Characterization of a *yjjQ* mutant of avian pathogenic *Escherichia coli* (APEC)

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Infections with extraintestinal avian pathogenic *Escherichia coli* (APEC) cause significant economic losses in the poultry industry worldwide. In a previous study we applied signature-tagged transposon mutagenesis and identified 28 virulence-associated genes in APEC strain IMT5155 (O2:H5:K1). One of them, *yjjQ*, encodes a putative transcriptional regulator whose function and role in pathogenesis are still unknown. In the present study, this mutant has been characterized. The *yjjQ*-defective mutant of IMT5155 (M18E10) was out-competed by the wild-type strain *in vivo*, and infection of chickens with this *yjjQ* mutant led to strongly reduced bacterial loads in several organs. Expression studies showed that transcription of *yjjQ* was significantly upregulated in M9 minimal medium. Correspondingly, the *yjjQ* mutant showed significantly reduced growth in M9 medium. Although the mutation could not be complemented, a *yjjQ* deletion mutant showed phenotypes similar to the transposon-generated mutant M18E10, whereas deletion and overexpression of the downstream gene *bglI* did not cause a growth defect in M9. To identify virulence genes regulated by YjjQ, one- and two-dimensional protein gel electrophoresis was performed. The proteomic analysis revealed that in the *yjjQ* mutant M18E10 the expression of several genes involved in iron uptake was downregulated and some other genes were upregulated. The regulation of genes involved in iron uptake was shown to occur at the transcription level using real-time RT-PCR. Taking the results together, this functional analysis strongly suggests that YjjQ is a regulator involved in virulence of APEC by affecting iron uptake.

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INTRODUCTION

Isolates of extraintestinal pathogenic *Escherichia coli* (ExPEC) cause infection in nearly every organ and anatomical site in humans and animals. Among ExPEC, avian pathogenic *E. coli* (APEC) strains bring about serious extraintestinal disease of poultry, causing high mortality and morbidity in chickens and turkeys, thus leading to great economic losses (Mokady *et al.*, 2005). To elucidate the pathogenesis of APEC, we adapted signature-tagged transposon mutagenesis to a 5-week-old chicken model and identified 28 virulence-associated genes (Li *et al.*, 2005). Among them, *yjjQ* encodes a putative transcriptional regulator since sequence analysis revealed that it harbours a helix–turn–helix (HTH) DNA-binding domain

shared by all members of the LuxR family regulators (Chirwa & Herrington, 2003; Crater & Moran, 2001; Pruneda-Paz *et al.*, 2004). The presence of *yjjQ* has been reported for commensal and various pathogenic bacteria such as enterohaemorrhagic *E. coli* (EHEC), uropathogenic *E. coli* (UPEC), *Shigella* and *Salmonella* by genomic sequencing. However, the function of YjjQ remains unknown.

To compete and survive in a changing environment, bacteria have developed elaborate global regulation networks to sense and respond to stimuli from outside. For example, guanosine tetraphosphate (ppGpp) downregulates rRNA synthesis during nutrient starvation (Chatterji & Ojha, 2001); the glutamate decarboxylase system together with AR1 and AR3 (arginine decarboxylase system) are jointly responsible for defence against acid stress (Foster, 2004); the challenge of oxidative stress is met by the OxyR and SoxR/S system (Storz & Imlay, 1999), while heat-shock proteins respond to elevation in temperature (Gophna & Ron, 2003); alternative sigma factors such as RpoS, RpoE and RpoH play an important role

Abbreviations: APEC, avian pathogenic *E. coli*; CI, competitive index; EHEC, enterohaemorrhagic *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*; UPEC, uropathogenic *E. coli*.

The GenBank/EMBL/DDBJ accession number for the sequence of *yjjQ* and its flanking region is AM184164.

in bacterial survival by directing the transcription of genes involved in starvation survival and resistance to oxidative, acid, thermal and osmotic stress (Kazmierczak *et al.*, 2005; Loewen & Hengge-Aronis, 1994). In addition, Fur affects iron uptake by binding to the promoter region of iron-regulated genes with ferrous iron as a cofactor, thus repressing the expression of these genes (Hantke, 2001). The virulence and pathogenesis of medically important organisms are also regulated by these regulators, and in recent years great progress has been made in identifying those that coordinate expression of virulence genes (Byrne & Swanson, 1998; Johnson *et al.*, 2006a; Lucas *et al.*, 2000; Mellies *et al.*, 2006; Pizarro-Cerda & Tedin, 2004). Perhaps not surprisingly, such regulators are almost as numerous as the environmental signals to which virulence gene expression has been found to respond (Dong *et al.*, 2005; Lucas & Lee, 2001; Sturme *et al.*, 2005), and many of them remain to be identified. In this study we aimed to elucidate the possible function of YjjQ as a regulator. We show that YjjQ is involved in virulence of APEC by regulating iron uptake.

METHODS

Bacterial strains, plasmids, oligonucleotide primers and growth conditions. All *E. coli* strains and plasmids used in this study are listed in Table 1. Oligonucleotide primers are given in Table 2. Growth of bacterial cultures was performed at 37 °C in Luria–Bertani (LB) broth, M9 medium or modified low-phosphate-glucose-salts medium (LPM) which lacks KCl (Sambrook & Russell, 2001; Sepuliere *et al.*, 2003). When necessary, antibiotics were added at the following concentrations: ampicillin, 50 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; nalidixic acid, 30 µg ml⁻¹; chloramphenicol, 25 µg ml⁻¹.

For growth kinetics, overnight cultures of *E. coli* strains were diluted 1:100 into LB medium and incubated at 37 °C with shaking at

225 r.p.m. until an OD₆₀₀ of 1.0 was attained. These cultures were again diluted 1:100 into fresh LB or M9 medium to give parallel cultures representing the test conditions and controls, and were then incubated at 37 °C until the stationary phase was reached. Growth was monitored by measuring OD₆₀₀ (Eppendorf Biophotometer) or c.f.u. ml⁻¹. To determine the plating efficiency, 0.1 ml volumes were plated onto LB agar, and colonies were counted after 18 h incubation at 37 °C.

