Identification of Avian pathogenic *E. coli* (APEC) genes important for the colonization of the chicken lung and characterization of the novel ExPEC adhesin I

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Summary

The extraintestinal pathogen, avian pathogenic *E. coli* (APEC), known to cause systemic infections in chickens, is responsible for large economic losses in the poultry industry worldwide. In order to identify genes, involved in the early essential stages of pathogenesis, namely adhesion and colonization, a lung colonization model of infection was established in 5-week old White leghorn specific-pathogen free (SPF) chickens, and Signature-tagged mutagenesis (STM) was applied to this model by generating and screening a total of 1,800 mutants of an APEC strain IMT5155 (O2:K1:H5; Sequence type complex 95).

The study led to the identification of new genes of interest, including adhesins, genes involved in capsule and LPS formation, regulators, transporters, metabolic enzymes and genes of putative function. Among the many genes identified was one coding for a novel APEC fimbrial adhesin (Yqi) not described for its role in APEC pathogenesis to date. Its gene product was temporarily designated ExPEC Adhesin I (EA/I). Deletion of the ExPEC adhesin I gene resulted in reduced colonization ability by APEC strain IMT5155 both *in vitro* and *in vivo*. Furthermore, complementation of the adhesin gene restored its ability to colonize epithelial cells *in vitro*. The ExPEC adhesin I protein was expressed as a fusion protein in *E. coli* BL21 *in vitro* during the log phase as shown by SDS-PAGE and western blotting, to reveal a protein with a molecular mass of ~ 39 kDa. Electron microscopy of an afimbriate strain *E. coli* AAEC189 over-expressed with the putative EA/I gene cluster revealed short fimbrial like appendages protruding out of the bacterial outer membrane.

We observed that this adhesin coding gene *yqi* is prevalent among extraintestinal pathogenic *E. coli* (ExPEC) isolates, including APEC (54.4%), uropathogenic *E. coli* (UPEC) (65.9%) and newborn meningitic *E. coli* (NMEC) (60.0%), and absent in all of the intestinal pathogenic *E. coli* strains tested, thereby validating the designation of the adhesin as ExPEC Adhesin I. In addition, prevalence of EA/I was most frequently associated with the *E. coli* phylogenetic group B2 and ST95 complex of the multi locus sequence typing (MLST) scheme, with evidence of a positive selection within this highly pathogenic complex.

This is the first report of the newly identified and functionally characterized ExPEC adhesin I and its role during APEC infection in chickens.

Keywords:

Avian pathogenic *E. coli* (APEC), Signature-tagged mutagenesis (STM), Chicken lung infection model, ExPEC adhesin I (EA/I)

Zusammenfassung

Aviäre pathogene *E. coli* (APEC) sind extraintestinale Pathogene, die beim Huhn systemische Infektionskrankheiten hervorrufen. APEC sind verantwortlich für erhebliche wirtschaftliche Verluste in der Geflügelindustrie. Zur Identifizierung bakterieller Gene, die an der Adhäsion und Kolonisierung des Wirtes beteiligt sind, wurde zunächst ein Lungen-Infektionsmodell in fünf Wochen alten spezifisch Pathogen-freien (SPF) Hühnern etabliert. In diesem Modell wurden 1.800 mittels Signature-tagged-Mutagenese (STM) hergestellten Mutanten des APEC Prototypstamms IMT5155 (O2:K1:H5; Sequenztyp-Komplex 95) auf ihre Fähigkeit zur Adhäsion und Kolonisierung im natürlichen Wirt getestet.

Die funktionelle Untersuchung führte zur Identifizierung bakterieller Gene, einschließlich Adhäsin-, LPS- und Kapsel-bildenden Genen, Regulator-, Transport- und Stoffwechselenzym-Genen, sowie Genen mit putativer Funktion. Die STM-Analyse erlaubte zudem die Identifizierung eines zuvor nicht charakterisierten putativen Fimbrien-bildenden Adhäsins (Yqi). Das Genprodukt wurde vorläufig als ExPEC Adhäsin I (EA/I) bezeichnet. Eine Deletion des EA/I-Gens führte zu einer Reduzierung der Adhäsionsfähigkeit des Wildtyp-Stammes IMT5155 sowohl *in vitro* in Zellkulturen als auch *in vivo* im Huhn. Eine Komplementierung des EA/I-Gens *in trans* resultierte in einer partiellen Wiederherstellung des Adhäsionsvermögens des Mikroorganismus an Epithel-Zellen *in vitro*. Das EA/I-Protein mit einer Größe von ~39 kDa wurde als Fusionsprotein in dem *E. coli*- Stamm BL21 *in vitro* exprimiert, und mittels SDS-PAGE und Western Blot nachgewiesen. Durch Überexpression des putativen EA/I-Operons in dem Fimbrien-negativen *E. coli*-Stamm AAEC189 konnten schließlich mittels elektronenmikroskopischer Aufnahmen Fimbrien-bildende Strukturen auf der äußeren Membran des Mikroorganismus dargestellt werden.

Das Vorkommen des *yqi* in den untersuchten extraintestinal pathogenen *E. coli* (ExPEC), einschließlich APEC (54.5%), uropathogenen *E. coli* (UPEC) (65.9%) und Neugeborenen-Meningitis-verursachenden *E. coli* (NMEC) (60.0%), bei gleichzeitigem Fehlen in allen untersuchten intestinal pathogenen *E. coli* bestätigt die Bezeichnung ExPEC Adhäsin I. Die Prävalenz des EA/I-Gens war am stärksten assoziiert mit Stämmen der B2-Phylogenetische-Gruppe sowie insbesondere mit Stämmen aus dem ST95-Komplex des Multi-Lokus-Sequenz-Typisierungs (MLST)-Schemas. DNA-Sequenzanalysen ergaben zudem erste Hinweise auf eine positive Selektion des EA/I-Gens innerhalb dieses ST95-Komplexes.

In der vorliegenden Promotionsarbeit gelang somit die Identifizierung und funktionelle Charakterisierung des neuen ExPEC Adhäsin I, welches bei der APEC-Pathogenese im natürlichen Wirt Huhn eine Rolle spielt.

Schlagworte:

Aviäre pathogene *E. coli* (APEC), Signature-tagged Mutagenese (STM), Lungen-Infektionsmodell, ExPEC Adhäsin I (EA/I)

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1 Introduction

1.1 *Escherichia coli* and the Extraintestinal pathogenic *E. coli* (ExPEC)

Escherichia coli typically colonize the gastrointestinal tract of human infants and warm-blooded animals within a few hours after birth. However, a large number of highly adapted *E. coli* pathovars have acquired specific virulence attributes, which confer an increased ability to adapt to new niches and allows them to cause a broad spectrum of disease (72). Pathogenic *E. coli* are also the increasing cause of a number of extraintestinal infections, apart from the classical intestinal infections they bring about in humans and animals (Fig. 1).

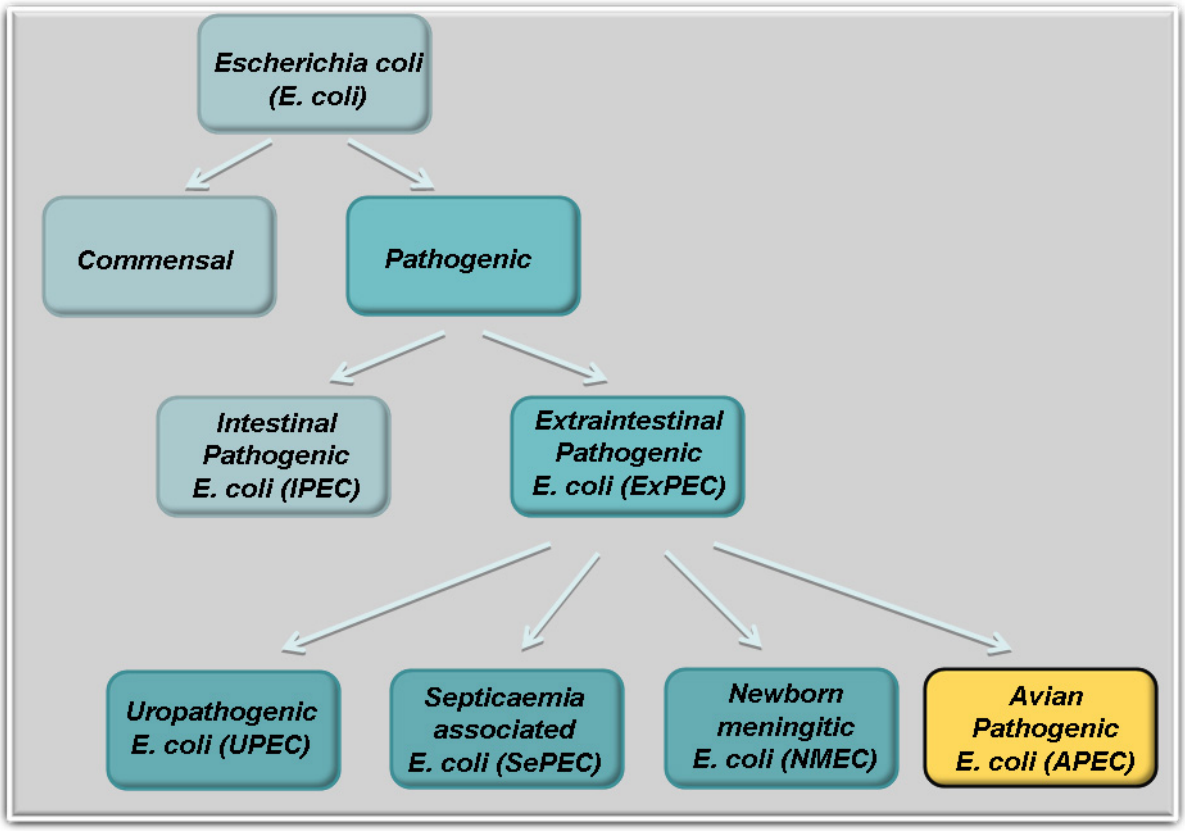


Figure 1: Schematic representation of *Escherichia coli* groups and pathotypes.

Extraintestinal pathogenic *E. coli* (ExPEC), as these pathotypes have now been classified, are responsible for a diverse spectrum of invasive human and animal infections, often leading to septicaemia (118). Molecular epidemiological analyses have led to the acceptance of ExPEC being distinct from other *E. coli*, as pathogenically versatile, thus reflecting the shared ability of the various ExPEC subsets to overcome or subvert host defences and cause disease at multiple anatomical sites in humans and animals (62). Unlike non-pathogenic commensal and intestinal pathogenic *E. coli*, ExPEC derive predominantly from *E. coli* phylogenetic group B2, and to a lesser extent, group D, and from specific clones within these groups, which can be recognized by their characteristic O:K:H serotypes (61).

ExPEC, which include uropathogenic (UPEC), newborn meningitic (NMEC) Septicaemia associated (SePEC) and avian pathogenic (APEC) *E. coli*, exhibit considerable genome diversity and possess a broad range of virulence-associated factors including adhesins, toxins, iron acquisition factors, lipopolysaccharides, polysaccharide capsules and invasins (30). These pathogens constitute an increasing problem for veterinary and human medicine, especially in patients that are immuno-compromised because of disease, chemotherapy or old age. They are also the leading cause of bloodstream infections in nursing homes, hospitals and young children especially newborns (151).

The pathotype known as uropathogenic *E. coli* (UPEC) is the major cause of community-acquired urinary tract infections (UTI) in humans and livestock, in addition they also cause uncomplicated UTI in companion animals like dogs and cats (71, 91). Multiple adhesins and toxins have been described for UPEC and these different virulence factors are found in varying percentages among different subgroups of UPEC and often located on pathogenicity islands (PAIs) (71). More than 80% of all pyelonephritogenic strains of *E. coli* express P fimbriae, which are recognized as a key determinant in promoting virulence of *E. coli* in UTI (162).

An important extraintestinal pathotype, the newborn meningitis *E. coli* (NMEC), is the most common cause of Gram-negative meningitis in neonates. *E. coli* strains with the K1 capsular polysaccharide are predominant (~80%) among isolates from neonatal *E. coli* meningitis (176). NMEC translocate from the blood to the central nervous system through an invasive process resulting in inflammation of the meninges (71). In a previous study it was shown that a feature that clearly distinguishes UPEC from NMEC and APEC, is the higher occurrence of a gene *neuC*, that is involved in the synthesis of the K1 capsule in NMEC as well as APEC, which is responsible for bacterial translocation (30).

It has however been previously reported, that although there are single genes or gene combinations with a higher linkage to a certain pathotype, there exists no host- or pathotype-specific virulence gene pattern, thus making a categorization of ExPEC strains into the pathotypes APEC, UPEC and NMEC impossible based just on virulence gene typing (30). In another study it was reported that there exists increasing evidence that virulence in *E. coli* extraintestinal infections is more likely linked to the phylogenetic background of a strain than to its ecological background (28). Therefore, since it is known that APEC infecting poultry, UPEC infecting humans and animals, and NMEC infecting infants, share common phylogenetic origins (30, 60,121), it is important to study an important pathogen like APEC, due to the likeliness of a potential zoonotic risk.

1.2 Avian pathogenic *Escherichia coli* (APEC) – a deeper insight

Avian pathogenic *E. coli* (APEC), the causal organism of infection in chickens, are responsible for large economic losses in the poultry industry worldwide. This would imply any localized or systemic infection caused entirely or partly by *Escherichia coli*, including coligranuloma, air sac disease and many others (7). APEC are said to be associated with infection of extraintestinal tissues in chickens, turkeys, ducks and other avian species.

The most important disease syndrome associated with APEC begins as a respiratory tract infection and is often known as aerosacculitis or the air sac disease, which in turn can evolve into a severe sepsis or systemic infection ultimately leading to death of the bird. Respiratory lesions are also observed, which include aerosacculitis with a serous to fibrinous exudate (Fig. 2), an initial infiltration with heterophils, and a subsequent predominance of mononuclear phagocytes (21). Sites of entry into the bloodstream are presumed to be the gas exchange region of the lung and the air sacs, which are relatively vulnerable to colonization and invasion by bacteria due to lack of resident macrophages (117).

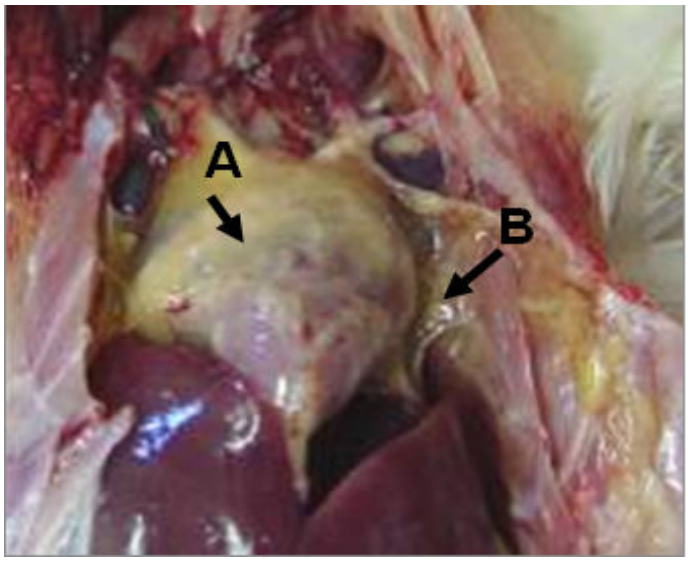


Figure 2: Pathomorphological changes of chicken organs during systemic APEC infection. (A) Acute pericarditis, (B) Air sacculitis, fibrinous exudate

The mechanisms of avian *E. coli* pathogenesis remain strikingly unclear to date, however, a number of virulence factors have been implicated in these extraintestinal diseases in avian species, including adhesins and genes related to bacterial adhesion (*afa*/*draB*, *bfp*, *csgA*, *bmaE*, *fimC*, *focG*, *gafD*, *hra*, *iha*, *nfaE*, *papAH*, *papC*, *papEF*, *papG* alleles II and III, *sfa*/*focCD*, *sfaS*, *tsh* and *mat*), iron acquisition systems (*chuA*, *fyuA*, *ireA*, *iroN*, *irp2*, *iucD*, *iutA*, *sitD* [chromosomal], and *sitD* [episomal]), serum resistance (*iss*, *kpsMTII*, *neuC*, *ompA* and *traT*), toxins and hemolysins (*astA*, *cnf1/2*, *sat*, *stx*, *vat* and *hlyA*), and invasion genes (*ibeA*, *gimB*, *tia*) (28, 29, 118). Without a doubt, there exist a number of virulence factors yet

to be identified and characterized, that would make a significant contribution to various stages of pathogenesis, thus shedding light on the nature of APEC virulence in its entirety.

APEC strains have been recently shown to have a number of similarities with human ExPEC strains (74), in that, most of the virulence genes they possess are similar to those identified in human ExPEC strains (30, 121). APEC, therefore, are currently gaining more and more importance, thereby increasing the quest for knowledge about incomprehensible virulence mechanisms of infectious diseases caused by such pathotypes. The common presence of certain virulence-associated genes among ExPEC strains as well as similar disease patterns and phylogenetic background are indeed an indication of a significant zoonotic risk of avian-derived *E. coli* isolates (30).

Further research is required in order to gain a thorough understanding about this pathogen and the disease it causes, one of the many reasons being the presence of only a single commercially available vaccine “Nobilis® *E. coli* Inac” manufactured by Intervet against *E. coli* infection whose effects are very limited due to the diversity of APEC. Furthermore, this vaccine is indicated for partial passive immunization of broiler chickens only, during their first seven weeks of life, by vaccination of broiler breeders. Therefore, laying hens are not covered by this vaccine, thereby still resulting in immense losses for this industry.

There is ample information on the virulence and pathogenicity mechanisms, including known virulence factors that play crucial roles in various infection stages; however, there is a pressing need for the identification of novel virulence factors and genes that would make a momentous contribution to the study of APEC virulence. This can be tackled in numerous ways. Molecular research has roughly reached its peak in the last decade, with many new methods and techniques being identified, that enable large-scale screening and identification of virulence genes in a limited time period. Some methods include IVET (*In vivo* expression technology) which can be adapted to study the induction of microbial genes in response to any condition and GAMBIT (Genomic analysis and mapping by *in vitro* transposition) which is used to identify essential genes in naturally transformable organisms whose genomes have been sequenced (18). In addition, the accessibility of complete genome sequences for most bacterial pathogens has considerably increased the number of genes with unknown function. Genome-wide approaches to functionally characterize these genes in the process of infection have turned out to be of immense value.

An interesting and important molecular technique, Signature-tagged mutagenesis (STM) (50), established in the last decade has gone a long way, in the identification of novel virulence factors in screens carried out in around 31 different bacterial species including APEC which has led to the identification of over 1,700 bacterial genes that are involved in virulence (153).

1.3 Adhesion and colonization as crucial steps during infection

Microbial pathogenicity is a complex phenomenon encompassing diverse mechanisms. There are, however, several common strategies that pathogenic organisms use to sustain themselves and overcome host barriers, one of them being the firm adhesion of the micro-organism to the host cells (88). The ability to adhere to host surfaces is by far the most vital step in the successful colonization by microbial pathogens. Colonization begins with the attachment of the bacterium to receptors expressed by cells forming the lining of the mucosa (174). A crucial event in colonization is surviving the mechanical cleansing of the mucosal surface. Long hair like extracellular appendages called fimbriae, produced by most Gram-negative pathogens, mediate specific attachment to the epithelial cell surface. Associated with the fimbria is a protein called an adhesin, which directs high-affinity binding to specific cell surface components (67) (Fig. 3).

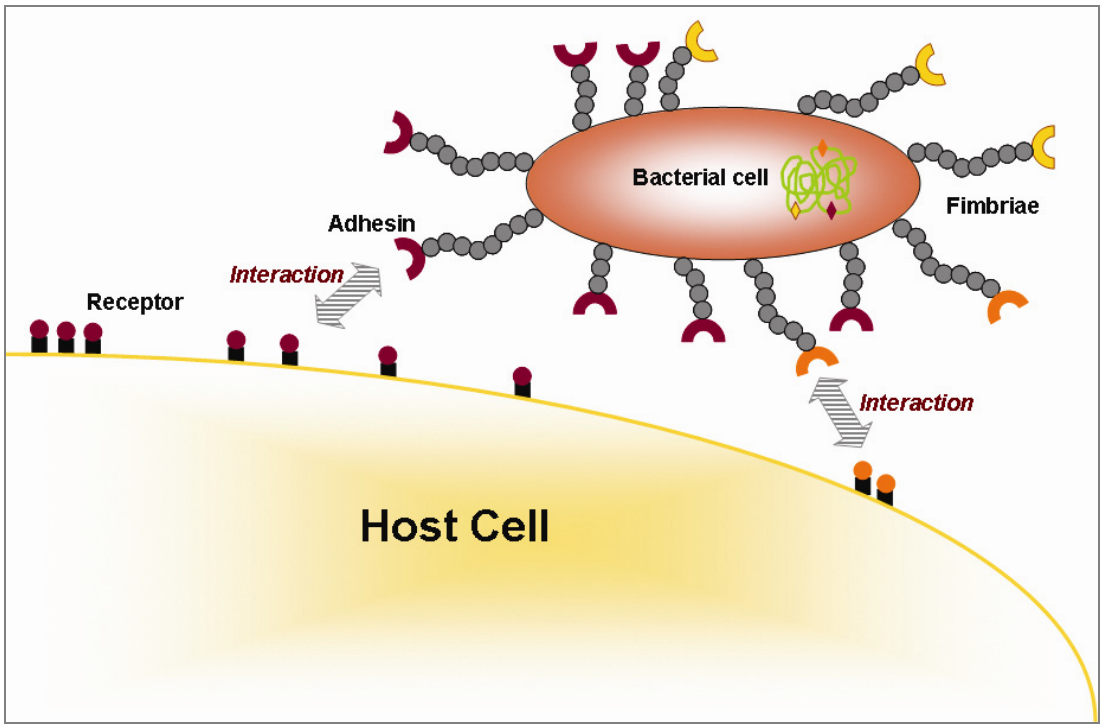


Figure 3: Schematic representation showing bacterial adhesion to a host cell by using specialized structures known as fimbriae. The host cell receptor specific binding unit is termed the adhesin which lies at the tip of the fimbria.

In the last couple of years, therefore, an enormous amount of research has been undertaken that deals with understanding how bacterial pathogens adhere to host cells. Remarkably, studies focused indefinitely on adhesins by comprehensively studying adhesin biogenesis, the regulation of adhesin factors, and even to some extent, the identity of host receptors that are the targets of microbial adhesin factors (32).

Not all adhesins are essential virulence factors and their specific role in disease has been surprisingly difficult to define, since a single pathogen expresses multiple adherence factors

(32). *E. coli* in all probability is one of the best studied free-living organisms. As with other pathogens, the initiation of many typical *E. coli* infections is thought to be mediated by molecular recognition between adhesins on the bacterial cell surface and specific receptor molecules on mucosal cells of the host (57). *E. coli* express long, thin filamentous protein appendages that are usually 5-10 nm in diameter and up to 2 µm in length (57). In order to understand bacterial attachment and adhesion a little more, a few of the essential fimbriae, particularly of the ExPEC group of pathogens will be discussed in greater detail.

1.4 Adhesins in ExPEC

Many bacteria have surface appendages of various sizes and appearances called pili and fimbriae. These terms have been used interchangeably. The term “pilus” (plural pili), however, should be reserved for appendages involved in bacterial conjugation, that is the transfer of genetic material, and the term “fimbria” (plural fimbriae) should be reserved for structures concerned with the adhesion of bacteria to various surfaces, including cell surfaces (53). On average, one to ten conjugative pili and up to more than 400 fimbriae may be present on the surface of a bacterial cell. Conjugative pili are longer than fimbriae and are composed mainly of a pilin protein organized into a tube-like structure, which allows the passage of genetic material during conjugation (53). Fimbriae have a similar structure but, because they are involved in cellular adhesion, the presence of strain-specific protein sub-units confers a variety of agglutination properties (53). Some of the well known adhesins present among ExPEC strains are Type 1 fimbriae (*fim*), P fimbriae or the pilus associated with pyelonephritis (*pap*), curli fibres (*csg*), S fimbriae or the sialic acid-specific fimbriae (*sfa*), F1C fimbriae (*foc*), Dr fimbriae (*dra*), afimbrial adhesins (*afa*), temperature-sensitive haemagglutinin (*tsh*) and novel adhesin gene clusters many of which remain to be characterized. A brief overview of the ExPEC adhesins is given in table 1 (4). A general description of each fimbria follows the tabular representation.

Table 1: Adhesins and fimbriae of extraintestinal pathogen *E. coli*. Information provided includes gene name, specific adhesin receptor, and adhesion properties to various cell lines in vitro and host tissue in infection models *in vivo*.

Adhesin	Gene	Receptor specificity	Cell adherence: *in vitro* and *in vivo* infection models
Type 1 fimbriae	*fim*	Mannose oligosaccharides (Mono- and Tri-mannose)	Human bladder epithelium, Chicken tracheal and gut explants, colonic and ileal enterocytes
P fimbriae	*pap*	P-blood group antigen-specific glycosphingolipids (α-D-*Galp*-(1-4)-ß-D-*Galp*)	Human kidney, colonic and ileal enterocytes
Curli	*csg*	Matrix and Plasma Proteins (Fibronectin, Laminin, Plasminogen, H-Kininogen)	Chicken tracheal and gut explants
S fimbriae	*sfa*	Neuraminic acid (Sialyl galactosides)	Human bladder and kidney epithelium, Brain endothelium, colonic and ileal enterocytes
F1C fimbriae	*foc*	Lactosylceramide containing glycolipids	Buccal epithelium, Collecting ducts and distal tubules of human kidney, renal tubulus cells
Dr fimbriae	*dra*	Dr blood group antigen, Decay accelerating factor (DAF)	Basement membranes of human and canine kidneys, Bowman's capsule, Bladder epithelium, colonic and ileal enterocytes
Afimbrial adhesins	*afa*	DAF	Uroepithelial cells
Temperature sensitive haemagglutinin	*tsh*	Haemoglobin, Fibronectin, Collagen IV	Chicken erythrocytes

1.4.1 Type 1 Fimbriae

Type 1 fimbrial structures were first noted in early electron microscopic investigations as non-flagellar, filamentous appendages of bacteria. They were first designated "fimbriae" by Duguid in 1955 (26) and termed "pilus" by Brinton almost 10 years later (13). Since then "pilus" has become a generic term used to describe all types of non-flagellar filamentous appendages and is often used interchangeably with the term "fimbriae". The fimbriae were also classified into types I-V (13) depending on agglutination and binding activity.

Fimbriae were thought to confer the ability to agglutinate erythrocytes and to attach to other cells of varying origin, while several other studies indicated a relation between adhesion and

virulence for bacteria inducing infection in relation to a mucous surface (27). It was also further demonstrated that there was specific binding of one *E. coli* strain to monkey kidney cells, mediated by purified type 1 pili. Moreover, a significant correlation was found between the presence of pili or fimbriae on *E. coli* and the ability of the bacteria to adhere to human urinary tract epithelial cells (27).

Type 1 fimbriae were initially associated with adhesive and pellicle-promoting activities which are inhibited by D-Mannose, that is, they are mannose sensitive (19). They were described as polymers of pilin subunits that consisted mainly of protein with a high content of hydrophobic amino acids (83). A single fimbria consists of approximately 100 identical protein subunits (82). The link between adhesion and virulence was not made immediately. A consensus started developing in the 80's that adhesion is important as a virulence factor in the establishment of infection and that attributes of both the host and the micro-organisms are important in this process (112). Further research showed that piliated strains of uropathogenic *E. coli* adhered to the polyethylene surface and formed small micro-colonies surrounded by small amounts of glycocalyx, whereas the non-piliated strains adhered only poorly and produced very little extracellular material (112).

The role of type 1 fimbriae and its effect on pathogenicity of APEC infections was first described in 1984, where an observation was made that the presence of adherence pili on the infecting bacteria affected both the number of chicks that developed disease as well as the severity of disease (125).

Over the years these fimbriae have been studied in depth, considering that these organelles provide an ideal model for the study of microbial adherence as stated by Orndorff and Falkow in 1984 (133). In *E. coli*, 50 – 70 % of all strains possess the chromosomally determined type 1 fimbriae (82). Later studies revealed that four genes designated *fimA*, *B*, *C* and *D* were involved in the synthesis of the fimbriae (81). The expression of type 1 fimbriae is phase variable that is, the bacteria shift periodically between a fimbriate and non-fimbriate state. It was found that the two regulatory *fim* genes, *fimB* and *fimE* control the phase variation of type 1 fimbriae in *E. coli* (78). Three additional genes *fimF*, *fimG* and *fimH* were further characterized and shown not to be necessary for the production of fimbriae but to be involved in the adhesive property and longitudinal regulation of these structures (79). The receptor-binding adhesin of the type 1 fimbriae was identified, characterized and purified in 1988 and this protein was found to be antigenically conserved among strains with different pilin serotypes, and located at the pilus tip (47, 48). *FimH* was later found to be the gene responsible for the mannose-specific or receptor-specific adhesin of the type 1 fimbriae (89) while *fimC* was found to be the periplasmic chaperone that directs assembly of type 1 pili (68). Very recently studies involving molecular evolutionary dynamics have shown that there is evidence for strong selection in the type 1 fimbrial adhesin *fimH* (171).

The type 1 fimbriae have thus been structurally and functionally characterized extensively over decades. There is ample evidence that type 1 pili play a significant role as mediators of attachment by *E. coli* infections, particularly UPEC infections (27). *FimH* being the receptor specific adhesin, has been utilized as a vaccine candidate in previous studies involving urinary tract infection caused by UPEC. One such study showed that immunization with *fimH* reduced *in vivo* colonization of the bladder mucosa by more than 99 percent in a murine cystitis model, and immunoglobulin G to *fimH* was detected in urinary samples from protected mice. Furthermore, passive systemic administration of immune sera to *fimH* also resulted in reduced bladder colonization by UPEC (98). The type 1 fimbriae best studied in *E. coli*, therefore, are of tremendous importance in the pathogenesis of Gram negative bacteria.

1.4.2 P fimbriae

The P fimbriae are morphologically indistinguishable from the type 1 fimbriae, however, they recognize and bind to the α-D-Gal*p*-(1-4)-ß-D-Gal*p* carbohydrate sequence occurring in the series of P-blood group antigen-specific glycosphingolipids (85) and hence the name. The genes encoding the P pilus type were termed the *pap* genes or pyelonephritis-associated pili genes since these were typical of strains isolated from human urinary tract infections (56). PapA was described to be the structural gene for the P fimbriae monomer (128) , that is, these pili, which are hair-like appendages consist of helically arranged subunits of the protein *papA*. The protein PapG is the digalactoside-specific adhesin and through immuno-electron microscopy it was found that the P pili are heteropolymers composed of the major pilin, PapA, the minor pilins, PapE and PapF, and the adhesin PapG (104), the last three proteins being located at the tip of the pilus. These findings were further confirmed by Lund et al (106), who showed that the major subunit PapA is not required for binding, but PapF and PapG are essential for adhesion.

There are several different serotypes of P fimbriae, which are said to differ in their serological differences due to structural variation within the central domain of the major pilus subunit (107). P pilus biogenesis has been studied extensively unravelling the function of each gene of the *pap* gene cluster or operon (105). A model of the pilus biogenesis which involves 11 genes organized in the *pap* gene cluster on the chromosome in clinical isolates expressing P pili has also been previously described (90). The PapD chaperone (D) interacts with each pilus protein subunit forming assembly competent complexes which are targeted to the PapC outer membrane assembly protein required for P pilus biogenesis. PapA subunits pack into a right-handed helical rod. PapH incorporation terminates polymerization of PapA and anchors the pilus to the cell. PapE subunits form open helical fibres called tip fibrillae.

The PapG adhesin, positioned at the distal end of the fibrillum, mediates binding to the Galα (1-4) Gal receptor determinant (90) or the globo series of glycolipids in the human kidney. PapG recognition of the galabiose receptor is thought to be a prerequisite for pyelonephritis (163).

It has been reported that more than 80% of all pyelonephritogenic strains of *E. coli* express P fimbriae, which are recognized as a key determinant in promoting the virulence of *E. coli* in urinary tract infection (UTI) (162). Furthermore, P-fimbriated *E. coli* interact poorly with neutrophils and resist their bactericidal actions *in vitro*. Studies involving different animal models of ascending UTI have found a variable role for P fimbriae, in particular PapGII-mediated adherence, a variant of the PapG adhesin, in the colonization of the mammalian kidney. Overall it appears that there is a subtle role for P fimbriae in mediating adherence to uroepithelial cells *in vivo* and establishing a robust inflammatory response during renal colonization, which in turn contributes to kidney damage during acute pyelonephritis (97). It is now fully established, that these P pili adhesive organelles are critical virulence factors, that mediate the recognition of and attachment to tissues of the kidney by the pathogen during UTI (101). It has also been previously shown that P fimbriae utilize the toll-like receptor 4 (TLR4)-dependent pathway to trigger mucosal inflammation (33).

