their drug resistance and tolerance were determined. The results showed that the deletion of *satP* gene did not affect the resistance of *PM* to fluoroquinolones, rather affected the time when *PM* developed resistance to fluoroquinolones. After 10 generations of drug induction, the MIC (minimum inhibitory concentration) of fluoroquinolones for wild strain was 64 μg/mL, while the MIC for *satP* gene deletion strain was only 8 μg/mL. The MDK_{99} test (time to kill 99% bacteria),agar diffusion test and mutation frequency test showed that the tolerance of *satP* gene deletion strain was significantly lower than that of wild strain. At the same time, the virulence of gene deletion strain and wild strain was tested, and about 400 times decreased virulence was observed for *satP* gene deletion strain. The mouse infection model confirmed that mice infected with *satP* gene deletion strains were more likely to be treated with ENR than mice infected with wild-type bovine *PM* strains. The results show that *satP* has potential to be a target of fluoroquinolone resistance inhibitors.

Keywords: Bovine respiratory disease; *Pasteurella multocida*; resistance; tolerance; *satP*.

1. Introduction

PM is a Gram-negative bacteria implicated in multi-host infections. Strains of their species are currently classified into type A, type B, type D, type E, and type F serogroups[1, 2]. *Pasteurella multocida* type A(*PMA*) is one of the primary pathogens of bovine respiratory disease in Asia, Middle East, Africa, and Europe[3-5]. The infection of cattle with *PMA* leads to tremendous economic losses for the cattle industry due to increased deaths and high treatment costs. BRD is mainly treated with different types of antimicrobials[6]. However, the abuse of antimicrobials for the prevention and treatment of BRD has increased the rate of antimicrobial resistance[7, 8].

Fluoroquinolones are the primary drugs for the treatment of *PMA* infections in animals. Recently, the mechanisms of *PMA* accounting for acquired antifungal fluoro-

quinolones resistance have been reported[9]. The best-documented mechanism is the substitution of amino acid targets. Our previous results indicated that *PMA* is extremely resistant to fluoroquinolones in vivo and in vitro, and the mechanism is mainly mediated by target mutation in *gyrA* and *parC*[10]. However, the development of antimicrobial resistance is a complicated multifactorial process that relies on tolerance factors[11, 12]. Thus, some genes such as *recA* are essential for increased tolerance to antibiotic treatment by enhancing repair of DNA damage that occurs directly by antibiotic-induced DNA damage. Related studies have confirmed that amino acid mutations are related to the generation of tolerance[13, 14]. Frequent usage of antibiotic treatments can enhance the antibiotic resistance of *PMA*[15]. Due to the importance of tolerance, there is an urgent need to identify new drug targets.

However, the mechanism of *PMA*'s tolerance to fluoroquinolones and the main targets for mediating tolerance has not been reported. To determine whether the resistance mechanism of *P. multocida* is caused by tolerance, it is crucial to work on a new tolerance mechanism and identify targets that can inhibit drug resistance.

2. Results

2.1.1 ***Selection of fluoroquinolone-resistant mutants***

Two strains（*PMS* and *PMR*）with the same molecular characteristics and different fluoroquinolone resistance phenotypes were isolated by 16S rRNA and PFGE (Supplementary **Figure. S1**). Using *PMR* as the parent strain, a highly resistant strain *PMHR* was obtained by in vitro induction with fluoroquinolones. *PMS* had no QRDR mutation, and *PMR* had three QRDR mutations (Ser83Ile in GyrA, Ser425Phe in GyrB and Glu84Lys in ParC). *PMHR* and the *PMR* have also the same QRDR mutations (Ser83Ile in GyrA, Ser425Phe in GyrB and Glu84Lys in ParC) (**Table 1**).

The parent strain and mutant-derived strains were tested for CCCP sensitive efflux. The MICs of ENR were determined using agar dilution method in the presence and absence of CCCP (4 μg/mL, MIC/2). These results indicated that efflux pump genes did not play any role in the quinolone resistance of *PM* mutants selected in vitro. The characteristic features of the three lineages are described in **Table 1**. We speculated that *PMHR* might be tolerant to fluoroquinolones, and tolerance mechanism should further be investigated in future studies.

2.1.2 ***Transcriptional profiling of PMS, PMR and PMHR by RNA sequencing***

In order to search for genes that play an important role in the formation of fluoroquinolone resistance in *PM*, we sequenced the transcriptome of four strains under the sub-inhibitory ENR concentration (Supplementary **Table S1**). The results show that more than 97% of the reads can be mapped to the reference genome sequence (GenBank: CP003022.1). Raw reads data were sequenced and filtered for further analysis, yielding approximately 1.76–2.15 billion clean reads. The average length of reads for *PMS*, *PMR* and *PMHR* was 300bp, and their GC content were 42.77%, 43.17% and 42.35%, respectively, indicating successful sequencing of the *PM* with different levels of ENR resistance (Supplementary **Table S2**).

