AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology

| Bacteriology | Full-Length Text

Biological characteristics of mechanosensitive channels MscS and MscL in *Actinobacillus pleuropneumoniae*

Jiajia Wan,[1] Lu Dai,[1] Huasong Xiao,[1] Wendie Zhang,[1] Rui Zhang,[1] Tingting Xie,[1] Yizhen Jia,[1] Xuejun Gao,[1] Jing Huang,[2] Feng Liu[1]

AUTHOR AFFILIATIONS See affiliation list on p. 10.

ABSTRACT *Actinobacillus pleuropneumoniae* is an important respiratory pathogen that can cause porcine contagious pleuropneumonia (PCP), resulting in significant economic losses in swine industry. Microorganisms are subjected to drastic changes in environmental osmolarity. In order to alleviate the drastic rise or fall of osmolarity, cells activate mechanosensitive channels MscL and MscS through tension changes. MscL not only regulates osmotic pressure but also has been reported to secrete protein and uptake aminoglycoside antibiotic. However, MscL and MscS, as the most common mechanosensitive channels, have not been characterized in *A. pleuropneumoniae*. In this study, the osmotic shock assay showed that MscL increased sodium adaptation by regulating cell length. The results of MIC showed that deletion of *mscL* decreased the sensitivity of *A. pleuropneumoniae* to multiple antibiotics, while deletion of *mscS* rendered *A. pleuropneumoniae* hypersensitive to penicillin. Biofilm assay demonstrated that MscL contributed the biofilm formation but MscS did not. The results of animal assay showed that MscL and MscS did not affect virulence *in vivo*. In conclusion, MscL is essential for sodium hyperosmotic tolerance, biofilm formation, and resistance to chloramphenicol, erythromycin, penicillin, and oxacillin. On the other hand, MscS is only involved in oxacillin resistance.

IMPORTANCE Bacterial resistance to the external environment is a critical function that ensures the normal growth of bacteria. MscL and MscS play crucial roles in responding to changes in both external and internal environments. However, the function of MscL and MscS in *Actinobacillus pleuropneumoniae* has not yet been reported. Our study shows that MscL plays a significant role in osmotic adaptation, antibiotic resistance, and biofilm formation of *A. pleuropneumoniae*, while MscS only plays a role in antibiotic resistance. Our findings provide new insights into the functional characteristics of MscL and MscS in *A. pleuropneumoniae*. MscL and MscS play a role in antibiotic resistance and contribute to the development of antibiotics for *A. pleuropneumoniae*.

KEYWORDS *Actinobacillus pleuropneumoniae*, mechanosensitive channels, MscL, MscS, biofilm formation, antibiotic resistance

Actinobacillus pleuropneumoniae, as one of the 10 most important pathogens in the swine industry, can cause porcine contagious pleuropneumonia (PCP) (1). PCP has caused tremendous economic losses to the global swine industry by increasing the mortality rate of swine, reducing feed efficiency, and driving up the costs of control measures such as antibiotic treatment and vaccines. According to the distinct capsular polysaccharides, 19 serotypes have been isolated (2). The maintenance of ion homeostasis by bacteria contributes to many fundamental cellular functions, including maintenance of cellular expansion pressure and cytoplasmic enzyme activation (3).The participation of potassium ions is necessary for pH homeostasis, membrane potential,

Editor Mohamed Y. El-Naggar, University of Southern California, Los Angeles, California, USA

Address correspondence to Feng Liu, liufeng68431@163.com.

The authors declare no conflict of interest.

See the funding table on p. 10.

Received 14 December 2023
Accepted 29 January 2024
Published 23 February 2024

Copyright © 2024 Wan et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

and cellular osmotic regulation (4). To meet these physiological functions, individual bacteria possess multiple ion-transporting systems.

Mechanosensitive channels (MS) are conserved ion channels that sense tension changes on the surface of the cell membrane and can convert the physical signals on the surface of the cell membrane into biochemical signals within the cell, thereby regulating the physiological functions of the cell (5, 6). During hypotonic stress, cells can rapidly open MS channels and release histocompatibility substances to reduce osmotic pressure in cells, preventing cell lysis due to excessive pressure (7).

Depending on the open pore, mechanosensitive channels can be divided into four categories: MscM (mini, M), MscS (small, S), MscL (large, L), and MscK (potassium, K) (8). So far, MscL and MscS are the most important and well-studied channels. MscL and MscS channels serve primarily to regulate cellar pressure in response to changes in environmental osmotic pressure (9). When *Escherichia coli* was exposed to hypotonic shock, MscS channels are activated first, and then MscL channels open when membrane tension approaches lysis tension to prevent cell lysis. Abnormal opening of MscL and MscS channels may lead to abnormal discharge of cell contents, resulting in slow cell growth and even death (10, 11).

In addition to osmotic pressure, MscL is also involved in regulating bacterial antibiotic sensitivity. Studies have shown that streptomycin and especially dihydrostreptomycin can enter *E. coli* cells through MscL channels (12). In addition, the novel compound K05, discovered by Wray et al., can bind directly to the MscL to regulate its gating, increasing the efficacy of common antibiotics (13). The compounds SCH-79797 and IRS-16 can directly bind and activate MscL, making the cell membrane permeable (14). Adding MscL activators to antibiotic compound can reduce the toxicity of antibiotics while preventing the development of antibiotic resistance (14).