In vitro competition and in vivo tests in chickens. For *in vitro* competition assays cultures of mutant and wild-type strain were mixed in a ratio of 1:1, incubated in LB broth for 4 h at 37 °C and then plated onto media with or without kanamycin. For *in vivo* competition assays (Li *et al.*, 2005), 5-week-old female specific pathogen-free chickens (Lohmann Selected White Leghorn; Lohmann Tierzucht) were inoculated with 10⁸ c.f.u. of this mixture (in a dose of 0.5 ml) by intratracheal application. Chicks were provided with food and water ad libitum. After 24 and 48 h of infection, the spleen, heart, liver, lungs and kidneys of five chickens were collected, weighed and homogenized, and serial dilutions were plated on LB medium with and without kanamycin for selection of mutants or total bacteria, respectively. A competitive index (CI) was calculated for the mutant by dividing the output ratio (mutant/wild-type) by the input ratio (mutant/wild-type).

For individual chicken tests, 10⁸ c.f.u. of the wild-type or mutant strain were inoculated into the trachea of 20 chickens. After 24 h and 48 h, 10 chickens were euthanized and examined for macroscopic lesions. The spleen, pericardium, anterior lobe of the liver, lungs and kidneys of 10 chickens were collected, weighed and homogenized. Bacterial loads were determined by plating serial dilutions of the homogenates on selective LB agar medium.

Statistical comparisons were performed by using Student's *t* test to determine the difference between two groups. A *P* value of <0.05 was considered significant.

DNA manipulation and data analyses. DNA manipulation and transformation were performed according to standard methods

Table 1. Bacterial strains and plasmids used in this study

Strain	Description	Reference
IMT5155	O2:K1:H5, <i>fyuA sitA chuA irp2 iroN ireA tsh iucD fimC iss ompA vat traT</i>	Li <i>et al.</i> (2005)
IMT5155Nal ^R	IMT5155 derivative, Nal ^R	Li <i>et al.</i> (2005)
S17λpir	<i>recA thi pro hsdR⁻ M⁺ RP4::2-Tc::Mu::Km Tn7</i> lysogenized with λpir phage	Li <i>et al.</i> (2005)
M18E10	IMT5155Nal ^R <i>yjjQ::mini-Tn5</i>	Li <i>et al.</i> (2005)
IMT5155 <i>lacZ⁻</i>	IMT5155Nal ^R <i>lacZ::pGP704</i>	This study
IMT5155P1	IMT5155 pPYJJQ1	This study
IMT5155P2	IMT5155 pPYJJQ2	This study
IMT5155P3	IMT5155 pKEAP1	This study
KEC361	IMT5155 Δ <i>bglJ</i> ::Cm ^R	This study
KEC363	M18E10 Δ <i>yjjQ</i> ::Cm ^R	This study
Plasmid		
pGP704	Ap ^r , suicide plasmid	Herrero <i>et al.</i> (1990)
pRS551	Ap ^r Km ^r , promoterless <i>lacZ</i>	Simons <i>et al.</i> (1987)
pKD46	Ap ^r , expresses λ red recombinase	Datsenko & Wanner (2000)
pKD3	<i>cat</i> gene	Datsenko & Wanner (2000)
pGP704 <i>lacYZ</i>	pGP704 derivative harbouring <i>lacYZ</i> fragment	This study
pPYJJQ1	pRS551 derivative, promoterless <i>lacZ</i> under control of P _{1yjjQ}	This study
pPYJJQ2	pRS551 derivative, promoterless <i>lacZ</i> under control of P _{2yjjQ}	This study
pKEAP1	<i>lacI^f tacOP bglJ</i> in pACYC, Km ^r	Paukner (2007)

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')	Annealing temp. (°C)
1433	AATGGTTGTCAGCACTATGGC	56
1434	GTTCAGTCAGGCAGGATAATCC	56
1406	TCCTGCCTGACTGAACTGG	56
1407	TAGCCACGCGACATAAAGC	56
1529	GGAAGATCTGTTCTCGCTATTATTCCAACCG	58
1530	ACGCGTCGACTAAGTAATTCGCCCATTGTCG	58
1780	CGATCAGAATTCATACAAAGCCGTGTTACGGC	58
1781	CGATCAGAATTCGGATAATAGATAGCTGGGCGC	58
1782	CGATAAGGATCCATTCCATTTTGCAGCATCC	58
2376	CCATCATAATTTTATAGACAGTGGATGTGGAGGAAATGTGTAGGCTGGAGCTGCTTCG	55
2377	CTGCGGCATCTCCATAAACAGAAGGCAAGTTAAGTTCATATGAATATCCTCCTTAGTTCCTATTCC	55
For RT-PCR*		
ChuA-F	CAGATAACAAAAGGAGCAAGGC	59
ChuA-R	CTGACGATAATACTACCGCC	59
SitA-F	TGTCGCCAGATAATGCTCTG	59
SitA-R	TAAGTATCGGCATTGCGTTG	59
DnaE-F	ACTTCCTTGGTTTGCGTACG	59
DnaE-R	TCTTATCATCCAGCGGGATC	59
FyuA-F	CAAACTCCCCAGAGTCTTGC	59
FyuA-R	CATCGACATACAGGGTGACG	59
IreA-F	GCAAATATCAGAGGGGTGGA	59
IreA-R	AGCGACTTGCCATTGAGACT	59

*Primers for real-time RT-PCR.

(Ausubel, 1994). All restriction and DNA-modifying enzymes were purchased from Roche Molecular Biochemicals. Sequence analysis was done by AGOWA GmbH, Berlin, Germany, by using the chain-termination sequencing technique and an ABI model 373A DNA sequencer. Sequence data and promoter analyses were performed by applying the HUSAR sequence analysis package from the German Cancer Research Centre (DKFZ) in Heidelberg, Germany, and Neural Network Promoter Prediction software.