In total, 14,055,756 reference genes were detected. Based on FDR threshold ≤0.05 and | log2 fold change (FC)| >1, a total of 1,499 significant DEGs were identified. Among them, 144 genes were up-regulated while the rest 292 genes were down-regulated in control group vs.*PMR* treated group, 181 genes were up-regulated while the rest 382

genes were down-regulated in control group vs. *PMHR1* treated group and 311 genes were up-regulated while the rest 244 genes were down-regulated in control group vs. *PMHR2* treated group (Supplementary **Figure S2-S3**).

The DEGs were also enriched in GO and KEGG pathways. There were total of 19 terms enriched in GO terms, among which, 7 terms were for biological process, 7 terms were for cellular component and 5 terms were for molecular function (Supplementary **Figures S1**). The top 20 of KEGG pathways were annotated. There were 630 DEGs belonging to microbial metabolism in diverse environments (Supplementary **Figure S4** and **Table S3**). Furthermore, in order to find the difference between *PMHR1* and *PMHR2*, significant expressed genes were screened with the improvement of drug resistance, and the *satP* (gene 1687), *focA*, *napF*, *glgP* and *napD* genes were significantly up-regulated (**Table 2**). Among them, *PMR* group was 17 times higher than *PMS* group, and *PMHR* group was 20 times higher than *PMS* group. The qRT-PCR analysis was performed to validate the expression of *satP* in *PM* with different levels of drug resistance. As shown in **Fig.2**, qRT-PCR results of *satP* agreed with the FPKM data. In contrast, the relative expression of *satP* in *PMR* group was significantly higher than *PMS* group and in *PMHR* group significantly higher than *PMR* group. The expression level of *satP* gene accords with the trend of drug resistance.

2.1.3 ***Construction and detection of satP and C-satP strains***

The knockout of *satP* in *PMS* and *PMHR* was conducted with the suicide vector pRE112. The complemented strain *C-satP* was constructed with the expression plasmids pBBR1-MCS by homologous recombination. To determine whether two *satP* deletion strains and one *C-satP* strain were successfully constructed, hereditary stability, PCR detection and qRT-PCR were performed. PCR showed that the *satP* gene of the deletion strain is 700bp less than that of the wild strain. Real-time qRT-PCR showed that no signal of *satP* gene was detected from the deletion strain and *satP* had 2.31-fold higher expression of C-*satP* than wild strain. Genetic stability showed that two *satP* deletion strains and C-*satP* were successfully constructed. The deletion of *satP* did not affect the MIC and growth of the strains (**Figure 2. A-C**).

2.1.4 ***Effect of satP deletion on drug resistance formation***

To verify the effect of *satP* gene on quinolone resistance of *PM*, the in vitro method of inducing drug resistance was used to detect the development time of drug resistance of gene deletion strain, wild strain and replenishment strain. The results showed that in 1-5th generation, the drug resistance of sensitive strains was the same as that of gene deletion strains, and the MIC was 0.5 μg/mL (increased by 3 times). The drug resistance formation time of sensitive strains and gene deletion strains was different in 5-10th generation. The resistance of wild strains developed rapidly, and the MIC was 64 μg/mL in the 10th generation. While the drug resistance of gene-deletion strains developed slowly, and the MIC was 16 μg/mL in the 10th generation (**Figure 3A**).

2.1.5 ***Effect of satP deletion on drug tolerance formation***

To verify the effect of *satP* gene on quinolone tolerance of ENR, the tolerance of *PMS* and △*PMS* to fluoroquinolones was determined. The result showed that the tol-

erance of *satP* gene deletion strain to ENR was significantly weakened. The tolerance decreased by 10 times with drugs treated for 2-3 h. The tolerance decreased by 100 times with drugs treated for 4h. The results of mutation frequency showed that the mutation frequency of deletion strains decreased significantly (**Figure 3B and Table 2**).

In order to further verify the effect of *satP* gene deletion on *PM* tolerance, MDK_{99} was measured. The result showed that the speed of ΔPMS, ΔPMR and $\Delta PMHR$ was faster than that of *PMS*, *PMR* and *PMHR*, which reached to 10^5 CFU/mL. The results showed that the deletion of *satP* gene could lead to the decrease of tolerance (**Figure 4A-C**).

2.1.6 ***Determination of median lethal dose***

The amount of bacteria and survival rate of 40 mice injected intraperitoneally are shown in Supplementary **Table S4**). The reasonable detection ranges are 10^5-10^7 CFU/mL and 10^9-10^{11} CFU/mL. According to the data in **Table S4**, the LD_{50} of *PMS* is $1.8x10^6$ CFU/mL, the 95% confidence limit is $1.3x10^6$-$2.5x10^6$ CFU/mL, the LD_{50} of *PMS* is $7.1x10^9$ CFU/mL and the 95% confidence limit is 5.3 $x10^9$-9.4 $x10^9$ CFU/mL.

2.1.7 ***Survival curve of mice***

In order to evaluate the effect of *satP* gene deletion on the infection model, we first constructed the infection model of *PMS* and ΔPMS in mice, and then intramuscularly injected ENR with clinical therapeutic dose into the experimental group and intramuscularly injected normal saline into the control group. The results are shown in **Fig. 5**. Compared with *PMS*, $\triangle PMS$ is more likely to be treated with ENR.