PCP, caused by infection with *A. pleuropneumoniae*, can result in significant economic losses annually. Furthermore, it remains unclear whether and what kind of function MscL performs in *A. pleuropneumoniae*. In this study, we identified and characterized the function of MscL and MscS in *A. pleuropneumoniae*. We constructed the deletion mutants of *mscL* and *mscS* and investigated the role of MscL and MscS in osmotic stress adaptation, antibiotic resistance, biofilm formation, and virulence of *A. pleuropneumoniae*.

RESULTS

Bioinformatics analysis of MscS and MscL

To investigate the role of MscL and MscS in *A. pleuropneumoniae*, we compared the amino acid sequence of MscL and MscS with those of *A. pleuropneumoniae* and *E. coli*. BlastP analysis revealed that the MscL sequence of *A. pleuropneumoniae* and *E. coli* had 76% amino acid sequence identity (Fig. 1A), while the MscS sequence of both species had 78% amino acid sequence identity (Fig. 1B). According to the consensus motif mentioned in the literature (5), two conserved motifs, TM1 and TM2, were found in the amino acid sequence of MscL, and the homology with *E. coli* was 90% and 84% (Fig. 1A). Additionally, three conserved motifs, TM1, TM2, and TM3, which respectively share 55%, 57%, and 89% homology with *E. coli*, were also found in MscS (Fig. 1B). SWISS-MODEL results suggested that MscL and MscS in *A. pleuropneumoniae* were polymeric porous structures composed of 5 and 7 monomers, respectively (Fig. 1C and D), which were similar to those found in *E. coli*. In order to gain a comprehensive understanding of the role of MscL and MscS in *A. pleuropneumoniae*, we used *A. pleuropneumoniae* S4074 [serovar 1 str, the wild-type (WT) strain] to construct the *mscL* mutant strain (Δ*mscL*), *mscS* mutant strain (Δ*mscS*), and both *mscL* and *mscS* mutant strains (Δ*mscL*-Δ*mscS*) (Fig. S1). The growth curve results showed that deleting *mscL* or *mscS* did not affect the growth rate of *A. pleuropneumoniae* (Fig. S2). The scanning electron microscopy (SEM) of Δ*mscL*, Δ*mscS*, and Δ*mscL*-Δ*mscS* cells revealed that *mscL* and *mscS* did not affect cell morphology (Fig. 1E).

FIG 1 Preliminary analysis of MscS and MscL. Multiple sequence alignments of MscL (A) and MscS (B) were performed using MEGA 11. The transmembrane helices (TM) were predicted by DTU/DeepTMHMM – BioLib. A computer model of the 3D structure predicted from the *A. pleuropneumoniae* S4074 (left), *E. coli* K12 (right) MscL protein (C), and MscS protein (D). (E) Observation of bacterial cell morphology in the WT, Δ*mscL*, Δ*mscS*, and Δ*mscL*-Δ*mscS* strains by SEM.

MscL is required for sodium hyperosmotic tolerance of *A. pleuropneumoniae,* but MscS is not

In order to investigate the function of MscS, MscL channels in *A. pleuropneumoniae*, we measured the activity of the WT, Δ*mscL*, complemented (C)Δ*mscL*, Δ*mscS*, complemented (C)Δ*mscS*, and Δ*mscL*-Δ*mscS* strains in the presence or absence of specific salt ions. In the medium containing 0.3 M Na^+, WT, Δ*mscS*, CΔ*mscS*, and CΔ*mscL* can form colonies efficiently, but Δ*mscL* and Δ*mscL*-Δ*mscS* cannot. In addition, the growth of Δ*mscL*, Δ*mscS*, and Δ*mscL*-Δ*mscS* was identical to that of WT, CΔ*mscS*, and CΔ*mscL* in the medium with 0.3 M K^+ supplemented or Na^+ removed (Fig. 2A). Furthermore, after overnight incubation in the medium supplemented with 0.3 M Na^+, the osmotic survival rates of Δ*mscL* (47.2%) and Δ*mscL*-Δ*mscS* (43.3%) were significantly decreased compared to WT, CΔ*mscS*, and CΔ*mscL*, while Δ*mscS* (100%) had no significant changes in osmotic survival rates (Fig. 2B). To further investigate the reasons for the decreased survival rate of Δ*mscL*, we used SEM to observe the changes in bacterial morphology and length. The results of SEM showed that the bacterial length of Δ*mscL* [mean = 2.40 µm; standard deviation (SD) = 1.575] increased significantly (P = 0.0029) compared to that of WT (mean = 1.85 µm; SD = 0.9968) when grown with the supplementation of 0.3 M Na^+ (Fig. 2C and D). These data demonstrated that MscL plays an essential role in sodium hyperosmolar tolerance.

MscL and MscS are required for some antibiotic resistance in *A. pleuropneumoniae*

Studies have shown that mechanosensitive channels play a decisive role in antibiotic sensitivity (12, 15). In order to investigate the role of MscS and MscL in antibiotic resistance of *A. pleuropneumoniae*, we performed antibiotic susceptibility tests on six strains. Compared with WT, CΔ*mscS*, and CΔ*mscL*, the sensitivity of Δ*mscL* and Δ*mscL*-Δ*mscS* to antibiotics such as chloramphenicol, erythromycin, and penicillin has declined. The sensitivity of Δ*mscL* to oxacillin has also declined, while the sensitivity of Δ*mscS* and Δ*mscL*-Δ*mscS* remains unchanged. Additionally, the sensitivity of Δ*mscS* to penicillin increased, but the sensitivity of Δ*mscL* and Δ*mscL*-Δ*mscS* decreased (Table 1). This indicated that MscL and MscS are required for some antibiotic resistance in *A. pleuropneumoniae*.