The P fimbriae are not only restricted to Uropathogenic *E. coli* (UPEC) causing UTI, but are also prevalent in newborn meningitic *E. coli* (NMEC) (65 %) and avian pathogenic *E. coli* (APEC) strains (25 %) (30). The importance of P fimbriae among APEC has gained a lot of interest over a period of time. It has been previously observed that the *pap* positive genotype was associated more frequently with pathogenic isolates from septicaemic chickens than from normal chickens, suggesting its function during septicaemic infection (25). Interestingly, sequencing of an APEC strain with serotype O1:K1 revealed that the *pap* operon which encodes the P pilus is located on a novel pathogenicity island, which also carries other putative virulence factors (73) substantiating its potential role in APEC pathogenesis. The potential of P pili as a vaccine candidate has also been studied and it was found that vaccination with Gal-Gal pili or the P fimbrial vaccines prevented pyelonephritis by piliated *E. coli* in a murine model as well as in monkeys (140, 149).

1.4.3 Curli Fibres

The name curli was proposed in 1989 to a third class of *E. coli* surface organelles in addition to the flagella and fimbriae, which were found to be coiled surface structures composed of a single type of subunit, the curlin, which differs from all known pilin proteins and is synthesized in the absence of a cleavable signal peptide (131). Most natural isolates of *E. coli* carry a transcribable curli gene, *crl*, however only certain strains are able to assemble the subunit protein into curli (131).

Curli bind several matrix and plasma proteins such as fibronectin, laminin, plasminogen, tissue plasminogen activator, and H-kininogen (132). Curli fibres are encoded on the *csg* (curlin subunit gene) gene cluster, comprised of two differently transcribed operons, one which encodes the *csgB*, *csgA* and *csgC* genes, and a second which encodes *csgD*, *csgE* and *csgG* (42). Curli in *E. coli* consist of polymers of a single 15-kDa protein encoded by the subunit gene *csgA* and production of the curli fibres requires expression of both operons (132). Assembly of curli fibres involves extracellular self-assembly of the subunit *csgA*, dependent on a specific nucleator protein *csgB*. *CsgD* is a transcriptional activator essential for expression of the two curli fibre operons, while *csgG* is an outer membrane lipoprotein involved in extracellular stabilization of *csgA* and *csgB* (42). The expression of genes coding for curli is complex and involves several control elements, such as H-NS, RpoS and OmpR which results in a great reduction in the expression of curli fibres at temperatures higher than 30 ºC and at high osmolarity in most strains (42). Recently a novel regulator, termed MlrA was found to be required for curli production and extracellular matrix formation (15). The ability of curli polymers to specifically interact with numerous human proteins such as the matrix proteins fibronectin and laminin, and proteins of the fibrinolytic and contact-phase systems, facilitates the adaptation of curli-expressing bacteria to different niches in the infected host (132). It has further been shown that curliated *E. coli* in human plasma absorbs plasminogen and tissue plasminogen activator, leading to the formation of proteolytically active plasmin which may promote bacterial spreading through tissue degradation (132).

Polymerization of curlin to fimbriae-like structures (curli) on the surface of *E. coli* differs from the prevailing model of fimbrial assembly, in that, it occurs extracellularly through a self-assembly process depending on a specific nucleator protein (45). Curli polymers are formed as a result of a conformational change of soluble *csgA* initiated by an interaction with a nucleating *csgB* protein and such an induced conformational change might involve a conversion from a partially disordered structure in the monomeric state to readily ordered secondary structures in the polymeric state (45).

Studies on the role of curli in avian pathogenic *E. coli* pathogenesis have also been carried out, and there is evidence that haemagglutination activity, fibronectin binding and curli production are co-expressed in an APEC strain and haemagglutination and fibronectin binding are recognized as virulence factors that may be important in the adherence of pathogens to host surfaces (143). In another study, it was seen that 99% *E. coli* isolated from diseased birds possessed the *csgA* gene responsible for curli biosynthesis (21). Furthermore curli fibres were found to be essential for the internalization of bacteria causing avian septicaemia as seen *in vitro* (42).

1.4.4 S Fimbriae

The S fimbriae were discovered as a group of fimbriae among pyelonephritogenic *E. coli* strains which recognized neuraminic acid (sialic acid) - containing structures other than mannosides or P antigens on human erythrocytes (137) and were termed the S fimbriae based on their receptor specificity, that is, their specific binding to sialyl galactosides (86). Morphologically, S fimbriae are similar to type 1 or P fimbriae of *E. coli*, that is, they are 1 to 2 µm in length, around 5 to 7 nm in diameter and their subunit size is equal to that of type 1 fibrillin of *E. coli* (86). The S-fimbriae adhesins (Sfa) were reported to be most often found among meningitis- and sepsis-associated *E. coli* isolates (134).

The *sfa* genetic determinant (6.5 kb) for these fimbriae was cloned and found to code for at least seven *sfa*-specific gene products. (134). This determinant represents a cluster of genes with a homogeneous genetic structure and consists of different regions involved in the production of fimbriae and adhesin, the biogenesis of fimbriae, and the control of transcription (136). *SfaS*, the minor subunit of the S fimbriae, a 14 kDa protein, localized at the distal end of the *sfa* gene cluster, was identified as the sialic acid - binding adhesin (119). The entire *sfa* gene cluster consists of *sfaA*, a major subunit protein of 16 kDa and three minor subunit proteins *sfaS* of 15 kDa, *sfaG* of 17 kDa and *sfaH* of 29 kDa, which together form the *sfa* complex (43). Expression of the *sfa* determinant is dependent on several environmental conditions, such as temperature, osmolarity, and the presence of glucose, while at the molecular level, regulation of the *sfa* determinant is mediated by two regulatory proteins *sfaB* and *sfaC* (6).

In a study on the prevalence of the S fimbriae among ExPEC strains, it was observed that 50% UPEC, 24% NMEC and 9.2% APEC strains harboured the *sfa* genes (30). In another study 79% of the septicaemic and diarrheic *E. coli* isolates from pigs tested were found to be positive for S fimbriae (23).

It has been shown that S-fimbriated bacteria and the purified S fimbriae bind specifically to human epithelia, for example, the vascular endothelium in both large vessels of kidney tissue, the capillary endothelium in the interstitium and the visceral epithelium of the glomerulus which are known to have a sialic acid coating (84). An important observation is that S fimbriae occur in some pyelonephritogenic *E. coli* strains but are mainly associated with strains causing neonatal sepsis and meningitis (84). S fimbriae have also been shown to bind the extracellular matrix components of fibronectin and laminin and sialoglycoproteins on brain microvascular endothelial cells, an interaction that may explain migration across physiological barriers (175).

1.4.5 F1C Fimbriae

A single F1C fimbria is a thin, 7-nm-wide, approximately 1µm long surface polymer which structurally closely resembles that of type 1 fimbriae (80). F1C fimbriae, with subunits of about 17K, confer no haemagglutination to erythrocytes from humans, oxen, horses, guinea-pigs or chickens; however, they adhere to buccal epithelial cells (155). Although these fimbriae are not haemagglutinating, they contribute to the adhesive properties of UPEC strains, in that they mediate specific adherence to the collecting ducts and the distal tubules of the human kidney (148), as well as to cultured renal tubulus cells (80). The *foc* (fimbriae of serotype 1C) gene cluster is involved in the synthesis of F1C fimbriae. This gene cluster which has been cloned and studied shows that six genes are involved in the biogenesis of F1C fimbriae, including *focA* which encodes the major fimbrial subunit, focC which encodes a product that is indispensable for fimbria formation, *focG* and *focH* which encode minor fimbrial subunits, and *focI* that encodes a protein which shows similarities to the subunit protein *focA* (148).

The F1C fimbriae (*foc*) genetic determinant is related to the S fimbriae (*sfa*) genetic determinant, both of which show a high degree of identity, in that, they show similarities in their DNA sequence composition and exhibit common epitopes on their corresponding fimbrial proteins; however, the Sfa and F1C antigens differ in their receptor specificities (135). It has been shown that *foc*-specific gene products are able to produce a wild-type phenotype in *sfa* insertion mutants and that hybrid DNAs consisting of *sfa*- and *foc*-specific sequences code for intact fimbriae after transformation into non fimbriated *E. coli* strains (135).

Until recently the exact receptor specificity of the F1C fimbriae was not known; however, in 2000, glycolipid receptors for purified F1C fimbriae were identified. TLC (thin-layer chromatography) fimbrial overlay analysis revealed the binding ability of purified F1C fimbriae only to glucosylceramide (GlcCer), ß1-linked galactosylceramide 2 (GalCer2) with non hydroxyl fatty acids, lactosylceramide, globotriaosylceramide, paragloboside (nLc_4Cer), lactotriaosylceramide, gangliotriaosylceramide (asialo-GM_2 [GgO_3Cer]) and gangliotetraosylceramide (asialo-GM_1 [GgO_4Cer]) (75). It has also been suggested that the disaccharide sequence GalNAcß1-4Galß of asialo-GM_2 (GgO_3Cer) which is positioned internally in asialo-GM_1 (GgO_4Cer) is the high-affinity binding epitope for the F1C fimbriae (75). It was further reported that F1C fimbriated bacteria selectively interact with two minor glycosphingolipids isolated from rat, canine, and human urinary tract, and comparison of the binding-active compounds with reference glycosphingolipids revealed that the receptor specificity is dependent on the ceramide composition (5).

1.4.6 Dr Fimbriae

Väisänen-Rhen et al. (167) originally described a mannose-resistant P blood group-independent haemagglutinin which was expressed by a number of UPEC strains belonging to serogroup O75; accordingly, this adhesin was named O75X (76). Nowicki et al. showed that the Dr blood group antigen, a component of the IFC blood group complex, is the receptor for the O75X fimbrial-like adhesin and the molecule recognized by the Dr haemagglutinin is a chloramphenicol-like structure (130). The name Dr haemagglutinin for the O75X fimbrial-like adhesin was therefore proposed (130). It was observed that Dr blood group substance was found in tubular basement membrane and Bowman's capsule of the human kidney (130) and Dr adhesins have been shown to bind preferentially to basement membranes of human and canine kidneys, Bowmans capsule and to a lesser extent to the bladder epithelium (76).

The Dr fimbriae or O75X fimbriae are chemically very similar but morphologically different from typical *E. coli* fimbriae, and electron microscopy has revealed that the purified proteins were shown to be arranged in a coil-like structure which consists of subunits with an apparent molecular mass of about 15 kDa (129). The Dr adhesin-encoding operon was identified and termed *dra* of which four genes, *draA*, *draC*, *draD* and *draE* are required for full expression of the mannose resistant haemagglutinin phenotype (76). *DraE* of the Dr operon encodes the major structural subunit that compose the respective fimbrial appendages and is also the adhesive subunit for the DAF (decay accelerating factor) receptor (168). The products of the *draB* and *draC* genes exhibit homology to chaperone-usher proteins belonging to the superfamily of PapD like chaperones (141).

A number of studies have assessed the role of Dr fimbriae in the pathogenesis of extraintestinal pathogenic *E. coli*. It has been reported that *E. coli* with Dr fimbriae persisted in the kidney tissue and were associated with significant tubulointerstitial nephritis, whereas an *E. coli* mutant without Dr fimbriae was gradually cleared from kidney tissue which displayed significantly less pathology (39). It was also observed that infections during pregnancy with *E. coli* bearing adhesins of the Dr family may pose a threat for patients due to bacterial invasive potential and pregnancy-associated up-regulation of DAF receptor (40).

Immunization of mice with the *E. coli* Dr fimbrial antigen reduced mortality associated with an experimental urinary tract infection due to a homologous strain bearing the Dr adhesin, while immune sera with high titers of anti-Dr antibody inhibited bacterial binding to bladders and kidneys but did not affect the rate of renal colonization (38).

1.4.7 Afimbrial adhesins

More than 20 years ago, it was observed that 10 % of the *E. coli* strains, which agglutinated human erythrocytes in the presence of D-mannose, also termed mannose-resistant haemagglutination (MRHA), did not show any fimbriae and still adhered to uroepithelial cells, suggesting the existence of afimbrial adhesins (94). It was also found that about 6.7 kb of DNA were required for the expression of the MRHA of human erythrocytes and to confer adhesion, and that this binding function was mediated by a 16 kDa protein named AFA-I (94).

The 6.7 kb insert expresses five polypeptides of molecular mass 13 kDa, 16 kDa, 18.5 kDa, 30 kDa, and 100 kDa, encoded, respectively, by the *afaA*, *afaE*, *afaD*, *afaB* and *afaC* genes which are localized and belong to the same transcriptional unit (93). The *afaE* gene encodes the adhesin or haemagglutinin AFA-I polypeptide, the *afaB* gene is also required for the MRHA expression, however, does not play an obvious role in the biosynthesis or the maturation of the AFA-I haemagglutinin, while the *afaC* gene codes for a polypeptide synthesized as a precursor and its gene product is transported through the cytoplasmic membrane by means of a signal sequence (93). Purification and characterization of the afimbrial adhesin AFA-I showed that it exists on the bacterial surface and free as a macromolecular aggregate in the supernatant of spent culture medium, and is composed of a single, repeating 16 kDa polypeptide subunit (169). Transformation of non adherent recipient pyelonephritic strains with recombinant plasmids carrying the *afa-I* operon confers binding specificities and biochemical properties different from those observed with strains expressing type 1, P fimbriae and S fimbriae (92).

It was later demonstrated that there exist gene clusters structurally related to the first *afa* operon which was described, but which encoded antigenically distinct afimbrial adhesins; experiments demonstrated that all the *afa* gene clusters harboured a highly conserved 4.1 kb DNA segment carrying the *afaB*, *afaC* and *afaD* genes and revealed heterogeneity for the *afaE* sequences (99). It was therefore proposed that there exist at least four different *afa* operons, *afa-1*, *afa-2*, *afa-3* and *afa-4* which encode variable adhesins designated AFA-I, AFA-II, AFA-III and AFA-IV respectively (99). AFA-III and AFA-I belong to a family of haemagglutinins which also include the Dr fimbrial adhesin, and this heterogeneous adhesin family is referred to as the Dr family (99). Two afa operons, designated *afa-7* and *afa-8* found in bovine isolates were cloned and analyzed, and *afa-8* was found to be widespread among bovine pathogenic *E. coli* strains associated with diarrhoea and septicaemia (96).

1.4.8 Temperature sensitive haemagglutinin

A mannose-resistant haemagglutinin of an avian pathogenic *E. coli* (APEC) isolate was identified, which was found to be best expressed at lower temperatures (142).

Haemagglutination activity was highest when cells were grown at 26 ºC and lower in cells grown at 37 ºC, while cells grown at 42 ºC lacked activity. This temperature-dependent haemagglutination phenotype was termed Tsh for temperature sensitive haemagglutinin (142). The gene responsible for the Tsh phenotype, *tsh*, was cloned and characterized and found to confer a haemagglutination-positive phenotype to *E. coli* K12 strains (142). It was found that *E. coli* K-12 strains containing a recombinant *tsh* gene produces two proteins, a 106 kDa extracellular protein and a 33 kDa outer membrane protein, and was able to agglutinate chicken erythrocytes (159). Further studies revealed that Tsh is synthesized as a 140 kDa precursor protein, whose processing results in a 106 kDa passenger or secreted domain and a 33 kDa ß- barrel domain (24, 87). The role of Tsh during pathogenesis of APEC has been studied. It was demonstrated that out of 300 avian *E. coli* isolates examined for the prevalence of the *tsh* gene, half of the isolates were *tsh* positive and *tsh* was specifically more frequent in high-lethality isolates compared to low-lethality isolates (24). In another study it was shown that the *tsh* gene was prevalent in more than 50 % of APEC, 4.5 % UPEC and 11.5 % NMEC isolates tested (30). It was further seen that in the *tsh* positive strains examined, *tsh* was always plasmid encoded and was linked to colicin V genes when they were present on the same plasmid (24). In an additional study it was also reported that purified Tsh secreted domain is capable of adhering to red blood cells, haemoglobin, and the extracellular matrix proteins fibronectin and collagen IV (87).

1.4.9 Novel adhesin gene clusters

With the availability of whole genome sequences for crucial pathogens, including many ExPEC prototypic isolates, many novel genes, including those encoding putative adhesins, have been found to be present on the genome of these strains and it has become clear that a single strain produces many different adhesins at one time or the other. The sequencing of a prototypic cystitis strain UTI89 revealed that the strain contains at least ten different chaperone-usher adhesin systems including *fim* and *pap* which still remain the only two adhesins best characterized (17, 175). Other putative adhesins like *auf*, *yad*, *yqi*, *yeh* and *fml* also found present in UTI89, have yet to be characterized for their role in UPEC pathogenesis (175). The presence of multiple pilus systems likely confers niche-adaptive advantages, and the combination of receptor specificity and tissue-specific receptor production will ultimately determine the site of action for a given pilus during infection (175). Understanding and functionally characterizing the roles of these putative adhesins in pathogenesis will most likely provide new targets for therapeutic intervention in the treatment and prevention of ExPEC infection.

1.5 Signature-tagged mutagenesis (STM)

Aside from characterization of putative virulence genes and the study of their role in virulence, new approaches have been undertaken to identify genomic regions specific for pathogenic strains. The use of genetic approaches for the identification of new virulence factors in APEC will greatly improve our knowledge of its pathogenic mechanisms.

One of the most important applications of genetic manipulation techniques, as applied to bacterial pathogens, is in the identification of genes that are required for virulence or pathogenicity *in vivo*. This knowledge serves the dual purpose of understanding virulence itself, as well as providing a route for rational attenuation of the pathogen for vaccine construction.

Signature-tagged mutagenesis (STM) was established by Hensel et al. and became a successful way of identifying genetic factors *in vivo*, that played a crucial role in virulence of a pathogen (50). It is a comparative hybridization technique that uses a collection of transposons, each one modified by the incorporation of a different DNA sequence tag (18). The tags are short DNA segments that contain a 40-bp variable central region flanked by invariant “arms” that facilitate the co-amplification and labelling of the central portions by PCR. Therefore, when the tagged transposons are used to mutate an organism, each individual mutant can in theory be distinguished from every other mutant based on the different tags carried by the transposons in its genome (18).

In STM, mutagenized bacterial strains are stored individually in arrays, and colony or dot blots are made from these arrays. Pools of mutants are subjected to a selective process like infection of an animal, and using PCR, labelled probes are prepared representing the tags present in the input and output pools (Fig. 4).

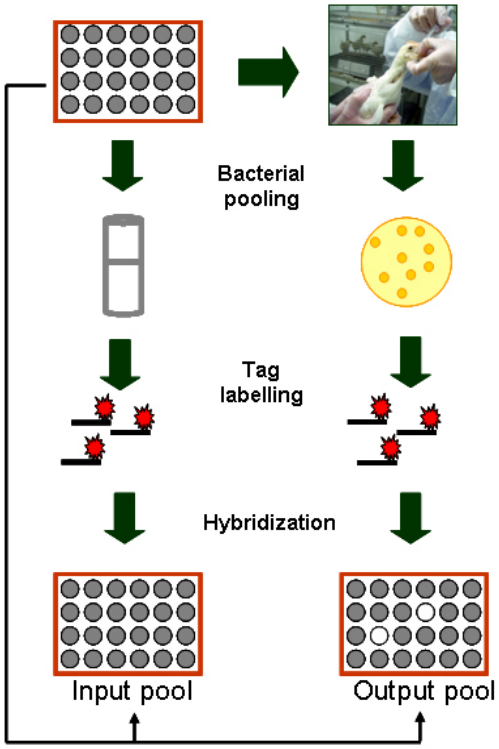


Figure 4: Schematic representation of the method of signature-tagged mutagenesis.

Hybridization of the tags from both pools permits the identification of mutants unable to survive the selective process, hence a negative selection. When pooled mutants are used to infect the animal, any mutants that are defective in essential virulence genes will fail to grow, and will therefore not be recovered in the final mixture. Those mutants that are absent (because of inactivation of a virulence or metabolic determinant) will be identified by the absence of hybridization (18).

One of the most important advantages of STM is that a large number of mutants can be analysed in a single assay, allowing genetic screens to be performed in biologically relevant animal models of disease (113). Besides, it does not require prior whole genome sequence information, yet can identify attenuated strains in a single step (41). Hence, STM has indeed become quite a legend as a genetic method widely used for the *in vivo* identification of virulence traits in pathogenic bacteria, while recent technical advances, mainly in the choice of negative selection systems and detection systems, have broadened its applicability and versatility as has been seen over the years (154).

2 Aim of the study

Host colonization begins with the attachment of the pathogen to specific receptors present on the host surface making this the most crucial step for the successful initiation of microbial infection. The availability of complete genome sequences for some ExPEC pathogens has drawn our attention to a large increase in the number of genes with unknown function, possibly involved in adhesion and colonization, and whose contribution to virulence on the whole is currently merely hypothetical. Fimbrial adhesins and colonization factors are known to play a vital role in adhesion and colonization of the host by the pathogen, thereby making this an interesting study aspect of host-pathogen interaction. The identification of new adhesins would therefore enhance our understanding of how a pathogen initiates and causes disease.

In a previous study at the Institute for Microbiology and Epizootics (IMT), Freie Universität Berlin, Signature-tagged mutagenesis (STM) was applied to an APEC strain IMT5155 (O2:K1:H5) isolated during an outbreak of septicaemia in Germany, in a chicken systemic infection model. The study led to the successful identification of genes crucial to systemic infection in chickens; however, due to the study design concentrating on the identification of APEC genes involved in systemic disease, no new adhesins were identified that are responsible for the initial stages of APEC infection.

In order to study the important early stages of APEC infection, particularly the host-pathogen interaction in the chicken lung, the first objective of my doctoral thesis was to establish a lung model of infection in the chicken using the strain IMT5155. The next step was to apply the method of signature-tagged mutagenesis to IMT5155, by generating an IMT5155 mutant library and screening 1,800 mutants in the chicken lung infection model, in order to try and identify novel genes, particularly adhesins, functionally involved in the adhesion and colonization of the chicken lung during the initial infection process. Finally, the STM screen led, among others, to the identification of a novel putative adhesin, Yqi, uncharacterized till date. This *bona fide* adhesin was therefore characterized for its role during APEC pathogenesis, and due to its predominant occurrence in a large collection of extraintestinal pathogenic *E. coli* and absence in intestinal pathogenic *E. coli*, temporarily designated ExPEC adhesin I.

3 Animals and Materials

3.1 Bacterial strains and plasmids

All *E. coli* strains and plasmids used are listed in table 2. The prototype strain IMT5155 used in this study, not yet completely sequenced, was obtained from internal organs of a laying hen clinically diagnosed with systemic APEC infection during an outbreak in the northern part of Germany in the year 2000 (59). The strain possesses a number of virulence-associated genes typical of ExPEC strains (*csgA*, *fimC*, *tsh*, *chuA*, *fyuA*, *ireA*, *iron*, *irp2*, *iucD*, *iutA*, *sit*, *neuC*, *ompA*, *ibeA*, *gimB*, *colV*) and is classified into the B2 phylogenetic group, and multi locus sequence type (ST) 140 of the ST95 complex (28, 30). IMT5155 (O2:K1:H5) belongs to one of the most common O-types among APEC strains and synthesizes a K1 polysaccharide capsule. IMT5155 was used for all genetic manipulation studies.

An additional *E. coli* strain, IMT11327 (Ont:H16; ST295) classified into the B1 phylogenetic group, isolated from the intestine of a clinically healthy chicken (A_{faecal} strain) and devoid of ExPEC related genes, was used as a non-virulent negative control during studies involving a chicken infection model *in vivo*. Strain IMT11327 has been found to harbour type 1 fimbriae, temperature sensitive haemagglutinin and curli fimbriae. This strain was also used as a negative control *in vitro* for adhesion assays involving a canine kidney epithelial cell line, as this strain served as a suitable negative control during preliminary tests while standardizing the adhesion assay protocol with this cell line during the study. *E. coli* - K12 strain (MG1655) was used as a negative control *in vitro* for adhesion assays involving a chicken fibroblast cell line, following previous standardization of the adhesion assays with this negative control strain. The strain harbours only the type 1 fimbriae. Both negative control strains do not harbour the putative adhesin identified in this study, namely ExPEC adhesin I.

All bacterial strains were routinely cultured at 37 ºC in Luria-Bertani (LB) broth and on LB agar plates with appropriate antibiotics when required in the following concentrations: Kanamycin (Kan), 50 µg/ml; Nalidixin (Nal), 30 µg/ml; Ampicillin (Amp), 50 µg/ml; Chloramphenicol (Cm), 30 µg/ml; Spectinomycin (Spec) 50 µg/ml. Micro-organisms were stored at -70 °C in LB broth with 10 % (v/v) glycerol until further use.

3.2 Animals

Five-week old White Leghorn Specific Pathogen Free (SPF) chickens (Lohmann Selected Leghorn; Lohmann Tierzucht GmbH, Cuxhaven, Germany) were used for the establishment of a lung infection model at the infectious disease facility of the Institute for Microbiology and Epizootics (IMT) at the Freie Universität, Berlin. A designated SPF flock is derived from chickens shown to be free from the following vertically-transmissable agents: Avian

adenoviruses group I, Avian encephalomyelitis virus, Avian infectious bronchitis virus, Avian infectious laryngotracheitis virus, Avian leucosis viruses, Avian nephritis virus, Avian orthoreoviruses, Avian reticuloendotheliosis virus, Chicken anaemia virus, Egg drop syndrome virus, Infectious bursal disease virus, Influenza A virus, Marek's disease virus, Newcastle disease virus, Turkey rhinotracheitis virus, Mycoplasma gallisepticum, Mycoplasma synoviae and Salmonella pullorum. The chickens were provided food and water ad libitum. In the study period from April 2005 to March 2008 a total number of 418 chickens were used for *in vivo* experiments as registered to the authorities.

3.3 Oligonucleotide primers

All oligonucleotide primers, their target regions, melting temperatures and primer references are listed in table 3.

3.4 Standard kits

All standard kits used are listed in table 4.

Enzymes and oligonucleotide primers were purchased from Invitrogen GmbH, Karlsruhe-Germany, New England Biolabs GmbH, Frankfurt am Main-Germany, Promega GmbH, Mannheim-Germany, Sigma-Aldrich Chemie GmbH, Munich-Germany, Operon Biotechnologies, Cologne-Germany.

Table 2: Strains and plasmids used in this study

Strain	Description	Reference
IMT5155	O2:K1:H5; *csgA*, *fimC*, *tsh*, *chuA*, *fyu*A, *ireA*, *iroN*, *irp2*, *iucD*, *iutA*, *sit*, *neuC*, *ibeA*, *gimB*, *colV*, *ompA*, ST140, STC95, B2	(103)
IMT5155 Nal^R	IMT5155 derivative, Nalidixin resistant	(103)
S17λpir	*recA thi* pro *hsdR*- M+ RP4::2-Tc::Mu::Km Tn7 lysogenized with λpir phage	(103)
IMT11327	Ont:H16; *fimC*, *crlA*, *ompA*, ST 295, B1	(3)
MG1655	F^-, Lam^-, Fim^+	(9)
EA7F9	IMT5155 Nal^R *yqi*::mini-Tn5	this study
IMT5155Δ*yqi*	IMT5155 Nal^R Δ*yqi*::Cm^R	this study
E. coli TOP10	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *recA1 araD*139 Δ(araleu)7697 *galU galK rpsL* (StrR) *endA1 nupG*	Invitrogen
pCR2.1-TOPO:*yqi*	*E. coli* TOP10 [pCR2.1 TOPO:*yqi*]	this study
pDSK602:*yqi*	*E. coli* TOP10 [pDSK602:*yqi*]	this study
Top10 (pGEX:*yqi*)	*E. coli* TOP10 [pGEX-6p-1:*yqi*]	this study
IMT5155Δ*yqi* (pDSK602:*yqi*)	IMT5155 Nal^R Δ*yqi*::Cm^R [pDSK602:*yqi*]	this study
IMT5155Δ*yqi* (pDSK602)	IMT5155 Nal^R Δ*yqi*::Cm^R [pDSK602]	this study
IMT5155 (pDSK602:*yqi*)	IMT5155 Nal^R [pDSK602:*yqi*]	this study
E. coli BL21	*E. coli* B F^-, *ompT*, *hsdS* (r_B^-, m_B^-), *gal*, *dcm*	Amersham
BL21 (pGEX)	*E. coli* BL21 [pGEX-6p-1]	this study
BL21 (pGEX:*yqi*)	*E. coli* BL21 [pGEX-6p-1:*yqi*]	this study
pCR2.1-TOPO:*yqi*_operon	*E. coli* TOP10 [pCR2.1 TOPO:*yqi*-5kb]	this study
Top10 (pKESK:*yqi*_4975_XB)	*E.coli* TOP10 [pKESK-22:*yqi*_4975bp_*XbaI-BamHI*]	this study
E. coli AAEC189	Δfim, Δlac, recA-, endA-, hsdR-, hsdM+	(10)
E. coli AAEC189 (pKESK: *yqi*_4975_XB)	*E.coli* AAEC189 [pKESK-22:yqi_4975bp:XbaI-BamHI]	this study
Plasmid		
pUTmini-Tn5km2	Kan^R, Amp^R	(103)
pKD46	Amp^R, expresses λ red recombinase	(20)
pKD3	*cat* gene	(20)
pCR2.1 TOPO	Kan^R, Amp^R, LacZα, T7 promoter	Invitrogen
pKESK-22	Neo^R, Kan^R, *tac* promoter	Schnetz
pDSK602	$Spec^R$, Sm^R, triple *lac* UV5, broad host range	(122)
pGEX-6p-1	Amp^R, GST-Tagged, $lacI^q$, *tac* promoter	Amersham

Table 3: Oligonucleotide primers used in this study

Primer number	Target region	Primer Sequence (5'-3')	Tm (ºC)	Reference
IMT2510	*cat* (pKD3)	AATCCCTCTGCCAAAGCTCTCCTGCTAAGAAGGGGAAAATGTGTAGGCTGGAGCTGCTTCGA	75	this study
IMT2511	*cat* (pKD3)	TAAATATGAAAATGCCGGGGTGTTCCCGGCATTTTGCTGTCATATGAATATCCTCCTTAG	71	this study
IMT2512	*yqi*	ATGCAATGGCAGTACCCTTC	60	this study
IMT2513	*yqi*	CTGGTGGCAACATCAAATTG	60	this study
IMT2558	221 bp upstream of *yqi*	ATTACCGTCGGTTATATCGGC	52	this study
IMT2559	110 bp downstream of *yqi*	ATAAACACAATATGGCGCTCG	50	this study
IMT2910	*yqi* with *EcoRI*	CGGATACGAATTCATGATTACGCTTTTTCGTT	59	this study
IMT2911	*yqi* with *HindIII*	TTCTCAAAAGCTTTGTCGTTCAGTTATAGTTTA	57	this study
IMT3045	*yqi* operon with *BamHI*	CGGATACGGATCCATGTTAAAAAAAACATTGTTATCTATGTTCGCAAC	56	this study
IMT3046	*yqi* operon with *HindIII*	TTCTCAAAAGCTTTCAGTTATAGTTTATTTTTACGATGAGATC	53	this study
IMT1560	Cloning vector pCR2.1 TOPO (-20)	GTAAAACGACGGCCAG	50	Invitrogen
IMT1561	Cloning vector pCR2.1 TOPO	CAGGAAACAGCTATGAC	50	Invitrogen
IMT1119	Flanking sequence Tn5	GGCCACGCGTCGACTAGTAC	58	(103)
IMT1118	Flanking sequence Tn5	GGCCACGCGTCGACTAGTACNNNNNNNNNNTACNG	58	(103)
IMT556	Signature tag	TACCTACAACCTCAAGCT	50	(103)
IMT557	Signature tag	TACCCATTCTAACCAAGC	50	(103)
IMT1035	Flanking sequence Tn5	CCTAGGCGGCCAGATCTGAT	61.4	(103)
IMT1048	Flanking sequence Tn5	CGCAGGGCTTTATTGATT	54.9	(103)
IMT718	*Cat*	TTATACGCAAGGCGACAAGG	57.3	(103)
IMT719	*Cat*	GATCTTCCGTCACAGGTAGG	59.4	(103)
IMT908	Expression vector pGEX-6P-1	GGGCTGGCAAGCCACGTTTGGTG	67.8	Amersham Biosciences

IMT909	Expression vector pGEX-6P-1	CCGGGAGCTGCATGTGTCAGAGG	67.8	Amersham Biosciences
IMT3138	Expression vector pKESK-22	AATGTGTGGAATTGTGAGCGG	60.6	this study
IMT3139	Expression vector pKESK-22	GCCGACATGATCCAACTGA	60.1	this study
IMT900	Mini-Tn5 *kan*	ATTCAACGGGAAACGTCTTG	55.3	this study
IMT901	Mini-Tn5 *kan*	ACTGAATCCGGTGAGAATGG	57.3	this study
IMT3706	*yqi*	AGTTAGGCTTTGTGGCGGAC	56.3	this study
IMT3707	*yqi*	GTTCACCGTCTATCTCCT	53.6	this study
IMT3259	*yqi* operon with *XbaI*	CGGTACTCTGAATGTTAAAAAAAACATTGTTAT CTATGTTCGCAAC	67.3	this study
IMT3260	*yqi* operon with *BamHI*	TTCTCGATCCTCAGTTTAGTTTATTTTTACGGG ATC	66.6	this study

Table 4: List of standard kits

Master Pure™ Genomic DNA Purification Kit	Epicentre, Biozym, Hessisch Oldendorf
High Pure™ PCR Product Purification Kit	Boehringer Mannheim GmbH, Mannheim
High Pure Plasmid Isolation Kit	Roche Diagnostics GmbH, Mannheim
PCR DIG Probe Synthesis Kit	Roche Diagnostics GmbH, Mannheim
TOPO TA Cloning ® Kit	Invitrogen GmbH, Karlsruhe
QIAquick Gel extraction Kit	Qiagen GmbH, Hilden

3.5 Nutrition media

Luria Bertani (LB) Broth

NaCl	5 g
Peptone (Casein)	10 g
Yeast extract	5 g

pH 7.0

Add to a volume of 1000 ml with distilled water. Autoclave, 121ºC, 25mins

Luria Bertani Agar

NaCl	5 g
Peptone (Casein)	10 g
Yeast extract	5 g
Agar	15 g

Add to a volume of 1000 ml with distilled water. Autoclave, 121ºC, 15mins

Brain Heart Infusion (BHI) Broth

Calf Brain infusion solids	12.5 g
Brain Heart infusion solids	5 g
Proteose peptone	10 g
Glucose	2 g
NaCl	5 g
Na_2PO_4	2.5 g

Add to a volume of 1000 ml with distilled water. Adjust pH to 7.4, Autoclave, 121ºC, 15mins

SOC medium

Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
KCl (20mM)	10 ml
NaOH (5N)	20 ml

Add to a volume of 1000 ml with distilled water. Autoclave, 121ºC, 15mins

After cooling add 5 ml $MgCl_2$ (2M) and 20 ml filter sterilized Glucose (1M).

