UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Análise epidemiológica de cepas APEC e Análise do regulador FNR na modulação da virulência de ExPEC.

Tese de Doutorado

Nicolle Lima Barbieri

Porto Alegre, março 2014

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Nicolle Lima Barbieri

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS, como parte dos requisitos para obtenção do título de Doutora em Ciências.

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a meus pais, meu irmão e

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Resumo

Escherichia coli é um bacilo Gram-negativo, anaeróbico facultativo e de distribuição cosmopolita. *E. coli* coloniza o intestino de humanos e outros animais endotérmicos logo após o nascimento, estabelecendo-se como um importante membro da microbiota intestinal. Algumas cepas de *E. coli* podem adquirir fatores de virulência, assumindo assim, uma natureza patogênica, como é o caso das *E. coli* patogênicas extraintestinais (ExPEC). As cepas ExPEC apresentam a capacidade de colonizar e se disseminar em diversos nichos no hospedeiro, e são divididas em UPEC (*E. coli* uropatogênica), NMEC (*E. coli* causadora de meningite neonatal) e APEC (*E. coli* patogênica para aves). UPEC, NMEC e APEC compartilham fatores associados à virulência. Para serem aptas a causar doença, cepas ExPEC devem apresentar pelo menos um fator associado à adesão, um fator para captação de ferro (sideróforo) e um fator de resistência ao soro, podendo, também, apresentar genes que codificam toxinas e invasinas. Embora sejam conhecidos muitos fatores de virulência associados à patogenicidade de cepas ExPEC, a regulação da expressão de tais fatores ainda não foi elucidada. **F**umarato-**n**itrato-**r**edutase (FNR) é uma proteína que atua como regulador global, agindo como um sensor da presença de oxigênio em bactérias gram-negativas. Já foi demonstrado que FNR está relacionada à regulação da virulência de bactérias patogênicas como *Shigella flexneri* e S*almonella enterica* serovar Typhimurium. Este trabalho teve como objetivo a análise epidemiológica e caracterização de cepas APEC, bem como a investigação do controle da expressão de fatores associados à virulência de ExPEC pelo regulador global FNR.

Os resultados da análise epidemiológica das cepas APEC mostram o perfil de resistência aos agentes antimicrobianos, a prevalência dos fatores de virulência e dos grupos filogenéticos (de acordo com a classificação EcoR) e a relação filogenética dos isolados, fornecendo um panorama da caracterização de *E. coli* patogênicas aviárias de lesões severas de celulite e de infecção sistêmica oriundas da região sul do Brasil.

Em relação ao FNR, este estudo mostrou a influência deste regulador sobre importantes fatores associados à virulência, estando envolvido no controle de várias etapas do estabelecimento da infecção por cepas ExPEC. A deleção de *fnr* na cepa UPEC CFT 073 reduziu a motilidade, a expressão das fimbrias tipo I e tipo P, reduziu a expressão da hemolisina e controlou a expressão da ilha de patogenicidade do α-cetoglutarato. Além disso, a deleção de *fnr* fez tornou as bactérias incapazes de invadir células dos rins e da bexiga e de causar doença *in vivo* em camundongos de 6 semanas. FNR também foi capaz de controlar as etapas da infecção por NMEC 56. Uma vez deletado, as bactérias perderam a capacidade de causar bacteremia, de crescer no fluido cerebrospinal e de causar doença *in vivo* em ratos de 5 dias de idade. A deleção de *fnr* em APEC O1 resultou na diminuição da expressão da proteína OmpT plasmidial, da fímbria do tipo I e do auto-transportador AatA.

A principal contribuição deste trabalho foi demonstrar que FNR atua na regulação da expressão de importantes fatores associados à virulência de cepas ExPEC (UPEC, NMEC e APEC), sendo importante para o estabelecimento da infecção por essas cepas. Neste trabalho, verificamos que, além da função já conhecida de regular os genes envolvidos na manutenção de um meio anaeróbico, FNR também atua no controle de genes associados à virulência de cepas ExPEC, refletindo na capacidade de causar doença que tais cepas apresentam.

Uni-termos: *Escherichia coli*; APEC; NMEC; UPEC; FNR; regulador global; colibacilose; celulite; colissepticemia; suscetibilidade a antimicrobianos; fatores de virulência; ARDRA; análise filogenética.

Abstract

Escherichia coli is a Gram-negative bacillus, facultative anaerobic and has cosmopolitan distribution. *E. coli* colonizes the intestine of humans and other endothermic animals immediately after birth, establishing as an important member of the intestinal microbiota. Some strains of *E. coli* can acquire virulence factors thereby assuming a pathogenic nature, as in the case of extraintestinal pathogenic *E. coli* (ExPEC). ExPEC strains have the ability to colonize and spread out in different niches of the host, and are divided into UPEC (uropathogenic *E. coli*), NMEC (newborn meningitis *E. coli*) and APEC (avian pathogenic *E. coli*). UPEC, NMEC and APEC share virulence factors. To be able to cause disease, ExPEC strains must produce virulence factors required for adherence, for iron uptake (siderophore) and for resistance to serum and may also contain genes encoding toxins and invasins. Although many virulence factors associated with the pathogenicity of ExPEC strains are known, the regulation of the expression of these factors has not yet been fully elucidated. Fumarate nitrate reductase (FNR) is a global regulatory protein, acting as a sensor of oxygen in Gram- negative bacteria. It has been shown that FNR relates virulence of pathogenic bacteria such as *Shigella flexneri* and *Salmonella enterica* serovar Typhimurium. The aim of this study was to do an epidemiological analysis and characterization of APEC strains as well as the investigation of regulation of ExPEC's virulence factors by the global FNR regulator.

The results of epidemiological analysis of APEC strains showed the profile of antimicrobial resistance , the prevalence of virulence factors and phylogenetic groups (according to the EcoR group) and the phylogenetic relationship of the isolates, providing an overview of the characterization of avian pathogenic *E. coli* causing severe cellulitis lesions and systemic infection originating from southern Brazil.

In relation to FNR, this study showed the influence of this important regulator of virulence factors that is involved in controlling various stages of establishment of infection by ExPEC strains. Deletion of *fnr* in UPEC strain CFT 073 reduced motility and expression of type I and type P fimbriae, reduced the expression of hemolysin and control the expression of the pathogenicity island of α -ketoglutarate. Furthermore, *fnr* mutant strains were unable to invade cells of kidney and bladder, and to colonize the urinary tract of 6 weeks-old mice. FNR was also able to control the stages of infection of NMEC 56. The *fnr* mutant lost its ability to cause bacteremia, grow in cerebrospinal fluid, cause disease in 5 days old rats. Deletion of *fnr* in APEC O1 resulted in decreased expression of genes corresponding to the plasmid encoded OmpT protein, type I fimbriae and autotransporter AatA.

The main contribution of this work was to demonstrate that FNR regulates expression of important virulence factors of ExPEC strains (UPEC, NMEC and APEC), which is important for the establishment of infection by these strains. In this work, we found that, besides the already known function in regulating genes involved in maintaining an anaerobic environment, FNR also acts in the control of virulence-associated genes of ExPEC strains, reflecting the ability of these strains to cause disease.

Key-words: avian pathogenic *Escherichia coli*; APEC; NMEC; UPEC; FNR; global regulator; colibacillosis; antibiotic susceptibility; virulence factors; phylogenetic grouping; ARDRA

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1. INTRODUÇÃO

Escherichia coli é um bacilo Gram-negativo, anaeróbico facultativo, em forma de bastonete curto e de distribuição cosmopolita. *E. coli* coloniza o intestino de humanos e outros animais endotérmicos logo após o nascimento, estabelecendo-se como um importante membro da microbiota intestinal. Essas cepas, conhecidas como comensais, raramente causam doença, exceto em pessoas imunodeprimidas ou quando as barreiras gastrintestinais são rompidas (Kaper *et al.*, 2004).

Algumas cepas de *E. coli* podem divergir das cepas comensais e assumir uma natureza patogênica ao adquirirem fatores de virulência (Yan and Polk, 2004). Esses fatores conferem à bactéria habilidade de causar doenças e são encontrados em elementos genéticos que podem estar presentes em diferentes combinações. Somente cepas com persistentes e importantes combinações de tais fatores de virulência causam doença em indivíduos saudáveis (Kaper *et al.*, 2004). De maneira geral, esses fatores de virulência devem prioritariamente permitir que a bactéria (1) se adapte e colonize eficientemente novos nichos no hospedeiro, (2) sobreviva e se multiplique no hospedeiro, (3) escape dos mecanismos de defesa do hospedeiro, e, finalmente, (4) seja transmitida para um novo hospedeiro suscetível, o que, em alguns casos, significa possuir aparatos para sobreviver em um ambiente externo antes de encontrar o novo hospedeiro (Falkow, 1991).

Escherichia coli podem ser divididas em três grandes grupos, de acordo com o local de onde foram isoladas, com a sintomatologia clínica e com a presença de genes de virulência. O primeiro grupo é constituído das cepas de *E. coli* comensais, que estão presentes na microbiota normal de humanos, mamíferos e aves saudáveis, e que coexistem pacificamente com o seu hospedeiro (Russo and Johnson, 2000).

O segundo grupo é constituído pelas cepas de *E. coli* enteropatogênicas, raramente encontradas na microbiota normal de animais saudáveis, que causam gastroenterites e colites quando ingeridas em quantidades suficientes. Os patotipos capazes de causar essas infecções incluem: EPEC (*E. coli* enteropatogênica), ETEC (*E. coli* enterotoxigênica), EIEC (*E. coli*

enteroinvasiva), EHEC (*E. coli* entero-hemorrágica), EAEC (*E. coli* enteroagregativa), DAEC (*E. coli* difusamente aderente), AIEC (*E. coli* aderente e invasiva), STEC (*E. coli* produtora de shigatoxina) (Russo and Johnson, 2000).

O terceiro e último grupo é constituído pelas cepas de *E. coli* que causam infecções extraintestinais (Ex-PEC, *E. coli* patogênica extra-intestinal). Neste grupo encontram-se as cepas UPEC (*E. coli* uropatogênica), NMEC (*E. coli* de meningite neonatal) e APEC (*E. coli* patogênica para aves) (Kaper, 2005).

1.1. Escherichia coli patogênica extraintestinal (ExPEC)

Escherichia coli patogênicas extraintestinais (ExPEC) apresentam a capacidade de colonizar e se disseminar em diversos nichos no hospedeiro. Em humanos, podem causar doenças quando presentes no sangue, no sistema nervoso central e no trato urinário; já em aves, podem causar doenças localizadas ou sistêmicas. Apesar disso, essas cepas são capazes de permanecer no intestino de forma assintomática sem causar dano ao hospedeiro (Wiles *et al.*, 2008).

Enquanto os patotipos entéricos de *E. coli* podem ser definidos pelos seus fatores de virulência ou pelo seu sorogrupo, as cepas classificadas como ExPEC compartilham vários fatores associados à virulência e não apresentam um patotipo definido, não estando estabelecidos os fatores de virulência que definem uma ExPEC ou distinguem uma da outra (Johnson *et al.*, 2008; Moulin-Schouleur *et al.*, 2007; Rodriguez-Siek *et al.*, 2005).

Por causarem doenças em sítios anatômicos tão diferentes, seria de se esperar que essas cepas bacterianas possuíssem fatores de virulência específicos para cada nicho a ser colonizado. Entretanto, nenhum fator ou perfil de fatores de virulência sítio-específico foi descrito até o momento. Isso não é tão surpreendente, uma vez que os mecanismos de defesa dos hospedeiros são comuns a vários sítios extraintestinais que podem ser infectados por diferentes cepas de *E. coli* (Russo and Johnson, 2000).

1.1.1. Escherichia coli patogênica do trato urinário (UPEC)

Infecções do trato urinário (UTI) são consideradas o segundo tipo de infecção mais comum em seres humanos em todo o mundo, sobretudo em mulheres (Nicolle, 2002). Apenas nos EUA, essas infecções resultam em sete milhões de visitas a consultório médico por ano, 1 milhão de atendimentos de emergência, 100.000 internações, e US$ 3,5 bilhões em custos de saúde, um encargo significativo para o sistema de saúde (Russo and JR, 2003).

Cepas de *Escherichia coli* são os agentes causadores responsáveis por mais de 80% de todas as infecções do trato urinário (UTI), incluindo bacteriúria assintomática e infecção sintomática (Hooton and Stamm, 1997; Stamm and Norrby, 2001; Svanborg and Godaly, 1997). As cepas UPEC que causam UTI sintomática colonizam o trato urinário, podendo ascender para a bexiga e causar cistite, o que normalmente está associado aos sintomas clássicos da infecção, como dor ao urinar, cistite (micção freqüente), súbito desejo compulsivo de urinar e bacteriúria (Foxman, 2003). No entanto, se essas infecções não forem tratadas, podem ascender a partir da bexiga, por meio dos ureteres, para os rins, causando pielonefrite, podendo causar danos renais irreversíveis e mesmo morte (Scholes *et al.*, 2005). Estima-se que metade das mulheres irão sofrer pelo menos um caso de UTI durante a vida, com uma taxa de recorrência de 25% no prazo de 6 meses, sendo que 5% resultam em infecções crônicas recorrentes (Engel and Schaeffer, 1998; Foxman *et al.*, 2000; Marrs *et al.*, 2005). Em 50% destas infecções recorrentes, a mesma cepa de UPEC pode provocar tanto a infecção primária quanto as infecções reincidentes (Ikaheimo *et al.*, 1996).

O desenvolvimento de UTI depende de fatores anatômicos e da integridade dos mecanismos de defesa do hospedeiro (Nicolle, 2002). O sucesso do estabelecimento da infecção bacteriana requer a adesão bacteriana a células epiteliais do trato urinário, a colonização do epitélio da bexiga, e, em certos casos, a invasão dessas células, seguida por multiplicação intracelular, e a eventual disseminação e persistência bacteriana para outros tecidos (Bien *et al.*, 2012). Tais habilidades foram bem desenvolvidas pelas cepas UPEC com a expressão de um largo espectro de fatores de virulência (Mulvey *et al.*, 1998). Cepas UPEC codificam fatores de virulência que permitem que as bactérias colonizem o trato urinário e persistam em face da

defesa do hospedeiro altamente eficaz. Além disso, cepas UPEC apresentam um alto grau de diversidade genética devido à presença de genes de virulência especializados localizados em elementos genéticos móveis chamados ilhas de patogenicidade (Oelschlaeger *et al.*, 2002; Wiles *et al.*, 2008).

1.1.2. *Escherichia coli patogênica aviária* (APEC)

As infecções extraintestinais causadas por cepas patogênicas de *E. coli* em aves (APEC) podem manifestar-se sob a forma de infecções localizadas ou sistêmicas, e são denominadas colibacilose. A colibacilose aviária é a doença mais comum e de maior custo para a indústria aviária em animais entre 2 a 12 semanas de idade (Arné *et al.*, 2000). Entre as manifestações mais comuns de infecções por APEC estão: celulite; doença respiratória que freqüentemente culmina em septicemia e lesões no fígado, sacos aéreos e coração; síndrome da cabeça inchada; onfalite (infecção do embrião); e salpingite (infecção do oviduto) (Dho-Moulin and Fairbrother, 1999; Ngeleka *et al.*, 1996).

A avicultura brasileira é afetada sobretudo pela celulite aviária (também chamada dermatite necrótica), uma infecção subcutânea que evolui para uma lesão inflamatória supurativa que pode atingir o músculo. Nos aviários, acredita-se que lesões de celulite sejam iniciadas por arranhões feitos por outros frangos, cuja ocorrência aumenta com o aumento na densidade da população de aves. Ademais, a chance desses arranhões ocorrerem é aumentada pela seleção de aves de alta conversão alimentar (rápido ganho de peso) que, entretanto, não apresentam a proporcional cobertura de penas. Quando a celulite não culmina em septicemia fatal (Peighambari *et al.*, 1995), a lesão é detectada apenas no momento do abate, acarretando a condenação de parte ou de toda a carcaça. Na avicultura, a produção de frangos de corte tende a ser mais afetada por lesões de celulite, enquanto que a criação de matrizes e galinhas poedeiras tende a ser mais afetada por infecções sistêmicas iniciadas pelo trato respiratório e salpingite.

A colibacilose, quando iniciada no trato respiratório superior, ocorre provavelmente devido à inalação de poeira contaminada. Fatores externos ao animal, como altas concentrações de amônia no ambiente, tornam as aves

predispostas à doença pelo fato de causar danos no epitélio ciliar do trato respiratório, facilitando a entrada da bactéria. A deficiência no sistema de ventilação, a exposição a temperaturas superiores a 25 ^{o}C, a limitação do espaço para a criação e a deficiência no processo de desinfecção também contribuem para a infecção das aves por APEC e para o estabelecimento da doença, pois oferecem à bactéria um ambiente mais propício ao seu desenvolvimento (Dho-Moulin and Fairbrother, 1999; Ferreira and Knöbl, 2000). Além disso, APEC podem estar presentes na microbiota intestinal de aves sadias, podendo assim, permanecer nas criações por um longo período, contaminando os alimentos e a água, que servirão como via de disseminação da bactéria para as aves. Acredita-se que a contaminação fecal da casca do ovo é uma das vias de transmissão da doença para os pintos (Ewers *et al.*, 2009).

Após a colonização dos pulmões e sacos aéreos da ave pela bactéria, observa-se o espessamento do epitélio e a presença frequente de um exsudato caseoso na superfície do trato respiratório. Microscopicamente, as primeiras mudanças são edemas e infiltrados de heterófilos no epitélio respiratório. Fagócitos mononucleares são frequentemente encontrados 12 horas após a infecção. Após, as células fagocitárias se tornam células gigantes, comumente encontradas pelas margens das áreas necróticas, e ocorre a proliferação de fibroblastos e acumulação de heterófilos necróticos no exsudato caseoso (Horn *et al.*, 2012). As lesões de doenças respiratórias são identificadas por aumento dos folículos linfóides, hiperplasia epitelial e presença de heterófilos nos epitélios aéreos (Barnes and Gross, 1997; Ferreira and Knöbl, 2000).

A colibacilose afeta todas as etapas da produção de aves comerciais, contribuindo para a diminuição da qualidade da produção. As perdas econômicas ocorrem devido à morbidade (perda de peso, redução do crescimento e da produção de ovos), ao aumento da condenação das carcaças durante o processamento e ao aumento da mortalidade (Dziva and Stevens, 2008).

1.1.3. *Escherichia coli patogênica causadora da meningite do recém-nascido* (NMEC)

Escherichia coli causadora de meningite neonatal é a principal bactéria gram-negativa causadora de sepse e meningite em recém-nascidos, e atrás somente de *Streptococci* do grupo B (Tseng *et al.*, 2012). Meningites bacterianas são caracterizadas pela inflamação das meninges, afetando a pia-máter, a aracnóide e o espaço subaracnóide (Kim, 2008). É reconhecida como uma das dez principais causas de morte relacionadas à infecção em todo o mundo. A mortalidade é estimada em 10% nos países desenvolvidos e em 40-58% nos países em desenvolvimento.

A meningite do recém-nascido afeta 2 a cada 10.000 bebês nascidos a termo, e 2 a cada 1.000 bebês nascidos abaixo do peso, afetando principalmente bebês prematuros (45-81%). Além disso, está associada a altas taxas de mortalidade e morbidade: cerca de 30% dos bebês infectados morrem, e cerca de 50% dos bebês sobreviventes ficam com sequelas, que incluem hidrocefalia, convulsões, retardo mental, paralisia cerebral e deficiência auditiva (Johnson *et al.*, 2002; Kim, 2008; Melllata, 2013).

As infecções por NMEC normalmente são adquiridas a partir do trato genital materno, no útero ou durante a passagem pelo canal do parto. Devido a isso, essas infecções já foram correlacionadas a infecções urinárias ao longo da gestação (Melllata, 2013). Em seguida, as bactérias necessitam ultrapassar a superfície epitelial (Fagan *et al.*, 2008) para atingir a corrente sanguínea, bem como sobreviver e se multiplicar no sangue. Após atingir um nível mínimo de bacteremia, ocorre a travessia da barreira hemato-encefálica, culminando com a invasão das meninges e do sistema nervoso central (Kim, 2012; Melllata, 2013; Tseng *et al.*, 2012).

1.2 Fatores associados à virulência

ExPEC compartilham fatores associados à virulência, comuns às cepas UPEC, NMEC e APEC (Ewers *et al.*, 2007).Para serem aptas a causar doença, cepas ExPEC devem ter pelo menos um fator associado à adesão, um fator para captação de ferro (sideróforo) e um fator de resistência ao soro, podendo, também, apresentar genes que codificam toxinas e invasinas (Barbieri *et al.*, 2013). Ainda não se sabe como tais fatores são regulados. Por outro lado, cepas ExPEC podem carecer de um ou mais desses fatores e ainda assim serem patogênicas. Excetuando a fímbria F1, encontrada na maioria das cepas de *E. coli*, os genes que codificam fatores associados à virulência em geral estão ausentes nos genomas de cepas não-virulentas de *E. coli* (Dozois *et al.*, 2003), sendo frequentemente encontrados em elementos genéticos móveis (via transferência horizontal de transposons, plasmídeos, bacteriófagos e ilhas de patogenicidade) (Johnson *et al.*, 2006; Mellata *et al.*, 2009; Tivendale *et al.*, 2009). Dessa forma, parecem existir estratégias comuns que tornam a bactéria um patógeno eficiente. Os fatores de virulência já descritos para ExPEC serão comentados brevemente a seguir.

1.2.1 *Adesinas*

A aderência da bactéria às células do hospedeiro é um passo importante para a colonização e ocorre nos primeiros momentos da interação bactéria-hospedeiro. *E. coli* possuem fatores de adesão específicos, que lhes permitem colonizar sítios em seu hospedeiro que provavelmente não seriam colonizados na falta desses fatores (Kaper *et al.*, 2004).

As estruturas que permitem a adesão da bactéria ao hospedeiro podem ser fimbriadas ou afimbriadas. Na maioria das vezes, as adesinas formam estruturas morfologicamente distintas, chamadas fímbrias ou pili. A adesão ao hospedeiro também pode ser realizada por proteínas da membrana externa da bactéria (La Ragione *et al.*, 2000).

As fímbrias F1 são filamentos de 1-2 µm de comprimento, presentes na superfície bacteriana, cuja extremidade contém uma lectina (FimH) de

reconhecimento de manose, que promove a aderência e a colonização bacteriana à superfície das mucosas. Já a fímbria P possui uma extremidade que reconhece receptores glicolipídicos presentes em eritrócitos e células renais (Wullt *et al.*, 2000; Yamamoto, 2007). Por sua vez, o Curli é uma adesina formada por uma estrutura enrolada encontrada na superfície externa da maioria das cepas de *E. coli,* e codificada pelo gene *crl* (La Ragione *et al.*, 2000).

1.2.2 *Resistência ao Soro*

Após a adesão à mucosa, as ExPEC devem resistir ao efeito bactericida do soro e evadir dos sistemas fagocíticos de defesa do hospedeiro. A resistência é conferida por uma conjunção de fatores, combinando os efeitos do LPS (lipopolissacarídeo), a produção de aerobactina, a presença da cápsula K, do antígeno O e de proteínas de membrana (Wooley *et al.*, 1992), como Iss, (*increased serum survival*), uma lipoproteína da membrana externa bacteriana.

1.2.3 *Sideróforos*

Outro componente essencial para a sobrevivência e crescimento de *E. coli* extraintestinais é o ferro, essencial à produção das proteínas da cadeia respiratória, que está presente nos meios fisiológicos dos animais em concentrações insuficientes para o crescimento bacteriano. Grande parte das bactérias patogênicas extraintestinais desenvolveu sistemas de alta afinidade para aquisição de ferro, que competem com os sideróforos dos hospedeiros e permite o crescimento bacteriano em locais de baixa concentração de ferro (Dho-Moulin and Fairbrother, 1999; Marrs *et al.*, 2005). Os sistemas sideróforos bacterianos incluem aqueles codificados pelos genes *iucD*, *iroN*, *sitD* e *irp2* (Sorsa *et al.*, 2003; Vokes *et al.*, 1999).

1.2.4 *Toxinas*

Outros fatores de virulência importantes na mediação de doenças causadas por *E. coli* são as toxinas. A α-hemolisina é uma proteína citolítica formadora de poros, que possibilita a inserção da bactéria na membrana de várias células eucarióticas, como eritrócitos, granulócitos, monócitos e células endoteliais (Trabulsi *et al.*, 1999). Outras toxinas já identificadas em ExPEC incluem *vat* (*vacuolating autotransporter toxin*), EAST1 (*heat stable cytotoxin associated with enteroaggregative E. coli*), *cnf 1/2* (*Cytotoxic necrotizing factor*) and *sat* (*secreted autotransporter toxin*) (Ewers *et al.*, 2007).

1.2.5 *Invasinas*

Uma forma pela qual as bactérias sobrevivem no interior dos seus hospedeiros é o processo de invasão celular, que proporciona aos micro-organismos uma forma de permanecer no hospedeiro sem estar em contato com os efeitos do soro. Com a invasão, a bactéria pode se manter no nicho celular a fim de se replicar ou apenas permanecer no interior do hospedeiro de forma quiescente. As invasinas conhecidas para ExPEC são Tia, IbeA e GimB (Kim, 2012).

1.3 Fatores mais comumente associados à virulência

1.3.1 *Fatores mais comumente associados à virulência de UPEC*

A fímbria P ocorre em cerca de 80% dos isolados de UPEC responsáveis por pielonefrite e representa uma das adesinas mais importantes e estudadas no contexto das infecções do trato urinário (Wullt *et al.*, 2000; Yamamoto, 2007). Outros fatores de virulência fortemente associados à UPEC são as adesinas do tipo 1, a toxina α-hemolisina - codificada pelo gene *hlyA* - (Marrs *et al.*, 2005), a toxina CNF, o sideróforo aerobactina e a protease autotranspotadora Sat.

1.3.2 *Fatores mais comumente associados à virulência de APEC*

Os fatores mais frequentemente associados à patogenicidade de APEC incluem as fímbrias de aderência F1, Tsh (hemaglutinina sensível à temperatura), o sistema sideróforo aerobactina, a proteína Iss (fator de resistência ao soro), a cápsula K e a produção de Colicina V (Delicato *et al.*, 2003; Ewers *et al.*, 2007; Tivendale *et al.*, 2004).

1.3.3 *Fatores mais comumente associados à virulência de NMEC*

O fator de virulência mais comumente associado à NMEC, presente em 80% dos isolados, é a cápsula K1 (Kim, 2012). Outros fatores fortemente relacionados à virulência de NMEC são fatores envolvidos na adesão (Tipo 1 e Fímbrias S), invasão (IbeA, gimB, OmpA e flagelina), resistência ao sistema imunológico do hospedeiro (K1 cápsula, O-LPS, Asla), produção de toxinas (CNF1) e sobrevivência (salmoquelina, enterobactina).

Apesar de serem conhecidos vários genes envolvidos nos diferentes passos da infecção, os mecanismos que permitem a sobrevivência de NMEC no sangue, a bacteremia e a travessia da barreira sangue-cérebro pela bactéria ainda não foram totalmente elucidados (Tseng *et al.*, 2012).

1.4. Regulação dos genes de virulência

Patógenos bacterianos utilizam mecanismos específicos para mediar as respostas adaptativas em diferentes ambientes; em situações de estresse no hospedeiro, a adaptação permite a sobrevivência nos diferentes nichos durante a infecção. Para se adaptarem a esse novo meio, algumas bactérias utilizam sistemas reguladores que traduzem os estímulos do ambiente, regulando a expressão de proteínas (Bertrand *et al.*, 2010).

Um grupo de mais de 250 fatores de transcrição controlam a expressão dos genes em *E. coli*. Alguns desses fatores são específicos de operons, enquanto outros são conhecidos como reguladores globais, que coordenam a expressão de dezenas de promotores em resposta a diferentes estímulos ambientais (Martinez-Antonio and Collado-Vides, 2003). Já foi relatado que sistemas reguladores interferem na aquisição de nutrientes, nos mecanismos de virulência, na resistência a antimicrobianos, entre outros mecanismos, em diferentes bactérias (Zhou *et al.*, 2003). No entanto, as funções específicas para cada um desses sistemas ainda não foram elucidadas, e mesmo aqueles sistemas cuja função já foi descrita podem apresentar outras funções ainda desconhecidas (Zhou *et al.*, 2003).

Embora se conheça muitos fatores de virulência associados à patogenicidade das ExPEC, a regulação da sua expressão ainda não foi elucidada.

1.4.1 Sistema regulador FNR

A **f**umarato-**n**itrato-**r**edutase (FNR) é uma proteína que atua como regulador global, agindo como um sensor da presença de oxigênio em bactérias gram-negativas. A FNR está presente em isolados patogênicos e não-patogênicos, e possui um papel importante na sobrevivência bacteriana em meios com restrição de oxigênio (Lazazzera *et al.*, 1993; Shan *et al.*, 2012).

Já foi demonstrado que a proteína FNR está relacionada com a regulação da virulência de bactérias patogênicas como *Shigella flexneri* e S*almonella enterica* serovar Typhimurium (Fink *et al.*, 2007; Marteyn *et al.*, 2010). Em *S.*

flexneri, a FNR foi associada à regulação de fatores relacionados à ilha de patogenicidade LEE (*locus of enterocyte effacement*). Esse estudo mostrou que o mutante para o gene *fnr* perdeu a capacidade de causar doença em camundongos (Marteyn *et al.*, 2010). Além disso, FNR também atua regulando diversos grupos de genes relacionados à virulência de *S. enterica* Typhimurium; outro estudo mostrou que o mutante para *fnr* não foi capaz de causar doença em camundongos infectados e persistir em macrófagos *in vitro* (Fink *et al.*, 2007). No entanto, não existem estudos sobre FNR relacionados à regulação de virulência em *E. coli* patogênicas.

2. OBJETIVO:

Frente ao que foi exposto, este trabalho teve como objetivos:

Realizar a análise epidemiológica de cepas de *Escherichia coli* patogênica aviária e

Determinar como FNR, um regulador global, controla a expressão de fatores associados à virulência de ExPEC.

2.1 Objetivos específicos:

1. Avaliar a suscetibilidade de isolados de *E. coli* recuperados de lesões de celulite e de infecções sistêmicas de frangos de corte em relação aos principais agentes antimicrobianos empregados na avicultura;

2. Detectar a presença dos principais fatores associados à virulência por Reação em Cadeia da Polimerase (PCR) Multiplex;

3. Realizar a tipagem filogenética através do método ECOR;

4. Analisar o perfil filogenético dos isolados de celulite e colissepticemia pelo método de variação do espaçamento intergênico.

5. Avaliar a virulência em modelo *in vivo* dos isolados de celulite e colissepticemia.

6. Caracterizar, por bioinfomática, os sítios em que a proteína FNR interage com o DNA das cepas ExPEC padrão (APEC O1, UPEC CFT073 e NMEC 58);

7. Analisar os fenótipos mais característicos de ExPEC (fímbria do tipo I, fímbria do tipo P, motilidade, hemolisina A, metabolismo do α-cetoglutarato, resistência a agentes antimicrobianos, entre outros) para cada par selvagem e mutante *fnr* de ExPEC;

8. Analisar a expressão desses genes cujos fenótipos foram observados;

9. Analisar os possíveis mecanismos de regulação da virulência;

10. Analisar, em modelo *in vivo*, a diferença na virulência dos mutantes em relação ao tipo selvagem, utilizando camundongos de 6 semanas de idade como modelo de infecção por UPEC, e ratos recém–nascidos como modelo de infecção por NMEC.

3. ARTIGO 1

Genotypes and Pathogenicity of cellulitis isolates reveal traits that modulate APEC virulence

Barbieri NL, de Oliveira AL, Tejkowski TM, Pavanelo DB, Rocha DA, Matter LB, Callegari-Jacques SM, de Brito BG, Horn F.

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Genotypes and Pathogenicity of Cellulitis Isolates Reveal Traits That Modulate APEC Virulence

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Abstract

We characterized 144 *Escherichia coli* isolates from severe cellulitis lesions in broiler chickens from South Brazil. Analysis of susceptibility to 15 antimicrobials revealed frequencies of resistance of less than 30% for most antimicrobials except tetracycline (70%) and sulphonamides (60%). The genotyping of 34 virulence-associated genes revealed that all the isolates harbored virulence factors related to adhesion, iron acquisition and serum resistance, which are characteristic of the avian pathogenic *E. coli* (APEC) pathotype. ColV plasmid-associated genes (*cvi/cva, iroN, iss, iucD, sitD, traT, tsh*) were especially frequent among the isolates (from 66.6% to 89.6%). According to the Clermont method of ECOR phylogenetic typing, isolates belonged to group D (47.2%), to group A (27.8%), to group B2 (17.4%) and to group B1 (7.6%); the group B2 isolates contained the highest number of virulence-associated genes. Clonal relationship analysis using the ARDRA method revealed a similarity level of 57% or higher among isolates, but no endemic clone. The virulence of the isolates was confirmed *in vivo* in one-day-old chicks. Most isolates (72.9%) killed all infected chicks within 7 days, and 65 isolates (38.1%) killed most of them within 24 hours. In order to analyze differences in virulence among the APEC isolates, we created a pathogenicity score by combining the times of death with the clinical symptoms noted. By looking for significant associations between the presence of virulence-associated genes and the pathogenicity score, we found that the presence of genes for invasins *ibeA* and *gimB* and for group II capsule *KpsMT*II increased virulence, while the presence of *pic* decreased virulence. The fact that *ibeA*, *gimB* and *KpsMT*II are characteristic of neonatal meningitis *E. coli* (NMEC) suggests that genes of NMEC in APEC increase virulence of strains.

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Introduction

Extraintestinal infections caused by avian pathogenic *Escherichia coli* (APEC) include omphalitis in embryos, salpingitis in laying hens, respiratory tract infections, and cellulitis [1]. Cellulitis is one of the most prevalent extraintestinal infections caused by APEC in broiler chickens, and is characterized by the presence of subcutaneous fibrinonecrotic plaques and inflammation of the overlying chicken skin, resulting in rejection of part or all of the carcasses at processing [2,3,4,5]. In Brazil, cellulitis lesions are estimated to cause the loss of 0.14 to 1.4% of poultry meat production [6], leading to losses of at least 18 thousand tons of meat in 2011 [7]. Depending on the virulence of the strain, the localized infections may become systemic [8].

The virulence genes that permit certain intestinal commensal *E. coli* to become APEC and infect extraintestinal sites include those encoding for the adhesins type 1 fimbriae and temperature-sensitive haemagglutinin (Tsh), iron-scavenging systems and the protectin Iss [9]. Most of these genes are often carried on Colicin V (ColV) or other large plasmids, and are thought to enable APEC strains to adhere to host tissues, survive within host fluids and resist host immune defenses [10,11,12,13]. Different APEC strains may have unique combinations of different virulence factors that have similar functions with regards to disease establishment. Despite our knowledge about the APEC pathotype, we still depend on *in vivo* assays to make sure that an *E. coli* isolate is able to cause an extraintestinal infection [14] and to determine the degree of virulence of the strain [8,15].

By genotyping a North American collection of APEC strains of known virulence in one-day-old chicks [16], Johnson et al. [17] identified five ColV-associated genes that distinguish an APEC from a non-pathogenic strain. Schouler et al. [14] combined the virulence genotyping of a large European collection with *in vivo* virulence tests in one-day-old chicks to identify four groups of virulence genes associated with APEC. The virulence traits proposed by Johnson et al. [17] and Schouler et al. [14] represent potentially efficient ways for screening APEC strains occurring

Table 1. Prevalence of VAGs in cellulitis isolates as detected by PCR.

Gene(s) or operon	Description	Size	n	% (*n* = 144)
Adhesins				
afa/draB	Afimbrial/Dr antigen-specific adhesin	809 pb	0	0
csgA	Cryptic curlin subunit	200 pb	144	(100.0)
crl	Curli fiber gene	249 pb	127	(88.2)
fimC	Type 1 fimbriae (D-mannose specific adhesin)	496 pb	132	(91.7)
hra	Heat-resistant agglutinin	540 pb	66	(45.8)
iha	Iron-regulated-gene-homologue adhesin	608 pb	18	(12.5)
papC	Pilus associated with pyelonephritis	500 pb	44	(30.5)
sfa/focCD	S fimbriae (sialic acid-specific) and F1C fimbriae	1222 pb	6	(4.2)
tsh[1]	Temperature sensitive hemagglutinin	823 pb	96	(66.6)
mat	Meningitis associated and temperature regulated fimbriae	898 pb	101	(70.1)
Iron acquisition				
chuA	Heme receptor gene (E. coli haem utilization)	278 pb	83	(57.6)
fyuA	Ferric yersinia uptake (yersiniabactin receptor)	773 pb	67	(46.5)
ireA	Iron-responsive element	384 pb	100	(69.4)
iroN[1]	Catecholate siderophore (salmochelin) receptor	846 pb	110	(76.4)
irp2	Iron repressible protein (yersiniabactin synthesis)	286 pb	96	(66.6)
iucD[1]	Aerobactin synthesis	710 pb	117	(81.2)
sitD chr.	Salmonella iron transport system gene	553 pb	21	(14.6)
sitD ep.[1]	Salmonella iron transport system gene	1032 pb	100	(69.4)
Protectins/Serum resistance				
cvi/cva[1]	Structural genes of colicin V operon (Microcin ColV)	597 pb	83	(57.6)
iss[1]	Increased serum survival	309 pb	114	(79.2)
neuC	K1 capsular polysaccharide	675 pb	31	(21.5)
kpsMT II	Group II capsule antigens	269 pb	53	(36.8)
ompA	Outer membrane protein	918 pb	137	(95.1)
traT[1]	Transfer Protein	429 pb	129	(89.6)
Toxins				
astA	EAST1 (heat stable cytotoxin associated with enteroaggregative E. coli)	110 pb	48	(33.3)
cnf1/2	Cytotoxic necrotizing factor	445 pb	0	0
sat	Secreted autotransporter toxin	666 pb	2	(1.4)
vat	Vacuolating autotransporter toxin	980 pb	51	(35.4)
hlyA	Hemolysin A	350 pb	1	(0.7)
Invasins				
gimB	Genetic island associated with newborn meningitis	736 pb	14	(9.7)
ibeA	Invasion of brain endothelium	341 pb	30	(20.8)
tia	Toxigenic invasion locus in ETEC strains	511 pb	26	(18.0)
Miscellaneous				
pic	Serin protease autotransporter	410 pb	38	(26.4)
malX	Pathogenicity-associated island marker	921 pb	11	(7.6)

[1]Genes associated with large virulence plasmids in APEC, such as pAPEC-O2-ColV [NC_007675], pTJ100 [AY553855], pAPEC-O1-ColBM [NC_009837], pAPEC-O1-R (NC_009838), pAPEC-O2-R NC_006671, pAPEC-O103-ColBM NC_011964, pAPEC-1 NC_011980.1.

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during poultry production. Neither work, however, allows the prediction of the degree of virulence of an APEC isolate.

In the present work we have genotyped 144 cellulitis isolates from broiler chickens in Southern Brazil and tested them for virulence in one-day-old chicks. We attributed a pathogenicity score to each isolate, which takes into account not only the number of deaths within 7 days, but also the clinical symptoms manifested before death and how quickly the infection kills birds. The pathogenicity score represents an improvement on the lethality test using the same number of animals, and may help to discriminate between different degrees of APEC virulence. We also characterized the isolates in terms of their resistance to 15 antimicrobial agents, their *E. coli* reference collection (ECOR) phylogenetic typing, and their clonal relationships.

Materials and Methods

Ethics statement

All animal experiments were approved by the Biosafety Committee of the Instituto de Pesquisas Veterinárias Desidério Finamor (CIB 004/08), and chickens were euthanized according to animal welfare norms.

Bacterial strains

One hundred and forty-four *E. coli* isolates were obtained between October 2006 and March 2007 from severe cellulitis lesions in 7-week-old broiler chickens at the time of their slaughter. The isolates were collected from different poultry flocks in 65 distinct farms in various locations within the southern Brazilian state of Paraná (PR). Farms could have more than one flock of broiler chickens: in order to ensure diversity of the strains, we collected only one isolate per flock. Biochemical tests (triple sugar iron, urease and MacConkey) were performed to confirm that all isolates were *E. coli* [18]. All strains were stored at –80°C in Luria-Bertani (LB) broth with 20% glycerol until they were needed.

Antibiotic resistance in APEC

The antimicrobial susceptibility of all APEC isolates was examined using the disc diffusion test according to the Clinical and Laboratory Standards Institute guidelines [18], using *Escherichia coli* strain ATCC 25922 as a control. The 15 antimicrobial agents tested were: ampicillin (10 U), bacitracin (10 U), cephalothin (30 μg), ceftiofur (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), enrofloxacin (5 μg), gentamicin (10 μg), neomycin (30 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), tetracycline (30 μg), sulphonamides (300 μg), trimethoprim (5 μg) and a combination of sulphonamides and trimethoprim (23.7 μg plus 1.3 μg). All antimicrobial discs were from CEFAR (São Paulo, Brazil). These antimicrobials were selected because they are, or were previously, employed in the poultry industry as growth promoters, for disease prevention and/or for treatment. The breakpoints were obtained from CLSI 2009 [18] for all antimicrobials, except for ampicillin, cephalothin, chloramphenicol and enrofloxacin [19], ceftiofur [20] and neomycin [21].

DNA extraction

Bacterial DNA was obtained from whole organisms by boiling [22]. The extracts were stored at 4°C, and the supernatants were used as templates for gene amplification.

Multiplex polymerase chain reactions

The presence of 33 virulence-associated genes in the isolates (Table 1) was investigated using multiplex polymerase chain reactions as described [23] with a few modifications, as outlined in [22].

Table 1. Prevalence of VAGs in cellulitis isolates as detected by PCR.

PCR-based classification into "ECOR" phylogenetic groups

All 144 isolates were classified using the multiplex PCR-based phylogenetic typing method of Clermont et al. [24], which groups strains into the four main phylogenetic groups shown in the reference strains in the ECOR collection [25]. Reactions were performed in a GenePro Thermal Cycler (Bioer Technology, China) as follows: denaturation for 4 min at 94°C, 30 cycles of 5 s at 94°C and 10 s at 59 °C, and a final extension step of 5 min at 72°C.

Phylogenetic analysis

Genetic data was obtained using the Amplified Ribosomal DNA Restriction Analysis (ARDRA) method [26]. This method is based on the variability of the ribosomal 16S-23S intergenic spacer region (ISR), which is well-distributed among isolates and has slow rates of mutation, and hence is considered useful for measuring intra-species diversity [26,27]. The ISR region was amplified and digested with restriction enzymes (*RsaI*, *HinfI* or *TaqI*) as previously described [22]. ARDRA restriction fragment length polymorphism profiles were analyzed by eye, and were converted into two-dimensional binary matrices according to the following criteria: 1 if a band was present, and 0 if it was absent. A matrix of distances was calculated and a dendrogram was produced using the NTSYS-pc program (version 2.0, Exeter Software, Setauket, NY). The Unweighted Pair Group Method with the Arithmetic Mean (UPGMA) was used.

Lethality and pathogenicity tests

Groups of 10 one-day-old Cobb female chicks were inoculated subcutaneously with 100 μL (10^8 CFU) of an overnight culture containing ~10^9 CFU/mL of each APEC isolate. A control group was inoculated with BHI broth. The animals were observed at 12 h intervals over 7 days, with all deaths being recorded. The lethality score (LS) was calculated according to the number of animals that died within this period with a range from 0 (no animal died) to 10 (all animals died) [2,28]. At 7 days post-infection, surviving chicks were killed by cervical dislocation, and clinical scores were recorded. Times of death and clinical scores were combined to give pathogenicity scores (PS), as described by Barbieri et al. [22]. Briefly, we performed postmortem examinations after chick deaths, looking for evidence of airsacculitis (A), pericarditis (P), perihepatitis (Ph), peritonitis (Pe) and cellulitis (C). The presence of a lesion was given the value 1, and its absence, the value 0. Pathogenicity scores (PS) were calculated from the equation PS = (TD x 5) + P + Pe + Ph + A + C, in which TD corresponds to the day of chick death, which has a value of 1 if the animal dies on the first day, and is reduced by 0.14 for each day that the animal survives up to day 7, which has the value 0. According to this equation, the PS can vary from 0 to 10.

Animals that died on the first day after inoculation had their livers dissected, homogenized and plated on lactose-containing MacConkey agar to identify *E. coli*; a PS = 10 score was attributed to these animals. The PS for each strain was calculated as the median PS for the 10 chicks infected with that particular strain.

RNA purification and quantitative real-time RT-PCR

E. coli strains PR001, PR013, PR017 and PR034 were grown overnight in BHI media. RNA from these strains was stabilized by RNAprotect Bacterial Reagent (QIAGEN) and extracted using an RNeasy Mini Kit (QIAGEN) with a one-hour in-tube DNase digestion (QIAGEN) to remove possible DNA contamination according to the manufacturer's instructions. Two biological replicates of each sample were prepared. The concentration of RNA was determined using a Spectrophotometer (ND-1000) (NanoDrop).

For quantitative real-time RT-PCR, melting curve analyses were performed after each reaction to ensure amplification specificity. Differences (n-fold) in transcripts were calculated using the relative comparison method, and amplification efficacies of each primer set were verified as described by Schmittgen et al. [29]. RNA levels were normalized using the housekeeping gene *tus* encoding DNA replication terminus site-binding protein as endogenous control [30]. Quantitative real-time RT-PCR (qRT-PCR) was performed with a Bio-Rad iQ5 iCycler detection system

using iScript one-step RT-PCR kit with SYBR Green (Bio-Rad) according to the manufacture's instruction [31].