Expression studies

Construction of a LacZ-negative mutant strain. An insertion mutation in the *lacZ* gene of IMT5155Nal^R was constructed by using suicide plasmid pGP704 (Herrero *et al.*, 1990). The *lacZY* fragment was amplified by PCR with primers 1529 and 1530. Underlined bases in the sequences listed in Table 2 indicate restriction cutting sites of *Bgl*II and *Sal*I. The recombinant plasmid of pGP704 with the *lacZY* fragment was transformed into *E. coli* strain S17 λ pir. Mating was performed between S17 λ pir/pGP704 and the recipient strain IMT5155Nal^R. The conjugants were selected on LB agar plates with 100 μ g ampicillin ml⁻¹, 30 μ g nalidixic acid ml⁻¹ and 1 mM X-Gal. One white colony was picked up as a LacZ-negative strain and designated IMT5155 *lacZ*⁻.

Construction of *lacZ* transcription fusions. P_{*yjiQ*}-*lacZ* fusions were constructed by cloning PCR fragments containing terminal *Eco*RI and *Bam*HI sites to *Eco*RI- and *Bam*HI-restricted pRS551 (Simons *et al.*, 1987). Primers 1781 and 1782 (Table 2) were used for amplification of fragment P_{*yjiQ1*} encompassing the *yjiQ* upstream region from position -398 to +28, relative to the translation start codon (accession no. AM184164). Cloning of this fragment resulted in plasmid pPYJJQ1. Fragment P_{*yjiQ2*} encodes the *yjiQ* upstream region from position -660 to +28 and was constructed by using primers

1780 and 1782. The plasmid carrying this cloned P_{*yjiQ2*} was named pPYJJQ2. Both plasmids were used to transform IMT5155 *lacZ*⁻, resulting in recombinants IMT5155P1 and IMT5155P2.

β -Galactosidase assays. These assays were carried out as described by Miller (1972). *E. coli* strains were grown overnight in LB broth at 37 °C and the cultures were then diluted 100-fold into fresh medium and grown to an OD₆₀₀ of 1.0. One-millilitre volumes of these fresh cultures were diluted into 50 ml LB, LB with 2 or 4% NaCl, LB with 500 or 100 μ M H₂O₂, M9 minimal medium with 0.4% glucose as sole carbon source, and modified LPM of pH 7.0 or 4.5. Samples for photometric quantification and for β -galactosidase assays were taken every hour and tests were performed in triplicate.

Mutant generation and *Bgl*II overexpression plasmid construction. The *yjiQ* gene was replaced with a chloramphenicol-resistance gene by using the λ red recombinase system (Datsenko & Wanner, 2000). The chloramphenicol acetyltransferase gene was amplified from plasmid pKD3 by PCR using primers 2376 and 2377 (Table 2). Underlined bases in the sequences indicate sequence homology to the *yjiQ* flanking region. The PCR product was gel purified and electroporated into M18E10 containing the λ red recombinase expression plasmid, pKD46. Following electroporation, the samples were incubated at 28 °C for 1 h in SOC broth (Sambrook & Russell, 2001) and plated on LB agar containing chloramphenicol for overnight growth at 37 °C to select for transformants and to induce the loss of pKD46. The *bgl*II deletion mutant KEC361 was generated from wild-type strain IMT5155 using the same strategy except that primers 2376 and 2377 were used (Table 2). The mutant was confirmed by PCR and Southern blotting.

The *Bgl*II overexpression plasmid pKEAP1 carries *bgl*II under control of the *tac* promoter. This pACYC-derived plasmid also carries the

lacI^q gene and a kanamycin-resistance gene (Paukner, 2007). Further, in *E. coli* K-12 induction of pKEAP1-encoded *bglJ* relieves the H-NS-mediated repression of the *bgl* promoter confirming that BglJ is expressed (Paukner, 2007). Plasmid pKEAP1 was transformed into wild-type strain IMT5155 to give IMT5155P3.

One-dimensional SDS-PAGE. Cells of IMT5155 wild-type, the *yjjQ* transposon-insertion mutant M18E10 and the $\Delta yjjQ$ mutant KEC363 were obtained from overnight cultures in LB and from cultures grown in M9 medium with 0.4% glucose for 6 h. Cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS) and resuspended in 200 μ l lysis buffer containing 9 M urea, 2% CHAPS (Sigma Aldrich), 70 mM dithiothreitol (Sigma Aldrich), and 2% ampholyte (pH 2.0–4.0; Amersham Biosciences). The resuspended cell pellet was six times frozen in liquid nitrogen, thawed and mixed. Then it was ultrasonicated six times for 20 s during a 30 min incubation at room temperature. Cell debris was removed by centrifugation, and the protein concentration in the aqueous supernatant was quantified photometrically. Ten-microgram samples of protein were separated on 15% SDS-PAGE gels by standard methods (Ausubel, 1994) and silver staining was carried out as described by Tsai & Frasch (1982).

Proteomic analysis. Proteins from wild-type IMT5155 and mutant M18E10 cultured in M9 minimal medium were extracted as described above. Protein samples (300 μ g) from both strains were separated by 2D-PAGE according to the method of Hochstrasser *et al.* (1988). Briefly, proteins were separated in the first dimension according to their isoelectric point (pI) by isoelectric focusing in a pH gradient ranging from 3.0 to 10.0. In the second dimension, the proteins were separated according to their molecular masses by SDS-PAGE and visualized by Coomassie blue. Cellular proteins of each tested strain were isolated in at least three separate experiments, and 2D-PAGE of each protein preparation was repeated at least three times. The 2D gels used for comparison analysis were digitized at a resolution of 150 d.p.i. using a PowerLook 2100XL with transparency adaptor. 2D image analysis was performed using the Proteomweaver software (Definiens).

Only proteins that could be reproducibly and clearly found to increase or decrease at least 2.5-fold in abundance, based on the intensity of the protein spots, were further analysed. The following procedures were commercially finished by the Proteome Factory (Berlin, Germany). Analysis of protein spots excised from 2D gels was performed by in-gel tryptic digestion. The generated peptides were applied to an Agilent 1100 nanoLC system with trap column online-coupled to an ion trap mass spectrometer Esquire3000plus (Bruker). The MS-MS data were searched against the NCBI nr protein database using the Mascot search engine.

