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Research Article

Adenylate Cyclase Affects the Virulence of Extraintestinal Pathogenic Escherichia coli Derived from Sheep Lungs

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Abstract

Escherichia coli is an important component of the normal bacterial community in humans and animals. However, in recent years, the pathogenicity of some virulent strains to the outside of the intestine has been confirmed in clinical medicine. The purpose of this study was to analyze the effect of *cyaA* gene knockout on the biological characteristics and pathogenicity of extraintestinal pathogenic *E. coli*. In this study, ExPEC strain (XJ10) was isolated from the lung tissue of sheep with respiratory tract infection, and the mutation strain and complementary strain of *cyaA* gene were constructed. Real-time PCR analysis showed that expression levels of most genes related to carbon source utilization and adhesion invasion were down-regulated in the *cyaA* mutant compared to the wild type; Quantification of the number of colonies showed that *cyaA* mutation results in the number of adhesion and invasive to TC-1 cells colonies decreased compared with wild type; Finally, as the *cyaA* gene mutated, the value of the LD_{50} increased as compared to wild type, based on the LD_{50} calculation. Taken together, mutations in the *cyaA* gene reduce growth rates, resulting in down-regulation of the transcription levels of genes associated with carbon source utilization and adhesion invasion, and a marked reduction in virulence.

Keywords: Adhesion and invasion, *cyaA*, Median lethal dose, XJ10

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Introduction

Escherichia coli can be divided into entero-pathogenic *E. coli* and extra-intestinal pathogenic *E. coli* according to their pathogenic characteristics. Through adaptive changes, Extraintestinal Pathogenic *E. coli* (ExPEC) have developed the ability to colonize extra-intestinal regions, causing pathologies in multiple organs and leading to diseases in humans and animals, including meningitis, septicemia, urinary tract infections, pneumonia, avian perihepatitis, and peritonitis. These infections are a serious human public health threat and thus have attracted the attention of researchers in China and abroad [1-3]. ExPEC can cause widespread infections in humans and animals, which are closely related to the presence of numerous virulence factors such as adhesins, toxins, protective factors, iron uptake systems, and pathogenicity islands. Each of these factors plays a different role during the different stages of infection [4-7].

Adenylate cyclase (AC), encoded by the *cyaA* gene, can enter a variety of eukaryotic cell and is mainly distributed on the plasma membrane, nuclear membrane, and endoplasmic reticulum membrane. When a cell receives a foreign signal molecule, the signal molecule binds to the corresponding membrane G protein-coupled receptors

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(GPCRs), thereby activating AC. Upon activation of the *cyaA* gene, ATP is catalyzed to form cyclic adenosine monophosphate (cAMP), which can affect the absorption and metabolism of carbohydrates, fats, amino acids, and esters. cAMP can regulate the expression of nutrients related to nutrition utilization by activating protein kinase A (PKA) related signaling pathways, thereby regulating the metabolism and utilization of nutrients [8]. cAMP binds and allosterically activates the transcriptional regulator cAMP receptor protein (CRP) and participates in regulating nearly 50% of the genes and operons in bacteria in the form of CRP-cAMP complexes [9,10]. CRP forms an active conformation only when it binds to cAMP. Moreover, the concentration of CRP-cAMP complex and its mediated biological response were affected by the intracellular concentration of cAMP. It was also found that the *cpdA* gene encoded phosphodiesterase could hydrolyze cAMP to 5'-AMP in vitro and in vivo. Hence, the concentration of cAMP in cells is determined by cAMP phosphodiesterase [11]. Studies have shown that deletion of the *cyaA* gene in the highly virulent strain *Salmonella choleraesuis*X3246 resulted in the loss of pathogenicity but maintenance of immunogenicity [12]. Yu [13] reported that *cyaA* gene deletion significantly reduced the virulence of *Salmonella typhimurium* but did not completely eliminate its pathogenicity in animals, with the growth rate of the mutant strain significantly lower than that of its parent strain. In recent years, our group isolated and identified ExPEC (XJ10) from the lungs of sheep that have died of respiratory infection [14].

In this study, by constructing a *cyaA* gene deletion strain of XJ10, we analyzed the role of the *cyaA* gene in ExPEC XJ10 in the regulation of pulmonary infection in the mouse model *in vitro* and *in vivo*.

Materials and Methods

Ethical Statement

This trial was approved by the Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine (Approval no: A2019-157).

Bacterial Strains, Plasmids, Media, and Growth Conditions

The bacterial strains and plasmids used in this study have been listed *(Table 1)*. Wild-type (WT) ExPEC strain XJ10 was isolated from the lungs of diseased sheep. Plasmid pDS132 was used as a homologous recombination vector. *E. coli* β2155 was used as the transfer host for pDS132. *E. coli* DH5α, pDS132, and plasmid pHSG396 were purchased from Takara Bio (Otsu, Japan). The strains stored in the -80°C refrigerator were inoculated on MacConkey (Haibo Bio, Qingdao, China) agar plate and cultured at 37°C for 18 h [15], the single colonies were collected and incubated in LB liquid medium at 37°C for 12 h, the bacteria were collected to prepare for the next test.

