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Mig-14 may contribute to *Salmonella enterica* serovar Typhi resistance to polymyxin B by decreasing the permeability of the outer-membrane and promoting the formation of biofilm

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ABSTRACT

Mig-14 is essential for *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) resistance to antimicrobial peptides, including polymyxin B (PB). However, the molecular mechanism is as yet unknown. In this study, we demonstrated that *mig-14* also played a crucial role in *Salmonella enterica* serovar Typhi (*S.* Typhi) resistance to PB. A series of genes associated with drug-resistance controlled by Mig-14 were identified in the presence of PB. Among which, *ompF* and *ompC* were up-regulated 8 and 6 folds in *mig-14* mutant (Δ*mig-14*) strains, respectively. Further, the deletion of *ompF* or/and *ompC* in Δ*mig-14* strains decreased their sensitivity to PB. Besides, the biofilm formation ability was reduced in Δ*mig-14* strains. Our results indicate that Mig-14 may contribute to PB resistance in *S.* Typhi by decreasing the permeability of the outer membrane and promoting biofilm formation.

1. Introduction

Typhoid fever causes hundreds of thousands of deaths annually worldwide. *S.* Typhi is the leading cause of the disease (Parry et al., 2002; Crump et al., 2004; Wain et al., 2015; Bhutta et al., 2018; Johnson et al., 2018). Little is known about the molecular mechanism of its survival in the host and its unique pathogenic features. The generation of multidrug-resistant *S.* Typhi caused by antibiotic drug abuse has brought about the burden of the increasing recurrence rate of typhoid fever (Qamar et al., 2014; Crump et al., 2015; Schwartz and Morris, 2018).

Antimicrobial peptides produced by host cells for innate immune defense are usually small cationic compounds with different structures (Hancock and Scott, 2000; Scott and Hancock, 2000). Their charge interaction with the anionic microbial membrane is a key step leading to microbial killing (Hancock and Scott, 2000; Scott and Hancock, 2000). The polymyxins have a strong antibacterial effect on many Gram-negative bacteria. Because of their resistance to most antibiotics and the lack of new antibacterial agents that are active against Gram-negative bacteria, they have been re-used as valuable supplements for antibacterial chemotherapeutics (Storm et al., 1977; Vaara, 1992; Trent et al., 2001).

Pathogenic bacteria are often resistant to antimicrobial peptides by modifying the structures of the lipopolysaccharides (LPS) located on the outer membrane, which may contribute to their virulence (Raetz et al., 1996; Brodsky et al., 2002). PB is an amphipathic cationic molecule, which can bind to LPS and permeabilize membranes of gram-negative bacteria (Storm et al., 1977; Vaara, 1992; Brodsky et al., 2002). A major mechanism of resistance to PB is the addition of 4-Aminoarabinose to the 4′ phosphate group of lipid A, an endotoxic component of LPS (Gunn et al., 1998; Brodsky et al., 2002).

mig-14, induced by the host, was first identified as a virulence gene in *S.* Typhimurium (Valdivia and Falkow, 1997; Bäumler, 1997; Bäumler and Heffron, 1998; Valdivia et al., 2000). Mig-14 has been shown to play a role in the long-term survival of *S.* Typhimurium in some tissues in infected mice (Brodsky et al., 2005). It is located in the centisome 61, which may be horizontally transmitted. After a long period of evolution, *mig-14* is only found in *S. enterica* subspecies I which can only infect the warm-blooded animals (Valdivia et al., 2000). *mig-14* is expressed in a *phoP*-dependent manner within cultured macrophages (Brodsky et al., 2005). In addition, *mig-14* is also *phoP*-independently up-regulated after treatment with PB (Brodsky et al., 2002). Compared with wild-type (WT) strains, Δ*mig-14* strains of *S.* Typhimurium were more susceptible to PB, the mammalian antimicrobial peptide protegrin-1 (PG-1) and cathelin-related anti-microbial peptide (CRAMP) (Brodsky et al., 2002, 2005). However, no

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detectable differences in the structures of lipid A, even the global LPS profiles were found between WT and Δ*mig-14* strains, indicating a new mechanism of *mig-14* exits that contributes to *Salmonella* resistance to PB (Brodsky et al., 2002, 2005).

Mig-14 contains the helix-loop-helix motif which is homologous to that of the AraC family. Our previous study also found that *mig-14* was up-regulated at the beginning of high osmotic stress and can regulate a series of genes expression under high-osmotic treatment (Sheng et al., 2013). Therefore, we hypothesize that Mig-14 may contribute to antimicrobial peptide resistance by regulating the expression of downstream genes involved in antibiotic resistance.

The outer membrane is the key element for permeation of the cell. OmpF and OmpC, which are regulated by EnvZ/OmpR, are two main porin proteins of the outer membrane in most Gram-negative bacteria (Nikaido and Nakae, 1979; Osborn and Wu, 1980; Nikaido, 2003). The small molecules are scattered rapidly by the pores formed by OmpF and OmpC (Nikaido and Nakae, 1979; Jaffe et al., 1982). *Escherichia coli* (*E. coli*) lacking OmpF showed increased resistance to chloramphenicol and tetracycline (Lutkenhaus, 1977; Pugsley and Schnaltman, 1978; Jaffe et al., 1982). OmpC plays a key role in accumulating cephalothin and cefoxitin in *E. coli*, thereby, increasing the effectiveness of cephalosporins in *S.* typhimurium (Medeiros et al., 1987; Mortimer and Piddock, 1993). All in all, porin-deficient mutants of *S.* Typhimurium and *E. coli* showed a great decrease in permeability of the outer membrane to many antibiotics.

