3 Methods

3.1 Cloning and propagation of DNA in *E. coli*

3.1.1 Bacterial culture

E. coli cultures were grown in LB medium supplemented, if necessary, with antibiotics. The cultures were grown at 37°C for 12-16 hrs, with shaking at 200 rpm.

Addition of antibiotics

Ampicillin (Amp)	50 µg/ml	Stock solution	50mg/ml in H_2O
Kanamycin (Kan)	50 µg/ml	Stock solution	50mg/ml in H_2O
Nalidixic acid (Nal)	30 µg/ml	Stock solution	30mg/ml in H_2O

3.1.2 Glycerol stocks

For long-term storage of bacterial strains, bacterial pellets were resuspended in 50% sterile glycerol/50% LB and kept at –80°C.

3.1.3 Preparation of competent cells

A single colony of bacteria from LB agar plate was inoculated into 20 ml LB, grown at 37°C with moderate shaking (200 rpm) to an OD600 = 0.2 to 0.8. The culture was inoculated into 100 ml LB and grown to an OD600 = 0.6. 100 ml of the culture were then inoculated into a sterile 2-liter flask containing 400 ml LB. As soon as the bacteria reached an OD600 = 0.6, the flask was put on ice to quickly cool the bacteria and stop further growth. The bacterial suspension was centrifuged in a GSARotor for 15 min at 4000 g (4°C). After discarding the supernatant, the bacterial pellet was resuspended carefully in 100 ml 0.1 M $CaCl_2$ solution and kept on ice for 15 min. This centrifugation was repeated, and the pellet resuspended in 0.1 M $CaCl_2$ solution and aliquoted (200 µl aliquots) into ice-cold Eppendorf tubes. The bacteria were frozen and stored at -80°C. Competency of the bacterial cells

was assessed by using 10 ng pUC19 to transform 100 µl of competent cells. Aliquots (1, 10, 25 µl) of the transformation culture were plated on LB/ampicillin agar plates and incubated at 37°C overnight.

3.1.4 Transformation of competent *E. coli*

An aliquot of 200 µl of chemical competent *E. coli* was thawed on ice, 2-5 µl plasmid or 10-20 µl of ligated DNA was added to the cells, mixed and kept on ice for 30 min. Then, bacterial cells were heat shocked by placing the tubes into a 42°C water bath for 90 seconds and left on ice for 5 min. 0.8 ml LB-medium was added to each tube. The tubes were placed on a roller drum at 200 rpm for 1 hr at 37°C. Aliquots of transformed cultures were placed on LB with appropriate antibiotic-containing plates, and incubated at 37°C for 12-16 hrs.

3.2 Plasmid isolation from *E. coli*

3.2.1 Mini-preparation plasmid isolation

1.5 ml of cells from mini culture was centrifuged (3500 g/10 min /RT) and the pellet was resuspended in 100 µl of solution I. 200 µl of solution II was added into each tube. After the addition of 150 µl of solution III buffer into the tube, the cellular debris were centrifuged (15.000 g/40min/RT) and the supernatant of each preparation was precipitated with 2.5 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate. After washing the DNA pellet with 70% ethanol, the DNA pellet was air dried and resuspended in 50 µl TE buffer (pH 8.0).

3.2.2 Preparation of plasmid DNA by lysis with SDS

The large virulence plasmids of IMT5155 were isolated by lysis with SDS. This method has advantages over others when dealing with large plasmids. 1 ml of late log-phase culture grown from a single colony was inoculated into 30 ml LB and the culture was incubated with vigorous shaking until the OD value reached 0.6. 25 ml of culture was