Construction of *cyaA* Mutants and Complementary Strains

Gene mutants were constructed by homologous recombination [16]. Using the primer (Beijing RuiboXingke Biotechnology Co., Ltd., Beijing, China) sets *cyaA*-F1/R1 and *cyaA*-F2/R2, the upstream and downstream regions of *cyaA* gene were amplified separately by PCR from the genomic DNA of the WT strain, which was then mixed for overlapping PCR. The primer set *cyaA*-F1/R2 was added to amplify the target fragment. The plasmid pDS132-Δ*cyaA* containing the target fragment was introduced into *E. coli* β2155 [17], which was co-cultured with the WT strain. Colony PCR was performed for transformants resistant to sucrose but sensitive to chloramphenicol, and deletion of the *cyaA* gene was confirmed using the primers *cyaA*-outF/outR and *cyaA*-inF/inR. The final selected strain was named XJ10/Δ *cyaA*.

To construct a *cyaA* gene complementary strain, the complete *cyaA* gene sequence was amplified by PCR from the genomic DNA of the WT strain using the primer set HF1/HF2 and then cloned into the pHSG396 vector.

Table 1. *Strains and plasmids used in this study*

Strain Or Plasmid		Relevant characteristics	Reference
Strain	XJ10	Wild-type (WT), sheep origin, separation and preservation in the author's laboratory	[14]
	XJ10/Δ*cyaA*	Mutant deleted a 2457-bp fragment from whole ORF of *cyaA* in XJ10	This study
	cyaA$^+$/Δ*cyaA*	XJ10/Δ*cyaA* mutant complemented with a copy of the *cyaA* via pHSG396	This study
	E. coli β2155	Conjugated transfer host of pDS132	Laboratory
Plasmids	pDS132	Cm^R Sac B, suicide vector	Laboratory
	pDS132-Δ*cyaA*	pDS132 vector inserted disrupted *cyaA* in *SmaI*	This study
	pHSG396	ori lac Z CmR	Takara
	pHSG396-*cyaA*	pHSG396 vector containing the full-length *cyaA*	This study

Table 2. Specific primers for the construction of cyaA gene deletion strain of XJ10

Primers	5′-3′ Sequence	Application
*cyaA*1	TTGTACCTCTATATTGAGACTCTGAAAC	Mutant
*cyaA*2	TCACGAAAAATATTGCTGTAATAGCG	
cyaA-F1	*TCCCCCGGGGTCCCAGACCTTGCGGGAA*	Mutant
cyaA-R1	ATGGCATCCAGTCTCTGTTTCAGAG	
cyaA-F2	CTGCCAATCAGGATCACGATACG	Checking PCR
cyaA-R2	CCCCCGGGCTGGTAGTTTCGTGATCGGCAC	
cyaA-outF	CGTTCAGCCAGTTGGCACTG	Checking PCR
cyaA-outR	CTTTCTATCATCGCAGGAGAAGACG	
cyaA-inF	CGTTGACCTGCTGTACCGCAACT	Checking PCR
cyaA-inR	CAGTTGCTGCACGCGAGTACG	
HF1	CCGCTCGAGTTGTACCTCTATATTGAGACTCTGAAAC	Complementation
HF2	CCGGAATTCTCACGAAAAATATTGCTGTAATAGCG	
HJ1	AACCGCTTCCGTCATAAT	Checking PCR
HJ2	GGTTTACCAGCGTCACTTTA	

Plasmid pHSG396-*cyaA* was transformed into the *cyaA* gene mutant by electroporation. Primer set HJ1/HJ2 was used to re-acquire a complete clone of the *cyaA* gene. The complementary strain was named *cyaA*$^+$/Δ*cyaA*. The primer sequences used in this study has been shown *(Table 2)*.

Growth Analysis

A growth curve was plotted to determine the effects of the *cyaA* gene on the growth of ExPEC. 10^4 cfu mL^{-1} of WT, XJ10/Δ*cyaA*, and *cyaA*$^+$/Δ*cyaA* strains were added to LB medium respectively and were inoculated at 37°C with 200 rpm oscillation. Samples were collected at 2, 4, 6, 8, 10, 12, 14, 16, and 18 h, placed in MacConkey solid medium, incubated at 37°C, and then counted separately. Data were obtained from three independent experiments, each in triplicate [18].

Analyses of Biochemical Characteristics and mRNA Levels of Various Genes Associated with Carbon Source Utilization, Adhesion, and Invasion

Use 0.45% NaCl solution to prepare bacterial suspensions from the single colonies of three bacteria cultured for 18 h, and set the concentration to 0.55 using a turbidimeter. Biochemical reaction characteristics of WT, XJ10/Δ*cyaA*, and *cyaA*$^+$/Δ*cyaA* were analyzed by the biochemical analyzer (Vitek2 compact, Meyrié) (including 47 biochemical reaction items involving sugar decomposition, amino acid utilization, and enzyme production) [19]. According to the OMG kit (OMEGA Bio-tek, Inc., Norcross, GA, USA) instructions, the 3 strains were cultured at 37°C for 12 h and collected at 10^7 cfu mL^{-1} for RNA extraction. The RNA was later reverse-transcribed into cDNA using of Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The Roche real-time fluorescence, quantitative PCR system (Basel, Switzerland) was used to measure the mRNA levels of eight genes: the pathogenicity island gene *(fyuA)*, QS system gene *(luxS)*, P pili gene *(pap)*, flagellum gene *(motA)*, and genes associated with carbon source utilization *(sucC*, *acs*, *gltA*, and *fabB)*. The complete list of primers used in this study has been shown *(Table 3)*, and the MIQE guidelines [20] associated with them are shown in the supplementary materials.