In this present study, in seeking for the molecules that mediate Mig-14 functions in antimicrobial peptide resistance, the genomic DNA microarray profiling was used to analyze WT strains and Δ*mig-14* strains upon exposure to PB. According to the results of microarray, we further constructed *mig-14-ompF* double mutant strain (Δ*mig-14-ompF*), *mig-14-ompC* double mutant strain (Δ*mig-14-ompC*), *mig-14-ompF-ompC* triple mutant strain (Δ*mig-14-ompC-ompF*), and compared their sensitivity to PB. Besides, biofilm formation analysis was performed on Δ*mig-14* strains under treatment of PB in comparison with WT strains.

2. Materials and methods

2.1. Bacteria, plasmids, and culture conditions

S. Typhi GIFU10007, a z66 flagellar antigen-positive WT strain was used as the parent strain for all mutants generated in this study. z66 flagellar antigen-positive WT strain was first discovered in *S.* Typhi strains collected from Indonesia and named by Guinee et al. in 1981. The *mig-14* (Δ*mig-14*) and the *ompF* mutant (Δ*ompF*) were constructed in our previous work (Sheng et al., 2013). Unless otherwise noted, the culture condition is at 37 °C with shaking (200 rpm). The information on bacteria and plasmids can be found in Table S1. Ampicillin (Amp) (100 μg/ml) was used for selecting strains when required.

2.2. Strain construction

The fusion protein (OmpC::3 × FLAG) was constructed as described previously (Zhang et al., 2015). In brief, two pairs of primers (*ompC*-F1A/1B and *ompC*-F2A/2B) were designed to amplify F1 (404 bp) and F2 (626 bp) fragments (Table S2) located in 5′-end and 3′-end of the *ompC* gene, respectively. In order to obtain the fragment (1005 bp) which includes the FLAG DNA (66 bp), the templates F1 and F2 were amplified by the primers *ompC*-F1A/F2B. The plasmid pGMB151, with the sucrose-sensitive *sacB* gene, was inserted at the BamHI site and transformed into the *S.* Typhi WT strains and the Δ*mig-14* strains by standard transformation method. The positive mutant strains were identified by PCR using *ompC*-F1A/F2B. These two strains were designated as WT-OmpC::3 × FLAG, and Δ*mig-14*-OmpC::3 × FLAG. Strains WT-OmpF::3 × FLAG and Δ*mig-14*-OmpF::3 × FLAG were constructed as described above using the primer pairs *ompF*-F1A/1B and *ompF*-F2A/2B.

Construction of Δ*mig-14-ompC*, Δ*mig-14-ompF* and Δ*mig-14-ompC-ompF* strains were performed as described previously (Khan et al., 1998; Huang et al., 2004). The primers used are listed in Table S2. The mutant bacteria were confirmed by PCR using *ompC*-F1A'/*ompC*-F2B' and P-*mig-14*-A/P-*mig-14*-B.

2.3. PB sensitivity assay

For PB sensitivity assay, 1 ml of LB at pH 7.4 was inoculated with a single clone and grown overnight. After, the culture (300 μl) was transferred into 30 ml pre-warmed (37 °C) fresh LB broth with 0.01, 0.05, 0.1, 0.15, 0.25 μg/ml of PB (Sigma). Then, the growth of bacteria under different conditions was measured per hour by using the Bio photometer, and all the experiments were performed for three times respectively.

2.4. RNA extraction

For RNA extraction, 1 ml of LB was inoculated with WT strains and Δ*mig-14* strains and grown overnight. PB was added into the cultures (0.1 μg/ml) at the log phase (OD_{600} 0.5) and further incubated for 5 h. Total RNA from bacteria was isolated with the RNeasy kit. The mRNA concentration and quality was checked with agarose gel and determined spectrophotometrically. The contaminating genomic DNA in the isolated total RNA was removed using the Ambion's DNA-free kit.

2.5. DNA microarray analysis to identify genes regulated by Mig-14

The synthesis of first strand cDNA and the fluorescence labeling of cDNA were performed from the total RNA. The analytical and statistical methods were described in previous studies (Tusher et al., 2001; Sheng et al., 2009). The value of the $\log_2$ ratio of Cy3/Cy5 intensity that was larger than 1 or smaller than -1 was considered as significant differential expression.

2.6. qRT-PCR analysis

cDNA was synthesized from 2 μg of total RNA using Reverse Transcriptase (Takara) with random and gene-specific primers. Then the product was used for qRT-PCR assay with specific primers (Table S2). 5S rRNA was used to normalize all samples. The comparative CT method was used to determine the relative mRNA levels. All the experiments were performed in triplicates.

2.7. Western Blot analysis

Bacteria were cultured as described in the RNA extraction section. Western blotting was performed as described previously with primary antibodies anti-FLAG (1:200, CMC Scientific) or anti-DnaK (1:5000, Enzo Life Sciences) at 4 °C. ImageJ64 (National Institutes of Health) was performed to quantify the intensities.