MscL is required for the biofilm formation in *A. pleuropneumoniae*, but MscS is not

To evaluate the effects of MscS and MscL on *A. pleuropneumoniae*, we measured biofilm formation of six strains at 42°C as previously reported. In crystal violet coloration, it can be seen that WT, Δ*mscS*, CΔ*mscS*, and CΔ*mscL* visibly form biofilms, while Δ*mscL* and Δ*mscL*-Δ*mscS* showed almost no biofilm formation (Fig. 3A). Quantitative analysis also showed that, compared to WT, CΔ*mscS*, CΔ*mscL*, and Δ*mscS*, biofilm formation of Δ*mscL* and Δ*mscL*-Δ*mscS* decreased significantly (Fig. 3B). These suggested that MscL is required for *A. pleuropneumoniae* biofilm formation.

MscL or MscS does not affect *A. pleuropneumoniae* virulence in mice

To further investigate the impact of *mscL* and *mscS* on the virulence of *A. pleuropneumoniae*, survival and colonization assays were performed *in vivo* in KM mice using WT, Δ*mscL*, CΔ*mscL*, Δ*mscS*, CΔ*mscS*, and Δ*mscL*-Δ*mscS* strains. As shown in Fig. S2, the survival rates for WT, Δ*mscL*, CΔ*mscL*, Δ*mscS*, CΔ*mscS*, and Δ*mscL*-Δ*mscS* groups were 20%, 30%, 20%, 20%, 20%, and 20%, respectively. There was no significant difference in survival rates between the six groups (Fig. S3A). The bacterial loads *in vivo* among the six groups also showed no significance difference (Fig. S3B). In summary, these results indicated that MscL and MscS do not affect the virulence of *A. pleuropneumoniae* in mice.

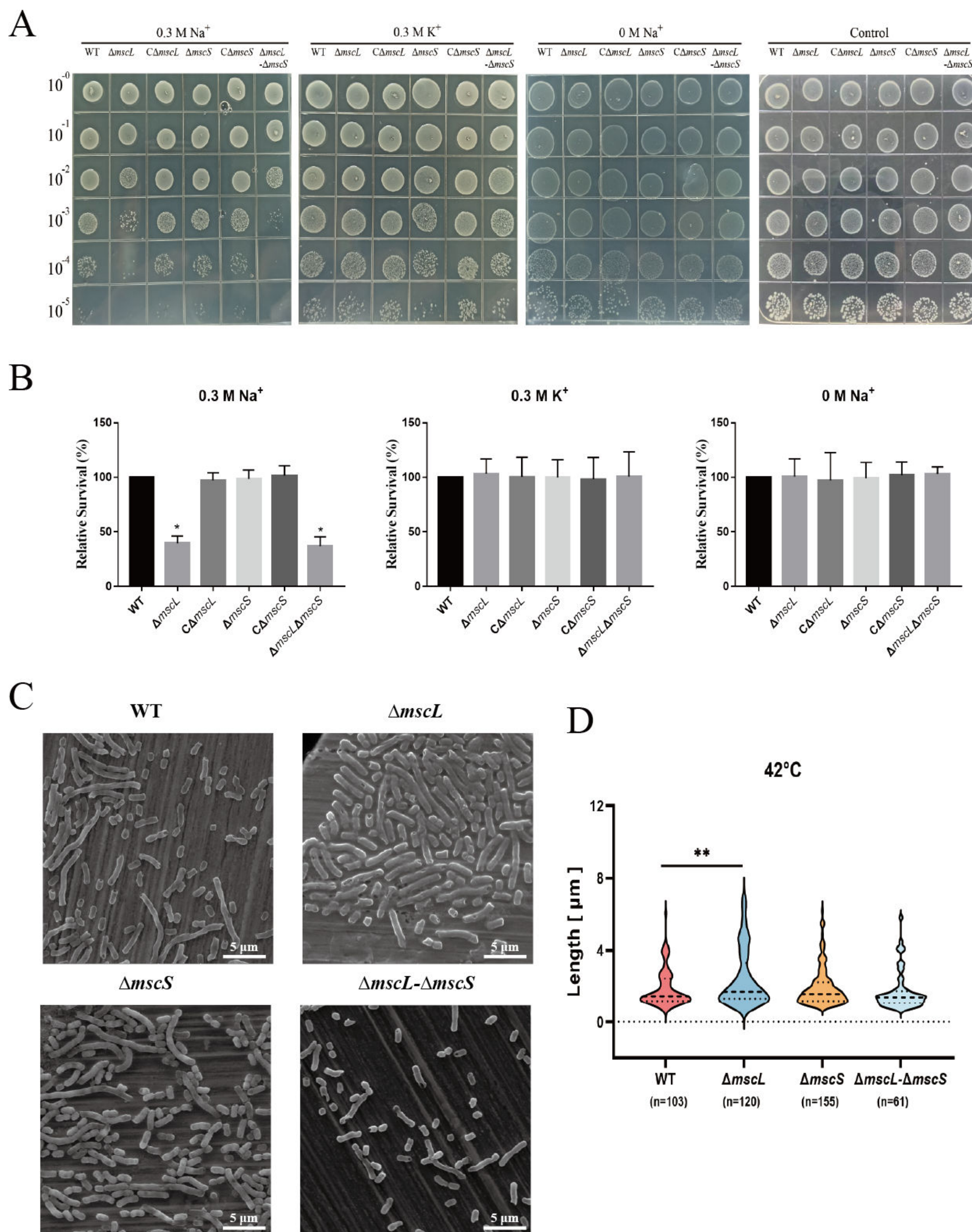

FIG 2 MscL is required for sodium hyperosmotic tolerance. Spot dilution assays (A) or relative survival (B) of WT, Δ*mscL*, CΔ*mscL*, Δ*mscS*, CΔ*mscS*, and Δ*mscL*-Δ*mscS* on TSA plates with or without ion treatment. The bacterial cell morphology and length of WT, Δ*mscL*, Δ*mscS*, and Δ*mscL*-Δ*mscS* strains grown with 0.3 mM Na^+ were observed by SEM (C) and measured using Image J (D). The number of bacteria measured was shown in brackets. **P* < 0.05. ***P* < 0.01.