RNA preparation and real-time RT-PCR. To restrict iron availability the cation-exchange resin Chelex 100 (Bio-Rad) was added to liquid media (batch method according to the supplier's manual) and glassware was soaked overnight in 10% HCl and then rinsed extensively in double-distilled deionized water. Ca^{2+} and Mg^{2+} were added to Chelex-treated LB to final concentrations of 0.2 and 0.1 M. The wild-type strain IMT5155, its *yjjQ* mutants M18E10 and KEC363, *bglJ* deletion mutant KEC361 and BglJ-overexpressing strain IMT5155P3 were grown in treated LB medium to the early exponential phase (OD_{600} 1.0) and total RNA was isolated using the High Pure RNA isolation kit (Roche Diagnostics). Treatment of total RNA with DNase and analysis on formaldehyde gels was performed as described by Puopolo *et al.* (2001). RNA concentrations were determined using a NanoDrop 1000 apparatus (NanoDrop Technologies), and 1 μ g total RNA was reverse transcribed in triplicate using random hexamers and M-MLV reverse transcriptase (Promega). Real-time PCR was done using the SYBR green PCR Master Mix (Applied Biosystems) according to the manufacturer's guidelines. Reactions (10 μ l) were performed using 1 μ l cDNA

template per reaction. A 7900HT Fast Real-time PCR machine (384-head) (Applied Biosystems) was used. PCR conditions consisted of an initial incubation step for 10 min at 95 °C, followed by 40 cycles for 30 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C. Melting curve analyses were performed after each reaction to ensure amplification specificity. Differences (*n*-fold) in transcript were calculated using the relative comparison method, and amplification efficacies of each primer set were verified as described by Schmittgen *et al.* (2000). RNA levels were normalized by using the housekeeping gene *dnaE* as control. Primers used for real-time RT-PCR analysis are listed in Table 2.

RESULTS

Mutation of *yjjQ* attenuates virulence of APEC *in vivo*

Previously we identified mutant M18E10 with an insertion in the putative regulator gene *yjjQ* to be attenuated in a chicken infection model (Li *et al.*, 2005). The capacity of mutant M18E10 to compete for growth in chicken tissues with wild-type strain IMT5155 (O2:H5:K1) was compared by *in vivo* and *in vitro* competition assays. The mutant showed moderately attenuated growth in lung and kidney, with competitive indices (CI) of 0.031 and 0.021 ($P < 0.01$), respectively; whereas in liver, spleen and heart, M18E10 was strongly out-competed by the wild-type (CI 0.0037, 0.001 and 0.0015, respectively, $P < 0.001$). In contrast, the *in vitro* CI was approximately 0.30 ($P < 0.05$).

Mutant M18E10 and wild-type IMT5155 were further tested individually in chickens by imitating the natural route of infection and administering a dose of 10^8 c.f.u. into the trachea of 5-week-old specific pathogen-free birds. All 10 birds that were challenged with the wild-type strain developed severe bilateral airsacculitis, moderate pericarditis, marked pulmonary congestion, severe hypertrophy and congestion of the spleen, and moderate perihepatitis covered by a thick yellow fibrous membrane. In contrast, M18E10 caused only moderate airsacculitis, mild pericarditis and pulmonary congestion, slight hypertrophy of the spleen and mild perihepatitis without yellow fibrous membrane. The capacity of M18E10 and the wild-type strain to colonize internal organs was studied by determining colony numbers in selected organs 24 and 48 h after infection. Both strains colonized the avian lung, heart, liver, spleen and kidney. Significantly, after 24 h maximum colonization of the lung as well as of the heart was observed for the wild-type, while M18E10 colonized only the lung at a maximum level. Minimal colony counts were obtained in liver (3.46×10^2 c.f.u. for M18E10) and spleen (8.61×10^5 c.f.u. for wild-type). After 48 h the results differed to some extent, with both the wild-type and the mutant strain colonizing the kidney maximally. Except in the kidney, c.f.u. levels were much lower at 48 h than at 24 h after infection, and minimum levels were seen in the liver for both strains. However, on average the M18E10 mutant strain was reisolated at levels 10–1000 times lower than the wild-type strain from all internal organs at both time points (Fig. 1).

Fig. 1. YjjQ mutant strain M18E10 is attenuated *in vivo*. Bacterial loads in different internal organs from 5-week-old chickens 24 h (a) and 48 h (b) after exposure to wild-type IMT5155 (white bars) and yjjQ::mini-Tn5 mutant M18E10 (black bars). The error bars indicate standard errors of the means from 10 chickens. At 24 h after infection the bacterial load differences in lung, liver and spleen between wild-type and mutant were significant ($P<0.05$); at 48 h after infection the differences in lung, liver, spleen and heart were significant ($P<0.05$).

Genetic analysis of yjjQ and its flanking sequences

Based on the nucleotide sequence of yjjQ in uropathogenic *E. coli* strain CFT073 (accession no. AE014075) PCR primers 1433 and 1434 were designed to amplify the yjjQ gene and its flanking region in APEC strain IMT5155 by PCR (Table 2). The products were sequenced commercially at AGOWA and all sequence data have been submitted to the EMBL database under accession number AM184164. Sequence analyses revealed that the upstream gene of yjjQ is yjjP, encoding a putative outer-membrane protein. Between yjjQ and yjjP, a 557 bp non-coding region is present which may harbour promoter and regulator elements for yjjQ as predicted by the Neural Network Promoter Prediction software. The gene immediately downstream of yjjQ is the regulator gene bglJ. BglJ, when constitutively expressed, activates expression of the β -glucoside operon, bgl (Giel *et al.*, 1996; Madhusudan *et al.*, 2005). The 5' end of bglJ shows a 42 bp overlap with the 3' end of yjjQ. In the yjjQ mutant strain M18E10, the Mini-Tn5 transposon was inserted 358 bp downstream of the yjjQ start codon.

The 726 bp yjjQ nucleotide sequence of *E. coli* IMT5155 is highly similar to that of *E. coli* strains MG1655, UPEC

CFT073 (O6:H5) and EHEC EDL933 (O157:H7), and *Shigella flexneri* (98–99%) (Blattner *et al.*, 1997; Hayashi *et al.*, 2001; Welch *et al.*, 2002), while there is lower similarity to that of *Salmonella* Typhimurium (67%) (McClelland *et al.*, 2001). Analysis of the amino acid sequence suggested that yjjQ encodes a putative protein of 242 amino acids which harbours a helix–turn–helix (HTH) DNA-binding domain shared by all regulators of the LuxR family, suggesting that yjjQ encodes a transcriptional regulator protein.