2.8. Biofilm formation assay

Biofilm formation assay was performed as described previously with some modifications (Stepanovic et al., 2004). Overnight cultures were diluted 1:100 in sterile TSB with 0.1 μg/ml of PB for incubation. At bacteria growth of OD_{600} 0.5, 0.02% (w/v), aliquots of 200 μl cell suspensions were transferred to a new 96-well plate with a lid. Some wells were filled with fresh TSB as blank controls. After incubation at 30 °C for 4 d without shaking, the planktonic cells were carefully removed. Unattached cells were removed by washing the wells twice with Phosphate-buffered saline. Adherent biofilm was treated with Methanol (200 μl) for 10 m and 1% (w/v) crystal violet (300 μl) for 10 m. The unbound dye was removed and the wells were washed for 3 times with clean water. The microtiter plate was air-dried for 2 h and stained

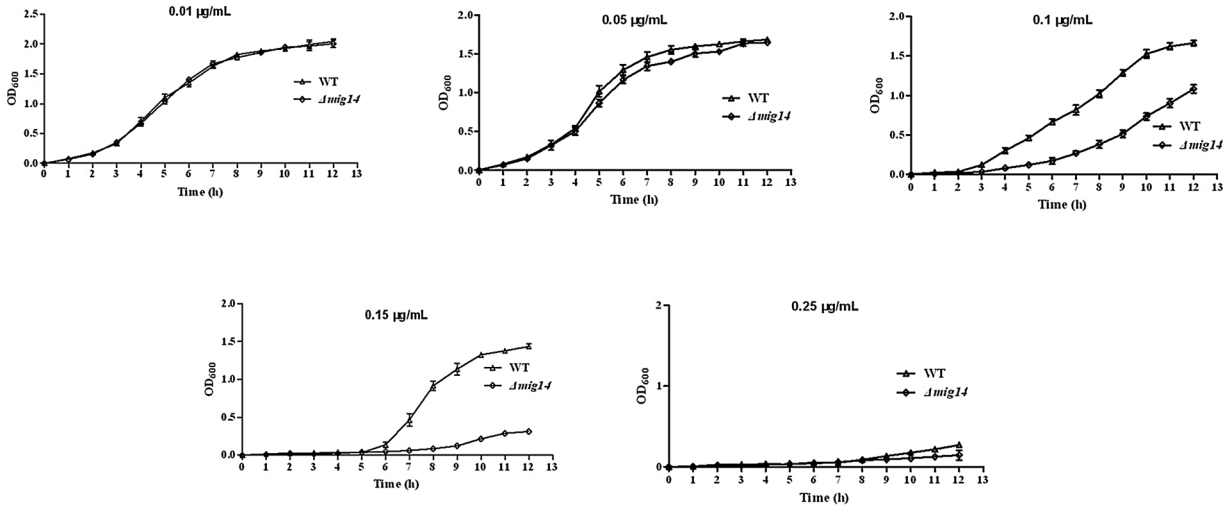

Fig. 1. Sensitivity of *S.* Typhi strains to polymyixn B (PB). WT and Δ*mig-14* were treated with 0.01, 0.05, 0.1, 0.15, 0.25 μg/ml of PB as described in *Materials and methods*, respectively. Bio Photometer was used to measure cell growth for three independent times. The sensitivity of Δ*mig-14* to PB was in a dosage dependent manner. 0.1 μg/ml PB was the selected concentration for later research.

biomass was treated by 200 μl 30% (v/v) acetic acid. After 10 min, OD_{570} was measured to quantify the biofilm formation ability. The assays were repeated for three independent times.

2.9. Statistical analysis

Data presented here are the means of 3 experiments ± standard error (SE). The student's *t*-test was used for statistical analysis. $P<0.05$ was regarded as statistically significant.

3. Results

3.1. Mig-14 is necessary for S. Typhi resistance to PB

mig-14 plays a crucial role in *S.* Typhimurium resistance to polymyxin (Brodsky et al., 2002). To verify whether *mig-14* is necessary for *S.* Typhi resistance to PB, the survival of WT strains and Δ*mig-14* strains under different concentration of PB treatment were measured. As is shown in Fig. 1, compared with WT strains, Δ*mig-14* strains were more sensitive to PB, which is similar to the results observed in *S.* Typhimurium. The sensitivity of Δ*mig-14* strains to PB was observed in a dosage-dependent manner. The growth of Δ*mig-14* strains was similar to that of WT strains when exposed to 0.01 and 0.05 μg/ml of PB. Both WT strains and Δ*mig-14* strains were killed by PB at the concentration of 0.25 μg/ml. The growth of Δ*mig-14* strains was much slower than that of WT strains when exposed to 0.10 and 0.15 μg/ml of PB, indicating that Δ*mig-14* strains were more sensitive to PB. A small number of Δ*mig-14* strains survived under exposure to high concentration of PB (> 0.15 μg/ml). Therefore, 0.1 μg/ml of PB was chosen to perform the subsequent research.

3.2. Identification of genes targeted by Mig-14 under PB exposure

Modification of the structures of LPS is not the mechanism that Mig-14 mediates *S.* Typhimurium resistance to antimicrobial peptides (Brodsky et al., 2002, 2005). The sequence analysis and our previous study showed that Mig-14 may be a regulatory protein (Sheng et al., 2013). To examine whether Mig-14 contributes to PB resistance by regulating the expression of genes related to antimicrobial resistance, we analyzed the transcriptome profiling of Δ*mig-14* strains and WT strains after treatment with 0.1 μg/mL of PB for 5 h. 604 genes showed different expression levels (Table 1), among which, 308 genes in Δ*mig-14* strains were down-regulated by exposure to PB. The invasion-associated genes (*invAEFGH*, *sopDE*, *iagAB*, *sigD*), effector genes (*sipABCD*, *prgJKH*), virulence genes (*spaIJKMNOPQRST*), and genes related to biofilm formation (*csgD*) were significantly down-regulated. Some genes with unknown function (Hypothetical protein *t1640*, *t1641*, *t1642*, *t1643*) were dramatically down-regulated (8-fold, 64-fold, 59-fold and 49-fold, respectively) in Δ*mig-14* strains. 296 genes of Δ*mig-14* strains were up-regulated compared with WT strains upon exposure to PB. The flagella genes (*flhABE*, *flgABCDEFGHIJ*, *fliFGHIJKLMOPQR*) were significantly up-regulated in Δ*mig-14* strains. Two genes *ompF* and *ompC*, encoding outer membrane proteins, were up-regulated 8-fold and 3-fold, respectively. These results indicated that Mig-14 can regulate a number of genes expression in the presence of PB.

qRT-PCR results were in line with the above results, which confirmed the regulation of 6 selected genes by Mig-14 (Fig. 2). These selected genes included 3 down-regulated genes (*invF*, *ssrB*, *csgD*) and 3 up-regulated genes (*flhA*, *ompC*, *ompF*).