TABLE 1 Susceptibility of *Actinobacillus pleuropneumoniae* strains to various antibiotics[a]

Antibiotics	MIC (μg/mL) of six *A. pleuropneumoniae* strains					
	WT	Δ*mscL*	CΔ*mscL*	Δ*mscS*	CΔ*mscS*	Δ*mscL*-Δ*mscS*
Chloramphenicol	0.5	50	0.5	0.5	0.5	10
Erythromycin	0.1875	7.5	0.1875	0.1875	0.1875	0.1875
Oxacillin	7.5	25	7.5	7.5	7.5	10
Penicillin	0.15625	2.5	0.15625	0.078125	0.15625	0.625

[a]MIC, minimal inhibitory concentration.

DISCUSSION

All living organisms need to detect and respond to both external stimuli and internal environmental changes, such as gravity and membrane deformation caused by osmotic pressure, to ensure proper growth, development, and reproduction, especially for microorganisms. Different proteins and their complexes in microbial cells can rapidly sense and respond to various mechanical forces to efficiently regulate life processes. Mechanosensitive channels play a crucial role in facilitating this process. As common mechanosensitive channels in bacteria, MscL and MscS participate in various life activities of bacteria.

In the present study, multiple sequence alignments showed that MscL and MscS possess homology with counterparts in *E. coli*. During the opening of MscL gated channels, the TM1 and TM2 domains tilt and rotate, resulting in thinning of membranes in the membrane plane (16–18). According to the consensus motif mentioned in the literature (5), two conserved motifs, TM1 and TM2, were found in the amino acid sequence of MscL (Fig. 1A). Additionally, three conserved motifs, TM1, TM2, and TM3, were also found in MscS (Fig. 1B). All of these motifs were similar to sequences found in *E. coli*. These data indicated that MscL and MscS are highly conserved.

As mechanosensitive channels, MscL and MscS primarily serve to protect cells from lysis when exposed to hypotonic environments, but few studies have been conducted on their role in hypertonic environments. In this study, the protective effect of MscL was demonstrated in the adaptation of *A. pleuropneumoniae* to hypertonic stress, whereas MscS did not appear to have this effect. In *E. coli,* the expressions of MscS and MscL were found to be upregulated in response to hypertonic stress. This upregulation is mediated by the stress sigma factor RpoS (19). Notably, *A. pleuropneumoniae* does not possess

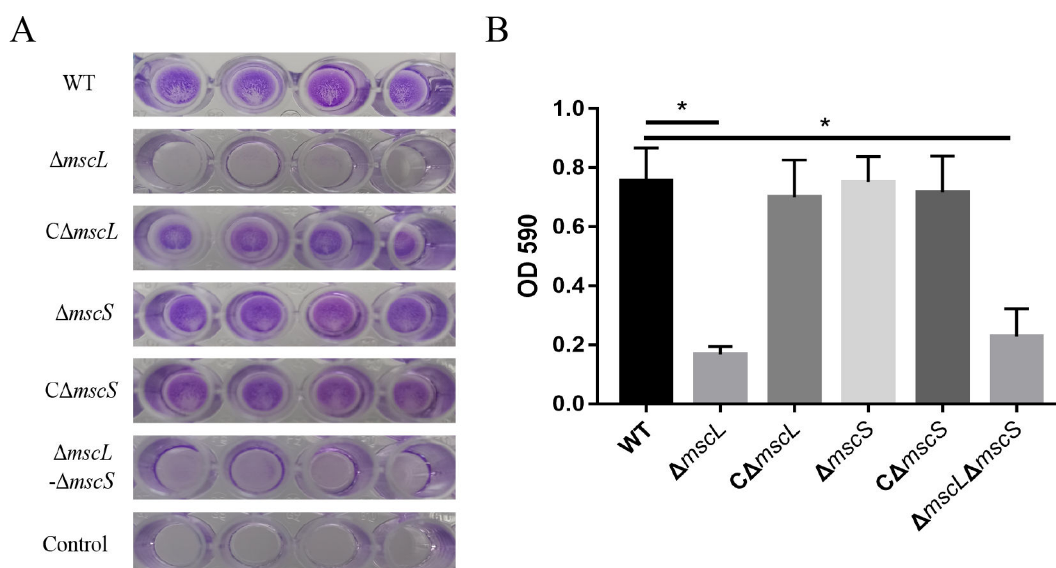

FIG 3 MscL is required for the biofilm formation. Crystal violet staining (A) and quantitative analysis (B) of biofilm formation in *A. pleuropneumoniae* (WT, Δ*mscL*, CΔ*mscL*, Δ*mscS*, CΔ*mscS*, and Δ*mscL*-Δ*mscS*). Data presented were the mean ± SD from three independent experiments performed in duplicate. *$P < 0.05$.

rpoS gene, and the deletion of *mscS* does not influence the survival of *A. pleuropneumoniae* in hypotonic environments. Consequently, the role and mechanism of MscL in *A. pleuropneumoniae* may not be entirely analogous to that in *E. coli*, which may be limited by its environmental niche.

