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The membrane transporter PotE is required for virulence in avian pathogenic *Escherichia coli* (APEC)

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ABSTRACT

Over the last few years, polyamines have been described as key-signal of virulence in pathogenic bacteria. In the current study, we investigated whether the knockout of genes related to polyamine biosynthesis and putrescine transport affected the virulence of an avian pathogenic *E. coli* (APEC) strain. One-week-old White Leghorn chickens were infected intratracheally with mutants in polyamine biosynthesis (*ΔspeB/C* and *ΔspeD/E*) and transport genes (*ΔpotE*) of a well-characterized APEC strain of ST117 (O83: H4). All polyamine mutants and the wild-type strain were able to infect chicken; however, we observed significantly fewer lesions in the lungs of the chickens infected with the polyamine mutants in comparison with chicken infected with the wild-type. Results derived from histology of infected lungs detected significantly fewer lesions in the lung of birds infected within particular the putrescine transport mutant (*ΔpotE*). A decrease in colonization levels was observed in the liver and spleen of birds infected with the putrescine biosynthesis mutant *ΔspeB/C*, and likewise, a decrease of the colonization levels of all organs from birds infected with the *ΔpotE* was detected. Together, our data demonstrate that the deletion of polyamine genes, and in particular the PotE membrane protein, attenuates the virulence of APEC during infection of chickens.

1. Introduction

Avian pathogenic *Escherichia coli* (APEC) strains represent an economic burden to many poultry farmers worldwide due to high morbidity and mortality (Schouler et al., 2012). These bacteria cause colibacillosis in a broad range of avian species, especially in chickens (Antão et al., 2008). Colibacilosis is mainly characterized by compromising the quality of hatching eggs and salpingitis in laying hens, omphalitis in embryos, and airsacculitis and respiratory problems in broiler chickens (Horn et al., 2012; Sadeyen et al., 2014; Olsen et al., 2016). The oral and respiratory routes seem to be significant modes of natural infection, which usually initiates as airsacculitis, leading to systemic infection (Antão et al., 2008; Dziva, 2010). Furthermore, APEC isolates may have an impact on public health due to the emergence of multidrug-resistant bacteria (Mellata, 2013). APEC isolates are genetically similar to human extra-intestinal *E. coli* strains, particularly to uropathogenic *E. coli*, and can potentially be transmitted to humans, especially through the food chain (Zhu Ge et al., 2014; Manges and Johnson, 2015).

Polyamines are small polycationic amines widespread in living organisms. They are essential for the normal cell cycle progression (Lee et al., 2009). Putrescine together with spermidine is the most prominent polyamines in bacterial cells. Putrescine can be synthesized from L-ornithine through ornithine-decarboxylation (OCD) due to redundant enzymes encoded by the genes *speC* and *speF*, or from arginine by sequential reactions involving the enzymes arginine decarboxylase and agmatinase, encoded by *speA* and *speB,* respectively (Kurihara et al., 2009). Spermidine is synthesized from putrescine and methionine, and the enzymes involved are encoded by the genes *speE* and *speD,* respectively (Shah and Swiatlo, 2008). Transport systems play an important role in the regulation of the polyamine intracellular levels. They can act as both importers and exporters. This is the case for the PotE membrane protein, encoded by the *potE* gene, which is involved in exchange reactions of putrescine and ornithine (Kashiwagi et al., 2000). They can also be ATP-binding-cassette (ABC) transporters consisting of two channel-forming proteins and a membrane-associated ATPase; in *E. coli*, this is the case for the spermidine-preferential and the putrescine-specific uptake systems (Igarashi and Kashiwagi, 1999). It has been

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shown that deletion of biosynthesis polyamine genes attenuates the virulence of *Salmonella* Typhimurium (Jelsbak et al., 2012; Schroll et al., 2014), *Francisella* (Russo et al., 2011) and *Shigella* (Barbagallo et al., 2011). Therefore, polyamines might be a potential target for the development of novel prophylactic or therapeutic treatments in bacterial infections (Shah et al., 2011).

In a recent study carried out by our group, mutations in genes involved in putrescine biosynthesis and transport were shown to cause a time-delay in the growth in minimal laboratory growth medium of an APEC *E. coli* strain and in some cases a reduced growth rate (Guerra et al., 2017, unpublished). To the best of our knowledge, no studies so far have investigated the virulence of APEC polyamine biosynthesis and transport mutants in a chicken challenge model. Thus, in the present work, we assessed for the first time the virulence of these mutants by airway challenge using in vivo chicken infection models.

2. Material and methods

2.1. Bacterial strains

All experiments were performed with an APEC *E. coli* strain (WT) of serotype O83: H4, phylogroup D and sequence type ST117. This strain is well characterized (Pires-dos-Santos et al., 2014). Double mutants for genes related to biosynthesis of putrescine (*speB/C*) and spermidine (*speD/E*) or to the transport of putrescine (*potE*) were constructed and quality controlled in a previous study (Table 1) (Guerra et al., 2017, unpublished). As part of the quality control process, all constructs were verified by PCR, and whole genome sequencing (WGS) confirming that no secondary mutations were present in the mutant strains. Also, the *potE* deletion mutant was functionally complemented for growth attenuation *in trans* using a cloned copy of the gene on a complementation plasmid (Guerra et al., 2017, submitted). Thus, the WGS approach allowed us to leave out laborious complementation experiments, a use of WGS that was first suggested by Bryant et al., 2012. Stock cultures were stored in 20% glycerol broth at −80 °C until use.