3.3. Mig-14 may contribute to PB resistance by affecting the permeability of the outer membrane in S. Typhi

OmpF and OmpC have been confirmed to participate in resistance to antibiotics by changing the permeability of the outer membrane (Jaffe et al., 1982; Nikaido, 2003). Our microarray and qRT-PCR results showed that deleting *mig-14* in *S.* Typhi increased the mRNA levels of *ompF* and *ompC* upon treatment with PB (Fig. 2). Western Blotting results showed that OmpC and OmpF proteins were also elevated when *mig-14* was deleted (Fig. 3A, C). By quantifying the relative intensities of the protein bands with the ImageJ software program, we found that the protein levels of OmpC and OmpF were increased 1.6 and 5.2 folds, respectively (Fig. 3B, D). Therefore, to examine whether OmpC or/and OmpF negatively mediates Mig-14 resistance to PB, we deleted *ompC* or/and *ompF* in Δ*mig-14* strains to construct Δ*mig-14-ompC* double mutant strains, Δ*mig-14-ompF* double deletion mutants and Δ*mig-14-ompC-ompF* triple deletion mutant strains, and compared their sensitivity to PB with WT strains and Δ*mig-14* strains. As is shown in Fig. 4, Δ*mig-14* strains were more sensitive to PB compared with WT strains. The deletion of *ompC* or/and *ompF* in Δ*mig-14* strains decreased the sensitivity to PB caused by the deletion of *mig-14* gene in *S.* Typhi. However, their growth was still slower than that of WT strains. When *ompC* or/and *ompF* were re-expressed in the complementary strains, they became sensitive to PB again which was similar to Δ*mig-14*. These results demonstrated that Mig-14 may contribute to PB resistance partially through down-regulating the expression of *ompF* and *ompC* to

Table 1
Gene expression changes in Δ*mig-14* strain compared to wild-type stain of *S.*Typhi upon PB exposure.

Functional kinds	Product function	Genes	Log $_2$ R*
Downregulated			
Invasion proteins			
	Invasion-associated secreted protein	*sopE*	−5.295
	Pathogenicity island 1 effector protein	*sipA*	−2.966
	Pathogenicity island 1 effector protein	*sipB*	−3.845
	Pathogenicity island 1 effector protein	*sipC*	−2.7
	Pathogenicity island 1 effector protein	*sipD*	−3.348
	Pathogenicity 1 island effector protein	*prgJ*	−3.457
	Pathogenicity 1 island effector protein	*prgK*	−2.333
	Pathogenicity 1 island effector protein	*prgH*	−3.419
	Possible virulence-associated secretory protein	*invA*	−2.217
	Cell invasion protein	*invE*	−3.316
	Cell invasion protein	*iagB*	−3.289
	Cell invasion protein	*sigD*	−3.103
	Oxygen-regulated invasion protein	*orgA*	−2.924
Regulators and putative regulators			
	Possible arac-family regulatory protein	*invF*	−3.425
	Invasion protein regulator	*iagA*	−3.255
	putative two-component response regulator	*ssrB*	−1.316
	Putative transcriptional regulator	*ygaE*	−3.083
	Putative regulatory protein	–	−2.548
	Cation transport regulator chab	*chaB*	−1.998
	Transcriptional regulatory protein	*ecnR*	−1.995
	Arac-family transcriptional regulator	–	−1.774
	Transcriptional activator caif	*caiF*	−1.608
	putative regulatory protein	*csgD*	−2.128
	Putative transcriptional regulator	*mig-14*	−3.303
	Flagellar transcriptional activator	*flhC*	−1.239
Flagella and chemotaxis proteins			
	Methyl-accepting chemotaxis citrate transducer	–	−1.556
	Methyl-accepting chemotaxis protein II	*tar*	−1.1
	Flagellar motor protein	*motA*	−1.093
	Flagellar biosynthesis protein	*fliC*	−1.053
Hypothetical proteins			
	Hypothetical protein t1640	–	−3.168
	Hypothetical protein t1641	–	−6.096
	Hypothetical protein t1642	–	−5.866
	Hypothetical protein t1643	–	−4.958
	Putative phage tail fiber protein	–	−4.037
	Putative periplasmic protein	–	−3.808
	Hypothetical protein t1479	–	−3.722
	Hypothetical protein t2036	–	−3.313
	Hypothetical protein t2782	–	−3.224
	Hypothetical protein t0682	–	−3.204
Metabolism-associated proteins			
	Trehalose-6-phosphate synthase	*otsA*	−2.734
	Trehalose phosphatase	*otsB*	−4.753
	Phosphate starvation-inducible protein psif	*psiF*	−4.281
	Fructose-bisphosphate aldolase	*fbaB*	−3.705
	Copper-zinc superoxide dismutase	*sodC*	−3.124
	Anaerobic sulfite reductase subunit A	*asrA*	−2.089
	Anaerobic sulfite reductase subunit B	*asrB*	−*3.091*
	Type III secretion system atpase	*spaI*	−2.409
	Thiosulfate reductase electron transport protein	*phsB*	−2.398
	Thiosulfate reductase precursor	*phsA*	−2.257
	Iron transport protein, periplasmic-binding protein	*sitA*	−2.279
Other functional proteins			
	Trpr binding protein wrba	*wrbA*	−4.045
	Antigen presentation protein span	*spaN*	−3.39
	Putative outer membrane protein	–	−2.317
	Curved DNA-binding protein	*cbpA*	−2.097
	Osmotically inducible lipoprotein B precursor	*osmB*	−1.819
	Osmotically inducible protein C	*osmC*	−1.707
	Osmotically inducible lipoprotein E precursor	*osmE*	−1.99
	Acidic protein msyb	*msyB*	−1.848
	Type-1 fimbrial protein, a chain precursor	*fimA*	−1.781
	Putative lipoprotein	*blc*	−1.326
Upregulated			
Hypothetical proteins			
	Hypothetical protein t2269	*ybdB*	2.388
	Hypothetical protein t0335	–	*1.11*
	Hypothetical protein t3506	*yijP*	2.092
Regulators and putative regulators			
	Regulatory protein soxs	*soxS*	1.781
	Transcriptional regulatory protein	*cII*	1.203
	Putative arac-family transcriptional regulator	*yijO*	1.3
Flagella-associated proteins			
	Flagellar biosynthesis protein	*flhA*	2.381
	Flagellar biosynthesis protein	*flhB*	1.165
	Flagellar protein flhe precursor	*flhE*	2.61
	Flagellar basal body rod protein	*flgB*	2.22
	Flagellar basal body rod protein	*flgC*	2.079
	Flagellar basal body rod modification protein	*flgD*	2.123
	Flagellar hook protein	*flgE*	2.079
	Putative flagellar basal-body rod protein flgf	*flgF*	2.488
	Flagellar basal-body rod protein flgg	*flgG*	1.539
	Flagellar L-ring protein precursor	*flgH*	2.423
	Flagellar P-ring protein precursor	*flgI*	2.124
	Flagellar biosynthesis protein	*flgJ*	2.172
	Flagellar motor protein	*fliG*	1.742
	Flagellar assembly protein	*fliH*	1.962
	Flagellum-specific ATP synthase	*fliI*	2.098
	Flagellar hook-length control protein	*fliK*	2.559
	Flagellar protein flio	*fliO*	2.048
	Flagellar biosynthesis protein	*fliP*	1.873
	Flagellar biosynthesis protein	*fliQ*	2.662
Molecular chaperone			
	Chaperonin groel	*groEL*	2.09
	Co-chaperonin groes	*groES*	2.632
Metabolism-associated proteins			
	Probable nitrate reductase	*napA*	3.445
	Cytochrome c-type protein napc	*napC*	1.988
	Quinol dehydrogenase periplasmic component	*napG*	3.263
	Quinol dehydrogenase membrane component	*napH*	3.218
	Cytochrome o ubiquinol oxidase subunit II	*cyoA*	3.69
	Cytochrome o ubiquinol oxidase subunit I	*cyoB*	2.979