3.6 Solutions

3.6.1 Solutions used for agarose gel electrophoresis

1.5 % Agarose Gel

Agarose	6 g
1x Tris Borate EDTA (TBE) buffer	400 ml
Ethidium Bromide	8 µl

4.5 % Low melting point (LMP) Agarose

LMP agarose	2.25 g
1x Tris Borate EDTA (TBE) buffer	50 ml
Ethidium Bromide	2.5 µl

5x Tris Borate EDTA (TBE) Buffer

Tris base	54 g
Boric acid	27.5 g
0.5M EDTA (pH 8.0)	20 ml

Add to a volume of 1000 ml with distilled water.

Loading dye

Glycerol	5 ml (50 %)
Bromophenol Blue	10 mg (0.1 %)
0.5M EDTA	2 ml (0.1 M)
Tris pH 8.0	0.1 ml (10 mM)
Distilled water	2.9 ml

3.6.2 Solutions used for Sodium Dodecyl Sulphate–Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS separating Gel (13.5%)

Polyacrylamide (30 %)	9 ml
Distilled water	5.6 ml
1.5 M Tris HCl (pH 8.8)	5 ml
TEMED	10 µl
SDS (10 %)	200 µl
APS (10 %)	1:100, added just before pouring the gel

SDS stacking Gel (5 %)

Polyacrylamide (30 %)	1.67 ml
Distilled water	5.63 ml
0.5 M Tris HCl (pH 6.8)	2.5 ml
TEMED	8 µl
SDS (10 %)	100 µl
APS (10 %)	1:100, added just before pouring the gel

2x Laemmli Buffer (SDS loading buffer)

0.5 M Tris HCl (pH 6.8)	2.5 ml (0.125 M)
SDS (10 %)	4 ml (4 %)
Glycerol	2 ml (20 %)
β – Mercaptoethanol	1 ml (10 %)
Bromophenol Blue	1 mg

5x Laemmli Buffer

0.5 M Tris HCl (pH 6.8)	2.1 ml (0.125 M)
SDS (10 %)	4 ml (4 %)
Glycerol	4 ml (40 %)
β – Mercaptoethanol	1 ml (10 %)
Bromophenol Blue	0.2 mg

10x SDS running buffer

Tris base	15 g
Glycine	72 g
Distilled water	480 ml
SDS	0.1 %
pH 8.3	

3.6.3 Solutions used for silver staining

Fixative

Ethanol	50 ml (40 %)
Glacial acetic acid	12.5 ml (10 %)

Add to a volume of 125 ml with distilled water.

Sensitizing solution

Ethanol	37.5 ml
Sodium acetate	5.15 g
Sodium thiosulphate	0.25 g

Add to a volume of 120 ml with distilled water. Just before use add 2.5 ml Glutaraldehyde.

Silver stain

Silver nitrate	312.5 mg

Add to a volume of 125 ml with distilled water. Just before use add 50 µl Formaldehyde.

Developing solution

Sodium carbonate	3.125 g

Add to a volume of 125 ml with distilled water. Just before use add 25 µl Formaldehyde.

Stop solution

EDTA	1.825 g

Add to a volume of 125 ml with distilled water.

Preserve solution

Glycerol	8 ml
Ethanol	60 ml

Add to a volume of 200 ml with distilled water.

3.6.4 Solutions used for DNA isolation

Proteinase K

Proteinase K	20 mg
Distilled water	1 ml

5M NaCl

NaCl	29.2 g
Distilled water	100 ml

CTAB/NaCl

CTAB	10 % (w/v)
NaCl	0.7 M

SDS (10 %)

SDS	10 g
Distilled water	100 ml

Other solutions

Chloroform:isoamyl alcohol, Phenol:chloroform:isoamyl alcohol, Isopropanol, 70 % Ethanol

3.6.5 Solutions used for DNA-DNA hybridization

20x SSC

Sodium citrate	88.23 g (300 mM)
NaCl	175.32 g (3 M)

pH 7.0

Add to a volume of 1000 ml with distilled water.

Pre-hybridization buffer

20x SSC	2.5 ml (5x SSC)
N-Lauryl sarcosine (10 %)	1ml (0.1 %)
SDS (10 %)	0.1 ml (0.02 %)
BRSS	5 ml (1 %)

Add to a volume of 50 ml with distilled water.

Stringent buffer I

20x SSC	40 ml
SDS (10%)	4 ml

Add to a volume of 400 ml with distilled water.

Stringent buffer II

20x SSC	2 ml
SDS (10 %)	4 ml

Add to a volume of 400 ml with distilled water.

Buffer I

1M Malic acid	100 ml
1M NaCl	150 ml

Tween 20	3 ml

pH 7.5

Add to a volume of 1000 ml with distilled water.

Blocking solution

Blocking reagent (10 %)	10 ml
Buffer I without tween	100 ml

Anti-Dig solution

Anti-Digoxigenin-AP	4 µl
Blocking solution	10 ml

Detection buffer

Tris HCl	12.11 g (100 mM)
NaCl	5.84 g (100 mM)

pH 9.5

Add to a volume of 1000 ml with distilled water.

CSPD solution

CSPD	5 µl
Detection buffer	500 µl

Keep in the dark

Stripping solution

NaOH (3 M)	33.3 ml
SDS (10 %)	5 ml

Add to a volume of 500 ml with distilled water.

3.6.6 Solutions used for Western blotting

Transfer Buffer

Tris base	6 g
Glycine	28.5 g
Methanol	500 ml

Add to a volume of 2000 ml with distilled water.

10x TBS wash buffer

Tris HCl (1 M)	100 ml
NaCl (5 M)	300 ml

Add to a volume of 1000 ml with distilled water.

TBST Buffer

Tween 20	500 µl
1x TBS wash buffer	1000 ml

Blocking solution

Skimmed Milk	1 g (5 %)
TBST buffer	20 ml

Primary antibody solution

Anti-GST antibody	4 µl (1:5000)
TBST buffer	20 ml

Secondary antibody solution

Anti-Goat IgG	2 µl (1:10000)
TBST buffer	20 ml

Solution A

Tris HCl (0.1 M, pH 8.6)	200 ml
Luminol	50 mg

Solution B

Coumaric acid	11 mg
Dimethylsulfoxide (DMSO)	10 ml

Enhanced chemiluminescence (ECL) reagent

Solution A	5 ml
Solution B	500 µl
Hydrogen peroxide (30 %)	1.5 µl

3.6.7 Solutions used for Southern blotting

20x SSC

NaCl	175.3 g
Tri-Sodium citrate-dihydrate	88.2 g

pH 7.0

Add to a volume of 1L with distilled water.

2x SSC

20x SSC	100 ml
Distilled water	900 ml

pH 7.0

250 mM HCl

HCl (concentrate)	20.8 ml

Add to a volume of 1L with distilled water.

Denaturation solution

NaOH (0.5M)	20 g
NaCl (1.5M)	87.83 g

Add to a volume of 1L with distilled water.

Neutralization solution

Tris HCl (0.5M)	60.55 g
NaCl (1.5M)	87.83 g

pH 7.0

Add to a volume of 1L with distilled water.

4 Methods

4.1 Generation of an APEC mutant library

A pool of tagged pUTmini-Tn5*km*2 plasmids kindly provided by David Holden was used to select 90 specific tags that did not cross-react with each other, and plasmids containing these specific tags were purified and transformed into donor strain *E. coli* S17-1 λ*pir* as described previously (103). Briefly, each tag contains a 40 bp random sequence flanked by two 20 bp invariable sequences. The invariable sequences serve as primers for amplification of the tags (50).

4.1.1 Generation of mutants by bacterial conjugation

To generate an APEC mutant library in this study, 90 separate conjugation experiments were carried out as follows. Briefly, overnight cultures of each single donor *E. coli* S17-1 λ*pir* (with specific tag) and recipient IMT5155 Nal^R were used to inoculate 4 ml LB broth which were then grown to an optical density at 600 nm of 1.0 at 37 ºC with aeration. 500 µl each of donor and recipient strains were then mixed together to give a ratio of 1:1 and centrifuged at 13,000 rpm for 30 secs. The same was repeated for negative controls of donor and recipient separately in a volume of 1 ml each. After discarding the supernatant, cells were resuspended in 150 µl of 0.01 M $MgSO_4$. Samples were centrifuged again at 13,000 rpm for 30 secs, 120 µl supernatant was discarded, and 10 µl from each sample was spot inoculated onto LB agar plates and incubated at 37 ºC for 4 h.

Colonies were then resuspended in 3 ml 0.01M $MgSO_4$ and transferred to 15 ml centrifuge tubes. Aliquots of 100 µl, 200 µl and 400 µl were plated onto selective LB medium containing Nalidixin and Kanamycin. After overnight incubation at 37 ºC, 20 single colonies were resuspended in 4 ml LB supplemented with Nalidixin and Kanamycin and incubated overnight at 37 ºC after which glycerol stocks were prepared using 25 % sterile glycerol and a total of 1,800 mutants were arrayed into 20 pools of 90 mutants each and stored at -80 ºC until further use. At every 10^{th} conjugation experiment, genomic DNA was extracted from the 20 mutants selected using a Master Pure™ Genomic DNA Purification Kit, Epicentre technologies in order to determine the presence of the Kanamycin gene in mutants using PCR using primers IMT900 and IMT901 listed in table 2.

PCR reaction mix:

10x Reaction buffer	2.5 µl
$MgCl_2$	1 µl
dNTP mix (10 mM)	0.5 µl

Forward primer IMT900 (10 pmol)	1 µl	
Reverse primer IMT901 (10 pmol)	1 µl	
Taq polymerase (5U/ µl)	0.1 µl	
Deionized water	18.9 µl	
Template	1 µl	

PCR reaction:

94 ºC	3 mins	1x
94 ºC	30 s	
58 ºC	30 s	30x
72 ºC	45 s	
72 ºC	7 mins	1x

PCR products were loaded on a 1.5 % agarose gel and samples positive for the kanamycin gene were determined by the presence of a 600 bp band.

4.1.2 Generation of a Kanamycin cassette labelled probe for southern blot hybridization

To generate a labelled probe of the kanamycin cassette, a PCR DIG Probe synthesis kit (Roche diagnostics GmbH, Mannheim, Germany) was utilized as per the manufacturer's instructions. The PCR product was excised from a 1 % agarose gel and purified using QIAquick gel extraction Kit, available from Qiagen.

4.1.3 Southern blotting

Southern blotting includes transferring of DNA fragments from an electrophoresis gel to a membrane support. The basic protocol describes southern blotting via upward capillary transfer of DNA from an agarose gel onto a nylon membrane, using a high-salt transfer buffer to promote binding of DNA to the membrane.

In order to determine that insertion of the Tn5*km2* transposon was random and unique in the IMT5155 genome, southern blot analyses were carried out with random mutants in a single conjugation as follows. After extraction of genomic DNA, using Master Pure^{TM} Genomic DNA Purification Kit available from Epicentre, Biozym, according to the manufacturer's instructions, the DNA was digested using restriction enzyme *EcoRI* for 2 h at 37 ºC on a sand bath.

Restriction digestion mix:

Deionized water	7.35 µl
Enzyme buffer	1.5 µl
Bovine serum albumin (BSA)	0.15 µl
DNA	5 µl
EcoRI (20U/ µl)	1 µl

The samples were run on a 1 % agarose gel for 3 h until the bands were well separated. The agarose gel was rinsed in distilled water and then transferred into a clean dish containing 250 mM HCl and left to shake at room temperature for 10 mins. Once again the gel was rinsed in distilled water, and then washed with denaturation solution twice for 15 minutes each. After a rinse in distilled water the gel was transferred to neutralization solution and washed twice for 15 mins each, after which the gel was transferred to 20x SSC solution for 10 mins.

A sponge, slightly larger than the gel, was placed in a plastic dish filled with enough 20x SSC solution to leave the soaked sponge half submerged in buffer. Two pieces of Whatman filter paper were placed on the sponge. The gel was transferred onto the Whatman paper carefully facing down, taking care to see that there were no air bubbles under the gel. Four strips of parafilm were placed over the edges of the gel. A piece pf positively charged Nylon membrane was placed on the gel, and one piece of Whatman paper was placed over the membrane. Paper towels were piled over the filter paper, over which came a glass plate. A weight was placed on the top to hold everything in place. The gel was left to blot overnight, then disassembled and the membrane was then rinsed with 2x SSC solution for 5 minutes. DNA was immobilized on the membrane by baking at 120 ºC in a pre-heated oven for 20 minutes and then pre hybridized using a kanamycin cassette probe (19µg/ µl) at 68 ºC. Details of DNA-DNA hybridization are described in 4.4.2.

4.2 Establishment of a lung colonization model of infection

4.2.1 Preparation of bacterial inoculum

To prepare *E. coli* cultures for infecting birds via the intra-tracheal route, one loopful of the stock culture was plated on Luria Bertani (LB) agar and incubated at 37 °C for 18h. A single colony of this fresh culture was inoculated in 5 ml LB broth and incubated at 37 °C for 18 h with agitation. On the day of infection, 18-hour cultures were diluted 1:50 in 100 ml fresh LB broth and grown at 37 °C with agitation to an optical density at 600 nm (OD_{600}) corresponding

to about 1-3 x 10^8 CFU/ml. After incubation, cultures were harvested by centrifugation at 5000 x *g* for 10 min and the cell pellet was resuspended in 100 ml Phosphate buffered saline (PBS) to a concentration of 3-6 x 10^8 CFU/ml. The estimated colony count was confirmed by plating 100 µl of a 10^{-6} and a 10^{-7} dilution of the final culture onto separate LB agar in duplicate and calculated by colony counting (144). To inoculate animals with a lower bacterial dose, bacterial cultures containing 10^8 CFU/ml were diluted 1:10 in 50 ml PBS until the desired bacterial concentration was reached.

4.2.2 Infection of animals

A lung infection model was established, such that no systemic infection was induced. In an initial infection trial, 5-week-old chickens were inoculated intra-tracheally, with the help of a buttoned Cannula 80mm long, with a 0.5 ml suspension containing 10^6, 10^5, 10^4 and 10^3 CFU of the virulent APEC strain IMT5155 respectively. Groups of 20 chickens were used for each dose for statistical analysis. At 24h and 48h post infection, 10 chickens each were killed, bacteria were re-isolated from the lungs and the spleen, and CFU was determined as described above.

In a succeeding trial, non-pathogenic avian *E. coli* strain IMT11327 was used as a negative control. Five chickens were infected intra-tracheally with a 0.5 ml suspension containing 10^6 CFU of the above mentioned strain. Chickens were killed 24h post infection and bacteria were isolated from the lungs and the spleen as previously described for quantitative determination.

4.2.3 Clinical and organ scoring

A clinical score was evaluated monitoring the development of experimental APEC infection ranging from score 0 (no symptoms) to score 4 (severe symptoms) taking into consideration both respiratory and general symptoms (Table 5). Chickens were monitored by the hour and birds observed with scores $\geq$ 3 were killed immediately according to animal welfare norms (Reg. 0220/06).

Table 5: Description of clinical scores [Antao et al. (3)]

Score	Respiratory symptoms	Systemic symptoms
0	none	none
1	Light breathing with slight movement of the chest	Ruffled feathers; alert
2	Heavy breathing with increased movement of the chest	Ruffled feathers; less reaction to acoustics
3	Heavy breathing with open beak and increased movement of the chest	Aloof; ruffled feathers; no reaction to acoustics; lesser balance; closed eyes
4	Secretion of oedema by exerting pressure on the chest; head/ neck move upwards to facilitate breathing	Aloof; ruffled feathers; no reaction to acoustics; closed eyes; reduced consciousness

Post-mortem examinations were performed immediately after the euthanization of chickens. Organs were aseptically removed, and the severity of the macroscopic lesions attributed to *E. coli* was scored according to an organ lesion score described in table 6. Mean additive lesion scores were determined for all respective groups and differences were calculated by the two-tailed *t* test, with *P* of < 0.05 considered significant. A maximum organ score is the sum of the highest scores for each organ, that is, air sacs (3), lungs (5), heart and pericardium (3), liver (2) and spleen (1) amounting to 14.

Table 6: Description of organ lesion scoring modified after Ginns et al (36) and Bree et al (12) [Antao et al. (3)]

Organ	Score	Description of organ lesions
Thoracic air sacs	1	slight opaque and/or thickened membranes ± slight amount of fibrin
	2	moderate opaque and/or thickened membranes ± moderate amount of fibrin
	3	severe opaque and/or thickened membranes ± severe amount of fibrin ± moderate to severe vascularization)
Lung	1	single small lesion, locally restricted (1/5 of the organ)
	2	multiple, locally restricted small lesions and/or one bigger lesion (2/5 of the organ)
	3	lesions cover about ½ of the organ
	4	lesions cover about ¾ of the organ
	5	complete organ covered with lesions
Heart and pericardium	1	vascularization, opacity
	2	excessive or cloudy fluid in the pericardial cavity
	3	thickened pericardium, acute pericarditis
Liver	1	decolourization and/or slight amounts of fibrinous exudates
	2	marked perihepatitis with high amounts of fibrinous exudates
Spleen	1	congestion

4.3 Screening of the STM mutant library *in vivo*

4.3.1 Infection of chickens with mutant pools

Twenty pools of APEC mutants – a total of 1,800 mutants – were screened in the lung infection model as follows. Mutant inoculants were prepared by sub-culturing frozen plates of pooled APEC mutants taken out of -80 ºC storage and transferring 10 µl from each well to a new 96-well round bottom plate containing 400 µl LB with Kanamycin and Nalidixin. Mutants were cultured overnight, after which each plate was pooled and 10 ml of pooled bacteria was added to 90 ml LB and incubated at 37 ºC with aeration to an optical density at 600 nm of 1.0. Bacteria were then diluted in PBS to approximately 10^6 bacteria per ml. An aliquot from

the infection dose was kept aside to form the “Input pool”. Groups of four 5-week old chickens each were infected with 0.5 ml of a single mutant pool. This was repeated for all 20 mutant pools. At 24 h post infection, chickens were killed and lungs were homogenized and plated onto selective LB agar with Kanamycin and Nalidixin. Plates were incubated overnight, after which at least 10,000 colonies were pooled together in 10 ml PBS to form the “Output pool”. The Input and Output pools were used to prepare genomic DNA.

4.3.2 Isolation of genomic DNA from pooled reisolated mutants

Bacteria were harvested from a 10 ml PBS suspension at 10,000 x *g* for 5 mins and the bacterial pellet was resuspended in 5 ml Tris EDTA (TE) buffer. 300 µl of 10 % SDS solution and 30 µl of Proteinase K solution was added to the bacterial suspension and incubated at 37 ºC for 1 h. One millilitre of 5M NaCl was then added and the suspension was mixed thoroughly by vortexing, after which, 800 µl of CTAB/NaCl was added and the suspension was incubated at 65 ºC for 10 mins. To extract DNA, 8 ml of chloroform:isoamylalcohol was added, mixed and centrifuged at 10,000 x *g* for 5 mins. The supernatant was then transferred to a fresh tube and 7.5 ml of phenol:chloroform:isoamylalcohol was added, mixed well and centrifuged at 10,000 x *g* for 5 mins to separate the phases. The upper aqueous phase was transferred to a fresh tube and isopropanol was added at a 0.6 volume of the total aqueous phase to precipitate the DNA. The sample was then centrifuged at 10,000 x g for 5 mins which led to a white clear DNA pellet. The pellet was washed once with 70 % ethanol and left to be air dried, after which the pellet was resuspended in 200 µl of TE buffer (pH 8.0).

4.4 Identification of mutants defective in colonization ability

4.4.1 Labelling of signature tags

To amplify signature tags, about 5 µg of genomic DNA from the input and output pools was used as a template in the first PCR with primers IMT556 and IMT557 as described by Hensel et al. (50).

PCR reaction mix:

Deionized water	80.1 µl
10x Reaction buffer	10 µl
Primer IMT556 (10 pmol)	1.2 µl
Primer IMT557 (10 pmol)	1.2 µl
$MgCl_2$	4 µl
dNTP mix (10 mM)	2 µl
Taq polymerase (5U/ µl)	0.5 µl

Template	1 µl	

PCR reaction:

95 ºC	4 mins	1 x
95 ºC	30 s	
50 ºC	45 s	30 x
72 ºC	10 s	
72 ºC	10 mins	1 x

One hundred microlitres of chloroform:isoamylalcohol was added to 100 µl of the PCR products to concentrate the amplified tag DNA. After mixing, samples were centrifuged at 13,000 rpm for 5 mins. Three hundred microlitres of ethanol was added to the supernatant, centrifuged at 13,000 rpm for 10 minutes and the precipitated DNA pellet was resuspended in TE buffer. The concentrated DNA sample was loaded on a 4.5 % LMP agarose gel after which an 80 bp band was excised from the gel, boiled for 5 mins at 65 ºC in 15 µl distilled water, 5 µl of which was to be directly used as a template in the labelling PCR reaction.

The amplified tags were labelled using digoxigenin (DIG)-dUTP from a PCR DIG probe synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany) as follows.

PCR reaction mix:

Deionized water	32.25 µl
10x Reaction buffer (with $MgCl_2$)	5 µl
Primer IMT556 (10 pmol)	1 µl
Primer IMT557 (10 pmol)	1 µl
Labelled dNTP mix (10 mM)	2.5 µl
Unlabelled dNTP mix (10 mM)	2.5 µl
Taq polymerase (5U/ µl)	0.75 µl
Template	5 µl

PCR reaction:

95 ºC	4 mins	1 x
95 ºC	30 s	
50 ºC	45 s	30 x
72 ºC	10 s	
72 ºC	10 mins	1 x

After the PCR reaction, 40 µl *Hind* III enzyme buffer and 310 µl distilled water was added to the PCR product. Ten microlitres of *HindIII* restriction enzyme was added to the reaction mix in order to digest the unspecific flanking regions of the signature tag. The reaction mixture was incubated at 37 ºC in a sand bath for 1 h. To purify the specific tag DNA, 50 µl of 4M LiCl and 1.5 ml chilled ethanol was added to the 400 µl mixture and incubated at -70 ºC for 30 mins. The sample was then centrifuged at 13,000 rpm for 15 mins, after which the pellet was washed with ice cold 70 % ethanol and after another round of centrifugation, the pellet was resuspended in 15 µl TE buffer. The sample was run on a 4.5 % LMP agarose gel, and 40 bp bands were excised from the gel, to be further directly used after resuspension in pre-hybridization buffer.

4.4.2 Selection of mutants by hybridization

The amplified tags from both the input pool and output pool were compared by hybridization. Briefly, plasmids from the original donor strains containing the cognate tags were used as templates for the amplification of these signature-tags with primers IMT556 and IMT557 in a PCR reaction as described in 3.4.1. Identical dot blots were prepared by transferring these amplicons onto positively-charged nylon membranes, by boiling the PCR products at 95 ºC for 5 mins, cooling quickly on ice, and then preparing dot blots with 5 µl of the product.

The membranes were pre-hybridized with pre-hybridization buffer at 42 ºC for 2 h in a rotating oven. The 40 bp excised region of the gel was transferred to 10 ml of pre-hybridization buffer to form hybridization solution and heated at 100 ºC for 10 mins, and then cooled on ice. Membranes were hybridized with this hybridization solution at 42 ºC for 20 h.

Membranes were then subsequently washed twice each with wash buffer I at 25 ºC for 5 mins and wash buffer II at 68 ºC for 15 mins. After the washes, membranes were equilibrated with buffer I for 1 minute at 25 ºC. Membranes were then blocked with blocking solution for 30 mins at 25 ºC and then incubated in anti-DIG solution for 30 mins at 25 ºC. After washing twice in buffer I for 15 mins each at 25 ºC, membranes were equilibrated with detection buffer for 2 mins before finally being processed with CSPD solution. Blots were exposed to X-ray films for visual evaluation. Mutants that showed strong hybridization signals with the input pool probe and poor or no signals with that of the output pool were selected as candidates for further analysis.

4.4.3 Identification of transposon insertion sites and sequence analysis

Transposon insertion sites were amplified from the genomic DNA of selected mutants by arbitrary PCR using arbitrary primer IMT1118 in combination with a transposon-specific

primer IMT1048. In a second nested PCR 1 µl of the first PCR product was used as a template with primers IMT1119 and transposon I terminus-specific primer IMT1035.

Arbitrary PCR reaction mix:

10x Reaction Buffer	5 µl
dNTP mix (10 mM)	1 µl
IMT1118 (10 pmol)	2 µl
IMT1048 (10 pmol)	1 µl
Template	1 µl
Ampli *Taq* Gold Polymerase (5U/ µl)	0.25 µl
Deionized water	39.75 µl

Nested PCR reaction mix

10x Reaction Buffer	5 µl
dNTP mix (10 mM)	1 µl
IMT1119 (10 pmol)	1 µl
IMT1035 (10 pmol)	1 µl
Template	1 µl
Ampli Taq Gold Polymerase (5U/ µl)	0.25 µl
Deionized water	40.75 µl

Arbitrary PCR reaction

95 ºC	8 mins	1 x
95 ºC	45 s	
30 ºC	30 s	6 x
72 ºC	1.5 mins	(5 s/cycle more)
95 ºC	45 s	
45 ºC	30 s	30 x
72 ºC	2 mins	(5 s/cycle more)
72 ºC	10 mins	1 x

Nested PCR reaction

95 ºC	8 mins	1 x
95 ºC	45 s	
55 ºC	45 s	35 x
72 ºC	1.5 mins	(5 s/cycle more)
72 ºC	10 mins	1 x

PCR products from the second round PCR were sequenced commercially (LGC's AGOWA Genomics, Berlin, Germany). Sequenced data were analysed using DNA and protein databases (www.ncbi.nlm.nih.gov), making use of BLASTX and BLASTN algorithms (1).

4.5 *In vitro* and *in vivo* competition assays

In vitro and *in vivo* competition assays were performed by mixing cultures of mutant and wild type pathogen IMT5155 in a ratio of 1:1. For *in vitro* assays, the bacterial mixture was incubated in LB broth at 37 ºC under aeration. The optical density (OD_{600}) was measured every 30 mins for 6 h. Bacterial cultures were plated out onto LB plates with and without Kanamycin and Nalidixin at a time point of 4 h. As controls, IMT5155 and mutant cultures were grown separately in LB to compare growth kinetics. For *in vivo* assays, four chickens were infected intra-tracheally with 10^6 CFU/ml of IMT5155:mutant bacterial mixture. At 24 h post infection chickens were euthanized, lungs homogenized and dilutions plated onto LB plates with and without Kanamycin and Nalidixin for selection of mutant and wild type strain respectively. A competitive index (CI) was calculated by dividing the output ratio (mutant: wild type) by the input ratio (mutant: wild type) at 0 h and 4 h or 24 h for *in vitro* and *in vivo* assays respectively. For mutants tested *in vivo*, CI<1, CI<0.5 and CI<0.1 were evaluated as slightly, moderately and highly attenuated with respect to their colonization ability of the chicken lung respectively.

4.6 Characterization of the putative adhesin (Yqi)

4.6.1 Generation of a *yqi* isogenic mutant

4.6.1.1 Preparation of electro-competent cells

To prepare competent cells, a single colony of bacteria was inoculated in 5 ml LB for all strains and grown overnight at 37 ºC with aeration. Subsequently when preparing electro-competent cells from strain IMT5155 [pKD46], a single colony was inoculated in 5 ml LB containing 100 µg Ampicillin and 10 mM Arabinose and grown overnight at 28 ºC with aeration. Four millilitres of the overnight culture was then inoculated in fresh medium and bacteria were grown under the same conditions described above until an OD_{600}=0.6 was reached. Culture flasks were then chilled on ice, and bacterial culture was transferred into 50 ml centrifuge tubes, and centrifuged at 3000 x *g* for 15 mins at 4 ºC. The supernatant was discarded and the pellet was resuspended in 50 ml ice cold distilled water. Bacteria were centrifuged again under the same conditions and the pellet was resuspended in 25 ml ice cold distilled water. After a third round of similar centrifugation, the bacterial pellet was resuspended in 2 ml ice cold 10 % glycerol and centrifuged a final time at 6000 x *g* for 15

mins at 4 ºC. Bacterial pellet was resuspended in ca. 200 µl of 10 % glycerol, and stored immediately in 50 µl aliquots in eppendorf tubes at -70 ºC.

4.6.1.2 Transformation of competent E. coli

An aliquot of electro-competent cells was defrosted and 50 µl of the cell suspension was loaded in an eppendorf tube chilled on ice. One microlitre (30 ng/ µl) of plasmid or 3 µl (10 ng/ µl) of template DNA was added to the cell suspension, mixed well and kept on ice for a few minutes. The mixture was then added to a pre-chilled 2 mm cuvette carefully without touching the aluminium electrodes and the cuvette was transferred into the slot on the electroporation unit. A 2500 volt pulse was immediately triggered and 1 ml of SOC medium was quickly added to the cuvette, mixed well and transferred to an eppendorf tube. The eppendorf tube was placed in a thermomixer at 37 ºC for 1 h in case of normal transformations and aliquots were then plated on LB agar with appropriate antibiotics for selection of clones. In case of gene deletion mutagenesis, the eppendorf tube was placed in a thermomixer at 28 ºC for 1 h, after which an aliquot was plated out on LB agar with the appropriate antibiotic, while the remaining mixture was left under aeration conditions at R.T. overnight and then plated out on LB agar with the appropriate antibiotic. All plates were incubated at 37 ºC overnight. Single clones were selected to test for successful transformation.

4.6.1.3 Deletion of the yqi gene in IMT5155

To generate a knock-out mutant of the putative adhesin in IMT5155, the *yqi* gene was replaced with a Chloramphenicol resistance cassette using the lambda red recombinase system (20). The Chloramphenicol acetyl transferase (CAT) gene was amplified from plasmid pKD3 using PCR with primers IMT2510 and IMT2511 (Table 2), part of which has sequence similarity 40 bp upstream and downstream of the *yqi* gene. The PCR product was purified on a 1 % agarose gel and 2 µl of the sample was transformed by electroporation into IMT5155 containing the lambda red recombinase expression plasmid pKD46. After electroporation, samples were incubated at 28 ºC for 1h in SOC broth and plated on LB agar with Chloramphenicol to select for positive clones (CAT). After overnight incubation at 37 ºC, transformants were selected and tested for loss of the *yqi* gene with primer pairs IMT718/IMT2558 and IMT719/IMT2559 (Table 2) by colony PCR.

PCR reaction mix (pKD3):

10x Reaction buffer	5 µl
$MgCl_2$	2.5 µl
dNTP mix (10 mM)	0.5 µl
Primer pair IMT2510/IMT2511 (10 pmol)	1.5 µl

Taq polymerase (5U/ µl)	0.15 µl	
Template plasmid (pKD3)	2 µl (20 ng/ µl)	
Deionized water	38.35 µl	

Eight reactions prepared per template.

PCR reaction:

95 ºC	3 mins	1 x
95 ºC	30 s	
55 ºC	30 s	35 x
72 ºC	1 min 20 s	
72 ºC	10 mins	1 x

PCR reaction mix (clone selection):

10x Reaction buffer	2.5 µl
$MgCl_2$	1 µl
dNTP mix (10 mM)	0.5 µl
Primer IMT718 or IMT719 (10 pmol)	1 µl
Primer IMT2558 or IMT2559 (10 pmol)	1 µl
Taq polymerase (5U/ µl)	0.1 µl
Template	1 µl
Deionized water	18.9 µl

PCR reaction:

94 ºC	4 mins	1 x
94 ºC	30 s	
56 ºC	30 s	30 x
72 ºC	1 min	
72 ºC	10 mins	1 x

4.6.2 Complementation of *yqi* in the isogenic mutant

4.6.2.1 Cloning of yqi into pCR2.1 TOPO vector

For complementation studies the *yqi* (1050 bp) gene of APEC strain IMT5155 was amplified and the PCR product was cloned into pCR2.1 TOPO vector according to the standard TOPO cloning manual (Invitrogen GmbH, Karlsruhe, Germany) using primers IMT2910 and IMT2911 with restriction enzyme cutting sites *EcoRI* and *HindIII*, and transformed into

electro-competent *E. coli* TOP10 cells via electroporation as described in 3.6.1. Positive colonies were selected on LB agar with Kanamycin and tested for the presence of *yqi* using standard primers IMT1560 and IMT1561.