2.2. Figures, Tables and Schemes

Figure 1. Characterization and verification of differentially expressed genes. (A) Differentially Expressed Genes analysis of resistance group (*PMS* vs *PMR*). (B) Differentially Expressed Genes analysis of resistance group (*PMS* vs *PMHR*). (C) *satP* expression level verification using qRT-PCR.

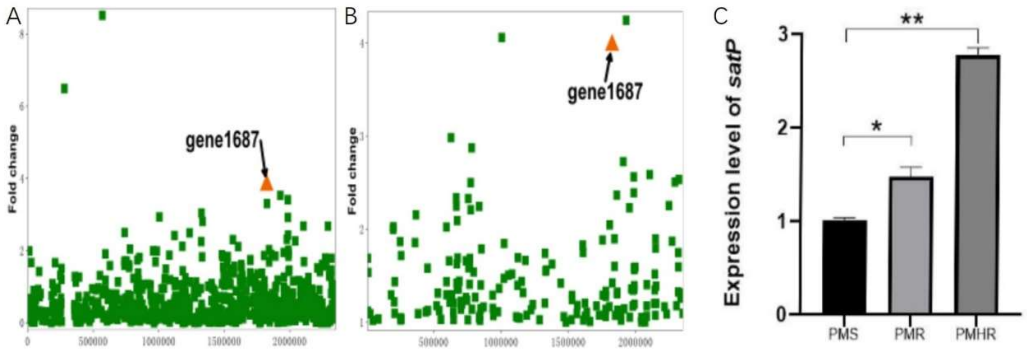


Table 1. QRDR mutation genotypes and fluoroquinolone MICs for *PM*

Strains	MIC range (μg/mL)			Substitution in			
	CIP	ENR	ENR+CCCP	GyrA	GyrB	ParC	ParE

PMS	0.25	0.25	0.125	-	-	-	-
PMR	4	4	1	Ser83Ile	Ser425Phe	Glu84Lys	-
PMHR	64	64	64	Ser83Ile	Ser425Phe	Glu84Lys	-

Figure 2. (A) Confirmation that the deletion *satP* strains were genetically stable beyond 40 generations by PCR. M, DL2000 Marker; 1–4, deletion strain *satP*; 5, wild-type strain.(B) The expression level of the *satP* gene in the wild-type S, deletion *satP*. (C) The expression level of the *satP* gene in the wild-type S and complemented C-*satP* (pACYC) strain.

Figure 3. Construction of deletion strain and complemented strain. (A) Confirmation that the deletion *satP* strains were genetically stable beyond 40 generations by PCR. M, DL2000 Marker; 1–4, deletion strain *satP*; 5, wild-type strain.(B) The expression level of the *satP* gene in the wild-type S, deletion *satP*.

