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TolC is important for bacterial survival and oxidative stress response in *Salmonella enterica* serovar Choleraesuis in an acidic environment

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ABSTRACT

The outer membrane protein TolC, which is one of the key components of several multidrug efflux pumps, is thought to be involved in various independent systems in Enterobacteriaceae. Since the acidic environment of the stomach is an important protection barrier against foodborne pathogen infections in hosts, we evaluated whether TolC played a role in the acid tolerance of *Salmonella enterica* serovar Choleraesuis. Comparison of the acid tolerance of the *tolC* mutant and the parental wild-type strain showed that the absence of TolC limits the ability of *Salmonella* to sustain life under extreme acidic conditions. Additionally, the mutant exhibited morphological changes during growth in an acidic medium, leading to the conflicting results of cell viability measured by spectrophotometry and colony-forming unit counting. Reverse-transcriptional-PCR analysis indicated that acid-related molecules, apparatus, or enzymes and oxidation-induced factors were significantly affected by the acidic environment in the null-*tolC* mutant. The elongated cellular morphology was restored by adding antioxidants to the culture medium. Furthermore, we found that increased cellular antioxidative activity provides an overlapping protection against acid killing, demonstrating the complexity of the bacterial acid stress response. Our findings reinforce the multifunctional characteristics of TolC in acid tolerance or oxidative stress resistance and support the correlative protection mechanism between oxygen- and acid-mediated stress responses in *Salmonella enterica* serovar Choleraesuis.

1. Introduction

During the course of infection, foodborne pathogens encounter a lethal acidic challenge in the stomach after the ingestion of contaminated food or water. Gastric fluid is extremely acidic characteristic ($pH < 2$) and is an important defense against infections (Smith, 2003). As an acid-sensitive bacterium, *Vibrio cholera* requires a higher infective dose to successfully infect a host, as compared to bacteria such as *Escherichia coli*, *Shigella* and *Salmonella enterica*, which can cause diseases when fewer than 100 cells are ingested (Schmid-Hempel and Frank, 2007). Moreover, the elderly, children, and people being administered acid suppression medicine are particularly susceptible to contamination and illness (Howell et al., 2010).

Salmonella enterica serovar Choleraesuis (*S.* Choleraesuis) is a nontyphoid serotype with a highly invasive character and is associated with bacteremia and systemic infection (Chiu et al., 2004). As a foodborne and waterborne organism, *Salmonella* uses various systems to survive in a wide range of pH values in both, natural and host environments (Riesenberg-Wilmes et al., 1996). Previous reports have suggested that *Salmonella* survives in a lethal acidic environment by inducing the adaptive acid tolerance response (ATR), which can be characterized by the capability to tolerate extreme acidity and enhance the survival rate after treatment with moderately acidic pH (Foster and Hall, 1990; Alvarez-Ordonez et al., 2011). Further studies provided evidence that there are at least two different types of ATRs used by *Salmonella* (Lee et al., 1994). The Log-phase ATR is induced in logarithmically growing cells and involves more than 40 acid-shock proteins that are distinct from stationary-phase acid-shock proteins (Lee et al., 1994). Interestingly, in addition to pH-inducible systems, *rpoS* also acts as a non-acid-inducible acid-shock protein during the stationary phase and has been studied in enteric bacteria (Fang et al., 1992).

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TolC is an important protein channel located on the bacterial outer membrane and participates in drug resistance. In *E. coli* and *Salmonella* Typhimurium (*S.* Typhimurium), *tolC* deficiency leads to increased sensitivity to a wide range of molecules, including bile salts, detergents, antibiotics, dyes, and organic solvents (Horiyama et al., 2010). The well-known AcrAB-TolC tripartite efflux pump, which belongs to the resistance-nodulation-division family of drug efflux systems, is composed of periplasmic lipoprotein AcrA, integral membrane protein AcrB, and outer membrane protein TolC (Poole, 2005). Additionally, TolC associates with a variety of inner membrane efflux proteins and recognizes diverse substrates and molecules (Horiyama et al., 2010). The role of TolC in *E. coli* physiology has been previously examined; the pleiotropic phenotypes of *tolC*, which are involved in drug resistance, outer membrane composition, virulence regulation, and acid tolerance, may be linked to the complex network of *tolC* and other regulatory factors (Zgurskaya et al., 2011).