(*continued on next page*)

Table 1 (*continued*)

Functional kinds	Product function	Genes	Log $_2$ R*
	Cytochrome o ubiquinol oxidase C subunit	*cyoD*	2.976
	Protoheme IX farnesyltransferase	*cyoE*	2.604
	Nitrite reductase large subunit	*nirB*	3.363
	Putative nitrite transporter	*nirC*	2.276
	Nitrite reductase small subunit	*nirD*	2.09
	Ketol-acid reductoisomerase	*ilvC*	3.435
	Isochorismatase	*entB*	2.076
	Isochorismate synthase	*entC*	1.695
	2,3-dihydroxybenzoate-AMP ligase	*entE*	2.412
	L-serine dehydratase 2	*sdaB*	1.76
	Putative serine transporter	*sdaC*	2.024
	Lon protease	*lon*	2.014
Other functional proteins			
	Outer membrane protein C	*ompC*	2.164
	Outer membrane protein F precursor	*ompF*	3.079
	30S ribosomal protein S3	*rpsC*	1.963
	30S ribosomal protein S12	*rpsL*	2.015
	30S ribosomal protein S19	*rpsS*	2.009
	50S ribosomal protein L16	*rplP*	1.976
	50S ribosomal protein L18	*rplR*	1.753
	50S ribosomal protein L29	*rpmC*	1.783
	Elongation factor EF-2	*fusA*	2.489

* Log_2 (Δ*mig-14*/wild).

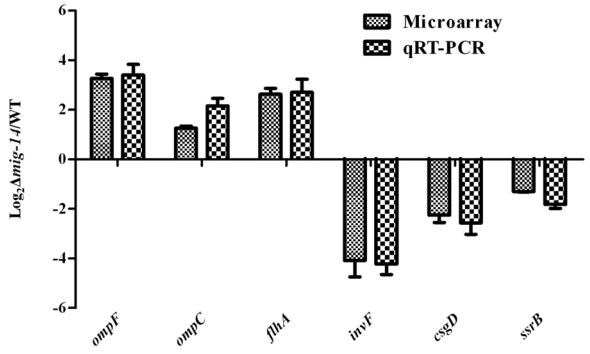

Fig. 2. qRT-PCR results were in line with the results of microarray. A set of genes including *ompF*, *ompC*, *flhA, invF*, *csgD*, *ssrB* were chosen to perform qRT-PCR to compare the expression difference between WT and Δ*mig-14* upon PB exposure. Relative mRNA levels were determined by calculating the threshold cycle (ΔCt) of each gene. 5S rRNA was used as the internal reference. All the experiments were performed triplicately.

decease the permeability of the outer membrane.