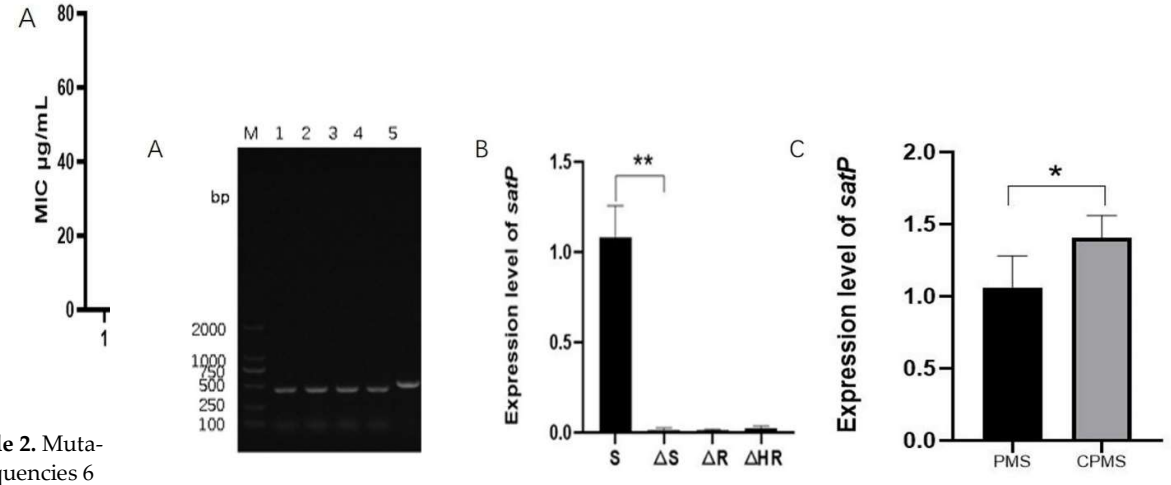


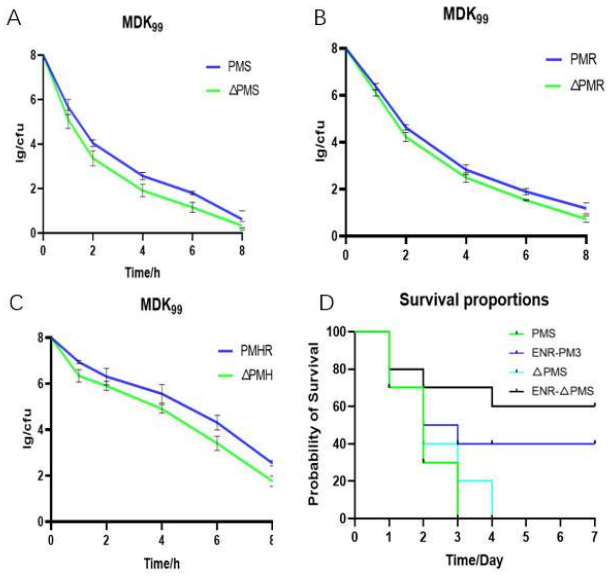
Table 2. Mutation frequencies of *PMS* strains to ENR

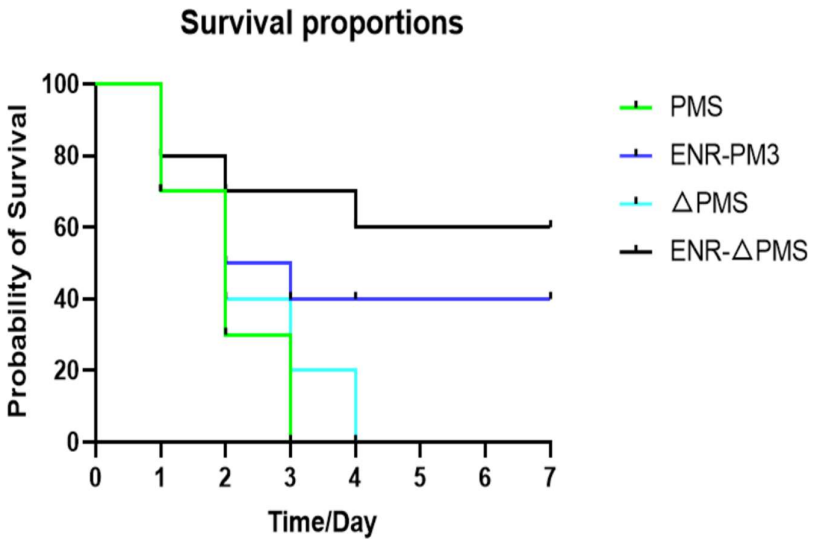
菌株	突变频率（×10⁸）（X±SD）
PMS	0.3±0.1

△PMS	-
PMR	6.4±2.8
△PMR	3.7±1.5
PMHR	14.0±5.8
△PMHR	8.2±1.6

Figure 4. (A) Confirmation that the deletion *satP* strains were genetically stable beyond 40 generations by PCR. M, DL2000 Marker; 1–4, deletion strainsatP; 5, wild-type strain.(B) The expression level of the *satP*gene in the wild-type S, deletion *satP*. MDK_{99} and Survival proportions. (A-C) MDK_{99} of *PMS*, *PMR*, *PMHR*, *△PMS*, *△PMR* and *△PMH*.(B) Survival proportions of *PMS* and *△PMS*

Figure 5. Survival rates of the mice treated with ENR infected with *PMS* and *△PMS*





3. Discussion

BRD has become one of the major diseases in the world cattle industry. One of the main pathogens causing the disease is *PM*[22]. At present, the treatment of *PM* mainly depends on antibiotics. However, it is easy to develop antibiotic resistance. Fluoroquinolones are usually applied in the treatment of *PM*, and *PM* has developed resistance to fluoroquinolones[10]. In recent years, multidrug-resistant strains of *PM* have been appeared, and *PMA* has caused huge economic losses to the cattle industry in Spain. Tang x et al. discovered that 93.2% in the 92 strains *PM* were resistant to fluoroquinolones[22]. The mutation of drug resistance target gene has a vital impact on the formation of drug resistance. Kong LC and others detected the target genes in quinolone resistant *PMA*, and found that the mutation of *gyrA* and *parC* genes has a significant influence on the generation of drug resistance[23].

In this study, three strains (*PMS*, *PMR* and *PMHR*) with different resistance phenotypes to fluoroquinolones were screened out. *PMS* had no QRDR mutation, while *PMR* had three QRDR mutations (Ser83Ile in GyrA, Ser425Phe in GyrB and Glu84Lys in ParC), and *PMHR* and the *PMR* have also the same QRDR mutations (Ser83Ile in GyrA, Ser425Phe in GyrB and Glu84Lys in ParC) (**Table 1**). The different levels of resistance between *PMHR* and *PMR* might be mediated by other resistance mechanisms or tolerance. Relevant researches show that tolerance is the first step for bacteria to develop drug resistance[10, 24]. With respect to the research question, we sequenced the transcriptome of three strains of bovine capsular type A *PM* with different levels of drug resistance treated by sub inhibitory concentration, and counted the differential genes. On the basis of ensuring stable and accurate transcriptome data, we analyzed the functional annotation and metabolic pathways, and analyzed and screened the genes that might be related to the formation of drug resistance. The results showed that with the increase of drug resistance, the expression of some genes namely *satP*, *focA*, *napF*, *glgP* and *napD* increased significantly. The most significant difference was the *satP* gene, which was up-regulated 17 times in the drug-resistant group and 20 times in the highly drug-resistant group compared with the sensitive group. The *satP* (also known as Yaah) is a representative member of AceTr family, which is preliminarily identified as succinate acetate/proton cotransporter transmembrane protein [24]. Indrajiths et al found that outer membrane protein ompk36 is an important influencing factor of cefoxitin and carbapenem resistance[25]. Yamamoto K et al. found that the membrane protein mmpl5 has an effect on the resistance of clofazimine, and it is also related to the tolerance of a variety of antibi-

