5 Discussion

5.1 Establishment of a chicken model of *E. coli*-septicemia

Several experimental models have been developed, allowing the evaluation of the pathogenicity of *E. coli* for chickens or turkeys. Pathogenic *E. coli* isolates are able to kill embryos or 1-day-old chick following subcutaneous inoculation (Dho and Lafont, 1984; Harry and Hemsley, 1965). Both models give rapid results and permit the measurement of the virulence of the isolates according to their 50% lethal dose. Other experimental models which reproduce natural disease in birds at susceptible ages that correspond to those of field disease (2-4 weeks old) have also been used. Bacteria can be aerosolized, inoculated into the naso-pharynx (Smith *et al.*, 1985), or directly inoculated into the trachea, following a preliminary challenge with a triggering agent such as virus (infectious bronchitis virus, or Newcastle virus), mycoplasma, or an increase in ammonia which impairs the natural defences of the respiratory tract.

So far, different chicken models revealed contrary published results concerning virulence properties of these strains as determined by LD_{50} for chickens with different ages. This urged us to establish a new infection model which imitates the natural route of infection, is not invasive and can be performed easily and reproducible. In contrast to the range of application methods mentioned above, applying the *E. coli* into the trachea confers bacteria with a circumvention of physical clearance and other local defense mechanisms of the avian upper respiratory tract.

We used a field strain IMT5155 (O2:H5) that had previously been shown to yield high mortality rates in 6-month-old chickens, leading to typical lesions of colisepticemia. With regard to vaccine development as a future perspective we selected this strain as it belongs to the most common O-group (O2) associated with colisepticemia worldwide and furthermore to a representative clonal lineage of APEC field isolates in Germany as determined by macro-restriction analysis (Ewers *et al.*, 2004). The reproduction of cellulitis and septicemia using clinical isolates of *E. coli* by intra-tracheal application was less suc-

cessful as described before. In this study, septicemia, cellulitis and other lesions like pericarditis, airsacculitis, perihepatitis in the same bird were well reproduced, which mimics the potential of bacteria as observed under field conditions. The bacteria could be reisolated from about 85% of infected chickens. Irrespective of the bacterial concentration used for inoculation, the control strain IMT5104 failed to be reisolated from any internal organ and chickens infected with this strain demonstrated neither clinical signs of colibacillosis nor any macroscopical lesions typical of the disease as shown by necropsy. Because further molecular work must be based on IMT5155Nal^R, we compared its virulence with that of its wild type and no differences were observed. As shown in the dynamic study of IMT5155Nal^R, more than 10^3 cfu/g bacteria from spleen, heart, liver, kidney and lung were detected as early as 3 h postinfection. At 48 h after inoculation, the bacterial numbers in internal organs reached the peak of 10^6 or 10^7cfu/g; soon afterwards, the number of bacteria dropped dramatically. When lesions were subacute by as early as day 7 post-infection, isolation of *E. coli* was rarely successful. This suggested that chickens were able to clear the infection rapidly and completely while fibrin deposits of inflammation remained.

5.2 Using Signature-tagged Transposon Mutagenesis to identify critical genes for chicken *E. coli*-septicemia

The virulence of bacterial pathogens is a complex, multifactorial process requiring the coordinated activity of various bacterial gene products as well as host responses. Infections are generally initiated with attachment to and colonization of the host, after which invasion of the host tissues or cells is followed by some pathogens. To multiply and persist within the host, a pathogen must then be able to circumvent the host's immune system and obtain nutrients for itself and may produce factors that cause damage to the host at any point during infection (Casadevall and Pirofski, 2003). A variety of in vitro systems have been developed that simulate certain aspects of the infectious process, enabling the development of screen to study bacterial gene expression and the behavior of mutant strains in physiologi-

cal conditions that reflect the situation in vitro. It is obvious that in vitro assays can not accurately reproduce all aspects of the host-pathogen interaction. Different genes may be required to be expressed at different times or in different localizations during pathogenesis. Therefore, a gene that seems important in an in vitro assay may not be important in vivo, and vice versa. For these reasons, in vivo experimental models are unavoidable which allow direct assessment of a pathogen's ability to colonize and survive in a living host and to cause disease or damage. Such infection models are particularly helpful, if they are performed in the same identical host they naturally occur in.