inoculated into 500 ml LB and incubated for 2.5 h at 37ºC with vigorous shaking. The bacterial cells were harvested by centrifugation at 2.700 g for 15 min at 4ºC and resuspended in 200 ml ice-cold STE. Again, the bacterial pellets were collected by centrifugation and then stored at –20ºC. To lyse bacteria, the frozen bacterial pellets were thawed at room temperature for 5-10 min and resuspended in 10 ml ice-cold Tris-sucrose solution. 2 ml of a freshly prepared lysozyme solution was added followed by 8 ml of 0.25 M EDTA solution addition (pH 8). The suspension was mixed by gently inverting the tube several times and kept on ice for 10 min. After addition of 4 ml of 10% SDS, the content of tubes was immediately mixed with a glass rod so as to disperse the solution of SDS evenly throughout the bacterial suspension. 6 ml of 5 M NaCl was added and the contents of tube were mixed once more and then placed on ice for at least 1 h. The high-molecular-weight DNA and bacterial debris were removed by centrifugation at 71.000 g for 30 min at 4ºC. The supernatant was precipitated with 2.5 volumes of absolute ethanol. After washing the DNA pellet with 70% ethanol, the DNA pellet was air dried and resuspended in 3 ml of TE buffer.

3.3 Isolation of genomic DNA

Bacterial chromosomal DNA for cloning and southern-blotting was prepared by using 'Master Pure Genomic DNA Purification Kit' (Epicentre Thechnologies) as recommended by the vendor.

A simple and fast method called "heat-lysis" was also used in this study to obtain template DNA for PCRs. A colony from an overnight cultured LB agar dish was inoculated into 5 ml LB media. Following overnight incubation at 37ºC, 50 µl bacterial culture was added into 1.500 µl of distilled water in a 1.5 ml tube. The mixed solution was incubated at 100ºC for 10 min in a water bath. After centrifugation at 15.000 g for 14 min, the supernatant was transferred into a new tube.

3.4 DNA analysis

3.4.1 DNA purification through phenol extraction and ethanol precipitation

The DNA to be purified was dissolved in TE, treated twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and finally with 1 vol. chloroform/isoamyl alcohol (24:1) to remove any remaining phenol in the watery phase. The upper TE phase was precipitated with 2.5 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2) at –80°C for 30-60 min. The pellet obtained after centrifugation at 15.000 g for 20 (see above) min was air-dried and resuspended in TE buffer.

3.4.2 DNA quantification

The concentration of a DNA solution in TE can be calculated through measuring DNA at OD_{260}. TE was used as the blank value. DNA concentration was calculated according to the formula 1 OD_{260} = 50 µg / ml DNA. Concentration of oligonucleotides was measured at OD_{260} and calculated as follows: ε260 (A) = 15.0; ε260 (G) = 1.8; ε260 (C) = 8.8 ; ε260 (T) = 7.4 ; Σε260 (oligonucleotide) = 1 µmol/µl

3.4.3 Agarose gel electrophoresis

1-4% agarose gels in TBE buffer were used to separate DNA. Agarose was suspended in TBE buffer and boiled in a microwave oven until the agarose was totally dissolved in the buffer. 0.5 µg/ml ethidium bromide was added to the agarose solution at about 60°C. The gel solution was poured into an appropriate gel form and left to cool and solidify. 6 x loading buffer was given to DNA dissolved in H_2O or TE and the DNA was loaded on the gel. The gel was run at 5-10 V/cm (length of the gel) in TBE buffer. The separated DNA bands were visible at a wavelength of 366 nm because of the inlay of ethidium bromide into DNA.

3.4.4 DNA extraction

DNA fragments were excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colourless tube and 3 volumes of buffer QG were added to 1 volume of gel (100 mg ~ 100 µl). The tube was incubated at 50°C for 10 min (or until the gel slice was completely dissolved). The gel was dissolved by vortexing the tube every 2 – 3 min during the incubation (after the gel slice has dissolved completely, the colour of the mixture should be yellow). 1 volume of isopropanol was added and the tube was mixed well. A QIAquick spin column was placed in a provided 2-ml collection tube. The sample was applied to the column for DNA binding and centrifuged for 1 min (3.000 g). The flow-through was discarded and the column was placed on the same tube again. To wash the column, 0.75 ml of buffer PE was added and the column in tube was centrifuged for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min. The column was then placed in a clean 1.5-ml tube. 50 µl of elution buffer EB (10 mM Tris-HCl, pH 8.5) or H_2O was added to the center of the column and the column was centrifuged for 1 min at maximum speed. Alternatively, for high DNA concentration, 30 µl of elution buffer was added to the center of the column and the column was centrifuged at maximum speed for 1 min.