Adhesion and Invasion Assay

The adhesion and invasion abilities of the three bacterial strains on mouse alveolar epithelial TC-1 cells (iCell Bioscience Inc, Shanghai, China) were determined. TC-1 cells were inoculated into 12-well tissue culture plates (NEST Biotechnology, Jiangsu, China) at approximately 10^5 cells per well. After the fusion degree of the cells reached 80%, TC-1 cells were infected at an MOI of 10. Low-glucose RMPI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) was added to every 1 mL of medium. The cells were incubated for 2 h at 37°C. After washing three times with PBS, the infected cells were lysed with 0.1% Triton X-100 (Solarbio, Beijing, China) for 3 min. Samples were diluted and plated on MacConkey agar plates to determine the number of bacterial (adherent cells and intracellular bacteria) colony-forming units (CFU). To measure the number of invading bacteria, the cells in the plates were washed with PBS after 2 h of incubation, and then further incubated with RMPI-1640 medium (include 25 μg mL^{-1} bleomycin, Thermo Fisher Scientific) for 90 min at 37°C to eliminate extracellular bacteria. Monolayer cells were washed with PBS, lysed with 0.1%

Table 3. *Primers used for real-time fluorescence quantitative amplification*		
Gene	**Primers**	**5′-3′ Sequence**
Adhesion-associated genes	*pap*1	TTTCTGCCTTACCTGTTTG
	*pap*2	TTTGCACCGTTCACCA
	*motA*1	TGCCGTTGCTGTTTCGTC
	*motA*2	TTCTGCCTCGCTTTCGTG
	*luxS*1	TGCGTGCCGAACAAAGAA
	*luxS*2	CAGCCCATTGGCGAGATA
	*fyuA*1	GCATCAGCCAACAACG
	*fyuA*2	CTCGCAGCAACTCCAC
Carbon source utilization related genes	*sucC*1	TTTGCCCGCTATGGCTTAC
	*sucC*2	CACGGATGTCTTCTTTGCTGTT
	*fbaB*1	CGCTAAGTTACCCGAATACC
	*fbaB*2	TGACTCTTCCGAGCCAAA
	*gltA*1	TCCTGCTGAATGGTGAAA
	*gltA*2	GGGTAAACAAATGGCTGA
	*acs*1	ACGCAGAAGTCCGCAACT
	*acs*2	TTCTCGACTACGCCAGGAT
Mouse lung tight junction and basement membrane gene	*occluding*1	GCTCTCTCAGCCAGCGTACT
	*occluding*2	GGCGATGCACATCACAATAA
	*mmp-9-*91	GCCAACTATGACCAGGAT
	*mmp-9-*92	TGCCACCAGGAACAGG
	*zo-1*1	AAAACCCGAAACTGAT
	*zo-1*2	CGCCCTTGGAATGTATG
Reference gene	*16SRNA*1	GAGCAAGCGGACCTCATA
	*16SRNA*2	ATTCACCGTGGCATTCTG
	*β-actin*1	TGCTATGTTGCTCTAGACTTCG
	*β-actin*2	GTTGGCATAGAGGTCTTTACGG

Table 4. *Biochemical reaction results*								
Parameter	**SAC**	**ADO**	**dMNE**	**dMAN**	**IARL**	**BGAL**	**dGLU**	**dSOR**
WT	+	+	+	+	-	+	+	+
XJ10/Δ*cyaA*	-	-	+	-	-	-	+	-
cyaA^{+}/Δ*cyaA*	-	+	+	+	-	+	+	-

Single colonies of the purified different strains were selected for biochemical identification
'+' indicates positive results; '-' indicates negative results
SAC: Saccharose; ADO: Adonitol; dMAN: D-maltose; BGA: β-galactosidase; dGLU: D-glucose; dSOR: D-Sorbitol; WT: wild type

Triton X-100, and plated to count invading bacteria. Each assay was performed in at least triplicate [21].

LD_{50} Determination

All animal experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals (1985). The total bacterial dose required for each group of mice was calculated according to the ratio of concentration difference between the minimum dose of all dead mice and the maximum dose of all surviving mice according to the modified Karber's method [22] (doses are shown in *Table 4*). The mice provided by the Xinjiang Medical University laboratory animal center were randomly divided into six groups of six mice each. Mice were infected with bacteria by intraperitoneal injection. The mice were then provided with a clean environment, clean water, and adequate food. The mice were observed continuously for 7 days, and the

number of dead mice in each group was recorded. At the end of the experiment, the surviving mice were treated humanely and harmlessly.

Effect of *cyaA* on mRNA Levels of Genes Related to Mouse Lung Tight Junction and Basement Membrane Inflammation

The bacterial count of the three strains was adjusted to 2/3 of the LD_{50} of XJ10 and the strains were injected intraperitoneally into mice. At 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, 96, and 108 h after CO_2 asphyxiation, the lung tissues of mice were collected and the corpses were treated innocuously. The OMEGA kit (Omega Bio-tek, Inc., Norcross, GA, USA) was used to extract total RNA from the harvested tissue, followed by reverse transcription of RNA into cDNA using the Thermo Fisher Scientific reverse transcription kit. The mRNA levels of transmembrane protein *(occludin* gene), accessory protein *(zo-1* gene), and matrix metalloproteinase *(mmp-9* gene) were measured using the Roche real-time fluorescence, quantitative PCR system. The primer sequences used in this study have been shown (*Table 3*), and the MIQE guidelines [20] associated with them are shown in the supplementary materials.

