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Functional characterization of *murB-potABCD* operon for polyamine uptake and peptidoglycan synthesis in *Streptococcus suis*

Wanquan Liu[a], Meifang Tan[b], Chunyan Zhang[a], Zhuofei Xu[a,c], Lu Li[a,c,d,*], Rui Zhou[a,c,d,*]

[a] State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China
[b] Institute of Animal Husbandry and Veterinary Science, Jiangxi Academy of Agricultural Sciences, Nanchang, China
[c] Cooperative Innovation Center of Sustainable Pig Production, Wuhan 430070, China
[d] International Research Center for Animal Diseases (Ministry of Science & Technology of China), Wuhan 430070, China

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ABSTRACT

Spermidine (Spd), spermine (Spm), and putrescine (Put), which are the most widely distributed cellular polyamines, are essential for normal growth and multiplication of both eukaryotic and prokaryotic cells. In this study, we identified the only putative polyamine transport system PotABCD in *Streptococcus suis*, a worldwide zoonotic Gram-positive pathogen causing lethal infections in humans and pigs. It was discovered that *S. suis* could uptake polyamines preferably Spd and Spm. By constructing a *potA* deleted mutant, we confirmed that PotABCD was responsible for polyamine uptake, and PotD bound to the protein of polyamines. The four PotABCD genes were co-transcribed with *murB*, a gene involved in peptidoglycan (PG) synthesis. Furthermore the roles of polyamine transport system in maintaining the PG structure were detected to understand the biological significance of this co-transcription. In contrast to the wild type, the mutant *ΔpotA* exhibited elongated chain length and abnormal cell division morphology. Phenotypic changes were attributed to be the up-regulation of genes involved in PG synthesis and hydrolysis in *ΔpotA*. Additionally, polyamines functioned not only as feedback regulators of PotA by inhibiting PotA activity but also as regulators on *potABCD* and genes involved in PG synthesis. This study reveals the functions of PotABCD in polyamine transport and the regulatory roles of polyamines in PG synthesis. Results provide new insights into the machineries contributing to normal growth and cell division of *S. suis*.

1. Introduction

Polyamines, such as spermidine (Spd), spermine (Spm), and putrescine (Put), are small, polycationic molecules containing a hydrocarbon backbone and multiple amino groups. Polyamines, which are positively charged at physiological pH, are required for optimal growth in all eukaryotes and most prokaryotes (Shah and Swiatlo, 2008; Shah et al., 2011). In eukaryotes, polyamines play important roles in cancer, synthesis and structure of proteins and nucleic acids, protection from oxidative damage, activity of ion channels, cell proliferation, cell differentiation, and apoptosis (Pegg, 2016). In prokaryotes, polyamines are involved in many biological processes, such as microbial carcinogenesis, bacteriocin production, escape from phagolysosomes, biofilm formation, toxin activity, and protection from oxidative, and acid stresses (Shah and Swiatlo, 2008). Polyamines are present inside mammalian tissues at the millimolar range. Polyamine uptake is critical to the survival of pathogens (Lin et al., 2017). Thus, components in polyamine biosynthesis and transporters are rational drug targets in both eukaryotic and prokaryotic cells (Shah and Swiatlo, 2008; Nowotarski et al., 2013).

Polyamine biosynthesis and transport in bacteria are a coordinated process, and intercellular polyamine levels are stringently regulated (Igarashi and Kashiwagi, 1999). Most prokaryotes possess a *de novo* synthesis pathway, in which polyamines are synthesized through enzymatic modification of precursor amino acids (Tabor and Tabor, 1985; Shah and Swiatlo, 2008). Furthermore, almost all bacteria can also utilize extracellular polyamines by the polyamine ATP-binding cassette transporter (PAT) encoded by an operon containing four genes (Shah et al., 2008). Some bacteria, such as *Escherichia coli*, possess two PATs (PotABCD and PotFGHI) (Kashiwagi et al., 1990; Pistocchi et al., 1993). A single operon *potABCD* exists in many other bacteria, such as *Streptococcus pneumoniae* and *Staphylococcus aureus* (Tettelin et al., 2001; Baba et al., 2002). In *E. coli*, PotD and PotF are the periplasmic substrate-binding proteins that bind to extracellular polyamine; meanwhile, PotA and PotG are membrane-associated cytosolic ATPases. The remaining proteins (PotB and PotC; PotH and PotI) contain membrane-

* Corresponding authors at: State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China.
E-mail addresses: lilu@mail.hzau.edu.cn (L. Li), rzhou@mail.hzau.edu.cn (R. Zhou).