3.4.5 DNA digestion

Purified DNA was digested by restriction endonuclease with their corresponding buffers. If necessary, BSA was added to the reagents. The digest was incubated at 37°C for 1-2 hrs and loaded onto an agarose gel after adding 6 x loading buffer.

3.4.6 DNA dephosphorylation

Restriction cloning vectors were dephosphorylated (Manitatis, 1982) with calf intestinal alkaline phosphatase (CIAP; Boehringer-Mannheim). 10 µg linearized plasmid DNA was dissolved in 90 µl 50 mM Tris-Hcl (pH 8.0) and incubated with CIP (0.1 units / µg) for 1 h at 37°C. Afterwards 1.0 µl of 0.5 M EDTA was added and the reaction was inactivated at 75°C for 10 min. Dephosphorylated DNA was then purified from agarose gel (3.4.4).

3.4.7 DNA ligation

5 µl vector DNA, 15 µl insert DNA, 6 µl T4-ligase-buffer, 1 µl T4-ligase, and 3 µl distilled H_2O were mixed and incubated at 16°C over night. 15 µl of ligated mixture was transformed into competent cells of *E. coli* DH5α and selected for antibiotics resistance. The transformation was performed as described in 3.1.3 and 3.1.4.

3.5 Polymerase Chain Reactions (PCR)

To detect *col*V and *met*V-island associated sequences, PCR with purified genomic DNA from *E. coli* or unpurified genomic DNA from *E. coli* (3.3) were performed with *Taq* DNA polymerase (10 Units/µl), primers are listed in *Materials*. PCR with ddH_2O were always used as controls.

The PCR reaction:

1.0 µl	10 × PCR buffer
1.5 mM	$MgCl_2$ (50mM)
0.2 mM each	dNTPs
0.5 µM	Primer each (10 pM)
0.05 U/µl	*Taq* polymerase
0.2 ng/µl	Template DNA
ad 20 µl	ddH_2O

The PCRs are performed as followed:

94 ºC	5min	1×
94 ºC	30s	
50-65 ºC	30s	30×
72 ºC	1min	
72 ºC	7min	1×

3.6 DNA labelling

Labelling was performed as recommended by the vendor's protocols utilizing the 'PCR DIG Probe Synthesis Kit'. PCR was performed as described in 3.5. An agarose gel electrophoresis was run to confirm if the labelling was successful. To purify the labelled probes, DNA fragments from the gel were isolated by 'Agarose Gel DNA Extraction Kit'.

3.7 Southern blotting

3.7.1 DNA transfer

Southern-blotting includes the transfer of DNA fragments from an electrophoresis gel onto a membrane. The transfer results in immobilization of DNA fragments, leading to a semi-permanent reproduction of the banding pattern of the gel. After immobilization, DNA can be subjected to hybridization analysis, enabling the identification of bands with sequence similarity to a labelled probe. Hybond N+-membrane, a positively charged nylon membrane, is ideally used for hybridization.

The agarose gel was rinsed in distilled water and placed in a clean glass dish containing 10 gel volumes of 0.25 M HCl where it was shaken slowly for 6 min at room temperature. HCl was discarded and the gel was rinsed with distilled water. The gel was then shaken in 10 volumes of 0.4 M NaOH for 40 min to denature the DNA. A sponge, with a size slightly larger than the gel, was placed in a plastic dish. The dish was filled with enough 0.4 M NaOH to leave the soaked sponge about half-submerged in buffer. Three pieces Whatman paper were cut and placed on the sponge. The gel was transferred onto the Whatman paper and air bubbles were squeezed out by rolling a glass pipet over the surface. A piece of Hybond N+-membrane was placed on the gel and 3 pieces of Whatman papers were put on the membrane, confirming non existence of air bubbles between membrane and gel. Paper towels were piled up on Whatman papers and a glass plate on top of the pile. A weight was placed on the top to hold everything in place. It was left overnight, disassembled and the membrane was rinsed with 2 x SSC the next day.