In recent years, genetic technologies have pathed the way for powerful tools to identify virulence genes from a genome-wide level by in vivo assays, leading to a deeper understanding of the molecular mechanism of pathogenicity and new molecular approaches to prevent infectious diseases caused by bacterial pathogens (Saunders and Moxon, 1998). Among these technologies are In vivo expression technology (IVET), Selective capture of transcribed sequences (SCOTS) and Signature-tagged transposon mutagenesis (STM). More recently, Suppression subtractive hybridization (SSH) has been used successfully to identify genes present in the genome of two APEC strains but absent in *E. coli* K-12 MG1655 (Stocki et al., 2002). Dozois et al. (2003) applied SCOTS to identify conserved genes in APEC strain χ7122 that are expressed in infected chicken tissues. Pathogen-specific DNA or cDNA such as putative adhesin, lipopolysaccharide core synthesis, iron-responsive, plasmid- and phage-encoded genes and genes of unknown function were successfully enriched and isolated by these authors (Dozois et al., 2003; Stocki et al., 2002). However, neither SSH nor SCOTS gives direct information about the significance of the isolated genes during pathogenesis in the host. STM allows high throughput screening of libraries of insertional mutants in animal models of disease. Mutants with transposon insertions in genes critical for pathogenesis exhibit an attenuated phenotype and are identified by negative selection. Therefore, we adapted STM to the newly established infection model using APEC wild type strain IMT5155 (O2:H5), which caused a severe outbreak of avian colisepticemia in Germany, in a chicken infection model.

5.2.1 Improvements for non-radioactive screening

In the original strategy of STM, the suitability of tags was checked before use by amplification, labelling and hybridization to colony blots representing the tags used to generate the probes. Mutants whose tags failed to yield clear signals on autoradiograms were discarded, and those that gave good signals were reassembled into new pools for animal infection studies. Because of the poor sensitivity of colony blotting, radioactive labelling hybridization must be applied in the original strategy (Hensel *et al.*, 1995). In our version of STM, a series of tagged transposons is selected before mutagenesis, based on efficient tag amplification and labelling and lack of cross-hybridization to other tags. Therefore, the tedious pre-screening process was avoided. Another advantage is that hybridization analysis can be done by tag DNA dot blots rather than colony blot because the identity of the tags in each mutant is known. The application of DNA dot blots dramatically increases the sensitivity of the system, thus enabling the use of non-radioactive detection methods which is desirable for safety, ease of use and reduction of hazardous materials.

5.2.2 Generation and characterization of the mutant bank

The most common procedure for insertion mutagenesis was described in 1994 (de Lorenzo and Timmis, 1994). The overnight donor and recipient cultures were mixed and incubated on LB without antibiotic at 37ºC for 8-18 h, then transferred onto selective media. By this method, too many duplicate mutants were generated, although it yielded a very high operational frequency. To ensure a high proportion of exconjugants resulting in fewer identical mutants, in this study we tried to lay the filter with mating mixture onto the surface of agar plates, containing minimal medium M9 and M63 devoid of carbon source. Both media were expected to allow conjugation but no further cell growth during the process of mating (Miller, 1992). The results showed that M9 and M63 without carbon source allowed neither conjugation nor further cell growth in our trial. Therefore, the exclusive choice was to shorten the incubation time in medium without antibiotics. Using 4 h instead of 8-18 h indeed reduced duplicate mutants in each single mating event. The results of southern blotting also suggested a broad distribution of transposon insertion

sites among the mutants (data not shown). A disadvantage of this approach is a much lower yield of operational frequencies: each mating test gave only 40-60 conjugants. However, this operational frequency proved to be sufficient for these experiments, as only 30 mutants were needed from each mating to generate a mutant bank of 2.700 mutants.