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spanning α-helices and form transmembrane channels for polyamine transport (Kashiwagi et al., 1990; Pistocchi et al., 1993). PotABCD, a Spd-preferential PAT, also binds Put, but the dissociation constant is considerably low (Kashiwagi et al., 1993). PotFGHI is a Put-preferential PAT, which only binds Put with a high binding affinity (Vassylyev et al., 1998). In *E. coli*, nonpolar mutations in any of the gene in the *potABCD* operon abolish polyamine transport (Furuchi et al., 1991). Spd uptake in cells is gradually inhibited in parallel with Spd accumulation. The binding of high-concentration Spd to PotA decreases the ATPase activity and inhibits polyamine transport (Kashiwagi et al., 2002). PotD can also down-regulate the transcription of the *potABCD* operon (Antognoni et al., 1999). Thus, both receptor and ligand can function as feedback regulators of polyamine transport.

In Gram-negative bacteria, Spd and Put have been found to be constituents of the cell wall peptidoglycan (PG); these molecules maintain the integrity of the cell surface structure and normal cell growth (Kamio and Nakamura, 1987; Hirao et al., 2000; Kojima et al., 2011). In Gram-positive bacteria, Put can substitute for amino alcohol choline in synthesis of the cell wall teichoic and lipoteichoic acids of *S. pneumoniae* (Ware et al., 2005; Potter and Paton, 2014). In addition, mutant strains lacking the ability to synthesize or transport Spd display a significant delay in autolysis onset (Potter and Paton, 2014). Autolysins are believed to play an important role in the cell wall metabolism and pathogenicity of bacteria (Berry et al., 1989). Bacteria produce several PG hydrolases, including autolysins, which can disintegrate their own PG saccules and lead to bacterial cell lysis under unfavorable conditions (Rigden et al., 2003).

Streptococcus suis is a worldwide zoonotic Gram-postive pathogen causing lethal infections in humans and pigs (Gao et al., 2016). As an emerging zoonotic pathogen, *S. suis* has caused large economic losses in the pig industry (Zhu et al., 2016). *S. suis* can also cause human infections, resulting in meningitis, septicemia, arthritis, toxic shock syndrome (STSS), and severe post-infection sequelae or death; as such, the bacterial species is a major public concern worldwide (Gottschalk and Segura, 2000; Lun et al., 2007; Ma et al., 2009). In 1998 and 2005, two large outbreaks of human *S. suis* infections were reported in China; in these outbreaks, 52 humans died of STSS or meningitis (Yu et al., 2006).

S. suis needs sufficient energy and nutrients to live and proliferate in the host. Nutrient acquisition is also crucial for the pathogenicity of *S. suis*. For instance, suffering from glucose starvation or the deletion of carbohydrate synthesis and transport genes remarkably affects the growth, morphology, and pathogenicity of *S. suis* (Tan et al., 2015; Koliwer-Brandl et al., 2016; Zhang et al., 2016). Investigations on nutrient transport systems are significant to understand the survival mechanism and pathogenesis of *S. suis* for development of novel drug targets and preventive strategies. To date, the functions of the polyamine transport system(s) of *S. suis* have not been discovered.