Statistical Analysis

All results of data analysis are presented as the mean ± standard deviation (SD). Data analysis was performed on independent samples using the *t*-test. Differences were considered as significant when $P \leq 0.05$. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad, Inc., La Jolla, CA, USA).

Results

Screening of Deletion Strain

The deletion strain was identified using primers *cyaA*-inF/inR. The amplified XJ10 produced a 193-bp band, whereas the deletion strain produced no bands *(Fig. 1-A)*. Using *cyaA*-outF/outR, the amplified XJ10 produced a 4139-bp band and the deletion strain produced a 1686-bp band *(Fig. 1-B)*. These results show that the deletion strain was successfully constructed.

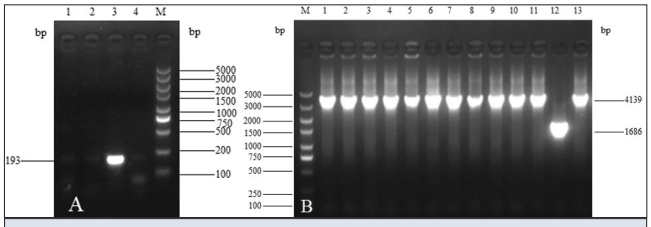

Fig 1. PCR results of homologous recombinant strains. A: (inF, inR) M: DL 5000 DNA Marker; 1,2,4: XJ10-Δ*cyaA*; 3: XJ10; B: (outF, outR) M: DL 5000 DNA Marker; 1-11,13: XJ10; 12: XJ10-Δ*cyaA*; The 3rd lane in A and 12th lane in B are the correct test results of the primers

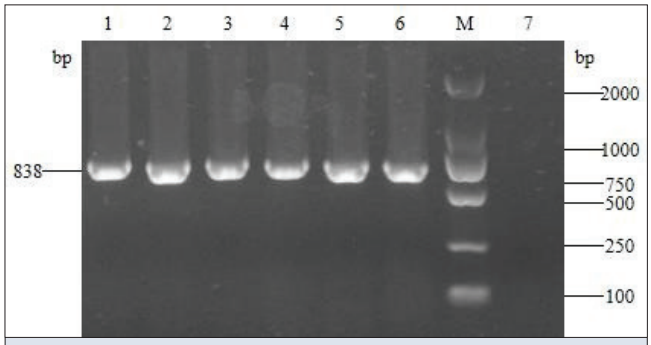

Fig 2. Complementary strain identification PCR results. 1-5: complementary strain; 6: positive control; 7: negative control; Using primers, complementary strains and WT showed amplified fragments of the same size

Screening Results of Complementary Strain

The full-length *cyaA* gene was amplified using HF1/2 and ligated with pHSG396 to construct the recombinant plasmid pHSG396-*cyaA*. The recombinant plasmid was transformed into XJ10-Δ*cyaA* via electroporation to construct *cyaA*$^+$/Δ*cyaA*. PCR identification was performed using primer HFJ1/2. Both XJ10 and *cyaA*$^+$/Δ*cyaA* amplified fragments 838 bp in size, whereas XJ10-Δ*cyaA* did not amplify any fragments *(Fig. 2)*, demonstrating successful construction of the complementary strain.

Growth Curve Measurement Results

The growth pattern of XJ10/Δ*cyaA* over 18 h was similar to that of XJ10 and *cyaA*$^+$/Δ*cyaA*, all of which reached a stable phase at 6 h. However, the number of live XJ10/Δ*cyaA* in the stable phase was significantly lower than that of XJ10 ($P<0.05$). The complementary strain *cyaA*$^+$/Δ*cyaA* did not reach the number of live XJ10 cells but was significantly higher than that of the gene deletion strain XJ10/Δ*cyaA* ($P<0.05$) *(Fig. 3)*.

Biochemical Identification and Analysis of mRNA Levels of Genes Related to Carbon Source Utilization, Adhesion, and Invasion

Biochemical identification of WT, XJ10/Δ*cyaA*, and *cyaA*$^+$/Δ*cyaA* was conducted using a biochemical analyzer.

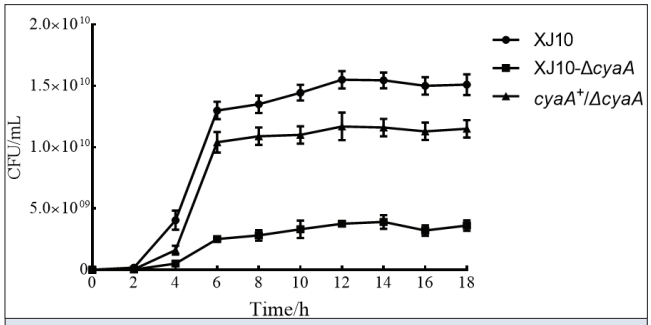

Fig 3. Growth curve. The WT strain (●), *cyaA* mutant (▪), and complemented strain (▲) were grown in LB medium at 37°C, respectively, and the number of live bacteria was measured every 2 h. The graph is representative of three independent experiments