Compared to *E. coli*, no studies have determined the relationship between TolC and acid tolerance in *Salmonella*, infection with which is a potential public threat. Our study showed that the TolC membrane protein is required for *S.* Choleraesuis survival, expression of adequate acid-mediated genes, and maintenance of its normal morphology in acidic environments. We further found that morphological changes in *tolC*-deficient *S.* Choleraesuis result from the presence of toxic reactive oxygen species (ROS), which are alleviated by antioxidants. Furthermore, we used antioxidants or cells exposed to hydrogen peroxide to demonstrate that an increased antioxidative capacity can upregulate acid resistance in *S.* Choleraesuis.

2. Material and methods

2.1. Strains, plasmids, and growth conditions

All *S.* Choleraesuis strains used in this study were derived from ATCC13312 (SCWT). The mutant was constructed using the λ red recombination system (Datsenko and Wanner, 2000) and confirmed by gene sequencing. The strains and plasmids used in this study are listed in Table 1. Cells were grown at 37 °C either in tryptic soy broth (TSB) or tryptic soy agar (TSA) at specific pH levels. The antibiotics ampicillin (100 μg/mL) and kanamycin (50 μg/mL) were also used.

2.2. Acid resistance assay

The acid resistance assay was induced as described previously (Lee et al., 1994) with some modifications. Briefly, overnight cultures grown at pH 7 were adjusted to McFarland 0.5, inoculated into fresh TSB at pH 3, and then incubated at 37 °C with shaking at 180 rpm for 30 min. Cultures designed for adaptation were cultivated at pH 5 for 16–20 h before challenge with pH 3 followed by incubation as mentioned above. After acid challenge, the samples were immediately diluted with saline (0.85% NaCl) and plated onto TSA. Surviving bacteria were investigated by determining the number of colony forming units (CFU) before acid challenge and after 24 h of incubation. Percent survival was calculated by comparing the CFU before and after challenge and the results were presented as percentages.

2.3. Quantitative reverse-transcriptional (RT)-PCR

Total RNA was extracted from *S.* Choleraesuis strains after acidic treatment. RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Next, 1 μg of RNA was reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) and suspended in 100 μL nuclease-free water. Quantitative RT-PCR was performed using the oligonucleotides described in the Supplementary material (Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.vetmic.2016.08.006%20), and primers specific for the *mdh* gene were used as an internal control.

2.4. Effects of antioxidant and hydrogen peroxide on S. Choleraesuis in acidic environment

To investigate whether ROS induced by acid was involved in morphological changes, a 1/100 dilution of the overnight culture was inoculated into TSB containing 1 mM *N*-acetyl-L-cysteine (NAC), pH 7 or 5, and incubated at 37 °C for 4 h. The morphology and viable counts were determined by microscopy and agar plate counting.

For the survival assay, *Salmonella* strains were examined to determine whether an increased antioxidative capability would protect cells from acid killing effects. Cells were challenged with TSB containing 1 mM NAC following overnight growth at pH 3 or 7 or cells cultured in the presence of 1 mM hydrogen peroxide overnight were challenged with TSB, pH 3. Surviving colonies were counted and the data are presented as percent survival.

3. Results

3.1. TolC plays a role in S. Choleraesuis acidic tolerance

In order to investigate the role of *tolC* in the survival of *S.* Choleraesuis, we initially used the *tolC*-deficient strain SCΔTolC to analyze the importance of *tolC* in acid resistance in *S.* Choleraesuis. Significant attenuation of the acid tolerance in the mutant was observed when SCΔTolC was exposed to lethal acidic challenge (pH

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Characteristic	Reference
Strain		
SCWT	Wild-type	ATCC 13312
SCΔTolC	*tolC* null mutant	This study
SCΔTolC + pKTC	SCΔTolC carrying pKTC8	This study
Plasmid		
pKD46	λ Red recombinase expression plasmid	Datsenko and Wanner, 2000
pKD4	Template for amplifying antibiotic cassette	Datsenko and Wanner, 2000
pCP20	FLP expression plasmid	Datsenko and Wanner, 2000
pKT25	Cloning vector	Karimova et al., 1998
pKTC8	*tolC* of SCWT cloned into pKT25	This study