Genetics and Molecular Research

GlpC gene is responsible for biofilm formation and defense against phagocytes and imparts tolerance to pH and organic solvents in *Proteus vulgaris*

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Genet. Mol. Res. 14 (3): 10619-10629 (2015)
Received February 10, 2015
Accepted June 17, 2015
Published September 9, 2015
DOI http://dx.doi.org/10.4238/2015.September.9.3

ABSTRACT. Biofilm-forming bacteria are highly resistant to antibiotics, host immune defenses, and other external conditions. The formation of biofilms plays a key role in colonization and infection. To explore the mechanism of biofilm formation, mutant strains of *Proteus vulgaris* XC 2 were generated by Tn5 random transposon insertion. Only one biofilm defective bacterial species was identified from among 500 mutants. Inactivation of the *glp*C gene coding an anaerobic glycerol-3-phosphate dehydrogenase subunit C was identified by sequence analysis of the biofilm defective strain. Differences were detected in the growth phenotypes of the wild-type and mutant strains under pH, antibiotic, and organic solvent stress conditions. Furthermore, we observed an increase in the phagocytosis of the biofilm defective strain by the mouse macrophage RAW264.7 cell line compared to the wild-type strain. This study shows that the *glp*C gene plays an important role in biofilm formation, in addition to imparting pH, organic solvent, and antibiotic tolerance, and defense against phagocytosis to *Proteus* sp. The results further clarified the mechanism of biofilm formation at the genomic

level, and indicated the importance of the *glp*C gene in this process. This data may provide innovative therapeutic measures against *P. vulgaris* infections; furthermore, as an important crocodile pathogen, this study also has important significance in the protection of Chinese alligators.

Key words: Biofilm; Chinese alligator; *glp*C; *Proteus vulgaris*

INTRODUCTION

Proteus vulgaris, an opportunistic pathogen found widely in nature, forms a part of the normal intestinal flora of many reptiles, birds, and mammals, including humans. *P. vulgaris* colonizes and causes diseases in these species through their virulence factors and ability to form biofilms. Many diseases caused by this pathogen have been reported; in addition, it has been recognized as major cross-infection pathogen in hospitals because of its strong adherence capacity and survival in so many intermediaries (Manos and Belas, 2006; Jacobsen and Shirtliff, 2011). More importantly, strains belonging to the *Proteus* sp. are often insensitive to the bactericidal action of normal human serum, which poses a clinical problem (Kwil et al., 2013).

Biofilm formation, which occurs under specific conditions, aids bacterial survival under many hostile conditions (Hall-Stoodley et al., 2004) and plays an important role in the persistence of infection (Costerton, 2001). One of the most intriguing and clinically relevant features of microbial biofilms is their significantly higher antibiotic resistance relative to their free-floating counterparts, which causes serious problems in the therapy of biofilm-associated infections. According to reports, the minimum inhibitory concentration (MIC) of antibiotics for biofilms can be 1000-fold higher than that for planktonic bacteria (Høiby et al., 2010). This warrants investigations of bacterial biofilms. Among the *Proteus* strains, the formation of biofilms by *P. mirabilis* on catheter material has been well-documented (Sabbuba et al., 2002; Liaw et al., 2003), although the gene responsible for biofilm development remains to be identified. Pratt and Kolter (1998) suggested that gene products that are important for biofilm development are also important for pathogenesis.

The Chinese alligator (*Alligator sinensis*), which is native to China, is a critically endangered species. In recent years, the number of Chinese alligators has increased markedly with the success of scale breeding. However, the number of Chinese alligators dying because of unknown reasons has also increased. The strain used in this study was isolated from a sick Chinese alligator at the Anhui Research Center of Chinese Alligator Reproduction. Evidence suggested that *P. vulgaris* was associated with the disease, although this was not confirmed. *Proteus* sp. associated with septicemia in crocodiles has been previously reported (Novak and Seigel, 1986). These observations highlight the threat posed to the health of Chinese alligators by *Proteus* sp. The aim of this study was to provide a reference for the prevention and treatment of Chinese alligators infected with this pathogen.