3.7.2 Hybridization of oligonucleotides

The membranes for southern blotting were prehybridized for 2h in 10 ml hybridization buffer at 68 ºC, and the denatured probe was then added and allowed to hybridize at 68ºC for 16h. Membranes were subsequently rinsed in stringent buffer I twice for 5 min each. Two further high stringency washes were performed in 100 ml of prewarmed Stringent buffer II at 68ºC for 15 min.

3.7.3 Visual evaluation

After post-hybridization washes, the membranes were equilibrated in washing buffer 1 for 1 min to allow the chemiluminescent substrate to come to room temperature. The membranes were then blocked by gently agitating it in blocking solution for 30 min using washed dishes. After pouring off the blocking solution, membranes were incubated in antibody solution for 30 min, containing Anti-Digoxigenin-AP diluted 1:10.000 in blocking solution. After washing twice in washing buffer 1 for 15 min each, the membranes were equilibrated in detection buffer for 2 min and finally processed with CSPD solution detection. Blots were exposed to X ray films for visual evaluation.

3.8 Selection of the tags and construction of the APEC mutant library

A pool of tagged pUTmini-Tn5*km2* plasmids (Amp^R, Km^R) was kindly provided by David Holden (Imperial College, London, UK). 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 (Hensel *et al.*, 1995). *E. coli* CC118 λ*pir* was transformed with pUTmini-Tn5*km2* containing signature tags as described in 3.1.3 and 3.1.4, and a total of 384 transformants were isolated for further screening by hybridization with their cognate labelled tags. After extensive cross-hybridization experiments, 90 clones that showed the strongest hybridization signals with single-labelled tags and that did not cross-react with each other were collected for further investigations (Fuller *et al.*, 2000). Finally, plasmids containing specific tags were purified from the clones and individually transformed into donor strain *E. coli* S17-1 λ*pir*. Independent matings were set up by growing

each *E. coli* S17-1 λ*pir* (with tag) clone and the IMT5155Nal^R to late log phase. To construct tagged transposon mutants of APEC, 400 µl donor and 400 µl recipient cells were mixed and centrifuged. After discarding the supernatant the cells were resuspended in 10 µl of a 10 mM $MgSO_4$ solution and plated onto LB agar plates and incubated at 37°C for 4 h. Colonies were collected and resuspended in PBS and plated onto LB agar containing kanamycin and nalidixic acid. Following selective overnight growth, single colonies were resuspended in 300 µl LB supplemented with nalidixic acid and kanamycin. After an overnight incubation, 53 µl glycerol (15%) was added and a total of 1.827 mutants were stored at -70ºC in microtiter plates.

3.9 Probe labelling and hybridizations for identification of mutants disappeared from recovered pools

For amplification of DNA tags, either 5 ng of plasmid DNA or 5 µg of genomic DNA from input and recovered pools was used as a template in first round PCRs using the conditions described as followed. The amplified tags from both the inoculum and the recovered pools were compared by labelling with digoxigenin (DIG)-dUTP using the PCR DIG probe synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer´s instructions. After purification as described in 3.4.1, the PCR products were labelled in a volume of 50 µl using 50 pmol of each primer P2 and P4, and a 1:1 mix of DIG-dUTP and 2.5 mM dNTP stock solution with the same cycle conditions as for the first round PCR (Fuller *et al.*, 2000). The labelled PCR products were digested with *Hind*III in a total volume of 400 µl, ethanol precipitated, and isolated from a 4% 3:1 NuSieve GTG:agarose gel (Cambrex Corporation, New Yersey, USA). The region containing the labelled tag was excised and the entire gel slice was directly used as probe in hybridizations. Plasmids containing the cognate tags from the original plate were used as templates for the amplification of these tags with primer pair P2 and P4. Identical dot blots were prepared by transferring these amplicons onto positively-charged nylon membranes followed by prehybridization and hybridization with easyhyb buffer at 42ºC. Other hybridization procedures are described in 3.7.2 and 3.7.3. Mutants showing hybridization signals with

the probe from the input pool but not with that from the recovered pool were selected for further analysis.