In principle, the suicide plasmid containing the transposon can integrate into episomal or chromosomal loci. Given the fact that pUT plasmids carry a *bla* gene external to the mini-transposon, absence of the delivery plasmid is readily confirmed by plating exconjugants onto medium containing a ß-lactam antibiotic. Authentic transposition results in the loss of the portion of the delivery plasmid containing the *bla* gene and therefore exconjugants should be sensitive to ampicillin as well as to other ß-lactams. In this study, nineteen mutants from 1.827 (about 1%) were actually resistant to ampicillin. Therefore, these mutants were excluded from the mutant bank. Unlike other transposons, the inserted sequence of mini-Tn5 excludes the transponase, the inserted sequence thus can not transpose any more. This character results in stable mutation and only one insertion site in each single mutant. Even though, control experiments by southern blotting were randomly performed to detect a possible integration into more than one site in one single mutant, always proving an integration in one site only.

5.2.3 Screening of the mutant bank in a chicken model

When STM is applied to a bacterial pathogen for the first time, several parameters must be taken into account to get reproducible identification of attenuated mutants from different chickens inoculated with the same pool of mutants. The route of administration of bacterial inoculum influences both the LD_{50} value and the numbers of bacterial strains that reach the target organs and tissues, hence the reproducibility of tag hybridization signals. Intra-air sac application is a well established and characterized chicken model for *E. coli*-colisepticemia. At the beginning, this model was used to screen for the most virulent strain choosen for further research. A very low LD_{50} value was observed in these screening tests. To represent a pool of 90 mutants yielding reproducible hybridization signals for the vast majority of strains recovered from the internal organs of infected animals, an inoculation

dose ranging from 10^6 to 10^8cfu was required in similar screens of other pathogens. Obviously, intra-air sac application could not meet this need. We chose the intratracheal application as it imitates the natural route of infection, is not invasive and can be performed easily. In contrast to a range of application methods that have been proposed for experimental APEC infection of poultry so far, a circumvention of physical clearance and other local defense mechanisms of the avian upper respiratory tract can only be encountered by applying the bacteria into the trachea. Therefore, a higher administration dose can be used in this model.

The inoculum dose is yet another highly important parameter for STM. If the inoculum dose is too low, there may be an insufficient number of bacteria of any one virulent mutant to initiate a successful infection. For instance, a given input pool containing two differentially marked wild type strains can yield markedly different output ratios of the two strains after an infection cycle initiated by a small inoculum. On the other hand, if the dose is too high, the chicken's immune defences may be overwhelmed, resulting in the growth of mutant strains that would otherwise be attenuated (Chiang *et al.*, 1999). Whether the inoculation dose is too high or too low can only be judged by the reproducibility of the results. Using intratracheal challenge doses of 10^5, 10^6, or 10^7 cfu of strain IMT5155 Nal^R, a very low percentage of animals developed typical signs of colibacteriosis. In contrast, the use of 10^9 cfu for intratracheal inoculation resulted in death of several chickens, even before reisolation of bacteria was initiated. The inoculation dose of 10^8 cfu not only gave clear and typical clinical symptoms and lesions of colisepticemia, but also yielded more than 10^4 cfu/g bacteria from spleens. Therefore, 10^8 cfu was preliminarily selected as inoculation dose and the dynamic of infection of this dose has been further studied to characterize this model. The consequent results confirmed that an inoculum dose of 10^8 cfu led to reproducible identification of attenuated mutants.