MATERIAL AND METHODS

Strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Both *Escherichia coli* and *P. vulgaris* strains were maintained in solid Luria-Bertani (LB) broth

(containing 1.5% agar) or in liquid LB. In organic solvent tolerance assays, glucose (0.1%) and $MgSO_4$ (10 mM) were added to the LB medium (LBGMg medium). The RAW264.7 cell line was laboratory stored. Dulbecco's modified Eagle's medium-high glucose (DMEM-HI) was obtained from HyClone (USA), fetal bovine serum (FBS) from Gibco (USA), and gentamicin (Gm; 100 μg/mL), 24-well tissue culture plates, and 96-well tissue culture plates (Corning, USA). When required, antibiotics were added at the following concentrations: 100 μg/mL ampicillin (Amp); 50 μg/mL kanamycin (Km); 25 μg/mL chloramphenicol (Cm); and 50 μg/mL polymyxin B. The antibiotics used here were purchased from Hangzhou Tianhe Microorganism Reagent Co. (China).

Table 1. Strains and plasmids.

Strains or plasmid	Description or/and relevant genotype	Source or reference
Escherichia coli strains		
S17-1 *λpir*	RP4-2-Tc::Mu *aphA*::Tn*5recA_pir*lysogen	Laboratory stored
SM10 *λpir*	Km^r, thi^{-1}, *thr*, *leu*, *ton*A, *lac*Y, *sup*E, *rec*A::RP4-2-Tc::Mu, *λpir*	Li et al., 2009
Proteus vulgaris strains		
XC 2	Amp^r	Laboratory stored
Plasmids		
PUT mini-Tn5 Km1	Amp^r, Km^r, ori_{R6K}, $oriT_{RP4}$	Li et al., 2009
PRK600	Cm^r, triparental mating helper plasmid	Li et al., 2009

Triparental conjugation and selection of markerless recombinants

Triparental conjugation was performed as described by Li et al. (2009) with some modifications. In brief, donor strains of *E. coli* S17-1 *λpir*, harboring the PUT mini-Tn5 vector, were mixed with helper strains of *E. coli* HB101 (pRK600) and recipient strains of *P. vulgaris* XC 2 at a ratio of 2:1:4. A 0.22-μm nylon membrane filter was placed on the surface of a LB plate, which was then spotted with the bacterial mix and incubated for 24 h at 37°C. Cells on the filter surface were then suspended in sterile H_2O. Two-fold serial dilutions of the suspension were plated onto media containing antibiotics for counter-selection of donor strains, and selection of recipient cells carrying the transposon marker.

Microtiter biomass assay

The biofilm biomass of each mutant strain was determined as previously described (O'Toole and Kolter, 1998; Merritt et al., 2005; Jin et al., 2006) with some modifications. Overnight inoculum (10 μL) was added to 200 μL medium on each well of sterile 96-well flat-bottomed polystyrene microplates, and then incubated at 37°C for 24 h. The medium was removed and the wells were washed thrice with 200 μL sterile PBS to remove planktonic bacteria. Absolute methanol (200 μL) was added for 15 min to fix the attached bacteria. After drying, the wells were stained with 200 μL (0.1%) crystal violet solution for 15 min. Excess stain was removed by washing with tap water; the wells were then allowed to dry before the addition of 200 μL 95% ethanol. The plates were shaken gently to ensure complete dissolution of the crystal violet and OD_{595} of each well was measured. Each strain was tested in triplicate; all tests were carried out thrice, and the average of the obtained results was determined. A wild-type strain (*P. vulgaris* XC 2) was used as a positive control, and the wells that had not been inoculated with bacteria were used as negative controls.

Determination of the insertion site

The sequence of the insertion site of the mutant was obtained using thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995). In this method, six specific primers and six degenerate primers were designed according to the transposon and bacterial genomic DNA sequences, respectively (Table 2). A third round of amplification was performed after the second amplification. Cycling conditions of TAIL-PCRs are listed in Table 3. Unpurified PCR products were directly used as the template for the next round of PCR after 20 dilutions (20X) with ddH_2O. The DNA fragments obtained after three sequential amplification steps were subjected to sequence analysis.

