2 Materials

2.1 Animals

5-week-old SPF chickens (Lohmann Selected White Leghorn) were used after they had been tested for absence of *Salmonella* and avian pathogenic *E. coli* (APEC). All experiments were performed on females, although no sex-related differences were found during preliminary studies (Registration number: Reg 0212/01).

2.2 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in table 1. *E. coli* strain IMT5155 (O2:H5) was used for infection trials, mutant construction and STM-analyses. This strain had been isolated from the internal organs of a four-month-old laying hen from Germany, which suffered from clinical symptoms and lesions of colisepticemia. Preliminary infection studies confirmed the virulence of the strain that caused severe symptoms and lesions of colibacillosis and high mortality rates in 6-month-old chicken. Furthermore, IMT5155 has been included in large scale phylogenetic analysis of 150 APEC strains and was grouped into one of the most representative clonal lineages in Germany (Ewers et al., 2004). The strain harbours the virulence associated genes *fyuA*, *irp2*, *colV*, *tsh*, *fimC*, *iss, ompA, traT, vat, astA, papC,* and *iucD* as shown by DNA-DNA-hybridization and PCR experiments. A spontaneous nalidixic acid-resistant mutant was generated by growing IMT5155 in the presence of this antibiotic and plating 10^9 cfu to solid media containing nalidixic acid with the concentration of 30 µg/ml. The nalidixic acid-resistant mutant was confirmed to retain full virulence in the chicken model. Strain IMT5104 (O8:H^-) served as a negative control during animal tests. This strain was isolated from the faeces of a healthy laying hen and was shown to harbour none of the virulence associated genes known so far for APEC. *E. coli* CC118 *λpir* was used for maintaining the tagged pUTmini-Tn5*km2*, and *E. coli* S17-1 *λpir* was used as the donor strain in conjugation experiments. A pool of tagged pUTmini-Tn5*km2* plasmids (Amp^r, Km^r) was kindly provided by D. 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 signature tags.

Table 1: *Escherichia coli* strains and plasmids used in this study

Plasmid/*E. coli* strain	Relevant features
pUTmini-Tn5*km2*	Amp^r, Km^r
pUC19	Cloning vector (Amp^r, ColE1-ori)
	Cloning vector (Cm^r, *lacZ*α, S ori)
S17-1 *λpir*	*recA thi pro hsdR* $^{-}M^{+}$ RP4::2-Tc::Mu::Km Tn7 lysogenized with *λpir* phage
C118 *λpir*	Δ(*are-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB arg*E *recA1* lysogenized with *λpir* phage
DH5α	*F`Φ80dlacZ Δ(lacZYA-argF)U169 recA1endA1 hsdR17*(r_K-m_K+)*supE44 λ- thi-1 gyrA relA1*
IMT5155	O_2 : H_5; *fyuA, irp2, tsh, iucD, fimC, iss, ompA, vat, traT*
IMT5155Nal^R	O_2 : H_5; *fyuA, irp2, tsh, iucD, fimC, iss, ompA, vat, traT*
IMT5104	O_8 : H^-; no known virulence factor identified

2.3 Chemicals

All chemicals, solutions and reagents used during this work are briefly listed in the following chapters. Additional comments are only made if necessary.

Kits used in this study:

- MasterPure™ 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
- Agarose Gel DNA Extraction Kit	Roche Diagnostics GmbH, Mannheim
- PCR DIG Probe Synthesis Kit	Roche Diagnostics GmbH, Mannheim
- DIG Luminescent Detetion Kit	Boehringer Mannheim GmbH, Mannheim
- QIAGEN® Plasmid Midi Kit (25)	QIAGEN, Hilden
- QIAGEN® Plasmid Maxi Kit (25)	QIAGEN, Hilden
- Roti®-Elutions-Kit	Carl Roth GmbH & Co, Karlsruhe

The enzymes and oligonucleotide primers were purchased from the following companies: Life Technologies™, Karlsruhe; New England Biolabs® GmbH, Schwalbach/Taunus; Promega GmbH, Mannheim; Stratagene, Amsterdam (NL) and MWG Biotech, Ebersberg.

Other organic und anorganic compounds used in this study were purchased from Acila GMNmbH, Walldorf; BioRad, München; Fluka, Hannover; Invitrogen, Groningen (NL); Merck AG, Darmstadt; Oxoid Unipath GmbH, Wesel; Roth® GmbH & Co., Karlsruhe; Serva, Heidelberg; and Sigma®, Deisenhofen.