Transcription of yjjQ is induced in M9 medium and mutant M18E10 shows reduced growth in this medium

To further characterize this putative regulator, we studied its expression under different environmental conditions, expecting that these results would provide clues for further functional analysis. Two fragments of the putative 5' regulatory promoter region of yjjQ were cloned and fused with promoterless lacZ, resulting in P1_{yjjQ}::lacZ, which encodes a fragment from –398 to +28, relative to the translation start, and P2_{yjjQ}::lacZ, encoding a fragment from –660 to +28 of the yjjQ promoter region. Expression analysis of transformants of strain IMT5155 lacZ[–] carrying these two fusions revealed that P1_{yjjQ}::lacZ expression was approximately two times higher than that of P2_{yjjQ}::lacZ under all conditions tested, including LB, M9, LB with 4% NaCl, LB at pH 5, and LB with 500 μ M H₂O₂ ($P<0.01$). These results suggested the existence of a negative regulatory element for yjjQ expression within the –398 and –660 region. Analysis of the promoter activity directed by P1_{yjjQ} showed that the activity was enhanced about twofold in M9 (2200 units) compared to LB medium (1000 units) ($P<0.01$).

As the transcription of yjjQ was shown to be upregulated in M9 medium, we further compared growth of the M18E10 mutant and wild-type in this medium. Interestingly, the mutant grew extremely slowly in M9 with 0.4% glucose as the sole carbon source. While the wild-type reached an OD₆₀₀ of 1.7 after 7 h, M18E10 reached an OD₆₀₀ of only 0.2 after the same period of time (Fig. 2). In contrast, the growth rates of wild-type and mutant strain in LB medium were nearly the same, with the mutant showing slightly slower growth during the exponential phase (data not shown). Since the transposon-insertion mutation could not be complemented, we constructed a yjjQ deletion mutant. The phenotypes of the wild-type strain IMT5155, the transposon-insertion mutant strain M18E10 and the yjjQ deletion mutant KEC363 (Table 1) were compared. As expected, the yjjQ-deleted mutant KEC363 could hardly grow in M9, like the original mutant M18E10 (Fig. 2). To rule out the possibility that the insertion of the mini-Tn5 exerts a polar effect on the downstream gene bglJ, we further constructed the bglJ deletion mutant KEC361 and BglJ-overexpressing strain IMT5155P3. No difference of growth in M9 was observed between these two new strains

Fig. 2. Growth of bacterial strains in M9. *In vitro* growth phenotypes of IMT5155 wild-type (□), M18E10 (△), KEC363 (×), KEC361 (◇) and IMT5155P3 (◆) in M9 medium with glucose as the sole carbon source; the values presented are the means and standard errors (not shown where smaller than symbols) of at least three independent experiments. KEC361 and IMT5155P3 grew as well as the wild-type in M9 (group 1), while M18E10 and KEC363 could hardly grow in M9 (group 2). The differences between group 1 and group 2 were significant ($P < 0.01$).

and the wild-type strain. These results corroborate our conclusion that the phenotypic changes in the mutants were due to loss of *yjjQ* function and not to effects on the downstream overlapping sensor-regulatory BglJ system (Fig. 2).

Proteins are differentially expressed in M9 medium in *yjjQ* mutants compared to the wild-type

Although YjjQ had been identified as a putative transcriptional regulator, the genes that it might regulate were not known. We therefore determined whether expression of

any protein was affected by loss of YjjQ using SDS-PAGE analysis of total proteins. Both the wild-type and mutant strain M18E10 were grown overnight in LB medium (to the late stationary phase) and proteins were extracted and subjected to SDS-PAGE. Three different bands were observed between the wild-type and the mutant grown in LB, indicating that YjjQ possibly influenced the expression of some proteins in the stationary phase (Fig. 3a). Due to the fact that growth differences between M18E10 and the wild-type strain were most obvious in M9 medium with glucose as the sole carbon source, extracted proteins from both strains grown in M9 were also examined by SDS-PAGE. In this analysis more than 10 differing protein bands were seen between wild-type and mutant strain, including ones representing both upregulated and down-regulated proteins (Fig. 3a). Furthermore, the transposon-insertion mutant M18E10 and the *yjjQ* deletion mutant KEC363 showed a similar expression profile when grown in M9 (Fig. 3b). The expression profiles of the *bglJ* deletion mutant KEC361, BglJ-overexpressing strain IMT5155P3 and the wild-type grown in M9 were also compared; no obvious difference was detected (results not shown). These results suggest that the observed differences between wild-type and mutant strain grown in M9 were indeed caused by mutation of the *yjjQ* gene.

To identify gene products possibly regulated by YjjQ, high-resolution 2D gel electrophoresis was performed. Wild-type and mutant strain M18E10 were again cultured in M9 minimal broth and proteins were extracted as described in Methods. We initially used either broad-range immobilized pH gradient (IPG) strips (pH 3.0–10.0) or narrow-range IPG strips (pH 4.0–7.0) and found that with the pH 4.0–7.0 strips several differential protein spots were missing. Therefore, pH 3.0–10.0 IPG strips were chosen for isoelectric

Fig. 3. *E. coli* protein expression is regulated by YjjQ at the stationary phase and during growth in M9 medium. (a) Lanes: 1 and 2, protein patterns of IMT5155 wild-type generated from overnight cultures in LB and M9 minimal medium, respectively; 3 and 4, protein patterns of mutant M18E10 generated from overnight cultures in LB and M9 minimal medium, respectively. Three different bands between 37 and 50 kDa were observed between the wild-type and the mutant grown in LB overnight and more than 10 different bands between 20 and 50 kDa were observed between the wild-type and the mutant grown in M9. (b) Protein patterns of IMT5155 wild-type (lane 1), mutant M18E10 (lane 2), mutant KEC363 (lane 3) generated from M9 minimal medium. No difference was observed between mutant M18E10 and mutant KEC363.

focusing in the following procedures. For each independent experiment duplicate analytical gels and one preparative gel were generated for protein samples from the wild-type and mutant strain obtained from the same cultures. Only proteins which were reproducibly and clearly found to be relatively increased or decreased in abundance based on their intensity of the protein spots were selected. In that way 16 protein spots were found to be downregulated and 9 to be upregulated at least threefold in the YjjQ mutant (Fig. 4) and were subjected to further analyses by nanoLC-ESI-MS(n). Two of the spots (14 and 25) possessed only low identification scores, while for two others (16 and 18) MS analysis did not yield an unequivocal identification. Among the remaining 21 protein spots, 14 were consistently enriched from the wild-type and 7 from the mutant strain (Table 3).