2.2. Inoculum preparation

For the chicken embryo lethality assay, bacterial strains were grown for 16 to 24 h with shaking (200 rpm) at 37 °C in Luria-Bertani (LB) (Oxoid) broth supplemented with trimethoprim (Tmp–10 μg/ml) (Sigma-Aldrich) and/or chloramphenicol (Cm- 5 μg/ml)(Sigma-Aldrich) as required (Table 1). Overnight cultures of wild-type and mutants were diluted (1:100) into fresh Brain-Heart infusion (BHI) broth (Oxoid) and incubated with shaking (180 rpm) at 37 °C until the cultures reached an OD_{600} = 1. Cultures were then harvested by centrifugation for 10 min at 6000*g* and resuspended in phosphate buffered saline (PBS) (Fisher Scientific). CFUs for the inoculum was determined according to the protocol described by Andersson et al. (2015), ranging from 100 to 500 CFU per 0.1 ml of PBS. For the infection of chickens, overnight cultures were diluted (1:50) in LB broth and grown at 37 °C, 180 rpm, until they reached OD_{600} = 1. Samples were centrifuged at 5000*g* for 10 min and resuspended in PBS to a final concentration of 3–6 × 10^9 CFU/ml.

Table 1
Strains and plasmids used in the study.

Strains	Relevant features[a]	Reference
E. coli 083:H4 (WT)	Virulence reference strain APEC strain	Pires-dos-Santos et al., 2014
Δ*speB/C*	Putrescine biosynthesis mutant: *speB* mutant, *speC* mutant, chl^R, tmp^R	Guerra et al., 2017, unpublished
Δ*speD/E*	Spermidine biosynthesis mutant: *speD* mutant, *speE* mutant, chl^R	Guerra et al., 2017, unpublished
Δ*potE*	Putrescine transport mutant: *potE* mutant, chl^R	Guerra et al., 2017, unpublished

[a] chl^R chloramphenicol resistant; tmp^R trimethoprim resistant.

2.3. Embryo lethality assay (ELA)

Chicken embryo lethality test was performed as described by Andersson et al. (2015). Briefly, embryonated eggs from Leghorn chicken were placed in a hatcher (Hova-Bator) at 37.5 °C. The hatcher provided heating air circulation with moisture level between 60% to 70% and automatic turning of eggs. After twelve days of incubation, eggs were removed from the incubator and rinsed with 70% ethanol. Carefully, 0.1 ml of the bacterial suspension or PBS (mock control) was inoculated into the allantoic cavity. Eggshells were sealed with warmed paraffin and tape; then the eggs were immediately placed back to the incubator. At two hours post-inoculation, all eggs were candled and their viability was scored by examining the presence of blood vessels and embryo movements. Deaths which occurred within the first two hours post-infection were assumed to be caused by lethal trauma occurred at inoculation and those embryos were excluded from the experiments. Every 12 h, the eggs were candled and the viability was scored. At 72 h post-infection, the embryos were killed. The liver was removed aseptically; macerated and serial 10-fold dilutions were spread on LB agar plates supplemented with trimethoprim and/or chloramphenicol when required. CFU counts were assessed after 18 h of incubation at 37 °C. Ten colonies from each egg were confirmed to correspond to the challenge strain by PCR. Primers are described in Table S1 (Supplemental material).

2.4. Infection of chickens

The virulence of WT and the mutants (Δ*speB/C*, Δ*speD/E*, and Δ*potE*) was assessed during infection of chickens, according to modified protocols (Antão et al., 2008; Ginns et al., 1998). A total of 200 one-week-old chicken White Leghorn chickens were used in the trial. All birds were provided from the same hatchery. Animals were fed ad libitum and acclimated under the same conditions for a week prior to infection. A total of 40 birds were included in each group, divided into five pens. Groups were challenged intratracheally with 0.1 ml of the bacterial suspension. A mock-infected control group was inoculated with 0.1 ml of PBS.

Animals were monitored over two days and clinical scores were recorded. Chickens that showed severe clinical signs were immediately euthanized, according to the welfare norms. At 48 h post-infection, all birds were euthanized by cervical dislocation. One-gram samples of lungs, liver, and spleens were aseptically collected, weighed, and homogenized. Ten-fold dilutions were spread on LB agar and/or LB agar supplemented with trimethoprim and/or chloramphenicol as appropriate. CFU counts were assessed after 16–18 h of incubation at 37 °C. Random colonies were examined by mass spectrometry (MALDI-TOF) and PCR to ensure that the re-isolated bacteria corresponded to the challenge strain, as mentioned above.

2.5. Animal ethical statement

All animal work has been conducted according to the national and international guidelines for animal welfare. The animal trials were approved by the Danish Animal Expectorate (license no. 2016-15-0201-00870).

2.6. Macroscopic scoring and histological analyses

Post-mortem examinations were conducted by three veterinarians and performed immediately after the birds were killed. All organs were dissected and aseptically removed. Lesions related to *E. coli* were scored using a scoring system modified after Antão et al. (2008); Ginns et al.