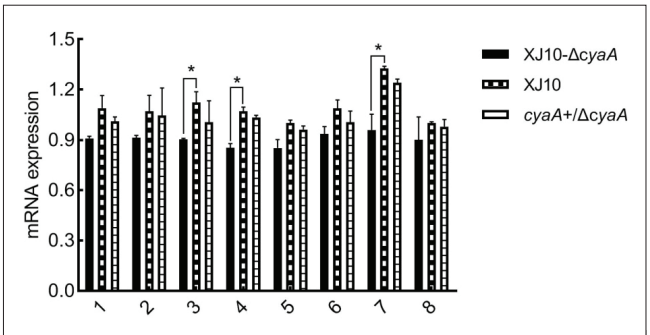

Fig 4. mRNA expression levels associated with carbon source utilization, adhesion, and invasion. (1: *sucC*; 2: *acs*; 3: *gltA*; 4:*fabB*; 5: *fyuA*; 6: *luxS*; 7: *pap*; 8:*motA*)

The mRNA expression levels of some genes related to carbon source utilization, adhesion, and invasion were determined. RNA extracted from the same WT, *cyaA* mutant, and complementary strain of CFU was reverse-transcribed into cDNA and subjected to real-time fluorescent quantitative PCR. All values are presented as the mean ± SD. Asterisks indicate significant differences between the mutants and WT strain (*P<0.05)

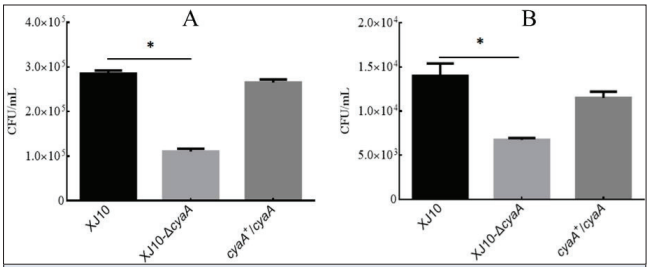

Fig 5. Effects of cyaA deletion on XJ10 adhesion and invasion mouse alveolar epithelial TC-1 cells. A: adhesion; B: invasion. Adhesion and invasion levels of ExPEC strains were measured. Cell associated bacteria (adherent + intracellular) were quantified after a 2 h infection period. Invasion was determined after bleomycin treatment for an additional 2 h. All values are presented as the mean ± SD. Asterisks indicate significant differences between the mutants and WT strain (*P<0.05)

The results are shown in *Table 4*. Compared to XJ10, XJ10/Δ*cyaA* lost the ability to utilize Saccharose (SAC), Adonitol (ADO), D-maltose (dMAN), β-galactosidase (BGAL), and D-Sorbitol (dSOR); *cyaA*$^+$/Δ*cyaA* regained the ability to utilize ADO, dMAN, and BGAL.

Compared to the WT strain, the mRNA expression levels of the eight genes in XJ10-Δ*cyaA* were all decreased. The expression levels of *gltA*, *pap*, and *fabB* were decreased

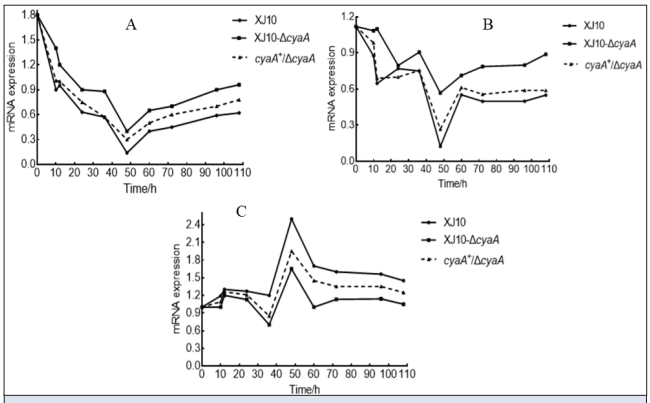

Fig 6. Effect of XJ10-*cyaA* on mouse lung tight junction and basement membrane-associated gene mRNA levels. A: *occludin*, B: *zo-1* (B), C: *mmp-9*. Animals were inoculated by intraperitoneal injection and lung samples were collected from the mice every 2 h. RNA was extracted from the samples and reverse-transcribed into cDNA for real-time PCR. All values are presented as the mean ± SD

significantly (P<0.05), whereas those of *fyuA*, *luxS*, *motA*, *sucC*, and *acs* were decreased but not significantly (P>0.05). Compared to XJ10, the mRNA expression levels of the eight genes in the complementary strain *cyaA*$^+$/Δ*cyaA* were all decreased, but the difference was not significant (P>0.05) *(Fig. 4)*.

Adhesion and Invasion Assay

Compared to XJ10, the adhesion and invasion abilities of XJ10-Δ*cyaA* for TC-1 cells were significantly lower (P<0.05) *(Fig. 5)*. The adhesion and invasion levels of XJ10-Δ*cyaA* for TC-1 cells were 2.58- and 2.07-fold lower those that of the WT strain, respectively. *cyaA*$^+$/Δ*cyaA* showed restoration of the adhesion and invasion abilities to some extent. These results demonstrate that the *cyaA* gene plays an important role in the cell adhesion and invasion abilities of ExPEC.