3.4. Mig-14 promotes biofilm formation in S. Typhi

The deletion of *ompC* or/and *ompF* in Δ*mig-14* strains only partially decreased the PB sensitivity caused by the deletion of *mig-14* gene in *S.* Typhi. It has been reported that antibiotic treatment has a low efficacy on bacteria biofilm (Scher et al., 2005; Turki et al., 2014; Singh et al., 2017), which encourages us to investigate whether Mig-14 promotes biofilm formation in *S.* Typhi under the treatment of PB. As is shown in Fig. 5, Δ*mig-14* strains had a lower biofilm level than that in the WT strains. The biofilm levels in complementary strains (Δ*mig-14*(**pBAD-mig-14-A**)) were restored near to that of the WT strains. The results demonstrated that Mig-14 can promote *S.* Typhi to form biofilms for surviving in the unfavorable environment. Therefore, promoting biofilm formation may be another mechanism that Mig-14 contributes to *S.* Typhi resistance to PB.

4. Discussion

As a mobile genetic element in *S. enterica*, *mig-14* was required for virulence in orally infected mice (Valdivia et al., 2000). The presence of mobile genetic elements is a recurrent theme in *Salmonella* pathogenesis, which can enhance the bacterium's virulence by seeking for a different host or increasing their own resistance (Ochman and Groisman, 1996; Bäumler, 1997; Miao and Miller, 1999). *mig-14* of *S.* Typhimurium plays no apparent role in the housekeeping functions or in the primary metabolism. *mig-14* expression can be induced by growing in different unfavorable environments, such as in acidic pH conditions, in minimal medium containing low magnesium, within macrophages, or when treated with antimicrobial peptides, indicating it is controlled by the global regulator PhoP (Brodsky et al., 2002, 2005). *mig-14* is essential for *S.* Typhimurium resistance to PB and protegrin-1 (Brodsky et al., 2002). However, Mig-14 cannot modify the structures of LPS under PB challenge (Brodsky et al., 2002). The mechanism that *Salmonella* resists to PB by Mig-14 still remains unknown.

Mig-14 contains the helix-loop-helix motif which is homologous to AraC family (Gallegos et al., 1997; Sheng et al., 2013). Our previous study also showed that Mig-14 of *S.* Typhi can regulate a lot of genes expression under increasing osmotic conditions for half an hour (Sheng et al., 2013). Therefore, genomic DNA microarrays were used to compare the transcriptome in WT strains and Δ*mig-14* strains to search the genes involved in antibiotic resistance. As expected, we first found that Mig-14 of *S.* Typhi also plays a key role in resistance to PB. Additionally, we identified some specific genes which were controlled by Mig-14 under the treatment of PB. Furthermore, we found that Mig-14 may contribute to PB resistance by decreasing expression of *ompF* and *ompC* to influence the outer membrane's permeability and promoting *S.* Typhi to form biofilm.

The results of DNA microarray assays showed that the virulence genes located in pathogenicity islands (SPI) in Δ*mig-14* strains were down-regulated in the presence of PB. *invF* which is the transcriptional activator of the type III secretion system (TTSS) and secreted effector proteins were down-regulated 8-fold in Δ*mig-14* strains in the presence of PB (Table 3). *ssrB* which controls the expression of genes located in SPI-2 was down-regulated 2.5-fold in Δ*mig-14* strains by exposure to PB (Table 3). In *S.* Typhi, SPI-1 encodes a TTSS1 involved in invading and infecting epithelial cells of the gut. SPI-2 encodes another TTSS2, which enables the pathogen to survive within tissue mononuclear cells (Johnston et al., 1996; Jones and Falkow, 1996). Although there are some functional differences between TTSS1 and TTSS2, *S.* Typhi needs effectors from both systems for virulence simultaneously. These results indicated that Mig-14 may activate the expression of virulence genes to help *S.* Typhi resist killing by antimicrobial peptides and contribute to their virulence. The regulatory mechanism needs further research.

The deletion of *mig*-14 in *S.* Typhi up-regulated the expression of genes related to motility like flagella, and genes encoding outer membrane pore proteins (*ompF* and *ompC*), indicating that Mig-14 negatively regulates their expression in the presence of PB. The increased protein levels of *ompC* and *ompF* in Δ*mig-14* strains caught our attention. OmpC and OmpF are two major porin proteins which regulate the outer membrane's permeability to small molecules (Nikaido and Nakae, 1979; Jaffe et al., 1982). The resistance to chloramphenicol, tetracycline and to copper were increased in *E. coli* mutants lacking OmpF (Lutkenhaus, 1977; Pugsley and Schnaltman, 1978; Jaffe et al., 1982). OmpC and OmpF can facilitate the diffusion of β-lactams into the outer membrane (Jaffe et al., 1982). The permeability of the outer membrane to antibiotics, such as ampicillin, cephaloridine, and 6-aminopenicillanic acid was significantly decreased in porin-deficient mutants of *S.* typhymurium and *E. coli* (Lutkenhaus, 1977; Pugsley and Schnaltman, 1978; Jaffe et al., 1982; Medeiros et al., 1987; Mortimer and Piddock, 1993). Therefore, to examine whether the decreased resistance to PB in Δ*mig-14* strains is mediated by the expression of OmpC or/and OmpF, we deleted *ompC* or/and *ompF* in Δ*mig-14* strains and compared their