Statistical analysis

Pathogenicity and lethality scores, resistance and number of virulence-associated genes (VAGs) were treated as quantitative variables and described by mean ± standard deviation (SD). Data was analyzed using non-parametric tests due to asymmetry in their distributions, except for number of VAGs. For comparisons among ECOR groups, one-way ANOVA and the Kruskal-Wallis methods were used. The relationship between the presence of a gene and the pathogenicity score was analyzed using the Mann-Whitney test, by comparing the scores in isolates with and without this particular gene. All statistical analysis was carried out with the Statistical Package for the Social Sciences (IBM SPSS v.18.0) or WinPEPI v.11.18 (Abramson, J.H. WINPEPI updated: computer programs for epidemiologists, and their teaching potential. Epidemiologic Perspectives & Innovations 2011, 8:1). Statistical significance was accepted at $p \leq 0.05$.

Results

Antibiotic resistance among the APEC isolates

All 144 APEC isolates were tested for susceptibility to 15 antimicrobial agents that have, at some point, been commonly employed in the Brazilian poultry industry, either as growth promoters, to prevent infection and/or for treatment. It was found that the APEC isolates were susceptible to the majority of antimicrobials. The frequency of resistance to all the antimicrobials was less than 30% except for tetracycline and sulphonamides (with frequencies of 69.4% and 59.7% resistance, respectively) (Fig.1). For the exact resistance values, see the shadowed boxes in Figure 2.

With respect to the sites of antibiotic action, 29.2% of the isolates were resistant to at least one of the antimicrobials that act on the cell wall (ampicillin, bacitracin, cephalothin, ceftiofur); 11.8% were resistant to at least one of the antimicrobials that inhibit nucleic acid synthesis (ciprofloxacin, enrofloxacin, norfloxacin); 68.0% were resistant to at least one of the antimicrobials that block protein synthesis (chloramphenicol, gentamicin, neomycin, tetracycline; but the percentage was only 15.3% if tetracycline is excluded); and 59.7% were resistant to at least one of the antimicrobials that target folate synthesis (sulphonamides, trimethoprim, sulpha + trimethoprim; but only 29.2% if sulphonamides are excluded).

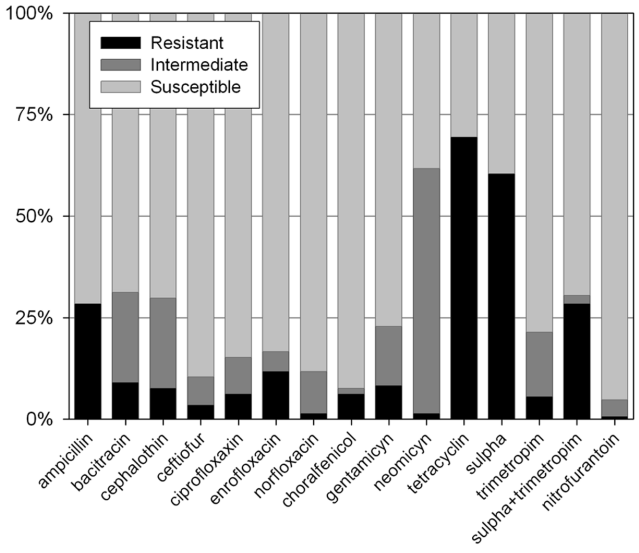

Figure 1. Antimicrobial susceptibility of cellulitis isolates. The susceptibility of 144 APEC isolates to 15 antimicrobials was tested individually using disc diffusion tests.
doi:10.1371/journal.pone.0072322.g001

When we analyzed multi-resistance patterns, we observed that 18% of all isolates were susceptible to, or had intermediate resistance to, all antibiotics tested. Twenty per cent were resistant to at least one agent; 17% to 2; 19% to 3; and 11% to 4. Fifteen per cent were resistant to 5 or more, and one strain (PR133) was resistant to 10. It is noteworthy that there was no antimicrobial agent to which all 144 APEC strains were susceptible. Figure 2 shows the percentage of strains with resistance to given pairs of antimicrobials.

Genotyping by multiplex polymerase chain reaction

The prevalence of virulence-associated genes (VAGs) among the APEC isolates is shown in Table 1. The factors *fimC, ompA, traT, csgA* and *crlA* were the most frequent VAGs. Overall, the isolates had an average of 15.2 VAGs.

Virulence factors related to adhesion, iron acquisition and serum resistance were present in all strains, with the exception of strain PR010 (median PS 9.1; Fig. S1), which did not contain any of the iron acquisition systems tested. ColV plasmid-associated genes (*iroN, iss, iucD, sitD, traT, tsh*) occurred in the majority of the isolates (from 89.6 to 66.6%), although the ColV-encoding genes *cvi/cva* were present in only 57.6% of the isolates (Table 1). The factors *afa/dra* and *cnf1/2* were not detected in any isolate, while *csgA* was detected in all the isolates. The VAGs harbored by each isolate are presented in Figure S1.

Lethality and pathogenicity tests

Lethality and pathogenicity tests in day-old chicks were used to evaluate the virulence of the APEC isolates as described in the Materials and Methods section, and the results for each strain are presented in Figure S1. Most isolates were lethal: 105 (72.9%) of the isolates killed all 10 chicks within 7 days (LS = 10); 14 isolates (9.7%) killed 9 chicks, 9 isolates (6.2%) killed 8 chicks, 5 isolates (3.5%) killed 7, 4 isolates (2.8%) killed 6, 2 isolates (1.4%) killed 5, 3 isolates (2.1%) killed 4, 1 isolate killed 3 chicks (0.7%), and 1 killed 1 chicken (0.7%). Overall, the isolates had a lethality score of 9.26. None of the chicks inoculated with BHI or MG1655 (the negative controls) died.

In addition to the LS, we also determined the pathogenicity scores. The PS takes into account the clinical symptoms and how quickly the infection kills birds, in addition to how many chicks die within 7 days [22]. Thus, while an LS of 10 means that all 10 chicks died within seven days, a PS of 10 means that most of the 10 chicks died on the first day. Fifty-six isolates (38.1%) had a median PS = 10; 36 (25%) had a 9.9 > median PS > 7.0; 42 (29.2%) had a 6.9 > median PS > 5.0; and 10 (6.9%) had a median PS lower than 5. Overall, the APEC isolates had a pathogenicity score of 8.01. Chicks inoculated with BHI displayed no signs of infection, while chicks inoculated with MG1655 displayed only small cellulitis lesions at the inoculation sites (Fig. 3). Figure 3 displays the data for a few isolates that illustrate different PS.

PCR-based classification into ECOR phylogenetic groups

We performed phylogenetic typing of the APEC isolates using the Clermont method [24]. This technique uses the *chuA* and *YjaA* genes and *TspE4C2* fragment to allocate *E. coli* strains to phylogenetic groups A, B1, B2 and D. In previous studies,

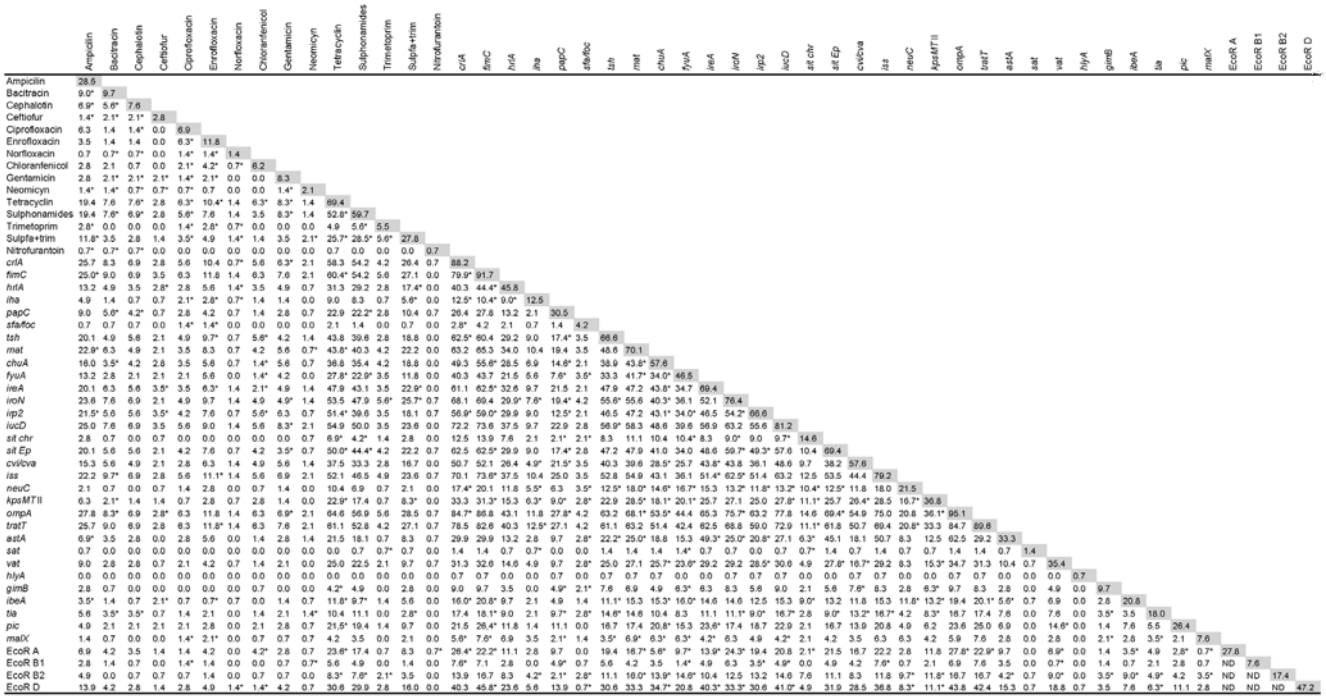

Figure 2. Association between resistance to 15 antimicrobials, presence of 31 VAGs[1] and ECOR group among 144 APEC isolates. Numbers indicate the percentage of isolates that have both traits, while numbers in shadowed boxes indicate the percentage of isolates that have the corresponding trait; * $p \leq 0.001$ using χ^2. ND, not determined.[1] *afa* and *cnf* are not included, since they were not present in any strain, and *csgA* is not included, since it was present in all strains.
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virulent extraintestinal strains were found to belong mainly to group B2 and, to a lesser extent, to group D, whereas most commensal strains belonged to group A [24,25]. The distribution of our APEC isolates among the four phylogenetic groups is shown in Table 2. As determined by PCR, most of the strains (47.2%) belonged to group D.

Table 2 also shows the mean number of antimicrobials to which the strains were resistant, and the mean number of VAGs and pathogenicity and lethality scores of strains according to their ECOR group. No statistical differences were observed among the phylogenetic groups in relation to resistance ($p > 0.10$). Isolates from group B2 possessed a significantly higher number of VAGs per strain, and the remaining groups did not differ in terms of the number of VAGs they possessed. Figure 2 shows the VAGs positively linked ($p \leq 0.001$) to ECOR groups A, B1, B2 and D. As for the lethality and pathogenicity scores, the B2 strains had on average the highest values, whereas the B1 and D groups had the lowest values.

In an attempt to find out what increases the virulence of APEC isolates in one-day-old chicks, we looked for significant associations between the presence of VAGs and pathogenicity scores (Table 3). We found that a higher PS was positively linked ($p \leq 0.05$) to the VAGs *kpsMTII*, *gimB* and *ibeA*, but negatively linked ($p \leq 0.005$) to *pic*.

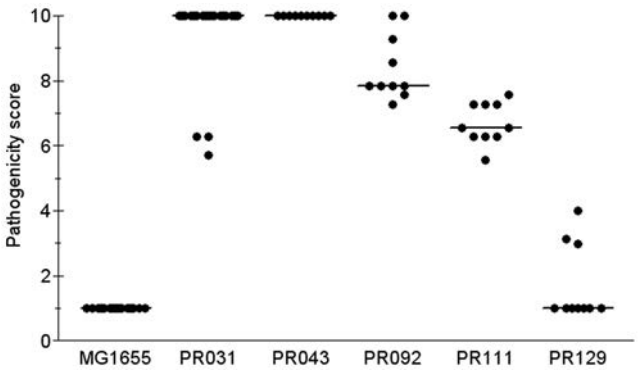

Figure 3. Pathogenicity scores for MG1655 and five cellulitis isolates. Ten one-day old chicks were infected with each isolate and observed for 7 days as described in the Materials and Methods section. Data points represent the PS for each chick, and horizontal bars represent the median PS for each isolate. Strain MG1655 was included as negative control.
doi:10.1371/journal.pone.0072322.g003

Expression of *kpsMTII*, *gimB*, *ibeA* and *pic*

We quantified the expression of *kpsMTII*, *gimB*, *ibeA* and *pic* in four strains that contain different combinations of these VAGs, namely PR01 (*pic*), PR013 (*kpsMTII*, *gimB* and *ibeA*), PR017 (*kpsMTII* and *gimB*) and PR034 (*gimB*, *ibeA* and *pic*), using *tus* as a housekeeping gene. Figure 4 shows that all strains expressed these genes.

Phylogenetic Analysis

We used the ARDRA method to evaluate genetic diversity among the 144 isolates. A similarity matrix was generated based on the presence or absence of restriction bands and strains were clustered accordingly. The ARDRA profiles of the strains are shown in Figure S2. We found 104 different ARDRA profiles among the 144 isolates; 82 of them had distinct band patterns, while the remaining 62 isolates fell into 22 groups each containing 2 to 6 strains with the same band pattern. The majority of isolates with similar band patterns came from different farms, and only 4

Table 2. ECOR groups among the 144 cellulitis isolates (within parentheses, number of strains) and respective mean and standard deviation for antimicrobial resistance, number of VAGs, lethality scores and pathogenicity scores.

	ECOR groups (number of isolates)				
	A (40)	**B1 (11)**	**B2 (25)**	**D (68)**	***p* value**
Resistance	2.9±1.9	2.7±2.2	1.8±1.8	2.5±2.1	0.144[3]
Resistance (-tetra,sulpha)[1]	1.4±1.6	1.4±1.9	0.9±1.2	1.2±1.6	0.441[3]
VAGs (34)	13.9±2.8 **a**	14.5±2.8 **a**	17.5±2.9 **b**	15.3±2.5 **a**	<0.001[4]
APEC VAGs (8)[2]	4.5±1.4	4.5±1.2	4.3±1.4	4.6±1.2	0.732[5]
Lethality score	9.6±0.9 **ab**	8.1±2.4 **b**	9.8±0.8 **a**	9.1±1.9 **b**	0.005[6]
Pathogenicity score	8.3±1.7 **ab**	7.3±2.7 **ab**	8.9±1.5 **a**	7.6±2.3 **b**	0.050[6]

[1]Mean number of antimicrobials to which strains were resistant, excluding tetracycline and sulphonamides.
[2]APEC VAGs: papC, tsh, irp2, iucD, cva/cvi, iss, astA and vat.
[3]One-way Kruskal-Wallis.
[4]One-way ANOVA; means indicated by the same letter did not differ using the SNK test (0.05 level).
[5]One-way ANOVA.
[6]One-way Kruskal-Wallis; means indicated by the same letter did not differ using the Kruskal-Wallis adjusted for multiple comparisons (0.05 level).
doi:10.1371/journal.pone.0072322.t001

of the 22 groups contained strains collected from the same farm (column "Origin" in Fig. S2). It is noteworthy that strains with the same band pattern have different virulence genotypes and could belong to distinct ECOR groups.

From the dendrogram, eight major clusters could be grouped at a similarity level of 80% or more (Fig. S2, right), with the majority of isolates falling into clusters A and B.

Discussion

Antimicrobial therapy is one of the primary control measures for reducing the morbidity and mortality caused by APEC infections. Since the indiscriminate use of antimicrobials leads to the selection of resistant isolates, they need to be used prudently in order to preserve their therapeutic usefulness in both animals and humans [32]. Among the 144 APEC strains isolated from cellulitis lesions in Brazil, the highest frequency of resistance was against tetracycline (69.4%), in agreement with the observations of others [33,34,35,36,37,38,39,40]. This is not surprising since tetracycline has been used extensively for several decades, primarily as a feed additive in the poultry industry, and is the oldest antimicrobial on the market that was tested in this work. As a consequence, resistance to tetracycline has increased over the years.

Apart from tetracycline and sulphonamides, the frequency of resistance to the other antimicrobials was fairly low. Despite that, resistance to at least one antimicrobial was observed in almost all isolates (85.9%), and about half (54.6%) exhibited resistance to three or more antibiotics, as observed in previous work [35,39,40]. Frequencies of resistance to nitrofurantoin and sulpha + trimethoprim were much lower in the strains collected between 2006-2007 (this work) than in strains collected between 1998-2000 within the same Southern Brazilian region [2]. We expect that the increasing restrictions on the use of antimicrobials in the poultry industry will, in the near future, result in a considerably lower frequency of resistance of APEC strains to antimicrobial agents.

Our phylogenetic typing results showed that the APEC mainly belong to *E. coli* reference collection (ECOR) group D, in contrast to studies in the USA, China and Germany, in which most isolates were found to be in group A [17,23,41,42], and in France where they were found to be in group B2 [43]. In all the cited studies, including ours, isolates belonging to group B1 were rare. Extraintestinal isolates from poultry seem, therefore, to be broadly distributed between groups A, B2 and D, but not group B1. It is important to note, however, that the multiplex-PCR method of Clermont et al. (2000) may sometimes classify strains actually belonging to group B1 as group A [24].

An APEC is defined as an *E. coli* isolated from an extraintestinal infection of birds. Since infections can be localized or systemic, and caused by more or less virulent strains, an APEC collection will include isolates with widely varying degrees of virulence. It can be argued that the higher the virulence of an APEC, the greater its potential to cause a systemic infection. Moreover, immunocompromised birds can be infected with less virulent APECs or even avirulent commensal *E. coli*. The genotyping of the 144 cellulitis isolates revealed that all of them harbor virulence factors related to adhesion, iron acquisition (with the exception of strain PR010) and serum resistance, which are characteristic of the APEC pathotype. Thus, as in previous reports [2,4,36], our results demonstrate that cellulitis APEC isolates are indistinguishable from septicemic APEC isolates on the basis of virulence factors. Yet the genotype does not guarantee that an extraintestinal *E. coli* is truly an APEC, so *in vivo* assays are necessary to confirm that the isolate does indeed cause infection.

Since it is not feasible to perform *in vivo* virulence tests on a large number of strains in 3- or 5-week-old chickens, large APEC collections are tested in one-day-old chicks [2,14,28,43]. To analyze the virulence of our strains, we used lethality tests (LS) on one-day-old chicks. The majority of our strains were lethal to these chicks within 7 days, strongly suggesting that they were virulent APEC.

To better analyze the virulence of a given strain, we combined an analysis of organ lesions with how quickly the infection killed the chicks, to provide a pathogenicity score (PS), which is more likely to detect differences in virulence among APEC strains than the lethality score. Mortality and organ lesions in one-day-old chicks have previously been used to classify APEC strains as having high, intermediate or low pathogenicity [16,44]. On the basis of their classification, the majority of our strains were found to be highly pathogenic; only ten had a PS lower than 5. Our phylogenetic analysis using ARDRA failed to identify a prevalent APEC clone. Instead, the population of cellulitis isolates proved to be diverse, with few strains belonging to the same clone.

We then analyzed which genes influenced the PS; we found that the presence of genes for invasins *ibeA* and *gimB* and for group II

Table 3. Relationship between APEC pathogenicity score (PS) and presence of different genes.

	Gene +		Gene -		
Gene[a]	Average PS	Number of isolates	Average PS	Number of isolates	p^b
crlA	8.11	127	7.26	17	0.300
fimC	7.92	132	9.01	12	0.100
hrlA	7.93	66	8.08	78	0.377
iha	8.28	18	7.97	126	0.925
papC	8.37	44	7.86	100	0.264
sfa/foc	8.88	6	7.98	138	0.377
tsh	7.87	96	8.31	48	0.269
mat	8.09	101	7.82	43	0.559
chuA	7.96	83	8.09	61	0.756
fyuA	7.84	67	8.16	77	0.282
ireA	7.84	100	8.40	44	0.204
iroN	8.01	110	8.01	34	0.886
irp2	7.87	96	8.29	48	0.344
iucD	8.03	117	7.93	27	0.770
sit chr	8.67	21	7.90	123	0.078
sit Ep	8.08	100	7.86	44	0.362
cvi/cva	8.24	83	7.71	61	0.176
iss	8.03	114	7.94	30	0.790
neuC	7.69	31	8.10	113	0.663
*kpsMT*II	8.61	53	7.66	91	0.004*
ompA	7.99	137	8.39	7	0.865
tratT	7.96	129	8.49	15	0.443
astA	7.89	48	8.08	96	0.864
sat	6.25	2	8.04	142	-
vat	7.84	51	8.11	93	0.475
hlyA	6.28	1	8.03	143	-
gimB	9.10	14	7.90	130	0.026*
ibeA	8.56	30	7.87	114	0.042*
tia	8.60	26	7.88	118	0.178
pic	7.11	38	8.34	106	0.006*
malX	8.09	11	8.01	133	0.844

[a]Genes that occurred in none (*afa/dra, cnf1/2)* or all (*csgA*) isolates are not listed.
[b]Exact *p* values for the Wilcoxon-Mann-Whitney test.
*$p \leq 0.05$.
doi:10.1371/journal.pone.0072322.t002

capsule *KpsMT*II increased virulence, while the presence of *pic* decreased virulence. The expression of these genes in BHI was confirmed by quantitative RT-PCR in four isolates. In agreement with these findings, it has been observed that APEC strains MT78 and IMT5155 caused a systemic infection when inoculated intratracheally into 5-week-old chickens, while UEL17 remained restricted to the lungs [8]. The main differences in the virulence genotypes among the three strains are the absence of *ibeA*, *gimB*, *neuC* and *KpsMT*II, and presence of *pic* in UEL17 [8]. Genes *ibeA* and *KpsMT*II have been associated with APEC virulence [45,46]. Although *ibeA*, *gimB* and *KpsMT*II were not found to be present in the majority of APEC strains (Table 1) [17,23,45,47] and cannot, therefore, be considered defining traits of APEC, they may be ''significant but minority traits'' in increasing APEC virulence [47]. Since *ibeA*, *gimB* and *KpsMT*II are characteristic of NMEC [23,48], we may conclude that the genes that render an APEC more similar to NMEC increase virulence, and the zoonotic risk. Interestingly, Mora et al. [49] observed that the extraintestinal *E. coli* clonal group O25b:K1:H4-ST131 harboring *ibeA* and *KpsMT*II has recently emerged among APEC isolates.

The gene *pic*, like *tsh*, encodes a serine protease autotransporter protein, and was included in the screening of VAGs in APEC isolates [15,23] because it had been implicated in UPEC virulence [50]. However, according to our results, the presence of *pic* was associated with decreased virulence of APEC in day-old chicks (Table 3). The construction of a *pic* mutant would help to elucidate its role in APEC virulence.

In summary, in this work, we genotyped and performed virulence tests *in vivo* on the largest number of APEC isolates from severe lesions of cellulitis described so far. In addition, our data provide a comprehensive overview of the susceptibility of cellulitis

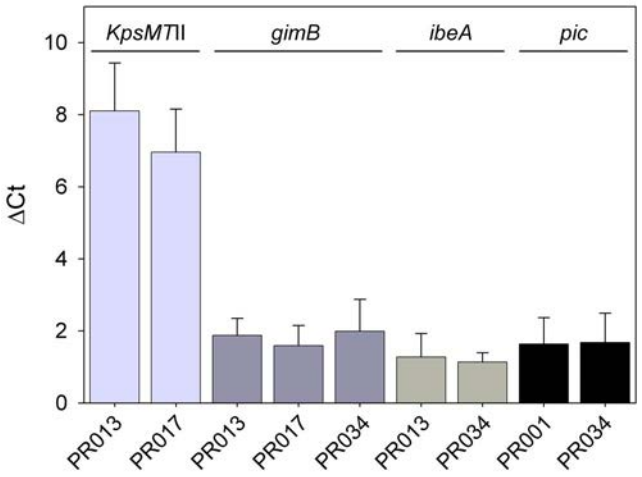

Figure 4. Expression of genes found to influence the PS. Real-time quantitative RT-PCR was used to analyze the expression of *kpsMTII*, *gimB*, *ibeA* and *pic* in PR001, PR013, PR017 and PR034. △ct expresses ct_{mean} subtracted of ct_{mean} of *tus* (housekeeping gene) of the respective isolate. Data represent the average ± SD of two experiments done in triplicates.
doi:10.1371/journal.pone.0072322.g004

isolates currently found in south Brazil to antimicrobials and their phylogenetic status.

Supporting Information

Figure S1 Characterization of 144 APEC isolates. Columns from left to right: S*train*, isolate designation; *Origin*, source of the isolate; *Resistance*, number of antimicrobials to which the isolate was resistant; the subsequent columns depict the PCR results for all VAGs tested, with presence indicated in black and absence indicated in white (except for *afa* and *cnf* ½, which were absent from all isolates); *no. VAGs*, total number of VAGs in each isolate; *LS*, lethality score; *PS median (range)*, median pathogenicity score (range); *PS mean ± SD*, mean pathogenicity score ± standard deviation; *ECOR*, ECOR phylogenetic group; *Cluster*, strains with 80% similarity were grouped into 8 genotypic clusterings (A to H). (PDF)

Figure S2 ARDRA profile of 144 APEC isolates. The ARDRA dendrogram was constructed by UPGMA based upon enzyme restriction digestion of amplified 16-23S DNA intergenic spacer regions. The column S*train* shows isolate designation; the column *Origin*, source of isolate, with 1 to 65 designating each of the 65 farms from which the isolates were collected. *Cluster* designates the 8 genotypic clusters (A to H) into which strains with 80% similarity were grouped. *E. coli* ATCC25922 was analyzed as a reference strain.
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: NLB BGB FH. Performed the experiments: NLB TMT. Analyzed the data: NLB DBP SMCJ FH. Contributed reagents/materials/analysis tools: FH. Wrote the paper: NLB FH. Performed the ECOR determinations: ALO. Performed PCR for VAGs: DAR. Supervised the antimicrobial susceptibility tests: LBM. Performed the in vivo experiments in day-old chickens: TMT. Performed the statistical analyses: DBP SMCJ.

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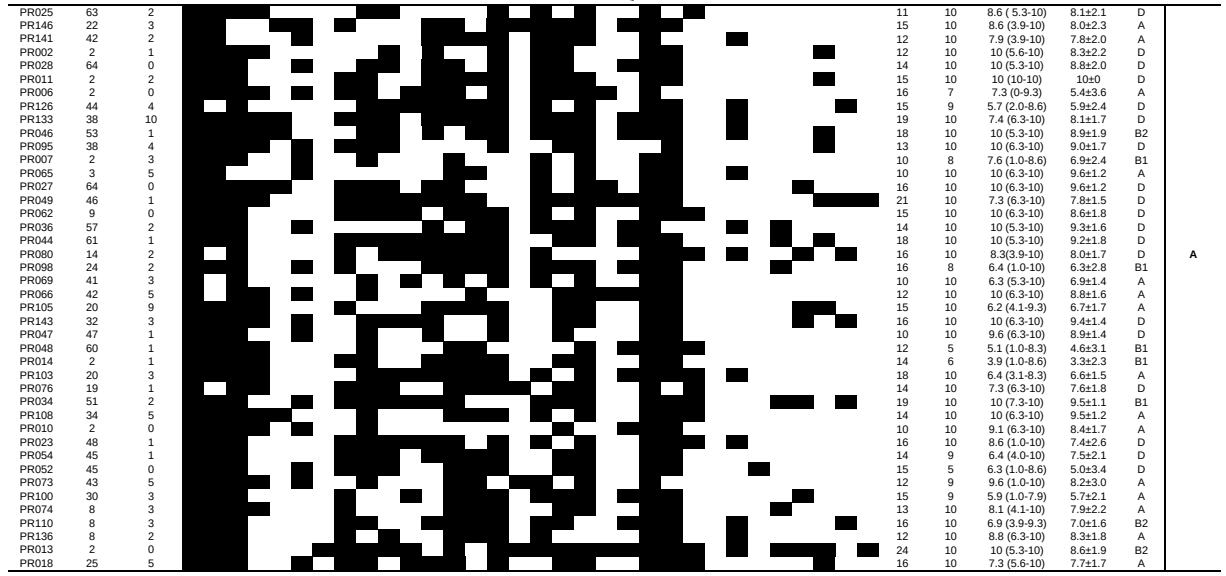
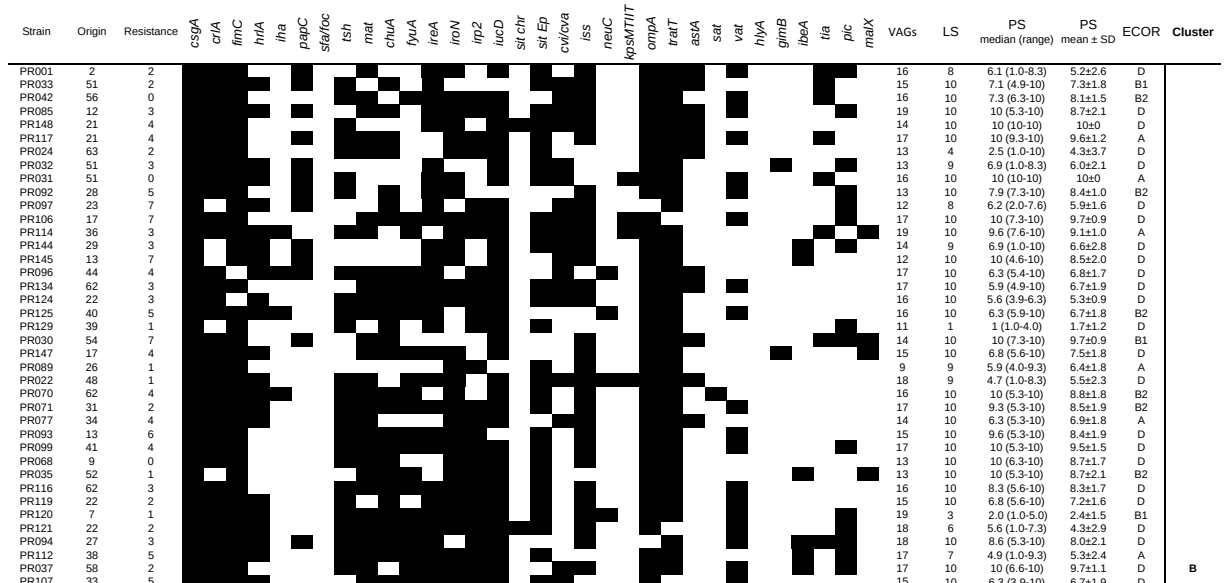


Strain	Origin	Resistance	csgA	crlA	fimC	hlyA	iha	papC	sfa/foc	tsh	mat	chuA	fyuA	ireA	iroN	irp2	iucD	sit chr	sit Ep	cvi/cva	iss	neuC	kpsMTIIT	ompA	traT	astA	sat	vat	hlyA	gimB	ibeA	tia	plc	malX	VAGs	LS	PS median (range)	PS mean ± SD	ECOR	Cluster
PR025	63	2																																	11	10	8.6 (5.3-10)	8.1±2.1	D	
PR146	22	3																																	15	10	8.6 (3.9-10)	8.0±2.3	A	
PR141	42	2																																	12	10	7.9 (3.9-10)	7.8±2.0	A	
PR002	2	1																																	12	10	10 (5.6-10)	8.3±2.2	D	
PR028	64	0																																	14	10	10 (5.3-10)	8.8±2.0	D	
PR011	2	2																																	15	10	10 (10-10)	10±0	D	
PR006	2	0																																	16	7	7.3 (0-9.3)	5.4±3.6	A	
PR126	44	4																																	15	9	5.7 (2.0-8.6)	5.9±2.4	D	
PR133	38	10																																	19	10	7.4 (6.3-10)	8.1±1.7	D	
PR046	53	1																																	18	10	10 (5.3-10)	8.9±1.9	B2	
PR095	38	4																																	13	10	10 (6.3-10)	9.0±1.7	D	
PR007	2	3																																	10	8	7.6 (1.0-8.6)	6.9±2.4	B1	
PR065	3	5																																	10	10	10 (6.3-10)	9.6±1.2	A	
PR027	64	0																																	16	10	10 (6.3-10)	9.6±1.2	D	
PR049	46	1																																	21	10	7.3 (6.3-10)	7.8±1.5	D	
PR062	9	0																																	15	10	10 (6.3-10)	8.6±1.8	D	
PR036	57	2																																	14	10	10 (5.3-10)	9.3±1.6	D	
PR044	61	1																																	18	10	10 (5.3-10)	9.2±1.8	D	
PR080	14	2																																	16	10	8.3(3.9-10)	8.0±1.7	D	A
PR098	24	2																																	16	8	6.4 (1.0-10)	6.3±2.8	B1	
PR069	41	3																																	10	10	6.3 (5.3-10)	6.9±1.4	A	
PR066	42	5																																	12	10	10 (6.3-10)	8.8±1.6	A	
PR105	20	9																																	15	10	6.2 (4.1-9.3)	6.7±1.7	A	
PR143	32	3																																	16	10	10 (6.3-10)	9.4±1.4	D	
PR047	47	1																																	10	10	9.6 (6.3-10)	8.9±1.4	D	
PR048	60	1																																	12	5	5.1 (1.0-8.3)	4.6±3.1	B1	
PR014	2	1																																	14	6	3.9 (1.0-8.6)	3.3±2.3	B1	
PR103	20	3																																	18	10	6.4 (3.1-8.3)	6.6±1.5	A	
PR076	19	1																																	14	10	7.3 (6.3-10)	7.6±1.8	D	
PR034	51	2																																	19	10	10 (7.3-10)	9.5±1.1	B1	
PR108	34	5																																	14	10	10 (6.3-10)	9.5±1.2	A	
PR010	2	0																																	10	10	9.1 (6.3-10)	8.4±1.7	A	
PR023	48	1																																	16	10	8.6 (1.0-10)	7.4±2.6	D	
PR054	45	1																																	14	9	6.4 (4.0-10)	7.5±2.1	D	
PR052	45	0																																	15	5	6.3 (1.0-8.6)	5.0±3.4	D	
PR073	43	5																																	12	9	9.6 (1.0-10)	8.2±3.0	A	
PR100	30	3																																	15	9	5.9 (1.0-7.9)	5.7±2.1	A	
PR074	8	3																																	13	10	8.1 (4.1-10)	7.9±2.2	A	
PR110	8	3																																	16	10	6.9 (3.9-9.3)	7.0±1.6	B2	
PR136	8	2																																	12	10	8.8 (6.3-10)	8.3±1.8	A	
PR013	2	0																																	24	10	10 (5.3-10)	8.6±1.9	B2	
PR018	25	5																																	16	10	7.3 (5.6-10)	7.7±1.7	A	



Strain	Origin	Resistance	csgA	crlA	fimC	hlyA	iha	papC	sfa/foc	tsh	mat	chuA	fyuA	ireA	iroN	irp2	iucD	sit chr	sit Ep	cvi/cva	iss	neuC	kpsMTIIT	ompA	traT	astA	sat	vat	hlyA	gimB	ibeA	tia	plc	malX	VAGs	LS	PS median (range)	PS mean ± SD	ECOR	Cluster
PR001	2	2																																	16	8	6.1 (1.0-8.3)	5.2±2.6	D	
PR033	51	2																																	15	10	7.1 (4.9-10)	7.3±1.8	B1	
PR042	56	0																																	16	10	7.3 (6.3-10)	8.1±1.5	B2	
PR085	12	3																																	19	10	10 (5.3-10)	8.7±2.1	D	
PR148	21	4																																	14	10	10 (10-10)	10±0	D	
PR117	21	4																																	17	10	10 (9.3-10)	9.6±1.2	A	
PR024	63	2																																	13	4	2.5 (1.0-10)	4.3±3.7	D	
PR032	51	3																																	13	9	6.9 (1.0-8.3)	6.0±2.1	D	
PR031	51	0																																	16	10	10 (10-10)	10±0	A	
PR092	28	5																																	13	10	7.9 (7.3-10)	8.4±1.0	B2	
PR097	23	7																																	12	8	6.2 (2.0-7.6)	5.9±1.6	D	
PR106	17	7																																	17	10	10 (7.3-10)	9.7±0.9	D	
PR114	36	3																																	19	10	9.6 (7.6-10)	9.1±1.0	A	
PR144	29	3																																	14	9	6.9 (1.0-10)	6.6±2.8	D	
PR145	13	7																																	12	10	10 (4.6-10)	8.5±2.0	D	
PR096	44	4																																	17	10	6.3 (5.4-10)	6.8±1.7	D	
PR134	62	3																																	17	10	5.9 (4.9-10)	6.7±1.9	D	
PR124	22	3																																	16	10	5.6 (3.9-6.3)	5.3±0.9	D	
PR125	40	5																																	16	10	6.3 (5.9-10)	6.7±1.8	B2	
PR129	39	1																																	11	1	1 (1.0-4.0)	1.7±1.2	D	
PR030	54	7																																	14	10	10 (7.3-10)	9.7±0.9	B1	
PR147	17	4																																	15	10	6.8 (5.6-10)	7.5±1.8	D	
PR089	26	1																																	9	9	5.9 (4.0-9.3)	6.4±1.8	A	
PR022	48	1																																	18	9	4.7 (1.0-8.3)	5.5±2.3	D	
PR070	62	4																																	16	10	10 (5.3-10)	8.8±1.8	B2	
PR071	31	2																																	17	10	9.3 (5.3-10)	8.5±1.9	B2	
PR077	34	4																																	14	10	6.3 (5.3-10)	6.9±1.8	A	
PR093	13	6																																	15	10	9.6 (5.3-10)	8.4±1.9	D	
PR099	41	4																																	17	10	10 (5.3-10)	9.5±1.5	D	
PR068	9	0																																	13	10	10 (6.3-10)	8.7±1.7	D	
PR035	52	1																																	13	10	10 (5.3-10)	8.7±2.1	B2	
PR116	62	3																																	16	10	8.3 (5.6-10)	8.3±1.7	D	
PR119	22	2																																	15	10	6.8 (5.6-10)	7.2±1.6	D	
PR120	7	1																																	19	3	2.0 (1.0-5.0)	2.4±1.5	B1	
PR121	22	2																																	18	6	5.6 (1.0-7.3)	4.3±2.9	D	
PR094	27	3																																	18	10	8.6 (5.3-10)	8.0±2.1	D	
PR112	38	5																																	17	7	4.9 (1.0-9.3)	5.3±2.4	A	
PR037	58	2																																	17	10	10 (6.6-10)	9.7±1.1	D	B
PR107	33	5																																	15	10	6.3 (3.9-10)	6.7±1.9	D	
PR109	18	6																																	19	9	5.93 (3.9-10)	6.0±1.2	D	
PR058	26	4																																	15	7	5.6 (1.0-9.3)	5.0±3.1	D	
PR061	4	2																																	14	9	10 (1.0-10)	8.0±3.0	A	
PR130	26	3																																	14	9	6.4 (1.0-9.3)	6.1±2.2	A	
PR016	2	1																																	10	9	7.6 (5.0-10)	7.5±1.5	D	
PR084	24	1																																	16	7	5.9 (1.0-8.3)	5.1±3.0	D	
PR087	20	2																																	16	8	5.4 (1.0-9.3)	5.3±2.7	D	
PR086	41	5																																	12	10	8.2 (6.6-10)	8.4±1.3	A	
PR055	45	1																																	13	10	10 (4.6-10)	9.1±1.9	D	
PR078	34	3																																	17	10	10 (10-10)	10±0	A	
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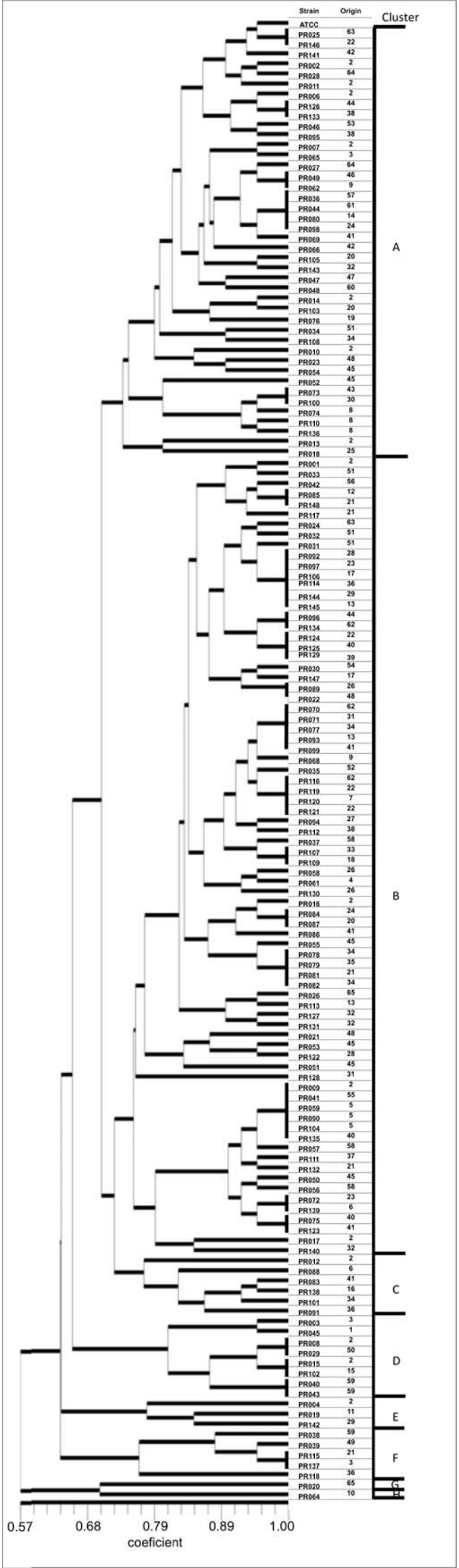
PR026	65	4																																	17	6	5.8 (1.0-8.3)	4.4±3.0	B2	
PR113	13	5																																	20	10	6.1 (3.9-10)	6.9±2.4	D	
PR127	32	4																																	17	10	6.9 (4.9-10)	7.7±2.1	D	
PR131	32	1																																	17	9	3.9 (1.0-5.6)	3.9±1.4	D	
PR021	48	3																																	13	9	9.3 (1.0-10)	7.8±3.0	A	
PR053	45	3																																	16	10	10 (6.3-10)	9.1±1.6	D	
PR122	28	2																																	15	4	2.5 (1.0-8.3)	3.3±2.6	D	
PR051	45	2																																	11	8	7.3 (1.0-10)	6.5±3.2	D	
PR128	31	1																																	22	10	10 (10-10)	10±0	A	
PR009	2	0																																	15	10	6.3 (5.3-10)	7.7±2.0	D	
PR041	55	0																																	18	10	10 (5.3-10)	9.1±2.0	D	
PR059	5	1																																	15	10	10 (6.3-10)	9.6±1.2	D	
PR090	37	0																																	20	10	10 (5.3-10)	8.7±1.8	B2	
PR104	5	1																																	20	10	10 (5.3-10)	9.1±1.6	B2	
PR135	40	2																																	16	10	10 (7.3-10)	9.5±1.1	B2	
PR057	58	0																																	19	10	10 (5.3-10)	8.4±2.1	B2	
PR111	37	2																																	20	10	6.6 (5.6-7.6)	6.7±0.6	B2	
PR132	21	1																																	17	10	10 (10-10)	10±0	A	
PR050	45	0																																	12	10	10 (10-10)	10±0	A	
PR056	58	0																																	19	10	6.3 (4.6-8.6)	6.3±1.5	B2	
PR072	23	4																																	17	10	10 (4.1-10)	8.7±2.3	B2	
PR139	6	2																																	12	10	10 (4.6-10)	9.0±1.9	A	
PR075	40	1																																	18	10	10 (5.3-10)	8.8±2.0	B2	
PR123	41	5																																	15	10	10 (5.3-10)	9.2±1.8	B2	
PR017	2	0																																	22	10	9.3 (7.6-10)	9.1±1.0	B2	
PR140	32	2																																	12	10	6.9 (3.9-10)	7.3±2.1	A	

Strain	Origin	Resistance	csgA	crlA	fimC	hlyA	iha	papC	sfa/foc	tsh	mat	chuA	fyuA	ireA	iroN	irp2	iucD	sit chr	sit Ep	cvi/cva	iss	neuC	kpsMTIIT	ompA	traT	astA	sat	vat	hlyA	gimB	ibeA	tia	plc	malX	VAGs	LS	PS median (range)	PS mean ± SD	ECOR	Cluster
PR012	2	0																																	15	10	6.9 (5.9-10)	7.3±1.5	A	
PR088	6	1																																	13	10	10 (5.6-10)	8.5±1.9	A	
PR083	41	4																																	18	10	10 (6.3-10)	9.6±1.2	D	C
PR138	16	1																																	12	10	7.4 (6.3-10)	7.9±1.6	B2	
PR101	34	0																																	16	8	6.4 (1.0-9.3)	5.4±2.8	D	
PR091	36	3																																	15	10	7.6 (4.6-10)	7.7±1.6	A	
PR003	2	5																																	15	10	6.3 (4.6-10)	6.4±1.5	A	
PR045	1	5																																	16	10	8.6 (5.3-10)	8.2±1.9	D	
PR008	2	0																																	20	4	2.5 (1.0-7.9)	3.7±3.1	D	
PR029	50	0																																	21	10	10 (5.3-10)	9.4±1.5	B2	D
PR015	2	2																																	14	6	5.6 (2.0-7.6)	5.1±2.1	D	
PR102	15	0																																	9	10	10 (5.6-10)	8.8±1.9	A	
PR040	59	0																																	19	10	10 (10-10)	10±0	B2	
PR043	59	1																																	19	10	10 (6.3-10)	9.4±1.4	B2	
PR004	2	4																																	16	10	10 (6.3-10)	9.2±1.4	D	
PR019	11	7																																	15	9	7.9 (1.0-9.3)	7.1±2.4	B1	E
PR142	29	2																																	13	10	10 (7.3-10)	9.7±0.9	B1	
PR038	59	0																																	19	10	6.3 (5.3-10)	6.7±1.8	D	
PR039	49	3																																	13	10	10 (5.3-10)	9.1±2.0	D	
PR115	21	3																																	14	10	10 (10-10)	10±0	B2	
PR137	3	0																																	12	10	6.8 (5.6-10)	7.5±1.8	D	
PR118	36	5																																	12	8	4.7 (0-8.6)	4.6±3.0	A	
PR020	65	3																																	11	7	7.8 (1.0-10)	6.9±3.5	A	G
PR064	10	0																																	11	10	9.6 (7.3-10)	9.3±0.9	D	H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PR044 61
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PR077 34
PR093 13
PR099 41
PR068 9
PR035 52
PR116 62
PR119 22
PR120 7
PR121 22
PR094 27
PR112 38
PR037 58
PR107 33
PR109 18
PR058 26
PR061 4
PR130 26
PR016 2
PR084 24
PR087 20
PR086 41
PR055 45
PR078 34
PR079 35
PR081 21
PR082 34
PR026 65
PR113 13
PR127 32
PR131 32
PR021 48
PR053 45
PR122 28
PR051 45
PR128 31
PR009 2
PR041 55
PR059 5
PR090 5
PR104 5
PR135 40
PR057 58
PR111 37
PR132 21
PR050 45
PR056 58
PR072 23
PR139 6
PR075 40
PR123 41
PR017 2
PR140 32
PR012 2
PR088 6
PR083 41
PR138 16
PR101 34
PR091 36
PR003 3
PR045 1
PR008 2
PR029 50
PR015 2
PR102 15
PR040 59
PR043 59
PR004 2
PR019 11
PR142 29
PR038 59
PR039 49
PR115 21
PR137 3
PR118 36
PR020 65
PR064 10
A
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4. ARTIGO 2

Genotypes and pathogenicity of colisepticemic isolates of avian pathogenic *Escherichia coli*

Nicolle Lima Barbieri, Aline Luísa de Oliveira, Thiago Moreira Tejkowski, Daniel Brisotto Pavanelo, Letícia Beatriz Matter, Sandra Regina Schincariol Pinheiro, Tânia Mara Ibelli Vaz, Benito Guimarães de Brito, Lisa K. Nolan, Catherine M. Logue, Fabiana Horn

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) are the etiological agent of localized or systemic extraintestinal infections in poultry, and both cause great economic losses to the poultry industry. We have determined the prevalence of 34 virulence-associated genes in 52 isolates from colisepticemic broiler chickens from South Brazil. All strains harbor virulence factors related to adhesion, iron acquisition and serum resistance, frequently found in APEC, and the most frequent factors were *fimC*, *csgA* and *crlA.* Here we performed the ECOR phylogenetic typing in isolates using the Clermont method. Most isolates (39%) belonged to group D; 29% belonged to group A; 15%, to group B2 and 17%, to group B1. We have also assessed their clonal relationship by phylogenetic analysis using the Amplified Ribosomal DNA Restriction Analysis (ARDRA) and pulse-field gel eletrophoresis (PFGE) methods. Our analysis did not identify a prevalent APEC clone; instead, the population of APEC isolates was diverse, with few strains belonging to the same clone, and we found that one animal could be infected with 2 distinct pathogenic strains. In one case, three distinct isolates were isolated from liver, heart and intestine of the same colisepticemic bird. All isolates were tested for virulence in one-day-old COBB chicks. The lethality score represents the number of animals that died within seven days, while the pathogenicity score combines the times of death with clinical evidence of air sacculitis, pericarditis, perihepatitis, peritonitis and cellulitis, as observed postmortem. According to lethality score, most isolates (67%) were lethal, and almost half of isolates (58%) were highly pathogenic to one-day-old chicks. Our results represent genotyping and *in vivo* virulence testing of colisepticemic isolates surveyed in a single analysis to date, and also we found for the first time that one animal could be infected with 2 distinct pathogenic strains.