Furthermore, the growth of *mscL* or *mscS* deletion mutant strains was not affected on TSA solid medium containing 0 M NaCl (Fig. 2A), probably due to the insufficient downward osmotic pressure applied. Regardless of the initial and final osmotic pressure, lysis of *mscL* or *mscS* deletion mutant strains only occurs when the osmolarity drops equivalent to >0.2 M NaCl (19). Commonly used TSA media have an osmolarity of ≈278 mOsm (equivalent to ≈0.14 M NaCl), and removing all NaCl of TSA (equivalent to ≈0.085 M NaCl) cannot cause lysis of *mscL* or *mscS*, or both *mscL* and *mscS* deletion mutant strains.

As the abuse of antibiotics, resistance as a self-defense mechanism against antibiotics has been popular around the world. To date, many bacterial antibiotic resistance mechanisms have been elucidated, including antibiotic target modification, envelope permeability reduction, efflux system activation, and enzymatic inactivation (20). MscL channels is considered as a potential drug target, and adding MscL-activating component to antibiotic compounds may help to evade antibiotic resistance (13, 14, 21). However, little information is available about MscL or MscS in *A. pleuropneumoniae*, which has hindered the development of antibacterial drug for this bacterium.

In this study, the deletion of *mscL* has declined the sensitivity to chloramphenicol, erythromycin, oxacillin, and penicillin. MscL functions as an emergency release valve, excreting cytoplasm solutes under hypotonic stress. Inappropriate opening of MscL allows solutes, even drugs, to pass into cytoplasm (22, 23). Therefore, MscL may allow chloramphenicol, erythromycin, and oxacillin to enter the cell and play a role. The deletion of *mscS* has increased the sensitivity to penicillin. Our findings indicate that MscS also plays a role in antibiotic resistance mechanisms. Moller et al. mentioned that MscL and MscS must act sequentially (24), and the presence of *mscL* may mitigate the deficiencies caused by the deletion of *mscS*. Consequently, we constructed strains that are deficient in both *mscL* and *mscS*. Compared to the deletion of *mscL*, deleting both *mscL* and *mscS* has increased the sensitivity to chloramphenicol, oxacillin, and penicillin. This seems to indicate that MscL and MscS play an antagonistic role, and MscL plays a dominant role. This could explain the differential effects of MscL and MscS on penicillin resistance. Additionally, in osmotic adaptation and biofilm formation, deleting *mscL* alone or deleting both *mscL* and *mscS* resulted in similar defects, while deleting *mscS* did not cause any defects, further confirming that *mscS* does not play a role in these physiological activities.

At present, no studies have shown that MscL is involved in biofilm formation. However, in the present study, we showed that MscL is required for the synthesis of biofilm in *A. pleuropneumoniae*. Berrier et al. suggested that MscL affects the secretion of thioredoxin, elongation factor Tu, and DnaK during osmotic shock (25). Morra et al. showed that proteins are released in a manner dependent on MscL. These findings suggested that MscL influences the secretion of cytoplasmic proteins (26). Biofilms are aggregated bacterial groups that are embedded in a matrix of extracellular polymeric substances (27). Biofilm contains extracellular polymeric substances (EPS), whose main components are extracellular DNA, polysaccharide, and proteins (28). The formation of biofilm involves a series of steps, and the secretion of exopolysaccharide matrix and proteins is an essential part of this process (29). In addition, biofilms served as a growth mode to help bacteria resist external stimuli, and the ability to form biofilm reflects the virulence of bacteria to a certain extent (28, 30). MscL, as a protein secretion system, should have an impact on bacterial virulence. In this study, the deletion of *mscL* resulted in decreased biofilm production, but it did not affect bacterial virulence. Detailed analysis of the molecular mechanism by which MscL affects *A. pleuropneumoniae* biofilm formation will be the focus of further research.

In conclusion, our research has provided valuable insights into the role of MscL and MscS in *A. pleuropneumoniae*, showing that MscL and MscS are involved in various physiological functions of *A. pleuropneumoniae*. Given the potential of MscL and MscS as targets for antibiotic development, our findings are expected to contribute to the advancement of antibiotic therapies targeting MscL and MscS. Subsequent research will aim to elucidate the mechanism by which MscL and MscS affects *A. pleuropneumoniae* resistance, thereby enhancing the understanding of MscL and MscS.

MATERIALS AND METHODS

Strains, plasmid, and growth conditions

The strains, plasmids, and primers used in this study were listed in Tables S1 and S2. *A. pleuropneumoniae* S4074 (WT) was used as a representative strain, grown at 37°C with shaking in Tryptic Soy Broth (TSB; Solarbio, China) containing 10% (vol/vol) fetal bovine serum (FBS; Everygreen, China) and 10 µg/mL nicotinamide adenine dinucleotide (NAD; Sigma-Aldrich, United States). *E. coli* β2155 was cultured in Luria-Bertani broth (LB) or agar (LA) with 25 µg/mL chloramphenicol (25 µg/mL) and 50 µg/mL diaminopimelic acid (DAP; Sigma-Aldrich, United States) at 37°C. Chloramphenicol (25 µg/mL) was added to LA or LB for the culture of *E. coli* DH5α at 37°C

Construction of mutant and complementary strains

To obtain upstream and downstream of the target gene by PCR amplification, the genome of *A. pleuropneumoniae* was used as template. The upstream and downstream fusion fragments from fusion amplification were ligated to the suicide plasmid pEMOC2 to create a recombinant plasmid, which was sequenced to ensure its precision. Then, the plasmid was transferred to *E. coli* β2155. After homologous recombination, the mutant strains of the target gene were selected by chloramphenicol screening.