otics[26]. Lu w et al. studied the function of membrane channel protein *lamB* and found that it can produce antibiotic resistance by reducing the pressure of protein translation and DNA replication. In order to further detect antibiotic resistance, *lamB* gene was knocked out and found to increase antibiotic resistance by reducing the activity of metabolic pathway. By comparing the function of wild strain and deletion strain after ciprofloxacin treatment, it was found that the down-regulation of *lamB* gene can increase the resistance of bacteria to antibiotics[27].

Based on the research background of *satP* gene, in order to further confirm the function of *satP* gene, we constructed the *satP* gene deletion strain of sensitive strain and drug-resistant strain. The results showed that the tolerance of *satP* decreased after the deletion of *satP* gene. Safi H and others have pointed out that tolerance is a temporary state that has the trend of drug resistance and has the ability to obtain drug resistance. Timely release of antibiotics can restore its tolerance, so the tolerance is reversible[28]. Repeated exposure will maintain the tolerance [29], which lays on the foundation for the functional study. During research of this gene in *Pasteurella multocida*, Westfall C et al. studied the mechanism of *MRSA* tolerance by using triclosan, an antibacterial agent synthesized by fatty acids, to induce the change of *MRSA* tolerance. MDK_{99} and agar diffusion method were used to detect its tolerance. The results showed that triclosan increased the tolerance of *MRSA* to bactericidal antibiotics by 10000 times and reduced the efficacy of antibiotics by 100 times. Genetic analysis showed that the antibiotic tolerance mediated by triclosan needs the cooperation of ppGpp[30]. However, up to now, there is no report about *satP* gene mediated tolerance; other membrane transporters have been confirmed to be involved in the formation of tolerance. Poudyal B found a MerR type transcriptional activator, which can activate the BrlR target encoding ABC transport system gene and affect the drug tolerance of bacteria[31]. One of the main stress responses of almost all bacteria depends on the rapid accumulation of ppGpp, an alarmone. Many scholars have found that ppGpp has a great impact on bacterial tolerance. Now, Ronneau S and others have studied ppGpp and found that the concentration of ppGpp is determined by a highly conserved and widely distributed protein family, and the family is known as Rela-Spot Homologs(RSH). Recent studies have found that RSH can affect not only the tolerance of bacteria, but also the drug resistance and virulence of bacteria[32].

One unanticipated finding was that the virulence of *satP* gene deletion strain was several times lower than that of wild strain, and we found that the treatment rate of mice infected with △*PMS* was higher. This findings broadly supports the work of other studies in this area which linked tolerance with virulence[33]. But there are also some reports contrary to this study. Ma D et al. knocked out the *MazEF* gene of three strains of bacteria, and compared with the wild strain and found that the tolerance of the deletion strain was decreased and the virulence was enhanced[34]. These findings may help us to understand the relationship between drug resistance and adaptive compensation. This finding has important implications for developing inhibitors. But there are still many unanswered questions about whether *satP* can be used as a target of drug resistance inhibitors and how effective it could be in clinical application. More attention should be paid to the study on *satP* and its inhibitors.

4. Materials and Methods

Antibiotics, culture media, bacterial strains and growth conditions

PMS and *PMR* were isolated from the lungs of dead cattle suffering from BRD, a highly resistant strain (*PMHR*, MIC of ENR is 32μg/mL) was obtained by continuous induction of drug resistance in vitro with *PMS* according to the methods reported in previous studies[10]. The *PMS*, *PMR* and *PMHR* have the same molecular type after PFGE typing as genome of *PMS* was completely sequenced in a previous study[8]. *PM* was cultured with Mueller-Hinton (MH, containing 5% bovine serum), BHI (Brain Heart

Infusion), nutrient broth (NB) and nutrient agar (NA) plate. Commercial antibiotics (ENR) were purchased from Sigma-Aldrich Company. Growth was monitored, by measuring the OD_{600} of the liquid cultures using a Biophotometer (Nanodrop2000, Thermo).