The PCR to amplify signature tags was performed as followed:

95 ºC	4 min	1×
95 ºC	30 s	
50 ºC	45 s	30×
72 ºC	10 s	
72 ºC	7 min	1×

3.10 Identification of transposon insertion sites and sequence analyses

Most of transposon insertion sites were amplified by arbitrary PCR (Merrell *et al.*, 2002). For the first round, arbitrary primers Arbi1, Arbi3, Arbi4, and Arbi5 were used in combination with the transposon-specific primer P9. Subsequently, 1 µl of each PCR product was used in a second round of nested PCR with primers Arbi2 and P6. Arbi2 is identical to the 5'-sequences of the above mentioned arbitrary primers and P6 is a transposon I terminus-specific primer. Genomic DNA from the wild-type strain served as negative control in all reactions. The second round PCR products were purified and sequenced. DNA sequencing was performed commercially (AGOWA GmbH, Berlin, Germany). Data analysis was performed using public DNA and protein databases (http://www.ncbi.nlm.nih.gov/BLAST) and employing the BLASTX and BLASTN algorithms (Altschul *et al.*, 1997). All DNA sequences were compared with sequences in the TIGR comprehensive microbial resource.

The PCR reaction:

5.0 µl	10×PCR buffer
0.2 mM each	dNTPs (100 mM)
1.5 mM	$MgCl_2$ (50 mM)
10 pM each	primers
0.05 U/µl	Golden AmpTag
5 µg genomic DNA/ 1µl PCR production	template
ad 50 µl	distilled water

The first round of PCR was performed as follows:

95 ºC	8 min	1×
95 ºC	45 s	
30 ºC	30 s	6×
72 ºC	1.5 min (5 sec/cycle more)	
95 ºC	45 s	
45 ºC	30 s	30×
72 ºC	2 min (5 sec/cycle more)	
72 ºC	10 min	1×

The second round of PCR was performed as follows:

95 ºC	8 min	1×
95 ºC	45s	
55 ºC	45s	30×
72 ºC	1.5min (5s/cycle more)	
72 ºC	10min	1×

3.11 LPS analysis

3.11.1 LPS isolation

The mutant and wild type strains were streaked on LB plates and incubated overnight at 37ºC. Single bacterial colonies were then scraped into 10.5 ml PBS respectively and aliquots transferred into 1.5 ml Eppendorf tubes. After centrifuged at 6.000×g at 4ºC for 8 min, bacterial pellets were suspended in 1 ml PBS. The SDS buffer (final concentration 1%) was added and the samples were incubated in a water bath at 100ºC for 10 min. Finally 50 µg Protease K was added and shaking overnight at 60ºC (Hitchcock and Brown, 1983).

3.11.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Shape banding of LPS components is achieved by using a discontinuous gel system, which is composed of stacking and separating gel layers that differ in either salt concentration or pH or both. Resolving and stacking mini-gel solutions for the preparation of two 7 x 10 cm gels with a thickness of about 0.75-1.00 mm were prepared. 10% ammonium persulfate (APS) and TEMED were added just prior to pouring the gel. The resolving gel mix was poured into assembled gel plates, leaving sufficient space at the top for the stacking gel to be added later. The gel mix was gently overlaid with isopropanol and the gel was allowed to polymerize for at least 15-30 min. After polymerization, the isopropanol layer was removed and the surface of the resolving gel was rinsed with water. The remaining space was filled with the stacking gel solution and the comb was inserted immediately. After the stacking gel had polymerized, the comb was removed and the wells were rinsed with water to remove unpolymerized acrylamid. At least 1 cm of stacking gel should be present between the bottom of the loading wells and the resolving gel.
LPS with 5 x loading buffer were heated at 95°C for 5 min and loaded onto the gel with a protein standard marker beside it. The gel was run in SDS gel running buffer at 200 V.

3.11.3 Silver staining

Initially the gel was soaked in 40% and 10% acetic acid in a shaker at room temperature. After overnight incubation, it was rinsed in 10% ethanol for 10 min and then in ddH_2O for 30 min with three changes. Subsequently the gel was incubated in 0.1% silver nitrate for 30 min and then developed in 3% $NaCO_3$, 0.02% formalin for 10 min until bands are visible. The reaction was finally stopped with 0.03% EDTA, the gel was washed with ddH_2O and preserved in 55% methanol and 10% glycerol (Tsai and Frasch, 1982).