Proteins regulated by YjjQ

The MS-MS data were searched against the NCBI nr protein database using the Mascot search engine. Among the wild-type-enriched spots, four were found to represent proteins involved in iron uptake (spots 1–4), indicating a notable role of YjjQ in the utilization of this metal (Table 3): SitA is

a periplasmic iron-binding protein which is encoded in a four-member operon that mediates manganese and iron transport (Hantke, 2001; Sabri *et al.*, 2006; Zhou *et al.*, 1999). The second downregulated protein, ChuA, is involved in haem uptake in EHEC, UPEC, APEC (Li *et al.*, 2005; Torres & Payne, 1997; Welch *et al.*, 2002) and several other bacterial pathogens. The TonB-dependent outer-membrane protein FyuA is encoded by the ferric yersiniabactin uptake system (Pelludat *et al.*, 1998), and the putative function of IreA is a siderophore receptor associated with ExPEC (Russo *et al.*, 2001). SitA, ChuA and FyuA were identified as unique protein spots in wild-type IMT5155 cultured in M9 medium, while IreA was approximately 10 times more abundant in this strain in comparison to mutant M18E10.

Several enzymes involved in amino acid synthesis were also identified as proteins repressed in the *yjjQ* mutant. TyrB and AroA are related to synthesis of aromatic amino acids (Ingraham *et al.*, 1987), with the former representing an aminotransferase that is involved in the last step of biosynthesis of both tyrosine and phenylalanine. The 5-enolpyruvylshikimate-3-phosphate synthase AroA is a key

Fig. 4. Comparison of 2D protein profiles from IMT5155 wild-type (a) and mutant M18E10 (b) grown in M9 minimal medium. A total of 25 spots were identified to differ at least 2.5-fold in expression. Nine were upregulated (arrows), and 16 were downregulated (circles) in mutant M18E10.

Table 3. Proteins regulated by YjjQ as identified by proteomic analysis

Spot no.	No. of matched peptides	pI observed	Mol. mass observed	Functional category and gene function	Gene	Differential abundance*
Induction						
1	60	7.7	35.1	Periplasmic iron-binding protein	<i>sitA</i>	Unique
2	68	4.5	70.9	Outer-membrane haem/haemoglobin receptor	<i>chuA</i>	Unique
3	23	6.5	75.3	Putative siderophore receptor	<i>ireA</i>	0.11
4	42	4.9	70.7	Outer-membrane protein, TonB-dependent	<i>fyuA</i>	Unique
5	56	6.0	46.6	Aromatic aminotransferase: tyrosine synthesis	<i>tyrB</i>	0.38
6	42	6.0	67.6	Amino acid biosynthesis: isoleucine, valine	<i>ilvD</i>	0.19
7	81	6.3	36.1	Amino acid biosynthesis: lysine	<i>dapD</i>	Unique
8	45	6.1	50.7	Amino acid biosynthesis: chorismate	<i>aroA</i>	0.33
9	45	4.7	74.1	Chaperone Hsp70	<i>dnaK</i>	0.32
10	66	5.4	9.6	Energy metabolism: anaerobic respiration	<i>yfiD</i>	0.18
11	72	5.1	13.0	Energy metabolism: anaerobic respiration	<i>yfiD</i>	0.119
12	33	5.3	30.8	Transcriptional regulator	Ecs1602	0.33
13	34	5.6	55	LPS core region biosynthesis	<i>rfaE</i>	0.40
14	6	7.5	40.5	OmpT, outer-membrane protein 3B	<i>ompT</i>	0.31
15	82	7.4	35	Glutamate/aspartate periplasmic binding protein	<i>ybeJ</i>	Unique
16	No match	5.2	62.5	Unknown	No hint	0.19
Repression						
17	107	4.8	97.8	H5 flagellin	<i>fliC</i>	Unique
18	17	5.2	54.2	Enzyme: surface polysaccharides and antigens	<i>lpxD</i>	4.51
19	66	6.9	45.9	Isocitrate lyase	<i>aceA</i>	4.02
20	119	6.3	55.7	Outer-membrane channel	<i>tolC</i>	3.12
21	16	5.9	73.2	2-hydroxy-3-oxopropionate reductase	<i>garR</i>	5.70
22	16	6.1	34.4	Phosphoenolpyruvate carboxykinase	Gi:1942693	3.05
23	54	6.1	72.1	Lysine biosynthesis	<i>asd</i>	Unique
24	10	4.7	9.7	Unknown	<i>yjgF</i>	3.44
25	2	5.1	73	Hypothetical protein	None	3.15

*Values are means of triplicate independent experiments.

enzyme in the aromatic amino acid biosynthetic pathway in micro-organisms. 2D signals of both enzymes from wild-type were about threefold stronger than those of mutant M18E10. Similarly, IlvD (dihydroxy acid dehydratase), which catalyses dehydration reactions of α,β -dihydroxy acids to yield the α -ketoacid precursors of isoleucine and valine (Ingraham *et al.*, 1987), was shown to be about five times more abundant in the wild-type compared to the mutant strain. Succinyltransferase DapD was targeted as a unique protein spot from the wild-type. This enzyme is involved in the succinylase pathway synthesizing DL-DAP, which is the direct precursor of L-lysine and an important constituent of the cell wall peptidoglycan (Fuchs *et al.*, 2000). To check whether addition of amino acids would improve the growth of mutants in M9, 0.4% Casamino acids (Sigma) was added. Indeed, the growth of both mutant strains M18E10 and KEC363 recovered to the level of wild-type in M9 with Casamino acids.