In this study, the known *de novo* synthesis pathway was not observed in SC-19 according to the genome of SC-19 strain. Only one operon encoding a polyamine transport system (*potABCD*) was identified in the *S. suis* genome. The *murB* gene involved in PG synthesis was located beside the operon *potABCD*. The polyamine transport ability of *S. suis* and the function of *potABCD* in polyamine transport and PG synthesis were detected. The regulatory roles of polyamine on polyamine transport and PG synthesis were also investigated.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* SC-19 was isolated from a diseased pig in Sichuan province of China in 2005 (Li et al., 2009). SC-19 and its genetically modified strains were grown in Tryptic Soy Broth (TSB; Difco, France) or plated on Tryptic Soy Agar (TSA; Difco) containing 5% (vol/vol) newborn bovine serum (Sijiqing, China) or chemically defined medium (CDM) (van de Rijn and Kessler, 1980) at 37 °C. *E. coli* strains used for gene cloning and protein expression were cultured in Luria-Bertani (LB) broth (Difco) or plated on LB agar plates at 37 °C or 18 °C.

Table 1
Summary of bacteria strains and plasmids used in this study.

Strain or plasmid	Characteristics and functions[a]	Sources or references
BACTERIAL STRAINS		
SC-19 Δ*potA*	*S. suis* serotype 2,wild-type (Strep^r)	(Li et al., 2009)
E. coli DH5α	*potA deletion mutant of SC-19*	This study
E. coli BL21 (DE3)	F-endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF) U169, hsdR17(rk-mK+), λ−	Trans
PLASMID	F-ompT hsdSB(rB-mB-) gal dcm (DE3)	Trans
pET28a	Expression vector (Kan^r)	Novagen
pSET4S	Temperature-sensitive *E. coli*—*S. suis* shuttle vector (Spc^r)	(Takamatsu et al., 2001)
pSET4S-P	Derived from pSET4s for deleting potA in SC-19	This study
pET28a-potA	pET-28a containing the CDS of *potA* cloned from SC-19 genome	This study
pET28a-potD	pET-28a containing the CDS of *potD* cloned from SC-19 genome	This study

[a] Strep^r, streptomycin resistant; Kan^r, kanamycin resistant; Spc^r,spectinomycin, resistant.

2.2. Construction of the potA knockout mutant

The thermosensitive suicide vector pSET4s was used for gene replacement in *S. suis* through homologous recombination (Takamatsu et al., 2001). All the primers were designed according to the genome sequence of *S. suis* SC-19 (GenBank accession number NZ_CP020863.1) and listed in Table 2. Two pairs of specific primers (PotAup-F/PotAup-R and PotAdown-F/PotAdown-R) were used in cloning the *potA* up- and downstream homologous regions and subsequently cloned into the pSET4s vector to create pSET4s:*ΔpotA*. The plasmid was confirmed by DNA sequencing and transformed into the SC-19 strain. The mutant strain was detected by PCR and RT-PCR (Tan et al., 2015) analyses with primer pairs listed in Table 2.

2.3. RT-PCR

The RT-PCR assays were performed according to our previous study (Gao et al., 2016). SC-19 and *ΔpotA* strains were grown overnight in TSB medium with 5% newborn bovine serum at 37 °C and diluted by 100-fold in the fresh medium. After 6 h of incubation (an OD_{600nm} of 0.6), cells in the exponential growth phase were collected. Total RNA was isolated and purified using Qiagen RNeasy Mini Kit (Qiagen, China) according to the manufacturer's instructions. cDNA was obtained using HiScript Q Select RT SuperMix (Vazyme, China) according to the manufacturer's instructions.

To confirm that the mutant strain *ΔpotA* and the up- and downstream of *potA* genes are expressed normally, we designed the primers murB-F/R, potA-F/R, potB-F/R, potC-F/R and potD-F/R for RT-PCR from cDNA (Table 2).

To confirm that the putative *potABCD* operon is co-transcribed with *murB*, we used the primer pairs of 1-F/R, 2-F/R, 3-F/R, 4-F/R, 5-F/R, and 6-F/R for RT-PCR from the cDNA of SC-19 according to the contiguous genes in the operon (Table 2). Products can be amplified from the two adjacent genes, which should be co-transcribed. Lengths of the expected products were 741 bp, 729 bp, 675 bp, 696 bp, 678 bp and 711 bp, respectively.