2.4 Nutrition medium

The following growth media were used throughout this work:

- LB-Medium (Luria Bertani Broth)	NaCl	5.0 g/l
	Peptone (Casein)	10.0 g/l
	Yeast extract	5.0 g/l
		pH 7.0±0.2

Autoclaved at 121°C, 15 min

- LB-Agar (Luria Bertani)	NaCl	5.0 g/l
	Peptone (Casein)	10.0 g/l
	Yeast extract	5.0 g/l
	1 *N* NaOH	1.0 ml
	Agar	15.0 g/l
		pH 7.0±0.2

Autoclaved at 121°C, 15 min

- Peptone water	Peptone	10.0 g/l
	NaCl	5.0 g/l
	Na_2PO_4	3.5 g/l
	KH_2PO_4	1.5 g/l
		pH 7.2±0.2

Autoclaved at 121°C, 15 min

- SOC-Medium	Tryptone	20.0 g/l
	Yeast extract	5.0 g/l
	NaCl	0.50 g/l
	250 mM KCl	10.0 ml
	5 N NaOH	0.20 ml
		pH 7.0

Autoclaved at 121°C, 15 min.

Cooled down to 60°C, then autoclaved, 2M $MgCl_2$ (5.0 ml) and filter sterilized 1M Glucose (20.0 ml) was added.

2.5 Solutions

2.5.1 Solutions used for agarose gel electrophoresis

The solutions utilized in this experimental work are outlined in the following tables:

Solution	Component	Concentration	Amount
- TBE buffer stock-solution (10 x)	Tris	890 mM	107.82 g
	Boric acid	890 mM	55.03 g
	EDTA-solution, pH 8,0	500 mM	18.62 g
	NaOH		in 100 ml
			40 to 1000 ml ddH_2O
- Stop-solution	Formamide		9.5 ml
	EDTA-solution, pH 8,0	500 mM	0.4 ml
	Bromphenol blue		5.0 mg
	Xylencyanol FF		5.0 mg
	ddH_2O		0.1 ml
- Agarose	Agarose		0.7 g
			in 100 ml 1 x TBE

- Ethidium bromide solution 1%

2.5.2 Solutions used for SDS-Polyacrylamid gel electrophoresis (SDS-PAGE)

Acrylamide solution	Acrylamide	30%
	N,N'-Methylen-Bisacrylamide	0.8%
Stacking gel solution	Acrylamide solution	1 ml
	Tris-HCl, pH 6.8 (1M)	0.75 ml
	10% SDS	65 µl
	H_2O	4.1 ml
	10% APS	100 µl
	TEMED (N,N,N',N'-Tetramethylethylenamine)	7 µl
Resolving gel solution	Acrylamide solution	5 ml
	Tris-HCl, pH8.8 (1M)	4.75 ml
	10% SDS	125 µl
	H_2O	3 ml
	10% APS	150 µl
	TEMED (N,N,N',N'-Tetramethylethylenamine)	15 µl
5 x loading buffer	SDS	10%
	ß-Mercaptoethanol	25%
	Glycerol	30%
	Tris-Hcl, pH 6.8	312.5 mM
	Bromophenol blue	0.01%
	Xylencyanol	0.01%
SDS gel running buffer	Tris	25 mM
	Glycin	192 mM
	SDS	0.1%

2.5.3 Solutions used for Polymerase chain reaction (PCR)

- 10 x PCR buffer[1]	Tris-HCl, pH 8.4	200 mM
	KCl	500 mM
- *Taq* DNA Polymerase[1]		5 U/µl
- Magnesium Chloride Solution[1]		50 mM
- dNTP[2]	PCR Nucleotide Mix:	
	dATP, dCTP, dGTP, dTTP	each 10 mM

[1] GIBCO BRL®, Life Technologies, Karlsrule

[2] Promega Corporation, Mannheim

- 10 x Herculase™ buffer		
- Herculase™ Enhanced DNA Polymerase		5 U/µl
- dNTP	PCR Nucleotide Mix:	
	dATP, dCTP, dGTP, dTTP	each 10 mM