Keywords: avian pathogenic *Escherichia coli*; avian cellulitis; virulence factors; phylogenetic grouping; *in vivo* virulence.

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) are the etiological agent of extraintestinal infections in poultry, known as colibacillosis. Colibacillosis is one of the main causes of morbidity and mortality in chickens, affecting all stages of production, and causing significant economic losses to the poultry industry worldwide. The infection normally initiates in the upper respiratory tract and affects other organs - such as lungs, air sacs, liver, heart and spleen - often culminating in a generalized infection (Barnes and Gross 1997; Dziva and Stevens 2008).

Brazil is the largest exporter and the third largest producer of poultry meat in the world (UBABEF 2013). Colibacillosis affects all stages of production, and it causes significant economic losses to the poultry industry (Dozois and others 1992; Johnson and others 2006). Considering the loss of colibacillosis as predicted by Fallavena and collaborators (Fallavena and others 2000) and the production of poultry meat in 2012 (UBABEF 2013), about 29 thousand tons of meat were lost just due to APEC infection in this year, accounting for almost 14 million dollars (Alves and others 2006).

One of the primary control measures for reducing morbidity and mortality caused by APEC infections is antimicrobial therapy. However, the constant monitoring of antimicrobial use is necessary to avoid the selection of resistant isolates and preserve these drugs for therapy both in animals and humans (Gyles 2008).

The virulence associated genes commonly associated with APEC are the fimbrial adhesins type 1 fimbriae and temperature- sensitive haemagglutinin (Tsh), iron-scavenging systems, outer membrane proteins, the protectin Iss; and the K1 capsule and O1, O2, O8, O11, O18, O25, O36, O78, O111 serogroups (Dziva and Stevens 2008; Rodriguez-Siek and others 2005b). The virulence genes permit APEC strains to adhere to host tissues, survive within host extraintestinal tissues fluids and resist host immune defenses (Brito and others 2003; Ewers and others 2007).

The fact that APEC strains have diverse combinations of virulence associated genes, makes it necessary to do *in vivo* assays to determine whether that an *E. coli* isolated from a diseased chicken is able to cause an extraintestinal infection (Schouler and others 2012), and also to determine the degree of virulence of the strain (Barbieri and others 2013).

The objective of this study was to analyze the prevalence of virulence associated factors and antimicrobial susceptibility in *E. coli* isolates from colisepticemic broiler chickens in Southern Brazil. We analyzed 52 isolates from 30 animals with clinical symptoms of colibacilosis for virulence associated genes, antimicrobial susceptibility, virulence in 1-day old chicks, serotype, phylogenetic typing, and performed ARDRA and PFGE to establish the clonal clonal relationships of these isolates.

We used phylogenetic analysis as a tool to measure the genetic diversity among isolates using molecular markers. It is an important tool to identify the differences among isolates in the same geographic region and among virulence associated genes. These approaches allowed us to characterize APEC strains from a specific geographic area (García-Martínez and others 1996; Lee and Maurer 2000). In addition, we found for the first time that one animal could be infected with 2 distinct pathogenic strains.

Material and Methods

Bacterial strains

Fifty two *E. coli* isolates were obtained from colisepticemic lesions from 30 7-week-old broiler chickens, at the time of their slaughter, from September 2007 to January 2008, and from January 2010 to May 2010. Separete sampling of strains were isolated from different organs from: heart, liver, air sacs, cellulitis and lesions of intestine.

The isolates were collected from different poultry flocks in 14 distinct farms in various locations within the southern Brazilian state of Rio Grande do Sul (RS). Biochemical tests (triple sugar iron, urease and MacConkey) were performed to confirm that all isolates were *E. coli* (CLSI 2009). All strains were stored at -80 ^{o}C in Luria-Bertani (LB) broth with 20% glycerol until they were needed.

Antimicrobial resistance in APEC

The antimicrobial susceptibility of all APEC isolates was examined using the disc diffusion test according to the Clinical and laboratory Standrds Institute guidelines (CLSI 2008; 2009; Sayah and others 2005; Up 2003) and using *E. coli* strain ATCC 25922 as a control, as described (Barbieri and others 2013). The antimicrobial aents tested were: ampicillin (10 U), bacitracin (10 U), cephalothin (30 µg), ceftiofur (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), neomycin (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), tetracycline (30 µg), sulphonamides (300 µg), trimethoprim (5 µg) and a combination of sulphonamides and trimethoprim (23.7 µg plus 1.3 µg).

DNA extraction and multiplex polymerase chain reactions and

Bacterial DNA was obtained from whole organisms by boiling (Barbieri and others 2012). The extracts were stored at 4^{o}C, and the supernatants were used as templates for gene amplification.

The presence of 34 virulence-associated genes in the isolates was investigated using multiplex polymerase chain reactions as described in (Barbieri and others 2012).

Serotyping

Serotyping was performed by tube agglutination with rabbit anti-*E. coli* immune sera produced against a panel of antigenic test strains of *E. coli* containing *E. coli* O groups 1–173 and *E. coli* H groups 1–56.

PCR-based classification into "ECOR" phylogenetic groups

All 52 isolates were classified using the multiplex PCR-based phylogenetic typing method of Clermont (Clermont and others 2000), which groups strains into the four main phylogenetic groups shown in the reference strains in the ECOR collection (Herzer and others 1990). Reactions were performed as described in (Barbieri and others 2012).

ARDRA Phylogenetic analysis

Genetic data was obtained using the Amplified Ribosomal DNA Restriction Analysis (ARDRA) method as previously described in Barbieri, 2013 (Barbieri and others 2013).

PFGE Phylogenetic analysis

All strains were subjected to molecular subtyping using PFGE. Isolates were analyzed using the method of Ribot (Ribot and others 2006). Briefly, frozen isolates were struck to TSA plates and incubated at 37 ^{o}C for 18–24 h. A single colony was inoculated onto a second TSA plate and incubated at 37 ^{o}C for 18–24 h. Colonies were transferred to 5-mL polystyrene round-bottom tubes containing 2 mL of cell suspension buffer (100mM Tris HCl [pH 8.0], Invitrogen; and 100mM EDTA [pH8.0],Gibco), adjusting the concentrations to an absorbance of 0.9–1.1 in a spectrophotometer at 610 nm. After that, the preparation, lysis, and washes of plugs, and then the *XbaI* enzyme restriction

digestion were performed according to the PulseNet protocol. Salmonella Branderup H9812 was used as a DNA marker. The electrophoresis was carried out in a Chef Mapper (Bio-Rad Laboratories) PFGE rig, with an initial switch time of 5 s, a final switch time of 40 s, and a total running time of 17 h 45 min.

After staining the gels with ethidium bromide, they were visualized using a UVP imager. Macrorestriction patterns were compared using the BioNumerics Fingerprinting software (Ver 6.5 Applied Math, Austin,TX). The similarity index was calculated using the Dice coefficient, a band position tolerance of 1%, and an optimization of 0.5%. The unweighted-pair group method with arithmetic averages was used to construct a dendrogram, and clusters were selected using a cutoff at 80% (Buyukcangaz E and others 2013).

Lethality and pathogenicity tests

Lethality and pathogenicity tests were performed as described (Barbieri and others 2013; Barbieri and others 2012). Briefly, groups of 10 one-day-old Cobb female chicks were inoculated subcutaneously with 100 µL (10^8 CFU) of an overnight culture containing ~10^9 CFU/mL of each APEC isolate. A control group was inoculated with BHI broth. The animals were observed at 12 h intervals over 7 days, with all deaths being recorded. The lethality score (LS) was calculated according to the number of animals that died within this period with a range from 0 (no animal died) to 10 (all animals died). At 7 days post-infection, surviving chicks were killed by cervical dislocation, and clinical scores were recorded. Times of death and clinical scores were combined to give pathogenicity scores (PS). We performed postmortem examinations, looking for evidence of air sacculitis (A), pericarditis (P), perihepatitis (Ph), peritonitis (Pe) and cellulitis (C). The presence of a lesion was given the value 1, and its absence, the value 0. Pathogenicity scores (PS) were calculated from the equation PS = (TD x 5) + P + Pe + Ph + A + C, in which TD corresponds to the day of chick death, which has a value of 1 if the animal dies on the first day, and is reduced by 0.14 for each day that the animal survives up to day 7, which has the value 0. According to this equation, the PS can vary from 0 to 10.

Animals that died on the first day after inoculation had their livers dissected, homogenized and plated on lactose-containing MacConkey agar to

identify *E. coli*; a PS = 10 score was attributed to these animals. The PS for each strain was calculated as the median PS for the 10 chicks infected with that particular strain.

Ethics statement

All animal experiments were approved by the Biosafety Committee of the Instituto de Pesquisas Veterinárias Desidério Finamor (CIB 004/08), and chickens were euthanized according to animal welfare norms.

RESULTS

Antibiotic resistance in APEC

We tested 52 APEC isolates for their susceptibility to 15 antimicrobial agents that used to be commonly employed in the poultry industry in Brazil as growth promoters, for infection prevention and/or treatment. We observed that APEC isolates were susceptible to the majority of antimicrobials, with less than 25% of the isolates being resistant to any antimicrobial, with the exceptions of antimicrobials that act in the cell wall: ampicillin (75%), bacitracin (81%), cephalothin (65%) and ceftiofur (56%); and antimicrobials that are for a long time in the market such as tetracycline and sulphonamides (with 69.4% and 59.7% respectively) (Fig.1).

Considering the site of antibiotic action, 94% of APEC isolates were resistant to at least one of the antimicrobials that act in the cell wall (ampicillin, bacitracin, cephalothin, ceftiofur); 15% were resistant to at least one of the antimicrobials that inhibit the nucleic acid synthesis (ciprofloxacin, enrofloxacin, norfloxacin); 65 % were resistant to at least one of the antimicrobials that act in inhibition of protein synthesis (chloramphenicol, gentamicin, neomycin, tetracycline; but only 33% if we exclude tetracycline); and 56% were resistant to at least one of the antimicrobials that antagonize the metabolic activity (sulphonamides, trimethoprim, sulpha + trimethoprim; but only 6% if we exclude sulphonamides).

When we analyze the multiresistance pattern, we observed that 31% of APEC isolates were resistant to at least one antimicrobial class; 19% of APEC isolates were resistant to 2; 38%, to 3; and 12%, to all 4 classes. It is noteworthy that there was no antimicrobial agent to which all 52 APEC strains were susceptible.

Genotyping by multiplex polymerase chain reaction

Prevalence of VAGs among APEC isolates is shown in Figure 2 (shadowed area). The genes *fimC, csgA* and *crlA* were present in all isolates, and *afa/dra* was not detected in any isolate. Figure 3 specifies the profile of

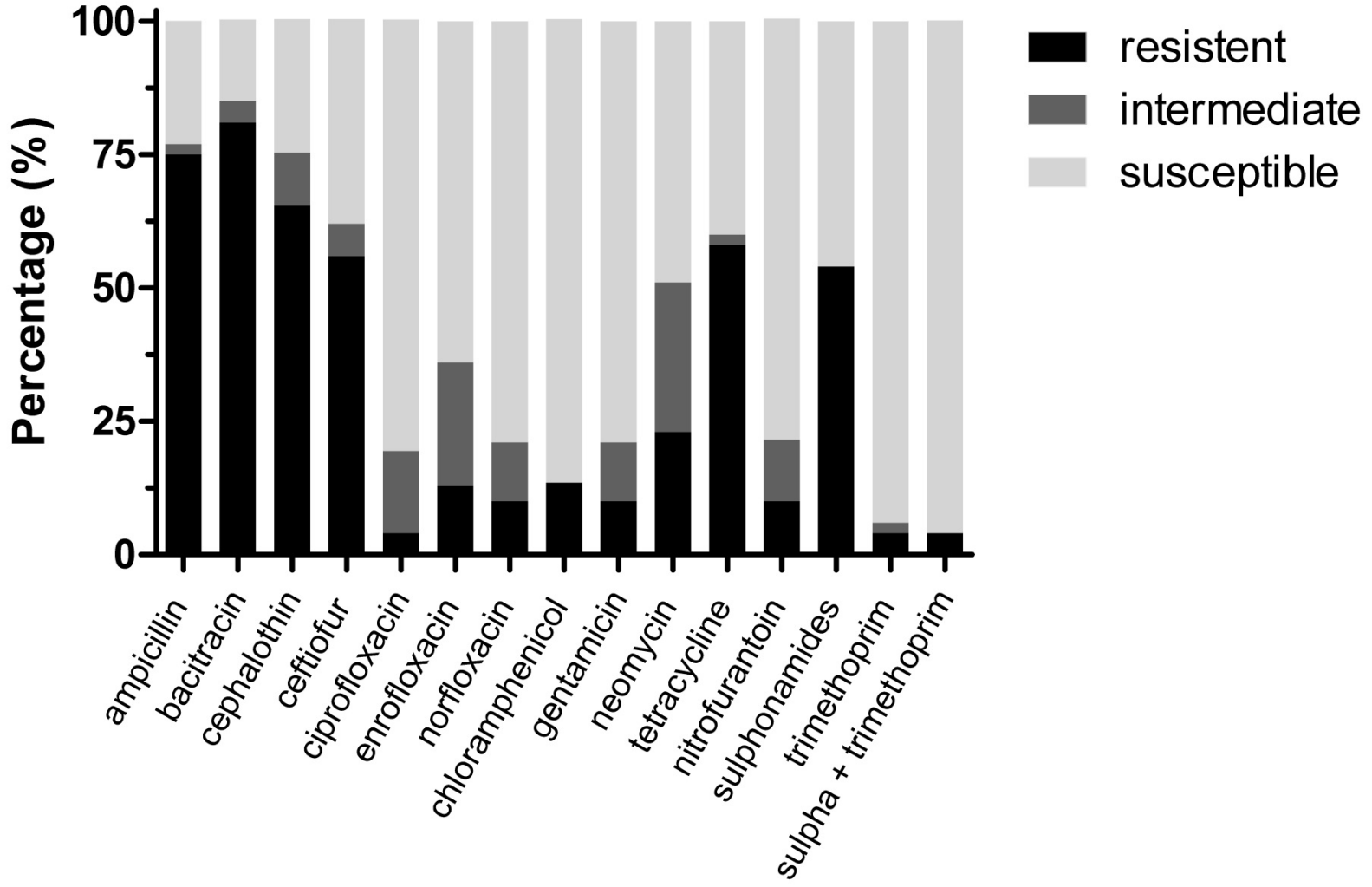


Figure 1. Antimicrobial susceptibility of colisepticemic isolates. The susceptibility of 52 APEC isolates to 15 antimicrobials was tested individually using disc diffusion tests

	sfa/foc	*malX*	*neuC*	*iha*	*hrlA*	*pic*	*kpsMTIIT*	*sitEp*	*ompA*	*iroN*	*gimB*	*sitChr*	*traT*	*ibeA*	*chuA*	*vat*	*tsh*	*iucD*	*cvi/cva*	*papC*	*irp2*	*iss*	*EAST1*	*mat*	*fyuA*	*tia*	*ireA*	*sat*	*cnf1/2*	*hlyA*
sfafoc	3.8																													
malX	1.9	7.7																												
neuC	1.9	7,7*	7.7																											
iha	1.9	3.8	3.8	21.2																										
hrlA	3.8	5.8	5.8	13,5*	32.7																									
pic	1.9	0.0	0.0	5.8	5.8	15.4																								
kpsMTII	3.8	7,7*	7,7*	9.6	17.3	11,5*	40.4																							
sitEp	1.9	1.9	1.9	7.7	17.3	9.6	15.4	46.2																						
ompA	3.8	7.7	7.7	21.2	30.8	15.4	40.4	46.2	92.3																					
iroN	3.8	3.8	3.8	5.8	15.4	7.7	23,1*	19.2	36.5	36.5																				
gimB	1.9	1.9	1.9	1.9	1.9	1.9	3.8	3.8	5.8	3.8	5.8																			
sit_chr	1.9	1.9	1.9	1.9	5.8	1.9	11,5*	7.7	15.4	15,4*	3.8	15.4																		
traT	3.8	7.7	7.7	19.2	25.0	13.5	32.7	32.7	71.2	23.1	1.9	5,8*	78.8																	
ibeA	0.0	3.8	3.8	0.0	5.8	1.9	9.6	5.8	13.5	9.6	1.9	5.8	7.7	13.5																
chuA	3.8	5.8	5.8	19.2	25.0	9.6	21.2	25.0	55.8	23.1	5.8	9.6	50.0	13,5*	61.5															
vat	0.0	1.9	1.9	5.8	5.8	1.9	11.5	1,9*	17.3	11.5	0.0	3.8	15.4	3.8	17.3	19.2														
tsh	3.8	3.8	3.8	7.7	17.3	7.7	25.0	19.2	44.2	25,0*	5.8	11.5	34.6	5.8	32.7	19,2*	48.1													
iucD	1.9	7.7	7.7	19.2	28.8	11.5	34.6	38.5	78.8	30.8	3.8	11.5	71.2	9.6	51.9	19.2	44.2	86.5												
cvi/cva	3.8	5.8	5.8	15.4	23.1	11.5	28.8	36,5*	59.6	23.1	3.8	9.6	50.0	3.8	28,8*	5,8*	26.9	55.8	61.5											
papC	1.9	1.9	1.9	7.7	11.5	5.8	9.6	11.5	23.1	9.6	3.8	5.8	23.1	3.8	21.2	3.8	7.7	17.3	13.5	25.0										
irp2	1.9	0.0	0.0	13.5	19.2	11.5	21.2	23.1	48.1	19.2	1.9	7.7	48.1	5.8	40.4	17,3*	30.8	51.9	34.6	15.4	55.8									
iss	3.8	7.7	7.7	19.2	28.8	13.5	38.5	38.5	80.8	36.5	5.8	15.4	67.3	13.5	53.8	17.3	44.2	76.9	57.7	17,3*	50.0	88.5								
EAST1	1.9	3.8	3.8	11.5	19.2	9.6	17.3	13.5	34.6	13.5	1.9	5.8	32.7	7.7	34,6*	13.5	21.2	32.7	15,4*	15.4	30,8*	40,4*	40.4							
mat	3.8	7.7	7.7	21.2	32.7	13.5	38.5	42.3	88.5	23.1	1,9*	13.5	78,8*	11.5	57.7	19.2	44.2	84.6	59.6	23.1	53.8	32.7	38.5	96.2						
fyuA	1.9	5,8*	5,8*	9,6*	15,4*	1.9	11.5	11.5	19.2	15,4*	1.9	5.8	11.5	1.9	9.6	5.8	13.5	19.2	17.3	5.8	7.7	84.6	3.8	19.2	19.2					
tia	1.9	1.9	1.9	9.6	15,4*	3.8	7.7	15.4	23.1	7.7	0.0	1.9	19.2	0.0	15.4	1.9	7.7	21.2	17.3	11.5	17.3	19.2	13.5	25.0	9.6	25.0				
ireA	1.9	7.7	7.7	17.3	23.1	9.6	26.9	34.6	63.5	30.8	1.9	11.5	55.8	9.6	40.4	15.4	32.7	63.5	44.2	13.5	36.5	63.5	21.2	69.2	19,2*	17.3	69.2			
sat	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	1.9	0.0	0.0	1.9	0.0	1.9	1.9	1.9	1.9	1.9	0.0	1.9	0.0	1.9		
cnf1/2	0.0	0.0	0.0	0.0	0.0	1.9	0.0	1.9	1.9	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	1.9	1.9	0.0	1.9	1.9	0.0	1.9	0.0	0.0	1.9	0.0	1.9	
hlyA	0.0	0.0	0.0	1.9	1.9	0.0	1.9	1.9	3.8	1.9	0.0	0.0	1.9	0.0	1.9	1.9	1.9	3.8	3.8	0.0	1.9	3.8	0.0	3.8	1.9	0.0	3.8	0.0	0.0	3.8

Figure 2. Association between presence of 30 VAGs among 52 APEC isolates.
Numbers indicate the percentage of isolates that have both traits, while numbers in shadowed boxes indicate the percentage of isolates that have the corresponding trait; * $p \leq 0.005$ using χ^2. ND, not determined.[1] *afa* is not included, since they were absent in all strain, and *fimC, crlA* and *csgA* is not included, since they were present in all strains.

Table 1. ECOR groups among the 52 colisepticemic isolates (whithin parentheses, number of strains) and respective mean for antimicrobial resistance, number of VAGs, lethality scores and pathogenicity scores.

	ECOR groups (number of isolates)			
	A (15)	**B1 (9)**	**B2 (8)**	**D (20)**
Frequency (%)	**29**	**17**	**15**	**39**
Resistance[1]	**4.7**	**5.0**	**6.1**	**6.7**
VAGs (34)	**12.9**	**13.1**	**16.3**	**14.9**
Lethality Score	**7.8**	**4.8**	**8.6**	**6.1**
Pathogenicity Score	**6.6**	**4.6**	**7.4**	**5.9**

[1] Mean number of antimicrobials to which the strains were resistant.

VAGs of each isolate; overall, the isolates had an average of 14.2 VAGs (range from 9 to 23 VAGs).

Virulence factors related to adhesion, iron acquisition and serum resistance were present in all strains. ColV plasmid associated genes (*cvi/cva, iroN, iss, iucD, sitD, traT, tsh*) occurred in the majority (from 88 to 36 %) of the isolates, and *cvi/cva* itself was present in 61% of the isolates (Fig 3).

The isolates have an average of 4.8 genes associated to adhesion (range from 3 to 8 of 9 tested), 3.9 associated to iron acquisition systems (range from 1 to 7 of 8 tested), 3.7 associated to serum resistance (range from 3 to 6 of 6 tested), 0.7 associated to production of toxins (range from 0 to 2 of 5 tested) and 0.4 genes associated to invasins (range from 0 to 2 of 3 tested).

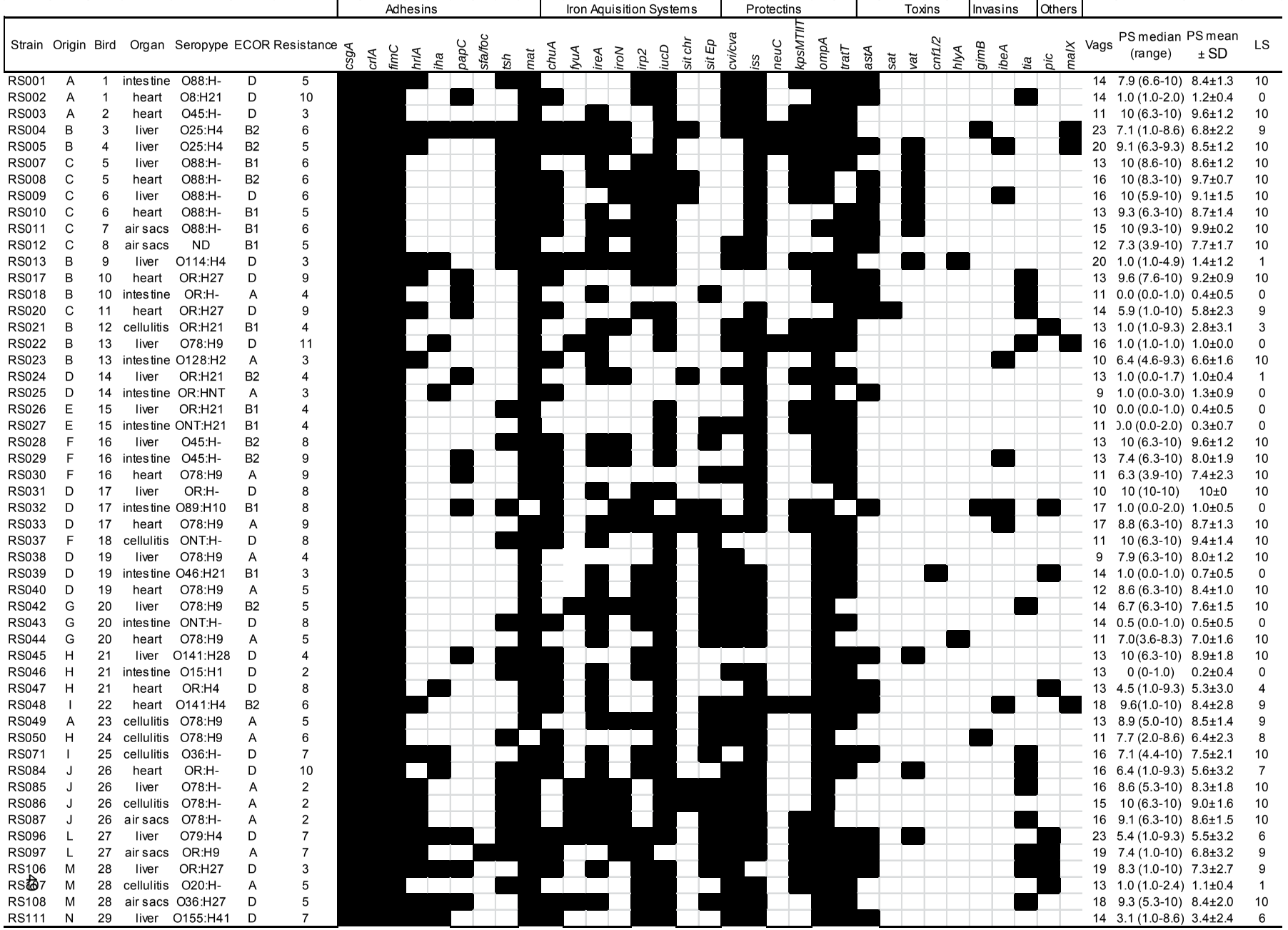
Serotyping

Among 51 serotyped APEC strains, 36 (69%) could be classified into a single O-type, while for 15 (29 %) of the strains the O-type could not be determined (Figure 3 and Table 2). Most of the typeable strains belonged to O-types O78 (23%), O88 (12%) and O45 (6%), while 28% were distributed among 12 different O-types. Only 3 isolates were non-typeable with any of O-serum used, but 12 isolates displayed a rough phenotype.

Moreover, 31 (60%) APEC strains could be classified into a single H-type. Most of strains belonged to H-types H9 (20%), H4 (12%), H21 (12%), and H27 (8%). Only 1 strain was non-typeable with any of H-serum used, whereas 19 isolates were non-motile.

Lethality and pathogenicity tests

Lethality and pathogenicity tests in 1 day-old chicken were used to evaluate the virulence of APEC isolates, and results for each strain are presented in Figure 3. Most isolates were very lethal: the number of isolates that killed 10 chickens within 7 days (LS = 10) was 28 (54 %), and the number of isolates that did not kill any chickens was 10 (19 %). Overall, the APEC isolates had a mean lethality score of 6.9.



Strain	Origin	Bird	Organ	Seropype	ECOR	Resistance	csgA	crlA	fimC	hrlA	iha	papC	sfa/foc	tsh	mat	chuA	fyuA	ireA	iroN	irp2	iucD	sit chr	sit Ep	cvi/cva	iss	neuC	kpsMTIIT	ompA	tratT	astA	sat	vat	cnf1/2	hlyA	gimB	ibeA	tia	pic	malX	Vags	PS median (range)	PS mean ± SD	LS
RS001	A	1	intestine	O88:H-	D	5																																			14	7.9 (6.6-10)	8.4±1.3	10
RS002	A	1	heart	O8:H21	D	10																																			14	1.0 (1.0-2.0)	1.2±0.4	0
RS003	A	2	heart	O45:H-	D	3																																			11	10 (6.3-10)	9.6±1.2	10
RS004	B	3	liver	O25:H4	B2	6																																			23	7.1 (1.0-8.6)	6.8±2.2	9
RS005	B	4	liver	O25:H4	B2	5																																			20	9.1 (6.3-9.3)	8.5±1.2	10
RS007	C	5	liver	O88:H-	B1	6																																			13	10 (8.6-10)	8.6±1.2	10
RS008	C	5	heart	O88:H-	B2	6																																			16	10 (8.3-10)	9.7±0.7	10
RS009	C	6	liver	O88:H-	D	6																																			16	10 (5.9-10)	9.1±1.5	10
RS010	C	6	heart	O88:H-	B1	5																																			13	9.3 (6.3-10)	8.7±1.4	10
RS011	C	7	air sacs	O88:H-	B1	6																																			15	10 (9.3-10)	9.9±0.2	10
RS012	C	8	air sacs	ND	B1	5																																			12	7.3 (3.9-10)	7.7±1.7	10
RS013	B	9	liver	O114:H4	D	3																																			20	1.0 (1.0-4.9)	1.4±1.2	1
RS017	B	10	heart	OR:H27	D	9																																			13	9.6 (7.6-10)	9.2±0.9	10
RS018	B	10	intestine	OR:H-	A	4																																			11	0.0 (0.0-1.0)	0.4±0.5	0
RS020	C	11	heart	OR:H27	D	9																																			14	5.9 (1.0-10)	5.8±2.3	9
RS021	B	12	cellulitis	OR:H21	B1	4																																			13	1.0 (1.0-9.3)	2.8±3.1	3
RS022	B	13	liver	O78:H9	D	11																																			16	1.0 (1.0-1.0)	1.0±0.0	0
RS023	B	13	intestine	O128:H2	A	3																																			10	6.4 (4.6-9.3)	6.6±1.6	10
RS024	D	14	liver	OR:H21	B2	4																																			13	1.0 (0.0-1.7)	1.0±0.4	1
RS025	D	14	intestine	OR:HNT	A	3																																			9	1.0 (0.0-3.0)	1.3±0.9	0
RS026	E	15	liver	OR:H21	B1	4																																			10	0.0 (0.0-1.0)	0.4±0.5	0
RS027	E	15	intestine	ONT:H21	B1	4																																			11	0.0 (0.0-2.0)	0.3±0.7	0
RS028	F	16	liver	O45:H-	B2	8																																			13	10 (6.3-10)	9.6±1.2	10
RS029	F	16	intestine	O45:H-	B2	9																																			13	7.4 (6.3-10)	8.0±1.9	10
RS030	F	16	heart	O78:H9	A	9																																			11	6.3 (3.9-10)	7.4±2.3	10
RS031	D	17	liver	OR:H-	D	8																																			10	10 (10-10)	10±0	10
RS032	D	17	intestine	O89:H10	B1	8																																			17	1.0 (0.0-2.0)	1.0±0.5	0
RS033	D	17	heart	O78:H9	A	9																																			17	8.8 (6.3-10)	8.7±1.3	10
RS037	F	18	cellulitis	ONT:H-	D	8																																			11	10 (6.3-10)	9.4±1.4	10
RS038	D	19	liver	O78:H9	A	4																																			9	7.9 (6.3-10)	8.0±1.2	10
RS039	D	19	intestine	O46:H21	B1	3																																			14	1.0 (0.0-1.0)	0.7±0.5	0
RS040	D	19	heart	O78:H9	A	5																																			12	8.6 (6.3-10)	8.4±1.0	10
RS042	G	20	liver	O78:H9	B2	5																																			14	6.7 (6.3-10)	7.6±1.5	10
RS043	G	20	intestine	ONT:H-	D	8																																			14	0.5 (0.0-1.0)	0.5±0.5	0
RS044	G	20	heart	O78:H9	A	5																																			11	7.0(3.6-8.3)	7.0±1.6	10
RS045	H	21	liver	O141:H28	D	4																																			13	10 (6.3-10)	8.9±1.8	10
RS046	H	21	intestine	O15:H1	D	2																																			13	0 (0-1.0)	0.2±0.4	0
RS047	H	21	heart	OR:H4	D	8																																			13	4.5 (1.0-9.3)	5.3±3.0	4
RS048	I	22	heart	O141:H4	B2	6																																			18	9.6(1.0-10)	8.4±2.8	9
RS049	A	23	cellulitis	O78:H9	A	5																																			13	8.9 (5.0-10)	8.5±1.4	9
RS050	H	24	cellulitis	O78:H9	A	6																																			11	7.7 (2.0-8.6)	6.4±2.3	8
RS071	I	25	cellulitis	O36:H-	D	7																																			16	7.1 (4.4-10)	7.5±2.1	10
RS084	J	26	heart	OR:H-	D	10																																			16	6.4 (1.0-9.3)	5.6±3.2	7
RS085	J	26	liver	O78:H-	A	2																																			16	8.6 (5.3-10)	8.3±1.8	10
RS086	J	26	cellulitis	O78:H-	A	2																																			15	10 (6.3-10)	9.0±1.6	10
RS087	J	26	air sacs	O78:H-	A	2																																			16	9.1 (6.3-10)	8.6±1.5	10
RS096	L	27	liver	O79:H4	D	7																																			23	5.4 (1.0-9.3)	5.5±3.2	6
RS097	L	27	air sacs	OR:H9	A	7																																			19	7.4 (1.0-10)	6.8±3.2	9
RS106	M	28	liver	OR:H27	D	3																																			19	8.3 (1.0-10)	7.3±2.7	9
RS107	M	28	cellulitis	O20:H-	A	5																																			13	1.0 (1.0-2.4)	1.1±0.4	1
RS108	M	28	air sacs	O36:H27	D	5																																			18	9.3 (5.3-10)	8.4±2.0	10
RS111	N	29	liver	O155:H41	D	7																																			14	3.1 (1.0-8.6)	3.4±2.4	6
				prevalence(%)			100	100	100	33	21	25	4	48	96	62	19	69	37	56	87	15	46	62	88	8	40	92	79	40	2	19	2	4	6	13	25	15	8				

Figure 3. Characterization of 52 APEC isolates. Columns from left to right: S*train*, isolate designation; *Origin*, source of the isolate; *Bird,* same numbers from the same animal; *Serotype*, O and H grouping serotype; *Resistance*, number of antimicrobials to which the isolate was resistant; the subsequent columns depict the PCR results for all VAGs tested, with presence indicated in black and absence indicated in white; *no. VAGs*, total number of VAGs in each isolate; *LS*, lethality score; *PS* pathogenicity score); *ECOR*, ECOR phylogenetic group;

Table 2. Most Prevalence O-type groups among the 52 colisepticemic isolates.

O type	Prevalence frequency %	n
O78	23.1	12
O88	11.5	6
O45	5.8	3
O141	3.8	2
O25	3.8	2
O36	3.8	2
O46	3.8	2
OR	28.8	15
ONT	5.8	3

We used the pathogenicity score to classify strains as highly pathogenic, which presented a PS from 7-10, intermediate pathogenic, with a PS from 3-7, and low pathogenic, with a PS from 0-3. According to this classification, most isolates were highly pathogenic to 1 day-old chickens (58% scored higher than 7) (Fig. 3) and, among these, 9 isolates scored 10. We observed that 14 strains (27%) had a PS lower than 3. Overall, the APEC isolates had a mean pathogenic score of 6.1.

Correlating the presence of VAGs to pathogenicity scores (Table 3), we found that a higher PS was positively linked (p≤0.05) to *cvi/cva* and *vat*, but negatively linked (p≤0.05) to *pic*. However, for the presence of VAGs and lethality scores (Table 4), we found that a higher LS was positively linked (p≤0.05) to the *ireA,* but negatively linked (p≤0.05) to *pic*.

ECOR grouping

We have performed phylogenetic typing of the APEC isolates using the Clermont method(Clermont and others 2000). The distribution of APEC isolates among the four phylogenetic groups is shown in Table 1. As determined by PCR, most of the strains (39%) belonged to group D.

Table 1 also shows the mean number of antimicrobials to which strains were resistant, mean number of VAGs, pathogenicity and lethality scores of strains according to different ECOR groups. We observed that isolates from group B2 had a higher number of VAGs per strain and higher pathogenicity scores, although not significant.

Phylogenetic Analysis

We used the ARDRA method to measure the genetic diversity among the 52 isolates obtained from 30 colisepticemic animals. This method is based on enzyme restriction digestion of amplified 16-23S DNA intergenic spacer regions that is a highly conserved DNA portion. The ARDRA profiles of the strains are shown in Figure 4. From the dendrogram, we found 49 different ARDRA profiles

Table 3. Relationship between APEC median pathogenicity score (PS) and the presence of different genes.

	Gene +		Gene -		
Gene[a]	Average PS	Number of isolates	Average PS	Number of isolates	*p*[b]
hlyA	4.39	2	6.16	50	0.486
hrlA	7.15	17	5.58	35	0.448
iha	4.85	11	6.43	41	0.054
papC	5.45	13	6.31	39	0.229
sfa/foc	7.69	2	6.03	50	0.757
tsh	6.50	25	5.71	27	0.184
mat	6.18	50	3.72	2	0.278
chuA	6.50	32	5.43	20	0.293
fyuA	6.51	10	5.99	42	0.877
ireA	6.56	36	5.03	16	0.191
iroN	6.98	19	5.58	33	0.083
irp2	6.28	29	5.85	23	0.408
iucD	6.45	45	3.76	7	0.059
sit chr	6.69	8	5.98	44	0.406
sit Ep	6.50	24	5.75	28	0.938
cvi/cva	5.57	32	6.93	20	0.036
iss	5.95	46	7.20	6	0.396
neuC	6.15	4	6.09	48	0.876
kpsMTIIT	5.49	21	6.43	31	0.376
ompA	5.93	48	8.05	4	0.258
tratT	5.89	41	6.83	11	0.539
astA	6.46	21	5.85	31	0.502
vat	7.66	10	5.72	42	0.027
gimB	4.74	3	6.17	49	0.272
ibeA	7.17	7	5.92	45	0.473
tia	6.12	13	6.08	39	0.672
pic	4.04	8	6.47	44	0.043
malX	6.15	4	6.09	48	0.876

[a] Gene that occurred in one or none (*afa/dra*, *cnf1/2, sat)* or all (*fimC, crlA, csgA*) isolates are not listed.

[b] Exact *P* values for the Wilcoxon-Mann-Whitney test.

*$p \leq 0.05$.

Table 4. Relationship between APEC median lethality score (LS) and presence of different genes

	Gene +		Gene -		
Gene[a]	Average LS	Number of isolates	Average LS	Number of isolates	*P*[b]
hlyA	5.50	2	6.80	50	1.000
hrlA	8.71	17	5.80	35	0.142
iha	5.64	11	7.05	41	0.091
papC	5.69	13	7.10	39	0.166
sfa/foc	9.00	2	6.67	50	0.843
tsh	6.84	25	6.67	27	0.841
mat	6.86	50	4.00	2	0.118
chuA	7.09	32	6.20	20	0.988
fyuA	7.60	10	6.55	42	0.430
ireA	7.44	36	5.19	16	0.047*
iroN	7.79	19	6.15	33	0.180
irp2	6.79	29	6.70	23	0.918
iucD	7.13	45	4.29	7	0.144
sit chr	7.50	8	6.61	44	0.470
sit Ep	7.50	24	6.11	28	0.446
cvi/cva	6.50	32	7.15	20	0.287
iss	6.78	46	6.50	6	0.913
neuC	7.00	4	6.73	48	0.691
kpsMTIIT	6.10	21	7.19	31	0.312
ompA	6.50	48	9.75	4	0.214
tratT	6.41	41	8.00	11	0.179
astA	6.76	21	6.74	31	0.609
vat	7.40	10	6.60	42	0.506
gimB	5.67	3	6.82	49	0.211
ibeA	8.43	7	6.49	45	0.258
tia	7.23	13	6.59	39	1.000
pic	4.00	8	7.25	44	0.009*
malX	7.00	4	6.73	48	0.691

[a] Genes that occurred in one or none (*afa/dra*, *cnf1/2, sat)* or all (*fimC, crlA,csgA*) isolates are not listed.

[b] Exact *P* values for the Wilcoxon-Mann-Whitney test.

*$p \leq 0.05$.

among the 52 isolates. We could separate the isolates into 2 major clusters at a similarity level of 70% or more (Fig. 4), with the majority of isolates belonging to cluster B. We observed that cluster B isolates had a higher number of VAGs (average of 15.1) and a higher PS (average of 6.5), when compared to cluster A, which had in average of 13.3 VAGs and an average PS of 5.8.

Using the ARDRA method we observed that, from the 30 birds sampled, 9 were infected with 2 or 3 different *E. coli* strains (Fig. 3), indicating that the cause of the disease were a sum of factors of two independent APEC strains.

To confirm that some birds were indeed infected with phylogenetic distinct APEC strains, we performed phylogenetic analysis using the PFGE method. This method uses the total chromosomal content of the bacteria to generate restrictions bands. The PFGE profiles of 48 strains are shown in Figure 5. We found 42 different PFGE profiles among the 48 isolates. From the dendrogram generated, 3 major clusters could be grouped at a similarity level of 60% or more (Fig. 5), with the majority of isolates falling into cluster B. We observed that cluster B isolates had a higher PS (average of 6.4) and higher LS (average of 7.7) when compared to cluster A and C.