Table 2. Primers used in thermal asymmetric inter-laced polymerase chain reaction (TAIL-PCR).

	Primer names	Sequences (5'→3')
Arbitrary degenerate primer (AD)	AD1	(G/C)TTGNTA(G/C)TNCTNTGC
	AD2	NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT
	AD3	(A/T)GTGNAG(A/T)ANCANAGA
	AD4	NGTCGA(G/C)(A/T)GANA(A/T)GAA
	AD5	TG(A/T)GNAG(G/C)ANCA(G/C)AGA
	AD6	AG(A/T)GNAG(A/T)ANCA(A/T)AGG
Upstream TAIL-PCR	SP1	CCGTGGCAAAGCAAAAGTTCAAAA
	SP2	GCAACACCTTCTTCACGAGGCAGA
	SP3	GCGCAGGGCTTTATTGATTCCATT
Downstream TAIL-PCR	SP1	ATCAACCGTGGCTCCCTCACTTTC
	SP2	GCCATCACGACTGTGCTGGTCATT
	SP3	GCCGGATCCTCTAGAGTCGACCTG

Primers SP1, SP2, and SP3 were used in the first, second, and third rounds of amplification, respectively. The intervals between SP1 and SP2, and that between SP2 and SP3 were approximately 100 bp; therefore, the target fragment was easily identifiable based on the 100 bp differences between the three arbitrarily primed PCRs.

Table 3. Arbitrarily primed polymerase chain reaction (PCR) procedure.

Reaction	Step	Cycle No.	Thermal condition
First	1	1	94°C (1 min), 98°C (1 min)
	2	5	94°C (30 s), 67.5°C (1 min), 72°C (2 min)
	3	1	94°C (30 s), 25°C (3 min), 72°C (2 min)
	4	15	94°C (30 s), 67.5°C (1 min), 72°C (2 min)
			94°C (30 s), 67.5°C (1 min), 72°C (2 min)
			94°C (30 s), 44°C (1 min), 72°C (2 min)
	5	1	72°C (10 min)
Second	6	15	94°C (30 s), 65°C (1 min), 72°C (2 min)
			94°C (30 s), 65°C (1 min), 72°C (2 min)
			94°C (30 s), 44°C (1 min), 72°C (2 min)
	7	1	72°C (10 min)
Third	8	15	94°C (30 s), 67.5°C (1 min), 72°C (2 min)
			94°C (30 s), 67.5°C (1 min), 72°C (2 min)
			94°C (30 s), 44°C (1 min), 72°C (2 min)
	9	1	72°C (10 min)

After the first reaction, the unpurified products were diluted (20X) with ddH_2O and used as the template in the second reaction; this process was repeated for the third reaction.

Minimal inhibitory concentration (MIC) assay for planktonic and biofilm cells

The MICs of gentamicin and chloramphenicol for planktonic and biofilm cells of the wild-type and mutant strains were determined using microtiter plate assays, as described

previously (Asako et al., 1997; Steinberg et al., 1997; Kavanaugh and Ribbeck, 2012) with some modifications. Antibiotics were diluted in growth medium and 200 µL of this was added to 10 µL bacteria on each well of the 96-well plate. Biofilm cells were cultured as previously described. Planktonic cells were washed with sterile PBS before the addition of 200 µL growth medium containing diluted antibiotics. The OD_{600} of each well was measured after incubation at 37°C for 16 h. Bacteria cultured in medium without antibiotics and medium alone were used as controls.

Different pH growth conditions

The pH of LB was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 using 1 M HCl or 1 M NaOH. The growth curve of bacteria under suboptimal pH conditions was obtained as described by Markkula et al. (2012) and Mangold et al. (2013). Prior to the experiment, LB broth containing the appropriate antibiotics was inoculated with the bacterial strains cultured on blood agar plates. Bacteria were cultured until an OD_{600} of 0.4-0.6 was reached. The OD_{600} of 50 mL fresh LB medium adjusted to different pH values was adjusted to 0.02 after inoculation with the bacterial cultures, and incubated at 37°C with shaking (180 rpm). The OD_{600} was monitored every hour for 16 h.