The time point after infection at which the bacteria are recovered from the organs to prepare tags for hybridization, is also crucial for the reproducibility of the STM approach. If this time is too short, virulent cells may have had insufficient time to outgrow the attenuated strains to a degree that is reflected in a clear difference in hybridization signal. On the

other hand if the time is too long, there may be a risk that some virulent strains may simply outgrow other virulent strains in a non-specific manner. In this study, reproducible hybridization results have been achieved when the infection duration was 48 h. Just as we expected, most of the mutants were present with the exception of 3 or 4 mutants absent from each recovered pool. This ratio coincides with the hypothesis that approximately 5% of the total number of genes of pathogenic *E. coli* is involved in virulence. While after 72 h of infection, too many mutants disappeared from the recovered pools, moreover the hybridization patterns of the same pool differed in individual chicken. This indicated that some virulent strains may have simply outgrown other virulent strains in a non-specific manner. That is why even after reproducibly identified mutants from the output pool, in addition outgrowth of the mutants was tested in vitro.

Pool complexity is also an important parameter for STM. A higher pool complexity is desirable not only because of animal welfare but also its lower cost. However, as the complexity of the pool (the number of different mutant strains tested in a single animal) increases, there is the probability that virulent mutants will fail to be recovered in sufficient numbers to yield hybridization signals, and this could lead to false identification of attenuated mutants. In this study, the pool complexitiy of 90 mutants gave reliable and reproducible results, in contrast to most of the other STM applications of non-radioactive hybridization using 48 mutants per pool. The efforts to improve the sensitivity of nonradioactive hybridization system as desribed in this study may contribute to the adoption of a higher pool complexity.

5.2.4 Determination of the insertion site of the transposon

Cloning is the commonly applied and classical method to identify the flanking sequences of integrated transposons. In this classical strategy a southern blotting is firstly employed to select an appropriate restriction enzyme. Genomic DNA of mutants is then digested with restriction enzymes, a 3-5 kb positive fragment purified and ligated to a suitable plasmid vector. Finally the resistance of the transposon is selected, and the flanking sequence can

be amplified by inverse PCR or directly sequenced. In this study, an innovative arbitrary PCR strategy was employed. By application of only two rounds of PCR, the transposon flanking region could be obtained by directly sequencing the product of the second round of PCR. Compared to the classical method of cloning, this arbitrary PCR is much easier, cheaper and far less time-consuming. Therefore this is a highly effective laboratory tool for molecular work. As an outcome, it took only about one month to obtain all sequences disrupted by the transposon.

5.2.5 Molecular characterization of the disrupted genes

5.2.5.1 Synthesis of different types of exopolysaccharides is necessary for APEC virulence

Several of the attenuated mutants had insertions in genes involved in the synthesis of extracellular polysaccharides. Two separate mutants were shown to have an insertion in the *kpsM* locus (M03A03, M03E05) while another was disrupted in the *kpsS* locus (M18H02). Both *kpsM* and *kpsS* encode for proteins required for translocation of *E. coli* group II capsular polysaccharide across the inner membrane (Clarke *et al.*, 1999). The degree of attenuation for these mutants was high (more than 100-fold reduction in certain organs), but varied for both the mutant and target organs, with the most severe attenuation observed in liver and heart (Tab. 4). Previous epidemiological studies noted that the K1 (group II) antigen is frequently associated with APEC, particularly serotype O1 and O2 (Gross, 1994a). More recently, a spontaneous K1-mutant of the APEC strain MT78 was found to show a decreased colonization potential in infected chickens and contributed to increased resistance to the bactericidal effects of chicken serum and phagocytosis by interaction with complement system (Mellata *et al.*, 2003a; Mellata *et al.*, 2003b). The isolation of three independent attenuated mutants by STM in this study therefore provides further evidence for the importance of K1 capsule biosynthesis in APEC pathogenicity.