Determination of MICs of fluoroquinolones **for *PMS* and *PMR***

The MICs were established by broth microdilution and two-fold AGAR dilution, as recommended by the Clinical and Laboratory Standard Institute guidelines in VET01-A4 (CLSI 2013)[16]. The MICs of Ciprofloxacin and ENR were tested in the range of 0.03 to 512 μg/mL. The *PMS* and *PMR* cells were inoculated into MH media and incubated for 24h at 37℃. Then, cells were harvested and washed three times with PBS. Each strain was inoculated at a density of 10^5 CFU/mL in MH containing 0.03 to 256 μg/mL of norfloxacin and grown for 24 h at 37℃. The breakpoints used for fluoroquinolone were adopted from CLSI document VET01-A4. Escherichia coli *ATCC25922* used as a quality control strain.

Amplification and sequencing of the QRDR genes

The *gyrA*, *gyrB*, *parC* and *parE* genes were amplified by polymerase chain reaction (PCR) and compared using the *gyrA*-F (5′- CCTTATCGTAAATCCGCTCGTA-3′), *gyrA*-R (5′-CGCAGGGACTTTGGTTGG-3′), *gyrB*-F (5′-GAAATGACCCGCCGTAA-3′), *gyrB*-R (5′-CTTGCCTTTCTTCACTTTGTA-3′), *parC*-F (5′-TACGAAGGCATTGAACAAAC-3′), *parC*-R (5′-CCACTGTCCCTTGCCCTA-3′), *parE*-F (5′-AATACGGTAAAGCGGTGGC-3′), *parE*-R (5′-GGATTCTGCTGGCGGTTC-3′) primers[8]. Amplified PCR products (312 bp, 456bp, 421bp and 331bp) were cloned into the pMD18-T easy vector via TA cloning and sequenced for identification of mutation positions.

RNA extraction, library construction, and sequencing

The cells were grown to exponential phase (OD_{600}≈0.5) and treated with ENR (Sub inhibitory concentration) over a period of 15 min. Total RNA extraction of *PMS*, *PMR* and *PMHR* were performed using an RNAiso Pure RNA Isolation Kit (TaKaRa, JP) according to the manufacturer's instruction. The quality and quantity of RNA was assessed and the library construction and sequencing were performed at Biomarker Technology (Beijing, China) using Illumina HiSeq 2500 platform. The genome sequence and annotation information of *PM* was obtained from the NCBI database (Accession No. NC_014259.1). Quality-filtered reads were aligned to the reference genome sequence using the CLC Genomics Workbench 4.0 (CLC Bio). Based on FDR threshold ≤0.05 and | log2 fold change (FC)| >1, significant DEGs were identified. Then, with the increase of drug resistance, differentially expressed genes were screened[17, 18].

Expression of *satP* gene at mRNA level was evaluated by qPCR. The cells were grown to exponential phase (OD_{600}≈0.5) and treated with ENR (Sub inhibitory concentration) over a period of 15 min. Total RNA extraction of *PMS*, *PMR* and *PMHR* was performed using an RNAiso Pure RNA Isolation Kit (TaKaRa, JP) according to the manufacturer's instruction, and was reversely transcribed to cDNA. Real-time PCR process was performed on Applied Biosystems 7500 Real-Time PCR System (United States). The primers of *satP*-F (CGCCAAAAGCAAACGCCATACC) and *satP*-R (TACGCCAATCCGGGTCCATTAGG) were designed. The specificity of the qRT-PCR results was confirmed by agarose gel and melting curve analysis, and the data were analyzed by the Applied Biosystems™ 7500 software. All qPCR reactions were performed with six replicates.

Construction of the *satP* mutant in the *PMS* and *PMHR* strains

The *satP* gene knockout was conducted with the suicide vector pRE112. Briefly, the upstream and downstream regions of *satP gene* were amplified by PCR with the primers UP-F/R and DOWN-F/R, respectively (Supplementary Table S2). Then, overlapping PCR based on that mixture of A and B as a template was performed. Amplified PCR products (1673 bp) were cloned into the pRE112 vector and sequenced for identification. Then, the plasmid pRE112-*satP* was successively transformed into DH5α-λpir and *E. coli WM3064*. Conjugation was conducted via biparental filter mating with the *DR1* strain, thus creating a *satP* mutant of the *PMS* and *PMHR* strains. At the same time, the complemented

strain C-*satP* was constructed with the expression plasmids pBBR1-MCS by homologous recombination.

Tolerance of wild type and the *satP* mutant

Tolerance was determined according to Westfall's method [19, 20]. The wild strain and the deletion strain were cultured to OD_{600}=0. 5 and colony count was performed, the bacterial solution was diluted to a final concentration of 10^6 CFU/mL according to the counting results. ENR with the final concentration of minimum inhibitory concentration was added to the culture medium, and the final system of 0.5 μg/mL per tube was subpacked into 2 mL sterile EP tube, and each strain was subpacked into 5 tubes for culture. Sampling was carried out at five time points of 0 h, 1 h, 2 h, 3 h, and 4 h. Take out 200 μL of culture solution was extracted from the tube and added to a 96-well plate, and the diluted bacterial solution was continuously diluted 10 times for 5 concentration gradients. The diluted bacterial solution was inoculated on the BHI solid plate through a multi-point inoculator. Cultured overnight, observed the growth of colonies the next day and recorded the results.