Cloning PCR reaction mix:

10x Reaction Buffer with $MgCl_2$	10 µl
dNTP mix (10 mM)	1 µl
Primer IMT2910 (10 pmol)	3.5 µl
Primer IMT2911 (10 pmol)	3.5 µl
Template	4 µl
Proof reading polymerase (PFU) (3U/ µl)	0.8 µl
Taq polymerase (5U/ µl)	0.4 µl
Deionized water	76.8 µl

Cloning PCR reaction:

94ºC	5 mins	1 x
94ºC	30 s	
62ºC	30 s	30 x
72ºC	1 min	
72ºC	7 mins	1 x

pCR2.1 TOPO cloning reaction:

PCR product	4 µl
Dilute salt solution	1 µl
pCR2.1 TOPO vector	0.5 µl

Mix gently and incubate at 25 ºC for 15 mins. Store overnight at -20 ºC.

4.6.2.2 Cloning of yqi into expression vector pDSK602

The plasmid pCR2.1-TOPO:*yqi* and expression vector pDSK602 were then digested with restriction enzymes *EcoRI* and *HindIII* for 1h at 37ºC on a sand bath, and ligated using T4 DNA ligase overnight at 4ºC.

Restriction digestion mix:

10x *EcoRI* buffer	4 µl
EcoRI (20U/ µl)	1 µl
HindIII (20U/ µl)	1 µl
Insert/Vector DNA (15ng/ µl)	10 µl

Deionized water	24 µl

Ligation mix:

Insert DNA	0.8 µl
Vector DNA	2.2 µl
T4 DNA ligase buffer	1 µl
Deionized water	5 µl
T4 DNA ligase (3U/ µl)	1 µl

Three microlitres of the ligation mix was then electroporated into electro-competent *E. coli* TOP10 cells as described in 4.6.1.2 and plated out on LB agar containing spectinomycin. Colonies were tested for the presence of *yqi* using PCR with primers IMT2910 and IMT2911.

Plasmid pDSK602:*yqi* was isolated from *E. coli* TOP10 and transformed into electro-competent IMT5155Δ*yqi* cells to complement the gene deleted and into IMT5155 to over-express the putative adhesin.

4.6.3 Cloning and sequencing of the putative Yqi adhesin operon in IMT5155

In order to sequence the *yqi* operon of strain IMT5155, the ~5 kb region was amplified using primers IMT3045 and IMT3046 and the PCR product was cloned into pCR2.1 TOPO vector according to the standard TOPO cloning manual (Invitrogen GmbH, Karlsruhe, Germany). The cloned product was transformed into *E. coli* TOP10 by electroporation and plated out on LB with Kanamycin to select for positive clones. Colonies were tested for the presence of *yqi* (5kb) insert using standard primers IMT1560 and IMT1561. The Modified plasmid containing the insert, pCR2.1 TOPO:*yqi*_operon, was isolated from the host strain using the High pure plasmid isolation kit, (Roche Diagnostics GmbH, Mannheim, Germany), and sequenced commercially by LGC's AGOWA Genomics, Berlin, Germany.

Cloning PCR reaction mix:

10x Reaction buffer with $MgCl_2$	5 µl
dNTP mix (10 mM)	0.5 µl
Primer IMT3045 (10 pmol)	2 µl
Primer IMT3046 (10 pmol)	2 µl
Proof reading polymerase (PFU) (3U/ µl)	0.6 µl
Taq polymerase (5U/ µl)	0.3 µl

Cloning PCR reaction :

94 ºC	5 mins	1 x
94 ºC	30 s	
63 ºC	30 s	30 x
72 ºC	4 mins	
72 ºC	7 mins	1 x

pCR2.1 TOPO cloning reaction:

PCR product	4 µl
Dilute salt solution	1 µl
pCR2.1 TOPO vector	1 µl

Mix gently and incubate at 25 ºC for 15 mins. Store overnight at -20 ºC.

4.6.4 Prevalence of the *yqi* gene among ExPEC, Intestinal pathogenic *E. coli* (IPEC) and commensals (A_{faecal} strains) using PCR

To determine the prevalence of *yqi* among *E. coli* strains, 406 avian pathogenic *E. coli* (APEC) strains, 138 uropathogenic *E. coli* (UPEC), 25 Newborn meningitic *E. coli* (NMEC), 19 Septicaemia associated *E. coli* (SePEC), 153 intestinal pathogenic *E. coli* (IPEC) and 159 faecal strains from clinically healthy chickens (A_{faecal}) were tested for the presence of the *yqi* gene using standard PCR reactions with primers IMT2512 and IMT2513 by way of amplification of a 400 bp region of the adhesin gene as described under PCR analyses. IMT5155 was used as a positive control, and IMT11327 as a negative control for all PCR reactions. Results were observed as a single clear band on an agarose gel and recorded as positive and negative for all strains respectively.

PCR reaction mix:

10x Reaction buffer	2.5 µl
$MgCl_2$	1 µl
dNTP mix (10 mM)	0.5 µl
Primer IMT2512 (10 pmol)	1 µl
Primer IMT2513 (10 pmol)	1 µl
Taq polymerase (5U/ µl)	0.1 µl
Template (20 ng/ µl)	3 µl
Deionized water	16.9 µl

PCR reaction:

94 ºC	5 mins	1 x
94 ºC	30 s	
60 ºC	30 s	30 x
72 ºC	1 min	
72 ºC	7 mins	1 x

4.6.5 Sequencing of the *yqi* gene and evolutionary analysis

A total of 77 strains representing multi locus sequence types (MLST) ST12, ST73, ST95, ST104, ST135, ST140, ST141, ST358, ST363, ST368, ST372, ST390, ST416, ST417 and ST421 were selected for sequencing of the *yqi* gene (1050bp), from a previous study at the Institute of Microbiology and Epizootics involving MLST analysis, using primer pairs IMT3706/IMT3707 and IMT2512/IMT2559. The PCR products were sequenced commercially by LGC's AGOWA Genomics, Berlin, such that a double stranded sequence was obtained for each strain. Sequences were analysed using Kodon software available from Applied Maths. A phylogenetic tree showing distances between strains of different STs was calculated by a maximum parsimony algorithm using Kodon Software from Applied Maths. The adhesin gene sequence of the strains APEC_O1 (APEC) (Accession: CP000468), CFT073 (UPEC) (Accession: AE014075), UTI89 (UPEC) (CP000243) was obtained from the available nucleotide database, and compared with sequenced strains in this study. Rates of non-synonymous (Dn) and synonymous (Ds) mutation were calculated using DnaSP 4.50.3 software (152) for the sequenced adhesin gene among strains belonging to the ST95 complex in order to determine the Dn/Ds ratio for each locus as described previously (171).

PCR reaction mix:

10x Reaction buffer	2.5 µl
$MgCl_2$	1 µl
dNTP mix (10 mM)	0.5 µl
Primer IMT2512/IMT3706 (10 pmol)	1 µl
Primer IMT2559/IMT3707 (10 pmol)	1 µl
Taq polymerase (5U/ µl)	0.1 µl
Template (20 ng/ µl)	3 µl
Deionized water	16.9 µl

PCR reaction:

94 ºC	5 mins	1 x
94 ºC	30 s	

56 ºC	30 s	30 x
72 ºC	1 min	
72 ºC	7 mins	1 x

4.6.6 Adhesion assays using Chicken Fibroblasts and polarized Madin-Darby-Canine-Kidney (MDCK-1) epithelial cells *in vitro*

4.6.6.1 Cultivation and passage of Fibroblasts and Epithelial cells

Chicken fibroblast (CEC) cells were used between passages 6 and 9 and were seeded into 12-well microtitre plates at a density of ~2 x 10^5 cells per well and incubated at 37 ºC in 5 % CO_2 without antibiotics prior to adhesion assays. Minimal essential cell culture medium (Pan^{TM} Biotech GmbH, Aidenbach, Germany) with 5 % Foetal calf serum (FCS) (Pan^{TM} Biotech GmbH, Aidenbach, Germany) was used to grow cells. Monolayers were used after 6 days incubation.

Madin Darby Canine Kidney (MDCK-1) cells, kindly provided by Alexander Karlas, MPI-IB, Berlin, Germany, were used between passages 1 and 5. Cells were grown in Dulbecco's modified eagle's medium (DMEM) (Pan^{TM} Biotech GmbH, Aidenbach, Germany) with 10 % FCS and were incubated at 37 ºC in 5 % CO_2. Transwell filter units (Costar) (Sigma-Aldrich Chemie GmbH, Munich, Germany) contained a 1.12 cm^2 porous filter membrane (0.4-µm pores) that had been treated for tissue culture. Filter units were incubated in 12-well microtitre plates (Costar) and were placed in DMEM containing 10% FCS for 1 h, at 37 ºC before seeding. Forty microlitres of a trypsinized MDCK cell suspension was added to each Transwell unit. Monolayers were used after four days incubation at which time there were around ~3 x 10^5 MDCK cells per filter.

To passage cells, the cell culture flask was removed from the incubator, and the entire medium was sucked out using a sterile pasteur pipette attached to a vacuum pump. The cell monolayer was then washed once with sterile PBS. Trypsin- EDTA solution was added to the monolayer and the culture flask was incubated at 37 ºC for 15 mins. Cells loosened in trypsin-EDTA were then transferred to a 15 ml centrifuge tube containing 7 ml fresh medium and centrifuged for 10 mins at 1000 x *g*. The supernatant was discarded and the cell pellet resuspended in 5 ml fresh warm medium. Around 30-50 µl of this suspension were added to 1 ml or 500 µl medium in each respective well of a 12-well cell culture plate and incubated at 37 ºC in 5 % CO_2 until a confluent monolayer was seen under the microscope.

4.6.6.2 Adhesion assays

Bacteria were added to the appropriate wells in triplicate in medium without FCS at an MOI of 100, that is, ~2-3 x 10^7 bacteria per well. Microtitre plates were centrifuged at 250 x *g* for 10 mins, and then incubated for 1.5 h and 3 h for CEC cells, and 3 h for MDCK cells, after which the supernatant was discarded; cells were washed thrice with 1 ml Phosphate buffered saline (PBS) and then plated out on LB agar to determine the number of adherent bacteria in each well.

4.6.7 Colonization assays of the chicken lung *in vivo*

4.6.7.1 Lung colonization model of infection

Groups of six 5-week-old chickens were infected with the virulent APEC strain IMT5155 and the non virulent isolate IMT11327, as positive and negative controls respectively. Chickens were inoculated intra-tracheally with 0.5 ml of 10^6 CFU / ml bacterial suspension, the dose chosen for successful lung colonization by APEC at 24 h post infection. Bacterial inoculum was prepared as described in 4.2.1. After 24 h of infection, chickens were euthanized and clinical scores were recorded according to table 4, while organ lesion scores were noted according to the classification in table 5. Bacteria were reisolated from the lung and spleen as follows. Tissue samples of the organs affected in a systemic infection, were weighed, suspended in phosphate-buffered saline (1 ml/g), and homogenized with an Ultra-Turrax apparatus. Serial 10-fold dilutions were plated out onto LB agar that was subsequently incubated at 37 °C for 24 h. Colonies were counted after 24 h incubation to determine the CFU per gram in each organ.

4.6.7.2 Systemic infection model

In this previously established infection model (3), groups of six chickens were infected with strains or mutants to be tested in order to determine to what extent a systemic infection could be caused by such test strains. The control strains used are described in 3.1. Birds were inoculated intra-tracheally with 0.5 ml of a bacterial suspension containing 10^9 CFU. Birds were monitored for 24 h and lethality rates were recorded. At 24 h post infection, chickens were euthanized and clinical scores and organ lesion scores were recorded as described in tables 4 and 5. Bacteria were reisolated from the lungs, heart, liver, kidneys, spleen and brain as described in 3.1. Bacterial loads were determined in each of the internal organs and an average from all chickens in the group was calculated.

4.6.8 Expression of the Yqi protein *in vitro*

4.6.8.1 Cloning of yqi into expression vector pGEX-6P-1

To express the *yqi* gene, a glutathione-S-transferase (GST) fusion protein was constructed by inserting the *yqi* gene into the multiple cloning site of a pGEX-6P-1 vector whose expression is under the control of the *tac* promoter (70). Briefly, the pCR2.1 TOPO:*yqi* and expression vector pGEX-6P-1 were digested with restriction enzyme *EcoRI* for 1 h at 37 ºC on a sand bath, and ligated using T4 DNA ligase overnight at 4 ºC.

Restriction digestion mix:

EcoRI Buffer	4 µl
Deionized water	28 µl
Insert/ Vector DNA	7 µl
EcoRI (20U/ µl)	1 µl

Ligation mix:

Insert DNA	1 µl
Vector DNA	0.5 µl
T4 DNA ligase buffer	1 µl
T4 DNA ligase (3U/ µl)	1 µl
Deionized water	6.5 µl

Three microlitres of the ligation mix were then electroporated into electro-competent *E. coli* TOP10 cells via electroporation as described in 4.6.1.2 and plated out on LB agar containing Kanamycin. Colonies were tested for the presence of *yqi* using PCR with primers IMT908 and IMT909 specific to vector pGEX-6P-1. In order to determine the directionality of gene *yqi* in the expression vector, plasmid pGEX:*yqi* was digested with restriction enzymes and incubated at 37 ºC for 1 h.

Restriction digestion mix I:

Enzyme buffer	4 µl
DNA	5 µl
BamHI (10U/ µl)	1 µl
HindIII (20U/ µl)	1 µl
BSA	0.4 µl
Deionized water	28.6 µl

Restriction digestion mix II:

Enzyme buffer	4 µl
DNA	5 µl
XhoI (20U/ µl)	1 µl
HindIII (20U/ µl)	1 µl
BSA	0.4 µl
Deionized water	28.6 µl

The presence of a 1050 bp band as seen by gel electrophoresis when digested with *BamHI* and *HindIII*, would suggest that gene *yqi* was in frame in vector pGEX-6P-1.

Plasmid pGEX:*yqi* was isolated from *E. coli* TOP10 and transformed into electro-competent *E. coli* BL21 for optimal expression of the fusion protein.

4.6.8.2 SDS-PAGE

SDS - polyacrylamide gel electrophoresis (PAGE) achieves separation of proteins, whereby migration of the proteins is determined not by intrinsic electric charge of the polypeptides, rather by their molecular weight. Sodium dodecyl sulphate (SDS) is an anionic agent that denatures proteins by wrapping the hydrophobic tail around the polypeptide backbone, thus conferring a net negative charge to the polypeptide in proportion to its length. Since molecular weight is essentially a linear function of the peptide chain length, the proteins separate by molecular weight (147).

SDS-PAGE was performed as described by Laemmli (95). Overnight bacterial cultures were diluted in LB broth to an OD_{600}=0.2. Bacteria were then grown at 37 ºC under aeration until they reached an optical density at 600 nm of 0.6. IPTG was added to the growing culture to a final concentration of 1mM and induction was allowed to take place for 1.5 h. Bacterial cultures without IPTG induction served as controls for *yqi* expression. After induction 1ml of bacterial culture was transferred to eppendorf tubes and centrifuged at 6000 x *g* for 2 mins. The supernatant was discarded and 100 µl of 2x Laemmli buffer (SDS gel loading buffer) was added to the pellet to give whole cell lysate proteins. After vortexing for a few seconds, samples were boiled at 95 ºC for 5 mins, centrifuged and kept on ice. Ten microlitres of the supernatant was loaded onto a 13.5 % SDS gel and the proteins in the samples were separated for 2 h at 100 -120 V. An all blue protein standard (Biorad GmbH, Munich, Germany) served as the protein marker.

4.6.8.3 Western blotting

The proteins were transferred to a nitrocellulose membrane by blotting with the BIO-RAD Tank Transfer system for 1 h at 100 V in transfer buffer. The blot was then blocked with 5 % skimmed milk in wash buffer (TBST). Anti-GST (GE Healthcare Europe GmbH, Munich,

Germany) was the primary antibody used to identify the GST:*yqi* fusion protein in a dilution of 1:5000 in TBST. The blot was then washed three times with TBST and a horseradish peroxidase-conjugated secondary antibody Anti-IgG (Sigma-Aldrich Chemie GmbH, Munich, Germany) against goat immunoglobulins was used as the secondary antibody at a dilution of 1:10000 in TBST. The blot was developed in the dark using enhanced chemiluminescence (ECL) reagent as per standard procedures.

4.6.8.4 Silver staining

In order to view proteins on the SDS gel, the gel was transferred into a chamber for staining purposes. First the gel was treated with 125 ml of fixative at 4 ºC overnight. After decanting the fixative, the gel was sensitized with 125 ml of sensitizing solution for 30 mins at room temperature. The gel was then washed thrice with distilled water for 5 mins in each round. Staining of the gel was then carried out with silver stain for 20 mins at room temperature after which the gel was once again washed twice for 1 min in each round. In order to develop the stain, developing solution was added to the gel for about 2 to 5 mins until clear protein bands were seen. The developing solution was then immediately decanted and stop solution was added for 10 mins at room temperature. A final washing of the gel was carried out with distilled water for 5 mins 3 times after which the gel was transferred into preserve solution for about 20 to 30 mins before scanning.

4.6.9 Purification of the Yqi adhesin

Purification of the Yqi protein was achieved by using Glutathione Sepharose 4B beads (GE Healthcare Europe GmbH, Munich, Germany) designed for rapid single step purification of recombinant derivatives of glutathione S-transferase proteins. Briefly a 50 % glutathione sepharose (GST) 4B slurry was prepared by equilibrating the beads with 1x PBS. *E. coli* BL21 containing the fusion protein on an expression plasmid, was grown in 200 ml LB medium until an OD_{600}=0.6, at which IPTG was added to the culture broth to a final concentration of 1mM to induce expression of the protein. Induction was carried out for 2 h at 37 ºC, after which the bacteria were harvested at 6000 x *g* for 5 mins.

The bacterial pellet was re-suspended in 10 ml of ice cold 1x PBS and lysozyme (Serva electrophoresis GmbH, Heidelberg, Germany) was added to a final concentration of 1 mg/ml and the suspension was incubated on ice for 30 mins. Ten millilitres of 0.2% Triton X-100 was added to the suspension and mixed well. DNase (Serva electrophoresis GmbH, Heidelberg, Germany) and RNase (Carl Roth GmbH, Karlsruhe, Germany) were added to a final concentration of 5 µg/ml and incubated for 10 mins at 4 ºC, after which the suspension was centrifuged at 3000 x *g* for 30 mins at 4 ºC. The supernatant was transferred to a fresh tube and dithiothreitol (DTT) (Biorad GmbH, Munich, Germany) was added to a final concentration

of 1 mM. 400 µl of the 50 % GST slurry was added to the clear supernatant and allowed to bind to the protein for 1h at room temperature with aeration.

The suspension was centrifuged at 500 x *g* for 5 mins at 4 ºC and the pellet (beads) was washed thrice with ice cold 1x PBS. The fusion protein was eluted with 200 µl elution buffer (reduced glutathione) for 10 mins at room temperature. After a final round of centrifugation, the supernatant was transferred to a fresh tube and samples were prepared with Laemmli buffer as described for SDS-PAGE. Samples were collected at different points during the purification procedure to confirm the presence of expressed protein in original bacterial lysates.

4.6.10 Over-expression of the 4975bp *yqi* adhesin gene cluster in fimbrial negative *E. coli* strain AAEC189

4.6.10.1 Cloning of the 4975bp yqi adhesin gene cluster into pCR2.1 TOPO vector

The 4975bp *yqi* adhesin gene cluster was amplified and the PCR product was cloned into pCR2.1 vector according to the standard TOPO cloning manual (Invitrogen GmbH, Karlsruhe, Germany) using primers IMT3259 and IMT3260 with restriction enzyme cutting sites *XbaI* and *BamHI*, and transformed into electro-competent *E. coli* TOP10 cells via electroporation as described in 3.6.1. Positive clones were selected on LB agar with Kanamycin and tested for the presence of the 4975bp gene cluster using standard primers IMT1560 and IMT1561.

Cloning PCR reaction mix:

10x Reaction Buffer	10 µl
dNTP mix (10 mM)	1 µl
Primer IMT3259 (10 pmol)	3.5 µl
Primer IMT3260 (10 pmol)	3.5 µl
Template (20 ng/ µl)	4 µl
Proof reading polymerase (PFU) (3U/ µl)	0.8 µl
Taq polymerase (5U/ µl)	0.4 µl
Deionized water	76.8 µl

Cloning PCR reaction:

94 ºC	5 mins	1 x
94 ºC	30 s	
58 ºC	30 s	30 x
72 ºC	4 mins	

72 ºC	7 mins	1 x

pCR2.1 TOPO cloning reaction:

PCR product	4 µl
Dilute salt solution	1 µl
pCR2.1 TOPO vector	0.5 µl

Mix gently and incubate at 25 ºC for 15 mins. Store overnight at -20 ºC.

4.6.10.2 Cloning of the yqi adhesin gene cluster into expression vector pKESK-22

The plasmid pCR2.1-TOPO:*yqi*_4975_XB and expression vector pKESK-22 were digested with restriction enzymes *XbaI* and *BamHI* for 1 h at 37 ºC on a sand bath, and ligated using T4 DNA ligase overnight at 4 ºC.

Restriction digestion mix:

10x Buffer 2	4 µl
BSA	0.4 µl
XbaI (12U/ µl)	1 µl
BamHI (10U/ µl)	1 µl
Insert/Vector DNA	6 µl
Deionized water	27.6 µl

Ligation mix:

Insert DNA	3 µl
Vector DNA	1 µl
T4 DNA ligase buffer	1 µl
Deionized water	4 µl
T4 DNA ligase (3U/ µl)	1 µl

Three microlitres of the ligation mix were then electroporated into electro-competent *E.coli* TOP10 cells as described in 4.6.1.3 and plated out on LB agar containing spectinomycin. Colonies were tested for the presence of the 4975 bp *yqi* adhesin gene cluster using PCR with primers IMT3138 and IMT3139.

Plasmid pKESK:*yqi*_4975_XB was isolated from *E. coli* TOP10 and transformed into electro-competent *E. coli* AAEC189 (afimbriate *E. coli* strain) cells, kindly provided by Ulrich Dobrindt, University of Würzburg, to over-express the adhesin gene cluster.

4.6.11 Electron Microscopy

E. coli strains AAEC189, AAEC189 (pKESK:*yqi*_4975_XB) and IMT5155 were grown in 5 ml Brain Heart infusion broth after inoculating with 150 µl of the respective overnight cultures. Strains AAEC189 and IMT5155 were used as negative and positive controls respectively. Strain AAEC189 (pKESK:*yqi*_4975_XB) was additionally given Kanamycin in its growth medium, and induced with 1mM IPTG after 30 minutes of growth. All three strains were grown to an OD_{600} of 2.5, allowing an induction time of 2.5 h for strain AAEC189 (pKESK:*yqi*_4975_XB). One millilitre of bacterial culture was centrifuged at 8000 x *g* and the bacterial pellet was washed thrice with 1x PBS and then resuspended in 500 µl 1x PBS. Twenty microlitres of bacterial suspension were applied to Formvar-coated 200- mesh copper grids and stained for 2 mins with 1 % Uranyl acetate. Negatively stained preparations were examined on a Zeiss EM900 microscope. Electron microscopic photographs were taken through the kind favour of Dr. Wilfried Bleiß, Department of Molecular Parasitology, Humboldt Universität zu Berlin.

4.7 Statistical analysis

All statistical analysis for *in vivo* animal experiments and *in vitro* cell culture experiments were carried out using the software SPSS (Statistical package for the social sciences), version 15.0 by carrying out the non-parametric Mann-Whitney U-Test and the students *t*-test at the 95% significance level ($p<0.05$).

5 Results

5.1 Generation of an IMT5155 (APEC O2:K1:H5) mutant library

A total of 90 uniquely tagged transposons were selected from a master pool of signature-tagged transposons, and used to construct the mutant library. The library was constructed by independent conjugation experiments using each of the 90 transposons, and the mutants generated were stored and arrayed in microtitre plates according to the tags they carried. A total of 1800 mutants were arrayed in 20 pools each containing 90 mutants. In order to confirm the insertion of the mini-Tn5*Km2* transposon in the IMT5155 genome, PCR was carried out in 20 random mutants after every 10th conjugation, using the kanamycin cassette primers as described in materials and methods and was confirmed by the presence of a 600 bp band in all samples (Fig. 5A). Southern blot analysis carried out with random mutants from a single conjugation experiment using the kanamycin-resistant cassette as a probe, revealed that all mutants tested were found to have a single transposon insertion at a unique site in the chromosome (Fig. 5B)

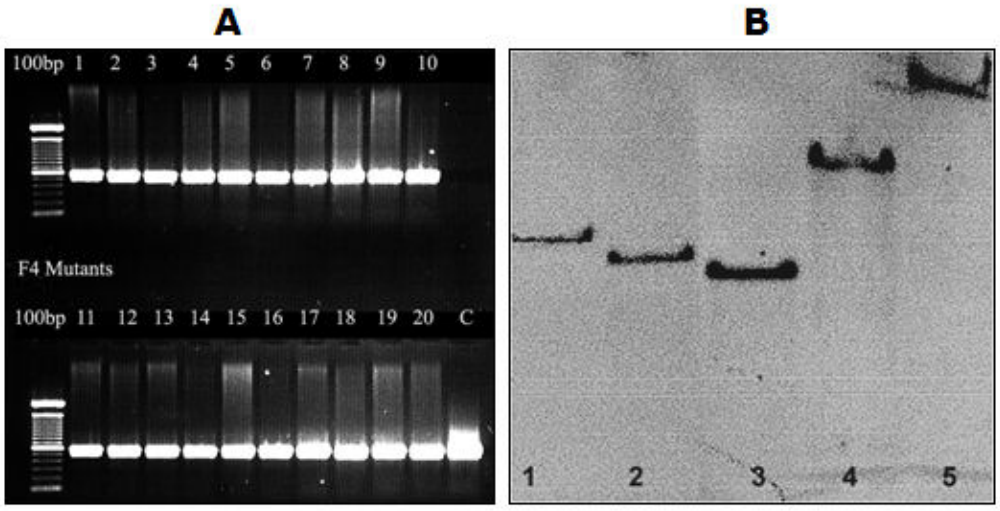


Figure 5: A) Photograph of agarose gel electrophoresis depicting IMT5155 transposon mutants with signature-tag F4 which are positive for the kanamycin cassette gene (600 bp). A 100 bp DNA ladder is loaded in the first slot. B) Autoradiograph depicting IMT5155 transposon mutants after souther blotting with DIG-labelled Kanamycin gene probe. The target gene kan is present at different positions in the genome of mutants tested.

5.2 Establishment of a lung colonization model of infection

A lung model of infection was effectively set up - modified from a previously established systemic infection model - for further STM screening studies. Ten chickens each were infected with the wild type pathogenic strain IMT5155, while only 5 chickens were infected

with the negative control strain IMT11327. Due to the low doses of infection that dominated this infection model, the chickens did not show any noticeable disease symptoms. At 24 h post infection, wild type strain IMT5155 was isolated from the lung in almost all chickens for all of the four doses in decreasing numbers (Fig. 6A). It was possible to isolate bacteria from the spleen in negligible amounts only with an infection dose of 10^6 CFU but these values do not correspond with the values of a standardized experimental systemic infection in chicken as was recently shown (3). Viable bacterial counts were significantly reduced in the lungs 48 h post infection up to 30 fold, while no bacteria were isolated from the spleen at lower infection doses.

When chickens were infected with non-pathogenic strain IMT11327, bacteria were isolated from the lungs 24 h post infection in reduced amounts as compared to the wild type pathogen, whereas no bacteria were isolated from the spleen. Results were reproducible for the pathogenic strain in 3 consecutive experiments and could be well compared with the non-pathogenic strain. IMT11327 was found to colonize the lung to a much lesser extent than IMT5155 accounting for more than a 100-fold decrease in colonization (Fig. 6B).

On examination of internal organs, lesions were observed on the lungs covering about two fifths of the organ. This is the first stage in the pathology of the disease. No other organs showed any pathomorphological signs of infection. A maximum lung organ score of 2 (Fig. 7) and thoracic air sac score of 1 was determined for 95 percent of the 20 chickens infected. The lung lesion scores corresponded to the bacterial numbers isolated from the infected organ. The spleen of infected chickens did not show any major clinical changes, aside from a mild swelling in a few cases.

For a successful STM analysis a re-isolated bacterial load of 10^5 CFU is necessary in order to heighten the chances that all mutants in a single pool are recovered after infection. Therefore, an infection dose of 10^6 CFU and a time point of 24h were selected for further infection studies, since this dose accomplished the requirements for STM screening.

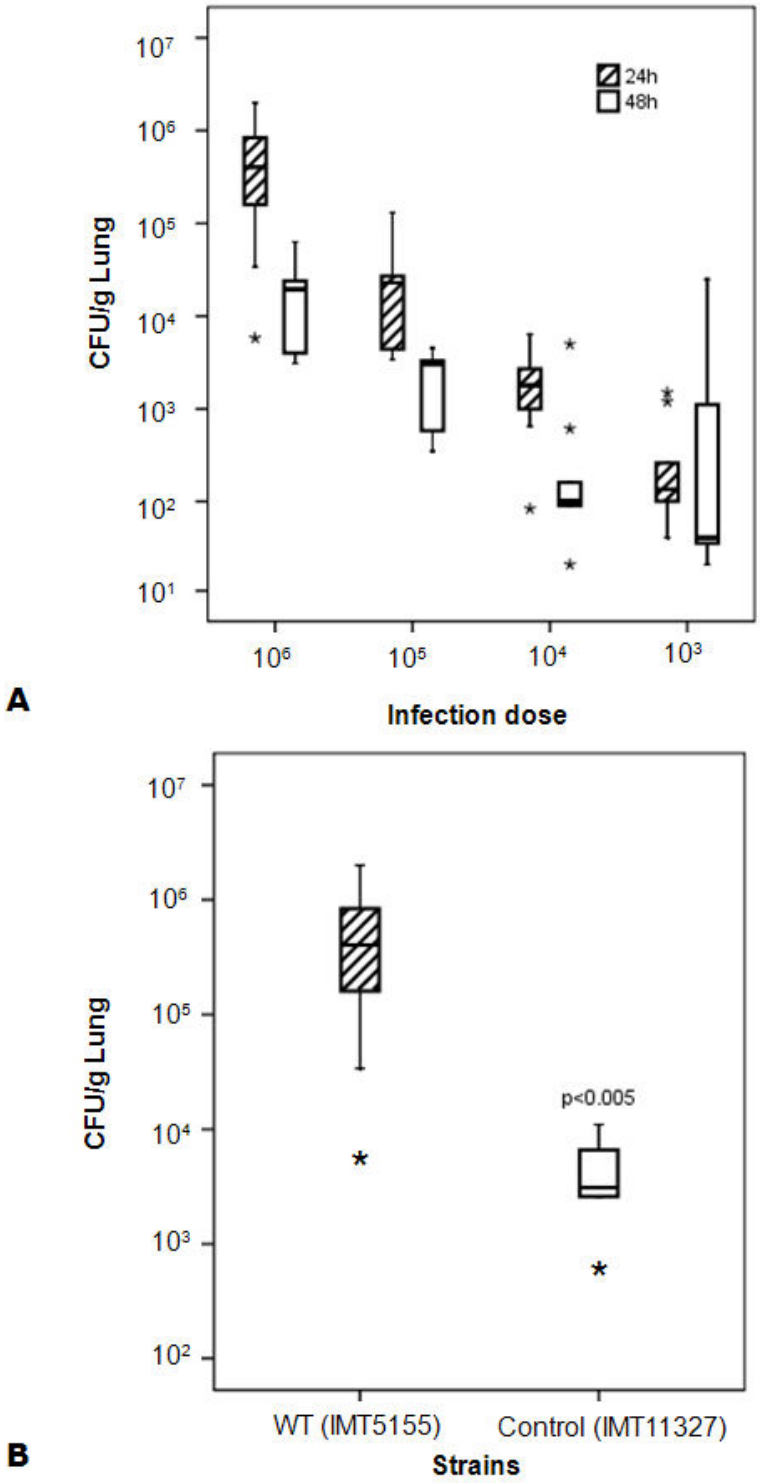


Figure 6: A) Bacterial re-isolation from the chicken lungs 24 h and 48 h after infection with IMT5155 at various infection doses measured in colony forming units (CFU) per gram organ (n=10 chickens; * = Values not included in statistical analysis). B) Bacterial re-isolation from the chicken lungs 24 h after infection with IMT5155 and IMT11327 with infection dose 106 CFU bacteria (IMT5155, n=10 chickens; IMT11327, n=5 chickens; * = Values not included in statistical analysis).