The results of the PFGE method confirmed the results obtained by ARDRA, indicating the presence of several clones among the isolates analyzed. Also PFGE agrees to ARDRA showing that 9 animals were infected with 2 or 3 distinct APEC strains. Among the 52 samples, three pairs of isolates in which the two strains had identical restriction pattern were found by ARDRA method. They are: (1) RS007 and RS008 (isolated from the same bird and with the same serotype), (2) RS 004 and RS 005 (isolated from different birds from the same flock with the same serotype), (3) RS021 and RS022 (isolated from different birds of the same flock and with different serotypes). Probably the first and second pair of isolates represents the same strain or two strains that have diverged recently, through horizontal gene transfer. The isolates of the third pair (RS021 and RS022), although they showed the same pattern of restriction by ARDRA method presented a more distant phylogenetic relationship (according to the PFGE), as well as a different serotype, which is sufficient to say that not represent the same strain.

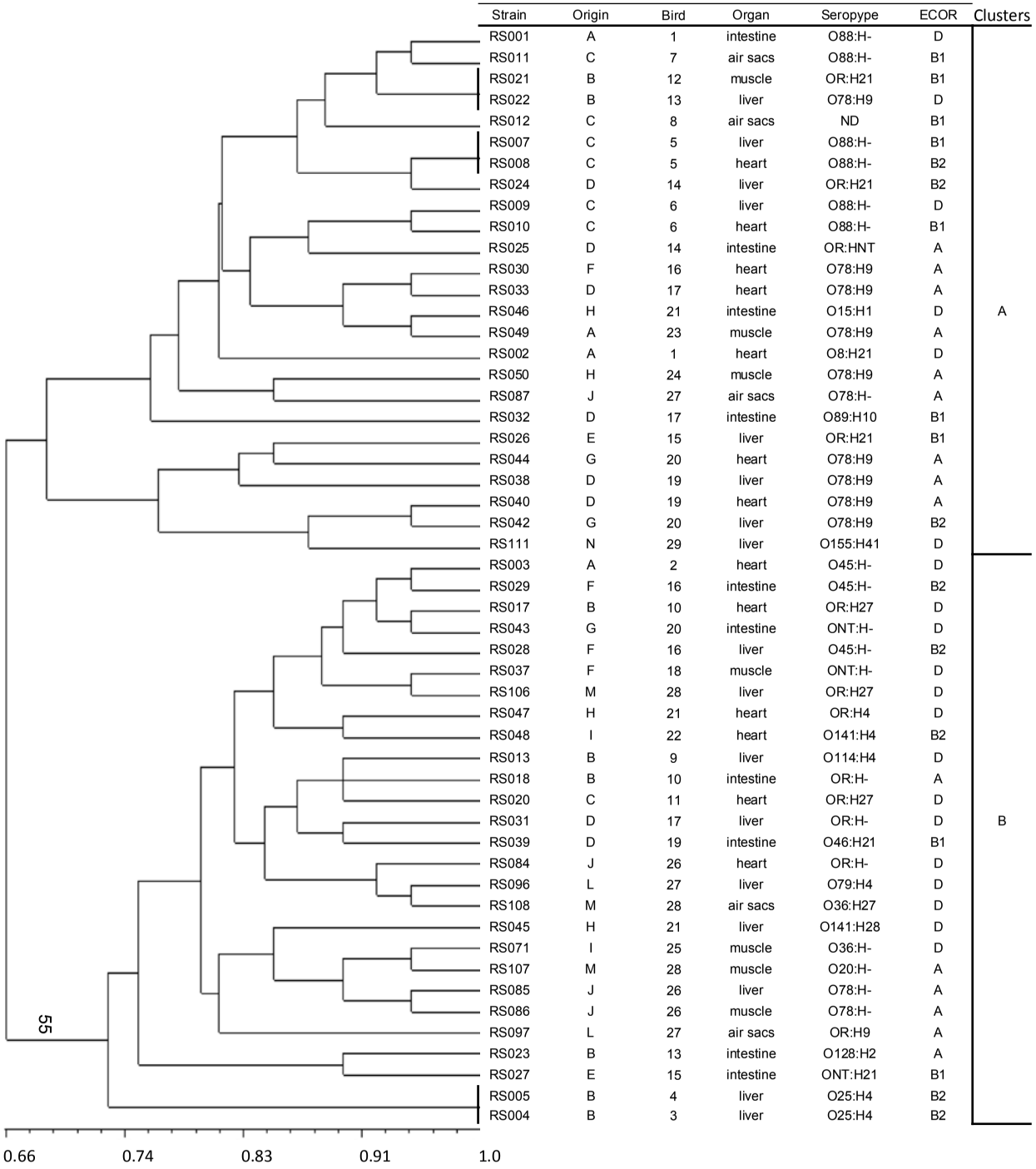
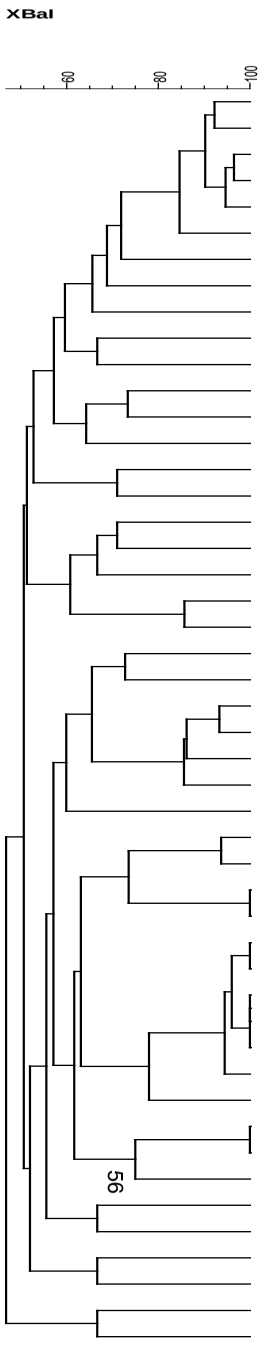
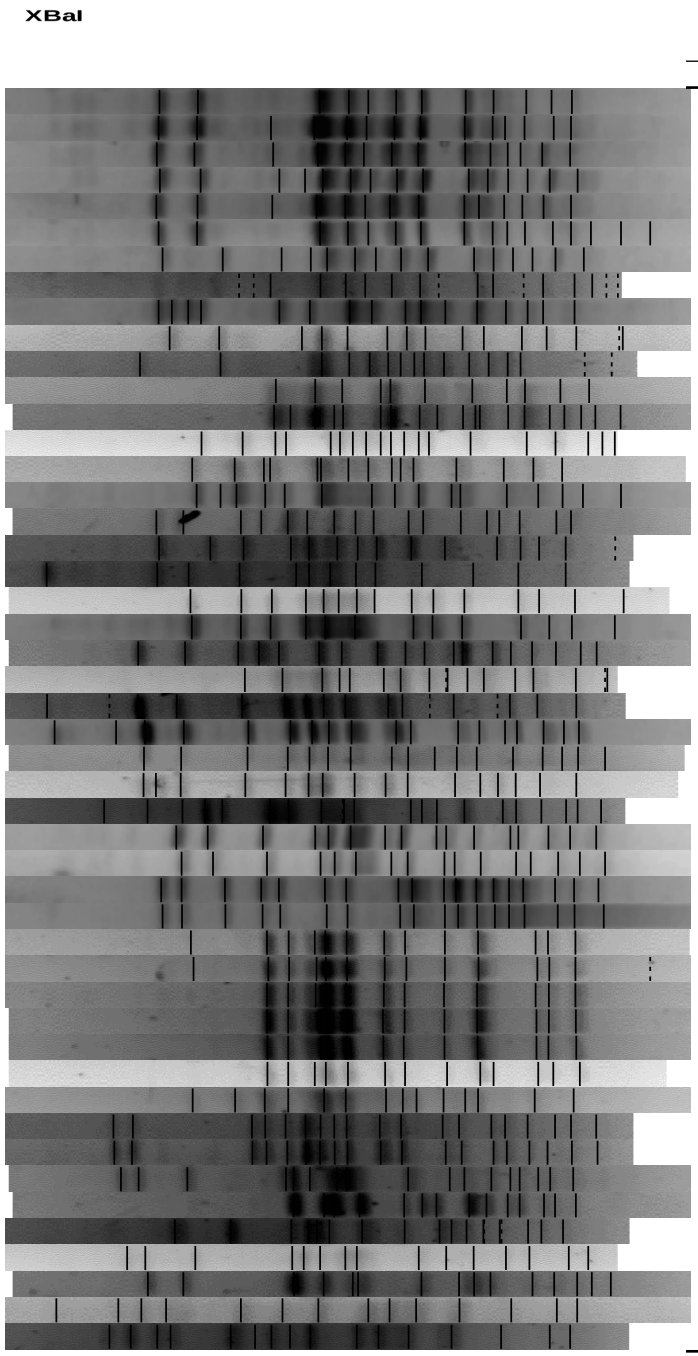


Figure 4. ARDRA profile of 52 APEC isolates. The ARDRA dendrogram was constructed by UPGMA based upon enzyme restriction digestion of amplified 16-23S DNA intergenic spacer regions. The column Strain shows isolate designation; the column Origin, source of isolate, with A to N designating each of the 65 farms from which the isolates were collected. Bird, same numbers from the same animal; Organ, organ of bacteria isolation; Serotype, O and H grouping serotype; ECOR, ECOR phylogenetic group; Cluster designates the 4 genotypic clusters (A to D) into which strains with 80% similarity were grouped. *E. coli* ATCC25922 was analyzed as a reference strain.





Strain	Origin	Bird	Organ	Seropype	ECOR	C
RS038	D	19	liver	O78:H9	A	
RS040	D	19	heart	O78:H9	A	
RS042	G	20	liver	O78:H9	B2	
RS049	A	23	muscle	O78:H9	A	
RS044	G	20	heart	O78:H9	A	
RS033	D	17	heart	O78:H9	A	
RS050	H	24	muscle	O78:H9	A	
RS030	F	16	heart	O78:H9	A	
RS045	H	21	liver	O141:H28	D	
RS107	M	28	muscle	O20:H-	A	
RS111	N	29	liver	O155:H41	D	A
RS022	B	13	liver	O78:H9	D	
RS024	D	14	liver	OR:H21	B2	
RS004	B	3	liver	O25:H4	B2	
RS005	B	4	liver	O25:H4	B2	
RS048	I	22	heart	O141:H4	B2	
RS025	D	14	intestine	OR:HNT	A	
RS097	L	27	air sacs	OR:H9	A	
RS086	J	26	muscle	O78:H-	A	
RS002	A	1	heart	O8:H21	D	
RS039	D	19	intestine	O46:H21	B1	
RS013	B	9	liver	O114:H4	D	
RS021	B	12	muscle	OR:H21	B1	
RS031	D	17	liver	OR:H-	D	
RS037	F	18	muscle	ONT:H-	D	
RS029	F	16	intestine	O45:H-	B2	
RS003	A	2	heart	O45:H-	D	
RS032	D	17	intestine	O89:H10	B1	
RS085	J	26	liver	O78:H-	A	
RS087	J	27	air sacs	O78:H-	A	
RS046	H	21	intestine	O15:H1	D	
RS047	H	21	heart	OR:H4	D	
RS007	C	5	liver	O88:H-	B1	
RS009	C	6	liver	O88:H-	D	B
RS001	A	1	intestine	O88:H-	D	
RS011	C	7	air sacs	O88:H-	B1	
RS012	C	8	air sacs	ND	B1	
RS010	C	6	heart	O88:H-	B1	
RS008	C	4	heart	O88:H-	B2	
RS106	M	28	liver	OR:H27	D	
RS108	M	28	air sacs	O36:H27	D	
RS017	B	10	heart	OR:H27	D	
RS018	B	10	intestine	OR:H-	A	
RS084	J	26	heart	OR:H-	D	
RS020	C	11	heart	OR:H27	D	
RS023	B	13	intestine	O128:H2	A	
RS071	I	25	muscle	O36:H-	D	C
RS096	L	27	liver	O79:H4	D	

Figure 5. Pulse-Field profile of 52 APEC isolates. The PFGE dendrogram was constructed by the unweighted-pair group method with arithmetic averages. The scale indicates levels of similarity within this set of isolates based upon *XbaI* enzyme restriction digestion of total bacterial DNA. The column *Strain* shows isolate designation; the column *Origin*, source of isolate, with A to N designating each of the 65 farms from which the isolates were collected. *Bird,* same numbers from the same animal; *Organ*, organ of bacteria isolation; *Serotype*, O and H grouping serotype; ECOR, ECOR phylogenetic group; Cluster designates the 5 genotypic clusters (A to E) into which strains with 60% similarity were grouped. *E. coli* ATCC25922 was analyzed as a reference strain. The *XbaI* could not digest the strains RS026, RS027, RS028 and RS043.

DISCUSSION

Antimicrobial use in the poultry industry is the first method of controlling morbidity and mortality caused by bacterial infections (Melllata 2013). Here we observed a higher resistance levels for antimicrobials that acts in the cell wall (ampicillim, bacitracin, cephalotin and ceftiofur), in comparison to our previously work (Barbieri and others 2013). However, since 2009 all antimicrobial use for growth promotion and prevention is forbidden in the Brazilian poultry industry (MAPA 2009). Because of that, we expect, in the near future, to observe a decrease in the resistance levels, since our isolates were collected before this prohibition.

Our strains harbored a number of VAGs that ranged from 9 to 23 (out of 34), with an average of 14.2 VAGs per strain. This result is in agreement to our previous work (Barbieri and others 2013). Virulence-associated factors related to iron scavenger systems are the most prevalent in all strains (Ewers and others 2007). The strains presented an average of 3.9 iron acquisition systems out of 8 tested. In fact, this apparent redundancy in iron-acquisition genes among APEC may indicate the importance of iron in the pathogenesis of avian colibacilosis. Genes related to adhesins also showed a significant association with APEC in this study. The strains presented an average of 4.8 adhesins out of 9 tested. Genes related to serum resistance are also important, and were present in all isolates. These results are not surprising because these virulence factors are the most important for survival in an extraintestinal environment.

When we look at the VAGs possessed by the strains classified according to the PS, we cannot discriminate high from intermediate pathogenic strains in terms of the total number of VAGs (out of 34) (Figure 3, Table 2).These data confirm that the presence of the virulence factors by themselves cannot explain why some strains are more virulent than others. Also, we found isolates that were isolated from intestine lesions had a high PS (RS1, RS 23, RS 29). However, other isolates were isolate from lesions in intestine had an low PS (RS18, RS25, RS27, RS32, RS39, RS43 and RS46). Our result showed that even isolates that presented a similar pattern of virulence-associated factors may present quite distinct PS.

The pathogenicity score is a more specific tool to evaluate virulence of APEC isolates (Barbieri and others 2013). Here we observed that the majority of the strains (54%) was lethal to one day-old chickens, and the pathogenicity score (PS) of the majority of the strains indicates that these isolates were highly pathogenic (58%). There was a close correspondence between lethality score (LS) and PS, but we believe the PS better reflects the virulence of a given strain. Since there is no *in vitro* test that could determine whether an *E. coli* isolate from a sick bird is pathogenic or not, pathogenicity score still is the best tool we currently have.

Type O78 was the most prevalent O-serotype observed in our strains. It is also the most prevalent O-serotype in United States (Rodriguez-Siek and others 2005b) and in Germany (Ewers and others 2007). However, we did not find any strain belonging to serotype O1 or O2, which are commonly reported among APEC.

Large conjugative ColV plasmids and the associated gene for colicin (*cvi/cva)* are well know virulence markers and commonly occur among APEC (Rodriguez-Siek and others 2005a; Skyberg and others 2006). Here we found that the presence of *cvi/cva* was associated with the most pathogenic strains (Table 3). This data indicates that *cvi/cva* it is not just a marker of large plasmid, but is also a virulence factor by itself. Within the isolates described here, we observed that *vat,* an autotransporter serine protease toxin, is also associated with pathogenic strains (Table 3). It has been previously associated to improve adherence to VERO cells (Frommel and others 2013), and was also related to increased pathogenicity to 1-day old chickens (Restieri and others 2007).

We observed in our collection 2 isolates belonging to serotype O25:H4 (RS004 and RS005). *E. coli* O25b:H4 has been described to be the cause of epidemiological outbreaks and are currently spread worldwide among humans, livestock and companion animals (Mora and others 2010). These *E. coli* strains belong to sequence type 131 (ST131) and frequently are multidrug resistance, because it usually express $bla_{CTX\text{-}M\text{-}15}$ and fluoroquinolone (FQ) resistance (Ahmed and others 2013). It is a reason for concern because it is the first time that this serotype is reported here in Brazil.

Analyzing the ARDRA and PFGE dendrograms, we observed that 9 of the 30 birds sampled showed an infection by more than one pathogenic strain. This is the first time that an infection by more than one pathogenic strain in the same chicken is described. Moreover, among the 52 samples analyzed, 3 pairs of isolates had identical restriction pattern, representing clones. The remaining 46 strains represent different samples, according to the method ARDRA, indicating the existence of a wide variety of strains within the same geographic region. This indicates that we do not have a single endemic clone.

In conclusion, our genotyping and *in vivo* virulence of isolates from colisepticemic chickens have shown for the first time that one chicken can be infected with more than one pathogenic strain at the same time. Our work also provides a panorama of the susceptibility to antimicrobials of colisepicemic isolates currently found in Southern Brazil.

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LEGENDS TO FIGURES

Figure 1. Antimicrobial susceptibility of colisepticemic isolates. The susceptibility of 52 APEC isolates to 15 antimicrobials was tested individually using disc diffusion tests

Figure 2. Association between presence of 30 VAGs among 52 APEC isolates.

Numbers indicate the percentage of isolates that have both traits, while numbers in shadowed boxes indicate the percentage of isolates that have the corresponding trait; * $p \leq 0.005$ using χ^2. ND, not determined.[1] *afa* is not included, since they were absent in all strain, and *fimC, crlA* and *csgA* is not included, since they were present in all strains.

Figure 3. Characterization of 52 APEC isolates. Columns from left to right: S*train*, isolate designation; *Origin*, source of the isolate; *Bird,* same numbers from the same animal; *Serotype*, O and H grouping serotype; *Resistance*, number of antimicrobials to which the isolate was resistant; the subsequent columns depict the PCR results for all VAGs tested, with presence indicated in black and absence indicated in white; *no. VAGs*, total number of VAGs in each isolate; *LS*, lethality score; *PS* pathogenicity score); *ECOR*, ECOR phylogenetic group;

Figure 4. ARDRA profile of 52 APEC isolates. The ARDRA dendrogram was constructed by UPGMA based upon enzyme restriction digestion of amplified 16-23S DNA intergenic spacer regions. The column Strain shows isolate designation; the column Origin, source of isolate, with A to N designating each of the 65 farms from which the isolates were collected. Bird, same numbers from the same animal; Organ, organ of bacteria isolation; Serotype, O and H grouping serotype; ECOR, ECOR phylogenetic group;

Cluster designates the 4 genotypic clusters (A to D) into which strains with 80% similarity were grouped. *E. coli* ATCC25922 was analyzed as a reference strain.

Figure 5. Pulse-Field profile of 52 APEC isolates. The PFGE dendrogram was constructed by the unweighted-pair group method with arithmetic averages. The scale indicates levels of similarity within this set of isolates based upon *XbaI* enzyme restriction digestion of total bacterial DNA. The column S*train* shows isolate designation; the column *Origin*, source of isolate, with A to N designating each of the 65 farms from which the isolates were collected. *Bird,* same numbers from the same animal; *Organ*, organ of bacteria isolation; *Serotype*, O and H grouping serotype; ECOR, ECOR phylogenetic group; Cluster designates the 5 genotypic clusters (A to E) into which strains with 60% similarity were grouped. *E. coli* ATCC25922 was analyzed as a reference strain. The *XbaI* could not digest the strains RS026, RS027, RS028 and RS043.

5. ARTIGO 3

FNR Regulates the Expression of Important Virulence Factors Contributing to the Pathogenicity of Uropathogenic *E. coli*

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FNR Regulates the Expression of Important Virulence Factors Contributing to the Pathogenicity of Uropathogenic *Escherichia coli*

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ABSTRACT

Uropathogenic *Escherichia coli* (UPEC) are responsible for the majority of urinary tract infections (UTIs), some of the most common bacterial infections in humans worldwide. In this study, we demonstrated that FNR (**F**umarate and **N**itrate **R**eduction), a well-known global regulator important for anaerobiosis, was involved in the expression of virulence factors in UPEC. A UPEC *fnr* deletion strain was highly attenuated in the mouse model of human urinary tract infection and showed a severe defect in adherence and invasion of bladder and kidney epithelial cells. Surprisingly, we found that these phenotypes were largely independent of the previously reported requirement of FNR for the expression of the anaerobic respiratory enzymes and transporters. Our results indicated FNR is a key regulator of multiple aspects of virulence affecting motility; regulating the expression of type I fimbriae and P fimbriae; modulating the expression of hemolysin; and controlling the expression of a novel pathogenicity island that is involved in α-ketoglutarate metabolism under anaerobic conditions. Our results indicated that FNR is a key global regulator of UPEC virulence.

KEY WORDS

UPEC, FNR, type I fimbriae, P fimbriae, hemolysin, and virulence regulation.

INTRODUCTION

FNR (for **F**umarate and **N**itrate **R**eduction) is known as a global regulator, which controls gene expression facilitating bacterial adaptation to anaerobic conditions. The *fnr* gene was first identified by Lambden and Guest in the mid-1970s in their pioneering work on the characterization of mutants that could not use fumarate or nitrate [1]. FNR is a member of a well-characterized global transcription factor family that has two conserved domains: the N-terminal ligand-binding domain for the O_2 signal and the C-terminal DNA-binding domain. The N-terminal sensory domain contains five cysteine residues, four of which (Cys20, Cys23, Cys29 and Cys122) were shown to be required for *in vivo* binding of either a $[4Fe\text{-}4S]^2$ or $[2Fe\text{-}2S]^2$ [2,3]. FNR is activated under anaerobic conditions by acquisition of one $[4Fe\text{-}4S]^2$ per protein, which promotes dimerization and enhances DNA-binding to target promoters. Under aerobic conditions, molecular oxygen triggers the conversion of the $[4Fe\text{-}4S]^2$ into a $[2Fe\text{-}2S]^2$. This conversion causes a conformational change within the FNR protein, becoming monomeric, that is the FNR inactive form, preventing DNA binding, and interactions with the transcription machinery [4].

The C-terminal DNA-binding domain recognizes specific FNR-binding sequences within FNR-controlled promoters. FNR binding sites can be located at variable positions within the promoter region [5] and can have only a partial match to the consensus sequence of TTGATnnnnATCAA. The FNR regulon has been well-studied in non-pathogenic *E. coli* and up to 125 regulon members have been identified so far [6]. These include genes encoding enzymes for the anaerobic oxidation of carbon sources such as the glycerol and formate dehydrogenases; enzymes for the anaerobic reduction of alternate terminal electron acceptors such as the nitrate, fumarate and dimethylsulfoxide (DMSO) reductases; and proteins for transport of these carbon sources or electron acceptors. FNR also represses synthesis of enzymes required for aerobic respiration, such as NADH dehydrogenase II. As a consequence, compounds such as fumarate, nitrate or other reducible substrates can replace oxygen as the terminal electron acceptors thus

providing alternate electron transport chains for generating energy via oxidative phosphorylation [6].

A role for FNR in bacterial virulence was first indicated by its requirement for *Salmonella typhi*'s replication within epithelial cells [7]. It has been further studied in *Salmonella enterica* serovar Typhimurium (ATCC 14028s), where FNR works as a positive regulator of genes involved in motility, flagellar biosynthesis and pathogenesis [8]. This regulation was confirmed by phenotype analysis with the *fnr* mutant being non-motile, lacking in flagella, unable to survive inside macrophages, and attenuated in a murine model of mucosal and acute infection. The inability of the mutant to survive inside macrophages was likely due to its sensitivity to the reactive oxygen species generated by NADPH phagocyte oxidase. In addition, many of the virulence genes in the *Salmonella* pathogenicity island 1 (SPI-1), as well as the *srfABC* operon were significantly down-regulated in the *fnr* mutant strain [8]. More recently, FNR was shown to modulate *Shigella* virulence [9]. Dysentery-causing *Shigella flexneri* encounters changes in oxygen tension as it progresses along the gastrointestinal tract, and its type three secretion system (T3SS), which is essential for cell invasion and virulence, is impacted by oxygen concentration. Expression of *spa32* and *spa33*, the virulence genes that regulate secretion through the T3SS, is regulated by FNR thereby affecting *Shigella*'s entry into cells in response to available oxygen *in vivo* [9].

Based on the importance of FNR for bacterial adaptation to anaerobic conditions and the many phenotypes shared by the *fnr* mutant in *Salmonella*, *Shigella* and *E. coli*, it has generally been assumed that FNR is important for pathogenic *E. coli's* virulence. However, experimental evidence for a more general role of FNR, i.e., beyond facilitating adaption to anaerobic environment, contributing to intramacrophage survival or affecting type-three secretion, has so far been lacking. To address these questions, we constructed and characterized an *fnr* mutant and a complemented strain in uropathogenic *E. coli* (UPEC). We found that deletion of *fnr* resulted in significantly decreased virulence *in vivo* and *in vitro*. These phenotypes, are associated with a loss of hemolytic activity and motility and reduced expression of type 1 and P

fimbriae, leading to decreased adhesion and invasion. In addition, FNR was found to target a novel pathogenicity island. The results indicate that FNR in UPEC FNR plays a much more extensive role than previously believed in regulating virulence gene expression, in addition to its established role as a regulatory switch between aerobic and anaerobic metabolism believed.

MATERIAL AND METHODS

Bacterial Strains, Culture Conditions and Plasmids

The wild-type strain used was UPEC CFT073, isolated from a patient that was admitted to the University of Maryland Hospital with acute pyelonephritis characterized by bacteriuria of 10^5 CFU/ml, pyuria, fever, and no other identified source of infection [10]. Strains and plasmids used in this study are listed in Table S1. Aerobic growth was achieved by shaking in air at 180 rpm and anaerobic growth by incubating in a Bactron chamber (Sheldon Manufacturing, Inc., OR) filled with gas mixture (N2, 90%; CO2, 5%; H2, 5%). For genetic manipulations, all *E. coli* strains were grown routinely in Luria Bertani (LB) broth medium. Selective antibiotics and IPTG were added when necessary at the following concentrations: ampicillin (Amp), 100 μg ml−1; kanamycin (Kan), 50 μg. ml−1; chloramphenicol (Chl), 25 μg.ml−1; IPTG, 0.1 mM [11].

Recombinant DNA Techniques

Polymerase chain reaction (PCR), DNA ligation, electroporation and DNA gel electrophoresis were performed according to Sambrook and Russell [12] unless otherwise indicated. All oligonucleotides primers were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa) and are listed in Table S2. All restriction and DNA-modifying enzymes were purchased from New England Biolabs (NEB, Ipswich, MA) and used based on the

suppliers' recommendations. Recombinant plasmids, PCR products, and restriction fragments were purified using QIAquick PCR purification kits or MinElute gel extraction kits (Qiagen, CA) as recommended by the supplier. DNA sequencing was performed at the DNA facility, Iowa State University.

Deletion mutants were constructed using the lambda red recombinase system described by Datsenko and Wanner [13]. Chromosomal transcriptional *lacZ* fusions were constructed by homologous recombination of the suicide plasmid pVIK112 carrying a fragment of complete 5′-region, 3′- region or internal fragment of the target gene [11,14] . For complementation, the coding sequences of genes plus their putative promoter regions were amplified from the CFT073 genome and independently cloned into pGEN-MCS [15] using *EcoRI* and *SalI* restriction sites.

Agglutination Assays

Tests for agglutination were performed as described earlier [16]. For analysis of the *fim* operon, suspensions (10%) of yeast cells (*Saccharomyces cerviciae*) were used, and for analysis of Pap operon, human red blood cells (RBCs) type "O" (10 %), washed 3 times in PBS, were used. Briefly, yeast cells or human RBCs were mixed on a glass slide with PBS or with bacteria in PBS, in the presence and absence of D-mannose. Agglutination was read after 10 min at room temperature. The strength of the agglutination was determined by titering of serial two-fold dilutions of bacterial suspensions in PBS.

Motility Assay

The motility of the wild type (WT), the *fnr* mutant, and the complemented strain ΔFNR+ pGEN-FNR were evaluated under anoxic conditions as described in [8] . Briefly, 10 µL of

anaerobically grown (16 h) cells were stabed onto LB-agar (0.25% agar) plates and incubated at 37°C for 16 h. The diameter of the growth halo was used as a measure of motility.

Determination of the Switch Orientation of *fim* ON and OFF-cells

The orientation of the *fim* invertible DNA fragment can be determined using a molecular approach as described previously [17]. In brief, a 669-bp DNA fragment, containing the *fim* invertible element, was amplified, subjected to *HinfI* restriction and analyzed on 2% agarose gel. Depending on the orientation of the *fim* invertible element, this method generates different sized fragments (415 and 254 bp when in the ON orientation, 526 and 143 bp when in the OFF orientation).

β-galactosidase Assays

Overnight LB cultures of *E. coli* containing the gene of interest fused to *lacZ* were washed with PBS once and then were diluted 1:100 in LB, or indicated media, and grown at 37°C. For analysis of *fim*, cultures were grown 48 h statically at 37°C [18]. For analysis of *pap*, cultures were grown on CFA media overnight without shaking at 37°C [17]. For analysis of *hly*, cultures were grown on blood agar plates overnight at 37°C under anaerobic conditions [10]. For analysis of *fli* transcriptional fusions, cultures were grown at 37°C in LB under shaking until OD 0.5 [19]. For analysis of α-ketoglutarate, cultures were grown anaerobically at 37°C in M9 medium with α-ketoglutarate overnight [11]. These cultures were collected with OD_{600} 0.4 and diluted 1:10 in Z buffer and assayed for β-galactosidase activity using ortho-Nitrophenyl-β-galactoside (ONPG) as a substrate as described previously [20]. β-galactosidase activity was consider the mean of 3 biological replicates, and two technical replicates.

Electrophoretic Mobility Shift Assays (EMSA)

We used the $(fnrD154A)_2$ protein variant because it displayed an DNA binding affinities and transcriptional regulatory activities to various FNR dependent promoters under aerobic conditions [21]. Protein expression was performed as described previously [5]. Briefly, *E. coli* BL21 with pET28a-$(fnrD154A)_2$ were grown in 200 mL of LB for 16 h at 25°C with 0.1 mM IPTG. To study the binding of FNR to the DNA probe, electrophoretic mobility shift assays (EMSAs) were performed as described elsewhere [21,22]. Briefly, FNR-His6 fusion protein was purified to homogeneity using Ni-NTA Spin Columns [5] and dialyzed against binding buffer. DNA probes were amplified using specific primers and purified using a QIAGEN MinElute gel extraction kit. EMSAs were performed by adding increasing amounts of purified $(FNRD154A)_2$-His6 fusion protein (0 to 20 ng) to the DNA probe (40 ng) in binding buffer (20 mM Tris (pH 6.8), 10 mM EDTA, 4 mM dithiothreitol, 50 mM NaCl, 10% glycerol, 0.5 mg ml−1 bovine serum albumin (NEB) for 30 min at 37°C. Reaction mixtures were then subjected to electrophoresis on a 6% polyacrylamide gel in 0.5×TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) at 200 V for 45 min. The gel was stained in 0.5×TBE buffer containing 1×SYBR Gold nucleic acid staining solution (Life Technologies, Grand Island, NY) for 30 min, and then the image was recorded.

Adherence and Invasion Assays

Cell adherence and invasion assays were performed as previously [23]. The cell lines HTB-1 (J82 bladder) and HTB-44 (A-498 kidney) (American Type Culture Collection) were cultured in Eagle's Minimum Essential Medium (GIBCO) containing 10% fetal bovine serum (FBS) (GIBCO), at 37°C and 5% CO_2.

Cells were plated in sterile 24-well plates with $1x10^5$ cells/well 48 h before experiment. Wild-type CFT073, mutant and complemented strains were cultured statically in LB medium for 16 h. Before infection, two wells of cultured cells were trypsinized and the cells counted to estimate cell number per well. Cells were washed once with 1 mL PBS and then were exposed

to bacteria, washed once with PBS, at a multiplicity of infection (MOI) of 10. The 24-well plates were centrifuged (500 *g* for 5 min) and incubated for 1 h. For the adherence assays, bacterial-exposed cells were washed four times with 1 ml of sterile PBS and then lysed with 1 mL 0.1% Triton-X 100. Serial dilutions of cell suspensions were spread onto MacConkey agar plates (Becton Dickinson & Co, Franklin Lakes, NJ), and CFU counts were obtained after overnight growth at 37°C. For the invasion assays, infected cells were washed 4 times with 1 ml of sterile PBS and then re-incubated with Eagle's Minimum Essential Medium containing 100 µg/mL gentamicin, and incubated for further 3 h. At 4 h post-infection, the cells were washed 4 times with 1 ml of sterile PBS and then lysed with 1 mL of 0.1% Triton-X 100. Serial dilutions of cell suspensions were spread onto MacConkey agar plates, and CFU counts were obtained after overnight growth at 37°C. The input dilution of bacteria was also plated to determine the CFU count for each inoculum.

***in vivo* Infection Studies**

Mouse infection studies were performed as described previously [24]. Female CBA/J mice (six to ten weeks of age) were anesthetized and inoculated via transurethral catheterization with a 20 µl (2×10^9 CFU) challenge inoculum per mouse. Overnight LB cultures of UPEC CFT073 and the mutant strain were pelleted and re-suspended in sterile PBS, mixed in equal volume and adjusted to make the combined challenge inoculum. To determine the initial CFU/mL, dilutions of each inoculum were plated onto LB plates with and without kanamycin. After 48 h, the mice were euthanized, and the bladder and kidneys were aseptically removed, weighed, and homogenized in PBS. Dilutions of the homogenized tissue were then plated on duplicate LB plates with and without kanamycin or on plates with different antibiotics to determine the bacterial concentration (CFU/g) per unit of tissue. After overnight incubation at 37^oC, colonies were counted. The numbers of colonies on kanamycin plates were subtracted from those on LB plates to obtain the number of WT bacteria. In the case of the complemented

strain, recovered bacteria were plated on LB plates with ampicillin and on LB plates with ampicillin plus kanamycin. Similarly, the numbers of colonies on ampicillin plus kanamycin plates were subtracted from those on LB plates with ampicillin to obtain the number of complemented bacteria. A group of 10 mice for each dual-strain challenge were used to determine alterations in fitness. The competition assay was performed once. For statistical analysis, a two-tailed Wilcoxon matched pairs test was used (Prism software, CA), and the threshold for statistical significance was a p value <0.05.

Ethics Statement

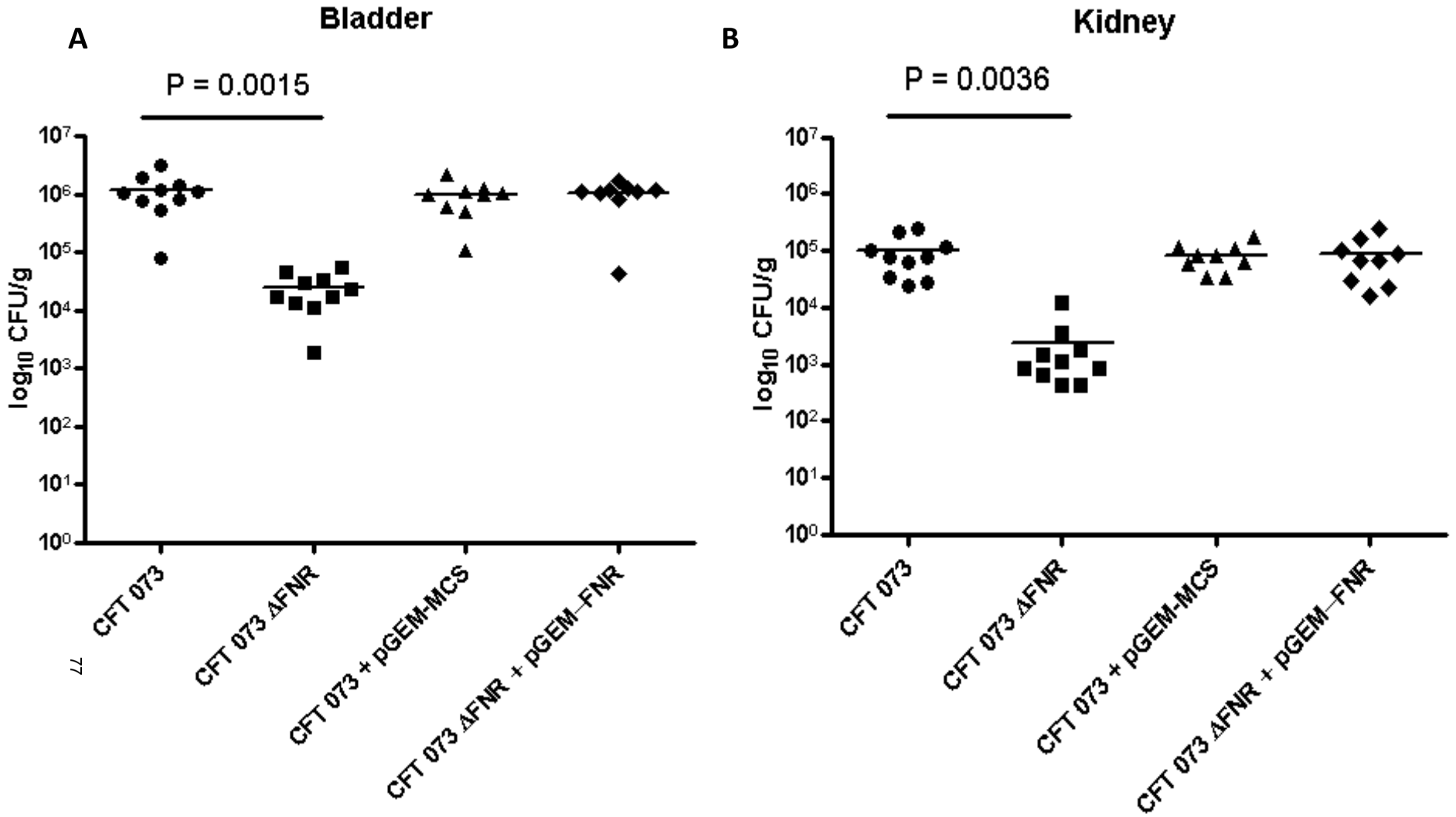
All animal procedures were conducted in accordance with NIH guidelines, the Animal Welfare Act and US federal law. The experimental protocol for handling animals was approved by the Institutional Animal Care and Use Committee at Iowa State University (Protocol number 4-11-7111-Z). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering. Further, procedures were done in the presence of a qualified veterinarian.

RESULTS

The *fnr* Mutation Attenuates Virulence in the Mouse Model of UTI

To address the role of FNR in UPEC virulence, we first compared the UPEC CFT073 wild type with its isogene *fnr* deletion mutant strain for the ability to colonize the mouse urinary tract at 48 hours post-inoculation using an *in vivo* competition assay. Though *fnr* mutant grew as well as the WT in LB, the *fnr* mutant showed significantly reduced colonization in both bladder (p<0.01) (Fig. 1A) and kidneys (p<0.01) (Fig. 1B) as compared to the WT. While the WT strains colonized the bladder at a level of 1.2 x10^6 CFU/g, the *fnr* mutant colonized at 2.5 x10^4 CFU/g, a 48-fold reduction in the median CFU/g in the mutant. Similar results were observed

Fig. 1.


A
Bladder
P = 0.0015
log10 CFU/g
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFT 073
CFT 073 ΔFNR
CFT 073 + pGEM-MCS
CFT 073 ΔFNR + pGEM–FNR
B
Kidney
P = 0.0036
log10 CFU/g
CFT 073
CFT 073 ΔFNR
CFT 073 + pGEM-MCS
CFT 073 ΔFNR + pGEM–FNR

247 for the kidney. The WT colonized at a level of 1.1 x10^5 CFU/g, while the *fnr* mutant colonized
248 at 2.4 x 10^3 CFU/g, a 47-fold reduction in the median CFU/g in the mutant.

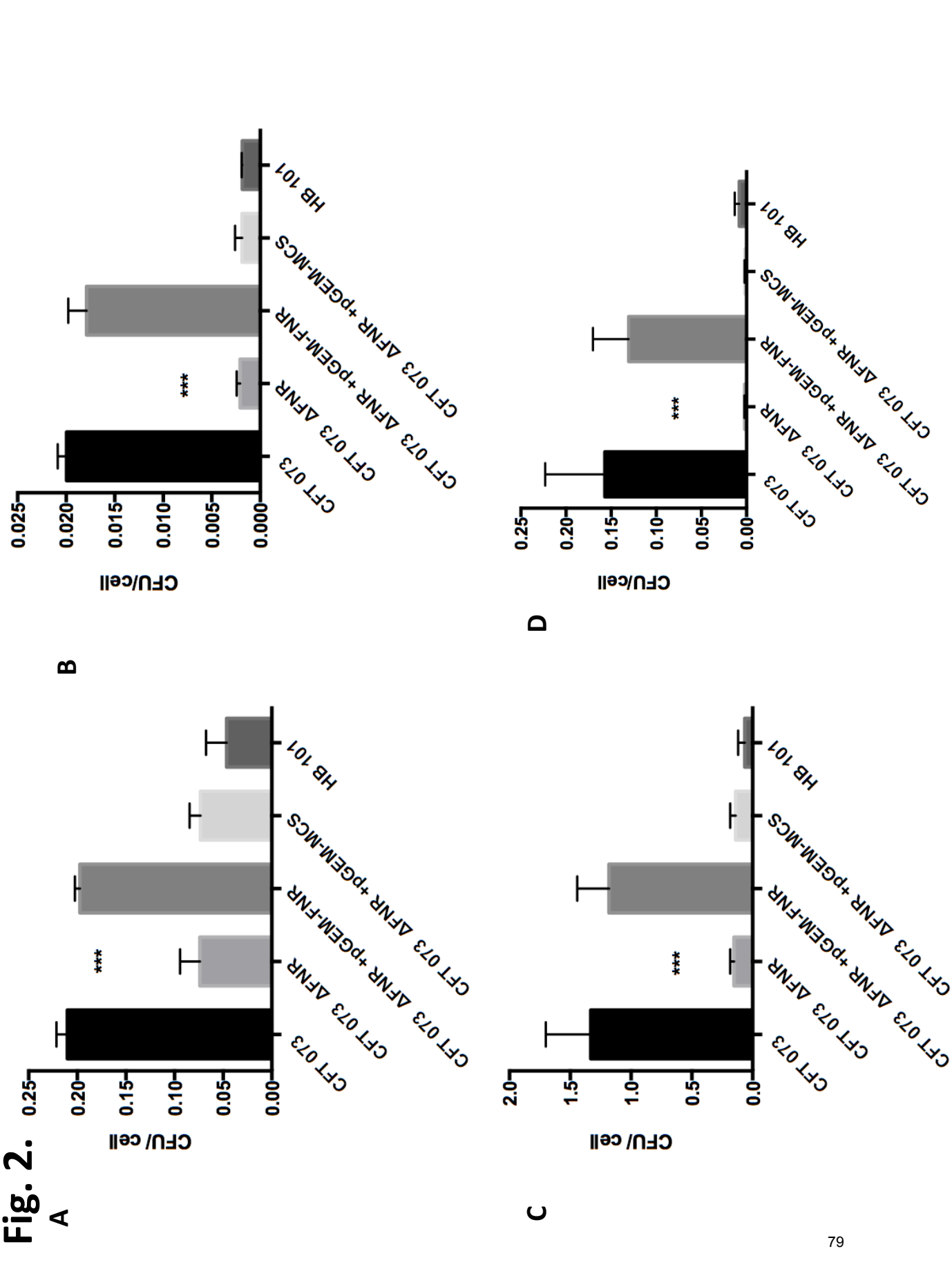
249 To verify that the impact on colonization is not due to a secondary mutation, *in vivo*
250 complementation experiments were performed. A stable low-copy plasmid pGEN-MCS [11]
251 was used to clone the coding region of *fnr* plus its predicted promoter region, and the obtained
252 plasmid was transformed into the mutant to construct the complemented strain. As shown in
253 Figure 1, reintroduction of *fnr* back into the mutants restored colonization in both the bladder
254 and kidney to WT levels. The *fnr* mutant containing the empty vector (pGEN-MCS)
255 demonstrated an expected colonization defect in bladder and kidney colonization as compared
256 to the WT with empty vector in bladder and kidney (not shown). These results demonstrate that
257 the *fnr* mutation attenuated virulence in the mouse model of UTI.

258

259 **The *fnr* Mutant was Impaired in Adherence and Invasion**

260 Successful colonization of the urinary tract relies on UPEC's ability to adhere to and
261 invade host cells and tissues. The cell models of UPEC adherence and invasion to bladder and
262 kidney epithelial cells have been established [23]. To determine the effect of the *fnr* mutation on
263 the adherence to and invasion rates of epithelial cells *in vitro*, cultured bladder epithelial cells
264 (HTB-1) were infected with WT, the *fnr* mutant, and the complemented strains. The results
265 showed that deletion of *fnr* significantly decreased UPEC's adherence to ($p < 0.01$) and invasion
266 of ($p < 0.01$) bladder cells (Fig. 2A and B); whereas, the WT strain displayed an association of
267 0.21 bacteria per HTB-1 cell, the *fnr* mutant displayed an association of 0.07 bacteria per cell, a
268 3-fold reduction in host cell adherence. Similarly, we observed that while the WT strain showed
269 an invasion of 0.02 bacteria per cell, the *fnr* mutant showed an invasion of 0.002 bacteria per
270 cell. When we express these values in percentage of bacterial invasion, which is calculated
271 dividing the number of intracellular bacteria at 4 h post infection by the number of the
272 corresponding adherent bacteria, taking the amount of associated bacteria as 100%, we found

Fig. 2.