In addition, several proteins involved in molecular chaperoning (DnaK) (Slepenkov & Witt, 2002), acid tolerance response (YfiD) (Stancik *et al.*, 2002), LPS core region biosynthesis (RfaE) (Valvano *et al.*, 2000), transcriptional

regulation and other functions were identified. Interestingly, YbeJ, a putative glutamate/aspartate periplasmic binding and transport protein (Tremoulet *et al.*, 2002) was represented by one of the five unique spots from the wild-type. While most of the identified proteins migrated as single spots on 2D gels, YfiD possessed two spots with different abundances less than 0.2, consistent with a wild-type bias for this protein.

Among the nine mutant-enriched protein spots, two were found to be unique for the *yjjQ* mutant, suggesting that these proteins may be repressed by YjjQ (Table 3). Two of them are directly or indirectly linked to amino acid synthesis: Asd is an aspartate-semialdehyde dehydrogenase involved in lysine biosynthesis and was identified as a unique spot in mutant M18E10. The abundance of YjgF in M18E10 was about 3.4 times greater than that in the parental strain; this protein is involved in isoleucine biosynthesis (Schmitz & Downs, 2004).

LpxD is an acyltransferase participating in lipid A biosynthesis while AceA is an isocitrate lyase (Rieul *et al.*, 1988; Vaara & Nurminen, 1999). Both LpxD and AceA were more than four times more abundant in mutant M18E10 when compared to the wild-type. TolC is a membrane channel

tunnel protein involved in enterobactin transport and multi-drug efflux (Bleuel *et al.*, 2005). This protein serves as an outer-membrane channel, and it was three times more abundant in mutant M18E10. Finally, FliC was identified as a further unique protein spot in mutant M18E10. The flagellar filament is composed of a single protein flagellin and most *E. coli* have a flagellin gene *fliC* (Wang *et al.*, 2003). However, when motility of mutant strains M18E10 and KEC363 and the wild-type was compared in LB medium with 0.3 % agar, no difference was observed. This suggests that the regulation of *fliC* by *yjjQ* might occur only under specific conditions.

Expression of iron-uptake genes is downregulated at the level of transcription

Because YjjQ is a putative transcriptional regulator, the regulation of the targeted iron-associated genes was confirmed by real-time RT-PCR at the level of transcription. Since the *yjjQ* mutant grew poorly in M9 medium and the cell density may be not sufficient to exhaust all the iron present in the medium, the iron-regulated genes may not be induced. To avoid overinterpreting the results of proteomic analysis, the total RNA was isolated from *yjjQ* transposon-insertion mutant strain M18E10, deletion mutant strain KEC363 and wild-type strain cultured in LB depleted of iron, instead of M9. Results of real-time RT-PCR showed that expression of all of four identified iron-uptake genes, *ireA*, *fyuA*, *chuA* and *sitA*, was downregulated in both transposon-insertion mutant strain M18E10 and *yjjQ* deletion mutant strain KEC363 when cultured in LB without iron. The maximum downregulation was observed for *chuA* (10.6-fold in M18E10 and 9.7-fold in KEC363); while the expression of *fyuA* was downregulated only 4.6-fold in strain M18E10 and 4.3-fold in strain KEC363. The mRNA abundances of *sitA* and *ireA* in the mutant M18E10 strain were about 6.6-fold and 5.4-fold, respectively, lower than those in the wild-type. Similarly, the mRNA abundances of *sitA* and *ireA* in strain KEC363 were 6.2 and 4.9-fold lower than those in the wild-type. Statistical significance was calculated on $\Delta\Delta C_t$ values (Schmittgen *et al.*, 2000) with a paired *t* test, and *P* values were all <0.0001. To determine if the downstream overlapping gene *bglJ* plays any role in the regulation of iron-uptake genes, we also compared the mRNA level of *ireA*, *fyuA*, *chuA* and *sitA* in *bglJ* deletion mutant KEC361, BglJ-overexpressing strain IMT5155P3 and the wild-type strain. No significant difference was found.

DISCUSSION

The *yjjQ* gene encodes a putative regulator, sharing sequence identities with members of the LuxR family of regulators. In the present study, YjjQ mutants were characterized. The transposon-insertion *yjjQ* mutant of APEC strain IMT5155 is attenuated and in a 5-week-old chicken model it colonizes poorly all chicken organs tested. Expression studies revealed

that transcription of *yjjQ* was upregulated when bacteria were grown in M9, and growth of both the $\Delta yjjQ$ mutant and the insertion mutant was reduced in M9 medium. Proteomic analysis revealed that the iron-uptake receptors SitA, FyuA, ChuA, and IreA were downregulated in the *yjjQ* mutant. Furthermore, their regulation occurs at the transcription level, as confirmed by real-time RT-PCR. We believe that this is the first characterization of a *yjjQ* mutant with defects in APEC virulence, apparently due to impaired iron uptake.

It was not possible to do a *yjjQ* complementation test, although great efforts were made. Fragments of *yjjQ* and *yjjQ-bglJ* were cloned into plasmid pKESK22 under control of promoter P_{tac} and plasmid pBAD18-Cm under control of promoter P_{BAD} . No evidence was found by SDS-PAGE that YjjQ was expressed when the plasmids thus obtained were transformed into transposon-insertion *yjjQ* mutant M18E10 and deletion mutant KEC363 respectively and then induced by arabinose or IPTG. Correspondingly, no *trans*-complementation of phenotypes was observed in *in vitro* tests in these plasmid-complemented mutant strains. The same was true when *yjjQ* and *yjjQ-bglJ* with their putative promoters (including 400 bp upstream sequence) were cloned into a low-copy plasmid derived from pACYC184. The failure of the complementation might be due to a clone/expression problem with *yjjQ*. Because the *in vitro* results were ambiguous, the *in vivo* virulence of those strains was not checked. To rule out a polar effect of the transposon insertion or another possible mutation, a *yjjQ* deletion mutant, a deletion mutant in the immediately downstream gene *bglJ* and a BglJ overexpression strain were generated. *In vitro* phenotypes such as growth in M9, protein expression profile and mRNA abundance of iron-uptake receptors were checked and none of the results hinted at any polar effect or a second mutation. Giel *et al.* (1996) previously showed in *E. coli* K-12 that a Tn10 insertion in *yjjQ* caused BglJ to be constitutively expressed, thus activating expression of the β -glucoside operon, *bgl*. However, deletion of the downstream gene *bglJ* from the transposon-insertion mutant M18E10 (IMT5155 *yjjQ::mini-Tn5 $\Delta bglJ::Cm$* or M18E10 *$\Delta bglJ::Cm$*) did not abolish the phenotype of defective growth in M9 (data not shown). These results corroborate our conclusion that the phenotypic changes in the mutants were probably due to loss of *yjjQ* function.