STRATAGENE®

- 10 x AmpiTaq Gold buffer I		
- AmpiTaq Gold Polymerase		5 U/µl

- dNTP	PCR Nucleotide Mix:		
	dATP, dCTP, dGTP, dTTP		each 10 mM

Applied Biosystems

2.5.4 Solutions used for Southern-Blotting

- Depurination solution	HCl	250 mM	10.8 ml
			500 ml
			HCl
- Denaturation solution	NaCl	1.5 M	87.66 g
	NaOH	500 mM	20 g
			ad 1000 ml ddH_2O
-Neutralization solution	Tris-HCl, pH 7.5	500 mM	60.57 g
	NaCl	3 M	175.32 g
			ad 1000 ml ddH_2O
- SSC (20 x)	Na-Citrate	300 mM	88.23 g
	NaCl	3 M	175.32 g
	pH 7		
			ad 1000 ml ddH_2O

2.5.5 Solutions used for DNA isolation

- Solution I	Glucose	50 mM
	Tris	25 mM
	EDTA	10 mM
	pH 8.0	
-Solution II	NaOH	0.2 M
	SDS	1%
-Solution III	potassium acetate	3 M
	glacial acetic acid	11.5 ml
-Tris-sucrose	Sucrose	10%
	Tris	50 mM
	pH 8.0	
-CTAB/NaCl solution	CTAB	10%(w/v)
	NaCl	0.7M

Other solutions: EDTA solution (pH 8.0) 0.25 M/L, SDS solution 10%, NaCl solution 5M, Ethanol, Phenol : chloroform (1 :1)

2.5.6 Solutions used for DNA-DNA-Hybridization

Solutions of the DIG Luminescent Detection Kit (Boehringer Mannheim GmbH, Mannheim)

- Maleic acid buffer	Maleic acid	100 mM	11.6 g
	NaCl	150 mM	8.8 g
	pH 7.5		
			ad 1000 ml ddH_2O
- Blocking reagent stock solution (BRSS) 10 x	Blocking reagent	10 %	10.0 g
			ad 100 ml Maleic acid buffer
heated until 65°C, autoclaved, stored at 4°C			
- SDS (10 %)	SDS	10 %	20.0 g
			ad 200 ml ddH_2O
- SSC (20 x)	Na-Citrate	300 mM	88.23 g
	NaCl	3 M	175.32 g
	pH 7.0		
			ad 1000 ml ddH_2O
- Hybridization buffer	SSC (5 x)	20 x	12.5 ml
	BRSS (1 %)	10 x	5.0 ml
	N-Lauroylsarcosine (0.1 %)	10 %	1.0 ml
	SDS (0.02 %)	10 %	0.1 ml
			ad 50 ml ddH_2O

- EasyHyb Buffer	SSC (5 x)	20 x	12.5 ml
	BRSS (2 %)	10 x	10.0 ml
	N-Lauroylsarcosine (0.1 %)	10 %	1.0 ml
	SDS (0.02 %)	10 %	0.1 ml
	Formamide	100%	25 ml
			ad 50 ml ddH_2O
- Stringent buffer I	SSC (2 x)	20 x	40.0 ml
	SDS (0.1 %)	10 %	4.0 ml
			ad 400 ml ddH_2O
- Stringent buffer II	SSC (0.5 x)	20 x	10.0 ml
	SDS (0.1 %)	10 %	4.0 ml
			ad 400 ml ddH_2O
- Wash buffer 1	Maleic acid buffer		100 ml
	Tween® 20	0.3 %	0.3 ml
- Blocking Solution (1 x)	BRSS	10 x	10.0 ml
			ad 100 ml Maleic acid buffer
- Detection buffer	Tris	100 mM	12.11 g
	NaCl	100 mM	5.84 g
	pH 9.5		
			ad 1000 ml ddH_2O
- Stripping buffer	NaOH	200 mM	4.0 g
	SDS (0.1 %)	10 %	5.0 ml
			ad 500 ml ddH_2O

The Anti-Dig-AP conjugate and CSPD® Solution were provided by DIG Luminescent Detection Kit (Boehringer Mannheim, Mannheim).