The gene was ligated to the shuttle plasmid pJF224-XN with corresponding restriction enzymes and transferred into the mutant strain by electroporation (2.5 KV; 25 µ FD; 800 Ω) to generate complementary strains. The mutant and complementary strains were verified by PCR and DNA sequencing (data not shown).

SEM

The SEM was performed to investigate the effect of *mscL* and *mscS* on bacterial cell morphology. Wild-type S4074 and its mutant derivatives were cultured on TSB medium (supplemented with 10% FBS and 10% NAD) with or without 0.3 M NaCl. Bacterial cells were harvested after centrifugation, fixed with 2.5% glutaraldehyde, and then deposited onto copper grids (200 mesh; Zhongjingkeyi, China). The air-dried copper grids were mounted on the sample stage using carbon conductive tape (Zhongjingkeyi, China) and then coated with a sputter-coater. Subsequently, samples were observed using the SEM (VEGA3; TESCAN, Czech Republic).

Osmotic shock

Survival rate after osmotic shock was examined according to the method previously described (9, 31). *A. pleuropneumoniae* (WT, Δ*mscL*, *C*Δ*mscL*, Δ*mscS, C*Δ*mscS*, and Δ*mscL*-Δ*mscS*) cultured overnight were transferred to TSB medium, shaken in 37°C, serially 10-fold diluted at $OD_{600\ nm}$ of 0.6, and spotted or plated onto different TSB plates. The survival rate was calculated (nshock/ncontrol) from the number of colony-forming units of cells that experienced osmotic shock (nshock) and those that did not experience the shock (ncontrol).

Biofilm assay

The biofilm experiment was carried out following the procedure outline in the literature (32). The overnight cultured *A. pleuropneumoniae* (WT, Δ*mscL*, *C*Δ*mscL*, Δ*mscS*, *C*Δ*mscS*,

and Δ*mscL*-Δ*mscS*) were diluted 1:100 in fresh TSB medium, transferred to 200 µL of diluent to each well in a 96-well microplate, and four replicated wells were used for each strain. Then, we incubated the microplate at 42°C for 48 h. After culture, we removed the bacterial solution, washed each well three times with sterile saline (0.9% NaCl, wt/vol), and stained it with 100 µL per well of crystal violet (0.1%, wt/vol) for 10 min at room temperature. We removed crystal violet solution and washed the wells under running tap water to remove the excess dye. The microplate was then inverted and allowed to dry at 37°C for 30 min. Once the microplate was completely dry, it was photographed. For quantitative analysis, 100 µL of glacial acetic acid solution (33%, vol/vol) was added to each well to dissolve the crystal violet. Subsequently, the dissolved solution was measured at $OD_{590\ nm}$ using Multi-Detection Microplate Reader.

Determination of minimum inhibitory concentrations (MIC)

MICs were measured on 96-well microplates using 2-fold broth dilution. To prepare the bacterial standard solution, *A. pleuropneumoniae* (WT, Δ*mscL*, *C*Δ*mscL*, Δ*mscS*, *C*Δ*mscS*, and Δ*mscL*-Δ*mscS*) were cultivated at 37°C to $OD_{600\ nm}$ of 0.6, adjusted to 0.5 McFarland standard with sterile saline (0.9% NaCl, wt/vol), and further diluted 1:100 with TSB. To each well, 100 µL of standard bacterial solution and 100 µL of antibiotics were added. A blank control was prepared by adding 100 µL of TSB and 100 µL of antibiotic to corresponding wells. A positive control was prepared by adding 200 µL of WT standard solution to the corresponding wells. The antibiotics used in the experiment were chloramphenicol (0.25–100 mg/mL), erythromycin (0.01–15 mg/mL), oxacillin (5–50 mg/mL), and penicillin (0.08–5 mg/mL). The 96-well plates were incubated at 37°C for 24 h. The minimum concentration that inhibits the visible growth was confirmed as MIC. All experiments were performed in triplicate.

Animal experiments

A total of 36 four-week-old female Kunming (KM) mice (CTGU University Laboratory Animal Center, Yichang, China) were randomly divided into six groups of six mice each. Each group of mice received an intraperitoneal injection of 1×10^7 CFU *A. pleuropneumoniae* (WT, Δ*mscL*, *C*Δ*mscL*, Δ*mscS*, *C*Δ*mscS*, and Δ*mscL*-Δ*mscS*) per mouse, and the remaining four mice were injected intraperitoneally with the same dose of normal saline (0.9% NaCl, wt/vol) as a blank control. Survival for each group was recorded once a day for 1 week following the injection.

Four-week-old female Kunming (KM) mice ($n = 6$ in each group) were used to assess colonization ability of *A. pleuropneumoniae* Δ*mscL*, Δ*mscS*, and Δ*mscL*-Δ*mscS*. The mice were administered intraperitoneally *A. pleuropneumoniae* WT, Δ*mscL*, *C*Δ*mscL*, Δ*mscS*, *C*Δ*mscS*, and Δ*mscL*-Δ*mscS* (1×10^7 CFU/mouse). At 6 h after injection, the mice were euthanized, and lungs were collected aseptically. Half of the lung was homogenized in normal saline (0.9% NaCl, wt/vol) and serially diluted. Appropriate diluents of lung tissue were plated on TSA plates for determination of bacterial load. The remaining lung was immersed in tissue fixative solution (Biosharp, Beijing, China) for histopathological analysis as previously described (33).