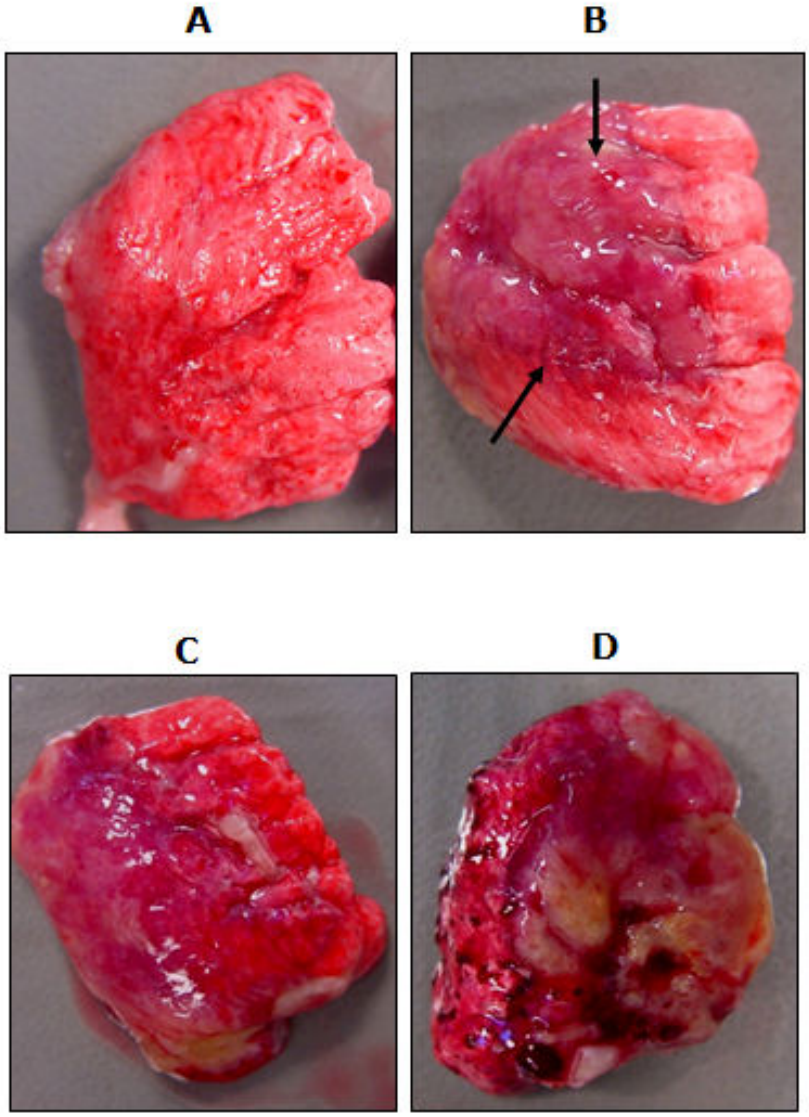


Figure 7: Photograph of chicken lung showing pathomorphological changes 24h after infection with IMT5155; A maximum lung lesion score of 2 was observed with infection dose 10^6 CFU/ml (A: Lung score 0, B: Lung score 2, C: Lung score 3, D: Lung score 4).

5.3 Identification of APEC genes required for the colonization of the chicken lung

After the chicken lung infection model had been established, twenty pools of IMT5155 STM-mutants were screened in this model with an infection dose of 10^6 CFU. At 24h post infection, bacteria were re-isolated from the chicken lungs and colonies were pooled together in order to extract genomic DNA to form the “Input pool” and the “Output pool”. Labelled probes were prepared from both pools to hybridize against original membranes. Mutants that showed hybridization signals with the probe from the input pool, but weaker or completely absent signals with that of the output pool were selected for further analysis. A representative pool is shown below (Fig. 8).

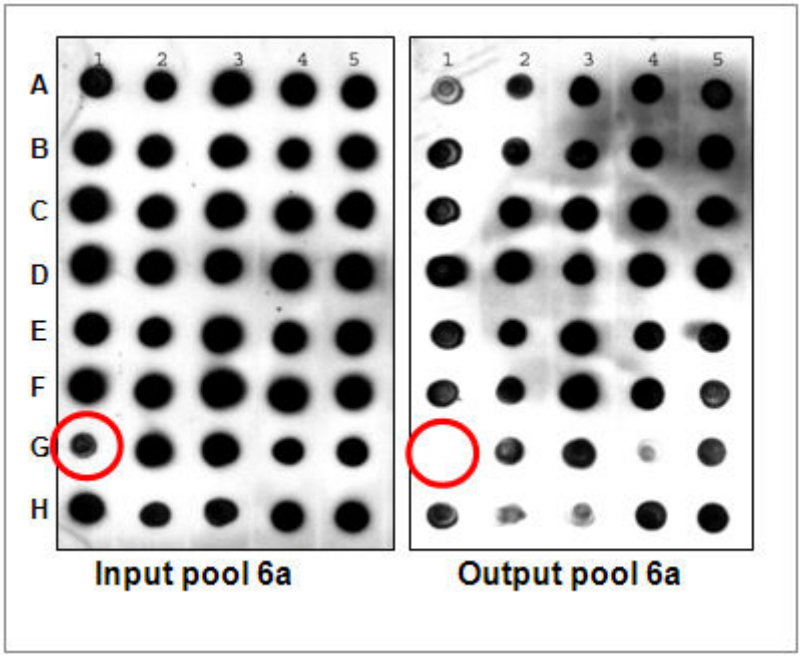


Figure 8: Representative autoradiograph of dot blots showing input pool 6a and output pool 6a. Mutant with tag G1 was selected as a candidate for arbitrary and nested PCR to identify gene disrupted by signature-tag.

After this first round of experiments a total of 38 mutants were selected for further analysis after screening 20 pools of mutants. Arbitrarily primed PCR was performed to determine the insertion sites of the mini transposon in the 38 selected IMT5155 mutants by amplifying their flanking DNA regions. Amplified DNA products ranged between 150 and 740 bp (Fig. 9). All amplified products were commercially sequenced and analysed using BLAST algorithms, whereby hits with the highest score and lowest *e* values were recorded (Table 7). All gene sequences were found to be different in mutants tested, that is, no gene was identified twice.

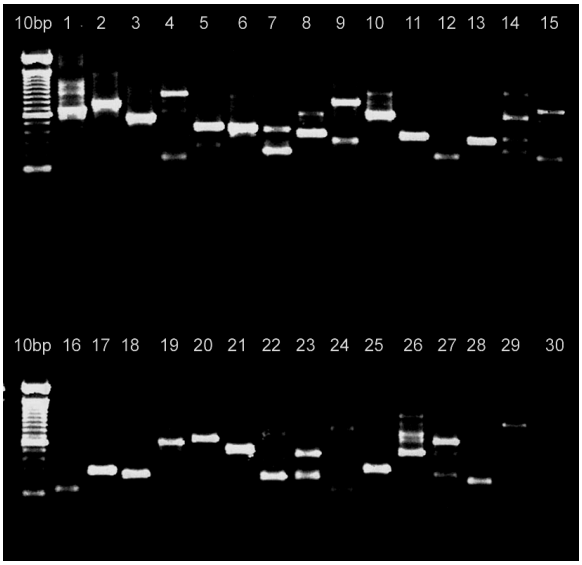


Figure 9: Photograph of agarose gel electrophoresis after arbitrary PCR of candidate mutants to identify genes disrupted by signature-tag. Most PCR products showed a single band on the agarose gel which were then sequenced.

Table 7: Identification of 38 mutants which were found to be attenuated after an initial screening of the mutant library in the chicken lung infection model 24h post infection with 20 mutant pools using arbitrary and nested PCR and BLAST algorithms

Functional class	Mutants	Gene name	Percentage of Identity	Accession Number
Adhesins	EA7F9	*APECO1_3367*	99%	CP000468.1
	EA7A2	*fimE*	97%	CP000468.1
Regulators	EA1H4	*leuO*	99%	CP000468.1
	EA16H4	*tdcA*	100%	CP000468.1
	EA7F8	*evgA*	100%	CP000468.1
LPS/ Capsule	EA16G1	*kpsF*	99%	CP000468.1
	EA10C1	*rfaJ*	98%	CP000468.1
	EA1A1	*YaiP*	99%	CP000468.1
Transport	EA1C4	*actP*	100%	AP009048.1
	EA7B2	*xapB*	100%	CP000468.1
	EA10F11	*pgtP*	99%	AJ617685.1
	EA11E1	*rbsA*	100%	CP000468.1
Metabolic Enzymes	EA1C2	*ycgC*	100%	CP000468.1
	EA7D8	*APECO1_2526*	100%	CP000468.1
	EA9G3	*ygiK*	99%	CP000468.1
	EA10G10	*hsdM*	100%	CP000468.1
	EA16H5	*yfiF*	99%	CP000468.1

	EA17F7	*carB*	99%	CP000468.1
	EA17H4	*treF*	100%	CP000468.1
	EA6G1	*APECO1_265*	98%	CP000468.1
	EA11B3	*metH*	99%	CP000468.1
Other	EA1B2	*purE*	99%	CP000468.1
	EA7A4	*ybcL*	100%	CP000468.1
	EA16H1	*iap*	100%	CP000468.1
	EA17H1	*APECO1_2450*	98%	CP000468.1
	EA18E1	*ECP_3411*	100%	CP000247.1
	EA18F1	*APECO1_2069*	100%	CP000468.1
	EA18G1	*bglF*	100%	CP000468.1
	EA17H5	*finO*	99%	DQ381420.1
Putative/Unknown	EA7A3	*yobB*	99%	CP000468.1
	EA7B4	*c5296*	99%	AE014075.1
	EA8B1	*c1248*	98%	AE014075.1
	EA9F4	*yjcS*	100%	CP000468.1
	EA10G11	*ydeH*	100%	CP000468.1
	EA2E10	*yabP*	98%	AP009048.1
	EA4H3	*APECO1_3370*	99%	CP000468.1
	EA1B12	*APECO1_1713*	100%	CP000468.1
	EA1A9	*ykgH*	100%	AP009048.1

CP000468: *Escherichia coli* APEC01, complete genome; Johnson et al (65)
CP000247: *Escherichia coli* 536, complete genome; Hochhut et al (54)
AP009048: *Escherichia coli* W3110 DNA, complete genome; Musso et al (123)
AJ617685: *Escherichia coli* pathogenicity island V, strain 536; Schneider et al (156)
DQ381420: *Escherichia coli* APEC01 plasmid pAPEC-01-ColBM, complete sequence; Johnson et al (64)
AE014075: *Escherichia coli* CFT073, complete genome; Welch et al (172)

In vitro and *in vivo* competition assays were performed on 20 mutants, representing each functional class, listed in table 7, to determine their growth rate in Luria-Bertani (LB) growth medium and to confirm the degree of attenuation of the mutant in chickens as described in materials and methods. The following 20 mutants were tested against wild type strain IMT5155: Adhesins EA7F9 (*APECO1_3367 annotated as a putative yqi fimbrial adhesin in the public database, hence the gene was named yqi*) and EA7A2 (*fimE*); Regulators EA1H4 (*leuO*), EA16H4 (*tdcA*), EA7F8 (*evgA*); LPS/capsule genes EA16G1 (*kpsF*), EA10C1 (*rfaJ*), EA1A1 (*yaiP*); transporters EA1C4 (*actP*), EA7B2 (*xapB*), EA10F11 (*pgt*P), EA11E1 (*rbsA*); metabolic genes EA1C2 (*ycgC*), EA16H5 (*yfiF*), EA17F7 (*carB*), EA1B2 (*purE*), EA18G1 (*bglF*); unknown genes EA7A3 (*yobB*), EA10G11 (*ydeH*), EA1A9 (*ykgH*). A competitive index (CI) was calculated for each mutant against IMT5155. Growth kinetics of all 20 mutants in LB medium was essentially similar, in that, no significant growth defect was observed *in vitro*. Figure 10 represents growth patterns of mutants EA7F9, EA7A2 and EA1A1 versus wild type strain IMT5155. Out of 20 mutants tested *in vivo*, 16 (80%) were out-competed by the wild type IMT5155 as seen in table 8. After comparison with *in vitro* competition data of the mutants, 11 mutants (55%) were confirmed to be attenuated in the chicken infection model, which are highlighted in bold in table 8.

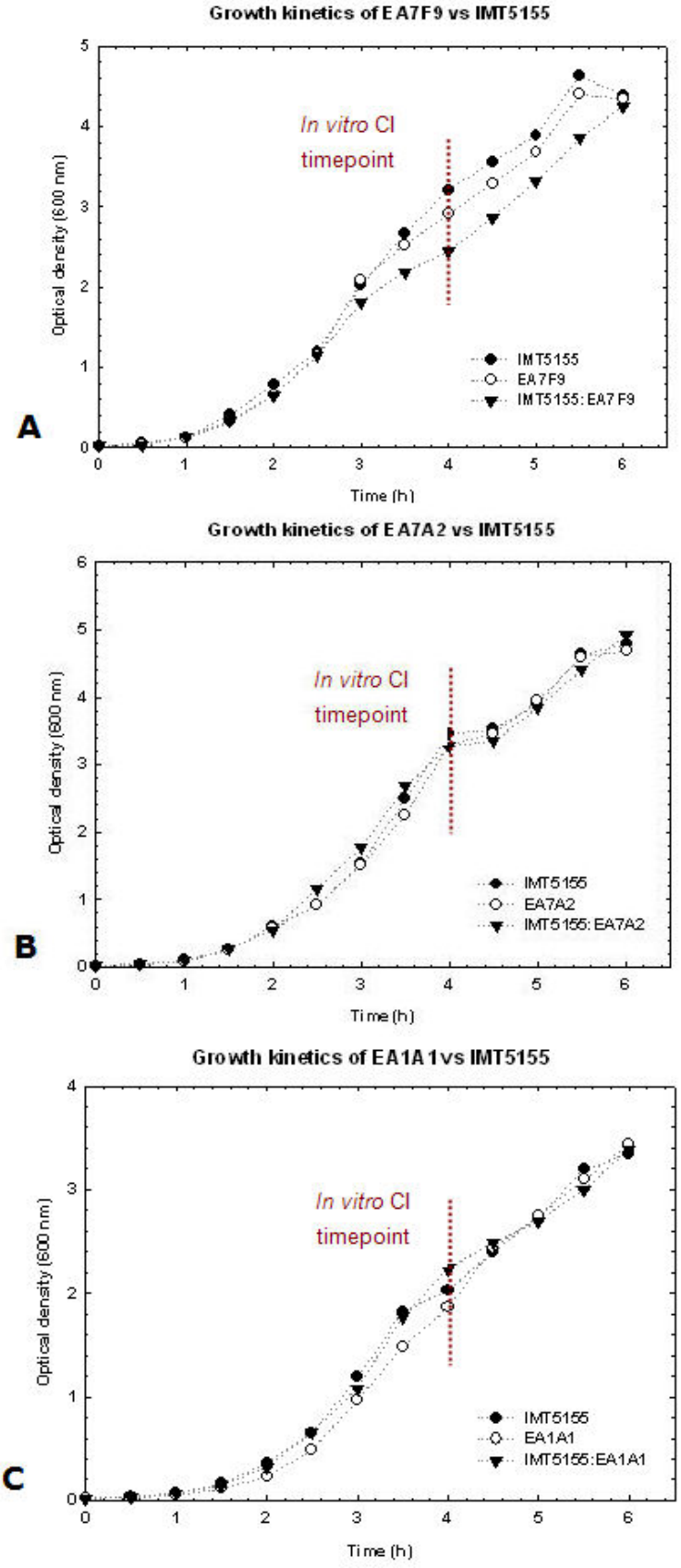


Figure 10: Graphic illustrating growth of mutants A) EA7F9 B) EA7A2 C) EA1A1 in competition with IMT5155 and separately in Luria Bertani (LB) growth medium. A competition index (CI) *in vitro* was calculated at a time point of 4 h.

Table 8: *In vitro* and *in vivo* competition indices (CI) of 20 mutants representing each function class of mutants identified in STM screen. (*In vitro* CI for mutant was calculated 4 h after growth in LB medium in competition with IMT5155; *In vivo* CI was calculated 24 h after infection as an average of 2 to 4 chickens infected with mutant strain in competition with IMT5155 after bacterial re-isolation from the chicken lungs). Mutants showing lower competition index *in vivo* than *in vitro* are highlighted confirming their attenuation.

Functional class	Mutant name	Gene	Putative function	*In vivo* CI[a]	*In vitro* CI[b]
Adhesins	EA7F9	*APECO1_3367 (yqi)*	Putative *yqi* fimbrial adhesin	**0.61**	**1.20**
	EA7A2	*fimE*	Type 1 fimbrial regulatory protein	**0.70**	**1.00**
Regulation	EA1H4	*leuO*	Leucine transcriptional activator	0.82	0.45
	EA16H4	*tdcA*	Transcriptional activator of tdc operon	4.20	0.73
	EA7F8	*evgA*	DNA-binding response regulator	0.41	0.28
LPS/Capsule	EA16G1	*kpsF*	Polysialic acid capsule synthesis	**0.36**	**0.67**
	EA10C1	*rfaJ*	LPS 1,2-glucosyltransferase	**0.08**	**5.70**
	EA1A1	*yaiP*	Putative glucosyltransferase	**0.16**	**1.00**
Transport	EA1C4	*actP*	Acetate transport	**0.20**	**0.60**
	EA7B2	*xapB*	Xanthosine transport	**0.71**	**1.90**
	EA10F11	*pgtP*	PgtP protein transport	**0.36**	**0.95**
	EA11E1	*rbsA*	Putative ribose transport	**0.14**	**1.20**
Metabolism	EA1C2	*ycgC*	Putative phosphoenol pyruvate kinase	0.64	0.48
	EA16H5	*yfiF*	tRNA/rRNA methyltransferase	**0.58**	**0.67**
	EA17F7	*carB*	Carbamoyl phosphate synthase	0.51	0.45
	EA1B2	*purE*	Phosphoribosylaminoimidazole carboxylase	2.64	1.50
	EA18G1	*bglF*	ß-glucoside specific IIABC compound	1.70	0.86
Unknown	EA7A3	*yobB*	Conserved hypothetical protein	**0.40**	**0.87**
	EA10G11	*ydeH*	Conserved hypothetical protein	0.42	0.25
	EA1A9	*ykgH*	Predicted inner membrane protein	1.40	1.40

5.4 Characterization of newly identified putative adhesin Yqi

Among the many genes identified as seen in table 8, two genes coding for adhesins were identified, including a novel putative adhesin *(yqi)* and type 1 fimbriae *(fimE),* both of which were found to be attenuated in the chicken as determined by in *vitro and in vivo* competition assays. These two genes were the most obvious targets of the STM screen being adhesins, the primary structures in direct contact with host tissue during infection. Type 1 fimbriae are

already well characterized for their role in ExPEC pathogenesis; however, yqi has until now never been described for its potential role during infection in ExPEC. Therefore, the yqi gene became the final target of this study, in that, it became important to know what role this gene plays in initiation of APEC infection. Hence the putative adhesin was characterized for its functional role in APEC pathogenesis by way of adhesion assays *in vitro and in vivo*, sequencing of the adhesin gene operon, prevalence studies among ExPEC strains, intestinal pathogenic *E. coli* and avian commensal *E. coli,* and finally protein expression and purification *in vitro*.

5.5 Sequencing of the adhesin gene cluster (*yqi*) region in IMT5155

The putative *yqi* adhesin gene cluster was completely sequenced in APEC strain IMT5155. Genomic organization of the IMT5155 *yqi* adhesin gene cluster was as follows: the putative outer membrane usher preceded the periplasmic chaperone, followed by the adhesin gene. A conserved hypothetical protein gene precedes the usher gene which may code for the adhesin subunit protein although this has not yet been confirmed (Fig. 11). This 4,975 bp region showed 99 percent sequence identity with the UPEC and APEC *yqi* adhesin gene cluster in sequenced strains UTI89 (Accession: CP000243) and APEC_O1 (Accession: CP000468) (Fig. 12). Comparing the sequence with genomes available in the public database, this adhesin gene cluster is only found among ExPEC strains including APEC, UPEC and NMEC and is not harboured by intestinal pathogenic *E. coli* like enteropathogenic *E. coli* (EPEC) or enterohaemorrhagic *E. coli* (EHEC) or non pathogenic *E. coli*.

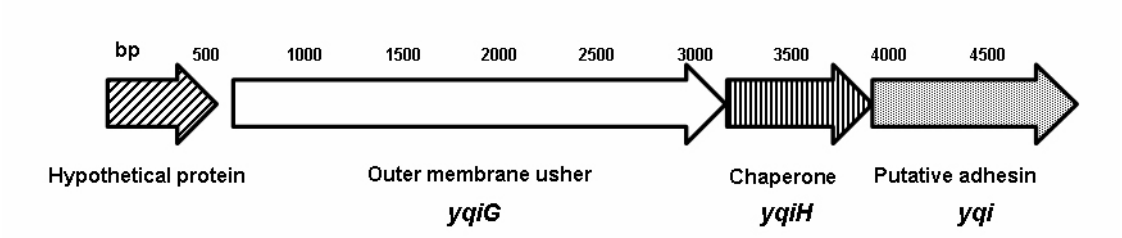
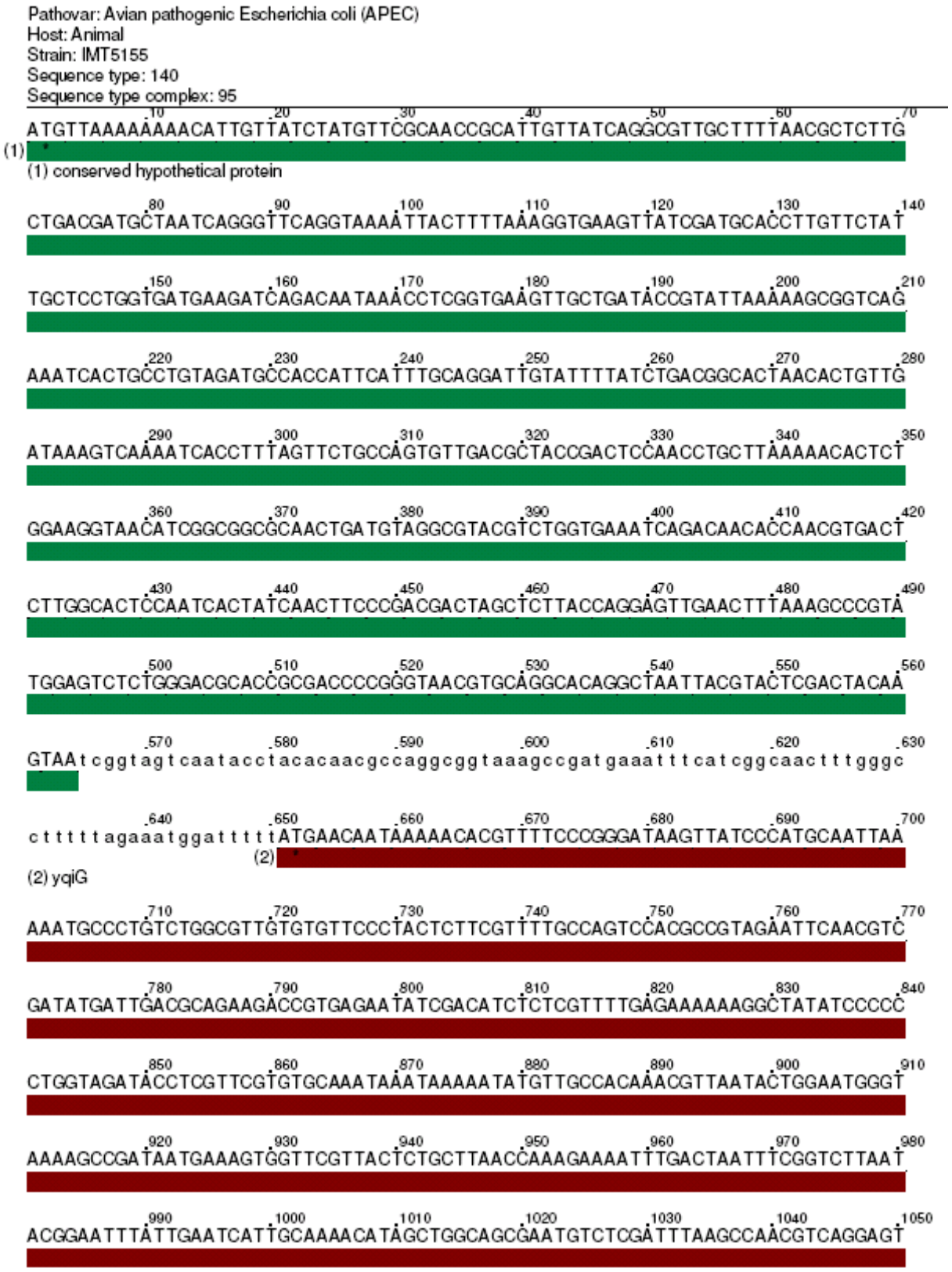


Figure 11: Physical map showing genomic organization of the *yqi* adhesin gene cluster in APEC strain IMT5155 depicted using the Kodon software available from Applied Maths. A hypothetical protein preceeds the putative outer membrane usher protein, followed by the putative chaperone and finally the putative adhesin.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Host: Animal
Strain: IMT5155
Sequence type: 140
Sequence type complex: 95
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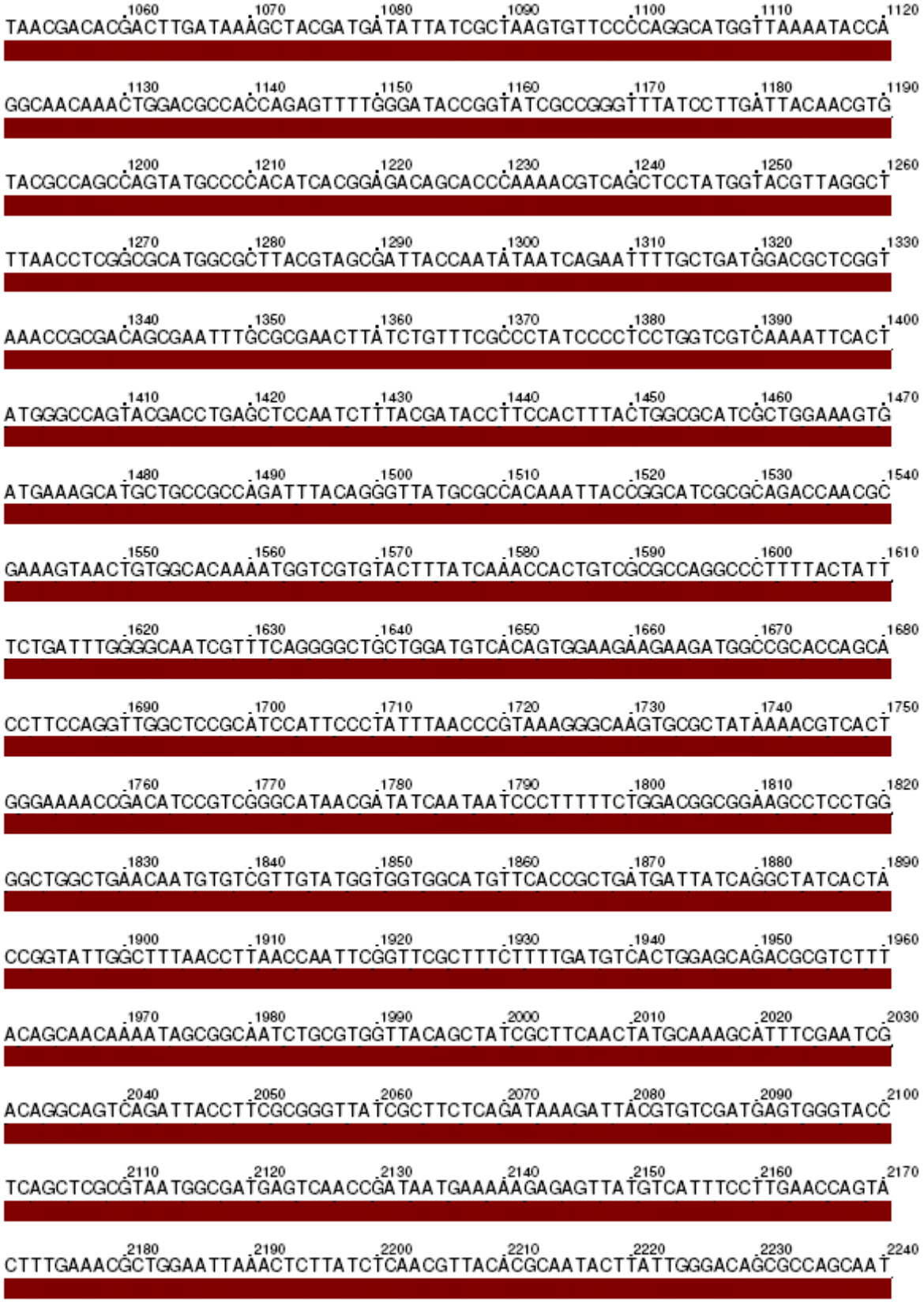
(2) yqiG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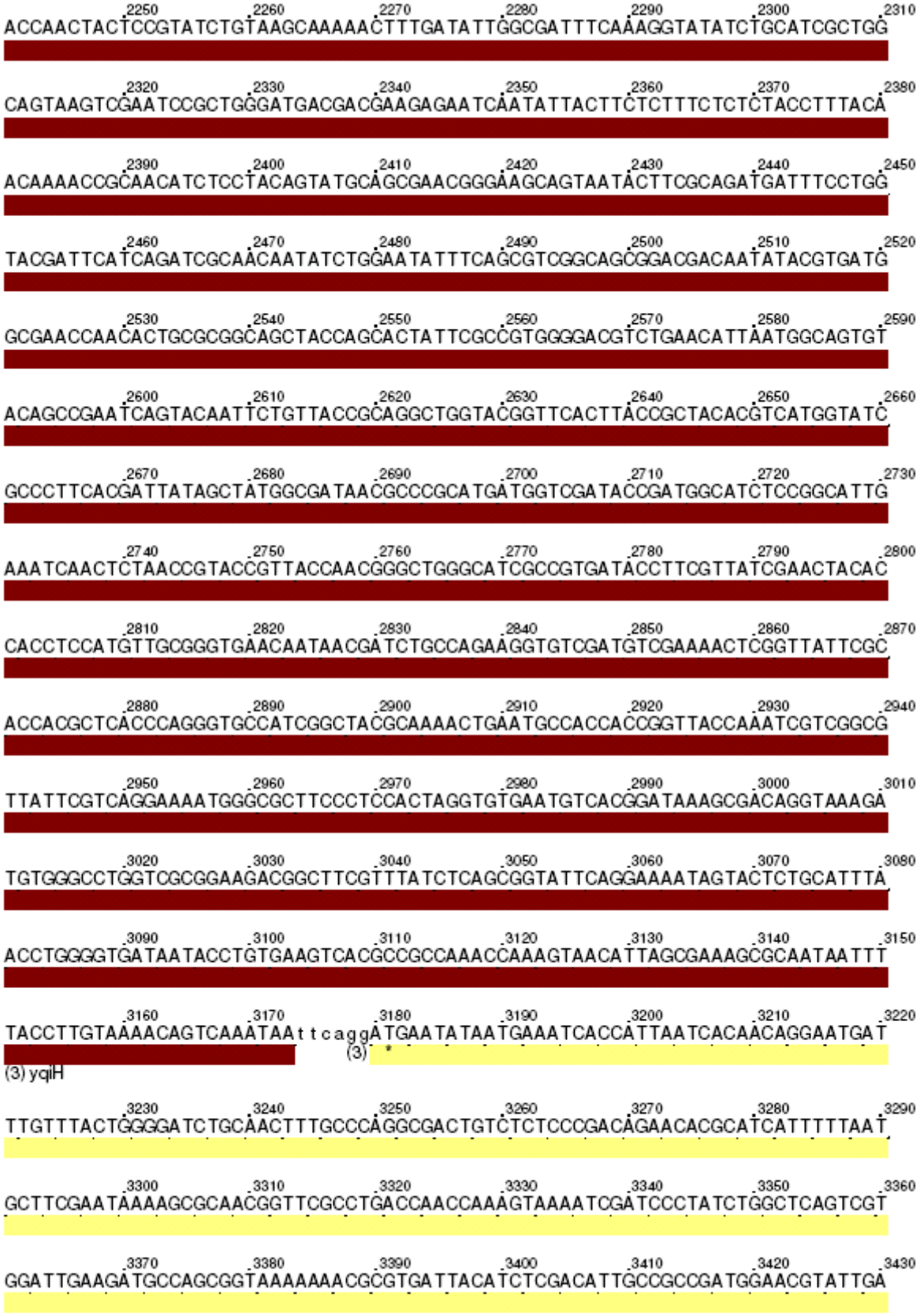
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(3)
(3) yqiH
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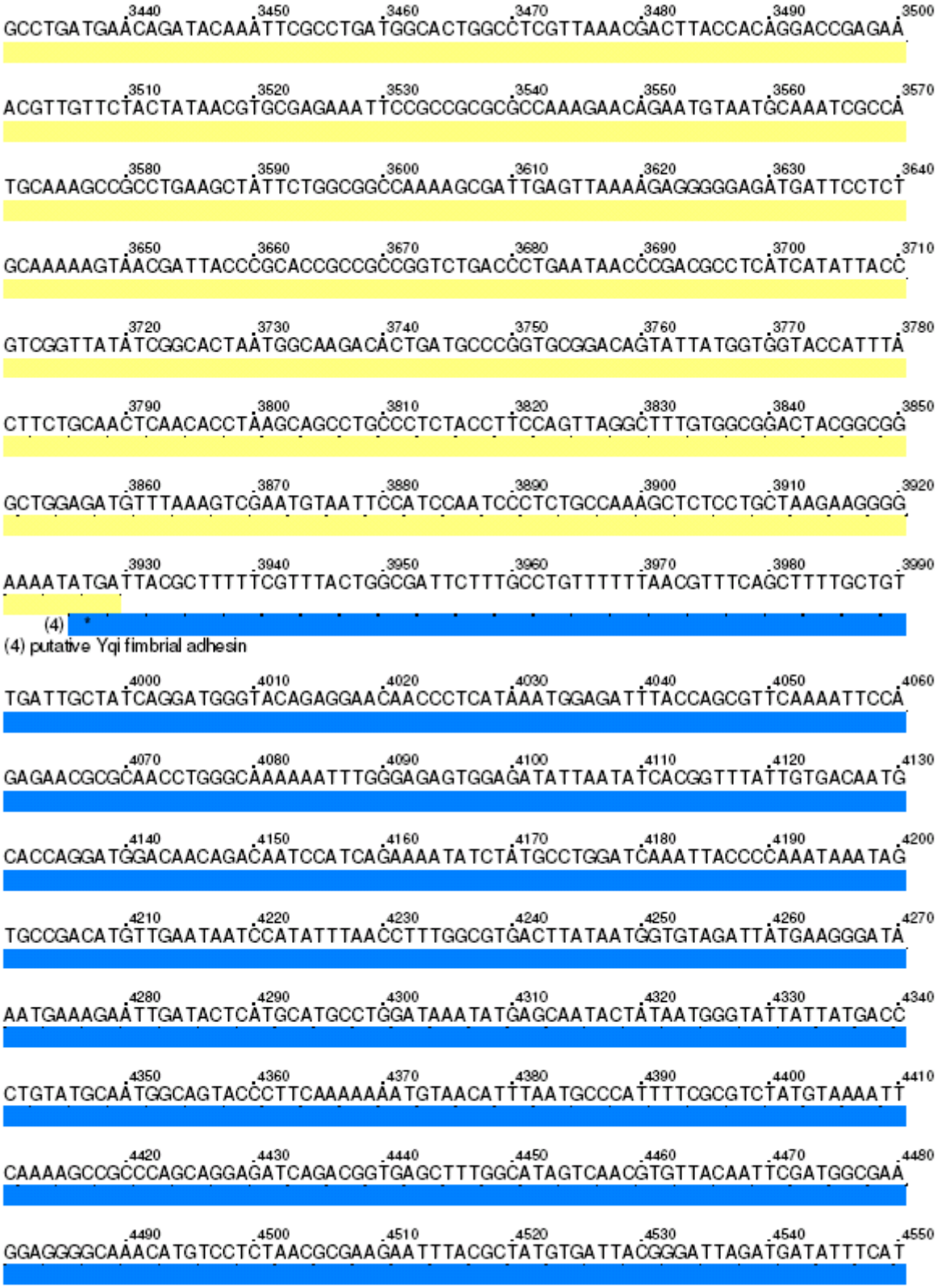
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3510 3520 3530 3540 3550 3560 3570
ACGTTGTTCTACTATAACGTGCGAGAAATTCCGCCGCGCGCCAAAGAACAGAATGTAATGCAAATCGCCA
3580 3590 3600 3610 3620 3630 3640
TGCAAAGCCGCCTGAAGCTATTCTGGCGGCCAAAAGCGATTGAGTTAAAAGAGGGGGAGATGATTCCTCT
3650 3660 3670 3680 3690 3700 3710
GCAAAAAGTAACGATTACCCGCACCGCCGCCGGTCTGACCCTGAATAACCCGACGCCTCATCATATTACC
3720 3730 3740 3750 3760 3770 3780
GTCGGTTATATCGGCACTAATGGCAAGACACTGATGCCCGGTGCGGACAGTATTATGGTGGTACCATTTA
3790 3800 3810 3820 3830 3840 3850
CTTCTGCAACTCAACACCTAAGCAGCCTGCCCTCTACCTTCCAGTTAGGCTTTGTGGCGGACTACGGCGG
3860 3870 3880 3890 3900 3910 3920
GCTGGAGATGTTTAAAGTCGAATGTAATTCCATCCAATCCCTCTGCCAAAGCTCTCCTGCTAAGAAGGGG
3930 3940 3950 3960 3970 3980 3990
AAAATATGATTACGCTTTTTCGTTTACTGGCGATTCTTTGCCTGTTTTTTAACGTTTCAGCTTTTGCTGT
(4)
(4) putative Yqi fimbrial adhesin
4000 4010 4020 4030 4040 4050 4060
TGATTGCTATCAGGATGGGTACAGAGGAACAACCCTCATAAATGGAGATTTACCAGCGTTCAAAATTCCA
4070 4080 4090 4100 4110 4120 4130
GAGAACGCGCAACCTGGGCAAAAAATTTGGGAGAGTGGAGATATTAATATCACGGTTTATTGTGACAATG
4140 4150 4160 4170 4180 4190 4200
CACCAGGATGGACAACAGACAATCCATCAGAAAATATCTATGCCTGGATCAAATTACCCCAAATAAATAG
4210 4220 4230 4240 4250 4260 4270
TGCCGACATGTTGAATAATCCATATTTAACCTTTGGCGTGACTTATAATGGTGTAGATTATGAAGGGATA
4280 4290 4300 4310 4320 4330 4340
AATGAAAGAATTGATACTCATGCATGCCTGGATAAATATGAGCAATACTATAATGGGTATTATTATGACC
4350 4360 4370 4380 4390 4400 4410
CTGTATGCAATGGCAGTACCCTTCAAAAAAATGTAACATTTAATGCCCATTTTCGCGTCTATGTAAAATT
4420 4430 4440 4450 4460 4470 4480
CAAAAGCCGCCCAGCAGGAGATCAGACGGTGAGCTTTGGCATAGTCAACGTGTTACAATTCGATGGCGAA
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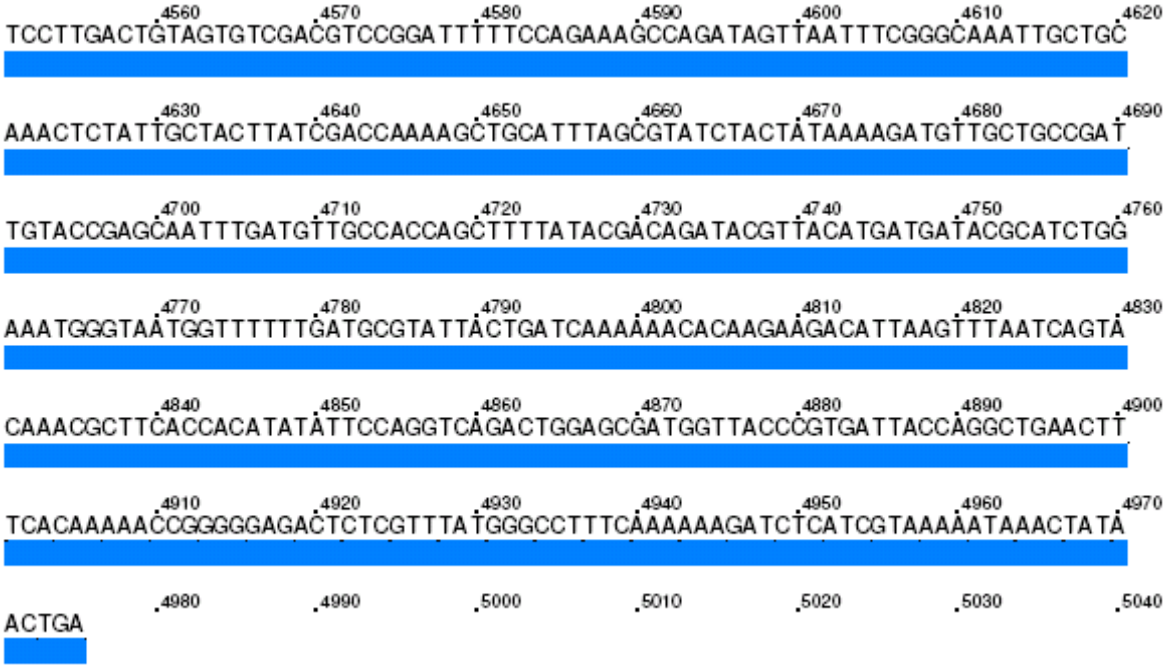