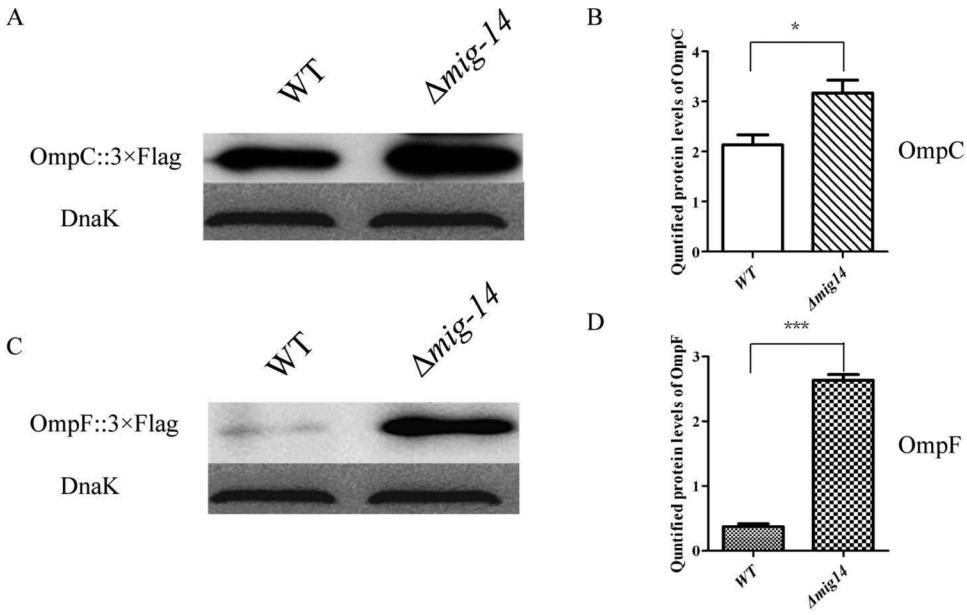

Fig. 3. Deletion of *mig-14* in *S.* Typhi increases the protein levels of OmpC and OmpF upon PB exposure. Western blot analysis of OmpC (A) and OmpF (C) in WT-OmpC::3 × FLAG strains and Δ*mig-14*-OmpC::3 × FLAG strains with anti-FLAG. Quantitation of OmpC protein levels (B) and OmpF protein levels (D). All the experiments were performed triplicately. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; NS: Not statistically significant.

sensitivity to PB with WT strains and Δ*mig-14* strains. The results showed that deleting *ompC* or/and *ompF* in Δ*mig-14* strains can partially increase the resistance to PB caused by deleting *mig-14* gene in *S.* Typhi. Thus, Mig-14 may contribute to PB resistance partially through down-regulating the expression of *ompF* and *ompC* to decrease the permeability of the outer membrane.

We noticed that either *ompC* or *ompF*, even *ompC* alone with *ompF*, can not entirely recover the resistance caused by deleting *mig-14* gene in *S.* Typhi. Previous studies reported that bacteria growing in a biofilm can resist many antibiotics, oxidative and acid stress, and benefits for their long-term survival (Scher et al., 2005; White et al., 2006; Joo and Otto, 2012; Turki et al., 2014; Singh et al., 2017). To kill microbes in a biofilm needs much higher antimicrobial concentrations, because biofilms decrease the antibiotic sensitivities up to a thousand folds (Costerton et al., 1995; Olson et al., 2002; Singh et al., 2017). CsgD, is a key regulator in biofilm formation which controls the production of curli fibers, cellulose and some other biofilm matrix. In this study, both microarray and qRT-PCR results revealed that the expression of *csgD* was down-regulated. Therefore, we further analyzed the biofilm formation ability of Δ*mig-14* strains and WT strains under the treatment of PB. The biofilm formation ability was found to be obviously lower in Δ*mig-14* strains than that of WT strains of *S.* Typhi. The complementary strains (Δ*mig-14*(**pBAD**-*mig-14-A*)) over-expressing the intact *mig-14* recovered and formed biofilms to the level of the WT strains. These results suggest that another mechanism that Mig-14 contributes to PB-resistance is to promote *S.* Typhi to form biofilm.

5. Conclusion

In conclusion, we first found that Mig-14 may contribute to PB resistance in *S.* Typhi by decreasing the outer membrane's permeability and promoting *S.* Typhi to form biofilm. Our results provided crucial downstream pathways and possible molecular mechanisms for Mig-14 resistance to PB, and deepen our understanding of the regulatory network of *S.* Typhi. Hypothetical protein t1640, t1641, t1642 and t1643 were dramatically down-regulated in Δ*mig-14* strains, suggesting they may also contribute to PB resistance, which is worth further research. Our future research work will focus on exploring the molecular mechanism of how Mig-14 regulates gene expression.

Summary points

Mig-14 is essential for S. Typhi resistance to PB

mig-14 plays a crucial role in inducing polymyxin resistance in *S.* Typhimurium. Here, we found that deleting *mig-14* in *S.* Typhi increased the sensitivity to PB.