LD_{50} Measurement Results

The mouse infection model was used to determine the role of the *cyaA* gene in the virulence of ExPEC *in vivo*. Compared to LD_{50} of the WT ($10^{9.45}$ CFU) and *cyaA*$^+$/Δ*cyaA* ($10^{9.58}$ CFU) strains, that of the XJ10-Δ*cyaA* ($10^{9.71}$ CFU) strain was increased by 1.8- and 1.3-fold, respectively *(Table 5)*.

Table 5. *LD_{50} of XJ10, XJ10/ΔcyaA and cyaA$^+$/ΔcyaA*

Inoculation Dose	XJ10	XJ10-Δ*cyaA*	*cyaA*$^+$/Δ*cyaA*
$10^{10.08}$	6/6	6/6	6/6
$10^{9.82}$	6/6	5/6	5/6
$10^{9.56}$	4/6	1/6	3/6
$10^{9.30}$	1/6	0/6	1/6
$10^{9.04}$	1/6	0/6	0/6
$10^{8.78}$	0/6	0/6	0/6

Animals were inoculated by intraperitoneal injection and observed for 7 days. The ratio indicated the number of dead mice/ number of mice infected

Effect of *cyaA* on Some Genes Related to Mouse Lung Tight Junction and Basement Membrane Inflammation

The mRNA levels of *occludin* and *zo-1* in the three strains showed an initial decrease during the test period, reaching minimum values at 48 h, and then increasing until 108 h. However, the mRNA expression levels of *occludin* and *zo-1* in XJ10-Δ*cyaA* were always significantly higher than those of XJ10 and *cyaA*$^+$/Δ*cyaA* throughout the experiment (P<0.05) *(Fig. 6-A,B)*. The mRNA levels of *mmp-9* for all three strains increased from 0 to 48 h, and reached their peak at approximately 48 h, after which the levels began decreasing until 108 h. However, the *mmp-9* mRNA level of XJ10-*ΔcyaA* was significantly lower than that of XJ10 and *cyaA*$^+$/Δ*cyaA* throughout the experiment (P<0.05) *(Fig. 6-C)*.

Discussion

ExPEC is capable of causing infections in many extra-intestinal systems, including the urinary tract, central nervous system, circulatory system, and respiratory system. This is caused by the joint action of the virulence factors carried by ExPEC, which not only enhances the virulence of ExPEC but also extraintestinal infection of the host [23,24]. In this study, we first evaluated the effect of the *cyaA* gene on ExPEC virulence using the sheep-derived clinical strain XJ10, deletion strain, and complementary strain. The *cyaA* gene deletion strain showed a lower growth rate than the WT and complementary strains, as well as defects in carbon source utilization, cell adhesion, and invasion. The LD_{50} indicated that the loss of gene attenuated the virulence of ExPEC in mice.

Liao [25] found that the *cya* gene mutant of *S. typhimurium* SL1344 lost its ability to utilize glycerol, maltose, and sorbitol. Wang [26] showed that the growth rate of the *cya* gene mutant of *E. coli* G8107 in goose yolk peritonitis was slower at each stage than that of the corresponding parent strain. The results of this study demonstrate that the growth rate of the *cyaA* gene mutant was significantly lower than that of WT and complementary strains. The *cyaA* gene deletion strain lost its ability to utilize some carbon sources, and the mRNA expression of genes related to sugar utilization was significantly decreased. These results suggest that the deletion of the *cyaA* gene led to a loss of efficiency in AC synthesis, resulting in the inability to catalyze the conversion of ATP into cAMP, in turn preventing activation of the receptor protein CRP. This further illustrates that the *cyaA* gene can regulate certain metabolic reactions in *E. coli*, thereby indirectly affecting its virulence. In addition, the experimental results showed that the *cyaA* gene knockout caused 4 genes related to carbon utilization to be down regulated, the growth curve of the bacteria also confirmed this result. Therefore, it is speculated that the deletion of the *cyaA* gene will affect the carbon source utilization of XJ10, thereby indirectly affect the growth of bacteria.

Liu [27] found that knocking out the *cya* gene in the *S. typhimurium* SL1344 strain resulted in decreased expression of genes related to the pili and cellulose as well as significantly reduced adhesion to and invasion of HeLa cells, whereas the deletion strain showed lower virulence for HeLa cells. In this study, deletion of *cyaA* gene significantly reduced the adhesion and invasion ability of XJ10 for mouse alveolar epithelial cells TC-1, and the mRNA levels of genes associated with adhesion and invasion were decreased significantly, whereas the complementary strain showed restored adhesion and invasion abilities to a certain extent. This supports that *cyaA* gene can regulate the expression of genes related to the flagellum, pili, or a series of surface proteins to affect the target cells, thereby reducing the virulence of XJ10. Lung tight junctions are composed of Occludins and accessory proteins (ZO-1). When these proteins are altered, the permeability of the intercellular space increases, causing tumor metastasis, destruction of organ physiological barriers, the spread of inflammation [28], etc. MMP-9 belongs to a group of matrix metalloproteinases expressed in a variety of cells and involved in physiological and pathological processes such as cell movement, inflammation, and tissue repair [29]. Jin found that the protein expression of ZO-1 in alveolar epithelial cells from mice was decreased following acute lung injury [30]. This study showed that the mRNA levels of the *occludin* gene and *zo-1* gene in the lung tissue of mice artificially infected with XJ10/Δ*cyaA* were significantly increased, whereas the expression of the inflammation-related *mmp-9* gene was decreased. The *cyaA* gene may be associated with XJ10 causing lung tissue pathological changes.