Figure 12: Genomic sequence of the *yqi* adhesin gene cluster in IMT5155 illustrated using Kodon software available from Applied Maths. Complete open reading frames including the start and stop codons of individual genes in the gene cluster are marked in colour as follows: Hypothetical protein: green, Outer membrane usher: brown, Chaperone: yellow, putative fimbrial adhesin: blue.

5.6 Prevalence of adhesin gene *yqi* among ExPEC strains reveals its potential as a factor of virulence

A collection of ExPEC strains, intestinal pathogenic *E. coli* and avian commensals available at the Institute of Microbiology and Epizootics, Freie Universität Berlin, was screened to determine the presence of the *yqi* gene among these strains. Out of a total of 588 ExPEC isolates tested for the presence of *yqi*, including 406 APEC, 138 UPEC, 25 NMEC and 19 SePEC, 368 isolates were found to be positive for *yqi*, which amounts to a total of 62.5% of pathogenic isolates harbouring the *yqi* gene (Table 9). Among APEC isolates alone, 221 isolates were found to be positive making it a total of 54.4% positive for *yqi* in this group. Among UPEC isolates 91 isolates were found to be positive in a total percentage of 65.9% positive for *yqi* and among NMEC isolates 15 were found to be positive amounting to 60.0% positive for *yqi*. Finally, among SePEC isolates, 10 were positive in a percentage of 52.6%. From a total of 159 A_{faecal} isolates tested, 31 were found to be positive for *yqi*, which accounts for only 19.4% of the non pathogenic strains tested.

Results were compared with MLST/EcoR data deposited in the publicly available database (**www.mlst.net**), of which 292 of the pathogenic isolates tested were known for their

sequence type (ST) and 607 were known for their EcoR groups according to Herzer et al (52), which was carried out as part of a separate study at the Institute of Microbiology and Epizootics. Of the isolates positive for *yqi*, 68 isolates belonged to the EcoR group A (18.9%), 4 to B1 (1.1%), 255 to B2 (70.8%) and 33 to D (9.1%) making B2 the predominant group for isolates harbouring *yqi*. Among isolates negative for *yqi*, 122 isolates belonged to EcoR group A (49.3%), 29 to B1 (11.7%), 42 to B2 (17.0%) and 54 to D (21.8%) where A is the predominant group for isolates lacking *yqi*.

When comparing the distribution of the *yqi* gene among ExPEC strains with available MLST data, it was observed that strains positive for *yqi* were allotted to sequence types 10,12, 14, 80, 68, 73, 95, 104, 115, 125, 135, 140, 141, 144, 348, 349, 352, 355, 357, 358, 363, 365, 371, 372, 390, 420, 913. A majority of the strains positive for *yqi* were associated with sequence type complex 95 known to harbour highly pathogenic ExPEC strains and sequence type complex 73 (Fig. 13). All strains belonging to sequence types 141, 372 and sequence type complex 95 were positive for *yqi*.

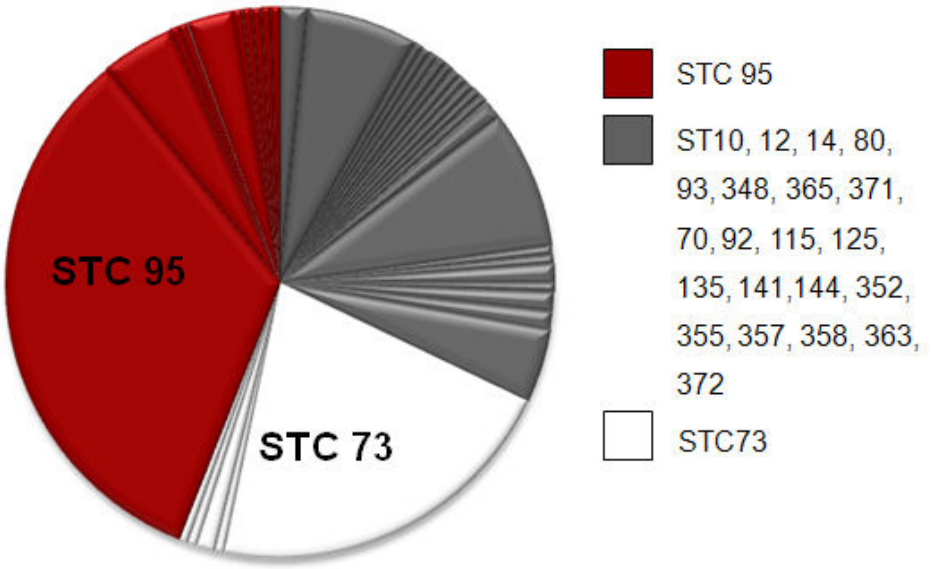


Figure 13: Pie chart showing a comparison of *E. coli* strains positive for *yqi* with MLST sequence types (ST) and sequence type complexes (STC); n=184 *E. coli* strains

Among 153 intestinal pathogenic *E. coli* tested in this study, none of the isolates were found to be positive for *yqi* as seen in table 9.

Due to the association of the putative fimbrial adhesin gene *yqi* with extraintestinal pathogenic *E. coli* (ExPEC), that is, its higher distribution among ExPEC isolates compared with non pathogenic *E. coli*, and complete absence in intestinal pathogenic *E. coli*, the adhesin is henceforth temporarily designated ExPEC adhesin I (EA/I).

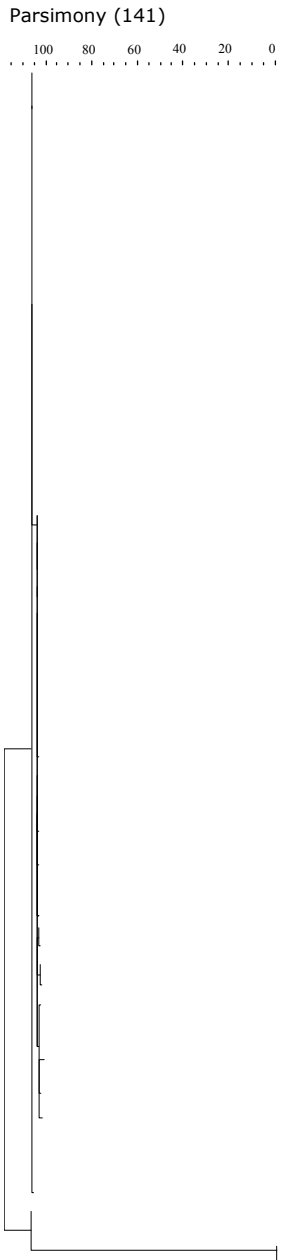
Table 9: Prevalence of ExPEC adhesin I coding gene *yqi* among pathogenic *E. coli* strains and non-pathogenic A_{faecal} strains; APEC=Avian pathogenic *E. coli*, UPEC=Uropathogenic *E. coli*, NMEC= Newborn meningitic *E. coli*, SePEC=Septicaemia associated *E. coli*, STEC=Shiga Toxin-producing *E. coli*, EHEC=Enterohaemorrhagic *E. coli*, EPEC=Enteropathogenic *E. coli*, aEPEC=Atypical Enteropathogenic *E. coli*, ETEC= Enterotoxigenic *E. coli*, EIEC=Enteroinvasive *E. coli*, EAEC=Enteroggregative *E. coli*.

***E. coli* Group**	**Isolates tested**	**Prevalence of yqi (%)**
APEC	406	54.4
UPEC	138	65.9
NMEC	25	60.0
SePEC	19	52.6
STEC	49	0.0
EHEC	46	0.0
EPEC	28	0.0
aEPEC	12	0.0
ETEC	8	0.0
EIEC	6	0.0
EAEC	4	0.0
Commensals/ (A_{faecal}) isolates	159	19.4

5.7 ExPEC adhesin I is evolving under positive selection

The ExPEC adhesin I gene (*yqi)* was sequenced in many strains and compared with existing Multilocus sequence typing (MLST) data. MLST is a nucleotide sequence based approach for the unambiguous characterization of isolates of bacteria using sequences of internal fragments of seven house-keeping genes (108). The population structure of microbial species with intermediate levels of recombination can thus be revealed by allele-based analyses (108). Wirth et al. described that sequence polymorphisms could define unique sequences for each of the seven house-keeping gene loci, which are referred to as alleles and each unique combination of alleles is assigned a sequence type (ST) number (173). Related STs are assigned to so-called ST complexes, using the principles of the eBurst algorithm (31): each ST complex includes at least three STs that differ from their nearest neighbour by no more than two of the seven loci while ST complexes differ from each other by three or more loci, and STs that did not match the criteria for inclusion within an ST complex are simply referred to by their ST designation (173).

Sequencing of the ExPEC adhesin I (*yqi*) gene in strains belonging to the various sequence types (ST) and sequence type complexes (STC) including STC10, STC12, STC73, STC95, ST141 and ST372, and calculation of the distances of the adhesin gene locus between these strains revealed distribution of the strains tested into two groups (Fig. 14). One group included strains belonging to the ST73 complex, while all other strains were assigned to a second group. Sequence homology was observed among strains belonging to a particular sequence type or sequence type complex as is the case with ST141, ST372, STC12 and STC73 within the sequence type or sequence type complex (Fig. 14). One exception to the rule was strain IMT15008, ST73, STC73 which showed variations in its *yqi* gene sequence compared to other strains in the ST73 complex, and could be better assigned to the group harbouring other strains tested. Interestingly, only strains belonging to sequence type complex 95 showed mutations in the *yqi* gene sequence. Therefore, we determined the ratio of the non-synonymous mutation rate (Dn) to the synonymous mutation rate (Ds) within this complex using DnaSP 4.50.3 software. Dn/Ds < or =1 indicates purifying or neutral selection, favouring amino acid substitutions (171). Our data show a non-synonymous mutation rate of 0.00095496 and a synonymous mutation rate of 0.00024 resulting in a Dn/Ds ratio of 3.979, indicating strong positive selection for structural evolution of the adhesin gene *yqi* within the sequence type 95 complex.



Strain	ST	STC	*E. coli* group	Host
IMT13833	10	10	UPEC	Human
IMT15010	12	12	UPEC	Animal
IMT8994	12	12	UPEC	Animal
IMT15025	12	12	UPEC	Animal
IMT14985	12	12	UPEC	Animal
IMT14974	12	12	UPEC	Human
IMT7916	12	12	UPEC	Human
IMT6744	12	12	SEPEC	Human
IMT13809	12	12	SEPEC	Human
IMT15030	372	None	UPEC	Animal
IMT14997	372	None	UPEC	Animal
IMT14984	372	None	UPEC	Animal
IMT9650	372	None	UPEC	Animal
IMT9713	372	None	APEC	Animal
IMT8995	358	None	APEC	Animal
IMT2248	358	None	APEC	Animal
IMT16064	141	None	Commensal	Animal
IMT8897	141	None	APEC	Animal
IMT5160	141	None	APEC	Animal
IMT15024	141	None	UPEC	Animal
IMT15016	141	None	UPEC	Animal
IMT13812	141	None	SEPEC	Human
IMT14989	141	None	UPEC	Animal
IMT5144	141	None	APEC	Animal
IMT8894	368	95	APEC	Animal
IMT9269	390	95	NMEC	Human
IMT2114	95	95	APEC	Animal
IMT2250	95	95	APEC	Animal
UTI189	**95**	**95**	**UPEC**	**Human**
IMT4525	95	95	APEC	Animal
IMT11962	416	95	Commensal	Human
APEC O1	**95**	**95**	**APEC**	**Animal**
IMT11981	95	95	UPEC	Human
IMT11088	95	95	NMEC	Human
IMT2099	95	95	APEC	Animal
IMT11973	390	95	UPEC	Human
IMT11978	95	95	Commensal	Human
IMT12023	95	95	NMEC	Human
IMT12024	95	95	NMEC	Human
IMT11967	417	95	UPEC	Human
IMT16083	95	95	Commensal	Animal
IMT13829	95	95	UPEC	Human
IMT11969	390	95	UPEC	Human
IMT13847	534	95	Commensal	Human
IMT9266	390	95	NMEC	Human
IMT9268	95	95	NMEC	Human
IMT11970	95	95	SEPEC	Human
IMT5214	95	95	APEC	Animal
IMT2545	95	95	APEC	Animal
IMT4534	140	95	APEC	Animal
IMT2492	140	95	APEC	Animal
IMT2288	140	95	APEC	Animal
IMT5155	**140**	**95**	**APEC**	**Animal**
IMT9285	140	95	UPEC	Human
IMT5128	140	95	APEC	Animal
IMT2272	140	95	APEC	Animal
IMT2470	95	95	APEC	Animal
IMT15000	95	95	UPEC	Animal
IMT11965	421	95	UPEC	Human
IMT15008	73	73	UPEC	Animal
IMT9300	135	None	APEC	Animal
CFT073	**73**	**73**	**UPEC**	**Human**
IMT7913	73	73	Commensal	Animal
IMT7911	74	73	UPEC	Human
IMT7908	73	73	Commensal	Animal
IMT13882	73	73	Commensal	Human
IMT13827	73	73	UPEC	Human
IMT13800	73	73	SEPEC	Human
IMT13797	73	73	SEPEC	Human
IMT13795	73	73	Commensal	Human
IMT15021	73	73	UPEC	Animal
IMT14967	73	73	UPEC	Animal
IMT9096	73	73	UPEC	Animal
IMT14976	73	73	UPEC	Animal
IMT14970	104	73	UPEC	Animal
IMT16101	73	73	Commensal	Animal
IMT10810	73	73	UPEC	Animal
IMT9089	73	73	UPEC	Human
IMT1200	353	73	UPEC	Human
IMT9402	104	73	UPEC	Animal
IMT9289	73	73	UPEC	Human

Figure 14: Dendrogram showing distances between *yqi* gene sequences among strains belonging to different sequence types (ST: Sequence type; STC: Sequence type complex)

5.8 ExPEC adhesin I is required for adhesion to chicken fibroblasts *in vitro*

In order to determine the role of the EA/I for APEC, adhesion assays were performed *in vitro* using chicken fibroblast cells. Strains EA7F9 with a disruption in the *yqi* gene via a transposon (STM generated mutant), and IMT5155Δ*yqi* devoid of the *yqi* adhesin gene were tested against the wild type pathogen IMT5155. An *E. coli* K-12 strain MG1655 was used as a negative control for the test, as this strain had been previously shown to be suitable as a control for adhesion to chicken fibroblast cells during standardization of the adhesion assay protocol (data not shown). Furthermore MG1655 did not harbour adhesin gene *yqi*. Adhesion of the bacteria to chicken fibroblast cells was determined at two different time points, at which a reduction in the ability of both EA7F9 and IMT5155Δ*yqi* to adhere to fibroblast cells was observed up to about forty percent of the total adhesion by IMT5155 (Fig. 15). MG1655 as expected also showed decreased adhesion ability when compared with IMT5155 indicating that the test was successful. An average CFU was calculated from three independent wells, and results were reproducible in 3 consecutive adhesion experiments. Statistical analysis of the CFU values showed a significant difference between wild type IMT5155 and mutants tested, with a $p<0.005$ and $p<0.05$ at 1.5h and 3h respectively (Fig. 15).

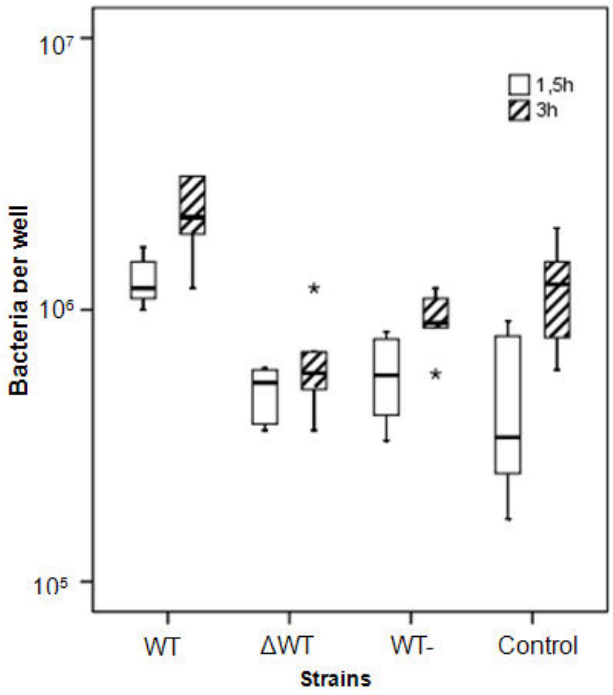


Figure 15: Bacterial adhesion to chicken fibroblast cells 1.5 h and 3 h after infection with an MOI=100 (100 bacteria/cell) measured as CFU bacteria/well (n=6 wells). WT: IMT5155, ΔWT: IMT5155Δ*yqi*, WT-: EA7F9, Control: MG1655. Differences between WT and ΔWT, and WT and WT- were statistically significant with a $p<0.005$ at 1.5h and $p<0.05$ at 3h; *= Values not included in statistical analysis.

5.9 Effect of ExPEC adhesin I in colonization of the chicken lung *in vivo*

Colonization of the chicken lung *in vivo* was studied by infecting 5-week old chickens intra-tracheally, and isolating bacteria from the lungs 24 h after infection. For this purpose, two different infection set ups were made use of, including the lung colonization model of infection and the systemic infection model. IMT5155 and IMT11327 served as positive and negative controls respectively. The results of the *in vivo* experiments confirmed the observations made *in vitro* in cell culture models described above. When chickens were infected with a dose of 10^6 CFU of IMT5155Δ*yqi* in a model used to study colonization abilities of the chicken lung by various strains, a distinct reduction in re-isolated bacterial numbers was observed as compared to IMT5155 as depicted in Fig. 16A. Differences between strains were statistically significant with a $p<0.05$. The average lung score in chickens infected with IMT5155Δ*yqi* was 1.63 in comparison to 1.71 and 1.21 in chickens infected with IMT5155 and IMT11327 respectively. All chickens infected did not exhibit any clinical symptoms.

Moreover, when chickens were infected with a higher dose of bacteria, that is, 10^9 CFU, in a model designed to induce systemic infection, there still was a significant difference in the colonization of the lung by strains IMT5155 and IMT5155Δ*yqi* as seen in Fig. 16B with a $p<0.02$. Chicken experiments were reproducible in 2 consecutive experiments and the number of chickens infected was always a minimum of six in a single experiment. The average lung score in chickens infected with IMT5155Δ*yqi* was found to be 1.52 in comparison to 2.4 and 0.6 in chickens infected with IMT5155 and IMT11327 respectively.

When chickens were infected with the complemented *yqi* mutant, IMT5155Δ*yqi* (pDSK602:*yqi*), the complemented mutant colonized the chicken lung to the same extent as the deletion mutant IMT5155Δ*yqi* and did not regain its ability to colonize the lung as much as the wild type strain IMT5155 (data not shown). In both infection models, the ability of IMT11327 to colonize the chicken lung was much less than both IMT5155 and IMT5155Δ*yqi* as seen in figures 16A and 16B.

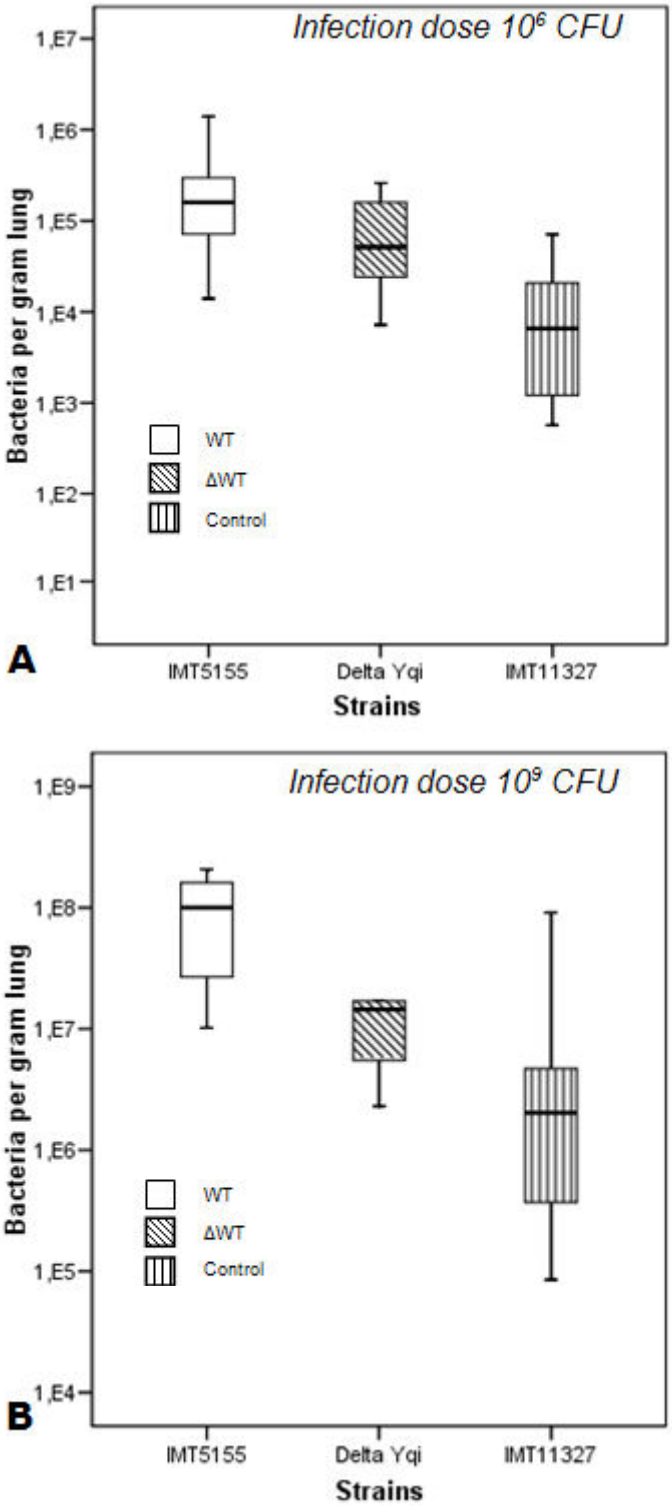


Figure 16: Bacterial colonization of the chicken lung *in vivo.* WT: IMT5155, ΔWT: IMT5155Δ*yqi*, Control: IMT11327.

A) Bacterial colonization of the chicken lungs 24 h after intra-tracheal infection with 10^6 CFU of bacteria (Lung infection model). Differences between WT and ΔWT, and WT and Control were statistically significant with a $p<0.05$ and $p<0.005$ respectively (n=6 chickens per group).

B) Bacterial colonization of the chicken lungs 24 h after intra-tracheal infection with 10^9 CFU of bacteria (Systemic infection model). Differences between WT and ΔWT, and WT and Control were statistically significant with a $p<0.02$ and $p<0.005$ respectively (n=6 chickens per group).

5.10 Effect of ExPEC adhesin I during systemic infection in chickens

Systemic infection can be induced in chickens by infecting 5-week old birds intra-tracheally with an infection dose of 10^9 CFU. As mentioned before, infection of chickens with this infection dose resulted in siginificant reduction in colonization of the chicken lung (Fig. 16B). A reduction in bacterial numbers was also seen when bacteria were re-isolated from chicken internal organs including kidneys, heart, liver and brain, when infected with IMT5155Δ*yqi* as compared to IMT5155 as seen in Fig. 17. Organ scores of the air sacs, lungs and the liver were considerably reduced in chickens infected with IMT5155Δ*yqi* as compared to chickens infected with IMT5155 as seen in table 10.

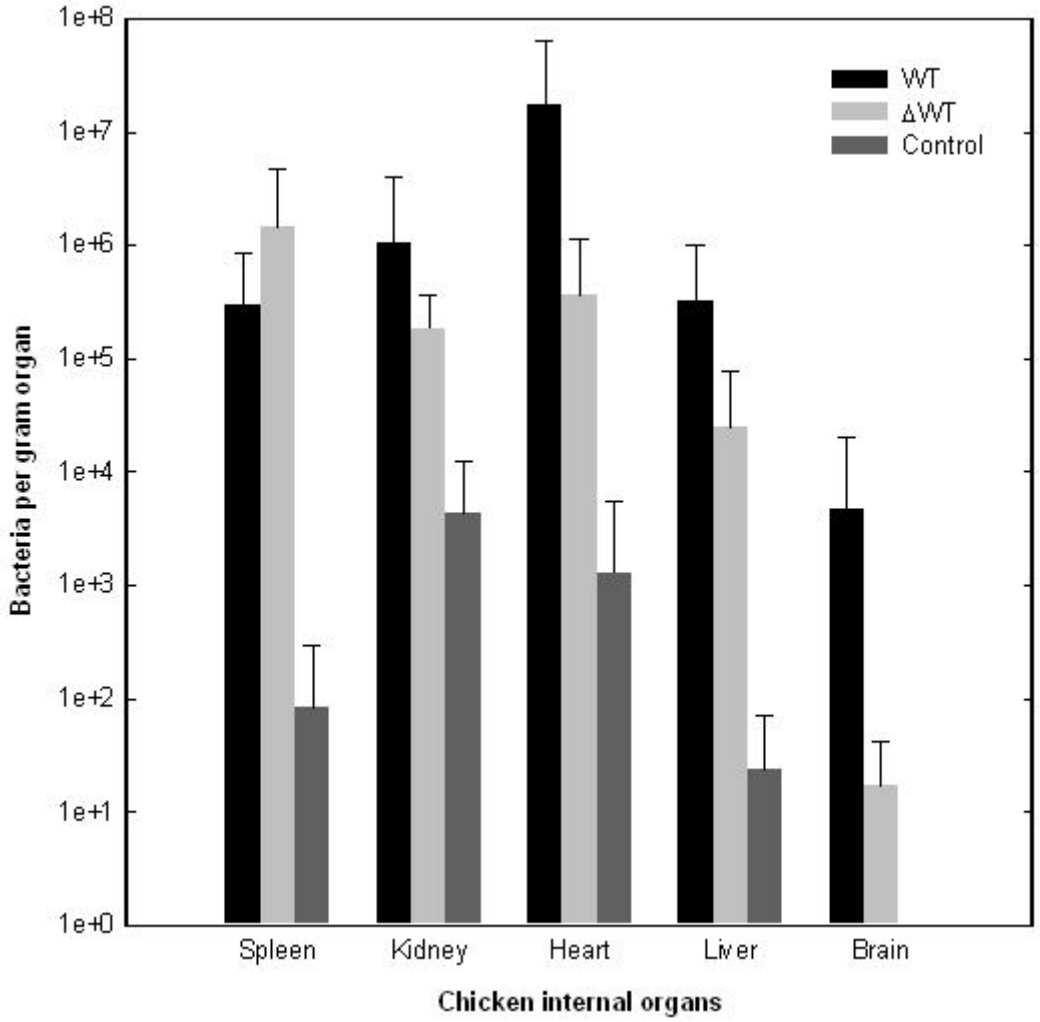


Figure 17: Bacterial re-isolation of WT: IMT5155, ΔWT: IMT5155Δ*yqi* and Control: IMT11327 from the spleen, kidneys, heart, liver and brain 24 h after intra-tracheal infection with 10^9 CFU of bacteria. Absence of a column indicates that no bacteria were isolated from the organ when infected with the respective strain.