Organic solvent tolerance assay

In the organic solvent tolerance assays, bacteria were cultured in LBGMg according to the method described by Asako (1997). Overnight cultures (100 µL) of the wild-type and mutant strains were inoculated in 5 mL of fresh LBGMg medium and incubated at 37°C with shaking (180 rpm). The culture was overlaid with a 10% volume of cyclohexane or *n*-hexane during the early exponential phase of growth. OD_{600} was measured at 16 and 24 h.

Phagocytosis assays

Phagocytosis assays of *P. vulgaris* XC 2 and the mutant strain were performed as described previously (Valentin-Weigand et al., 1996; Segura et al., 1998; Chen et al., 2008) with some modifications. Bacterial suspensions (5 x 10^5 CFU/mL) were prepared in DMEM supplemented with 2% FBS. Monolayers of RAW264.7 cells (approximately 5 x 10^5 cells per well) were plated on 24-well tissue culture plates. After washing twice with warm PBS, 1 mL of the bacterial suspension was added to the cell monolayers, and incubated at 37°C in a humidified 5% CO_2 incubator for 1 h. After incubation, the cells were washed twice with warm PBS (again), and incubated for a further 1 h with medium containing gentamicin (100 µg/mL) to kill the extracellular bacteria. The cell monolayers were then washed twice with warm PBS and 1 mL of sterile distilled water was added to every well to lyse the cells. The numbers of intracellular bacteria were confirmed by quantitative plating of serial 10-fold dilutions on LB agar. For spot assays (Yu et al., 2012; Holdsworth and Law, 2013), aliquots (10 µL) of cell lysates of every dilution were spotted onto LB plates, in order to estimate the numbers of viable wild-type strain *P. vulgaris* XC 2 and mutant strain bacteria phagocytized by RAW264.7 cells. Each strain was tested in triplicate; all tests were performed thrice and the average of the results obtained was determined. Bacteria in the absence of RAW264.7 cells and cell monolayers without bacteria were set up as controls.

Statistical analysis

Each test was performed at least in triplicate, and results are reported as means ± standard error (SE). Differences were analyzed for significance using the Student *t*-test. P values less than 0.05 were considered to be statistically significant.

RESULTS

Triparental conjugation and selection of markerless recombinants and microtiter biomass assay

Approximately 500 transposon mutants were obtained. In the subsequent biofilm detection, each strain was tested in triplicate and the average of the results of three tests was obtained. A mutant strain (OD_{595}: 0.5550 ± 0.0883) exhibiting significantly different biomass compared to *P. vulgaris* XC 2 (OD_{595}: 1.4361 ± 0.1646) was obtained (**P < 0.01). This mutant strain was used in further studies.

Thermal asymmetric interlaced PCR and sequencing

The DNA sequence flanking the insertion elements was determined in order to identify the disrupted gene. Thermal asymmetric interlaced PCR method was used to amplify the target DNA fragment, which was purified for sequencing after the third round of PCR. Approximately 580 bp of DNA sequence located in the upstream region of the transposon insertion of the mutant strain was obtained. Homologous nucleotide sequences were searched for in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). The 580-bp DNA fragment obtained was found to exhibit 84% homology with the *glp*C and *glp*B genes of *P. mirabilis* (the genome of *P. vulgaris* remains unpublished to date) and the insertion element of the mutant was located in the *glp*C gene, encoding subunit C of *sn*-glycerol-3-phosphate dehydrogenase.

Analysis of antibiotic sensitivity

The degree of chloromycetin and gentamicin sensitivity of planktonic and biofilm cells was analyzed by the MIC assay (Table 4). Though the biofilm cells and planktonic cells of two strains showed a similar MIC as that of gentamicin, we observed a difference in the MIC to chloromycetin, that is, the MIC of biofilm cells of *P. vulgaris* XC 2 was greater than that of the planktonic and biofilm cells of the mutant strain *P. vulgaris* XC 2.