Acute toxicity test in mice

Twenty mice, half male and half female, were randomly divided into 5 groups with 4 mice in each group. After 3 days of adaptive feeding, the mice were reared without food and water for 12 hours, and then *PMS* with bacterial counts of 10^8, 10^7,10^6,10^5 and10^4 CFU/mL were intraperitoneally injected into the mice. The state of mice was monitored, and the minimum dose of 100% (4/4) death and the maximum dose of 0% (0/4) death were recorded to provide a reasonable concentration range for the formal test. The operation of △*PMS* pre-experiment was the same as that of *PMS* pre-experiment.

Fifty mice, half male and half female, were randomly divided into 5 groups with 10 mice in each group. After adaptive feeding for 3 days, the mice were reared without food and water for 12 hours. According to the results of the preliminary experiment, each strain of bovine type A *PM* was diluted to different concentrations, and each strain was set with five concentration gradients. The infection was carried out by intraperitoneal injection, and each mouse was infected with 100 μL. The control group was injected with the same dose of sterile PBS buffer. After 48 hours of observation, the death rate was counted. The LD_{50} of each strain to mice was calculated by bliss method using SPSS 26.0 software.

Therapeutic curve of mice

Forty mice, half male and half female, were randomly divided into 4 groups with 10 mice in each group. After 3 days of adaptive feeding, the mice were kept without food and water for 12 hours. The bacterial culture medium was removed with normal saline, and the mice were injected with twice the concentration of LD_{50}. The first and second groups were injected with *PMS* bacterial solution, and the third and fourth groups were injected with △*PMS* bacterial solution.

One hour after bacterial injection, mice in the first and third groups were intramuscularly injected with 100 μL of ENR (2.5 mg/kg body weight) twice a day. The second and the fourth groups were injected with the same dose of normal saline intramuscularly, and the survival conditions of mice in each group were observed and recorded, and plotted by Prism software according to the methods reported in previous[20, 21].

5. Conclusions

In this study, based on transcriptome sequence of three strains of *PMA*, *satP* gene was obtained, whose expression was significantly up-regulated with the increase of drug resistance. Through the study of the formation of *satP* gene of drug resistance, the deletion of *satP* gene in *PMA* can lead to faster growth rate, longer development time of drug resistance, and reduced tolerance. In addition, due to the deletion of *satP* gene, its virulence was 400 times reduced than wild strain. The results indicated that *satP* might be the target of fluoroquinolone resistance inhibitors.

Supplementary Materials:

Author Contributions: Conceptualization, G.X., HAQMAL MOHAMMAD AMAN.; funding acquisition, L. K; H.M.; supervision, H.M.; project administration, H.M.; investigation,C.H.,Y.J., H.Z .and Y.W.; validation, Y.X., X.L., I.M. and J.X. All authors have read and approved this final version of the manuscript.

Funding: This work was supported by the Jilin Province Science and Technology Department Theme Guide Project (20180101003JC), National Natural Science Foundation of China (31702293), Jilin Natural Science Foundation (20190201294JC).

Conflicts of Interest: The authors declare that there is no conflict of interest.