By reducing the utilization of certain nutrients such as SAC, ADO, dMAN, BGAL, and dSOR, deletion of the *cyaA* gene can reduce the growth rate of XJ10, affect its adhesion and invasion abilities in sensitive cells, and influence certain factors related to cell barrier and inflammation in the lung tissue, thereby decreasing the virulence of XJ10. However, the complementary strain cannot utilize D-sorbitol and sucrose, possibly because the construction of the complementary strain only expresses the *cyaA* gene in the bacterium through plasmid expression, without reintegrating the *cyaA* gene into the bacterial genome. Therefore, the relevant biological characteristics of the complementary strains have not fully recovered to be consistent with those of the wild strains. Based on these results, the LD_{50} of the bacteria in mice was determined. The results showed that the LD_{50} of XJ10 with *cyaA* gene deletion was 1.8-fold higher than that of WT, and its virulence was significantly reduced. The construction of

the XJ10/Δ*cyaA* mutant strain in this experiment provides a basis for comparing the virulence intensity of the XJ10/Δ*cyaA* mutant and wild strains, conducting functional analysis of the *cyaA* gene, and exploring novel approaches for developing attenuated live vaccines against *E. coli*.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding author/s upon reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

This trial was approved by the Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine (Approval no: A2019-157).

Author Contributions

Conceived of or designed study: X Z, ML H, X H; Performed research: YJ C, XX G; Analyzed data: FG Z, J L; Contributed new methods or models: TZ W, XX Z; Wrote the paper: YJ C, YN S, XL W.

References

1. Adorján A, Thuma Á, Könyves L, Tóth I: First isolation of atypical enteropathogenic *Escherichia coli* from geese *(Anser anser domestica)* and first description of atypical EPEC from turkeys and pigeons in Hungary. *BMC Vet Res*, 17:263, 2021. DOI: 10.1186/s12917-021-02968-w

2. Huang WC, Lin CY, Hashimoto M, Wu JJ, Wang MC, Lin WH, Chen CS, Teng CH: The role of the bacterial protease Prc in the uropathogenesis of extraintestinal pathogenic *Escherichia coli. J Biomed Sci*, 27 (1):14, 2020. DOI: 10.1186/s12929-019-0605-y

3. Nielsen DW, Klimavicz JS, Cavender T, Wannemuehler Y, Barbieri NL, Nolan LK, Logue CM: The impact of media, phylogenetic classification, and *E. coli* pathotypes on biofilm formation in extraintestinal and commensal E. coli from humans and animals. *Front Microbiol*, 9:902, 2018. DOI: 10.3389/fmicb.2018.00902

4. Pakbin B, Bruck WM, Rossen JWA: Virulence factors of enteric pathogenic *Escherichia coli:* A review. *Int J Mol Sci*, 22 (18):9922, 2021. DOI: 10.3390/ijms22189922

5. Manges AR, Johnson JR: Reservoirs of extraintestinal pathogenic *Escherichia coli. Microbiol Spectr*, 3 (5): 2015. DOI: 10.1128/microbiolspec.UTI-0006-2012

6. Biran D, Ron EZ: Extraintestinal pathogenic *Escherichia coli. Curr Top Microbiol Immunol,* 416, 149-161, 2018. DOI: 10.1007/82_2018_108

7. LeCuyer TE, Byrne BA, Daniels JB, Diaz-Campos DV, Hammac GK, Miller CB, Besser TE, Davis MA: Population structure and antimicrobial resistance of canine uropathogenic *Escherichia coli. J Clin Microbiol*, 56 (9):e00788-18, 2018. DOI: 10.1128/JCM.00788-18

8. Zurita-Martinez SA, Cardenas ME: Tor and cyclic AMP-protein kinase A: Two parallel pathways regulating expression of genes required for cell growth. *Eukaryot Cell*, 4 (1): 63-71, 2005. DOI: 10.1128/EC.4.1.63-71.2005

9. Gárate F, Dokas S, Lanfranco MF, Canavan C, Wang I, Correia JJ, Maillard RA: cAMP is an allosteric modulator of DNA binding specificity in cAMP receptor protein from *Mycobacterium tuberculosis. J Biol Chem*, 296:100480, 2021. DOI: 10.1016/j.jbc.2021.100480

10. Mcdonough KA, Rodriguez A: The myriad roles of cyclic AMP in microbial pathogens: From signal to sword. *Nat Rev Microbiol*, 10 (1): 27-38, 2012. DOI: 10.1038/nrmicro2688

11. Imamura R, Yamanaka K, Ogura T, Hiraga S, Fujita N, Ishihama A, Niki H: Identification of the *cpdA* gene encoding cyclic 3',5'-adenosine monophosphate phosphodiesterase in *Escherichia coli*. *J Biol Chem*, 271 (41): 25423-25429, 1996. DOI: 10.1074/jbc.271.41.25423

12. Kelly SM, Bosecker BA, Curtiss R: Characterization and protective properties of attenuated mutants of *Salmonella choleraesuis. Infect Immun*, 60 (11): 4881-4890, 1992. DOI: 10.1128/iai.60.11.4881-4890.1992

13. Zhuhua Y, Songbiao C, Chunjie Z, Xiangchao L, Yinju L, Lei H, Chuan Y, Jing L, Yanyan J: Comparison of biological characteristics of 5 strains of *Salmonella typhimurium* gene deletion strains. *Chin J Vet Med*, 35 (8): 1275-1279, 2015. DOI: 10.16303/j.cnki.1005-4545.2015.08.14

14. Qiangqiang G, Jingya K, Yingjin C, Xin H, Mengli H, Xingxing Z, Tongzhong W, Xia Z, Fagang Z: Isolation and identification of pulmonary pathogenic *Escherichia coli* from sheep. *Microbiol China*, 44 (12): 2805-2811, 2017.