Bioinformatic and statistical analysis

The amino acid sequences of *mscS* and *mscL* were downloaded from National Center for Biotechnology Information. MEGA 11 (Mega Limited, New Zealand) was used to compare their homology, prediction of transmembrane domains (TM) was preformed using DTU/DeepTMHMM – BioLib, and the results were processed using GeneDoc 2.7 (FSF Inc., USA). The three-dimensional structures of MscL and MscS were predicted using SWISS-MODEL. The statistical analyses of experimental data were performed using two-tailed Student's *t*-tests through GraphPad (GraphPad Inc., USA). All data were presented as means ± SD. Statistical significance was determined if the P value is <0.05.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (32002252).

AUTHOR AFFILIATIONS

[1]College of Animal Science and Technology, Yangtze University, Jingzhou, Hubei, China
[2]College of Arts and Sciences, Yangtze University, Jingzhou, Hubei, China

AUTHOR ORCIDs

Feng Liu http://orcid.org/0000-0001-8671-1687

FUNDING

Funder	Grant(s)	Author(s)
MOST | National Natural Science Foundation of China (NSFC)	32002252	Feng Liu

AUTHOR CONTRIBUTIONS

Jiajia Wan, Data curation, Investigation, Methodology, Software, Writing – original draft | Lu Dai, Investigation, Methodology | Huasong Xiao, Investigation, Methodology | Wendie Zhang, Investigation, Methodology | Rui Zhang, Investigation, Methodology | Tingting Xie, Investigation, Methodology | Yizhen Jia, Investigation, Methodology | Xuejun Gao, Writing – original draft | Jing Huang, writing - review and editing.

ETHICS APPROVAL

All animal experiments were approved by the Animal Ethics Committee of Yangtze University.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Figure S1 (JB00429-23-s0001.tif). Identification of mutant strains.
Figure S2 (JB00429-23-s0002.tif). MscL or MscS does not affect the growth rates in *A. pleuropneumoniae*.
Figure S3 (JB00429-23-s0003.tif). MscL or MscS does not affect *A. pleuropneumoniae* virulence in mice.
Supplemental legends (JB00429-23-s0004.doc). Legends for Figs. S1 to S3.
Table S1 (JB00429-23-s0005.docx). Bacterial strains and plasmids used in this study.
Table S2 (JB00429-23-s0006.docx). Primers used in this study.

REFERENCES

1. VanderWaal K, Deen J. 2018. Global trends in infectious diseases of swine. Proc Natl Acad Sci U S A 115:11495–11500. https://doi.org/10.1073/pnas.1806068115
2. Stringer OW, Bossé JT, Lacouture S, Gottschalk M, Fodor L, Angen Ø, Velazquez E, Penny P, Lei L, Langford PR, Li Y. 2021. Proposal of *Actinobacillus pleuropneumoniae* serovar 19, and reformulation of previous multiplex PCRs for capsule-specific typing of all known serovars. Vet Microbiol 255:109021. https://doi.org/10.1016/j.vetmic.2021.109021
3. Su J, Gong H, Lai J, Main A, Lu S. 2009. The potassium transporter Trk and external potassium modulate *Salmonella enterica* protein secretion and virulence. Infect Immun 77:667–675. https://doi.org/10.1128/IAI.01027-08
4. Stautz J, Hellmich Y, Fuss MF, Silberberg JM, Devlin JR, Stockbridge RB, Hänelt I. 2021. Molecular mechanisms for bacterial potassium homeostasis. J Mol Biol 433:166968. https://doi.org/10.1016/j.jmb.2021.166968
5. Balleza D, Gómez-Lagunas F. 2009. Conserved motifs in mechanosensitive channels MscL and MscS. Eur Biophys J 38:1013–1027. https://doi.org/10.1007/s00249-009-0460-y
6. Martinac B, Buechner M, Delcour AH, Adler J, Kung C. 1987. Pressure-sensitive ion channel in *Escherichia coli*. Proc Natl Acad Sci U S A 84:2297–2301. https://doi.org/10.1073/pnas.84.8.2297
7. Booth IR, Edwards MD, Black S, Schumann U, Miller S. 2007. Mechanosensitive channels in bacteria: signs of closure? Nat Rev Microbiol 5:431–440. https://doi.org/10.1038/nrmicro1659