Table 4. Minimum inhibitory concentration (MIC) of antibiotics against biofilm and planktonic bacteria (μg/mL).

Bacteria	*Proteus vulgaris* XC 2		Mutant strain	
	Biofilm	Planktonic	Biofilm	Planktonic
Chloromycetin	100	12.5	12.5	6.25
Gentamicin	25	12.5	12.5	12.5

Bacterial growth under varying pH conditions

P. vulgaris XC 2 and mutant strain were cultured in LB adjusted to pH 5.0, 6.0, 7.0, 8.0, and 9.0. Growth was monitored by measurement of OD_{600} at 1 h intervals for 16 h (Figure 1). The two strains exhibited similar growth at pH values of 6.0, 7.0, and 8.0, reaching a stationary phase at approximately the same time. The growth of the mutant strain was slightly better than that of the wild-type cells once the stationary phase was reached (Figure 1A, B, C, D); however, the phenomenon was revised at pH 5.0. As the pH increased to 9.0, the lag phase of *P. vulgaris* XC 2 growth was greatly increased, while the growth of the mutant strain was inhibited, compared to *P. vulgaris* XC 2.

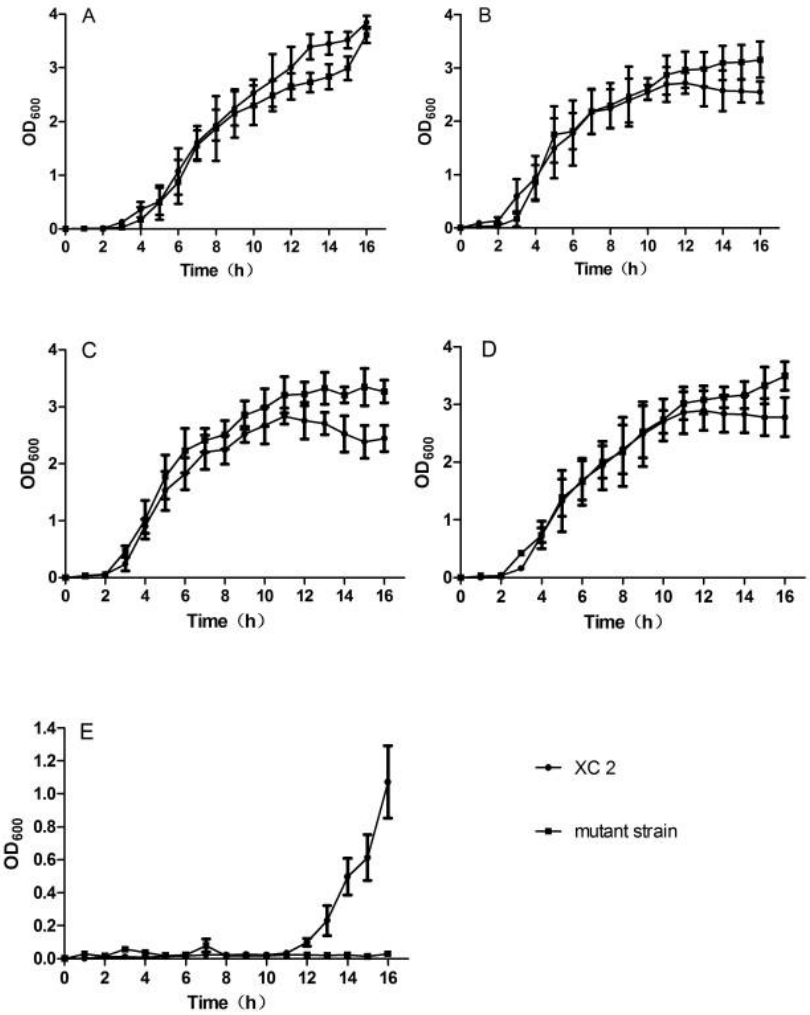

Figure 1. Growth curve of *Proteus vulgaris* XC 2 (XC 2) and mutant strain under different pH conditions. (**A**) pH 5.0, 37°C; (**B**) pH 6.0, 37°C; (**C**) pH 7.0, 37°C; (**D**) pH 8.0, 37°C; (**E**) pH 9.0, 37°C. Results are reported as means ± standard error (SE).