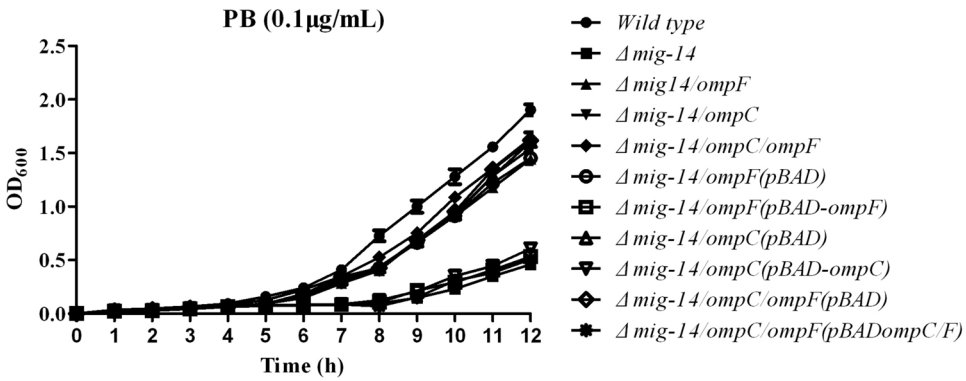

Fig. 4. Deletion of *ompF* and/or *ompC* decreases the sensitivity of Δ*mig-14* strains to PB. WT strains, Δ*mig-14* strains, Δ*mig-14-ompC* strains, Δ*mig-14-ompF* strains, Δ*mig-14-ompC-ompF* strains, Δ*mig-14-ompF*(pBAD) strains, Δ*mig-14-ompF*(pBAD-*ompF*), Δ*mig-14-ompC*(pBAD), Δ*mig-14-ompC*(pBAD-*ompC*), Δ*mig-14-ompC-ompF*(pBAD), and Δ*mig-14-ompC-ompF*(pBAD-*ompC/F*) strains were treated with 0.1 μg/ml of PB as described in *Materials and methods*, respectively. Bio Photometer was used to measure cell growth for three independent times. Δ*mig-14* strain was more sensitive to PB compared with WT strain. The deletion of *ompC* or/and *ompF* in Δ*mig-14* strains decreased their sensitivity to PB.

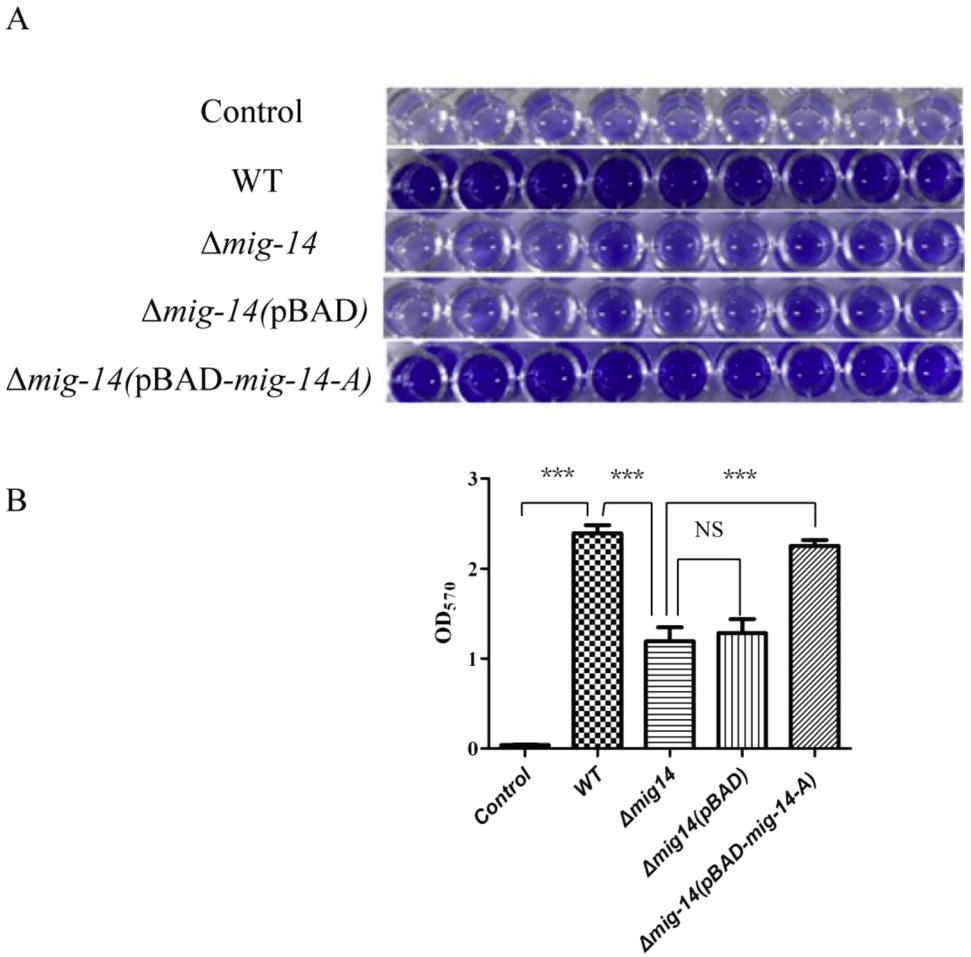

Fig. 5. Deletion of *mig-14* suppresses biofilm formation in *S.* Typhi. (A) Biofilm formation analysis of Control (Blank), WT strains, Δ*mig-14* strains, Δ*mig-14*(pBAD) strains and Δ*mig-14*(pBAD-*mig-14-A*) strains. The experiment was performed in triplicate. (B) Quantitation of biofilm formation by measuring the OD_{570} value. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; NS, Not statistically significant.

Gene-expression profiling regulated by Mig-14 under PB exposure

A whole-genome microarray analysis showed that 604 genes showed significant difference between Δ*mig-14* strains and WT strains of *S.* Typhi. Among which, genes located in SPI-1/2, and genes associated with biofilm were down-regulated. However, the expression of flagellar, *ompF/C* genes were up-regulated in Δ*mig-14* strain.

Mig-14 contributes to PB resistance by affecting the permeability of the outer membrane in S. Typhi

qRT-PCR and Western Blotting analysis confirmed that the expression levels of *ompC* and *ompF* were elevated when *mig-14* was deleted in *S.* Typhi. Deleting *ompF* or/and *ompC* in Δ*mig-14* strain increases their resistance to PB, but do not arrive at the same level to that of the WT strain.

Mig-14 promotes biofilm formation in S. Typhi

Deletion of *mig-14* in *S.* Typhi decreased the biofilm formation ability in Δ*mig-14* strains compared with that in the WT.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.01.001.

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