8. Kung C, Martinac B, Sukharev S. 2010. Mechanosensitive channels in microbes. Annu Rev Microbiol 64:313–329. https://doi.org/10.1146/annurev.micro.112408.134106
9. Levina N. 1999. Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity. EMBO J 18:1730–1737. https://doi.org/10.1093/emboj/18.7.1730
10. Sidarta M, Baruah L, Wenzel M. 2022. Roles of bacterial mechanosensitive channels in infection and antibiotic susceptibility. Pharmaceuticals (Basel) 15:770. https://doi.org/10.3390/ph15070770
11. Wang J, Blount P. 2023. Feeling the tension: the bacterial mechanosensitive channel of large conductance as a model system and drug target. Curr Opin Phy 31:100627. https://doi.org/10.1016/j.cophys.2022.100627
12. Iscla I, Wray R, Wei S, Posner B, Blount P. 2014. Streptomycin potency is dependent on MscL channel expression. Nat Commun 5:4891. https://doi.org/10.1038/ncomms5891
13. Wray R, Wang J, Iscla I, Blount P. 2020. Novel MscL agonists that allow multiple antibiotics cytoplasmic access activate the channel through a common binding site. PLoS One 15:e0228153. https://doi.org/10.1371/journal.pone.0228153
14. Wray R, Wang J, Blount P, Iscla I. 2022. Activation of a bacterial mechanosensitive channel, MscL, underlies the membrane permeabilization of dual-targeting antibacterial compounds. Antibiotics 11:970. https://doi.org/10.3390/antibiotics11070970
15. Zhao Y, Lv B, Sun F, Liu J, Wang Y, Gao Y, Qi F, Chang Z, Fu X. 2020. Rapid freezing enables aminoglycosides to eradicate bacterial persisters via enhancing mechanosensitive channel MscL-mediated antibiotic uptake. mBio 11. https://doi.org/10.1128/mBio.03239-19
16. Rui H, Kumar R, Im W. 2011. Membrane tension, lipid adaptation, conformational changes, and energetics in MscL gating. Biophys J 101:671–679. https://doi.org/10.1016/j.bpj.2011.06.029
17. Pivetti CD, Yen M-R, Miller S, Busch W, Tseng Y-H, Booth IR, Saier MH. 2003. Two families of mechanosensitive channel proteins. Microbiol Mol Biol Rev 67:66–85. https://doi.org/10.1128/MMBR.67.1.66-85.2003
18. Blount P, Iscla I. 2020. Life with bacterial mechanosensitive channels, from discovery to physiology to pharmacological target. Microbiol Mol Biol Rev 84:e00055-19. https://doi.org/10.1128/MMBR.00055-19
19. Stokes NR, Murray HD, Subramaniam C, Gourse RL, Louis P, Bartlett W, Miller S, Booth IR. 2003. A role for mechanosensitive channels in survival of stationary phase: regulation of channel expression by RpoS. Proc Natl Acad Sci U S A 100:15959–15964. https://doi.org/10.1073/pnas.2536607100
20. Peterson E, Kaur P. 2018. Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. Front Microbiol 9:2928. https://doi.org/10.3389/fmicb.2018.02928
21. Wray R, Herrera N, Iscla I, Wang J, Blount P. 2019. An agonist of the MscL channel affects multiple bacterial species and increases membrane permeability and potency of common antibiotics. Mol Microbiol 112:896–905. https://doi.org/10.1111/mmi.14325
22. Booth IR, Blount P. 2012. The MscS and MscL families of mechanosensitive channels act as microbial emergency release valves. J Bacteriol 194:4802–4809. https://doi.org/10.1128/JB.00576-12
23. Ewis HE, Lu C-D. 2005. Osmotic shock: a mechanosensitive channel blocker can prevent release of cytoplasmic but not periplasmic proteins. FEMS Microbiol Lett 253:295–301. https://doi.org/10.1016/j.femsle.2005.09.046
24. Moller E, Britt M, Schams A, Cetuk H, Anishkin A, Sukharev S. 2023. Mechanosensitive channel MscS is critical for termination of the bacterial hypoosmotic permeability response. J Gen Physiol 155. https://doi.org/10.1085/jgp.202213168
25. Berrier C, Garrigues A, Richarme G, Ghazi A. 2000. Elongation factor Tu and Dnak are transferred from the cytoplasm to the periplasm of *Escherichia coli* during osmotic downshock presumably via the mechanosensitive channel *mscL*. J Bacteriol 182:248–251. https://doi.org/10.1128/JB.182.1.248-251.2000
26. Morra R, Del Carratore F, Muhamadali H, Horga LG, Halliwell S, Goodacre R, Breitling R, Dixon N. 2018. Translation stress positively regulates MscL-dependent excretion of cytoplasmic proteins. mBio 9:e02118-17. https://doi.org/10.1128/mBio.02118-17
27. Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167–193. https://doi.org/10.1128/CMR.15.2.167-193.2002
28. Zhang Q, Peng L, Han W, Chen H, Tang H, Chen X, Langford PR, Huang Q, Zhou R, Li L. 2023. The morphology and metabolic changes of *Actinobacillus pleuropneumoniae* during its growth as a biofilm. Vet Res 54:42. https://doi.org/10.1186/s13567-023-01173-x
29. Carniello V, Peterson BW, van der Mei HC, Busscher HJ. 2018. Physico-chemistry from initial bacterial adhesion to surface-programmed biofilm growth. Adv Colloid Interface Sci 261:1–14. https://doi.org/10.1016/j.cis.2018.10.005
30. Zhang T, Zhu J, Xu J, Shao H, Zhou R. 2020. Regulation of (p)ppGpp and its homologs on environmental adaptation, survival, and pathogenicity of *Streptococci*. Front Microbiol 11:1842. https://doi.org/10.3389/fmicb.2020.01842
31. Owada N, Yoshida M, Morita K, Yoshimura K. 2019. Temperature-sensitive mutants of MscL mechanosensitive channel. J Biochem 166:281–288. https://doi.org/10.1093/jb/mvz035
32. O'Toole GA. 2011. Microtiter dish biofilm formation assay. J Vis Exp 47:2437. https://doi.org/10.3791/2437
33. Guo Y, Xiao Z, Wang Y, Yao W, Liao S, Yu B, Zhang J, Zhang Y, Zheng B, Ren B, Gong Q. 2018. Sodium butyrate ameliorates streptozotocin-induced type 1 diabetes in mice by inhibiting the HMGB1 expression. Front Endocrinol (Lausanne) 9:630. https://doi.org/10.3389/fendo.2018.00630