Insertional mutation of *yjjQ* led to virulence attenuation of APEC IMT5155 *in vivo* and showed that mutant M18E10 was attenuated 30- to 1000-fold in internal organs. Likewise, individual tests demonstrated that the bacterial loads of the mutant in each organ were much lower than that of the wild-type. Among the proteins that were shown to be repressed or downregulated in mutant strain M18E10, four are parts of previously described iron-uptake systems and their regulation by YjjQ has been confirmed at the transcription level using real-time RT-PCR. The acquisition of iron is crucial for bacterial survival and infection, since this metal is necessary for a series of cellular functions and

the concentration of free iron is exceedingly low in host tissues. Therefore bacteria have developed several strategies to sequester this essential element (Perkins-Balding *et al.*, 2004) and iron-acquisition systems have been associated with bacterial virulence especially for bacteria causing septicaemia (Janakiraman & Schlauch, 2000; Li *et al.*, 2005; Russo & Johnson, 2000; Schaible & Kaufmann, 2004; Torres & Payne, 1997). One of the downregulated genes, *sitA*, is part of the four-member *sit* operon which is included within SPI1 of *Salmonella* Typhimurium and is believed to be located on a pathogenicity island in *Shigella flexneri* as well (Runyen-Janecky *et al.*, 2003; Zhou *et al.*, 1999). Previous studies have shown that the *Sit* system is required for full virulence of *Salmonella* Typhimurium and that a *sitB* mutant of APEC strain IMT5155 is markedly attenuated for survival in the chicken as demonstrated by *in vivo* competition assays (Janakiraman & Schlauch, 2000; Li *et al.*, 2005). Only recently, Sabri *et al.* (2006) demonstrated that, unlike other *SitABCD* metal-type transporters identified in *Salmonella* Typhimurium and *Shigella flexneri*, the *sitABCD* operon of APEC strain χ 7122 is located on a virulence plasmid and not only mediates transport of iron and manganese but also is involved in oxidative stress defence of this pathogen. The *sitABCD* operon was also identified on large plasmids in two other APEC strains of serotypes O1 and O2 (Johnson *et al.*, 2006b, c), and moreover IMT5155 was confirmed to harbour two copies (chromosomal and plasmid) of particular operons (unpublished data from our laboratory). Duplication of the *sit* operon in this pathogen may hint at an enhanced capacity to transport manganese or iron. The particular relevance of this metal-acquisition locus for APEC is furthermore displayed by its high prevalence (Ewers *et al.*, 2007), and the fact that this system is more commonly detected in clinical isolates than in strains from healthy poultry or environmental samples (Rodriguez-Siek *et al.*, 2005a, b). Likewise, the second downregulated gene encoding the membrane receptor for yersiniabactin, *FyuA*, is significantly more often detected in outbreak isolates than in isolates from healthy layers (Rodriguez-Siek *et al.*, 2005a, b). The third protein identified and belonging to this category is *ChuA*, which is an outer-membrane acceptor involved in haem uptake in a series of bacterial pathogens, e.g. EHEC, UPEC, *Neisseria meningitidis* and *Shigella dysenteriae* (Mills & Payne, 1995; Torres & Payne, 1997; Welch *et al.*, 2002). The ability to use haem/haemoglobin as iron source has been implicated as an important determinant of virulence and it is assumed that these compounds provide the requisite amounts of iron necessary for the expression of virulence factors such as haemolysins, proteases or cytotoxins (Lee, 1995). Furthermore, *ChuA* has been confirmed as playing a role in the pathogenesis of APEC (Li *et al.*, 2005) and 52.1 % out of 436 APEC strains were shown to harbour this gene (Ewers *et al.*, 2007). The fourth identified protein, *IreA*, is a novel iron-responsive element first identified in ExPEC and its amino acid sequence has 48–56 % similarity to that of previously identified siderophore receptors. Its expression was upregulated extensively in human urine, in human ascites and in human blood relative to expression in

LB medium (Russo *et al.*, 2001) and it was recently identified in a novel pathogenicity island in an APEC strain (Kariyawasam *et al.*, 2006b). The *ireA* gene is predominantly present in APEC rather than in avian commensal *E. coli* (Kariyawasam *et al.*, 2006a).

The master regulator of bacterial iron homeostasis is *Fur*, which can directly bind to the promoter region of iron-regulated genes with ferrous iron as a cofactor, thus repressing the expression of these genes (Hantke, 2001). In certain cases, *Fur* indirectly regulates iron-uptake systems by regulating three types of activators: two-component signal transduction systems, *AraC*-like regulators of the synthesis of siderophores and their uptake systems, and extracytoplasmic function (ECF) sigma factors (Hantke & Braun, 1998). Our results showed that *YjjQ* probably regulates iron-uptake receptors *SitA*, *FyuA*, *ChuA* and *IreA*, and its regulation has been confirmed at the transcription level by real-time RT-PCR. Whether *YjjQ* plays a role in *Fur* regulation or vice versa requires further study.

Several proteins with other functions were identified by proteomic analysis, such as transcriptional regulation, LPS and outer-membrane protein biosynthesis, energy metabolism, molecular chaperoning and amino acid synthesis. The putative regulatory function of *YjjQ* in those phenotypes requires further study. EHEC, UPEC, *Shigella* and *Salmonella*, as well as APEC, also contain a *yjjQ* gene. Future studies will hopefully lead to a more comprehensive understanding of *YjjQ* pathogenesis in these important human pathogens.

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