Table 10: Score values for severity of organ lesions ± standard deviation in respiratory and other organs after infection with IMT5155, IMT5155Δ*yqi* and IMT11327 at infection dose 10^9 CFU. Differences in organ scores between IMT5155 and IMT5155Δ*yqi* and between IMT5155 and IMT11327 were statistically significant for air sacs, lungs and liver with a $p<0.05$.

Strain	Average Organ Score				
	Air sacs	Lungs	Liver	Heart	Spleen
IMT5155	2.0 ± 1.0	2.4 ± 0.9	0.8 ± 0.8	1.8 ± 1.3	1.0 ± 0.0
IMT5155Δ*yqi*	1.1 ± 0.7	1.5 ± 0.6	0.1 ± 0.3	0.7 ± 0.8	0.9 ± 0.3
IMT11327	1.6 ± 0.5	2.0 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.5

5.11 ExPEC adhesin I plays a role during adhesion to kidney epithelial cells *in vitro*

Since APEC are known to be similar to other ExPEC strains in many ways, particularly in the similarities in their genotypes, we aimed to study the role of ExPEC adhesin I in the adhesion to cell lines beyond the chicken host cell type. Therefore, we studied the role of *yqi* in APEC *in vitro*, using adhesion assays with a polarized Madin-Darby Canine-Kidney (MDCK-1) epithelial cell line. This cell line was chosen, as uropathogenic *E. coli* (UPEC) causing urinary tract infection are known to adhere to kidney and bladder cells. Strains IMT5155Δ*yqi* and IMT5155Δ*yqi* (pDSK602:*yqi*), a strain carrying an expression plasmid with the *yqi* gene, that is, the complemented mutant, were tested against IMT5155, the positive control, and IMT11327, a negative control strain of avian origin lacking the gene *yqi*. Adhesion was monitored 3h after infection. A reduction in colonization by IMT5155Δ*yqi* was seen, up to twenty percent of the total adhesion by IMT5155 (Fig. 18). The complemented strain IMT5155Δ*yqi* (pDSK602:*yqi*) regained its ability to adhere to MDCK-1 cells by introduction of the *yqi* gene when compared with its deletion mutant IMT5155Δ*yqi* with a statistical significance of $p<0.04$.

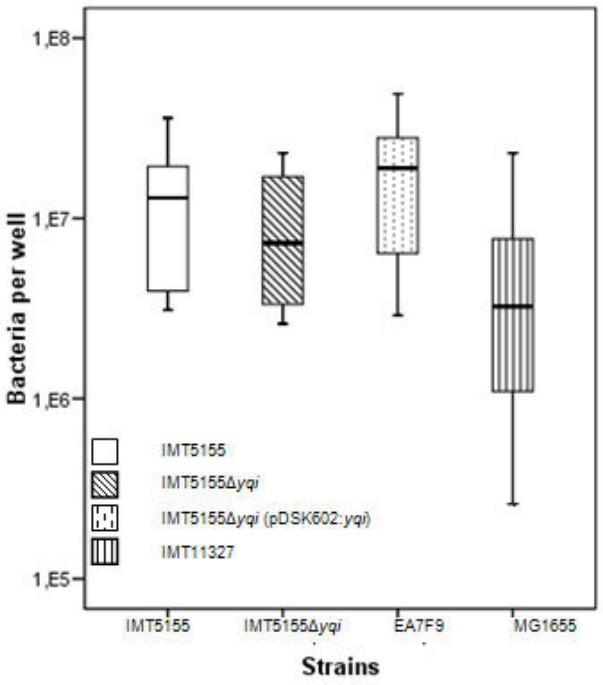


Figure 18: Bacterial adhesion to polarized Madin Darby canine kidney (MDCK-1) cells 3 h after infection with an MOI=100 (100 bacteria/cell) measured as CFU bacteria/well (n=6 wells). The difference between IMT5155Δ*yqi* and IMT5155Δ*yqi* (pDSK602:*yqi*) was significant with a *p*<0.04.

5.12 Expression of *yqi in vitro* reveals a ~39 kDa protein

The ExPEC adhesin I protein was expressed *in vivo* by constructing a glutathione S-transferase (GST) fusion protein by cloning the *yqi* gene into pGEX-6P-1 expression vector. The GST gene fusion system is a versatile system. GST is a 26 kDa moiety, which is located at the amino terminus of a fusion protein while the protein of interest lies at the carboxyl terminus. The *yqi* gene was successfully cloned into pGEX-6P-1 as confirmed by PCR reactions. To ensure optimal protein expression, the modified plasmid containing the GST:*yqi* fusion protein was successfully transformed into *E. coli* BL21. To check for expression of the protein, SDS-PAGE was carried out followed by silver staining which revealed a distinct band of the fusion protein with a molecular weight of around 65 kDa when induced with IPTG in whole cell lysate samples, which was not detected in the absence of IPTG (Fig. 19). These results were further confirmed by performing a western blot, using anti-GST as the primary antibody for detection of the GST protein, and hence also the fusion protein. The GST protein alone, expressed in *E. coli* BL21 was used as a control. A strong band was seen at 65 kDa in whole cell lysate samples induced with IPTG (Fig. 20). Of the 65 kDa, GST makes up 26 kDa, thereby confirming the presumed molecular weight of the Yqi protein, namely 39 kDa.

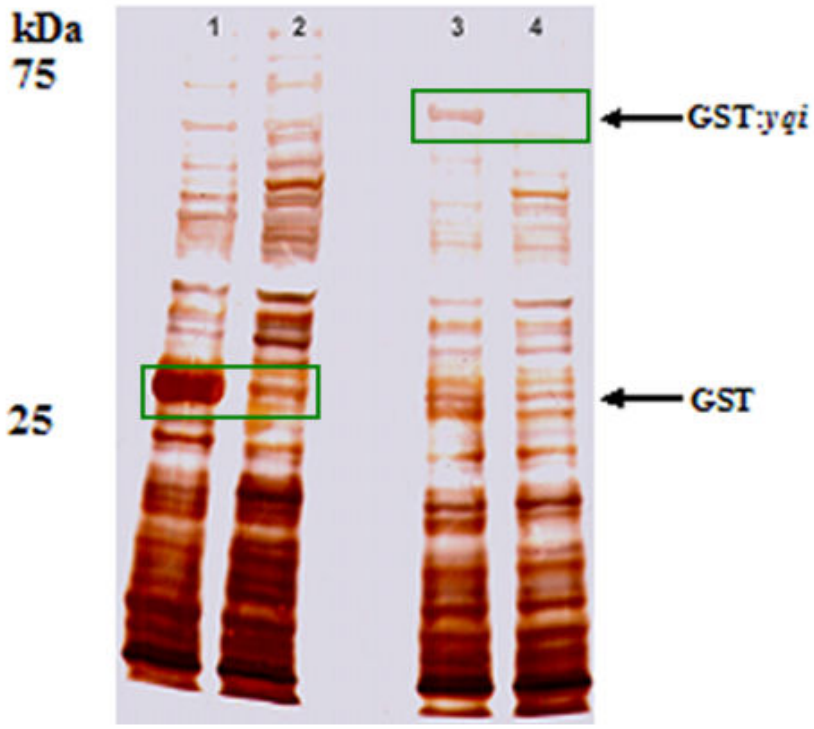


Figure 19:_ Photograph of SDS-PAGE gel stained with silver stain showing expression of *yqi* as a recombinant GST fusion protein in *E. coli* BL21. Lane 1: *E. coli* BL21 [pGEX] induced with IPTG for 2 h; Lane 2: *E. coli* BL21 [pGEX] not induced with IPTG; Lane 3: *E. coli* BL21 [pGEX:*yqi*] induced with IPTG for 2 h; Lane 4: *E. coli* BL21 [pGEX:*yqi*] not induced with IPTG.

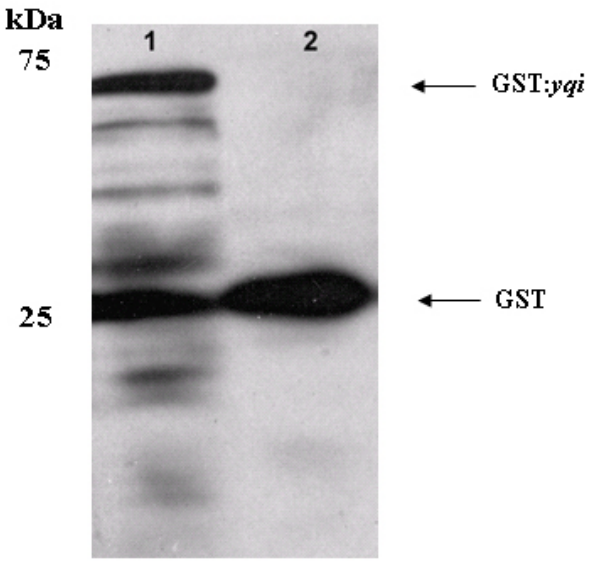


Figure 20: Immunoblot showing expression of *yqi* as a recombinant GST fusion protein in *E. coli* BL21 when treated with GST-specific antibody Anti-GST. Lane 1: *E. coli* BL21 [pGEX:*yqi*] induced with IPTG for 2 h; Lane 2: *E. coli* BL21 [pGEX] induced with IPTG for 2h.

5.13 Purification of the ExPEC adhesin I protein

The GST:*yqi* fusion protein was purified from a bacterial lysate of *E. coli* BL21 (pGEX:*yqi*) by using glutathione immobilized to a sepharose matrix. When applied to the affinity medium, the fusion protein bound to the ligand and impurities were removed by washing with PBS. The protein was eluted from the glutathione sepharose under mild, non-denaturing conditions in order to preserve both protein antigenicity and function.

When heated to 95 ºC in SDS-PAGE sample buffer (Laemmli buffer), purified GST:*yqi* migrated at its calculated molecular mass of 65 kDa which was observed by the presence of a single clear band at ~65 kDa on the SDS gel after silver staining (Fig. 21). This confirmed the results seen with western blotting. A strong protein band was also seen at ~26 kDa which was presumed to be the GST protein detached from the fusion protein with a high affinity to glutathione, its natural substrate.

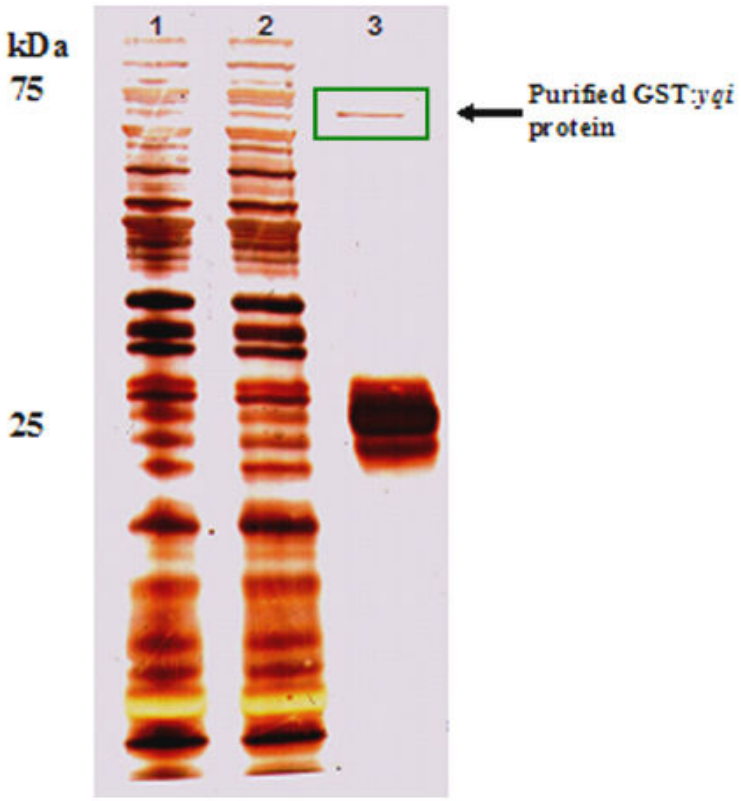


Figure 21: Photograph of SDS-PAGE gel stained with silver stain showing the purification of the Yqi protein as a recombinant GST fusion protein using GST beads as a binding substrate. Lane 1: *E. coli* BL21 [pGEX:*yqi*] after induction with IPTG for 2 h, Supernatant; Lane 2: *E. coli* BL21 [pGEX:*yqi*], Supernatant, Post-binding; Lane 3: *E. coli* BL21 [pGEX:*yqi*], Purified GST:*yqi* protein

5.14 Electron microscopy reveals fimbrial like appendages associated with ExPEC adhesin I (*yqi*) gene cluster

The ExPEC adhesin I (*yqi*) 4975bp gene cluster coding for the putative subunit, chaperone, usher and adhesin was cloned and over-expressed in an afimbriate *E. coli* K12 strain AAEC189 using a suitable expression vector. Negative staining of strain AAEC189 (pKESK:*yqi*_4975_XB) revealed the expression of short fimbrial like appendages forming on the outer membrane of the bacterial cell (Fig. 22A-C). These appendages were about 0.04 µm long and 0.005 µm thick and were not detected in the afimbriate strain AAEC189, that is, the negative control (Fig. 22D). The wild type strain IMT5155, harbouring other adhesins like type 1 fimbriae and curli, in addition to ExPEC adhesin I, was used as a positive control for the staining method, and long fimbriae with a length of about 0.5 µm (Fig. 22E) were observed which were morphologically different from the fimbrial structures observed in strain AAEC189 (pKESK:*yqi*_4975_XB).

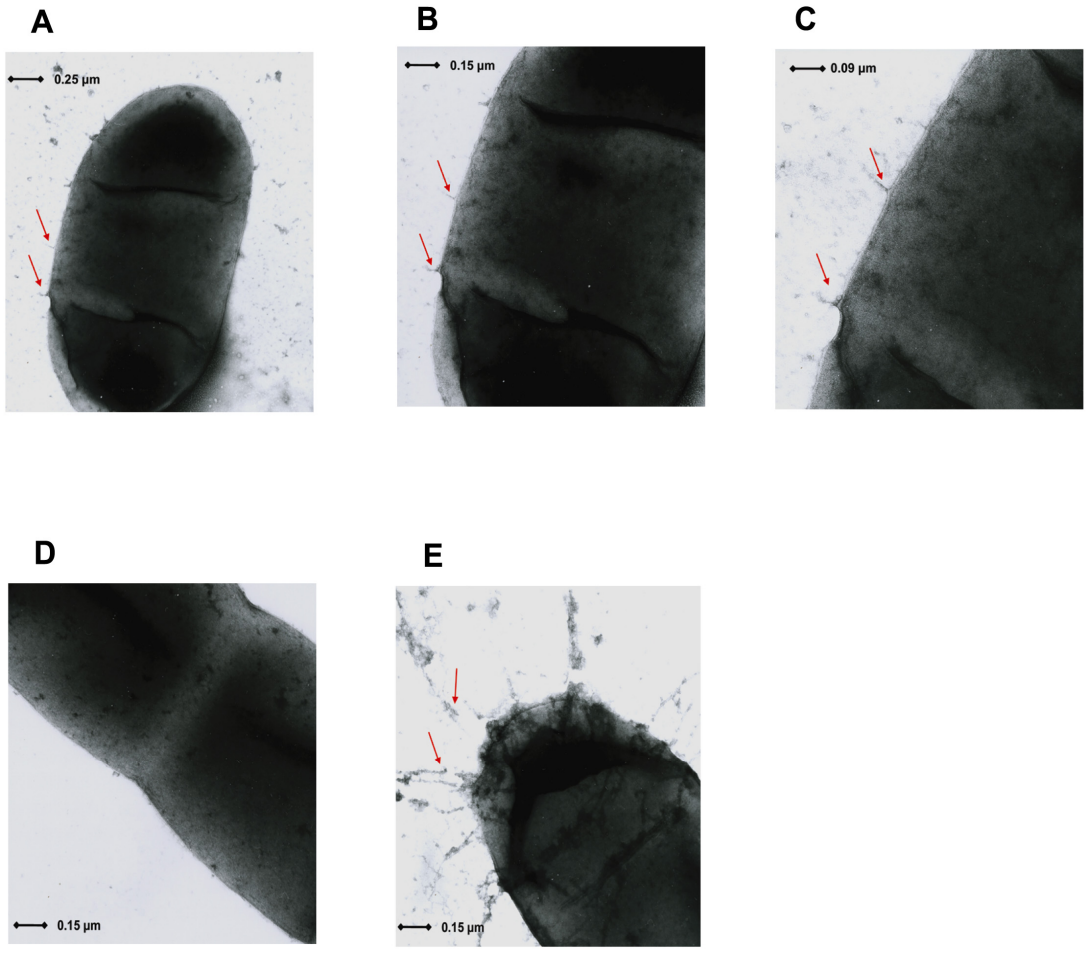


Figure 22: Expression of the ExPEC adhesin I gene cluster *in vitro*. Electron micrographs show negatively stained afimbriate strain *E. coli* AAEC189 (pKESK:*yqi*_4975_XB) overexpressed with the *yqi* adhesin gene cluster at a magnification of 45,000x, 65,000x and 100,000x (A-C), negative control strain *E. coli* AAEC189 (D) and wild type fimbriae *E. coli* strain IMT5155 (E). The arrows indicate the location of the fimbriae. Photograps were taken by Dr. Wilfried Bleiß (HU Berlin).

6 Discussion

Research on avian pathogenic *E. coli* (APEC) has progressed to a great extent over the last couple of years. However, despite many efforts made to understand APEC pathogenesis, little is still known about how infection is initiated in the respiratory tract of chickens, which is one of the first and the most important disease syndrome associated with APEC (21). A necessary step in the successful colonization and production of disease by most microbial pathogens is the ability to adhere to and colonize host surfaces (32). In search for adhesion and colonization factors, that may still be unknown for APEC, the present study therefore made use of a newly established lung colonization chicken infection model in order to identify APEC genes specific to early stages of infection including adhesion to and colonization of the host by its pathogen via signature-tagged mutagenesis (STM). For this purpose infection was successfully reproduced in 5-week old chickens, the natural host model of APEC infection, using a highly virulent APEC strain IMT5155 (O2:K1:H5), which belongs to multi locus sequence type 95 complex, to which a large group of ExPEC strains are allotted. This phylogenetic group is an excellent representative of the APEC population according to current knowledge (74). The prototype strain IMT5155 was also used to successfully generate an STM mutant library for screening purposes.

6.1 The “Lung Infection Model” is appropriate for STM in the identification of genes involved in colonization

In this study, a lung infection model was established, which to the best of our knowledge, is the first of its kind in chicken (3). The most important aim of the lung infection model was to use it for STM analyses *in vivo* in order to select stage-specific genes during infection. Therefore, a modified model of infection based on the previously existing systemic infection model (103) was established to induce effective colonization of the chicken lung, but simultaneously no systemic infection. This was possible, in that, the infection dose was reduced up to a 1000-fold. Various infection doses were experimented with, leading to the selection of a dose, which would not only enable colonization of the avian lung by APEC, but would also be the ideal infection dose for further experimental purposes. After several trials, an infection dose of 10^6 CFU and a time point of 24h post infection were selected, based on a number of factors essential for successful STM analysis, for example, pool complexity, recovery of mutants and genes targeted.

With regard to the pool complexity when testing STM mutants simultaneously, if the inoculum dose is too low, there may be insufficient cells of any one virulent mutant to initiate a successful infection. On the other hand if the dose is too high, the animal’s immune defences

may be overwhelmed, resulting in the growth of mutant strains that would otherwise be attenuated (18).

During the STM screen, the genes targeted in this lung infection model were those that played a role in the initial stages of infection, namely adhesion and colonization of the lung. Hence, the lung became the target organ in this model, making the route of inoculum administration in turn another vital aspect. The infection route of bacterial inoculum also influences the numbers of bacterial strains that reach the target organ and tissues, hence the reproducibility of tag hybridization signals (18). Previous studies have made use of various inoculation routes including intra-air sac, intra-peritoneal, subcutaneous, and intravenous to name a few, however, most of these routes bypass the upper respiratory tract, which is the natural route of entry for APEC (3). It is generally accepted that the most important natural route of *E. coli* infection for birds is the inhalation of faecally contaminated dust (36). Therefore, some studies have previously reported experimental reproduction of disease via aerosol infection, imitating natural infection in the field (36, 138, 164). Aerosol infection can under standardized conditions ensure that the birds infected inhale the bacteria under normal conditions of respiration, besides making sure that the defence mechanisms of the upper respiratory tract are not bypassed. Even though this is advantageous for natural experimental purposes, this challenge method would be disadvantageous for successful STM analysis, given that each experimental animal is infected with a pool of mutants, all of which would have to reach the target organ. This would give rise to problems pertaining to accuracy of bacterial inoculum, which is so crucial for an STM screen. In this study, therefore, chickens were infected via the trachea, representing a near to natural mode of infection. This challenge method is indeed a very crucial step for the infection model, in that; host defence as well as primary adhesion begins at the tracheal epithelium and thereby primary host defence mechanisms, including aerodynamic filtration, mucociliary clearance and phagocytosis would be accounted for (145).

The lung infection model is fascinating due to the fact that it can replace the systemic model in many considerable aspects of pathogenesis studies particularly STM studies. The systemic model bypasses the early stages of the infection process including adherence to and colonization of epithelial surfaces, followed by subsequent invasion of host cells; hence, some bacterial factors important to these stages in pathogenesis will not be identified using this model (66). This is due to the high dose of bacterial inoculum used in the systemic infection model, which overwhelms the chicken lung thus obstructing specific bacterial adhesion. In order to identify factors associated with the adhesion and colonization of the chicken respiratory tract by APEC, be it through STM or site directed mutagenesis, such a systemic model would not hold good, and therefore the lung infection model would aid in the identification of specific factors in mutants that fail to survive during the early stages of the

infection process. This study has indeed proven this hypothesis and it was seen that many factors involved in colonization including a new APEC adhesin, have been identified using this strategy.

6.2 Identification of genes that play a role in APEC colonization of the chicken lung during infection

Genes important to the early stages of infection by APEC were identified by the STM screen in this study and were found to belong to different classes based on their function, including adhesins, regulators, LPS/capsule genes, metabolic genes and genes of unknown function.

6.2.1 Bacterial regulators play a role in APEC colonization of the lung *in vivo*

Most bacterial genes are known to be regulated either positively or negatively by other genes or regulators. Three mutants were identified with gene disruptions in known bacterial regulators. Mutant EA1H4 that was attenuated *in vivo* had a disruption in gene *leuO*, a relatively well characterized LysR-like regulator, whose putative function is DNA dependent intracellular regulation of transcription. *LeuO* has been previously shown to be a positive regulator of *ompS2* in Salmonella, a gene encoding quiescent porins, whereby strains with a mutation in *leuO* were found to be attenuated for virulence in mice (150).

It has been reported that porins play a role in virulence. In *Shigella flexneri*, outer membrane protein (OMP) mutants were found to be associated with important steps of pathogenesis. Furthermore, previous results point toward the role of *ompS1* and *ompS2* genes, regulated by *leuO*, in the initial stages of infection (150). Interestingly *ompA*, a major outer membrane protein in *E. coli* has been found to contribute to *E. coli* K1 invasion of brain endothelial cells *in vitro* (170). *E. coli* K1 is known to be the most common Gram-negative organism causing neonatal meningitis (NMEC), that is, it is one of the main pathovars of the ExPEC group of pathogens. Wang et al. have previously demonstrated that OmpA is indeed a critical determinant for NMEC penetration of the CNS *in vivo* (170). It is possible that *leuO* may be involved in the regulation of OmpA in ExPEC, a gene important for later infection stages; however, it may also be likely that *leuO* regulates genes involved in initial infection stages which are yet to be determined, but would explain its identification in this study.

leuO has also been shown to be involved in the translational regulation of *rpoS*, which encodes the general stress sigma factor in *E. coli*, by affecting the expression of the small regulatory *dsrA*-RNA (77). Whether other important virulence factors, especially those required for early stages of infection namely attachment and colonization by APEC, are regulated by *leuO* is yet to be determined; however, it still remains a possibility.

A second mutant EA7F8, disrupted in the *evgA* gene, a DNA binding response regulator in a two-component regulatory system (115), was also found to be moderately attenuated *in vivo*. One of the major mechanisms of signal transduction in bacteria, leading to specific gene expression is the two-component system as seen in *E. coli* which consists of response regulator EvgA and sensor kinase EvgS. EvgA and EvgS are highly similar to the Bordetella virulence genes (*bvg*) BvgA and BvgS, respectively, which control the expression of adhesins and other virulence factors in *Bordetella pertussis* (115). *Bordetella bronchiseptica* is also an important pathogen known to infect most mammalian species, but rarely humans, whose virulence determinant (filamentous haemagglutinin, fimbriae, pili) expression, all of which are adhesins, is regulated by the *bvg* locus particularly *bvgA* and *bvgS* (116).

Macroarray analysis was previously used in *E. coli* to study the expression of genes under the control of EvgA. Interestingly, a number of genes were found to be down-regulated by EvgA, including those involved in motility, *fimA*, *fimF*, *fimH*, *fimI*, *fliA* and *fliC*, whereby decreased expression of these genes was observed (126). It has also been previously shown that EvgA activates the expression of genes which are required for the full efficiency of locus of enterocyte effacement (LEE) repression (124). The LEE is required by enteric pathogens for effective colonization of eukaryotic cells of the intestinal tract, and therefore LEE-positive bacteria form typical attaching and effacing (A/E) lesions on cells, which are characterized by the effacement of the enterocyte microvilli, an intimate attachment of bacteria to the eukaryotic cell, and an increased assembly of actin filaments underneath the attaching site of the bacteria (termed actin pedestal) (69). It has further been found that over-expression of recombinant *evgA* caused a reduction of actin pedestal formation which has led to the conclusion that EvgA down-regulates LEE expression in Enteropathogenic *E. coli* (EPEC) (124). It is likely that there are a number of genes whose EvgA dependence is not yet known with respect to APEC adhesion and virulence which would be of interest for future studies.

Another mutant EA16H4 with a transposon disruption in the *tdcA* gene was identified. It is known that *tdcA* is a transcriptional activator of the Tdc operon involved in the transport and metabolism of threonine and serine during anaerobic growth (44). It has nevertheless been shown, that *tdcA* accounts for adherence in Enterohaemorrhagic *E. coli* (EHEC) by way of it being a mediator of hyperadherence which might explain the high competitive index value of mutant EA16H4 *in vivo*. Expression of *ompA*, an important adherence factor in EHEC, was increased in a mutant with an insertionally inactivated *tdcA* gene as previously published (165, 166).

It is interesting to note, that prior to this study, regulators *leuO*, *evgA* and *tdcA* had not been associated, either directly or indirectly, with adherence and colonization of APEC.

6.2.2 Biosynthesis of LPS and exopolysaccharide has an effect on APEC colonization

The outer membrane of Gram negative bacterial pathogens serves as an excellent interface between the environment and the cell interior. Lipopolysaccharides (LPS) are one of the major constituents of the outer membrane, and are known to be a key factor responsible for the pathogenesis of severe Gram-negative infections (58). Belanger et al. were one of the first to propose that bacterial LPS may also act as an adhesin (8) after which, a number of studies showed that LPS plays a role in adhesion and colonization (58). In LPS of *Enterobacteriaceae*, the core oligosaccharide (OS) links a highly conserved lipid A to the antigenic O-polysaccharide (49). One could therefore assume that mutations in the chromosomal regions containing the major core OS assembly operons would lead to deformation of the outer membrane with an eventual loss of function. One such important chromosomal region involved in assembly of the core oligosaccharide is the *waa* (formerly *rfa*) operon (146). The *waaJ* (*rfaJ*), *waaT* (*rfaJ*) and *waaR* (*rfaJ*) genes of the *waa* operon encode α-1, 2- glycosyltransferases involved in the synthesis of the outer core region of LPS of *E. coli* (102). Previously it has been shown that a *waaR* mutant defective in outer core synthesis was isolated and found to affect cell surface expression of K5 and K1 capsules, probably by altering retention of the capsular polysaccharide on the cell surface (161).

Earlier studies involving STM in a systemic model of APEC infection resulted in the identification of genes involved in LPS biosynthesis essential to systemic infection in chickens, including w*aaW* and w*aaL* (103). The role of LPS in systemic infection could therefore be associated with bacterial resistance to serum complement as has been previously shown (14). Interestingly, we identified three mutants EA16G1, EA10C1 and EA1A1 with mutations in genes involved in polysialic acid capsule synthesis (*kpsF*), LPS 1,2-glucosyltransferase (*rfaJ*) and a putative glucosyltransferase (*yaiP*) respectively, which were all attenuated in a lung colonization model of infection in this study. The *rfaJ* mutant was highly attenuated as compared to the wild type pathogenic strain suggesting its more prominent role in APEC colonization during infection. In comparison with the role of LPS in systemic infection, the lung infection model gives new insight to the role of LPS in colonization of the chicken lung by APEC.

6.2.3 Colonization by APEC is influenced by genes involved in bacterial metabolism

Bacterial virulence is largely influenced by their adaptation to the host environment. In this study, a number of genes were identified, that are associated with transport and other metabolic functions. Four mutants EA1C4, EA7B2, EA10F11, EA11E1 had insertions in

genes whose activity involves sugar transport in the bacterial system, namely *actP*, *xapB*, *pgtP* and *rbsA* respectively, all of which were confirmed to be attenuated in the chicken model of infection. The roles of these genes in APEC virulence have not been previously described; however, it is likely that these genes are indefinitely required by the pathogen for *in vivo* survival in the host during infection. Sixteen genes were further identified, of which five were tested for *in vivo* competition, and mutant EA16H5 (*yfiF*), encoding an RNA methyltransferase was confirmed to be attenuated as determined by competition assays *in vivo*. In a previous study, it was shown that a *yfiF* mutant of *Yersinia pseudotuberculosis*, an important human pathogen was also found to be attenuated in a mouse infection model (35). This gene may contribute to bacterial survival in the host environment, thus potentially influencing initial stages of APEC infection.

6.2.4 Genes of unknown function are potentially required for APEC colonization

Ten mutants were identified with transposon insertions in genes of unknown or putative function of which three were further tested for *in vivo* competition. Mutant EA7A3 had an insertion in gene *yobB* which is a putative amidohydrolase (111), while EA10G11 had an insertion in gene *ydeH* which has a putative conserved domain similar to the Diguanylate-cyclase domain, which is involved in regulating cell surface adhesiveness in bacteria (111). Recently it has been shown that the diguanilate-cyclase *ydeH*, is required for full biofilm upregulation in *E. coli*, in response to translation inhibition (11). Mutant EA7A3 was confirmed to be attenuated in the chicken infection model suggesting the probable role of this novel gene in the initial stages of an APEC infection. Three other mutants EA1A9, EA9F4 and EA2E10 also had insertions in genes of unknown function, namely, *ykgH*, a predicted inner membrane protein (111), *yjcS*, a predicted hydrolase of the beta lactamase family (111) and *yabP*, a gene with unknown function; however, these still need to be confirmed for their attenuation during APEC infection.

6.2.5 STM in a chicken lung infection model leads to the identification of adhesins

Adhesins are known to facilitate host colonization by mediating the earliest interaction with host tissue (175). In certain cases pathogens have been reported to have a substantial amount of different adhesins expressed at one time or another (32). In *E. coli*, two of the best characterized adhesins are the type 1 fimbriae and the P fimbriae which are regulated by the *fim* and *pap* operons (47). As already stated, genome sequencing of prototypic cystitis UPEC strain UTI89 revealed ten different chaperone-usher adhesin systems, among which the putative adhesin *auf*, *yad*, *yfc*, *yqi*, *yeh* and *fml* operons still remain to be characterized (17,

175). Furthermore comparative genome analysis of two UPEC O6 strains 536 and CFT073 revealed the presence of 14 and 13 putative fimbrial operons respectively (16)

Interestingly, the *yqi* adhesin gene found in UPEC strain UTI89, now termed ExPEC adhesin I (EA/I) in this study, and *fimE*, a *gene* encoding the type 1 fimbrial regulatory protein were identified in the STM screen specially designed to detect factors that would be involved in the initiation of APEC infection, which were found to be attenuated in the chicken. Type 1 fimbriae are already well characterized for their role in ExPEC pathogenesis; however, ExPEC adhesin I has until now never been described for its functional role during infection, either in UPEC or in APEC. Therefore, the *yqi* gene was further studied for its role in the initiation of APEC infection.