An additional STM mutant (M03G02) was found to harbor a disruption in a sequence related to *wcaE*, a putative colanic acid (CA) glycosyl transferase. This CA mutant was attenuated for survival in different organs with the most severe defect in heart tissue

(Tab. 4). Besides capsule biosynthesis, *E. coli* produces a variety of exopolysaccharides (EPS), e.g. colanic acid (CA). CA contains L-fucose, D-glucuronic acid, D-galactose, D-glucose, and pyruvate and forms a thick, mucoid matrix on the cell surface. Similar to the group IA capsular polysaccharides, CA is characterized by high molecular weight, specific sugar composition, and a chromosomal location near the *his* operon of the responsible biosynthetic genes (Stevenson *et al.*, 1996). Hanna *et al*. (Hanna *et al.*, 2003) found that the capsular polysaccharide colanic acid of an uropathogenic *E. coli* did not contribute to bacterial adhesion to bladder epithelium as had been previously suggested, but rather blocked the establishment of specific binding as well as time-dependent interactions between the uropathogenic strain and inert substrates. A CA-deficient mutant of *E. coli* O157:H7 demonstrated a reduction in heart and acid tolerance (Mao *et al.*, 2001), suggesting this exopolysaccharide confers a protective effect against host environmental stresses, such as body temperature and acid environment in macrophages (Cheville *et al.*, 2001). This might be advantageous for APEC which survive and replicate in an environment of 42°C in birds, the normal body temperature. APEC have also been demonstrated in macrophages in the air sac of chickens 24 h after infection where they appear to be resistant to the acidic, intracellular environment (Pourbakhsh *et al.*, 1997a). Thus, for the first time, direct evidence is provided for an association of colanic acid with virulence and fitness of this pathogen. Furthermore, mutants with insertions in genes involved in the synthesis of several distinct extracellular polysaccharide structures including group II capsule are attenuated in the ability to cause septicemia in a chicken model.

5.2.5.2 Lipopolysaccharide (LPS) contributes to viability of APEC in chickens

LPS is one of the major components of the bacterial outer membrane. This amphipathic molecule is composed of lipid A, a core oligosaccharide, and a polysaccharide chain termed O-specific antigen. Four of the isolated STM mutants were shown to have transpositional insertions in genes involved in biosynthesis of LPS. M03D10 had a disruption in a gene encoding a surface polymer ligase termed *waaL* which joins newly synthesized O-polysaccharide (O-PS) to the lipid A core (David *et al.*, 1998). In addition, two independent mutants (M13F03 and M12D10) had different transposon insertion sites in the same

gene which showed similarity to *waaW*. This gene is involved in LPS R1 core biosynthesis and functions as a (galactosyl) LPS alpha-1,2 galactosyltransferase (Heinrichs *et al.*, 1998). The R1 core structure is the most prevalent among clinical isolates of *E. coli* (Appelmelk *et al.*, 1994; Gibb *et al.*, 1992). The in vivo tested mutant M12D10 showed moderate attenuation in heart, and slight attenuation in lung, spleen, and kidney but not in the liver. Clones corresponding to genes involved in the synthesis of the R1-type core LPS have recently been identified by Selective capture of transcribed sequences (SCOTS)-analysis (Dozois *et al.*, 2003). The O78-antigen of the APEC strain χ7122 used in this prior study was previously shown to be required for virulence by increasing the bacterial resistance to serum (Brown and Curtiss, 1996). We identified another mutant, M00G10, with a disruption in a gene locus of unknown function which does not share similarity with *E. coli* K12, CFT073 (UPEC), or EDL933 (EHEC) strains. Immediately downstream of this gene, there is a locus encoding a putative protein similar to glucose-1-phosphate thymidylyltransferase (RmlA), which is involved in O-antigen biosynthesis of *Shigella boydii* and *E. coli* (D'Souza *et al.*, 2002; Wang *et al.*, 2001).

In order to confirm defects in LPS synthesis, LPS of the four mutant and wild-type strains was isolated and subjected to SDS-PAGE and silver staining. Compared to the parental strain, all four mutants showed reduced LPS production. As expected, M13F03 and M12D10, both being disrupted in *waaW*, also showed altered banding patterns for the LPS core region compared to wild-type strain. Mutant M03D10 (*waaL*) had alterations in both O-antigen and core region, whereas M00G10 (no similarity to any known gene) demonstrated altered banding patterns only in the O-antigen region (Fig. 16). These results indicated that the *waa*-orthologues and the gene interrupted in M00G10 most likely function in different steps of LPS-biosynthesis and are required for the expression of a complete LPS molecule, as was also shown in a previously STM study investigating the gastrointestinal colonization of infant rats by an *E. coli* K1-strain (Martindale *et al.*, 2000). Our findings of four attenuated LPS mutants by STM further confirm the importance of LPS, including O-antigen in the pathogenesis of APEC.