GlpC is responsible for organic solvent tolerance

The OD_{600} values of *P. vulgaris* XC 2 and the mutant strain were measured at 16 and 24 h (Figure 2). The growth of the wild-type and mutant strains was inhibited at varying degrees when cultured in the presence of different organic solvents. *P. vulgaris* XC 2 and the mutant strain showed similar tolerance to the organic solvents at 16 h. Following continued culturing up to 24 h, the OD_{600} of *P. vulgaris* XC 2 was found to be apparently greater than that of the mutant strain in media containing cyclohexane (**P < 0.01), although there was no significant difference in the growth curves of the two strains cultured in media containing *n*-hexane.

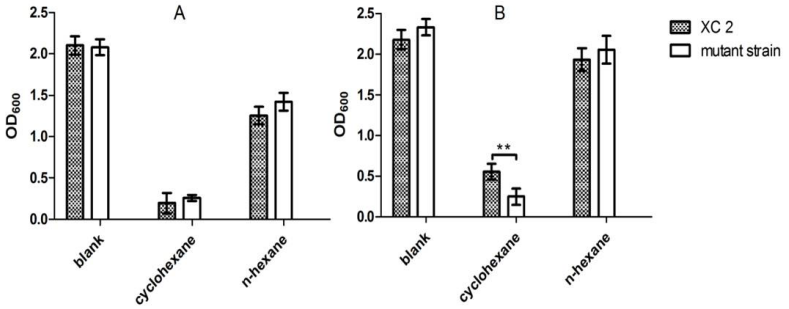

Figure 2. Organic solvent susceptibility of *Proteus vulgaris* XC 2 (XC 2) and mutant strain. OD_{600} values at (**A**) 16 h and (**B**) 24 h are shown. The group without any organic solvent was used as a control (blank). Results are reported as means ± SE.

Phagocytosis assays

Phagocytosis of the two strains was analyzed by measuring the number of viable intracellular bacteria after incubation with RAW264.7 cells. The percentage of *P. vulgaris* XC 2 and mutant strains phagocytized was calculated using the equation reported by Chen (2008). Phagocytosis of the mutant strain by RAW264.7 was more than 2.5-fold greater than that of *P. vulgaris* XC 2 (31.7 ± 5.7 *vs* 13.0 ± 0.76%, respectively; *P < 0.05) (Figure 3A). This trend was also observed in the spot assay (Figure 3B).

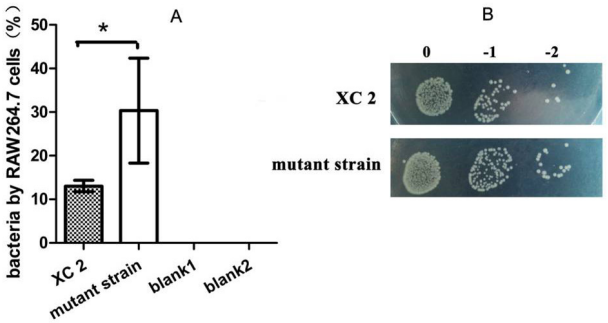

Figure 3. Phagocytosis assays. (**A**) Phagocytic uptake of *Proteus vulgaris* XC 2 (XC 2) and the mutant strain by RAW264.7 cells (*P < 0.05). Blank 1: *P. vulgaris* XC 2 without RAW264.7 cells (0.020 ± 0.0046%); blank 2: mutant strain without RAW264.7 cells (0.017 ± 0.0015%). (**B**) A serial dilution assay was performed to evaluate the number of viable bacteria. Dilutions of the bacteria (10^0, 10^{-1}, and 10^{-2}) were spotted (10 µL) onto the plate. Results are reported as means ± SE.