A
CFU/ cell
0.25
0.20
0.15
0.10
0.05
0.00
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR
CFT 073 ΔFNR +pGEM-MCS
HB 101
B
CFU/cell
0.025
0.020
0.015
0.010
0.005
0.000
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR
CFT 073 ΔFNR +pGEM-MCS
HB 101
C
CFU/ cell
2.0
1.5
1.0
0.5
0.0
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR
CFT 073 ΔFNR +pGEM-MCS
HB 101
D
CFU/cell
0.25
0.20
0.15
0.10
0.05
0.00
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR
CFT 073 ΔFNR +pGEM-MCS
HB 101

that 9.5% of the adherent WT cells actually invaded HTB-1 cells, but only 2.8% of the adherent *fnr* mutant strain were able to invade the cells. Thus, the mutant strain showed a three-fold reduction in invasion of bladder epithelial cells as compared to the WT. Complementation of the mutant by reintroduction of *fnr* restored adherence and invasion to WT levels (Fig. 2A and B). These results suggest that mutation of *fnr* reduces adherence and invasion of UPEC to bladder cells.

We next determined the impact of the *fnr* mutation on adherence to and invasion of kidney epithelial cells (HTB-44). Deletion of *fnr* significantly decreased UPEC's adherence to and invasion of kidney cells ($p<0.01$) (Fig. 2C and D) at even higher fold changes than seen with bladder epithelial cells. Whereas, the WT strain displayed adherence of 1.33 bacteria per HTB-44 cell, the *fnr* mutant displayed an adherence of 0.15 bacteria per cell, a 9-fold reduction as compared to the WT. Similarly, the WT invaded at a rate of 0.16 bacteria per cell, while the *fnr* mutant showed an invasion rate of 0.002 bacteria per cell. When we express these values in percentage bacterial invasion, we found that 11.8% of the adherent WT actually invaded HTB-44 cells, but only 1.6% of the adherent *fnr* mutant cells were able to invade cultured kidney epithelial cells. Thus, the mutant strain showed a 7-fold reduction in invasion of kidney epithelial cells as compared to the WT. Also, reintroduction of *fnr* back into the mutant restored WT levels of adherence and invasion (Fig. 2C and D). All of these results suggest that *fnr* mutation impairs the adherence and invasion capabilities of UPEC to bladder and kidney cells.

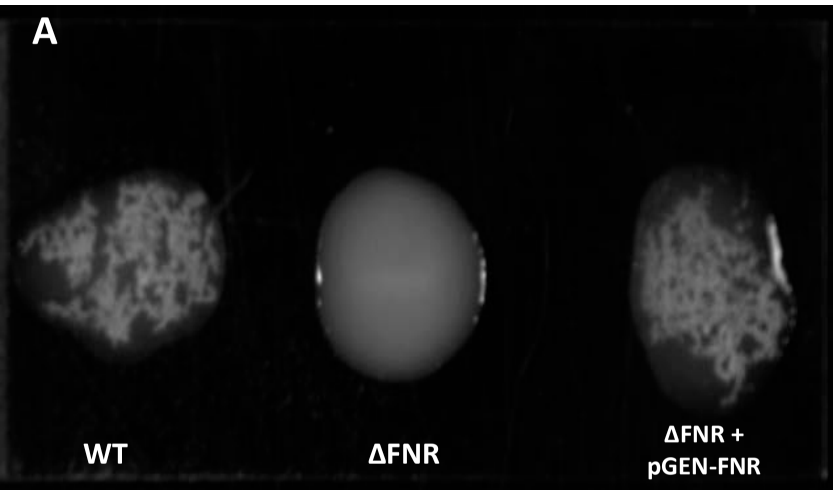
Deletion of *fnr* Reduced Expression of Type 1 Fimbriae in UPEC

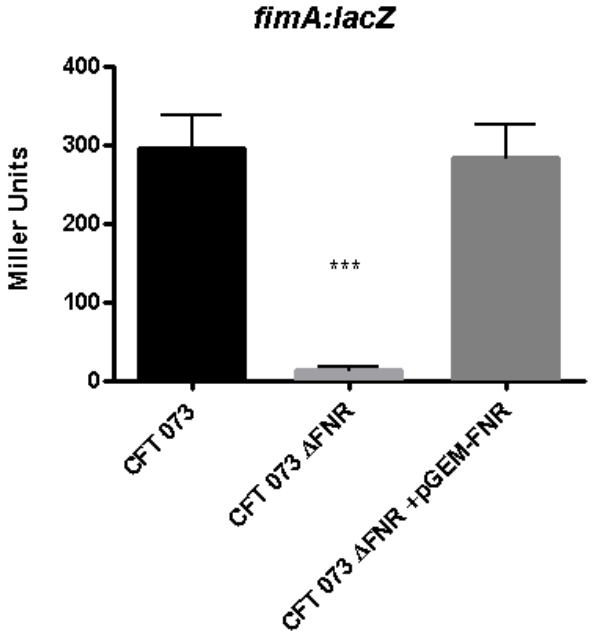
We have shown that deletion of *fnr* significantly decreased UPEC's colonization *in vivo* and also significantly reduced attachment to and invasion to human bladder and kidney epithelial cell culture lines. These pathogenic mechanisms require expression of diverse virulence factors including fimbrial and afimbrial adhesins. Among them, type 1 fimbriae are prominent for their role in the establishment of a UTI [25,26]. We therefore analyzed whether

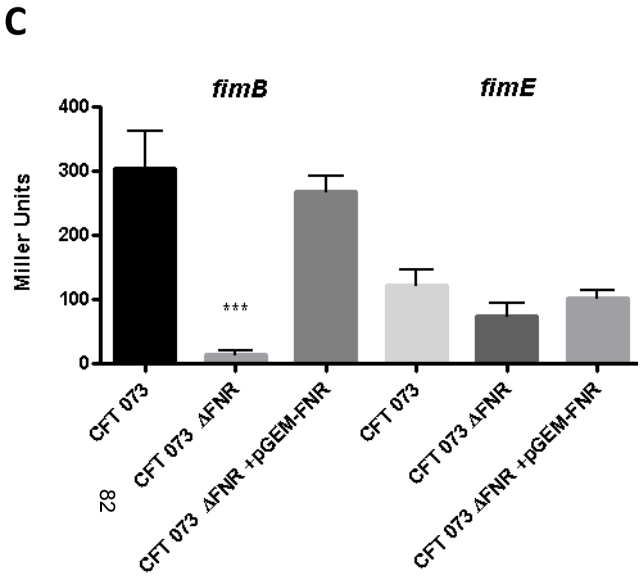
299 *fnr* deletion affects expression of type 1 fimbriae, as detected by the mannose-sensitive yeast
300 agglutination (MSYA), which tests the ability of bacteria to bind mannoside-containing
301 receptors on the surface of yeast cells. Growth of the WT and *fnr* mutant was analyzed in
302 various culture media (LB, TBA, CFA, and TSA; data not shown) under different culture
303 conditions (data not shown), and the medium and conditions in which they showed the same
304 growth rates were chosen for the semi-quantitative MSYA. Using serially diluted bacteria
305 suspended in PBS, we observed agglutination of yeast cells with a higher dilution of WT cell
306 suspensions (16-fold, i.e., containing 16-times more bacterial cells) when compared to the *fnr*
307 mutant; the complemented strain recovered the agglutination ability to the WT level (Fig. 3A).
308 These results indicate that the *fnr* mutation caused a substantial decrease in the expression of
309 type 1 fimbriae.

310 The *fimA* gene encodes the major subunit of the type 1 fimbriae. Transcriptional studies
311 were firstly performed to compare the expression of the *fim* operon in phase variation proficient
312 WT and *fnr* mutant that carry the same *fim-lacZ* in the chromosome. A significant down-
313 regulation of *fimA* was observed in the *fnr* deletion background as compared to the WT (Fig.
314 3B), suggesting that FNR enhances type 1 fimbriation at the transcriptional level. Expression of
315 *fim*A is phase variable [17]. In order to examine whether *fnr* played a role in regulation of *fimA*
316 expression independent of phase variation, , we next performed expression studies in phase
317 variation deficient WT (CFT073 ON and CFT073 OFF) and *fnr* mutant. Due to mutations in
318 *fimB* and *fimE* (encoding the site-specific recombinase), both strains have invertible elements
319 that are locked in the ON orientation (Fig. 3D). A significantly lower *fimA* promoter activity
320 was detected in the *fnr* mutant, suggesting *fnr* up-regulates *fimA* promoter activity itself.

321 To determine if FNR directly regulates *fimA* expression, an electrophoretic mobility shift
322 assay (EMSA) was performed. The promoter region of *fimA* was predicted by the BProm
323 program (http://linux1.softberry.com) [45]. The potential binding site of FNR was identified
324 with the Patser software (version 3d) [8]. DNA fragments containing the potential binding site
325 were then PCR amplified for use as probes (202 nucleotides in size, starting from -158 to +44


A
WT
ΔFNR
ΔFNR +
pGEN-FNR


B
fimA:lacZ
Miller Units
400
300
200
100
0
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR


C
fimB
fimE
Miller Units
400
300
200
100
0
***
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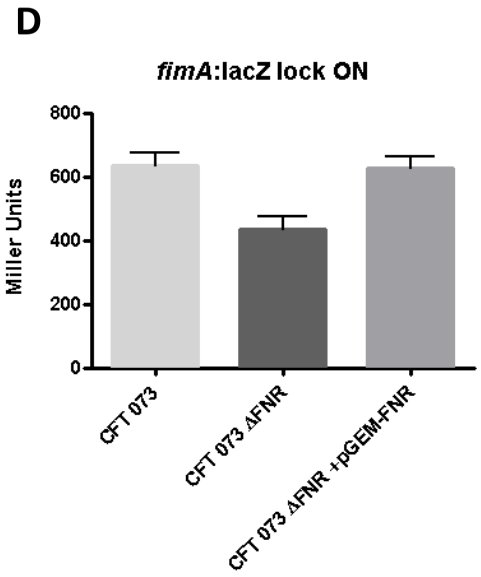
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR


D
fimA:lacZ lock ON
Miller Units
800
600
400
200
0
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR

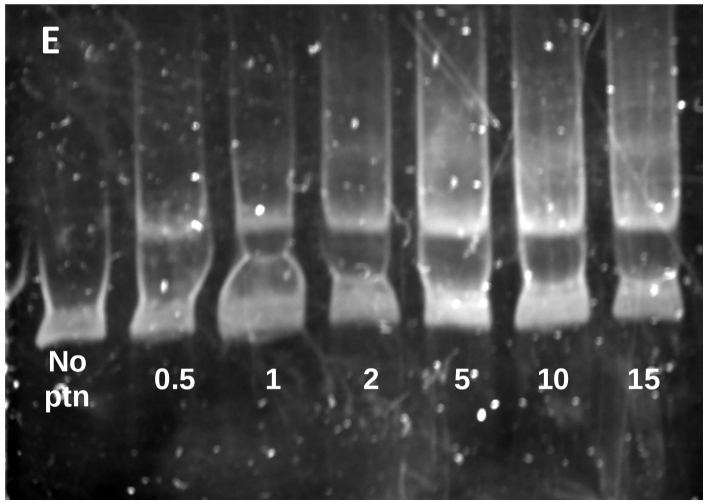

E
No ptn
0.5
1
2
5
10
15
nM

Fig. 3.

relative to translational start codon). Fragments amplified from the coding region of *fimA* were used as negative controls. As shown in Fig. 3E, the FNR fusion protein was able to shift the promoter fragment of *fimA*, but not the control fragment. These results demonstrate that FNR directly regulated the expression of *fimA*.

FNR Affected Phase Variation of Type 1 Fimbriae

Deletion of *fnr* resulted in much higher fold-change of *fimA* expression in phase variation proficient strains compared to the phase variation deficient strains (Fig. 3). This suggests that FNR may also affect phase variation of type 1 fimbriae. To test this hypothesis, we tested the phase ON/OFF switch. Our results showed that *fnr* mutant strain blocks the phase switch in the lock off phase as compared to the wild type. Furthermore, complementation of the *fnr* mutant restored the ON orientation to the parent level (Fig. 4A). These results demonstrate that deletion of *fnr* affected phase variation of type 1 fimbriae.

The recombinases FimB and FimE have been previously associated with phase variation of type 1 fimbriae in *E. coli* [27]. We analyzed the expression of the *fimB* and *fimE* using *fimB-lacZ* and *fimE-lacZ* fusions in the chromosome. Our results showed that *fimB* transcription was down-regulated 20-fold in the *fnr* mutant (Fig. 4B) and *fimE* transcription was down-regulated 2-fold as compared to the wild type. Expression of *fimB* and *fimE* was restored to wild type level in the complemented strain (Fig. 4B and C). Thus, our results showed that FNR affected phase variation of type 1 fimbriae by up-regulating the transcription of recombinases *fimB*.

Deletion of *fnr* Decreased Expression of Type P Fimbriae in UPEC

P fimbriae appeared to be especially important in *E. coli* pyelonephritis and its subtle roles in uroepithelial cell culture models have been previously described [17]. We thus also

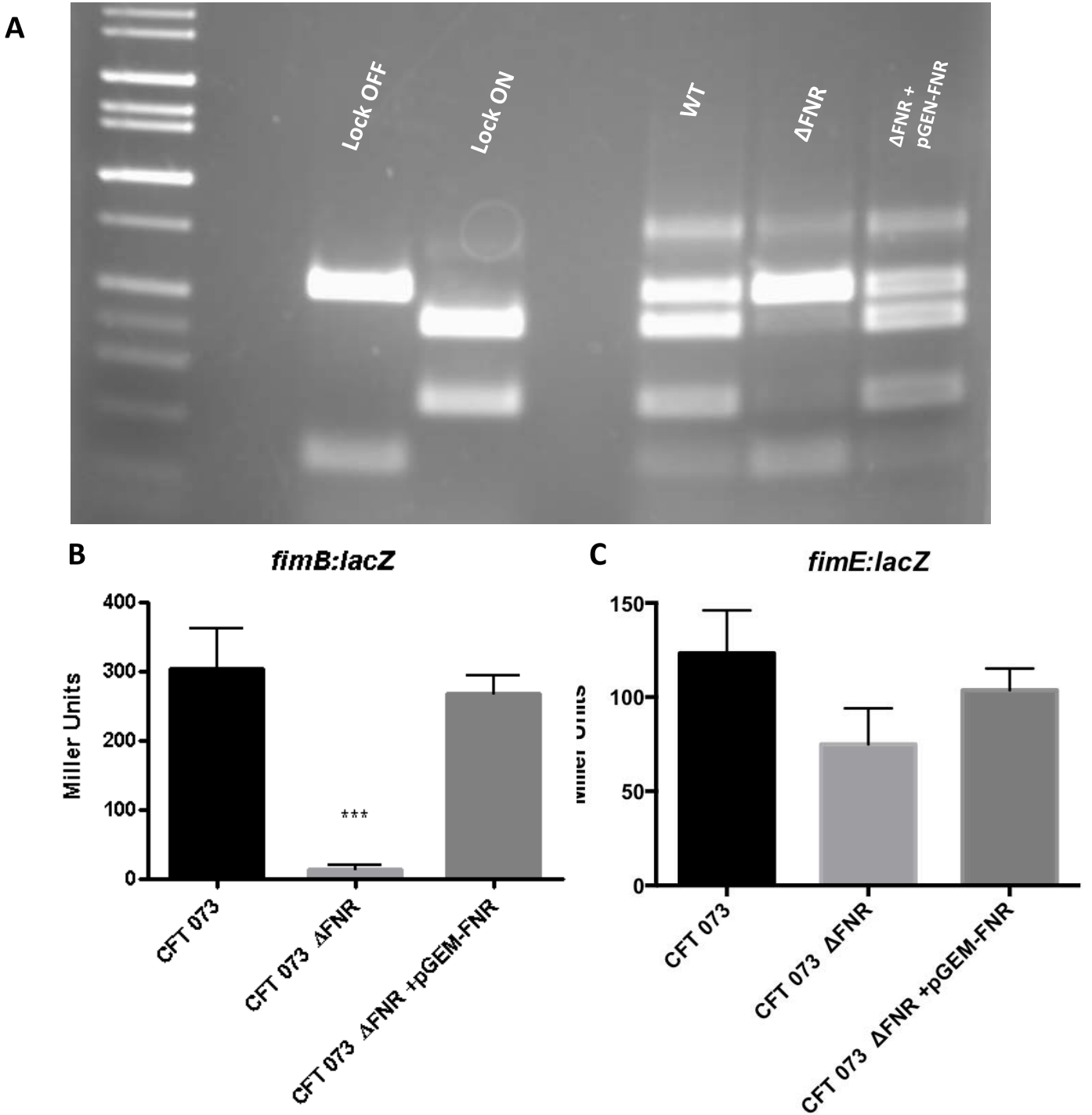

A
Lock OFF
Lock ON
WT
ΔFNR
ΔFNR + pGEN-FNR
B
fimB:lacZ
Miller Units
400
300
200
100
0
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR
C
fimE:lacZ
150
100
50
0
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR

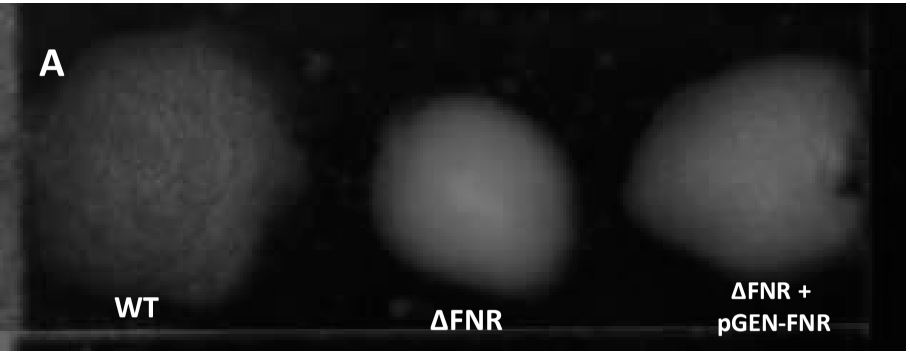
Fig. 4.

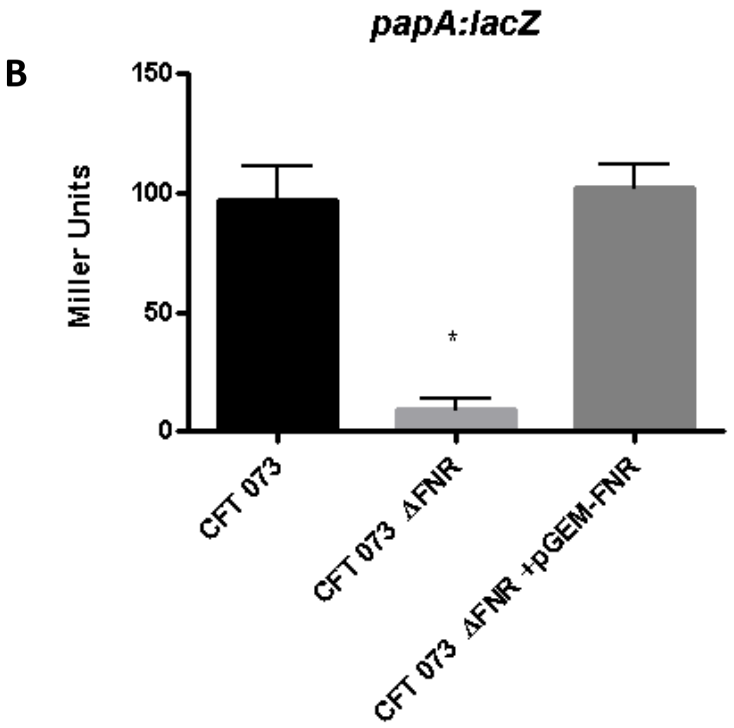
analyzed whether deletion of *fnr* affects the expression of P fimbriae. P-fimbriated *E. coli* specifically bind to receptors on the surface of human red blood cells, and so the expression of P fimbriae can be detected by the human red blood cell agglutination assay [16]. The agglutination assay showed that the UPEC wild type CFT073 agglutinated human red blood cells (serotype O) in a mannose-independent way, and the agglutination titers of wild type were 32-fold higher than those obtained for the *fnr* mutant (Fig. 5A). Reintroducing the *fnr* gene into the mutant recovered the agglutination ability of the *fnr* mutant to the wild type level. These results suggest that the *fnr* mutation significantly decrease the expression of P fimbriae.

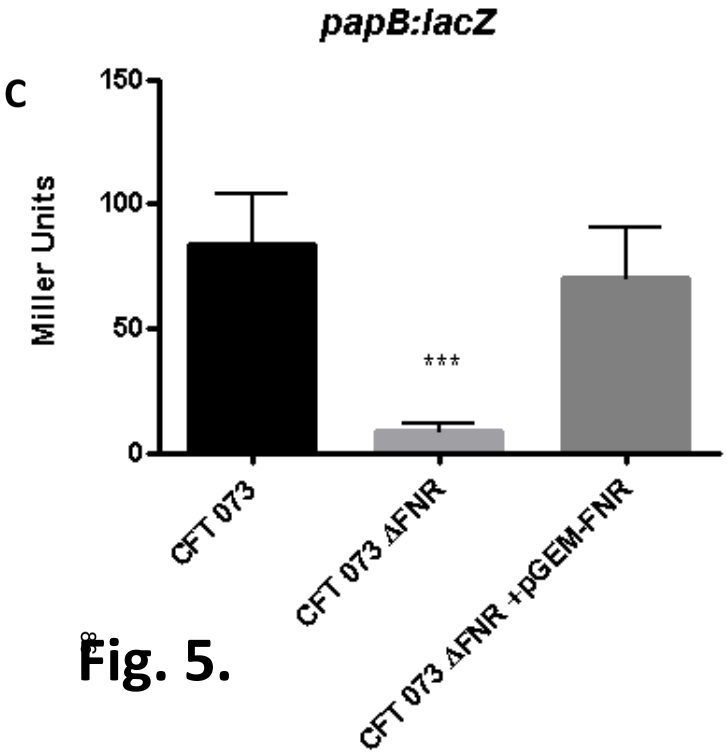
papA encodes the major subunit of P fimbriae. Using CFT073 wild type and *fnr* mutant carrying *papA*-lacZ fusion in the chromosome, we showed that *papA* gene expression was significantly down-regulated in the *fnr* mutant at the transcriptional level, and the reintroduction of the *fnr* gene back to the *fnr* mutant restored *papA* expression (Fig. 5B). We also evaluated the *papB* expression, and also observed that *papB* gene expression was significantly down-regulated in the *fnr* mutant, indicating that FNR mutation affects not just papA in the P-fimbriae operon. A potential binding site of FNR was identified in the promoter region of *papA* (-44 to -23) using Patser software (version 3d) [8]. To determine if FNR directly regulates *papA* expression, EMSA was performed. DNA fragments containing a potential binding site of FNR were then PCR amplified for use as probes, and fragments amplified from the coding region of *papA* were used as negative controls. As shown in Fig. 4D, FNR fusion protein was able to shift the promoter fragment of *papA*, but not the control fragment. These results demonstrate that FNR directly regulated the expression of *papA*.

FNR Affects Motility in UPEC

Bacterial flagella likely facilitate the establishment and spread of infection by microbial pathogens within the host, and expression of flagella was coincident with UPEC ascension to the upper urinary tract [15]. To correlate FNR with UPEC flagella expression, we compared the


A
WT
ΔFNR
ΔFNR +
pGEN-FNR


papA:lacZ
B
Miller Units
150
100
50
0
*
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR


papB:lacZ
C
Miller Units
150
100
50
0
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR

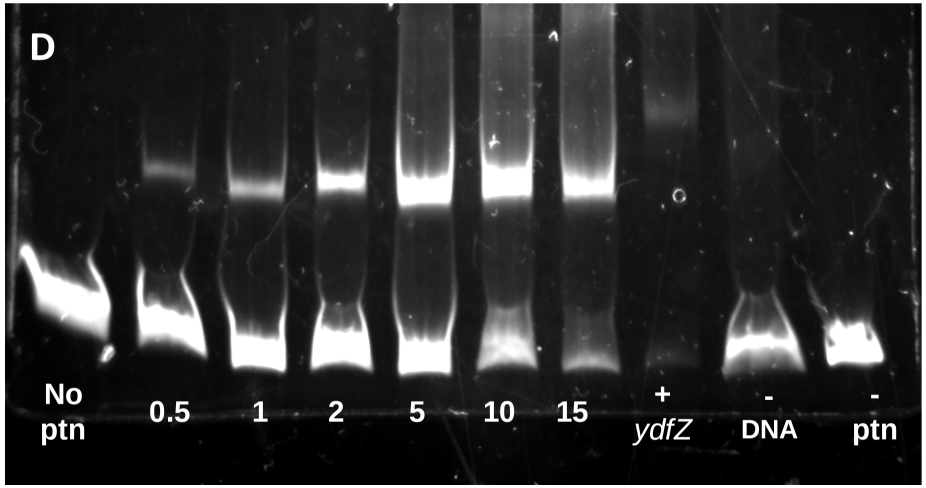

D
No ptn
0.5
1
2
5
10
15
+ ydfZ
- DNA
- ptn
nM

Fig. 5.

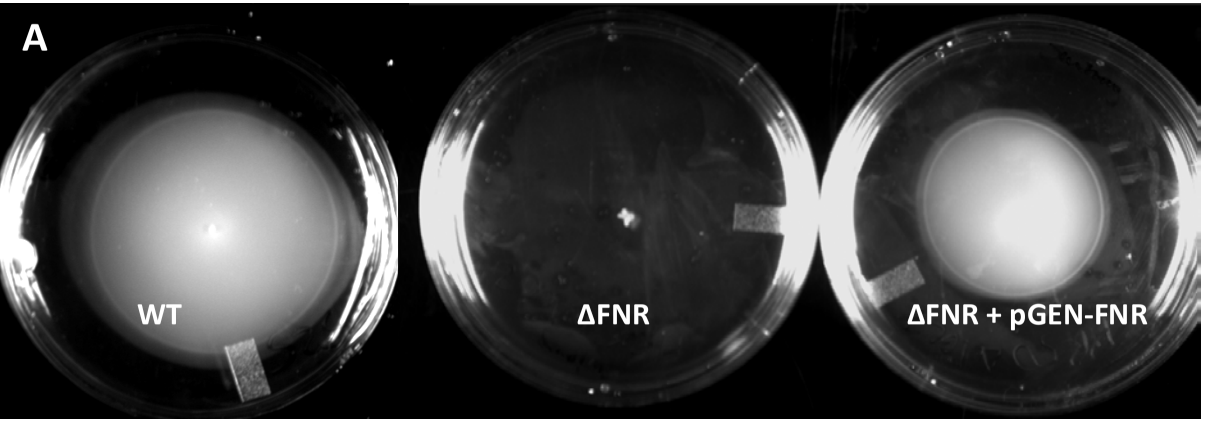
377 motility of wild type, *fnr* mutant, and the *fnr* complemented strain using soft-agar plates. We
378 observed that deletion of *fnr* resulted in a non-motile phenotype in UPEC CFT073 and
379 reintroduction of *fnr* into the mutant restored its motility capability (Fig. 6A). To determine
380 which gene(s) was (were) down-regulated by FNR in the *fli* and *flh* operons, we constructed
381 *lacZ* fusions with *fliA*, *fliC* and *flhDC*. We observed that expression *of fliA* and *fliC* were
382 significantly down-regulated in the *fnr* mutant strain compared to that in the wild type (Fig. 6B
383 and C respectively), but no significant difference was found for the expression of *flhDC* (Fig.
384 6D) between the wild type and mutant strains. A potential binding site (-84 to -105) for the
385 global regulator FNR was found in the promoter region shared by both *fliA* and *fliC* genes, and
386 the direct regulation of flagellar gene(s) by FNR was also confirmed by EMSA (Fig. 6E).

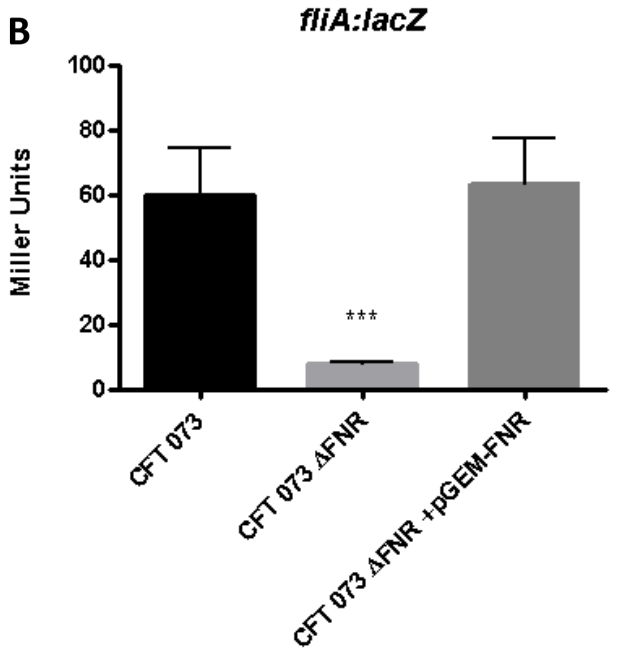
387

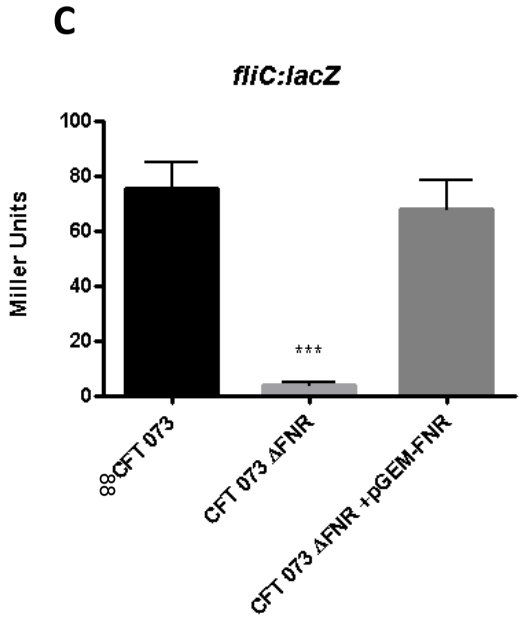
388 **FNR Regulates Expression of a Novel Pathogenicity Island**

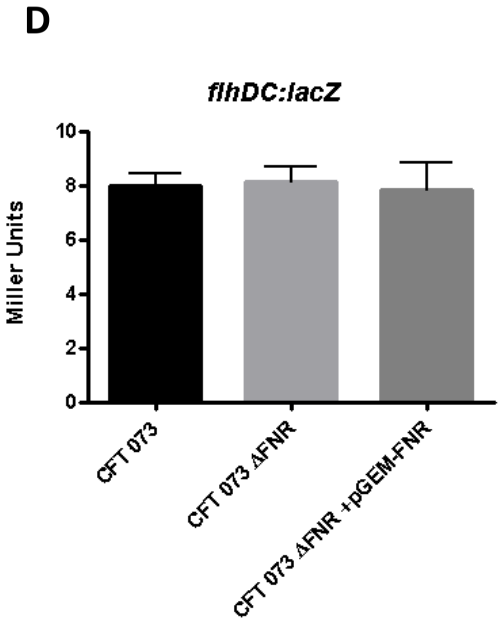
389 Utilization of α-ketoglutarate under anaerobic conditions mediated by a pathogenicity
390 island has been described as a novel metabolic trait, which increased adaptability and
391 competitiveness of UPEC *in vivo* [11]. Since FNR is a well-known global regulator mediating
392 bacterial adaptation to the anaerobic environment, we analyzed whether FNR affects the
393 utilization of α-ketoglutarate under anaerobic conditions. Our growth curves showed that *fnr*
394 deletion significantly reduced the anaerobic growth of UPEC in the M9 minimum medium with
395 α-ketoglutarate as the sole carbon source; in contrast, the *fnr* mutant did not show a significant
396 growth defect in the M9 minimum medium supplemented with glycerol as the sole carbon
397 source as compared to the wild-type (Fig. 7A and B). Therefore, we compared the expression of
398 the pathogenicity island genes among wild type, *fnr* mutant, and the mutant complemented
399 strain. We observed that *fnr* deletion resulted in a significant decreased expression of
400 pathogenicity island genes including the α-ketoglutarate transporter gene (*c5038*), the kinase
401 gene and the response regulator gene of the novel two-component signal transduction system

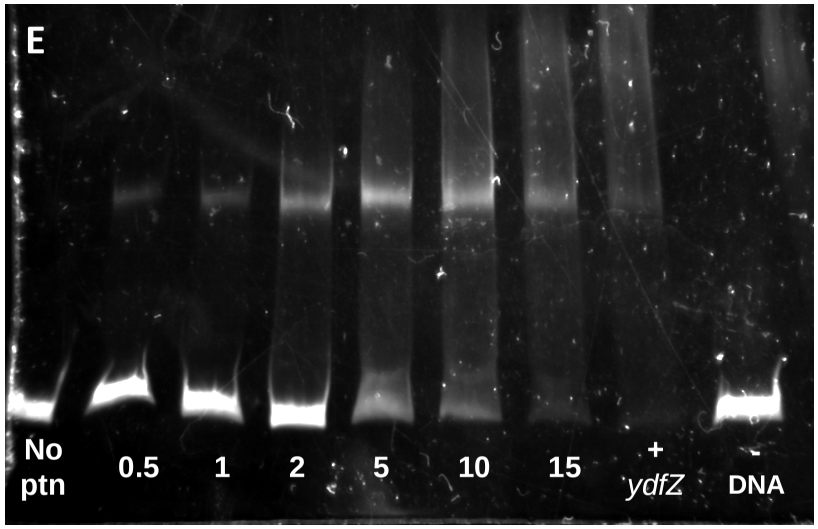
Fig. 6.


A
WT
ΔFNR
ΔFNR + pGEN-FNR


B
fliA:lacZ
Miller Units
100
80
60
40
20
0
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR


C
fliC:lacZ
Miller Units
100
80
60
40
20
0
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR


D
flhDC:lacZ
Miller Units
10
8
6
4
2
0
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR


E
No ptn
0.5
1
2
5
10
15
+ ydfZ
- DNA
nM

(*kguS*/*kguR*). The complementation assay demonstrated that reintroduction of *fnr* into the mutant restored the expression of these targeted genes (Fig. 7C).

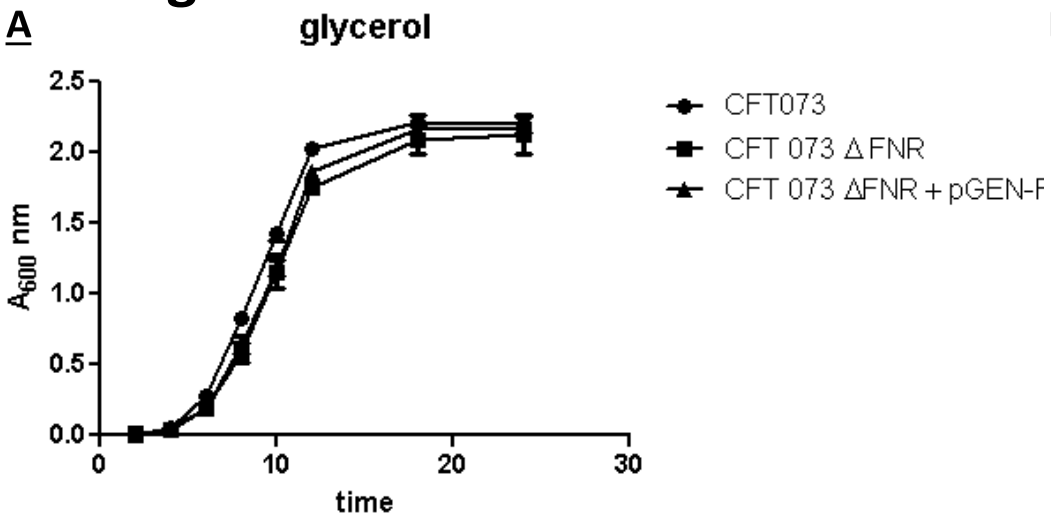
By bioinformatics analysis, we found an FNR binding motif in the promoter region of the signal transduction system KguS/KguR. EMSA was therefore performed using DNA fragments containing the promoter region of the *kguS/kguR* genes. As shown in Fig. 7D, the FNR fusion protein was able to shift the promoter fragment of *kguS/kguR*, but not the control fragment. These results confirmed that FNR directly regulates the expression of the two-component signal transduction system KguS/KguR. It has been reported that KguS/KguR directly regulates the transport gene *c5038* [11]; therefore, it is very likely that FNR indirectly regulates other pathogenicity island genes, such as *c5038* through KguS/KguR.

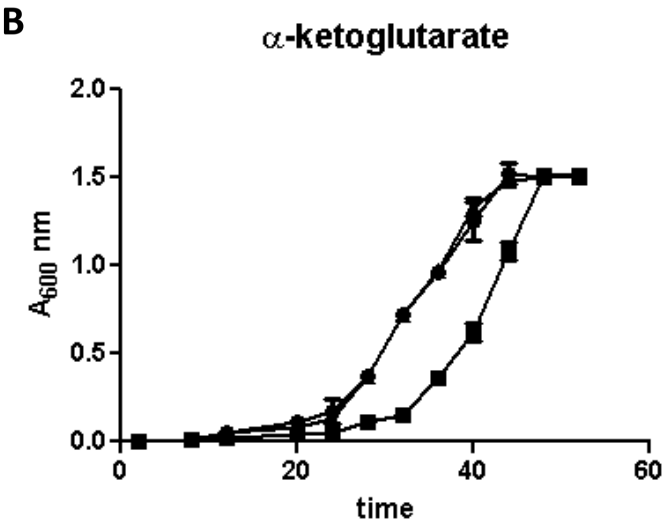
Deletion of *fnr* Results in Down-Regulation of Hemolysin encoding genes

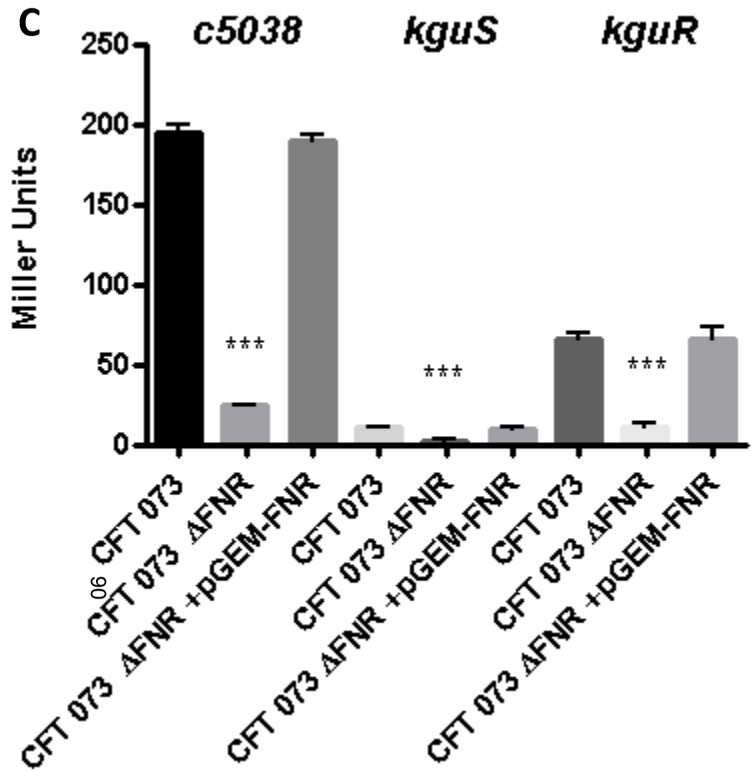
Hemolysin is a very important virulence factor of UPEC [28,29]. We had previously found that UPEC CFT073 showed much stronger hemolysis on blood agar under anaerobic conditions than under aerobic conditions, and the upregulation of *hlyCABD* was responsible for this hemolytic phenotype (data not published). We reasoned that FNR might be involved in the regulation of *hlyA* expression. To verify this hypothesis, we compared the hemolytic activity of the wild type with that of the *fnr* mutant grown on blood agar under anaerobic conditions. As expected, the *fnr* mutation abolished the hemolytic activity, and complementation rescued this phenotype level (Fig. 8A). A quantitative assay for the hemolytic activity further confirmed that the *fnr* mutant showed significantly weaker hemolysis than the wild-type strain under anaerobic conditions (Fig. 8B).

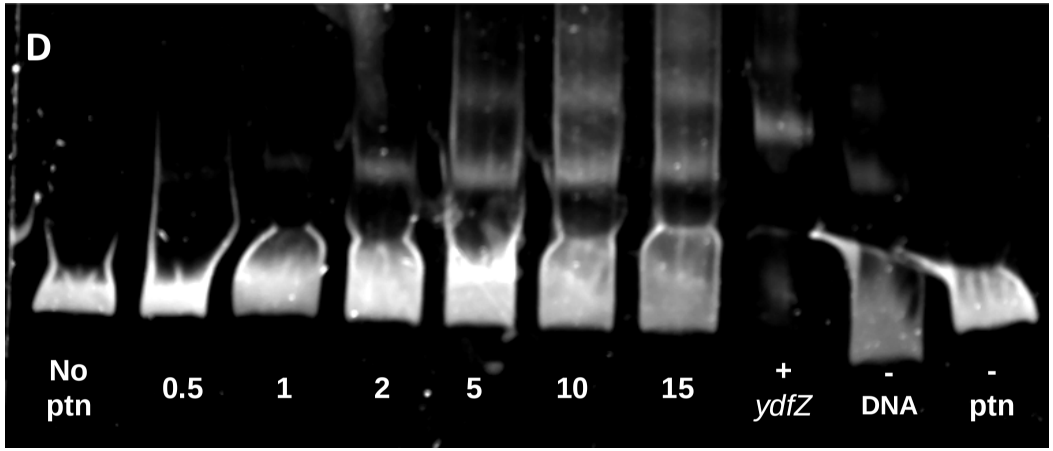
We then checked if FNR affected the expression of *hlyCABD*. Since it was reported that *hlyC*, *hlyA*, *hlyB*, and *hlyD* shared only one promoter and there was an attenuator localized between *hlyA* and *hlyB*, we therefore constructed *hlyA-lacZ* and a *hlyD-lacZ* fusions in the chromosome. The *fnr* mutation significantly down-regulated the expression of both *hlyA* (Fig.

Fig. 7.


A
glycerol
CFT073
CFT 073 ΔFNR
CFT 073 ΔFNR + pGEN-F
A_{600} nm
time


B
α-ketoglutarate
A_{600} nm
time


C
c5038
kguS
kguR
Miller Units
***
***
***
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR


D
No ptn
0.5
1
2
5
10
15
+ ydfZ
- DNA
- ptn
nM

8C) and *hlyD* (Fig. 8D). The molecular regulatory mechanisms of *hlyCABD* regulation by FNR are currently being examined in our laboratory.

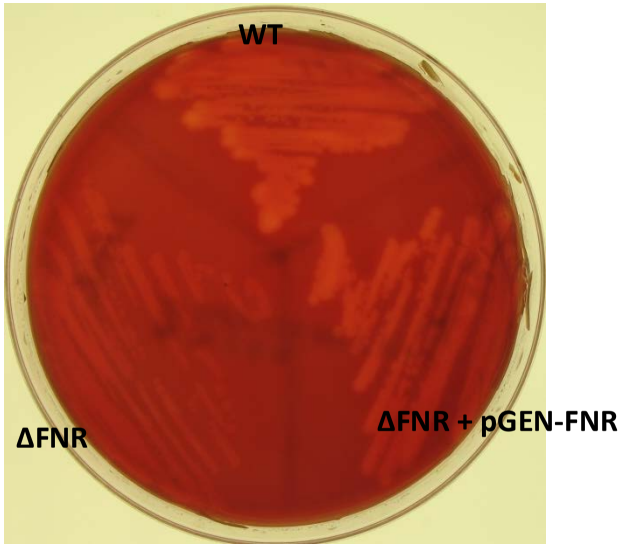
DISCUSSION

The global regulator, FNR, has been recognized as a major transcription factor of bacterial gene expression, which participates in numerous regulatory pathways allowing facultative anaerobes to adjust to oxygen deprivation [30]. FNR is likely to be important for virulence of bacterial pathogens that encounter hypoxic and anoxic niches within their host. It serves as the O_2 sensor to reprogram bacterial metabolism by activating genes required for anaerobic respiration and fermentation and inhibiting genes necessary for aerobic respiration. Accordingly, previous studies demonstrated that FNR of *Bordetella pertussis*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium were required for *in vivo* optimal growth and survival. Proteomic analyses also showed that *S. dysenteriae* type 1 switched from aerobic respiration *in vitro* to anaerobic catabolism *in vivo*. In addition to facilitating adaptation to hypoxic and anoxic metabolism during host colonization and infection, FNR also triggers virulence gene expression and coordinates the function of a Type III secretion system, thus being involved in bacterial pathogenesis. In this study we have explored the role of the global regulator FNR in the virulence of UPEC beyond facilitating adaption to anaerobic metabolism.