2.5.7 Solutions used for ligation

- 5 x T4 DNA Ligase Buffer	Tris-HCl, pH 7.6	250 mM
	$MgCl_2$	50 mM
	ATP	5 mM
	DTT	5 mM
	Polyethylenglycol-8000	25 % (w/v)
- T4 DNA Ligase		1 U/µl

Invitrogen® Life Technologies, Karlsrule

2.5.8 Solutions used for the Restriction digestion of DNA

- Enzyme buffer for *Eco*RI[1]	Tris-HCl, pH 8.0	50 mM	0.60 g
	NaCL	100 mM	0.58 g
	$MgCl_2$	10 mM	0.10 g
			ad 100 ml ddH_2O

GIBCO BRL , Life Technologies, Karlsrule

- Enzyme buffer for *Bam*HI[1]	Tris-HCl, pH 8.0	50 mM	0.60 g
	NaCL	100 mM	0.58 g
	$MgCl_2$	10 mM	0.10 g
			ad 100 ml ddH_2O

GIBCO BRL , Life Technologies, Karlsrule

- Enzyme buffer	Tris-HCl, pH 7.9	10 mM	0.12 g
for *Hind*III	NaCL	50 mM	0.30 g
	$MgCl_2$	10 mM	0.10 g
	DTT	1 mM	0.02 g
			ad 100 ml ddH_2O
	Bovine Serum Albumine		0.1 mg/ml

NEW ENGLAND BioLabs®Inc., Frankfurt an main

2.6 Oligonucleotide-Primers

All oligonucleotide primers listed in the below table were synthesized by MWG Biotech Ebersberg.

Table 2: Oligonucleotide primers used in this work

Primer	Sequence (5´- 3´)	Target region
P2	TACCTACAACCTCAAGCT	Signature tag
P4	TACCCATTCTAACCAAGC	Signature tag
P6	CCTAGGCGGCCAGATCTGAT	Flanking sequence of Tn5
P7	GCACTTGTGTATAAGAGTCAG	Flanking sequence of Tn5
P9	CGCAGGGCTTTATTGATTC	Flanking sequence of Tn5
Arbi1	GGCCACGCGTCGACTAGTAC$(N)_{10}$GATAT	Flanking sequence of Tn5
Arbi2	GGCCACGCGTCGACTAGTAC	Flanking sequence of Tn5
Arbi3	GGCCACGCGTCGACTAGTAC$(N)_{10}$TGACG	Flanking sequence of Tn5
Arbi4	GGCCACGCGTCGACTAGTAC$(N)_{10}$ACGCC	Flanking sequence of Tn5
Arbi5	GGCCACGCGTCGACTAGTAC$(N)_{10}$TACNG	Flanking sequence of Tn5
P19	ATTCAACGGGAAACGTCTTG	Km^r gene
P20	ACTGAATCCGGTGAGAATGG	Km^r gene
Left-F	CTGGTGGTGTCATACGCTAA	*prrA*
Left-R	GTAACGCTGTCGGAAGAGGC	*prrA*

Primer	Sequence (5´- 3´)	Target region
PrrA-F	TACGACCCAACTAAACGATCG	prrA
PrrA-R	CACGACATCGACATTATCGG	prrA
FepC-F	TACCTGGATAATGCTGTCGG	*fepC*
FepC-R	ATGGTGTTGATGGGGCTGGC	*fepC*
HP1-F	AGTGGCTCAGGCTCTCATTT	*hP1*
HP1-R	TGGCATCATCGTTGGTCGTG	*hp1*
HlyD-F	TATTGCATAAACAGGCAATTGC	*hlyD*
HlyD-R	CCAGAGCAGTAACCTCCAGC	*hlyD*
PapG-F	TTTGCGAGTGGAGTGTATTT	*papG*
PapG-R	TACCTAACCCAACCGAAAAT	*papG*
PapC-F	TGATATCACGCAGTCAGTAGC	*papC*
PapC-R	CCGGCCATATTCACATAA	*papC*
R14-15-F	GCCAGTGACACATACTGAGAGC	*r14* plus *r15*
R14-15-R	CAGATGTACAGTGGCGCG	*r14* plus *r15*
R3-F	AGGGTACAGTCGTTGGCAAC	*r2* plus *r3*
R3-R	GCAGTTCTTCTTCCGACCAG	*r2* plus *r3*
R1 pus R2-F	AGCCTTTCTGTTTTGAGCAT	*r1* plus *r2*
R1 plus R2-R	TCGCTACTATTGATTCTTGC	*r1* plus *r2*
R1 plus f447-F	CCGCAAGAATCAATAGTAGC	*r1* pus *f447*
R1 plus f447-R	CTGGCGAGAAGGGGATAATG	*r1* pus *f447*