6.3 ExPEC adhesin I, a novel putative adhesin plays a role during APEC infection

The newly identified putative fimbrial adhesin encoding the gene *yqi*, will be temporarily called ExPEC adhesin I (EA/I) until the specific host receptor for this adhesin has been identified in order to allow better classification and nomenclature of the novel *E. coli* fimbrial adhesin. EA/I was found to show sequence identity with the *yqi* gene present in UPEC strain UTI89 (Accession: CP000243) and is located on a ~5 kb adhesin gene cluster yet uncharacterized. On sequencing the *yqi* adhesin gene cluster, we observed a resemblance to chaperone-usher fimbrial adhesin systems in ExPEC (Fig. 23). Fimbrial adhesins share common genetic organization, in that, the adhesin regulatory genes precedes the major subunit gene, which is followed by the periplasmic chaperone, outer membrane usher, and finally the adhesin genes (175). This gene cluster organization is seen in type 1 fimbriae (*fim*), S fimbriae (*sfa*), F1C fimbriae (*foc*) and the Dr-antigen recognizing fimbriae (*dra*) (175). Organization of the *yqi* adhesin gene cluster differs by having the positions of the usher and chaperone inverted, which is also true for the *pap* adhesin gene cluster of the P fimbriae (175), although the reason for this is still unclear.

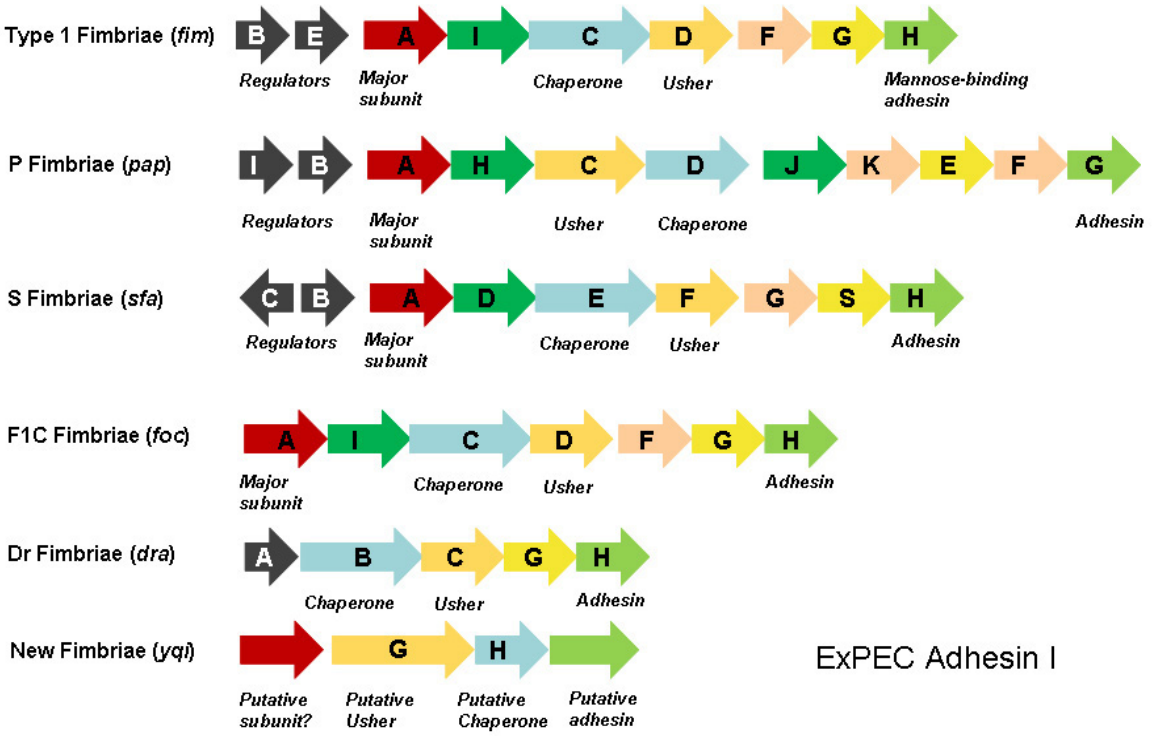


Figure 23: Schematic representation of chaperone-usher fimbrial adhesin systems in ExPEC.

To date there are no studies implying the role of either the UPEC or APEC adhesin coded by the *yqi* gene in colonization of host tissues during infection. Previously, it has been shown that the *fimH* adhesin of the type 1 fimbriae, and the *papG* adhesin of the P fimbriae are the receptor specific binding proteins that are located at the tip of the pilus structure (89). It has also been reported that deletion of the *papG* gene had no effect on pilus formation; however, the pili isolated from a *papG* deletion strain were not adhesive (67). This study now provides evidence which shows that deletion of the *yqi* adhesin gene in APEC strain IMT5155 results in a significant decrease in the adhesion to chicken fibroblasts and epithelial cells *in vitro* and reduction in colonization of the chicken lung *in vivo*. It is possible that the Yqi adhesin is the receptor-specific protein responsible for initial attachment to host cells; however, this receptor is unknown and still to be identified. One of the ways to identify this receptor in future experiments is by haemagglutination inhibition tests as described previously (37). A number of compounds known to be receptor structures for different bacterial fimbriae like Galactose α- (1-4)-galactose, glycophorin A, N-acetyl-glucosamine, N-acetylneuraminic acid, N-acetylneuraminlactose, fucose, fetuin, asialofetuin, α-acid glycoprotein (orosomucoid), gelatin, collagens type I, II, IV and V, chloramphenicol, Trihexosylceramide, globoside, fibronectin and laminin could be tested as putative inhibitors of mannose-resistant haemagglutination (MRHA) (37). Furthermore, binding of bacteria to glycolipids either separated by thin-layer chromatography (TLC) or immobilized on microtiter plates has also

been described as a convenient way of detecting and characterizing carbohydrate receptors, which was the method used for the identification of the F1C fimbriae receptor (75). More recently, a new method has been described for the rapid detection and analysis of protein-protein interactions known as SPINE (Strep-protein Interaction Experiment) (51). The adhesin of interest would have to be co-purified with the putative receptor protein followed by mass spectrometry in order to identify the specific receptor (51).

APEC strain IMT5155 has been previously tested for the presence of important adhesins like *fim*, *pap*, *csg*, *sfa*, *tsh*, *afa*, *dra*, *foc* and others, and has been found to harbour only type 1 fimbriae (*fim*), curli (*csg*) and temperature-sensitive haemagglutinin (*tsh*) genes (30). The absence of P, F1C, S, Dr fimbriae in IMT5155, which are known for their role in UPEC pathogenesis (175), made it apparent that there might in fact be unidentified adhesins that participate during initiation of infection. It was observed that in both infection models used in this study, there always was a significant reduction in numbers of viable bacteria in the chicken lung when chickens were infected with an APEC strain IMT5155Δ*yqi* lacking the adhesin. Frommer et al reported many years ago that piliation may be a critical pre-colonization factor that determines whether an *E. coli* strain will gain access to the host and cause infection (34). Our results provide initial evidence that ExPEC adhesin I (EA/I) plays a role in the colonization of the chicken lung by APEC strain IMT5155 during infection. It is possible that EA/I adhesin makes up for other adhesins that are absent, and which are usually known to be required for pathogenesis, which in turn validate the results obtained in this study.

It was also observed that there was only a slight reduction in bacterial numbers isolated from internal organs of chickens when infected with IMT5155Δ*yqi* in the systemic infection model. This is not unique, because IMT5155 harbours a number of known virulence genes like iron acquisition genes (*chuA*, *fyuA*, *ireA*, *sitD*), serum resistance genes (*iss*, *ompA*, *traT*) and invasins (*gimB*, *ibeA*) which play a role in the initiation and prolongation of disease and which may on the whole contribute to systemic infection in chickens (30). Besides, with an infection dose of 10^9 CFU as used in the systemic infection model, there is very little room left for specific adhesion to take place in contrast to the lung infection model as already described. This could also be a reason for the larger numbers of bacteria in internal organs during systemic infection.

Complementation of the *yqi* adhesin gene was achieved *in vitro*, where the strain regained its ability to adhere to canine kidney epithelial cells, which confirms the adhesive function of EA/I and rules out the possibility of a potential polar effect. It was not possible to demonstrate complementation in the chicken infection model, which could be due to lack of expression of the adhesin protein *in vivo*, since it was rather difficult to induce the promoter during the 24 h infection period. The use of a more suitable vector in the future may eventually provide these

data. Additionally, the use of a different assay *in vivo* like Fluorescence in situ Hybridisation (FISH) (120) which would enable studying adhesion of APEC to the chicken lung, may be more suitable for testing the complemented mutant. Additionally this kind of assay, would also give preferred evidence about the role of ExPEC adhesin I during adhesion and attachment of APEC to the chicken host cells.

An interesting result was the distribution of the ExPEC adhesin I (*yqi*) gene among a relatively large collection of strains, particularly its high incidence among ExPEC strains like APEC, UPEC and NMEC strains, infrequent occurrence among non pathogenic strains and complete absence among intestinal pathogenic *E. coli* strains. Although the incidence of *yqi* among ExPEC strains only reaches a maximum of 65%, this figure is important when considering the phylogenetic background of these isolates. It has been previously reported that distinctive strains of *E. coli* responsible for most cases of urinary tract infection, sepsis and newborn meningitis are derived predominantly from *E. coli* phylogenetic group B2 (63). Additionally, diverse studies show that virulent clonal groups are derived mainly from phylogenetic group B2 and to a lesser extent from group D (30, 62). Our observations show that 70.8% of the isolates positive for *yqi* belong to the phylogenetic group B2 in contrast to only 17.0% of isolates negative for *yqi* belonging to this group. Furthermore, we found that all of the isolates belonging to sequence types 95, 140 and 141 were positive for *yqi*. It is known that famous pathogens are associated with specific sequence types (STs) or sequence type complexes (STCs), for example, ST95 complex contains related pathogenic bacteria of serogroups O1, O2, and O18 that express the K1 polysaccharide, that is , the K1 invasive isolates known to be associated with septicaemia (171, 173). Of interest, therefore, was the evolutionary analysis of the ExPEC adhesin I gene (*yqi*) sequence from strains belonging to different sequence types, which showed that within a particular sequence type complex (STC), the adhesin sequence showed complete sequence homology which is the case for STC12 and STC73, as well as other sequence types like ST372, ST141 and ST358 with a single exception, strain IMT15008. Since this strain had no unique characteristic that differentiated it from other strains within the ST73 complex, a possible explanation could be that the *yqi* gene in strain IMT15008 is the result of a recombination event.

Interestingly, within the ST95 complex, the adhesin gene sequence showed the presence of single nucleotide polymorphisms (SNPs) which resulted in amino acid substitutions in the gene, and which were confirmed as a positive selection on the gene within this complex as already described in the results section. Mutations producing functional modifications are called pathogenicity-adaptive or pathoadaptive, and are often SNPs producing amino acid replacements in proteins essential for a pathogen's success, and it has been shown that two major adhesins of extraintestinal pathogenic *E. coli* (ExPEC) – type 1 and P fimbrial adhesins – acquire structural SNPs at a rapid rate, and this adaptation constitutes a major factor in the

pathoadaptive microevolution and genetic diversification of groups of clonally related ExPEC strains (171). It has also been reported that the amino acid sequences of FimH, the type 1 fimbrial adhesin, exhibit several mutations considered as potentially pathoadaptive for UPEC (55, 157). It is possible, that ExPEC adhesin I also undergoes structural mutations or pathoadaptive mutations, particularly, among strains belonging to the ST95 complex, which further confirms the importance of this adhesin within this highly pathogenic complex.

Therefore, taken together, it is likely that *yqi* is associated with highly pathogenic ExPEC strains, and may serve as a predictor of this group of isolates. ExPEC adhesin I could perhaps play a very specific role in ExPEC pathogenesis, and we have evidence of the potential role of the adhesin during APEC infection; however, the same still needs to be functionally proven for UPEC and NMEC strains. However, an interesting addition to this story is the decrease in adhesion to kidney epithelial cells as shown by *in vitro* adhesion experiments. This may indicate the importance of *yqi* in UPEC, which particularly colonize the urinary tract including the kidney epithelium and bladder epithelium during urinary tract infection (UTI), thus extending the field of study of ExPEC adhesin I beyond APEC infection. Furthermore, it provides evidence for the zoonotic potential of APEC, a topic of great interest in the field of ExPEC.

The presence of a single virulence factor hardly ever makes a strain virulent, while a combination of virulence factors usually determines its ability to cause disease (118). ExPEC probably have to possess specific genes to outcompete commensals during intestinal colonization and to adapt to different specific niches encountered during infection (16). Therefore much like countless other genes that are classified into the class of virulence factors when exceedingly prevalent among pathogens, the ExPEC adhesin I gene (y*qi*) could also eventually be an addition to this category based on its regular presence in highly pathogenic strains in contrast to non-pathogenic strains. One must keep in mind, that the meager group of A_{faecal} strains that were found to be positive for *yqi* in this study are no classical non-pathogenic strains as seen in a previous study (28). Ewers et al reported that a number of A_{faecal} *E. coli* strains have characteristics typical of human and animal ExPEC, and that some nonoutbreak strains are capable of causing systemic disease in immunocompetent 5-week old chickens, suggesting the avian intestine reservoir hypothesis (28). ExPEC can stably colonize the host intestine, but in contrast to intestinal pathogenic *E. coli*, host acquisition of an ExPEC is not sufficient to cause infection, and instead, bacteria have to reach an extraintestinal site of the host (16). Brzuszkiewicz et al reported that since extraintestinal colonizing sites are unlikely to provide a selective advantage in terms of transmissibility, so-called “extraintestinal virulence factors” have probably evolved to enhance surivival in the gut, and transmission between hosts, and therefore will be shared with at least some commensal strains (16). Furthermore it was reported that these strains pose a

zoonotic risk because they could be transferred directly from birds to humans or serve as a genetic pool for ExPEC strains. Therefore, as already mentioned, ExPEC adhesin I may serve as a predictor of pathogenic isolates, among commensals isolated from the intestinal tract.

Expression of the adhesin protein was achieved by constructing a fusion protein with a known tag, GST. Induction of the fusion protein resulted in strong expression of the protein *in vitro*, which was not seen in non-induced bacterial cultures. Purification of the fusion protein was also successfully carried out using an affinity matrix glutathione sepharose. However, the purified protein yield was fairly low, when compared with the expressed protein in whole cell lysates. Purification of adhesins is generally difficult because most adhesins are proteolytically degraded when expressed as independent moieties (174). It is likely that the GST:*yqi* fusion protein yield could be increased by optimizing culture conditions, in particular incubation temperature and induction conditions. Furthermore, expression of the adhesin protein together with the chaperone protein may allow better purification of the adhesin, as in chaperone-usher fimbrial systems, the adhesin is transported through the bacterial membrane when bound to the chaperone protein (57).

In order to purify this protein for specific antibody production, higher concentrations of the protein are required. However, since we were not successful in purifying the adhesin protein in large amounts and obtaining specific antibody against EA/I in this study, we resorted to different means of visually detecting this novel fimbrial adhesin using electron microscopy. We hypothesized that cloning of the putative adhesin gene cluster coding for the putative subunit protein, putative usher and chaperone proteins and putative adhesin (Fig. 23), which together are believed to be responsible for expression of fimbrial structures, when successfully cloned in an afimbriate *E. coli* strain would enable the expression of EA/1 fimbriae visible under the electron microscope. Using negative staining and transmission electron microscopy, our hypothesis was confirmed, in that, short fimbrial like structures were detected in the afimbriate strain overexpressed with the adhesin gene cluster, which were not observed in the afimbriate strain, or negative control. These structures will have to be confirmed using specific antibody and immunogold staining in the future; however, this study provides preliminary evidence for the fimbrial structures of this newly identified fimbrial adhesin. ExPEC adhesin I is therefore confirmed to be a novel fimbrial adhesin, worthy of further studies, which will indeed enable a better understanding of the interactions between adhering ExPEC and their host cells.

6.4 The *in vivo* infection model influences the types of genes identified by STM

The negative selection system plays a central role in the STM technique, and therefore, the screening systems of choice are *in vivo* systems which have a high selection pressure (154). *In vivo* systems are known to be broadly used and applied in STM studies.

In a previous study in our lab, STM was used to identify genes that were essential for the survival of APEC systemically inside its host, the chicken (103). This study was the first of its kind involving infection of the chicken respiratory tract by *E. coli* using a well established chicken systemic infection model. Genes involving LPS (*waaW*, *waaL*), capsule (*kpsM*, *kpsS*) and iron acquisition systems (*sitB*, *chuA*) among others, were found to be important as systemic infection-associated virulence determinants in APEC (103). In addition, a number of novel genes and regulators were identified and found to play a role in the pathogenesis of APEC for the first time *in vivo* (103).

Interestingly, no adhesins or colonization factors, particularly pili or fimbriae of any kind were identified using the systemic infection model. On the other hand, the present study made use of a lung colonization chicken model as already described in detail, in order to identify genes specific to early stages of infection. Pertaining to the theory of a stage-specific model being used to identify stage-specific genes involved in infection, it was possible to identify adhesins, including a well characterized fimbrial protein, and a novel adhesin, genes not previously captured in the systemic infection model.

Another interesting point is that genes involved in iron acquisition by pathogenic bacteria were not identified in this study, in contrast to these genes being previously identified in the systemic model of infection. The ability of pathogens to acquire iron in the host is an important determinant of both their virulence and the nature of the infection produced. Pathogenic bacteria that cause systemic and disseminating infections have been found to produce greater quantities of siderophores which stimulate microbial growth in low-iron media (139). This could strongly suggest the role of iron acquisition systems in infections leading to sepsis in the host, and hence genes involved in iron acquisition could be said to be specific to an infection stage involving dissemination of bacteria through the entire bloodstream. This can be confirmed by the results obtained in this study, in that, no iron acquisition genes were identified using the lung colonization model of infection, a model restricted to the initial stages of infection.

Genes that were common to both the systemic and lung infection models include those involved in LPS and capsule synthesis. This result is not unexpected, since LPS and exopolysaccharides have been found to play a role in the survival of pathogens within the host, under different stress conditions which would explain its identification in a systemic

infection model. In an earlier study, it was reported that the exopolysaccharide colanic acid, may serve as a protective barrier for the pathogen *E. coli* O157:H7 for its survival in the human gastrointestinal tract, that is, the pathogen is thus able to combat acid stress (110). In another study it was observed that colanic acid confers to *E. coli* O157:H7 a protective effect from the environmental stresses of heat and acid (109). Therefore it is very likely that the exopolysaccharides produced by ExPEC strains would enable them to survive and persist within host tissue during systemic infection. At the same time, a number of studies provide evidence for the role of LPS and exopolysaccharides in biofilm formation and adhesion of pathogens to host cells. Studies with *Salmonella enterica serovar* Typhimurium describe a disruption in biofilm formation in Hep-2 cells and chicken intestinal tissue through a mutation in the colanic acid biosynthetic gene *wcaM* (100). It has also been demonstrated that colanic acid plays a role in adhesion by blocking the establishment specific-binding as well as time-dependent interactions between uropathogenic *E. coli* (UPEC) and inert substrates (46). Thus, it is evident that the LPS of ExPEC strains could under the given conditions enable these pathogens to better adhere to host cells and tissues and initiate infection.

Taken together, these results validate the hypothesis, which assumes that in order to screen for and identify genes involved in a specific stage of infection, particularly colonization of specific host tissue, be it the lung, gastrointestinal tract, urinary tract etc, a model has to be appropriately established targeting these tissue regions in the host, where pathogenic bacteria first make their contact in different infection processes.

6.5 ExPEC adhesin I as a potential vaccine candidate?

The ultimate aim of a vaccine is to produce long-term protective immunity against a pathogen. Since for most bacteria, the first encounter with their host involves attachment to a eukaryotic cell surface, which results in colonization of the host prior to disease, induced antibody responses at the mucosal surface could prevent attachment and abrogate colonization (174). The ideal target, therefore, for such antibodies are the surface proteins or adhesins which mediate microbial attachment to host tissue (158). This study has identified an adhesin that plays a role in the initial stages of APEC infection.

Previous studies with other adhesins, like the *fimH* adhesin, have shown that antibodies elicited against the adhesin can impede colonization, block infection and prevent disease, that is, prophylactic vaccination with adhesins can inhibit bacterial infections (174). In other studies, it was found that vaccination with Gal-Gal pili or the P fimbrial vaccines prevented pyelonephritis by piliated *E. coli* in a murine model and in monkeys (140, 149). It would indeed be interesting to study the effects of an ExPEC adhesin I immunization in chickens. A way to do this could perhaps be an *in ovo* immunization, a method that has been successfully tried out with other diseases like avian influenza, new castle disease,

coccidiosis and others (22, 114, 160). Studies have shown that *in ovo* vaccination of 18-day-old embryonated chicken eggs provided 90% and 80% protection against a highly stringent lethal challenge with new castle disease virus and H5N1 virus, respectively (160). Furthermore it was shown that *in ovo* oral vaccination with *Campylobacter jejuni* led to the early development of intestinal immunity in chickens (127). In a separate study, vaccination with the *Eimeria tenella* EtMIC2 gene led to protective immunity against infection by *Eimeria acervulina* and *Eimeria maxima* (22). *In ovo* vaccination offers many advantages, among which, protection of chickens from as early as 3 weeks of age (160). This method of vaccination might therefore be useful in attempting to prevent APEC infection, by immunizing chickens with the ExPEC adhesin I protein, thus potentially enabling earlier immunity as compared to post-hatch vaccinations. The only vaccine currently available against APEC infections is the "Nobilis® *E. coli* Inac" vaccine developed and manufactured by Intervet as already described. It is an inactivated vaccine containing the F11-antigen and FT-antigen of *E. coli* per dose in a water-in-oil emulsion. It protects against postnatal colibacillosis, caused by fimbrial F11-antigen and flagellar FT-toxin containing *E. coli*, in broilers. This vaccine has its limitations, particularly because a large group of poultry, namely the egg layers, cannot be vaccinated with the available vaccine. Therefore, it would be of great value to have an alternative vaccine against APEC infections in poultry.

One of the key aspects of proving the potential efficacy of an adhesin-based vaccine *in vivo* is the development of an animal model of disease that relies on bacterial colonization of the mucosal epithelium mediated by the specific adhesin of interest (174). Here, a chicken model has been successfully established which solely allows the colonization of the chicken lung epithelium without inducing systemic infection. This model serves as a suitable challenge model, since it has now been shown that infection with a strain devoid of the newly identified Yqi adhesin, results in decreased colonization of the chicken lung as compared to its wild type counterpart.

Even though vaccination trials in *in vivo* models may seem straight-forward, testing for protection could be difficult for various reasons including high doses of *in vitro* grown bacteria usually required to establish mucosal colonization, the lack of specific glycoprotein receptors for some adhesins, and the difficulty in purifying bacterial adhesins, particularly tip adhesins associated with whole pili (174). It is possible to eventually overcome these problems during future vaccination trials with ExPEC adhesin I due to the following reasons: the lung infection model requires a much lesser infection dose of bacteria in order to allow colonization of the lung, as compared to the infection dose required for a complete systemic infection. Though the specific receptor of the Yqi adhesin is not yet known, the chances of the receptor being present on the lung tissue are good, which can be assumed from the fact that there are differences in colonization of bacterial strains harbouring and lacking the Yqi adhesin

respectively. Finally, the Yqi adhesin has been expressed *in vitro* and successfully purified on a small scale, which confirms the potential of this adhesin to be purified on a larger scale in appropriate amounts for the purpose of specific antibody production and for use as an antigen in vaccination studies.

6.6 Outlook

Signature-tagged mutagenesis (STM) of a highly virulent APEC strain IMT5155, led to the identification of many interesting candidate genes, which were found to play a role in the colonization of the chicken lung during APEC infection. To date, no whole genome sequence of APEC strain IMT5155 is available to screen for putative genes that might be essential during the infection process. Therefore, a screen making use of a well known molecular method like STM has proven to be successful in the identification of novel genes not described previously for their role in APEC infection. Furthermore, no genome sequence analysis can replace the *in vivo* functional data of important genes obtained from an STM analysis in the natural host.

A number of genes of interest of APEC were identified and confirmed to be attenuated in the natural host, the chicken. These include genes encoding adhesins, genes involved in LPS and capsule formation and those involved in bacterial transport and metabolism. In addition genes involved in regulation have also been identified in the study. It would be interesting to know what functional role these genes play during APEC, or even ExPEC infection. Deletion of the candidate genes and testing of the isogenic mutants in the natural host, that is, chicken lung infection model that has already been established would be one of the ways to identify the role that these genes play in early stages of APEC infection, by studying the adhesion and colonization abilities *in vivo*. Furthermore it would be interesting to find out whether these genes are involved in the regulation of known and unknown adhesins of APEC which could be achieved by carrying out microarray analyses and over-expression studies *in vitro*. All these data will help us to understand various aspects of infection caused by ExPEC strains, many of which remain unclear to date, making the functional characterization of these newly identified genes extremely important for future studies.

One of the findings in this study which was of most interest to us was the novel adhesin termed ExPEC adhesin I (Yqi), since adhesins affect the initial stages of APEC infection, namely adhesion and colonization, this is the first report of the role of ExPEC Adhesin I during early stages of APEC infection. It is one of many adhesins not characterized till date for its role in pathogenesis of infection, be it APEC, UPEC or NMEC infection. The initial steps have now been taken to describe this adhesin and its function, and deletion of the adhesin in IMT5155 has led to decreased adhesion ability of the strain (2). It has been determined, that the Yqi adhesin is associated with highly pathogenic ExPEC isolates of

different sources, as compared to non pathogenic isolates. Furthermore the adhesin gene was found to be absent in all of the intestinal pathogenic *E. coli* strains tested.The prevalence of Yqi among a few non-pathogenic isolates has helped in the identification of potential virulent strains among the A_{faecal} isolates.

Most adhesins function by binding to a specific receptor on the host tissue during infection as already described. Therefore, a single pathogen may make use of multiple adhesins, with affinities to different specific receptors which enable the pathogen to adapt to different host tissues during initiation of infection. The receptor for ExPEC adhesin I is still unknown, and the identification of such a receptor would indeed be of great value in future prevention therapies, for example, by blocking of the receptors to prevent the pathogen from attaching to host surfaces. In addition it will also allow for appropriate nomenclature of this adhesin in the future.

Finally, all results point to the fact that ExPEC adhesin I could possibly be a good vaccine candidate against APEC infections among poultry as already described, and could moreover be used to identify strains belonging to highly virulent phylogenetic groups. It would be worthwhile to carry out for vaccination and challenge assays using the purified adhesin protein. Such a study might in fact be very promising, in that, it moves a step closer to the ultimate goal of any research in the field of infectious pathogens, namely the prevention of infection and disease.

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List of Publications

Part of this work has been published

Publications:

1. **Antão EM** et al. The Chicken as a natural model for extraintestinal infections caused by avian pathogenic *E. coli* (APEC). *Microb. Pathog.* 2008
2. **Antão EM** et al. Signature-tagged mutagenesis in a chicken infection model leads to the identification of a novel avian pathogenic *E. coli* Fimbrial adhesin. *PLoS ONE.* 2009
3. **Antão EM** et al. Adhesive threads of extraintestinal pathogenic *E. coli*. *Gut Pathogens* 2009

Poster Abstracts and Scientific Talks:

1. **Antão EM**, Ewers C, Diehl I, Gröbler L, Li G and Wieler LH. Contribution of a putative adhesin to virulence of avian pathogenic *E. coli* (APEC). *EMBO-FEMS-Leopoldina Symposium, "E.coli - Facets of a versatile pathogen", Kloster Banz, Bad Staffelstein, Germany*. 2007 (Poster)
2. **Antão EM**. Signature-tagged mutagenesis of avian pathogenic *E. coli* (APEC) identifies genes essential to a chicken lung infection. *Berliner Doktoranden Symposium 2007, FU Berlin*. 2007 (Talk)
3. **Antão EM**, Li G, Gröbler L, Ewers C and Wieler LH. Identification of genes essential to an avian pathogenic *E. coli* (APEC) lung infection. *2nd Annual Symposium on Host Pathogen interactions, Berlin*. 2007 (Poster)
4. **Antão EM**, Li G, Ewers C and Wieler LH. Avian pathogenic *E. coli* and Signature-tagged mutagenesis: Understanding virulence better. *Berliner Doktoranden Symposium 2006, FU Berlin*. 2006 (Poster)
5. **Braganca EM**, Li G, Alt K, Laturnus C, Ewers C and Wieler LH. Molecular interactions between avian pathogenic *E. coli* (APEC) and the avian lung during APEC infection. *1st Annual Symposium on Host Pathogen interactions, New York*. 2005 (Poster)

Additional Co-author Publications

1. Wieler LH, **Antão EM**, Semmler T. Research on Zoonoses: Central element of the “One-Health” initiative. *Berl. Münch. Tierärtzl. Wochenschr*. 2009
2. Ewers C, **Antão EM**, Diehl I, Philipp HC, Wieler LH. Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. *Appl. Environ. Microbiol.* 2009

3. Li G, Ewers C, Laturnus C, Diehl I, Alt K, Dai J, **Antão EM**, Schnetz K and Wieler LH. Characterization of a *yjjQ* mutant of avian pathogenic *E. coli* (APEC). *Microbiology* 2008
4. Ewers C, Li G, Wilking H, Kiessling S, Alt K, **Antão EM**, Laturnus C, Diehl I, Glodde S, Homeier T, Böhnke U, Steinrück H, Philipp HC and Wieler LH. Avian pathogenic, Uropathogenic and newborn meningitis-causing *E. coli*: how closely related are they? *Int. J. Med. Microbiol.* 2007

Abbreviations

aEPEC	Atypical Enteropathogenic *E. coli*
afa	Afimbrial adhesin
APEC	Avian pathogenic *E. coli*
Bfp	Bundle forming pili
BHI	Brain Heart Infusion
bp	Base pairs
BSA	Bovine Serum Albumin
Cat	Chloramphenicol acetyl transferase
CFU	Colony Forming Unit
CI	Competition Index
Cnf	Cytotoxic necrotising factor
ColV	Colicin V
Csg	curlin subunit gene
CSPD	Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricycle [3.3.1.1 3,7] decan}-4-yl) phenyl phosphate
CTAB	Cetyltrimethylammoniumbromide
DAF	Decay accelerating factor
DIG	Digoxigenin
DMSO	Dimethyl Sulphoxide
Dn	Non-synonymous mutation
DNA	Deoxyribonucleic Acid
dNTP	Deoxy Nucleotide-tri-phosphate
Dra	Dr antigen-specific adhesin
Ds	Synonymous mutation
EAEC	Enteroaggregative *E. coli*
EA/I	ExPEC Adhesin I
EcoR	*E. coli* Reference Collection
EDTA	Ethylenediaminotetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
FCS	Foetal Calf Serum
Foc	Fimbriae of serotype 1C

Fyu	Ferric yersinia uptake
Gim	Genetic island associated with newborn meningitis
GST	Glutathione-S-Transferase
h	Hours
Hly	Haemolysin A
Hra	Heat-resistant agglutinin
Ibe	Invasion of brain endothelium
IFC	Inab-Freiberger Cromer
Iha	Iron-regulated-gene homologue adhesin
IPTG	Isopropyl-thio-ß-D-galactopyranoside
Ire	Iron-responsive element
Irp	Iron-repressible protein
Iss	Increased serum survival
Kan	Kanamycin
kb	Kilobase
LB	Luria-Bertani
LEE	Locus of Enterocyte Effacement
LMP	Low Melting Point
LPS	Lipopolysaccharide
M	Molar
Mat	Meningitis-associated and temperature-regulated fimbriae
MDCK	Madin-Darby Canine-Kidney
Mins	Minutes
ml	Millilitre
MLST	Multilocus Sequence Typing
MOI	Multiplicity of Infection
MRHA	Mannose-resistant Haemagglutination
Nal	Nalidixin
Nfa	Non-fimbrial adhesin
NMEC	Newborn Meningitic *E. coli*
OMP	Outer Membrane Protein
PAGE	Polyacrylamide Gel Electrophoresis
Pap	pyelonephritis-associated pili
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Proof Reading Polymerase
pMol	Picomole

RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
Secs	Seconds
SePEC	Septicaemia associated *E. coli*
Sfa	Sialic acid (S)-fimbriae adhesins
Sit	Salmonella iron transport system gene
SNP	Single Nucleotide Polymorphism
SOC	Super Optimal Broth with Catabolite repression
SSC	Standard Sodium Citrate
ST	Sequence Type
STC	Sequence Type Complex
STEC	Shiga Toxin-producing *E. coli*
STM	Signature-tagged transposon Mutagenesis
TBE	Tris-borate-EDTA
TBS	Tris-buffered Saline
TBST	Tris-buffered Saline with Tween 20
TE	Tris EDTA
TEMED	N, N, N', N'-tetramethylenediamine
Tra	Transfer protein
TRIS	2-Amino, 2-hydroxymethyl-1,3-propandiol
Tsh	Temperature sensitive haemagglutinin
U	Units
µl	Microlitre
UPEC	Uropathogenic *E. coli*
UTI	Urinary Tract Infection
Vat	Vacuolating autotransporter toxin

Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde.

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