DISCUSSION

In this study, we observed an evident decrease in the biofilm-forming ability of the mutant strain obtained by random transposon insertion mutation, with the OD_{595} value (measured in biomass assays) reduced by more than 50%. Thermal asymmetric interlaced PCR was used to determine the insertion site using six arbitrary degenerate primers, in order to increase the achievement ratio. Furthermore, three rounds of amplification were conducted to enrich the flanking DNA. Finally, the *glp*C gene was identified as the insertion site in the obtained biofilm defective strain. In *E. coli*, *glp*C has been demonstrated to play an important role in increasing the organic solvent tolerance, through analysis of gene expression profiles before and after exposure to organic solvents (Shimizu et al., 2005).

The biofilm cells of *P. vulgaris* XC 2 demonstrated significantly greater resistance to chloromycetin than planktonic cells. In this study, the tolerance of biofilm cells of *P. vulgaris* XC 2 to chloromycetin was greater than that to gentamicin. This was speculated to be a result of differences in the penetration of biofilm by the two antibiotics, and/or differences in the mechanisms by which the antibiotics function.

Maintenance of a circumneutral intracellular pH is important for any organism (Mangold et al., 2013). According to Finlay and Falkow (1997) and Hoyle and Costerton (1991), biofilms are complex aggregations of planktonic microorganisms. It is not clear whether the aggregations were related to the large OD_{600} of the biofilm defective strain at pH 6.0, 7.0, and 8.0. The growth of *P. vulgaris* XC 2 was increased compared to that of the mutant strain, as the pH of the culture medium was reduced from 7.0 to 5.0, demonstrating the greater tolerance of *P. vulgaris* XC 2 to the acidic environment. Growth of the mutant strain within 16 h was negligible at pH 9.0; however, at 24 h, the OD_{600} value of the mutant strain reached 0.41 (data not shown). These observations demonstrated the greater tolerance of *P. vulgaris* XC 2 to alkaline environments compared to the mutant strain, thereby indicating the importance of *glp*C in pH tolerance.

Organic solvents are toxic to most microorganisms (Ni et al., 2013). In this study, the effects of inactivation of the *glp*C gene in *P. vulgaris* yielded similar results to those obtained by Shimizu (2005). The sensitivity of the mutant strain to *n*-hexane stress was unchanged compared to that of the wild-type strain. However, in response to cychlohexane stress, the growth of both strains was evidently inhibited over the first 16 h of culture. With prolonged culture under these conditions, the greater tolerance of wild-type strain became more apparent. Therefore, it can be hypothesized that *glp*C does not function under *n*-hexane tolerance, or that the tolerance of *P. vulgaris* to *n*-hexane is achieved by simultaneous changes in the expression of many related genes. Furthermore, the role of *glp*C in cychlohexane tolerance was clearly demonstrated with continued culture, indicating that other relevant genes may be activated. The biomass of *P. vulgaris* XC 2 was also measured at different times (data not shown); this showed that the biofilm formation was not completed during the early stages of culture. However, biofilm formation was found to be accelerated after 16 h, with the gradual formation of a mature biofilm. This might explain the differences in the growth of *P. vulgaris* XC 2 and the mutant strain, in media containing cychlohexane. Therefore, the relationship between biofilm formation and organic solvent tolerance should be further investigated.

In view of the role of biofilms in bacterial escape from the host immune system (Pearson et al., 1997), the interactions of *P. vulgaris* XC 2 and the biofilm defective strain

with mouse macrophage RAW262.7 cells were studied. Phagocytosis of the mutant strain by RAW264.7 cells was significantly greater than that of the wild-type strain, indicating that *glp*C plays an important role in bacterial resistance to phagocytosis by RAW264.7 cells. However, it is not clear whether this effect is caused directly by the disruption of *glp*C or indirectly by the decrease in biofilm formation.

The detailed mechanism of biofilm formation and the relationship between biofilms and tolerance to changes in pH and organic solvent stress require further investigation. Differential gene and protein expression analyses will provide important insights into the mechanisms underlying these effects, and provide the basis for the development of novel approaches for the protection and treatment of this pathogen in Chinese alligators (Novak and Seigel, 1986).

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We are grateful to the Laboratory of Microbiology in College of Veterinary Medicine, Nanjing Agricultural University for providing the experimental equipment. Research supported by grants from the Special Fund for State Forestry Administration, and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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