15. Kiponza R, Balandya B, Majigo MV, Matee M: Laboratory confirmed puerperal sepsis in a national referral hospital in Tanzania: Etiological agents and their susceptibility to commonly prescribed antibiotics. *BMC Infect Dis*, 19 (1):690, 2019. DOI: 10.1186/s12879-019-4324-5

16. González-Torres P, Rodríguez-Mateos F, Antón J, Gabaldón T: Impact of homologous recombination on evolution of prokaryotic core genomes. *MBio*, 10 (1):e02494-18, 2019. DOI: 10.1128/mBio.02494-18

17. Sáenz Y, Briñas L, Domínguez E, Ruiz J, Zarazaga M, Vila J, Torres C: Mechanisms of resistance in multiple-antibiotic-resistant Escherichia coli strains of human, animal, and food origins. *Antimicrob Agents Chemother*, 48 (10): 3996-4001, 2004. DOI: 10.1128/AAC.48.10.3996-4001.2004

18. Rui C, Zhongxing W, Min J, Xiangkai Z, Jianjun D: sRNA chaperone protein Hfq controls the virulence of avian pathogenic *Escherichia coli. Anim Husb Vet Med*, 54 (5): 105-112, 2022.

19. Plakhova XI, Petrova NP, Nikonorov AA, Kubanov AA: Biochemical atypia in the modern Russian strains of *Neisseria gonorrhoeae. Klin Lab Diagn*, 65 (8): 507-511, 2020.

20. Bustin, SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*, 55 (4): 611-622, 2009. DOI: 10.1373/clinchem.2008.112797

21. Cong D, Jiale K, Meishang L, Yanfen L: Effect of IUCA gene deletion on the proliferation, adhesion, invasion and colonization of urinary tract pathogenic *Escherichia coli. Acta Univ Med Anhui*, 57, 177-181, 2022.

22. Zongying W, Yonghao C, Guangjin Z, Chunmiao Y, Shengjing L: Anti-inflammatory and analgesic effects of acanthus acanthus acanthus alkaloid a and its derivative acetyl acanthus acanthus alkaloid A. *Chin Hosp Pharm J*, 31 (10): 807-810, 2011.

23. Hossain M, Tabassum T, Rahman A, Hossain A, Afroze T, Momen AMI, Sadique A, Sarker M, Shams F, Ishtiaque A, Khaleque A, Alam M, Huq A, Ahsan GU, Colwell RR: Genotype-phenotype correlation of β-lactamase-producing uropathogenic *Escherichia coli* (UPEC) strains from Bangladesh. *Sci Rep*, 10 (1):14549, 2020. DOI: 10.1038/s41598-020-71213-5

24. Phan MD, Peters KM, Sarkar S, Lukowski SW, Allsopp LP, Moriel DG, Achard MES, Totsika M, Marshall VM, Upton M, Beatson SA, Schembri MA: The serum resistome of a globally disseminated multidrug resistant uropathogenic *Escherichia coli* clone. *PLoS Genet*, 9 (10):e1003834, 2013. DOI: 10.1371/journal.pgen.1003834

25. Liao C: Construction and biological characteristics of Salmonella typhimurium Δcrp and *Δcya* deletion strains, *MSc Thesis*, Henan University of Science and Technology, 2011.

26. Kai W, Zhiqiang Z, Chunhong Z, Luo J, Guoqiang Z: Construction of cya and crp double gene deletion strains from E. coli yolk peritonitis and analysis of some biological characteristics. *Chin J Vet Med*, 33 (9): 49-54, 2013.

27. Shasha L, Yanyan J, Chunjie Z, Songbiao C, Chengshui L, Yadong Y, Erxin W, Xiangchao C: Detection of biological characteristics of *Salmonella typhimurium* SL1344 strain *cya* gene deletion strain. *Chin J Immunol*, 32 (9):4, 2016.

28. Yujun H, Xinmin Q, Ming H, Ligong Q: Research and progress of myelin basic protein. *J Guangxi Univ Chin Med*, 7 (2):5, 2004. DOI: 10.3969/j.issn.1008-7486.2004.02.044

29. Yazheng Z, Yuanyuan Z, Junping K, Boyang Y: Advances in the role of tight junctions in tumor metastasis. *J Cross-Strait Pharm*, 29 (11): 1-5, 2017. DOI: 10.3969/j.issn.1006-3765.2017.11.001

30. Jin K: The mechanism of tight junction proteins Claudin-4 and ZO-1 in the protective barrier of oleic acid-induced lung injury in the lungs of the torches, *MSc Thesis*, Ningxia Medical University, 2012.