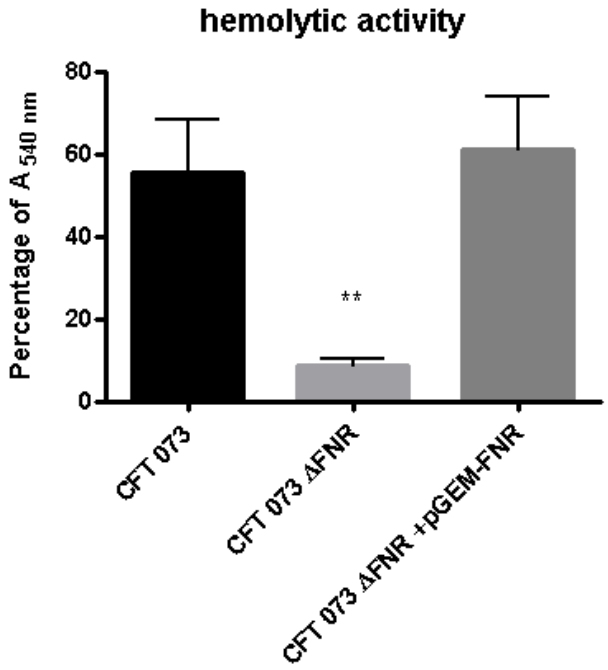
The virulence-associated phenotype observed was the significantly reduced adherence and invasion of the *fnr* mutant to bladder and kidney cells. The ability of UPEC to adhere and invade cultured bladder and kidney epithelial cells is dependent on the expression of fimbrial and afimbrial adhesins. Type 1 fimbriae are among the most important adhesins of UPEC, and have been shown to mediate bacterial adherence and invasion of cells *in vitro* and to play a major role in the *in vivo* colonization process associated with urinary tract infection. In the presence of oxygen, FNR was thought to be inactive. Interestingly, our study showed that FNR could activate the expression of type 1 fimbriae under both aerobic and anaerobic conditions (data not shown) and notably played a dual role in the up-regulation of type 1 fimbriae by

Fig. 8.

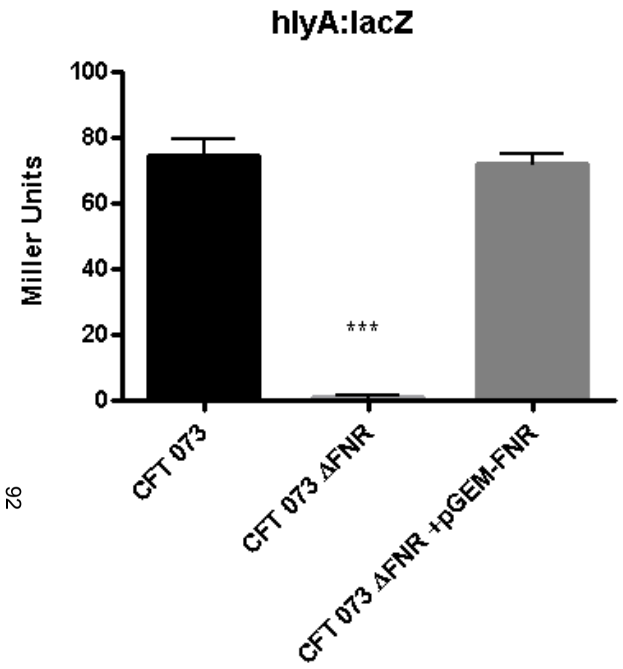
A


WT
ΔFNR
ΔFNR + pGEN-FNR

B


hemolytic activity
Percentage of A 540 nm
80
60
40
20
0
**
CFT 073
CFT 073 ΔFNR
CFT 073 ΔFNR +pGEM-FNR

C


hlyA:lacZ
Miller Units
100
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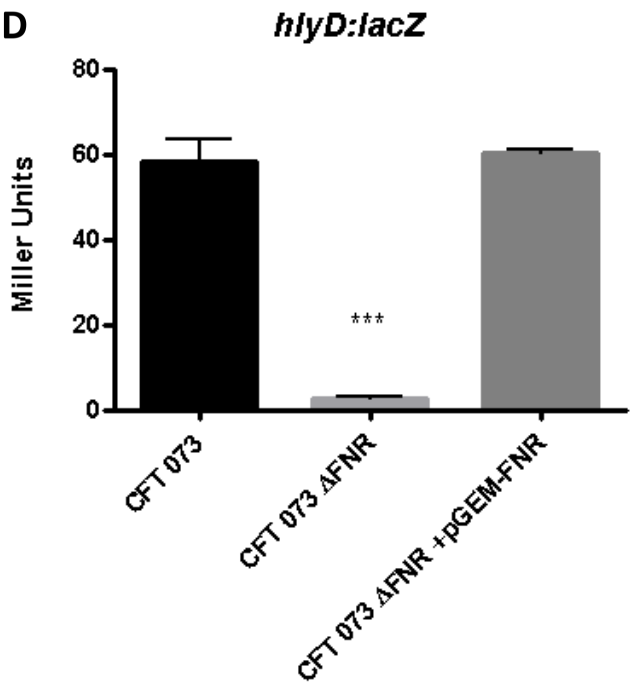
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promoting both promoter activity and phase variation. The FNR protein could bind to the promoter region of the *fimA* gene, which encodes the main subunit of the fimbriae, and directly up-regulated the expression of *fimA*. Also, FNR could activate FimB and FimE-mediated recombination, thus promoting phase OFF to ON switching. At this time, the mechanisms of how FNR affected the expression of *fimB* and *fimE* are still unknown, and further studies are required to fully understand the underlying mechanisms by which FNR affects both levels of regulation of type 1 fimbriae.

P fimbriae play an important role in the pathogenesis of ascending UTIs and pyelonephritis in humans [31,32] by promoting epithelial attachment and resistance to filtrate flow, facilitating early bacterial multiplication prior to the onset of ischemia and infiltration of immune cells [33]. Using the human blood red cell agglutination assay, we could demonstrate that deletion of *fnr* significantly reduced the expression of P fimbriae; in addition, decreased transcription of the main structural gene *papA* was detected in the *fnr* mutant, suggesting that FNR activates the expression of P fimbriae. The regulation of *papA* by FNR appears to be direct since the FNR protein could bind to the promoter region of the *papA* gene as shown by the EMSA. Finally, we conclude that the reduced adherence and invasion ability of the *fnr* deletion mutant was presumably caused by the decreased expression fimbriae such as type 1 and P fimbriae.

The kidney is a critical site in serious urinary tract infections, and is characterized by metabolites not found elsewhere in UPEC life cycle. For example, the proximal tubule cells have 10-40 fold higher levels of α-KG than any other cells [34]. We identified the first metabolic trait, which is encoded by a pathogenicity island, to increase UPEC's adaptability and competitiveness during kidney infection [11]. The pathogenicity island encodes a novel two-component signal transduction system KguS/KguR and its target genes are involved in anaerobic utilization of α-KG. The oxygen tension was shown to modulate expression of island genes directly via the KguS/KguR and oxygen deficiency up-regulated the expression of KguS and KguR, suggesting that oxygen modulates expression of island genes by controlling

KguS/KguR expression. However, no sensory domain of oxygen was found in either KguS or KguR, and the mechanisms by which oxygen tension regulates the expression of KguS and KguR remains unknown. Here, we demonstrated that deletion of *fnr*, which usually mediates the regulation of oxygen in *E. coli* [6], substantially reduced the growth of UPEC in M9 medium with α-KG as the sole carbon source under anaerobic conditions, and deletion of *fnr* significantly decreased the expression of pathogenicity island genes including *kguS* and *kguR*. Our evidence also showed that FNR directly regulated the expression of the two-component signal transduction system KguS/KguR, thus likely indirectly regulating other pathogenicity island genes such as *c5038* through KguS/KguR. It has been well known that the global regulator FNR controls *E. coli* K12-common genes mediating adaptation to host anaerobic niches. To the best of our knowledge, this is the first report showing that FNR controls the expression of a metabolism island and contributes to bacterial fitness *in vivo*.

Alpha-hemolysin has been shown to be an important virulence factor of UPEC [35]. This toxin can lyse eukaryotic cells providing an opportunity for UPEC deeper invasion and supplying bacteria with nutrients released from host cells [36]. In addition, it has been recently demonstrated that α-hemolysin is not only a pore forming toxin, but also triggered proteolysis of host proteins to disrupt cell adhesion, inflammatory, and survival pathways [37]. The *hly* operon contains four genes *hlyCABD* encoding the toxin precursor HlyA and other components necessary for the toxin activation and secretion [38]. Several environmental stimuli, for example anaerobiosis, have been reported to induce *hly* expression. However, the underlying mechanisms are not completely understood [39,40]. UPEC CFT073 displayed much higher hemolytic activity under anaerobic conditions than aerobic conditions (data not shown), while deletion of *fnr* totally abolished the hemolytic activity of CFT073 under anaerobic conditions. The restoration of *fnr* with its native promoter in the *fnr* mutant, and comparison of hemolytic activity in the resulting complemented strain and in the wild-type strain revealed that the abolished hemolytic activity is indeed due to deletion of the *fnr* gene. Expression studies further confirmed that deletion of *fnr* reduced the transcription of *hly* operon. These results indicated

510 that the global regulator FNR, a well-known oxygen sensor, mediates the anaerobiosis induction
511 of the *hly* operon.

512 Deletion of *fnr* also reduced the expression of UPEC flagellum, whose expression was
513 coincident with UPEC ascension to the upper urinary tract [15] and significantly enhanced the
514 pathogenesis of urinary tract infection caused by UPEC [41].

515 In summary, our results indicate that, in addition to mediating bacterial adaptation to
516 anaerobic metabolism *in vivo*, the global regulator FNR contributes to the virulence of UPEC by
517 regulation of the expression of important virulence genes of UPEC. Several known global
518 regulators such as H-NS [42], RpoS [43], RfaH [44], Hfq [45], and CRP-cAMP [17] have been
519 reported to regulate virulence of UPEC, and now FNR can be included in the growing list of
520 regulatory networks controlling UPEC virulence. The mechanisms by which these global
521 regulators interplay among each other, thus orchestrating virulence gene regulation cascades in
522 response to host environment, are still unknown and require further studies.

523

524 **ACKNOWLEDGEMENTS**

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529 Mobley and the plasmid pET28a-(fnrD154A)$_2$ from Dr Aixin Yan.

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658

659 **Figure legends**

660 **Figure 1. Deletion of *fnr* attenuates virulence in the mouse model of UTI by UPEC**
661 **CFT073.** (**A**) The WT and *fnr* mutant strains and wild-type strain containing empty vector
662 (e.v.) pGEN-MCS and mutant strain containing complementation plasmid (pGEN-FNR) were
663 mixed to a 1:1 ratio and approximately 2×10^9 CFU were transurethrally inoculated into female
664 mice. Two days after infection, the mice were sacrificed and their bladders (**A**) and kidneys (**B**)
665 were aseptically removed. WT and *fnr* mutant bacteria were recovered by plating homogenized
666 tissue samples on LB or LB containing kanamycin and their viable counts were determined.
667 Wild-type strain containing empty vector (e.v.) pGEN-MCS and mutant strain containing
668 complementation plasmid (pGEN-FNR) were recovered by plating homogenized tissue samples
669 on LB with ampicillin or LB with both ampicillin and kanamycin. Each dot represents
670 $\log_{10}$CFU/g in the bladder or kidney from an individual animal and the detection limit is 1000
671 CFU/g. Bars indicate the median $\log_{10}$CFU/g. A two-tailed Wilcoxon matched pairs test was
672 performed, and the difference in colonization levels of WT and mutants was considered
673 statistically significant if $p < 0.05$.

674 **Figure 2. Adherence to and invasion of bladder HTB-1 cell line and HTB-44 kidney**
675 **cell line by CFT073 and its mutants.** HTB-1 cells (**A** and **B**) and HTB-44 cells (**C** and **D**)
676 were infected at MOI of 10 CFU/cell as described in Material and Methods. For the association
677 assays (**A** and **C**), cells were lysed at 1 h post-infection, and the extracts plated onto LB agar for
678 enumeration, For the invasion assays (**B** and **D**), at 1 h post-infection, cells were washed with
679 PBS and incubated for further 3 h in the presence of gentamicin. Cells were then lysed, and the
680 extracts plated onto LB agar for enumeration. *E. coli* HB101 was used as a negative control.
681 Significant differences are indicated by asterisks (***, P<0.0001compared to the WT and
682 mutant).

683 **Figure 3. UPEC type I fimbriae regulation**. **A**, yeast agglutination assay with dilutions
684 of WT 1:16; ΔFNR 1:1 and ΔFNR + pGEN-FNR 1:16. Bacterial were grown statically for 48 h,
685 agglutination was read after 10 min at room temperature, and the strength of the agglutination
686 was determined by tittering of serial two-fold dilutions in PBS of the bacterial suspensions;
687 *fimA-lacZ* transcriptional fusion strains were grown in LB medium statically for 48 h at 37°C. β-
688 galactosidase activity was measured, and the values shown are means plus standard deviations
689 of triplicate samples. **B**, β-galactosidase activity assay for expression of *fimA.;* **C**, β-
690 galactosidase activity assay for expression of *fimA* in *fimB* and *fimE* costructs*;* **D**, β-
691 galactosidase activity assay for expression of *fimA* in locked ON strains **E**, Non-radioactive
692 EMSA studying the binding of $(FNRD154A)_2$ - His_6 to the promoter regions. PCR product of
693 *fimA* promoter region was used as probes. Purified $(FNRD154A)_2$ -His_6 fusion protein was

added at different concentrations in each reaction mixture as indicated and MBP-His_6 was used as a negative control at 300ng per each reaction. For the lane on the right on each panel, a negative control DNA fragment amplified from the *kguR* coding region was used, and as positive controls *ydfZ* were used. DNA fragments were stained with SYBR green. Significant differences are indicated by asterisks (***, P<0.0001compared to the WT and mutant).

Figure 4. UPEC type 1 fimbriae phase variation. A. Phase variation electrophoresis, PCR products were purified and digested with *HinfI* for 4 h, and analyzed on 2 % agarose gel.; *fimB-lacZ* and *fimE-lacZ* transcriptional fusion strains were grown in LB medium statically for 48 h at 37°C. β-galactosidase activity was measured, and the values shown are means plus standard deviations of triplicate samples **B.** β-galactosidase activity assay for expression of *fimB;* **C.** β-galactosidase activity assay for expression of *fimE.* Significant differences are indicated by asterisks (***, P<0.0001compared to the WT and mutant).

Figure 5. UPEC type P fimbriae regulation. A, RBC agglutination assay with dilutions of the WT 1:32; ΔFNR 1:1 and ΔFNR+pGEN-FNR 1:32. Bacteria were grown statically for 48 h, agglutination was read after 10 min at room temperature and the strength of the agglutination was determined by titering of serial two-fold dilutions in PBS of the bacterial suspensions; *papA-lacZ* and *papB-lacZ* transcriptional fusion strains were grown in CFA media overnight without shaking at 37°C. β-galactosidase activity was measured, and the values shown are means plus standard deviations of triplicate samples. **B,** β-galactosidase activity assay for expression of *papA*; **C,** β-galactosidase activity assay for expression of *papB;* **D**, Non-radioactive EMSA studying the binding of $(FNRD154A)_2$ - His_6 to the promoter regions. PCR products of *papA* promoter region were used as probes. A negative control DNA fragment, amplified from the *kguR* coding region, was used, and *ydfZ* were used as positive controls. DNA fragments were stained with SYBR green. Significant differences are indicated by asterisks (*, P<0.05 compared to the WT and mutant ; ***, P<0.0001compared to the WT and mutant).

Figure 6. Motility regulation. A, Soft agar motility assay; bacterial cultures were stabbed in the middle of each soft-agar plate and incubated at 37°C for 16 h; *fliA-lacZ, fliC-lacZ* and *flhDC-lacZ* transcriptional fusion strains were grown at 37°C in LB with shaking until OD 0.5.β-galactosidase activity was measured. The values shown are means plus standard deviations of triplicate samples. **B,** β-activity assay for expression of *fliA*. **C,** β-activity assay for expression of *fliC*. **D,** β-activity assay for expression of *flhDC*. **E,** Non-radioactive EMSA studying the binding of $(FNRD154A)_2$ - His_6 to the promoter regions. PCR products of *fliC* promoter region were used as probes. A DNA fragment amplified from *kguR* coding region was used as a negative control, and *ydfZ* was used as a positive control. DNA fragments were stained

with SYBR green. Significant differences are indicated by asterisks (***, P<0.0001compared to the WT and mutant).

Figure 7. UPEC α-ketoglutarate metabolism regulation. *in vitro* growth of *fnr* mutants in M9 medium containing α-KG as the sole carbon source (**A**) or M9 medium containing glycerol as the sole carbon source (**B**). Optical density of the UPEC CFT073 WT and mutants during growth in M9 medium containing α-KG as the sole carbon source or glycerol under anaerobic conditions was determined. Growth bars represent the average measurement at each time point from triplicate experiments; *c5038 -lacZ, kguS-lacZ* and *kguR-lacZ* transcriptional fusion strains were grown anaerobically at 37°C in M9 medium with α-KG overnight. β-galactosidase activity was measured, and the values shown are means plus standard deviations of triplicate samples. **C,** β-galactosidase activity assay for expression of *c5038, kguS* and *kguR.* **D,** Non-radioactive EMSA studying the binding of $(FNRD154A)_2$ - His_6 to the promoter regions. PCR products of *kguR* promoter region were used as probes. A DNA fragment amplified from the *kguR* coding region was used as a negative control, and *ydfZ* was used as a positive control. DNA fragments were stained with SYBR green. Significant differences are indicated by asterisks (***, P<0.0001compared to the WT and mutant).**Figure 8. UPEC hemolysin regulation. A,** Blood agar hemolytic assay. Bacteria were inoculated into blood agar plates overnight at 37°C under anaerobic conditions; **B,** hemolytic activity quantification, bacterial strains were grown in the presence of RBC cells overnight at 37°C under anaerobic conditions and OD 540_{nm} recorded, 100% are RBC cells lysed with Triton X-100 1 %. For analysis of *hlyA-lacZ* and *hlyD-lacZ* transcriptional fusions, strains were grown on blood agar plates overnight at 37°C under anaerobic conditions. **C,** β-galactosidase activity assay for expression of *hlyA.* **D,** β-galactosidase activity assay for expression of *hlyD.* Significant differences are indicated by asterisks (**, P<0.001 compared to the WT and mutant ; ***, P<0.0001compared to the WT and mutant).

Supplementary material:

Figure. S1. Logo graph of the information matrix obtained from the consensus alignment of FNR motif sequences for UPEC CFT073. The total height of each column of characters represents the amount of information for that specific position, and the height of each character represents the frequency of each nucleotide.

761 **Table S1. Strains and plasmids.** The genotypes of all strains of *E. coli* used or
762 constructed in this study and information about the plasmids used in this study are presented
763 here.

764 **Table S2. Oligonucleotides.** Oligonucleotide sequences used as PCR primers.

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Fig. S1.

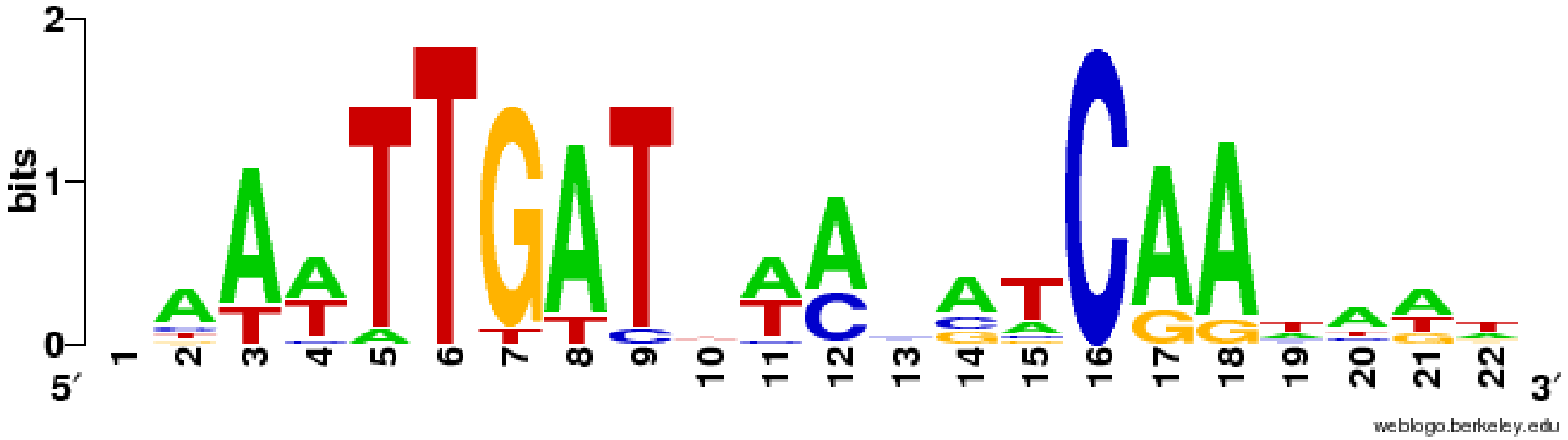
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Table S1. Strains and plasmids. The genotypes of all strains of *E. coli* utilized or constructed in this study and information about the plasmids used in this study.

Bacterial strains and plasmids	Genotype or relevant characteristics	Source or Reference
Bacterial strains		
E. coli DH5α	Plasmid propagation strain	Invitrogen
E. coli S17-λpir	RK2 tra regulon, pir, host for pir-dependent plasmids	[1]
E. coli fnr-771(del)::kan	F-, Δ(*araD-araB*)567, Δ*lacZ*4787(::rrnB-3), λ-, Δ*fnr*-771::kan,rph-1, Δ(*rhaD-rhaB*)568, *hsdR*514	[2]
UPEC CFT073	Blood isolate from a patient with acute pyelonephritis	[3]
LMP10	CFT073 Δ*lacZYA*	[4]
LMP11	CFT073 Δl*acZYA*::Chlr	[4]
	CFT073 Δ*fnr*	This study
	CFT073 Δ*fnr::kan*	This study
CFT073 ^{OFF}	IRL, fim invertible element locked off	[5]
CFT073 ^{ON}	IRL, fim invertible element locked on	[5]
	CFT073 Δ*lacZYA* Δ*fnr*	This study
	CFT073 Δ*lacZYA fimA-lacZ*	This study
	CFT073 Δ*lacZYA* Δ*fnr fimA-lacZ*	This study
	CFT073 Δ*lacZYA fimB-lacZ*	This study
	CFT073 Δl*acZYA* Δ*fnr fimB-lacZ*	This study
	CFT073 Δ*lacZYA fimE-lacZ*	This study
	CFT073 Δl*acZYA* Δ*fnr fimE-lacZ*	This study
	CFT073 Δ*lacZYA papA-lacZ*	This study
	CFT073 Δ*lacZYA* Δ*fnr papA-lacZ*	This study
	CFT073 Δ*lacZYA papB-lacZ*	This study
	CFT073 Δl*acZYA* Δ*fnr papB-lacZ*	This study
	CFT073 Δ*lacZYA* c5038-lacZ	[4]
	CFT073 Δ*lacZYA* Δ*fnr* c5038-lacZ	This study
	CFT073 Δ*lacZYA kguS-lacZ*	[4]
	CFT073 Δl*acZYA* Δ*fnr kguS-lacZ*	This study
	CFT073 Δ*lacZYA kguR-lacZ*	[4]
	CFT073 Δl*acZYA* Δ*fnr kguR-lacZ*	This study
	CFT073 Δ*lacZYA hlyA-lacZ*	This study
	CFT073 Δ*lacZYA* Δ*fnr hlyA-lacZ*	This study
	CFT073 Δ*lacZYA* c3565-lacZ	This study
	CFT073 Δ*lacZYA* Δ*fnr* c3565-lacZ	This study
	CFT073 Δ*lacZYA fliA-lacZ*	This study
	CFT073 Δ*lacZYA* Δ*fnr fliA-lacZ*	This study
	CFT073 Δ*lacZYA fliC-lacZ*	This study
	CFT073 Δ*lacZYA* Δ*fnr fliC-lacZ*	This study
	CFT073 Δ*lacZYA flhDC-lacZ*	This study
	CFT073 Δ*lacZYA* Δ*fnr flhDC-lacZ*	This study

Plasmids		
pET28a-(fnrD154A)2	expression plasmid of FNR protein	[6]
pVIK112	suicide plasmid for chromosomal lacZ transcriptional fusion	[7]
pGEN-MCS	low copy plasmid for complementation	[8]
pGEM-FNR	pGEN-MCS carrying fnr coding region and 500bp upstream promoter region	This study
pKD3	template for λ-Red Chlr cassette	[9]
pKD4	template for λ-Red Kanr cassette	[9]
pCP20	encodes FLP recombinase for removal of resistance cassette	[9]
pKD46	λ-Red recombinase expression	[9]

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Table S2. Oligonucleotides. Oligonucleotide sequences used as PCR primers.

Primers	Sequence (5'-3')
General PCR for cloning	
pGEN-FNR-F	GTACCATATGGATCGAATCCCATCAGCATC
pGEN-FNR-R	CCGAGTCGACAGGATCGATAACAACGAGCA
For lacZ fusion	
fimA-lacZ-5'-F	CACGGAATTC GTTGATGCAGGCTCTGTTGA
fimA-lacZ-5'-R	GCAGTCTAGA TTATTGATACTGAACCTTGA
fimB-lacZ-5'-F	GTCGGAATTCCCGATTGAGGATTTCGGATA
fimB-lacZ-5'-R	CCAGTCTAGACTATAAAACAGCGTGACGCT
fimE-lacZ-5'-F	GACGGAATTCGGCATGGGATGCGTATTAGT
fimE-lacZ-5'-R	CGAGTCTAGATCAAACTTCTTCTCTTTTTA
papA-lacZ-5'-F	CACGGAATTCATCAGTCGGTCAGGAAATGC
papA-lacZ-5'-R	GCAGTCTAGAGAGCAGCATATGCACCAAAA
papB-lacZ-5'-F	CACGGAATTC GGCCCCTGGATATATGCTTC
papB-lacZ-5'-R	GCAGTCTAGA CTCCATCATGCCTGTTCAGA
c5038-lacZ-5'-F	AGTCGAATTCTGGTGGTAATGCGGAAGAAC
c5038-lacZ-5'-R	ATCGTCTAGATATCGCCCAGTGGCAGAAGG
kguS-lacZ-5'-F	ATGCGAATTCTTCGCTTTCTGGCGAGAAGG
kguS-lacZ-5'-R	GCTGTCTAGAGAAACCGCGAGCATGATAAG
kguR-lacZ-3'-F	ATCGGAATTCTGTTATTGCAGCGACCAAGG
kguR-lacZ-3'-R	GCACTCTAGATTAGCTGGATGATTCTGGTC
hlyA-lacZ-5'-F	ATCGGAATTCTATTGATTTCCGGGAT
hlyA-lacZ-5'-R	ACCGTCTAGATTATGCTGAGCTGTC
c3565-lacZ-5'-F	GCTGTCTAGAGAAACCGCGAGCATGATAAG

c3565-lacZ-5'-R	ATCGGAATTCTGTTATTGCAGCGACCAAGG
hlyD-lacZ-5'-F	CACGGAATTC TTCGGGAAAAGTTCAGCAAC
hlyD-lacZ-5'-R	GCAGTCTAGA TTAACGCTCATGTAAACTTTCT
fliA-lacZ-3'-F	CACG CCCGGG ATCAGGCCTACAAGGGGAAT
fliA-lacZ-3'-R	GCAGTCTAGA GCGTTCGACGGCATTAAGTA
fliC-lacZ-3'-F	CACGGAATTC CGACACGTAAAACGAATACCG
fliC-lacZ-3'-R	GCAGTCTAGA CGCAGACTGGTTCTTGTTGA
flhDC-lacZ-3'-F	CACGGAATTC CGGTGAGACCGCATAAAAAT
flhDC-lacZ-3'-R	GCAGTCTAGA CCCAGGTCATAAACCAGTCG

For Deletion[a]

Del-fnr-F	GACGGTTATGCCAGACCACT
Del-fnr-R	AAGCGACAAGCTTCGTGAAT

For ON/OFF switch

fimEin F	GGCATGCTTGTGGTTATGAA
fimAin R	TTTTCATGCTGCTTTCCTTT

For EMSA

Inside negative control-For1	ATCTGTGTGGTAAGAGAATC
Inside negative control-Rev1	TGGTGCGCCATGGGATATTG
PromfimI-For	TTTGCAGAGCCAGTACGTTG
PromfimI-Rev	GTTGATGCAGGCTCTGTTGA
PrompapI-For	TTCACCCGTTTTTCAGAAGC
PrompapI-Rev	AAAATCCGCACACTGACCAT
PromkguR-For	AAGCCATAACGTTCCGCTTC
PromkguR-Rev	TTGCTACTGTTTGCCGCTAC

Prom3565-For	CAGCGTAACCACAGAGGATG
Prom3565-Rev	CGCAACAGAGCTGCAATATC
PromfliC-For	CGCAGACTGGTTCTTGTTGA
PromfliC-Rev	GGGAATAAGGGGCAGAGAAA
PromydfZ-For	GCGACTGGTTTAGCGAAGAG
PromydfZ-Rev	TGGTGATTGCGTTACGGTTA

- Underlined are restriction cutting sites;
- Capital letters represent homologous fragments of the deleted genes.

6. ARTIGO 4

FNR Regulates the Expression of Important Virulence Factors Contributing to the Pathogenicity of Avian Pathogenic *E. coli* APEC O1

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FNR Regulates the Expression of Important Virulence Factors Contributing to the Pathogenicity of Avian Pathogenic *E. coli* APEC O1

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) are the etiological agent of colibacillosis, which is one of the main causes of morbidity and mortality of chickens and is worldwide disseminated. Though many virulence factors associated with APEC pathogenicity are known, the regulation of virulence in these organisms remains unclear. FNR (fumarate and nitrate reduction) is a well-known global regulator that works as an oxygen sensor and has previously been described as a virulence regulator in pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium and *Shigella flexneri*. The objective of this study was to examine the role of FNR in the regulation of virulence genes in APEC O1. We found that FNR is involved in the regulation of some important genes related to APEC pathogenicity, such as plasmidial OmpT (outer membrane protein), type I fimbriae and AatA (autotransporter system). Loss of FNR resulted in a decreased expression of these virulence factors. Our results indicate that FNR is important for virulence regulation in APEC.

KEY WORDS

APEC, FNR, type I fimbriae, outer membrane protein, genes regulation, virulence regulation.

INTRODUCTION

Escherichia coli is an important gastrointestinal inhabitant. It is present in the microbiote of human and birds (1). *E. coli* is usually a nonpathogenic bacteria, however, some strains of *E. coli* acquired specific virulence factors (via DNA horizontal transfer by transposons, plasmids, bacteriophages, and pathogenicity islands) and became pathogenic. The virulence factors confer to the bacteria the ability to adapt to new niches and allow the bacteria to improve its ability to cause a broad spectrum of diseases. Extraintestinal pathogenic *E. coli* (ExPEC) strains may exist in the gut without causing disease to the GI tract, but have the capacity to disseminate and colonize other host niches including the urinary tract, the blood, and the central nervous system, resulting in disease (2).

Avian pathogenic *Escherichia coli* (APEC) are the etiological agent of colibacillosis, which is one of the main causes of morbidity and mortality of chickens, and is worldwide disseminated. It normally initiates in upper respiratory tract and affects other organs - such as lungs, air sacs, liver and spleen - and can culminate in a generalized infection (3, 4).

The APEC most common virulence associated factors include genes that enable APEC strains to adhere to host tissues, survive within host fluids, resist to host immune defenses and make the bacteria able to infect extraintestinal sites (3, 5, 6).

More than 250 transcription factors are known to regulate gene expression in *E. coli*. Some of these factors are operon-specific while others, known as global regulators, coordinate the expression of scores of promoters in response to specific environmental cues (7). Although many virulence factors are known to be associated with APEC pathogenicity, the regulation of their expression is still not understood.

The transcription factor FNR (for **F**umarate and **N**itrate **R**eduction) plays a major role in altering gene expression between aerobic and anaerobic conditions to facilitate such changes in energy metabolism. The gene encoding FNR (*fnr*) was originally discovered by Lambden and Guest in the mid-1970s from their pioneering work on the characterization of mutants that failed to carry out fumarate and nitrate reduction (8). It has been shown to be a global regulator of anaerobic metabolism in non-pathogenic *E.*

coli, controlling the synthesis of up to 125 genes (9). These include the expression of genes encoding enzymes for the anaerobic oxidation of carbon sources, enzymes for the anaerobic reduction of alternate terminal electron acceptors and proteins for transport of these carbon sources or electron acceptors (9).

FNR has been described as a virulence regulator in pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium (ATCC 14028s), in which FNR worked as a positive regulator of motility, flagellar biosynthesis and pathogenesis (10). In *Shigella flexneri* strains (M90T), it was observed that M90TΔfnr was substantially attenuated for gastrointestinal colonization compared to the wild-type strain, whereas FNR binding motifs were observed upstream of genes required for T3SS function, that are crucial to *Shigella* virulence (11).

OmpT, outer membrane protein T, is characterized as a protease (12), and it is associated with the degradation of α-helical antimicrobial peptides. Antimicrobial peptides are usually small cationic peptides that are secreted into the extracellular environment by epithelial cells, and have both bactericidal and immunomodulatory properties. These peptides are key players in the innate immune response to infection. They bind to the anionic cell membrane and lyse bacterial cells by forming pores as a mechanism of action. They also act as a bridge between the innate and adaptive immunity by recruiting immune cells to the site of infection (13). The LL-37 is one example of antimicrobial peptide and is expressed by different cell types, including neutrophils, bone marrow cells and epithelial cells of the lung and intestine. LL-37 derives from its biological precursor of human cationic antimicrobial protein 18 (hCAP18), which is processed into the biologically active peptide by the serine protease proteinase 3 (13, 14). Previous reports showed that EHEC OmpT readily degraded and inactivated antimicrobial peptides to promote bacterial survival, whereas EPEC OmpT was found to have a more marginal role in antimicrobial peptides degradation (13). APEC O1 encodes two copies of *ompT* gene, one in the chromosome and other in the plasmid (15), but until now, its role in the pathogenesis of avian colibacillosis remains unknown (12).

Type 1 fimbriae is the most important adhesin of APEC strains. It is reported as virulence factors in animal models of colibacillosis. The type 1 fimbriae have been shown to enhance bacterial survival, to stimulate mucosal inflammation, and to promote

invasion and growth in UPEC strain (16). Type 1 fimbriae mediate colonization of the center of the tubule via a mechanism that involves inter-bacterial binding and biofilm formation (17, 18).

The majority of APEC strains harbor large virulence plasmids that encode both known and unknown phenotypes that contribute to virulence. Autotransporters have been associated with diverse functions, including adhesion, cytotoxicity and lipase or protease activity. AatA is a novel autotransporter first identified in the PAI of APEC O1's virulence plasmid, pAPEC-O1-ColBM, which mediates adherence to chicken fibroblasts and contributes to virulence (19).

Due to these observations, the objective of this study was to examine the role of FNR in the regulation of certain known virulence genes in APEC O1. We observed the influence of *fnr* mutant in relation to antimicrobial resistance, outer membrane protein OmpT, type I fimbriae and AatA autotransporter.

MATERIAL AND METHODS

Bacterial Strains and Culture Conditions

The wild type strain used was APEC O1 isolated from lesions of chickens clinically diagnosed with colibacillosis, from United States. APEC O1 is a O1:K1:H7 strain whose genome is available (15).

Strains and plasmids used in this study are listed in Table S1. Aerobic growth was achieved by shaking at 180 rpm. For genetic manipulations, all *E. coli* strains were grown routinely in Luria Bertani (LB) broth medium. Selective antibiotics and IPTG were added when necessary at the following concentrations: ampicillin (Amp), 100 µg ml−1; kanamycin (Kan), 50 µg. ml−1; chloramphenicol (Chl), 25 µg.ml−1; IPTG, 0.1 mM (20).

Bioinformatics Analysis

The information matrix for the generation of the FNR logograph was produced by using the alignment of the *E. coli* FNR binding sequences as done in (10). To account for differences in nucleotide usage or slight variations in consensus sequences, a second alignment was built for *E. coli* APECO1 using the regions of the homologous genes originally used to build the *E. coli* information matrix. The alignment was used to prepare a new information matrix using the Patser software (version 3d). A graphical representation (Fig. S1) of the matrices through a logograph was obtained with Weblogo software (version 2.8.1).

Recombinant DNA Techniques

Polymerase chain reaction (PCR), DNA ligation, electroporation and DNA gel electrophoresis were performed according to Sambrook and Russell (21), unless otherwise indicated. All oligonucleotide primers were purchased from Integrated DNA Technologies (Iowa, USA) and are listed in Table S1. All restriction and DNA-modifying enzymes were purchased from New England Biolabs and used based on the suppliers' recommendations. Recombinant plasmids, PCR products, and restriction

Table 1. Strains and plasmids. The genotypes of all strains of *E. coli* used or constructed in this study and information about the plasmids used in this study.

Bacterial strains and plasmids	Genotype or relevant characteristics	Source or Reference
Bacterial strains		
E. coli DH5α	Plasmid propagation strain	Invitrogen
E. coli S17-λpir	RK2 tra regulon, pir, host for pir-dependent plasmids	(44)
E. coli fnr-771(del)::kan	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, Δfnr-771::kan,rph-1, Δ(rhaD-rhaB)568, hsdR514	(45)
APEC O1	isolate from lesions of chickens clinically diagnosed with colibacillosis	(15)
	APECO1 ΔlacZYA	This study
	APECO1 ΔlacZYA::Chlr	This study
	APECO1 Δfnr	This study
	APECO1 Δfnr::kan	This study
	APECO1 ΔlacZYA Δfnr	This study
	APECO1 ΔlacZYA fimA-lacZ	This study
	APECO1 ΔlacZYA Δfnr fimA-lacZ	This study
	APECO1 ΔlacZYA aatA-lacZ	This study
	APECO1 ΔlacZYA Δfnr aatA-lacZ	This study
	APECO1 ΔlacZYA ompTchr-lacZ	This study
	APECO1 ΔlacZYA Δfnr ompTchr-lacZ	This study
	APECO1 ΔlacZYA ompTplas-lacZ	This study
	APECO1ΔlacZYA Δfnr ompTplas-lacZ	This study
	CFT073 ΔlacZYA Δfnr flhDC-lacZ	This study
Plasmids		
pET28a-(fnrD154A)2	expression plasmid of FNR protein	(29)
pVIK112	suicide plasmid for chromosomal lacZ transcriptional fusion	(23)
pGEN-MCS	low copy plasmid for complementation	(24)
pGEM-FNR	pGEN-MCS carrying fnr coding region and 500bp upstream promoter region	This study
pKD3	template for λ-Red Chlr cassette	(22)
pKD4	template for λ-Red Kanr cassette	(22)
pCP20	encodes FLP recombinase for removal of resistance cassette	(22)
pKD46	λ-Red recombinase expression	(22)

fragments were purified using QIAquick PCR purification kit or MinElute gel extraction kit (Qiagen, CA) as recommended by the supplier. DNA sequencing was performed at the DNA facility, Iowa State University.

Deletion mutants were constructed using the lambda red recombinase system described by Datsenko and Wanner (22). Chromosomal transcriptional *lacZ* fusion was constructed by homologous recombination of the suicide plasmid pVIK112 carrying a fragment of the complete 5′-region, 3′- region, or internal fragment of the target gene (23), as described in (20) . For complementation, the coding sequences of genes plus their putative promoter regions were amplified from APEC O1 genome and independently cloned into pGEN-MCS (24) using EcoRI and SalI restriction sites.

Agglutinattion Assays

Tests for agglutination were performed as described earlier (25). For analysis of the *fim* operon, bacterial strains were grown at 37^{o} C in LB broth without shaking for 48 h, then were pelleted and concentrated to OD_{600nm} of 5.0.Suspensions (10% w/v) of yeast cells (*Saccharomyces cerevisiae*) were washed 3 times in PBS prior to use. Briefly, yeast cells were mixed on a glass slide with PBS or with bacteria in PBS, in the presence and absence of D-mannose. Agglutination was read after 10 min at room temperature. The strength of the agglutination was determined by titering of serial two-fold dilutions of the bacterial suspensions in PBS (25).

β-galactosidase Assays

Overnight LB cultures of *E. coli* containing the gene of interest-lacZ fusions were washed with PBS once and then diluted 1:100 in LB or indicated media and grown at 37°C. For analysis of *fim*, cultures were grown for 48 h without shaking at 37°C (26). For analysis of *ompT* and *aatA*, cultures were grown in LB under shaking until OD 0.5. These cultures were diluted 1:10 in Z buffer and assayed for β-galactosidase activity using ortho-Nitrophenyl-β-galactoside (ONPG) as a substrate as described previously (27).

Electrophoretic Mobility Shift Assays (EMSA)

Protein expression analysis was performed as describe in (28). Briefly, BL21 with pET28a-(fnrD154A)2 were grown in 200 mL of LB for 16 h at 25°C with 0.1 mM IPTG. To study the binding of FNR to the DNA probe, electrophoretic mobility shift assays (EMSAs) were performed as described in (29, 30). Briefly, FNR-His6 fusion protein was purified to homogeneity using Ni-NTA Spin Columns (28) and dialyzed against binding buffer. DNA probes were amplified by PCR using specific primers, and the gel was purified using a QIAGEN MinElute gel extraction kit.

EMSAs were performed by adding increasing amounts of purified (FNRD154A)2 -His6 fusion protein (0 to 20 ng) to the DNA probe (40 ng) in binding buffer (20 mM Tris (pH 6.8), 10 mM EDTA, 4 mM dithiothreitol, 50 mM NaCl, 10 % glycerol, 0.5 mg ml−1 bovine serum albumin (NEB) for 30 min at 37°C. Reaction mixtures were then subjected to electrophoresis on a 6% polyacrylamide gel in 0.5×TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) at 200 V for 45 min. The gel was stained in 0.5×TBE buffer containing 1×SYBR Gold nucleic acid staining solution for 30 min, and then the image was recorded.

Fragments amplified from the coding region of *kguR* (20) were used as negative controls, and fragments amplified the gene *ydfZ* as describe previously (29) were used as positive controls.

Minimal inhibitory concentrations (MIC)

Minimal inhibitory concentrations (MIC) were determined in 96-well microtiter plates using the broth microdilution method (31). Briefly, bacterial cells were grown to an optical density at 600 nm (OD600) of 0.5 in N-minimal medium, diluted to $5x10^5$ CFU/ml in the same medium, and aliquoted into rows of wells. Twofold serial dilutions of the tested antimicrobials were added to each row of wells. The plates were incubated at 37°C for 18 h. The lowest concentration of antimicrobial that did not permit any visible growth, determined by the absence of turbidity, was the MIC. Determination of MIC values was repeated at least three times. The antimicrobial test concentrations ranged from: LL-37 (128-0.002 μg/ml), Polymyxin B (128-0.002 μg/ml) and Lysozyme (5000-2.4 μg/ml).

RESULTS

In *silico* prediction of FNR binding sites

To determine whether a binding site for FNR might be present in the region upstream of the candidate FNR-regulated genes, we performed an *in silico* search of the 5′ regions of these genes for the presence of a putative FNR-binding motif, using *E. coli* APEC O1 specific logograph (Fig. 1).

We predicted 516 binding sites in the whole APEC O1 chromosome (NC_008563.1), 19 binding sites in the plasmid pColBM (NC_009837.1) and 21 binding sites in the plasmid pColR (NC_009838.1). We predicted binding sites upstream of virulence genes and operons such as genes encoding the plasmid allele of the OmpT protein, type I fimbriae, and the autotranspoter AatA.

Deletion of *fnr* reduced the expression of outer membrane protein OmpT from plasmid but not of OmpT chromossomal in APEC.

It was previously reported that *ompT* is an important gene for resistance to antimicrobial peptides (13). Since we found an in silico predicted FNR binding motif in the promoter region of the *ompT* gene, we investigated whether FNR affects *ompT* expression.

Two copies of the *ompT* gene are present in the APEC O1 strain. One copy is in the chromosome (APECO1_1482) and the other copy is in the pColBM plasmid (APECO1_O1CoBM192). Through *in silico* prediction, we observed a FNR binding motif in the promoter region of the plasmidial *ompT* (from - 81 to -60), but not in the chromosomal *ompT*. We constructed *ompT-lacZ* fusions to test gene expression of the chromosome and plasmid *ompT* genes independently. We observed that expression of chromosomal *ompT* did not alter with *fnr* mutation (fig. 2A). However, the expression of plasmid-encoded *ompT* was significantly reduced with *fnr* mutation (fig. 2B), and plasmid-encoded *ompT* expression was restored in the *fnr* complemented strain.

Fig. 1.