3.12 Infection study

Groups of chickens of five weeks old were randomly allocated into individual cages, and acclimatized for 7 days before inoculation. Water and ration were provided ad libitum. Birds were provided with 12 h lighting period, and room temperature was maintained at 20-22°C. Filtered, nonrecirculated air was provided and air pressure differentials and strict sanitation conditions were maintained. To sum up, all birds were maintained at highest standards for human treatment of experimental animals. Moribund birds were immediately killed by cervical dislocation or carbon dioxide asphyxiation (Registration number: Reg 0212/01).

3.12.1 Establishment of a chicken infection model

In the first chicken test, 0.5 milliliter of bacteria was given by intratracheal application. For both *E. coli* strains IMT5155 and IMT5155 Nal^R strains, five groups of birds of five weeks old were inoculated respectively with 1×10^9, 1×10^8, 1×10^7, 1×10^6, 1×10^5 colony-forming units (cfu) of *E. coli* in 0.5 ml of saline. While only three groups of birds were applied for *E. coli* strain IMT5104 with 1×10^9, 1×10^7, 1×10^5 cfu of bacteria, respectively. The control group was inoculated with 0.5 ml of saline. The actual number of bacteria per ml was determined by plating 10-fold serial dilutions of suspension in duplicate. All groups contained 4 birds and groups of birds receiving virulent *E. coli* and the control group were housed in separate rooms.

To study the dynamics of bacteria in vivo, 10 groups of chicken, each containing 6 chickens, were inoculated by intra-tracheal application of 1×10^8 cfu of IMT5155 Nal^R in 0.5 ml of saline. At time point 3, 6, 12 hours and days 1, 2, 3, 4, 5, 7, 14, one group of chicken was killed. The lungs, spleens, hearts, kidneys and livers were collected. For bacterial isolation and number counting, 100 mg of each internal organ was homogenized in 1 ml sterile PBS and then the actual number of bacteria per millilitre were determined by plating 10-fold serial dilutions of suspension in duplicate.

3.12.2 Screening of the mutant bank

For preparation of mutant inoculants, frozen plates of pooled APEC mutants were taken out of -70 ºC storage and subcultured by transferring 10 µl from each well to a new 96 well round bottom plate containing 300 µl LB with kanamycin and nalidixic acid. Following overnight culture, each plate was pooled and 10 ml of pooled bacteria was added to 90 ml LB and then incubated at 37 ºC shaking at 250 rpm/min until OD_{600} reached approximately 1.0. The culture was diluted appropriately in PBS to a dose of approximately 2×10^8 cfu/ml. 0.5 ml of each mutant pool was used to infect at least four 5-week-old SPF chickens per mutant pool via the intra-tracheal application route. After 48 h and/or 72 h of infection, chickens were killed and bacteria were re-isolated from spleens. The organs from at least two chickens were homogenized in sterile PBS and then plated onto LB agar plates supplemented with kanamycin and nalidixic acid. Following overnight incubation, at least 10.000 colonies (5.000 colonies from each chicken) were pooled in 10 ml PBS to form the 'output pool'. A 5 ml aliquot of this pool was used to prepare genomic DNA. Mutants with reduced output hybridization signal were subsequently selected for further study.

3.12.3 In vivo and in vitro competition assay

For both in vitro and in vivo competition assays, cultures of mutant and wild type strain were mixed at a ratio of 1:1. For in vitro competition, the bacteria were incubated in LB broth for 4 hours at 37ºC and then plated onto media with or without kanamycin. For in vivo competition, four chickens (76 chickens for 19 mutants) were infected with 10^8 cfu of

this mixture. After 48 h of infection, spleen, heart, liver, lung, and kidney were collected, weighed and homogenized, and the dilutions were plated on media with or without kanamycin for selection of mutants or total bacteria, respectively. A competitive index (CI) was calculated for each mutant by dividing the output ratio (mutant/wild type) by the input ratio (mutant/wild type). Due to this CI, mutants being out-competed up to 10-fold were evaluated as slightly, up to 100-fold as moderately and more than 100-fold as highly attenuated.