References

1. Townsend, K.M., et al., Genetic organization of Pasteurella multocida cap Loci and development of a multiplex capsular PCR typing system. J Clin Microbiol, 2001. 39(3): p. 924-9.
2. Christenson, E.S., H.M. Ahmed and C.M. Durand, Pasteurella multocida infection in solid organ transplantation. Lancet Infect Dis, 2015. 15(2): p. 235-40.
3. Zhao, G., H. He and H. Wang, Use of a recombinase polymerase amplification commercial kit for rapid visual detection of Pasteurella multocida. BMC Vet Res, 2019. 15(1): p. 154.
4. Stanford, K., et al., Antimicrobial Resistance in Members of the Bacterial Bovine Respiratory Disease Complex Isolated from Lung Tissue of Cattle Mortalities Managed with or without the Use of Antimicrobials. Microorganisms, 2020. 8(2).
5. Elsayed, M., et al., Virulence Determinants and Antimicrobial Profiles of Pasteurella multocida Isolated from Cattle and Humans in Egypt. Antibiotics (Basel), 2021. 10(5).
6. Peng, Z., et al., A capsule/lipopolysaccharide/MLST genotype D/L6/ST11 of Pasteurella multocida is likely to be strongly associated with swine respiratory disease in China. Arch Microbiol, 2018. 200(1): p. 107-118.
7. Zhu, D., et al., Emergence of a multidrug-resistant hypervirulent Pasteurella multocida ST342 strain with a floR-carrying plasmid. J Glob Antimicrob Resist, 2020. 20: p. 348-350.
8. Kong, L.C., et al., Antimicrobial susceptibility and molecular typing of Pasteurella multocida isolated from six provinces in China. Trop Anim Health Prod, 2019. 51(4): p. 987-992.
9. Michael, G.B., et al., ICEPmu1, an integrative conjugative element (ICE) of Pasteurella multocida: analysis of the regions that comprise 12 antimicrobial resistance genes. J Antimicrob Chemother, 2012. 67(1): p. 84-90.
10. Kong, L.C., et al., Fluoroquinolone resistance mechanism of clinical isolates and selected mutants of Pasteurella multocida from bovine respiratory disease in China. J Vet Med Sci, 2014. 76(12): p. 1655-7.
11. Cao, L., et al., Transcriptional analysis reveals the relativity of acid tolerance and antimicrobial peptide resistance of Salmonella. Microb Pathog, 2019. 136: p. 103701.
12. Shin, B., C. Park and W. Park, Stress responses linked to antimicrobial resistance in Acinetobacter species. Appl Microbiol Biotechnol, 2020. 104(4): p. 1423-1435.
13. Alam, M.K., et al., RecA Inhibitors Potentiate Antibiotic Activity and Block Evolution of Antibiotic Resistance. Cell Chem Biol, 2016. 23(3): p. 381-91.
14. Beaber, J.W., B. Hochhut and M.K. Waldor, SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature, 2004. 427(6969): p. 72-4.
15. Thomas, E., et al., Field evaluation of efficacy and tolerance of a 2% marbofloxacin injectable solution for the treatment of respiratory disease in fattening pigs. Vet Q, 2000. 22(3): p. 131-5.
16. Tang, X., et al., Isolation, antimicrobial resistance, and virulence genes of Pasteurella multocida strains from swine in China. J Clin Microbiol, 2009. 47(4): p. 951-8.
17. Kong, L.C., et al., Antimicrobial susceptibility and molecular characterization of macrolide resistance of Mycoplasma bovis isolates from multiple provinces in China. J Vet Med Sci, 2016. 78(2): p. 293-6.
18. Sun, P., et al., Crystal structure of the bacterial acetate transporter SatP reveals that it forms a hexameric channel. J Biol Chem, 2018. 293(50): p. 19492-19500.
19. Indrajith, S., et al., Molecular insights of Carbapenem resistance Klebsiella pneumoniae isolates with focus on multidrug resistance from clinical samples. J Infect Public Health, 2021. 14(1): p. 131-138.
20. Yamamoto, K., et al., Coexpression of MmpS5 and MmpL5 Contributes to Both Efflux Transporter MmpL5 Trimerization and Drug Resistance in Mycobacterium tuberculosis. mSphere, 2021. 6(1).
21. Li, W., et al., An integrated quantitative proteomic and metabolomics approach to reveal the negative regulation mechanism of LamB in antibiotics resistance. J Proteomics, 2019. 194: p. 148-159.
22. Safi, H., et al., Phase variation in Mycobacterium tuberculosis glpK produces transiently heritable drug tolerance. Proc Natl Acad Sci U S A, 2019. 116(39): p. 19665-19674.
23. Petes, C., et al., The effects of CD14 and IL-27 on induction of endotoxin tolerance in human monocytes and macrophages. J Biol Chem, 2018. 293(45): p. 17631-17645.

24. Westfall, C., et al., The Widely Used Antimicrobial Triclosan Induces High Levels of Antibiotic Tolerance In Vitro and Reduces Antibiotic Efficacy up to 100-Fold In Vivo. Antimicrob Agents Chemother, 2019. 63(5).
25. Poudyal, B. and K. Sauer, The ABC of Biofilm Drug Tolerance: the MerR-Like Regulator BrlR Is an Activator of ABC Transport Systems, with PA1874-77 Contributing to the Tolerance of Pseudomonas aeruginosa Biofilms to Tobramycin. Antimicrob Agents Chemother, 2018. 62(2).
26. Ronneau, S. and R. Hallez, Make and break the alarmone: regulation of (p)ppGpp synthetase/hydrolase enzymes in bacteria. FEMS Microbiol Rev, 2019. 43(4): p. 389-400.
27. Niu, Y., et al., Antibacterial Effect of Caffeic Acid Phenethyl Ester on Cariogenic Bacteria and Streptococcus mutans Biofilms. Antimicrob Agents Chemother, 2020. 64(9).
28. Ma, D., et al., The Toxin-Antitoxin MazEF Drives Staphylococcus aureus Biofilm Formation, Antibiotic Tolerance, and Chronic Infection. mBio, 2019. 10(6).
29. Zhu, D., et al., Emergence of a multidrug-resistant hypervirulent Pasteurella multocida ST342 strain with a floR-carrying plasmid. JOURNAL OF GLOBAL ANTIMICROBIAL RESISTANCE, 2020. 20: p. 348-350.
30. Langmead, B., et al., Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol, 2009. 10(3): p. R25.
31. McKenna, A., et al., The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res, 2010. 20(9): p. 1297-303.
32. Westfall, C., et al., The Widely Used Antimicrobial Triclosan Induces High Levels of Antibiotic Tolerance In Vitro and Reduces Antibiotic Efficacy up to 100-Fold In Vivo. Antimicrob Agents Chemother, 2019. 63(5).
33. Cirz, R.T., et al., Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biol, 2005. 3(6): p. e176.
34. Zuluaga, A.F., et al., Neutropenia induced in outbred mice by a simplified low-dose cyclophosphamide regimen: characterization and applicability to diverse experimental models of infectious diseases. BMC Infect Dis, 2006. 6: p. 55.