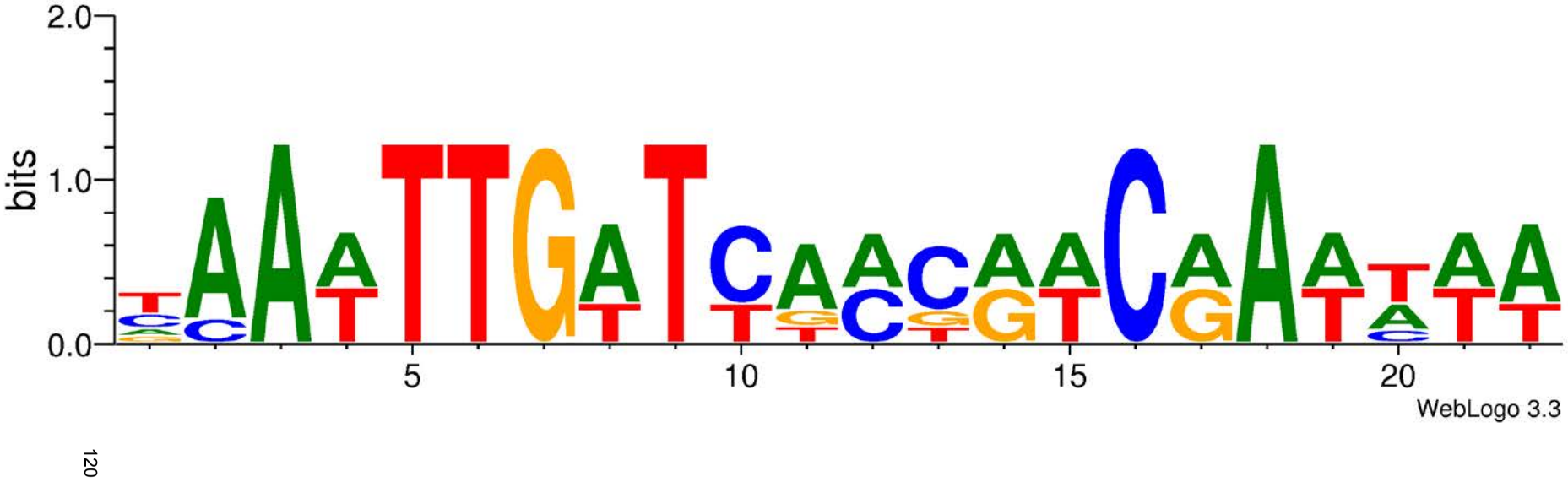
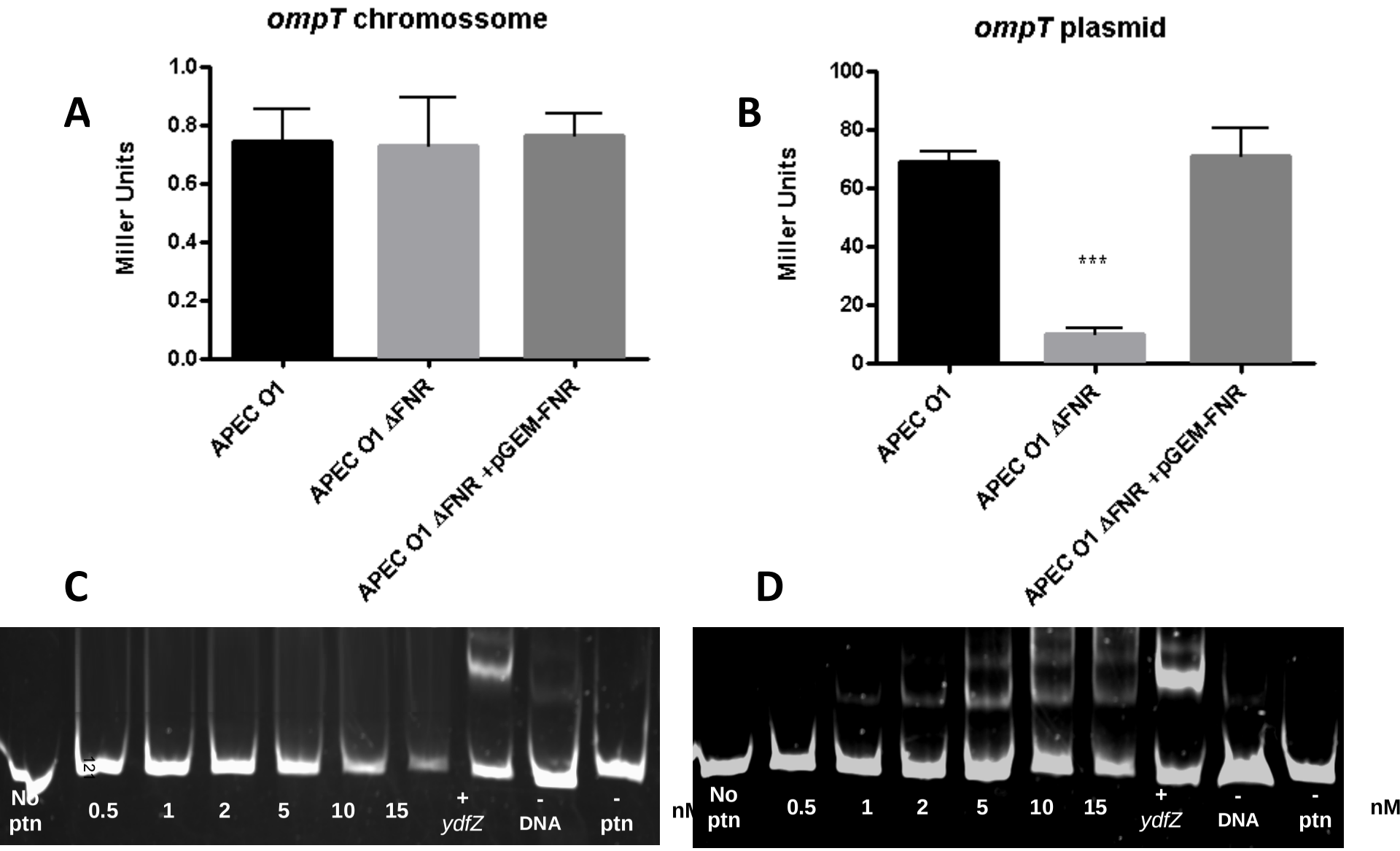


Fig. 2.


A
ompT chromossome
Miller Units
1.0
0.8
0.6
0.4
0.2
0.0
APEC O1
APEC O1 ΔFNR
APEC O1 ΔFNR +pGEM-FNR
B
ompT plasmid
Miller Units
100
80
60
40
20
0
***
APEC O1
APEC O1 ΔFNR
APEC O1 ΔFNR +pGEM-FNR
C
No ptn
0.5
1
2
5
10
15
+ ydfZ
- DNA
- ptn
nM
D
No ptn
0.5
1
2
5
10
15
+ ydfZ
- DNA
- ptn
nM

To determine whether FNR directly regulates plasmidial *ompT* expression, electrophoretic mobility shift assay (EMSA) was performed. The promoter regions of chromosomal *ompT* and plasmidial *ompT* were predicted by BProm program (http://linux1.softberry.com). A potential binding site of FNR was identified by bioinformatic analysis. DNA fragments containing the potential binding site were then amplified by PCR for use as probes (259 nucleotides in size, starting from -137 to +122 relative to translational start codon). As shown in Fig. 3C, FNR did not shift the promoter fragment of chromosomal *ompT*. However, FNR directly bound to the plasmidial *ompT* regulatory region. These results indicate that FNR directly binds to the regulatory region of plasmidial *ompT*, but not to the regulatory region of chromosomal *ompT*.

FNR contributes to antimicrobial peptide resistance in APEC

It was previously reported that pathogenic *E. coli* strains is resistant to antimicrobial peptides such as LL-37, lysozyme and polymyxin B (13). Here we observed that APEC O1 strain was resistant to antimicrobial peptide LL-37, having a MIC of 128 μg/ml (Table 2, figure 3A). For the *fnr* mutant, the MIC was 32 μg/ml, 4-fold more susceptible to LL-37 peptide than the wild type strain. Moreover, the wild type phenotype was recovered when APEC O1 Δ *fnr* was complemented with pGEN-FNR, having a MIC of 128 μg/ml.

We also tested the minimal inhibitory concentration in relation to lysozyme. Our results showed that APEC O1 had an MIC of 2500 μg/ml, APEC O1 Δ *fnr* had an MIC of 312 μg/ml, and the phenotype was recovered in APEC O1 Δ *fnr* + pGEN-FNR, showing an MIC of of 2500 μg/ml (Table 2, Fig. 3B). APEC O1 Δ *fnr* was 8-fold more susceptible to lysozyme, indicating that FNR is important for this phenotype.

However, for the antimicrobial peptide polymyxin B we did not observe any difference among the wild type, mutant and complemented strains (Table 2, figure 3C). All of them presented a MIC of 4 μg/ml.

Deletion of *fnr* reduced the expression of type 1 fimbriae in APEC

Fig. 3.

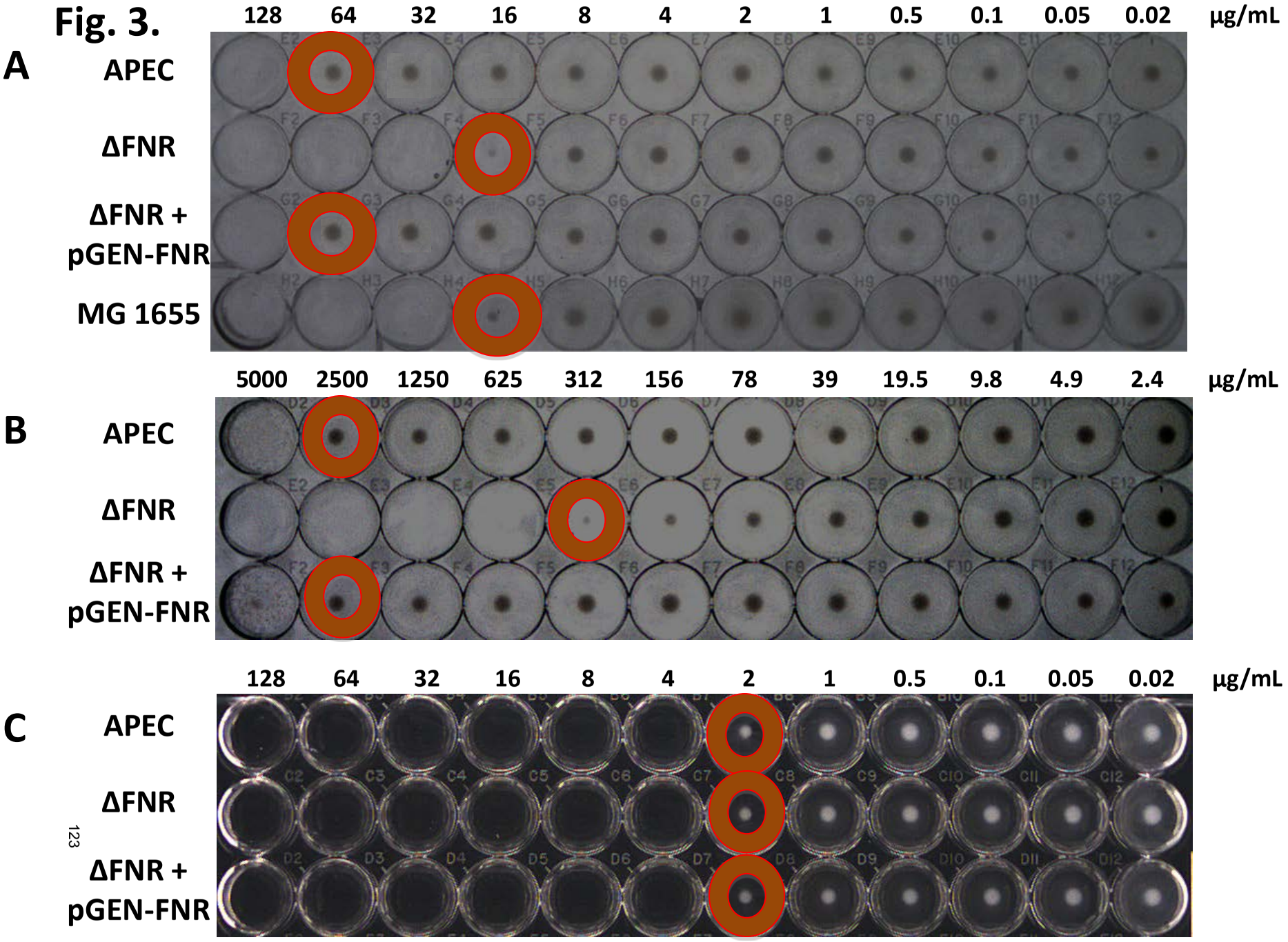

A
128 64 32 16 8 4 2 1 0.5 0.1 0.05 0.02 µg/mL
APEC
ΔFNR
ΔFNR + pGEN-FNR
MG 1655
B
5000 2500 1250 625 312 156 78 39 19.5 9.8 4.9 2.4 µg/mL
APEC
ΔFNR
ΔFNR + pGEN-FNR
C
128 64 32 16 8 4 2 1 0.5 0.1 0.05 0.02 µg/mL
APEC
ΔFNR
ΔFNR + pGEN-FNR

Table. 2. FNR affects MIC in APEC strains

Strain	MIC µg/ml		
	LL-37	Lysozyme	Polymyxin B
APEC O1	128	2500	4
APEC O1 ΔFNR	32	312	4
APEC O1 ΔFNR +pGEN-FNR	128	2500	4
MG1655	32	312	4

The expression of type 1 fimbriae can be detected by mannose-sensitive yeast agglutination (MSYA), which attests the ability of type 1 fimbriated bacteria to bind to mannose-containing receptors on the surface of yeast cells. Using serially diluted assays, we observed that APEC O1 agglutinated yeast cells even at an 8-fold dilution of the wild type cell suspension (8-fold); in contrast, the *fnr* mutant was unable to agglutinate yeast cells; the complemented strain recovered the agglutination ability to the wild type level (Fig. 4A). These results indicate that *fnr* mutation caused a substantial decrease in the expression of type 1 fimbriae on the cell surface.

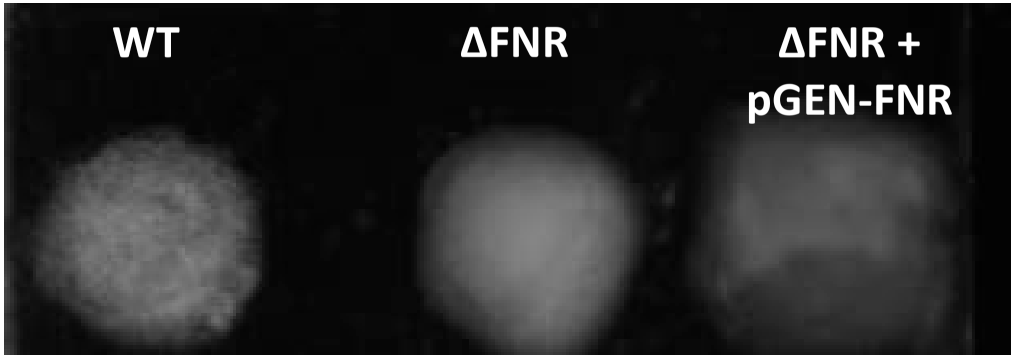
To confirm if this phenotype was related with the type 1 fimbriae genes, we evaluated the gene expression of *fimA*, which encodes the major subunit of type 1 fimbriae. We tested the gene expression by β-galactosidase assays, using *fim-lacZ* construction in the chromosome. A significant down-regulation of *fimA* was observed in the *fnr* deletion strain as compared with wild type (Fig.4B), suggesting that FNR enhance type 1 fimbriation at the transcriptional level, consistent with our agglutination data.

Deletion of *fnr* reduced the expression of AatA autotransporter in APEC

Our group has recently described an AatA autotransporter system in APEC O1 (APECO1_O1CoBM96), and found that this protein is important for APEC virulence *in vivo* (19). A potential binding site of FNR in the promoter region of the *aatA* gene (from - 89 to -68) was identified by bioinformatic analysis. To determine if deletion of *fnr* affects *aatA* expression, we tested the gene expression by β-galactosidase assays. We observed that *aatA* expression was significantly reduced in the *fnr* mutant strain as compared to the wild type strain, and its expression was restored in the complemented strain (Fig.5A).

To determine whether FNR directly regulates *aatA* expression, an electrophoretic mobility shift assay (EMSA) was performed. DNA fragments containing the potential binding site were then amplified by PCR for use as probes (245 nucleotides in size, starting from -141 to +104 relative to translational start codon). As shown in Fig. 5B, FNR fusion protein was able to shift the promoter fragment of *aatA*. These results demonstrate that FNR directly binds to the promoter of *aatA*.

Fig. 4.



	WT	ΔFNR	ΔFNR +pGEN-FNR
dilution	1:8	1:1	1:8

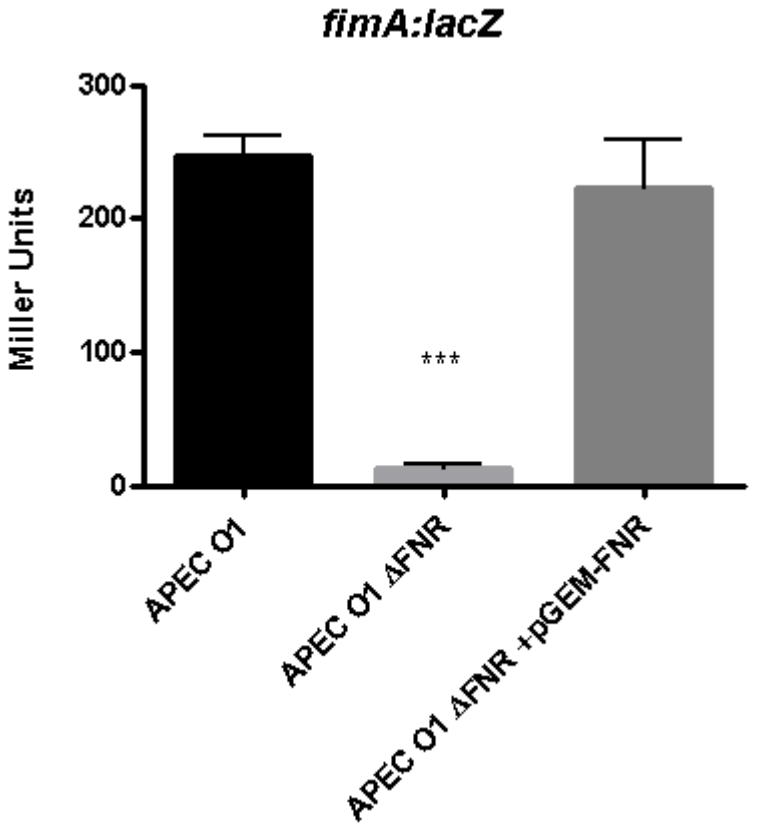
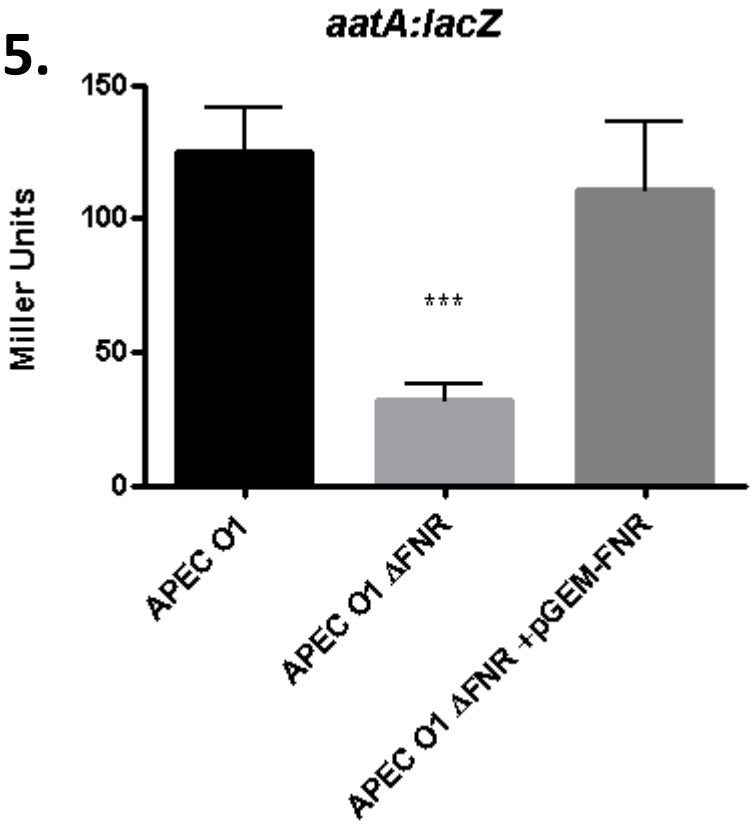
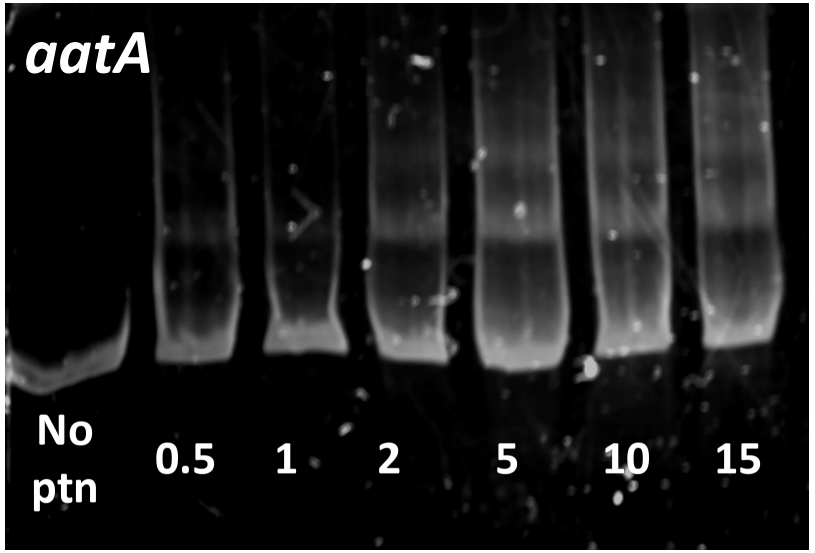


Fig. 5.



Gel shift de *aatA* (EMSA)



DISCUSSION

The regulator of fumarate and nitrate reduction (FNR) is related to transcription activators that control the expression of networks of *E. coli* genes in response to oxygen starvation observed in nonpathogenic strains such as *E. coli* K-12 (MG 1655) (32). In anaerobic conditions, FNR was able to bind to specific DNA targets at promoters and modulate transcription (33). It is known that FNR is related to regulation of the expression of genes encoding enzymes for respiratory processes that utilize alternative electron acceptors, such as nitrate and fumarate, or that promote fermentation under anaerobic conditions (9).

This work is the first to test the global capability of FNR regulation in an avian pathogenic *E. coli* strain. Other works were performed using K-12 (MG 1655) as model (33).

By bioinformatic analysis, we identified 516 FNR binding sites in the whole APEC O1 chromosome, 19 binding sites in the plasmid colBM (NC_009837.1) and 21 binding sites in the plasmid colR (NC_009838.1). We observed genes related to metabolic functions, transport of small molecules, iron metabolism, regulatory genes and related to outer membrane protein. The fact that some genes bound by FNR are not related to the switch to aerobic and anaerobic environmental, we believe that *fnr* is more than just an oxygen sensor, but is also an important player in APEC virulence.

Proteolytic degradation of α-helical antimicrobial peptides, as the protein conformation present in LL-37 antimicrobial peptide, was done by outer membrane proteases of the omptin family (*omp*). This mechanism of resistance has been shown to be used by Gram-negative pathogens (13). As we observed by bioinformatic analysis, the *ompT* present in the pColBM plasmid has a FNR binding motif in its promoter region. We observed that plasmidial *ompT* was down-regulated in the *fnr* mutant strains (Fig. 2B) compared to the wild type. In contrast, chromosomal *ompT* expression was not altered in the mutant strain (Fig. 2A). These data indicate that the resistance phenotype to antimicrobial peptides was due to a FNR gene regulation of the plasmidial *ompT* gene, suggesting that FNR influences the metabolism of antimicrobial peptides.

Also, by the EMSA assay, we confirmed that only the plasmidial copy of *ompT* was able to bind FNR protein. These data clearly indicate that FNR could regulate just one of two copies of the same gene. We also observed that outer membrane protein OmpT has 2 different promoter regions in their different copies. Based on this, we can infer that there may exist different biological functions for OmpT that could be activated by different environmental stimuli.

Antimicrobial peptides are an important new family of molecules developed for treatment of infection by multi-resistant bacteria. Here, we tested APEC O1 susceptibility to LL-37, lysozyme and polymyxin B (Fig. 3, Table 2). We observed a resistance for this new LL-37 peptide. APEC O1 Δ *fnr* was more susceptible than wild type strains, and this increase in susceptibility to LL-37 is due to a gene regulated by FNR unrelated to its oxygen sensor activity, but related to the bacteria metabolism, as previously described (33).

Type I fimbriae, which are present in almost all APEC isolates (6, 34) are one of the most common virulence associated factors in APEC strains. In our findings, we observed that the *fnr* mutant was unable to agglutinate yeast cells extracts at a high concentration, and also *fimA* expression was reduced in the mutant strain (Fig. 4). This confirms that *fnr* mutant were less able to express the type I fimbriae. Previous works have shown that the loss of type I fimbriae caused reduction of adhesion to human pneumocytes (A549) and hepatocytes (LM) (35), reduction of biofilm formation (18), reduction in bacterial adhesion to eukaryotic cells (36) and a reduction of pathogenicity *in vivo* (37), and *fimH* is required by UPEC to invade bladder epithelial cells (38). It has also been demonstrated that the reduction of type I fimbriae expression caused by a mutation of other virulence genes, such as type 6 secretion system (39) and *ibeA* (40, 41) caused a reduction in the adherence process and virulence. All of this data showed that type I fimbriae are very important for the maintenance of the APEC and its expression is important for bacterial adherence and development of the infection process. The FNR regulation of type I fimbriae expression underscores that type I fimbriae is an important virulent associated gene.

AatA is an autotransporter encoded by the APEC autotransporter gene (*aatA)*, which has been localized at the PAI found in the virulence plasmid pColBM of APEC O1 (19). The gene *aatA* is present in 32-40 % of APEC isolates (19, 42). AatA is a

protein that contributes to an adherence phenotype (42), and it is also related to improvement of the pathogenesis of APEC O1 *in vivo* (19). Here we observed that *aatA* was upregulated by FNR, and that FNR binds to the promoter region of *aatA* gene (FIG. 5), thus being directly regulated by FNR.

Large plasmids are highly associated with virulence of APEC strains, and their transfer to non-pathogenic strains can render them pathogenic or increase their virulence potential (43). These plasmids are also associated with horizontal gene transfer, because they can be transferred to another bacteria by conjugation process, and because they usually encode virulence associated genes they confer to the bacteria the ability to survive in a new environment. This is the first time that FNR, a protein encoded by the chromosome, is reported to regulate genes present in plasmids, such as outer membrane plasmid *ompT* and auto-transporter *aatA*. Here we describe the FNR regulation of the APEC virulence-associate genes plasmidial *ompT*, *fim* and *aatA* autotransporter system. All of these specific virulent-associated genes required FNR for expression. Our data indicate that FNR is not just an oxygen sensor regulator, but may also act as a very important regulator of virulence-associated genes in APEC.

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Figure legends:

Figure. 1. Logograph of the information matrix obtained from the consensus alignment of FNR motif sequences for *E. coli* APEC O1. The total height of each column of characters represents the amount of information for that specific position, and the height of each character represents the frequency of each nucleotide.

Figure. 2. FNR affects antimicrobial resistance in APEC. MIC assay for antimicrobial resistance to (**A**) LL-37 peptide (**B**) lysozyme (**C**) polymyxin B. Bacterial cells were grown to an optical density of 0.5 at 600 nm (OD600) in N-minimal medium, diluted to $5x10^5$ CFU/ml in the same medium, and aliquoted into rows of wells. Two-fold serial dilutions of the tested antimicrobials were added to each row of wells. The plates were incubated at 37 °C for 18 h. The lowest concentration of antimicrobial that did not permit any visible growth, determined by absence of turbidity, was the MIC.

Figure. 3. APEC outer membrane protein OmpT regulation. *ompTchr-lacZ* and *ompTplas-lacZ* transcriptional fusion strains were grown in LB under shaking until OD 0.5 at 37°C. β-galactosidase activity was measured and the values shown are means plus standard deviations of triplicate samples. **A**,β-galactosidase activity assay for expression of *ompT* chromossome; **B**,β-galactosidase activity assay for expression of plasmidial *ompT*; Non-radioactive EMSA studying the binding of $(FNRD154A)_2$ - His_6 to the promoter regions. PCR products of *ompT chr* **(D)** and *ompTplas* **(C)** promoter region were used as probes. Purified $(FNRD154A)_2$ -His_6 fusion protein was added in different concentrations in each reaction mixture as indicated and MBP-His_6 was used as negative control at 300ng per each reaction. For the lane on the right on each panel, a negative control DNA fragment amplified from *kguR* coding region was used, and as positive controls *ydfZ* were used. DNA fragments were stained with SYBR green. Significant differences are indicated by asterisks (***, P<0.0001compared to the WT and mutant).

Fig. 4. APEC type I fimbriae regulation. A, yeast agglutination assay with dilutions of wild-type 1:8; ΔFNR 1:1 and ΔFNR + pGEN-FNR 1:8; Bacterial were grown statically for 48 h, agglutination was read after 10 min at room temperature and the strength of the agglutination was determined by titering of serial two-fold dilutions in PBS of the bacterial suspensions; *fimA-lacZ* transcriptional fusion strains were grown in LB medium statically for 48 h at37°C. β-galactosidase activity was measured and the values shown are means plus standard deviations of triplicate samples **B**, β-galactosidase activity assay for expression of *fimA.* Significant differences are indicated by asterisks (***, P<0.0001compared to the WT and mutant).

Fig. 5. APEC autotransporter AatA regulation. *aatA-lacZ* transcriptional fusion strains were grown in LB under shaking until OD 0.5 at 37°C. β-galactosidase activity was measured and the values shown are means plus standard deviations of triplicate samples **A**,β-galactosidase activity assay for expression of *aatA*; **C.** Non-radioactive EMSA studying the binding of $(FNRD154A)_2$ - His_6 to the promoter regions. PCR products of *aatA* promoter region was used as probes. A negative control DNA fragment amplified from *kguR* coding region was used, and as positive controls *ydfZ* were used. DNA fragments were stained with SYBR green. Significant differences are indicated by asterisks (***, P<0.0001compared to the WT and mutant).

Table 1. Strains and plasmids. The genotypes of all strains of *E. coli* used or constructed in this study and information about the plasmids used in this study.

Table. 2. FNR affects MIC in APEC strains

Table S1. Oligonucleotides. Oligonucleotide sequences used as PCR primers.

Table S1. Oligonucleotides. Oligonucleotide sequences used as PCR primers.

Primers	Sequence (5'-3')
General PCR for cloning	
pGEN-FNR-F	GTACCATATGGATCGAATCCCATCAGCATC
pGEN-FNR-R	CCGAGTCGACAGGATCGATAACAACGAGCA
For lacZ fusion	
fimA-lacZ-5'-F	CACGGAATTC GTTGATGCAGGCTCTGTTGA
fimA-lacZ-5'-R	GCAGTCTAGA TTATTGATACTGAACCTTGA
ompTgen-lacZ-5'-F	CACGGAATTCATTGCGAGGCCTTATGTGTC
ompTgen-lacZ-5'-R	GCAGTCTAGAGCCTCCTTCTTCGGCTAGAT
ompTplas-lacZ-5'-F	GACGGAATTCGGCCTTATTCGCAGTTACCA
ompTplas-lacZ-5'-R	GTCGTCTAGATCAGGCAGATAAACCCGTTC
aatA-lacZ-5'-F	CACGGAATTCGGGAATATCTACGGCAGCAA
aatA-lacZ-5'-R	GCAGTCTAGATGCACCACCGATTGATGTAT
For Deletion[a]	
Del-fnr-F	GACGGTTATGCCAGACCACT
Del-fnr-R	AAGCGACAAGCTTCGTGAAT
For EMSA	
Inside negative control-For1	ATCTGTGTGGTAAGAGAATC
Inside negative control-Rev1	TGGTGCGCCATGGGATATTG
PromompTgen-For	TCAGGCAGATAAACCCGTTC
PromompTgen-Rev	AAACAAACGTTAAACAAAACAGCA
PromompTplas-For	AAACAAACGTTAAACAAAACAGCA
PromompTplas-Rev	TCAGGCAGATAAACCCGTTC
PromaatA-For	CCAGACGTCTCGTGATACTCC
PromaatA-Rev	CAGAGGCGTTCGAGCATTAT
PromydfZ-For	GCGACTGGTTTAGCGAAGAG
PromydfZ-Rev	TGGTGATTGCGTTACGGTTA

- Underlined are restriction cutting sites;
- Capital letters represent homologous fragments of the deleted genes.

7. ARTIGO 5

FNR Regulates the Expression of Important Virulence Factors Contributing to the Pathogenicity of Newborn Meningitis *E. coli* strain NMEC 58

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FNR Regulates the Expression of Important Virulence Factors Contributing to the Pathogenicity of Newborn Meningitis *E. coli* strain NMEC 58

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ABSTRACT

Escherichia coli is the most common Gram-negative causative agent of neonatal bacteremia and meningitis. Newborn meningitis *E. coli* (NMEC) is recognized as one of the top ten causes of infection-related death worldwide. Mortality is estimated at 10% in developed countries and 40–58% in developing countries (1). The regulation of virulence of NMEC is still unclear. FNR (fumarate and nitrate reductase) is a well-known global regulator that acts as an oxygen sensor and has been described as a virulence regulator in pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium and *Shigella flexneri*. The objective of this study was to examine the role of FNR in the pathogenicity of NMEC 58. Using *in vivo* model of 3-day-old rats, we observed that the FNR mutant was less virulent then the wild type strain. The *fnr* mutants strains were not able to cause bacteremia and did not reach the cerebrospinal fluid nor cause meningitis as the wild type did. FNR also affected motility in NMEC, and *fnr* mutant were more susceptible to LL-37 peptide, Human Platelet factor IV 18 peptide and lysozyme. Our results confirm that FNR is important for the regulation of virulence in NMEC.

KEY WORDS

NMEC, FNR, pathogenicity, *in vivo* assay, motility, antimicrobial susceptibility and virulence regulation.

INTRODUCTION

Escherichia coli strains causing extraintestinal disease are known as extraintestinal pathogenic *E. coli* (ExPEC), and include: the uropathogenic *E. coli* (UPEC), that cause urinary tract infections; avian pathogenic *E. coli* (APEC), that causes systemic infections in birds; and neonatal meningitis *E. coli* (NMEC) (2), that is the most common Gram-negative organism that causes neonatal bacteremia and meningitis (3).

Bacterial meningitis is characterized by inflammation of the meninges that affects the pia, arachnoid, and the subarachnoid space (1). It is recognized as one of the top ten causes of infection-related death worldwide. Mortality is estimated at 10% in developed countries and 40–58% in developing countries (1). *E. coli* primarily affects preterm babies (45–81%) causing high mortality and morbidity. Most infant survivors (30–50%) have sequelae including hydrocephalus, seizures, mental retardation, cerebral palsy, and hearing loss (4, 5).

Infants acquire NMEC from the maternal genital tract or uterus during birth, which is thought to enter the bloodstream by trauma during birth (5). Once in the bloodstream, NMEC are phagocytized by monocytes and resist the killing respiratory burst and begin intracellular replication. Once sufficient and broad bacteremia has been established, NMEC will adhere to and invade human brain microvascular endothelial cells and traverse the blood-brain barrier, causing inflammation in the central nervous system and brain (3, 6).

Up until now, the set of virulence factors know for NMEC have include: adhesins (Type 1 and S fimbriae), invasins (IbeA, flagellin, OmpA), resistance or evasion of the host immune system (K1 capsule O-LPS, AslA), toxins (CNF1), and nutrient uptake (salmochelin, enterobactin). The most common virulence associated factor is the capsule K1, that is present in 80% of the isolates (6). However, the microbial determinants that contribute to neonatal meningitis *E. coli* (NMEC) survival in the blood and bacteremia and how circulating bacteria cross the blood-brain barrier are not completely understood (3).

Flagellum, an organelle responsible for bacterial motility, is involved in the interaction of various pathogenic *E. coli* strains with epithelial cells. In NMEC pathogenesis, it is involved in contact between bacteria and endothelium or epithelium. Flagella contribute to virulence factors by, allowing bacteria to ascend in the

bloodstream and reach the blood-brain barrier (7). *E. coli* strains may invade endothelial epithelium through flagellin, and flagellin acts as an invasin in this process (6, 8).

Epithelial cells can secrete to the extracellular environment antimicrobial peptides. These peptides are small cationic molecules that have both bactericidal and immunomodulatory properties, acting as key players in the innate immune response to infection. The antimicrobial action of these peptides involves binding to the anionic cell membrane and lysing bacterial cells by forming pores, resulting in bacterial death. One known human antimicrobial peptide is LL-37, which belongs to the cathelicidin family. It is synthesized as the precursor of human cationic antimicrobial protein 18 (hCAP18), which is processed into the biologically active peptide by the serine protease proteinase 3 (9). However, bacterial pathogens have evolved different mechanisms to resist the killing action of antimicrobial peptides such as proteolytic degradation and extrusion by efflux pumps.

It had been described a group of more than 250 transcription factors hav been identified to control gene expression in *E. coli* (10). These regulators genes could act as operon-specific, and as global regulators, coordinating the expression of scores of promoters in response to specific environmental changes. Although many virulence factors are known to be associated with NMEC pathogenicity, the regulation of their expression is still not fully understood.

The transcription factor FNR (for **F**umarate and **N**itrate **R**eductase) plays a major role in altering gene expression between aerobic and anaerobic conditions to facilitate such changes in energy metabolism. The gene encoding FNR (*fnr*) was originally discovered by Lambden and Guest in the mid-1970s from their pioneering work on the characterization of mutants that failed to carry out fumarate and nitrate reduction (11). Since then, FNR has been shown to be a global regulator of anaerobic metabolism in non-pathogenic *E. coli*, controlling the synthesis of up to 125 genes (12). It has also been described as a virulence regulator in pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium (ATCC 14028s), in which FNR acts as a positive regulator of motility, flagellar biosynthesis, and pathogenesis (13). Furthermore, it has been observed that, in *Shigella flexneri* strains (M90T), FNR is a positive regulator of the T3SS pathogenicity island which confers to the bacteria the ability to invade epithelial cells (14).

Based on the importance of FNR for the bacterial adaptation to anaerobic conditions and on the many phenotypes shared by the *fnr* mutant in *Salmonella*,

Shigella and *E. coli*, it has generally been assumed that FNR would be important for pathogenic *E. coli* virulence. To address these questions we constructed and characterized a *fnr* mutant strain of NMEC 58. We observed the role of *fnr* mutant for virulence *in vivo* and we also tested this mutant for antimicrobial susceptibility and motility phenotypes.

MATERIAL AND METHODS

Bacterial Strains and Culture Conditions

The wild type strain NMEC 58 was isolated from the cerebrospinal fluid of an anonymous newborn (less than 28 days old) with newborn meningitis in The Netherlands Reference Laboratory for Bacterial Meningitis (Amsterdam) (4). The isolate is characterized as a O18:K1 strain, belonging to phylogenetic group B2 and MLST group ST 416 (2).

Strains and plasmids used in this study are listed in Table 1. Aerobic growth was achieved by shaking in air at 180 rpm. All *E. coli* strains were grown routinely in Luria Bertani (LB) broth medium. Selective antibiotics were added when necessary at the following concentrations: ampicillin (Amp), 100 μg ml−1; kanamycin (Kan), 50 μg. ml−1; chloramphenicol (Chl), 25 μg.ml−1 (15).

Bioinformatics Analysis

The information matrix for the generation of the FNR logograph was produced using the alignment of *E. coli* FNR binding sequences as done in (13). To account for differences in nucleotide usage or slight variations in consensus sequences, a second alignment was built for *E. coli* NMEC 58 using the regions of homologous genes originally used to build the *E. coli* information matrix. The alignment was used to construct a new information matrix using the Patser software (http://stormo.wustl.edu/software.html). A graphical representation (Fig. 1) of the matrices and a logograph was obtained with Weblogo software (version 2.8.1).

Recombinant DNA Techniques

Polymerase chain reaction (PCR), DNA ligation, electroporation and DNA gel electrophoresis were performed according to Sambrook and Russell (16) unless otherwise indicated. All oligonucleotides were purchased from Integrated DNA Technologies (Iowa) and are listed in Table 2. All restriction and DNA-modifying enzymes were purchased from New England Biolabs and used based on the suppliers' recommendations. Recombinant plasmids, PCR products and restriction fragments were purified using QIAquick PCR purification kit or MinElute gel extraction kit (Qiagen, CA) as recommended by the supplier. DNA sequencing was performed at the DNA facility, Iowa State University. Deletion mutants were constructed using the lambda red

Table 1. Strains and plasmids. The genotypes of all strains of *E. coli* used or constructed in this study and information about the plasmids used in this study.

Bacterial strains and plasmids	Genotype or relevant characteristics	Source or Reference
Bacterial strains		
E. coli DH5α	Plasmid propagation strain	Invitrogen
E. coli fnr-771(del)::kan	F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, Δfnr-771::kan,rph-1, Δ(rhaD-rhaB)568, hsdR514	(23)
NMEC 58	Newborn meningitis *Escherichia coli* isolate from a patient with meningitis	(4)
	NMEC 58 Δfnr	This study
	NMEC 58 Δfnr::kan	This study
Plasmids		
pGEN-MCS	low copy plasmid for complementation	(18)
pGEM-FNR	pGEN-MCS carrying fnr coding region and 500bp upstream promoter region	This study
pKD3	template for λ-Red Chlr cassette	(17)
pKD4	template for λ-Red Kanr cassette	(17)
pCP20	encodes FLP recombinase for removal of resistance cassette	(17)
pKD46	λ-Red recombinase expression	(17)

Table 2. Oligonucleotides. Oligonucleotide sequences used in PCR.

Primers	**Sequence (5'-3')**
General PCR for cloning	
pGEN-FNR-F	GTACCATATGGATCGAATCCCATCAGCATC
pGEN-FNR-R	CCGAGTCGACAGGATCGATAACAACGAGCA
For Deletion[a]	
Del-fnr-F	GACGGTTATGCCAGACCACT
Del-fnr-R	AAGCGACAAGCTTCGTGAAT

- Underlined are restriction cutting sites;
- Capital letters represent homologous fragments of the deleted genes.

recombinase system described by Datsenko and Wanner (17). For complementation, the coding sequences of genes plus their putative promoter regions were amplified from the CFT073 genome and independently cloned into pGEN-MCS (18) using *EcoRI* and *SalI* restriction sites.

Motility Assay

The motility of NMEC 58, *fnr* mutant, and complemented ΔFNR+ pGEN-FNR strains was evaluated under anoxic conditions as done in (13). Briefly, 10 µl of anaerobically grown (16 h) cells were spotted onto LB-agar (0.25% agar) plates and incubated at 37 °C for 16 h. The diameter of the growth halo was used as a measure of motility.

MIC determination

Minimal inhibitory concentrations (MIC) were determined in 96-well microtiter plates using the broth microdilution method (19). Briefly, bacterial cells were grown to an optical density of 0.5 at 600 nm (OD600) in N-minimal medium, diluted to $5x10^5$ CFU/ml in the same medium, and aliquoted into rows of wells. Two-fold serial dilutions of the tested antimicrobials were added to each row of wells. The plates were incubated at 37 °C for 18 h. The lowest concentration of antimicrobial that did not permit any visible growth, determined by absence of turbidity, was the MIC. Determination of MIC values was repeated at least three times. The antimicrobial test concentrations ranged from: Lysozyme (5000- 2.4 µg/ml); LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) (128-0.002 µg/ml); Human Platelet factor IV 18, C18G (128-0.002 mg/ml); Polymyxin B (128-0.002 µg/ml).

***In vivo* Rat neonatal meningitis model**

Groups of approximately 12 specific-pathogen-free (SPF) (range of 6-14) 5-day-old Sprague-Dawley rat pups were infected by the intraperitoneal route with approximately 200 CFU (range, 48 to 244 CFU) of log grow, 0.5 at 600 nm (OD600) *E. coli* (2). After 24 hours, the pups were euthanized using sodium pentobarbital (Sleepaway; Fort Dodge Laboratories). For bacterial counting, blood was collected from heart vein and plated on MacConkey agar to indicate septicemia. Cerebrospinal fluid (CSF) was collected by cisternal punctureand plated on MacConkey agar to indicate meningitis. The minimum detection dose was 100 CFU/ml (20). For statistical analysis,

a two-tailed Wilcoxon matched pairs test was used (Prism software, CA) and the threshold for statistical significance was a p value <0.05.

To confirm that all isolates derived from animals maintained their status, one colony of blood and CSF isolates from each animal was retested for the FNR mutation amplification gene.

Ethics Statement

All animal procedures were conducted in accordance with NIH guidelines, the Animal Welfare Act and US federal law. The experimental protocol for handling animals was approved for Institutional Animal Care and Use Committee at Iowa State University (IACUC) (Protocol number 4-11-7111-Z). All efforts were made to minimize suffering. All the procedures were done with the presence of a well-qualified veterinarian doctor.

RESULTS

The *fnr* mutation attenuates virulence in newborn rats infection model of sepsis meningitis

To study the role of FNR in virulence of NMEC *in vivo*, we compared the NMEC 58 wild-type strain with its NMEC 58 ΔFNR deletion mutant strain for the ability to reach bloodstream and cerebrospinal fluid in 3-day-old rats infected intraperitoneally for 24 hours (the *in vivo* model developed for NMEC). The deletion mutation was constructed by replacement of *fnr* with the *cat* cassette conferring kanamycin resistance.

We observed a significant difference in mortality (p value ≤0.001): of 14 animals infected by NMEC 58, 11 animals died, whereas none of the ten animals infected with NMEC 58 ΔFNR died. Reintroduction of *fnr* back into the mutants restored mortality: of 13 animals infected with complemented strain, 12 animals died.

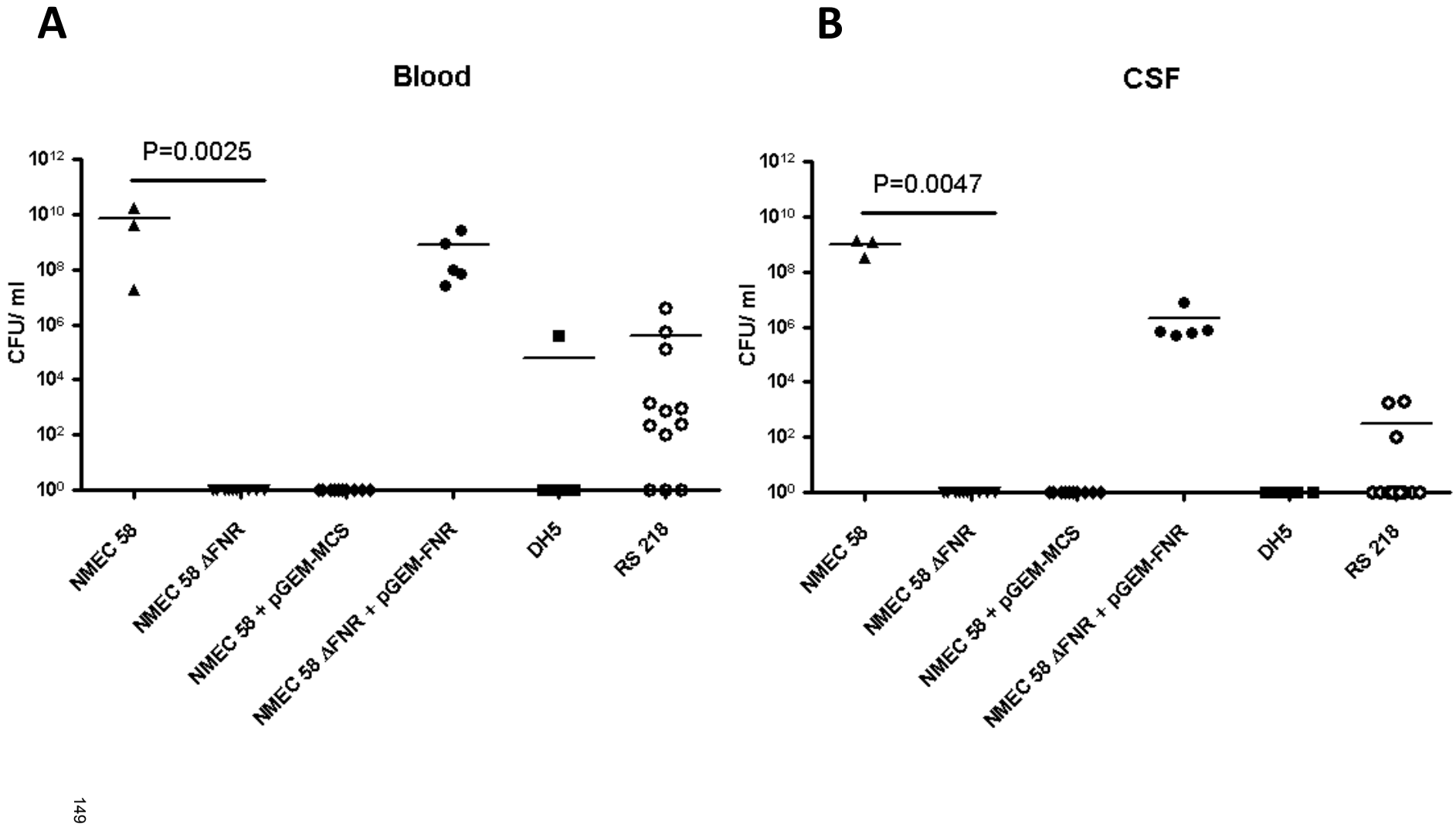
We did not observe detectable blood or CSF bacterial grow (p value ≤0.001) in *fnr* mutant strains 24 hours post-infection (Fig. 1A and B respectively). Animals infected with wild type strain presented a blood bacterial counting of 3.9 x10^9 CFU/ml, whereas *fnr* mutant strain was unable to reach the bloodstream (<100 CFU/ml). This indicates that *fnr* is essential for bacterial growth in blood. For cerebrospinal fluid counting, we observed similar results: wild type counting was 9.8 x10^8 CFU/ml, whereas *fnr* mutant was unable to traverse the blood-brain barrier (<100 CFU/ml).

Reintroduction of *fnr* back into the mutants restored infection in both blood and CSF to wild-type levels, and we did not observe influence of the empty plasmid vector, pGEN-MCS (Fig. 1).

FNR bioinformatics binding motif

Based on the *in vivo* results, we determined whether a binding site for FNR might be present in the region upstream of the candidate FNR-regulated genes. We searched *in silico*, in the 5′ regions of these genes, for the presence of a putative FNR-binding motif using an *E. coli* NMEC 58 specific logograph (Fig. 2).

Fig. 1.


A
Blood
P=0.0025
CFU/ ml
10^{12}
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
NMEC 58
NMEC 58 ΔFNR
NMEC 58 + pGEM-MCS
NMEC 58 ΔFNR + pGEM-FNR
DH5
RS 218
B
CSF
P=0.0047
CFU/ ml
10^{12}
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
NMEC 58
NMEC 58 ΔFNR
NMEC 58 + pGEM-MCS
NMEC 58 ΔFNR + pGEM-FNR
DH5
RS 218

We identified 524 binding sites in NMEC 58 chromosome (NC_011742.1), and 16 binding sites in the plasmid (NC_011747.1). We notably observed binding upstream of an important flagellar operon.

FNR affects motility of NMEC

Bacterial flagella likely facilitate the establishment and spread of infection by microbial pathogens within the host, and expression of flagella was coincident with the ability of NMEC to traverse the blood-brain barrier (18). To correlate FNR with NMEC's flagella expression, we compared the motility of wild type, *fnr* mutant, and *fnr* complemented strain using soft-agar plates. We observed that deletion of *fnr* resulted in a non-motile phenotype in NMEC 58, and reintroduction of *fnr* into the mutant restored its motility (Fig.3). This result indicates that *fnr* mutant strain loses its motility phenotype.

FNR affects antimicrobial resistance in NMEC

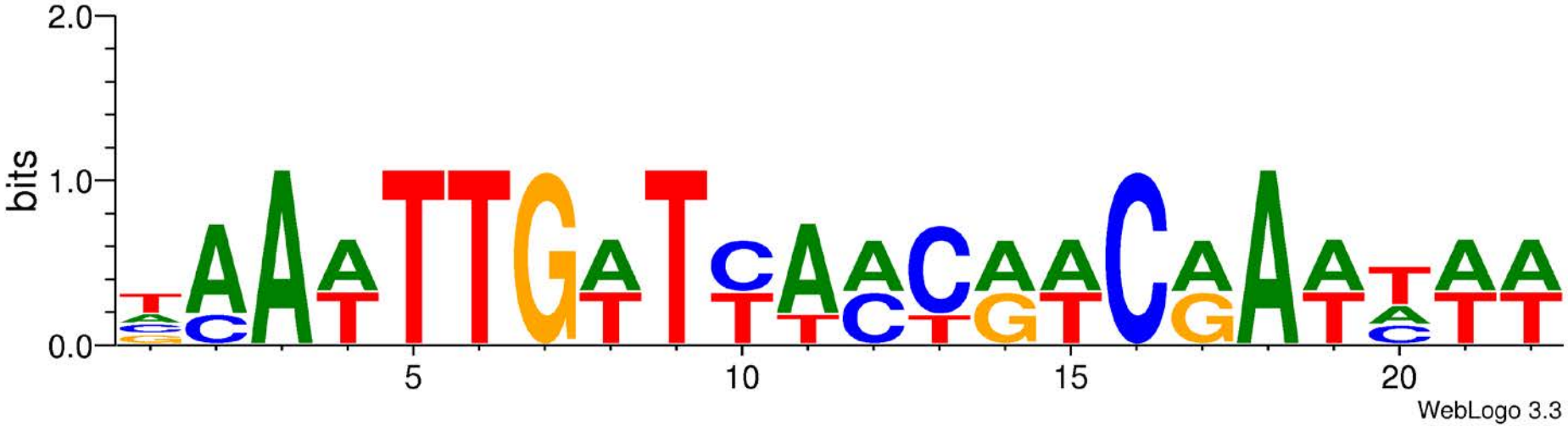
Pathogenic *E. coli* strains were reported to be resistant to antimicrobial peptides such as LL-37, lysozyme and Human Platelet factor IV 18, C18G (9).

Here we observed that NMEC 58 strain was resistant to antimicrobial peptide LL-37, having a MIC of 32 μg/ml (Table 3, Figure 4A). For the *fnr* mutant, the MIC obtained was 8 μg/ml, 4 fold more susceptible to LL-37 peptide when compared to the wild type strain. Moreover, the phenotype was recovered when NMEC 58Δ *fnr* was complemented with pGEN-FNR.

We also tested the minimal inhibitory concentration in relation to lysozyme. Our results showed that NMEC 58 had a MIC of 5000 μg/ml, NMEC 58 Δ *fnr* had a MIC of 625 μg/ml, and the resistance was recovered in the NMEC 58 Δ *fnr* + pGEN-FNR, which had a MIC of 5000 μg/ml (Table 3, figure 4B). The NMEC 58 Δ *fnr* was 8 fold more susceptible to lysozyme, indicating that FNR is important for this phenotype.

Furthermore, we tested the minimal inhibitory concentration in relation to Human Platelet factor IV 18, C18G. Our results showed that NMEC 58 had a MIC of 32 μg/ml, NMEC 58 ΔFNR, a MIC of 4 μg/ml, and the phenotype was recovered in the

Fig. 2.



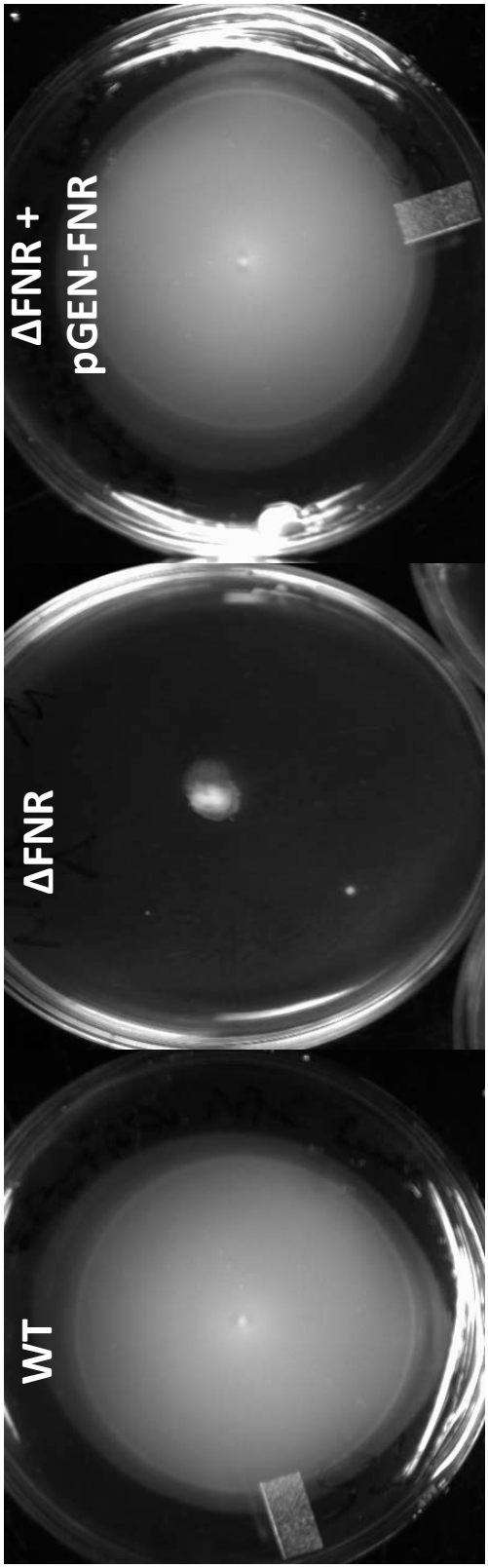


Fig. 3.

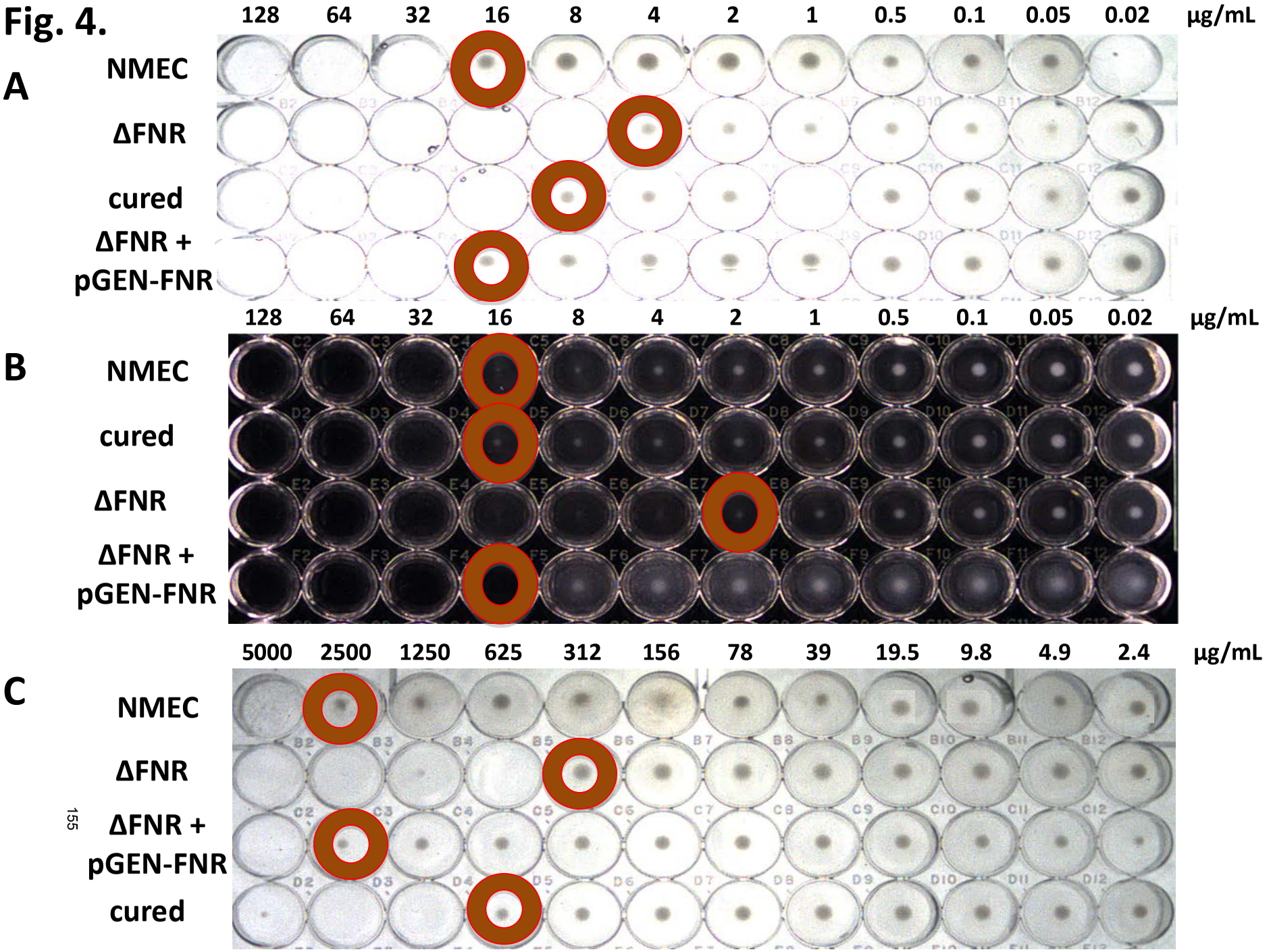
complemented strain (Table 3, figure 4C). The NMEC 58 Δ *fnr* mutant was 8 fold more susceptible to Human Platelet factor IV 18, indicating that FNR is also important for this phenotype.

However, we did not observe any difference among the wild type, mutant and complemented strains for the antimicrobial peptide polymyxin B (Table 3), with all presenting a MIC of 4 μg/ml.

Table. 3. FNR affects MIC in NMEC strains

	MIC µg/ml		
Strains	LL-37	C18G	Lysozyme
NMEC 58	32	32	5000
NMEC 58ΔFNR	8	4	625
NMEC 58 ΔFNR +pGEN-FNR	32	32	5000
NMEC 58 cured	16	32	1250

Fig. 4.


A
128 64 32 16 8 4 2 1 0.5 0.1 0.05 0.02 µg/mL
NMEC
ΔFNR
cured
ΔFNR +
pGEN-FNR
B
128 64 32 16 8 4 2 1 0.5 0.1 0.05 0.02 µg/mL
NMEC
cured
ΔFNR
ΔFNR +
pGEN-FNR
C
5000 2500 1250 625 312 156 78 39 19.5 9.8 4.9 2.4 µg/mL
NMEC
ΔFNR
ΔFNR +
pGEN-FNR
cured

DISCUSSION

Most cases of *E. coli* meningitis develop as a consequence of blood-brain barrier crossing by circulating bacteria in the bloodstream. To accomplish the penetration of this barrier, two major pathogenesis steps are required for the pathogen: induction of a high-level bacteremia and binding to and invasion of microvascular endothelial cells (3).

Our results using an NMEC infection model demonstrated that an *fnr* mutant strain was not able to cause meningitis, since bacteria could not be isolated from the cerebrospinal fluid, nor even reach the bloodstream and cause bacteremia in the infected animals. All animals infected with the wild type strain presented symptoms of disease such as hypothermia, cyanosis and seizures. In contrast, animals infected with the *fnr* mutant strains appeared to be healthy and did not show any symptoms of infection. Our *in vivo* results indicate that virulence of the *fnr* mutant is attenuated. As observed in other works (13, 14), FNR regulates many virulence associated factors, whereas the *fnr* mutant is unable to cause infection in the newborn rat bacteremia-meningitidis model.

The regulator FNR is related to transcriptional activators that control the expression of networks of *E. coli* genes in response to oxygen starvation, as observed in the non-pathogenic strain MG 1655 (21). In anaerobic conditions, FNR was able to bind to specific DNA targets at promoters and modulate transcription (22). Here, we did not link this absence of infection to the regulation of a specific gene or group of genes.

We observed, *in silico*, that FNR has 524 putative sites in the chromosome and 16 sites in the plasmid of NMEC 58. We observed binding sites in genes related to metabolism, transport of small molecules, motility, iron metabolism, antimicrobial peptide resistance and outer membrane proteins. Considering that FNR binds other genes not related to the switch from aerobic to anaerobic environment, *fnr* may serve additional functions other than its role as an oxygen sensor. Instead, FNR appears to also regulate a large family of genes that modulate physiological changes in response to various environmental and metabolic challenges, allowing the NMEC strain to cause disease. In order to confirm the bioinformatics results, we selected some genes to test the phenotypes *in vitro*.

Flagella are associated with NMEC infection because it allows the bacteria to ascend the bloodstream and reach the blood-brain barrier; the flagellin is also suggested to be an invasin (5). The structure of the flagellum is well developed and involves many

groups of genes (13). Our data indicates that FNR positively regulates the expression of genes involved in flagellum biosynthesis and motility (Fig. 3). We observed that the *fnr* mutant strain was not able to swarm, presenting a non-motile phenotype. This lack of motility likely contributes to reduce of the virulence of the strain.

Antimicrobial peptides are an important family of molecules developed for treatment of infection with multi-resistant bacteria. Here, we tested the susceptibility of NMEC 58 to LL-37, lysozyme, human platelet factor IV 18 peptide and polymyxin B (Fig. 4, Table 3). NMEC 58 was resistant to LL-37 peptide, lysozyme, and human platelet factor IV 18 peptide. NMEC 58 Δ *fnr*, in contrast, showed an increased susceptibility to LL-37, lysozyme, human platelet factor IV 18 peptide when compared to the wild type, and this susceptibility could be due to a gene regulated by FNR. Such a phenotype is not related to an oxygen sensor activity, but related to bacterial metabolism. In agreement with our results, it has been previously described that FNR can also affect genes related to metabolism of small peptides (22).

Among Gram-negative bacteria, NMEC is the pathogen most frequently associated with neonatal meningitis (6). For the infection to initiate, bacteria need to reach the bloodstream. As an invasin, the flagellum helps and makes the bacteria able to reach the blood-brain barrier. In addition, for bacteria to persist in the bloodstream, they need to resist the innate immune system and be resistant to the antimicrobial peptides to finally cause meningitis. Here we show that both flagellum expression and antimicrobial resistance were regulated by *fnr*, and as a consequence the *fnr* mutant lost its ability to cause bacteremia, to grow in the cerebrospinal fluid and to cause disease in the neonatal rat model.

Here we show for the first time that the global regulator FNR plays a critical role for virulence of neonatal meningitis causing by *E. coli* pathogenic. We also observed, by *in silico* analysis, that FNR is involved in the regulation of important genes associated to the NMEC pathogenicity, such as flagella and antimicrobial resistance genes. All of these specific virulence-associated genes were upregulated by *fnr*. In addition, the *fnr* mutant was not able to cause infection *in vivo*. Finally, we observed that in NMEC, FNR controls genes involved in anoxic maintenance as well as genes involved in virulence.

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Figure legends:

Figure. 1. *In vivo* virulence assay. Three day-old rats were inoculated intraperitoneally with 200 CFU of NMEC 58 and its mutants, 24h after infection, the pups were sacrificed and blood (A) and CSF (B) were aseptically removed. WT and *fnr* mutant bacteria were recovered by plating on LB agar and their viable counts were determined. Each dot represents $\log_{10}$CFU/g in blood and CSF from an individual animal and the detection limit is 100 CFU/g. Bars indicate the median $\log_{10}$CFU/g. A two-tailed Wilcoxon matched pairs test was performed, and the difference in colonization levels of WT and mutants was considered statistically significant if $p < 0.05$. DH5α and RS218 were included as negative control and reference strain, respectively. Significant differences are indicated by asterisks (***, P<0.0001compared to the WT and mutant).

Figure. 2. Logograph of the information matrix obtained from the consensus alignment of FNR motif sequences for *E. coli* NMEC58. The total height of each column of characters represents the amount of information for that specific position, and the height of each character represents the frequency of each nucleotide.

Figure. 3. FNR affects motility in NMEC. Baterial cultures were grown until stationary fase, 10 µL of inocullum were stabbed in the middle of each soft-agar plates and incubated at 37°C for 16 h .

Figure. 4. FNR affects antimicrobial reistance in NMEC. MIC assay for antimicrobial resistance for (A) LL-37 peptide (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), I(B) Human Platelet factor IV 18 peptide, (C) lysozyme. Bacterial cells were grown to an optical density of 0.5 at 600 nm (OD600) in N-minimal medium, diluted to $5x10^5$ CFU/ml in the same medium, and aliquoted into rows of wells. Two-fold serial dilutions of the tested antimicrobials were added to each row of wells. The plates were incubated at 37 °C for 18 h. The lowest concentration of antimicrobial that did not permit any visible growth, determined by absence of turbidity, was the MIC.

Table 1. Strains and plasmids. The genotypes of all strains of *E. coli* used or constructed in this study and information about the plasmids used in this study.

Table 2. Oligonucleotides. Oligonucleotide sequences used in PCR.

Table. 3. FNR affects MIC in NMEC strains

8. DISCUSSÃO

8.1 *Epidemiologia de APEC*

Os resultados obtidos com o presente trabalho mostram o perfil de resistência aos agentes antimicrobianos, a prevalência dos fatores de virulência e dos grupos filogenéticos EcoR e a análise filogenética dos isolados de *E. coli* patogênica aviária de lesões severas de celulite e de infecção sistêmica. Esses resultados fornecem um panorama geral dos perfis de suscetibilidade, da virulência e da filogenia de cepas APEC da região sul do Brasil.

O tratamento com antimicrobianos é uma das formas de diminuir o impacto da colibacilose. No entanto, a emergência e a disseminação da resistência a antimicrobianos em amostras de *E. coli* pode dificultar o tratamento das infecções em humanos e outros animais, uma vez que os genes de resistência podem ser transferidos horizontalmente, gerando um aumento de isolados resistentes, acarretando na ineficácia dos medicamentos atualmente utilizados tanto para animais quanto para humanos (Ewers *et al.*, 2007). Nosso trabalho fornece dados sobre o perfil de resistência de isolados APEC, que pode ser útil para o monitoramento e o desenvolvimento de medidas preventivas contra a disseminação de APEC nos estágios iniciais da doença.

As cepas de *E. coli* causadoras de celulite e de infecções generalizadas em aves apresentaram fatores de virulência em comum. Analisando-se as cepas utilizadas nesse estudo, todas apresentaram fatores de virulência relacionados a: (1) adesão, (2) aquisição de ferro (com a exceção da cepa PR010) e (3) resistência ao soro. Tais fatores são característicos do patotipo de APEC (Ewers *et al.*, 2007). Isso sugere que a mesma cepa de *E. coli* possa ser capaz de causar tanto infecção localizada (celulite) quanto infecção sistêmica (colissepticemia), dependendo da via de entrada da bactéria no seu hospedeiro. Até o momento não foi identificado um fator de virulência capaz de distinguir uma cepa patogênica de uma não patogênica.

Baseando-se apenas no genótipo de uma cepa de *E. coli* extraintestinal isolada de uma ave, não se pode afirmar que esta se trata de uma APEC. Para isso são necessários os ensaios *in vivo*, que mostram se determinada cepa é capaz ou não de causar doença (Schouler *et al.*, 2012). Neste trabalho, foram

realizados testes de virulência *in vivo* com o maior número de APEC isoladas de lesões graves de celulite até o momento.

Para analisar de forma mais ampla a virulência, utilizamos um método que combina a análise de lesões nos órgãos das aves infectadas e a rapidez com que as aves morrem após infectadas. Essa combinação resulta em um índice de patogenicidade, que consiste em uma ferramenta mais específica para avaliar a virulência de isolados APEC (Barbieri *et al.*, 2013). Através deste método, a maioria dos nossos isolados foram considerados altamente patogênicos.

Através da análise filogenética, verificou-se uma alta diversidade na população de cepas estudada, bem como a ausência de clones endêmicos na região de coleta dos isolados (Sul do Brasil), embora um mesmo clone possa infectar 2 ou mais aves do mesmo bando. Além disso, observou-se que uma mesma ave pode ser infectada por duas ou mesmo três cepas patogênicas distintas, podendo estas apresentar uma relação filogenética mais ou menos distante.

Uma vez que o controle da colibacilose depende de vários fatores, a incidência da colibacilose poderia ser diminuída através da associação entre manejo eficiente e desinfecção dos galpões de criação (Dho-Moulin and Fairbrother, 1999; Ferreira and Knöbl, 2000). Assim como foi sugerido por Ewers *et al.* (Ewers *et al.*, 2007), as cepas de APEC apresentam um elevado potencial zoonótico, pois cepas isoladas de carne de frango e seus derivados mostraram-se semelhantes a cepas ExPEC isoladas de humanos (Russo and JR, 2003). Dessa forma, deve-se garantir que a carne de frango e seus derivados cheguem ao consumidor sem apresentar risco para a saúde pública.

8.2 *Regulação dos fatores de virulência em ExPEC*

O regulador global FNR tem sido reconhecido como o principal fator de regulação da expressão de genes bacterianos, atuando como um sensor da presença de oxigênio em bactérias gram-negativas. FNR está presente em isolados patogênicos e não-patogênicos, e desempenha um papel importante para a sobrevivência bacteriana em meios com restrição de oxigênio (Lazazzera *et al.*, 1993; Shan *et al.*, 2012). Este estudo mostra a capacidade global de regulação da FNR em cepas de *E. coli* patogênicas extraintestinais.

Observamos, neste trabalho, que FNR está envolvido no controle de várias etapas do estabelecimento da infecção por UPEC. No início da infecção, a expressão da fímbria do tipo I é necessária para que a bactéria colonize o trato urinário e ascenda para a bexiga, causando a cistite. Se a infecção não for tratada, as bactérias podem ascender para os ureteres e, por conseguinte, para o rim. Isso é possível devido à motilidade conferida à bactéria pelos flagelos. As fímbrias P são necessárias para estabelecer uma infecção secundária e causar pielonefrite aguda, com a possibilidade de causar danos irreversíveis aos rins, levando à insuficiência renal. A α-hemolisina atua danificando as células epiteliais do trato urinário, permitindo que as bactérias atinjam a corrente sanguínea e causem uma infecção sistêmica, e o sistema kguS/KguR permite que a bactéria seja capaz de metabolizar o α–cetoglutarato, metabólito abundante nos rins. Todos esses genes associados à virulência tiveram a suas expressões reduzidas quando a *fnr* foi deletado, o que indica que esses genes são regulados positivamente pela FNR. Além disso, a deleção da *fnr* fez com que as bactérias não fossem mais capazes de invadir células dos rins e da bexiga, ou causar doença *in vivo*.

Aqui também descrevemos a regulação da virulência de APEC O1 pela FNR. Os fatores associados à virulência, como a proteína OmpT plasmidial, a fímbria do tipo I e o auto-transportador AatA tiveram sua expressão reduzida quando o gene *fnr* foi deletado, o que indica que esses genes são regulados positivamente pela FNR.

FNR também foi capaz de controlar as etapas da infecção por NMEC. No início da infecção, as bactérias devem atingir a corrente sanguínea e alcançar a barreira hemato-encefálica, capacidade conferida em parte pelos flagelos. Para persistirem na corrente sanguínea do hospedeiro, é necessário resistir ao sistema imunológico inato e aos peptídeos antimicrobianos, para, então, causarem meningite. Quando o gene *fnr* foi deletado, as bactérias perderam a capacidade de causar bacteremia, de crescer no fluido cerebrospinal e de causar doença *in vivo*.

A principal contribuição deste trabalho foi determinar a regulação da expressão de importantes fatores associados à virulência em ExPEC por FNR, que pode ser considerado um determinante de virulência de cepas UPEC, APEC e NMEC. Neste trabalho, verificamos que, em ExPEC, FNR não controla

apenas os genes envolvidos na manutenção de um meio anaeróbico, mas também aqueles associados à virulência, refletindo na capacidade que tais cepas apresentam de causar doença.

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Linhas de pesquisa

1. Análise da Presença dos Genes do Sistema de Secreção do Tipo 6 em cepas de Escherichia coli patogênica aviária (APEC) isoladas de colissepticemia em frangos de corte do Rio Grande do Sul

Objetivos:Embora o Sistema de Secreção Tipo 6 tenha sido descrito recentemente em Vibrio cholerae, a presença de genes componentes desse sistema tem sido detectada em diversos microrganismos, incluindo várias linhagens de Escherichia coli e algumas espécies de outros gêneros, relacionados evolutivamente ou não. Entre as linhagens de E. coli positivas para os genes do T6SS estão as cepas patogênicas aviárias APEC O1 e MT78, esta uma cepa sabidamente invasiva. Além disso, mais recentemente, foi demonstrada a influência do T6SS sobre a virulência da cepa SEPT 362, isolada de um frango com colissepticemia. Nessa cepa, a deleção dos genes componentes do T6SS levou a uma diminuição da virulência. Entretanto, ainda são poucos os estudos que investigam a possível relação do T6SS com a virulência de uma cepa bacteriana.Diante desse panorama, considerou-se importante investigar a presença do T6SS nas cepas de E. coli patogênicas aviárias que constituem a nossa coleção.

2. Resistência a Antimicrobianos, Prevalência dos Fatores Associados à Virulência, Tipagem Filogenética e Perfil Filogenético de isolados de Escherichia coli patogênica aviária (APEC)

Objetivos:Escherichia coli patogênica aviária (APEC) é a causa de doenças extra-intestinais em aves, que se manifestam na forma de doenças localizadas ou infecções sistêmicas, ge- rando grandes perdas econômicas para a indústria aviária. O objetivo desse trabalho foi Avaliar a suscetibilidade de isolados de E. coli recuperados de lesões de celulite e isolados de infecções sistêmicas de frangos de corte em relação aos principais agentes an- timicrobianos empregados na avicultura;2. Detectar a presença dos principais fatores associados à virulência por Reação de Polimerase em Cadeia (PCR) Multiplex;3. Realizar a tipagem filogenética através do método ECOR;4. Analisar o perfil filogenético dos isolados de celulite e colissepticemia pelo método de variação do espaçamento intergênico.

3. Verificação da Adesão, Invasão e Ativação de Morte Celular por Apoptose e Citotoxidade de Cepas de E. coli patogênicas aviárias, usando como a modelo macrófagos e fibroblastos aviários

Objetivos:Este projeto tem como objetivo geral estudar a interação (adesão, invasão, citotoxicidade, ativação de caspases) entre Escherichia coli patogênica aviária e células eucarióticas, buscando entender melhor o patotipo e a colibacilose aviária. De uma maneira mais específica pretende-se determinar os fatores de virulência essenciais para o mecanismo patogênico..

4. Análise do Regulador FNR na Modulação da Virulência de ExPEC

Objetivos:A principal contribuição esperada com o desenvolvimento deste projeto é a descoberta de interações que podem ocorrer entre o sistema regulador FNR e os fatores de virulência presentes em isolados ExPEC. A regulação por esse sistema poderá ser um determinante de virulência para isolados ExPEC. O desenvolvimento deste projeto também irá observar como os fatores associados à virulência de ExPEC estão sendo expressos, o que permitirá elucidar o mecanismo dos isolados no estabelecimento da infecção. Além disso, ainda poderemos identificar uma qualidade ubíqua em todos os isolados ExPEC e,assim, definir melhor esse patotipo. Ainda, poderemos estudar os mecanismos de regulação da expressão e de modulação do regulador

Áreas de atuação

1. Biologia Molecular
2. Microbiologia
3. Bioquímica

Projetos

Projetos de pesquisaProjetos de pesquisa
2012 - Atual Análise do Regulador FNR na Modulação da Virulência de ExPEC
Descrição: A principal contribuição esperada com o desenvolvimento deste projeto é a descoberta de interações que podem ocorrer entre o sistema regulador FNR e os fatores de virulência presentes em isolados ExPEC. A regulação por esse sistema poderá ser um determinante de virulência para isolados ExPEC. O desenvolvimento deste projeto também irá observar como os fatores associados à virulência de ExPEC estão sendo expressos, o que

permitirá elucidar o mecanismo dos isolados no estabelecimento da infecção. Além disso, ainda poderemos identificar uma qualidade ubíqua em todos os isolados ExPEC e,assim, definir melhor esse patotipo. Ainda, poderemos estudar os mecanismos de regulação da expressão e de modulação do regulador

Situação: Em andamento Natureza: Projetos de pesquisa

Alunos envolvidos: Doutorado (4);

Integrantes: Nicolle Lima Barbieri; Fabian Horn; CAI, WENTONG; NICHOLSON, BRYON; LOGUE, CATHERINE M.; NOLAN, LISA K.; LI, GANWU (Responsável); Ashraf Hussein

2008 - Atual Colibacilose aviária: Epidemiologia molecular e análise da resistência a antimicrobianos de cepas de Escherichia coli patogênicas aviárias e estudo do estabelecimento da infecção em aves

Descrição: Escherichia coli patogênicas aviárias (APEC) causam infecções extra-intestinais em aves, conhecidas como colibacilose. A doença pode instalar-se em diferentes locais anatômicos da ave e apresentar sintomatologia variada. A manifestação mais grave da doença é a colissepticemia que, geralmente, é iniciada pelo trato respiratório superior e culmina com a morte da ave. A colibacilose tem causado grande prejuízo econômico na indústria aviária devido ao comprometimento no desenvolvimento da ave ou na produção de ovos, ao descarte da carcaça ou pela morte da ave. O Brasil é o terceiro maior produtor de carne de frango e o primeiro maior exportador, o que faz com que seja de interesse nacional medidas que garantam a qualidade e a segurança na produção e no produto. O objetivo deste projeto é realizar a epidemiologia de cepas APEC (duas a três centenas) isoladas de aves com colissepticemia ou celulite na região Sul do Brasil; a epidemiologia será feita através da genotipagem dos fatores mais freqüentemente associados à virulência de APEC, da avaliação molecular do perfil filogenético por ARDRA e sorotipagem dos sorotipos mais prevalentes entre APEC. A caracterização genotípica dessas cepas será feita por quatro Reações de Polimerase em Cadeia Multiplex, para amplificar um total de 33 genes associados à virulência em APEC e em outros patógenos gram-negativos. Outro objetivo do projeto é determinar a suscetibilidade das cepas APEC a um total de quinze antimicrobianos, que incluem aqueles usados como aditivos na avicultura brasileira. Além do levantamento epidemiológico, analisaremos a histologia do pulmão de galinhas infectadas com 3 a 4 cepas APEC selecionadas por sua suposta virulência e com uma cepa aviária não-patogênica. Com isso, esperamos elucidar como as APEC causam a infecção sistêmica a partir dos pulmões e o que distingue, na interação com o hospedeiro, uma cepa patogênica de uma não-patogênica. Os resultados deste projeto vão (1) fornecer um panorama da diversidade

Situação: Em andamento Natureza: Projetos de pesquisa

Alunos envolvidos: Graduação (2); Mestrado acadêmico (2); Doutorado (1);

Integrantes: Nicolle Lima Barbieri; Roberta Reis; Fabian Horn (Responsável); Letícia Matter; Benito Guimarães de Brito; Daniel Brisotto Pavanelo; OLIVEIRA, ALINE LUÍSA DE

Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq

2007 - 2010 avaliação de suscetibilidade a antimicrobianos e a prevalência de fatores de virulência em cepas de Escherichia coli patogênica aviária

Situação: Concluído Natureza: Projetos de pesquisa

Alunos envolvidos: Graduação (1); Mestrado acadêmico (1); Doutorado (1);

Integrantes: Nicolle Lima Barbieri; Fabian Horn (Responsável); Letícia Matter; Aline Luísa de Oliveira

2006 - 2008 Investigação da resistência de cepas patogênicas aviárias de Escherichia coli à fagocitose por macrófagos de galinha

Descrição: O projeto propõe investigar por que cepas patogênicas aviárias de Escherichia coli (APEC) não são eliminadas pelas células de defesa das aves. Cepas APEC são o agente etiológico de colibacilose em aves, uma doença extraintestinal caracterizada por lesões em vários órgãos, que pode evoluir para septicemia, freqüentemente fatal. A colibacilose é responsável por perdas econômicas substanciais na avicultura brasileira e mundial. Dados do nosso e de outros grupos mostram que APEC são internalizadas por macrófagos, ou seja, elas não escapam da fagocitose, porém, uma vez internalizadas, não são eficientemente destruídas. Para elucidar como APEC resistem à ação bactericida de células de defesa, investigaremos

principalmente (a) a viabilidade e a localização intracelular de APEC em macrófagos de galinha; e (b) se infecção por APEC ativa programas de morte do macrófago aviário. Estas são estratégias comumente usadas por bactérias patogênicas, e a citotoxicidade de cepas APEC, com concomitante ativação de caspases apoptóticas, já foi observada em macrófagos de camundongo.

Situação: Concluído Natureza: Projetos de pesquisa

Alunos envolvidos: Graduação (2); Doutorado (1);

Integrantes: Nicolle Lima Barbieri; Fabian Horn (Responsável); Francine Azeredo; Letícia Matter

Idiomas

Inglês Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Espanhol Compreende Bem , Fala Razoavelmente , Escreve Razoavelmente , Lê Bem

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Curso de Férias: Microrganismos mocinhos ou bandidos?, 2009. (Extensão, Curso de curta duração ministrado)

Demais produções técnicas

1. Pelegrini, A., **BARBIERI, Nicolle Lima**, CARLINI, C.
Curso de Férias : De onde vem as células?, 2010. (Extensão, Curso de curta duração ministrado)

2. Pelegrini, Alessandra L., **BARBIERI, Nicolle Lima**, MARGIS, R., CARLINI, C.
Curso de Férias - Microrganismos: O mundo que não enxergamos, 2009. (Extensão, Curso de curta duração ministrado)

3. Pelegrini, A., **BARBIERI, Nicolle Lima**, MARGIS, R., CARLINI, C.
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Orientações e Supervisões

Orientações e supervisões

Orientações e supervisões concluídas

Trabalhos de conclusão de curso de graduação

1. Aline Luisa de Oliveira. **Análise da Presença dos Genes do Sistema de Secreção do Tipo 6 em cepas de Escherichia coli patogênica aviária (APEC) isoladas de colissepticemia em frangos de corte do Rio Grande do Sul**. 2011. Curso (Ciências Biológicas) - Universidade Federal do Rio Grande do Sul

Eventos

Eventos

Participação em eventos

1. Apresentação de Poster / Painel no(a) **27 Congresso brasileiro de microbiologia**, 2013. (Congresso)
FNR CONTROLS EXPRESSION OF MAJOR VIRULENCE GENES IN UROPATHOGENIC ESCHERICHIA COLI.

2. Apresentação de Poster / Painel no(a) **112th General meeting ASM**, 2012. (Congresso)

Prevalence of Virulence Factors, Phylogenetic analysis and Pathogenicity Score in Strains of Avian Pathogenic Escherichia coli.

3. Apresentação de Poster / Painel no(a) **25 Congresso brasileiro de microbiologia**, 2011. (Congresso)

Resistência a antimicrobianos, prevalência de fatores associados à virulência, análise filogenética e índice de patogenicidade em isolados de colissepticemia de Escherichia coli patogênica aviária (APEC).

4. Apresentação de Poster / Painel no(a) **39a Reunião Anual da SBBq**, 2010. (Congresso)

Phylogenetic analyses of avian pathogenic Escherichia coli isolates (APEC).

5. Apresentação de Poster / Painel no(a) **XX Congreso Latinoamericano de Microbiologia**, 2010. (Congresso)

Resistência a antimicrobianos, prevalência de fatores associados a virulência, análise filogenética e índice de patogenicidade em isolados de E. coli patogência.

6. Apresentação de Poster / Painel no(a) **109th general meeting of American Society for microbiology**, 2009. (Congresso)

Antibiotic Resistance and Prevalence of Virulence Factors in Strains of Avian Pathogenic Escherichia coli (APEC).

7. Apresentação de Poster / Painel no(a) **25 Congresso brasileiro de microbiologia**, 2009. (Congresso)

Resistência a antimicrobianos, prevalência de fatores associados à virulência, análise filogenética e índice de patogenicidade em isolados de colissepticemia de Escherichia coli patogênica aviária (APEC).

8. Apresentação de Poster / Painel no(a) **XXXVII Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology**, 2008. (Congresso)

Antibiotics resistance and Virulence factors prevalence in strains of Avian pathogenic Escherichia coli (APEC).

9. Apresentação de Poster / Painel no(a) **XXXVI Reunião Anual da SBBq e 10 IUMB conference**, 2007. (Congresso)

AVIAN FIBROBLAST INFECTION WITH APEC DID NOT RESULT IN CASPASE 3/7 ACTIVATION.

10. Apresentação Oral no(a) **XIX Salão de Iniciação Científica da UFRGS**, 2007. (Outra)

VERIFICAÇÃO DA ATIVAÇÃO DE CASPASE POR CEPAS PATOGÊNICAS AVIÁRIAS DE Escherichia coli EM MACRÓFAGOS AVIÁRIOS HD11.

11. Apresentação de Poster / Painel no(a) **XXXV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Celular**, 2006. (Congresso)

Strains of avian pathogenic Escherichia coli (APEC) induce caspase 3 activation in HD11 cell line of chicken macrofages.

12. Apresentação Oral no(a) **XVIII Salão de Iniciação Científica**, 2006. (Outra)

Verificação da Ativação da Caspase 3/7 por cepas Patogênicas Aviárias de Escherichia coli.

13. Apresentação de Poster / Painel no(a) **XIX Congresso Brasileiro de Parasitologia**, 2005. (Congresso)

XIX Congresso Brasileiro de Parasitologia.

14. Apresentação Oral no(a) **XVII Salão de Iniciação Centífica**, 2005. (Outra)

XVII Salão de Iniciação Científica.

15. Apresentação de Poster / Painel no(a) **XXXIV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular**, 2005. (Congresso)
XXXIV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular.

16. Apresentação Oral no(a) **XVI Salão de Iniciação Científica**, 2004. (Outra)
XVI Salão de Iniciação Científica.

17. **II Jornada de farmácia hospitalar**, 2004. (Encontro)
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18. **Ciclo de palestras em toxicologia**, 2003. (Simpósio)

Citações

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Nome(s) do autor utilizado(s) na consulta para obter o total de citações:
Barbieri, Nicolle Lima

Totais de produção

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Apresentações de trabalhos (Simpósio).....1
Apresentações de trabalhos (Outra).....4

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Participações em eventos (simpósio).....1
Participações em eventos (encontro).....1
Participações em eventos (outra).....4