5.2.5.3 Several iron uptake systems ensure bacterial survival in different host environments

Two mutants with insertions in genes likely to be involved directly or indirectly in iron uptake were also isolated. Both mutants (*sitB* and *chuA*) showed moderate attenuation in different tissues. Sequestration of iron by host compounds results in an extremely low free iron concentration in vivo, and has been suggested to limit bacterial growth in this environment. Consistent with this, iron acquisition systems have been associated with bacterial virulence especially for bacteria causing septicemia. *Chu*A encodes an outer membrane protein involved in haem uptake in EHEC and UPEC strains (Nagy *et al.*, 2001; Stocki *et al.*, 2002; Torres and Payne, 1997), and has been implicated as a determinant of virulence in *E. coli* (Griffiths, 1987). C*huA* has previously been identified by SSH in APEC without any further functional characterization (Stocki *et al.*, 2002). Thus, the isolation of a *chuA* mutant and its attenuation in vivo is the first and most compelling evidence so far that this gene is important for the pathogenicity of APEC.

The second iron uptake protein identified in this study is highly similar to *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* SitB, which is encoded in a four-member operon that mediates manganese and iron transport (Runyen-Janecky *et al.*, 2003; Zhou *et al.*, 1999). In *Salmonella*, the *sit* iron acquisition locus is encoded within a pathogenicity island and is required for full virulence of this pathogen (Janakiraman and Slauch, 2000; Zhou *et al.*, 1999). A *sitA* mutant of *Shigella flexneri* showed reduced in vitro growth compared to the wild-type, and *sitA* is likely located on a pathogenicity island in this species as well (Runyen-Janecky *et al.*, 2003). More recently, the SitABCD system has been identified in an APEC strain MT512 by genomic subtraction (Schouler *et al.*, 2004). However, no direct evidence was shown that Sit is involved in the virulence of APEC by these authors. Here, again we provide the first striking evidence for a role of this factor in the pathogenesis of avian colibacillosis.

In addition to the *chu* and *sit* gene loci, IMT5155 possesses aerobactin and yersiniabactin as iron transport systems (Ewers *et al.*, 2004). The occurrence of multiple iron transport systems in APEC suggests the importance of this metal ion for bacterial survival, possibly

helping the bacteria to adapt to various host environments during the infection process (Torres *et al.*, 2001).

5.2.5.4 Membrane and periplasmic proteins contribute to the virulence of APEC

Several genes with high similarity (93% - 100%) to genes coding for diverse membrane and periplasmic proteins were also identified by STM. The mutant M08E08 showed a slight 4- to 6-fold attenuation in lung, liver, heart, and spleen and was shown to harbor a disruption in a gene similar to *sbmA*. SbmA is predicted to be an inner membrane transport protein and functions in the uptake of microcins B17 and J25 and bleomycin in *E. coli* (Lavina *et al.*, 1986). A *sbmA* orthologue mutant of *Sinorhizobium* (*S.*) *meliloti* exhibited increased sensitivity to agents such as hydrophobic dyes and detergents indicating a role for SbmA in maintaining envelope integrity (Ferguson *et al.*, 2002). In *S. meliloti* and *Brucella abortus*, orthologues of SbmA have also been shown to be important for long-term survival within host cells (LeVier *et al.*, 2000).

Mutant M17F12 was found to harbor a disruption in a gene with sequence similarity (97%) to *mppA*, encoding a periplasmic protein essential for import of bacterial cell wall peptide L-alanyl-γ-D-glutamyl-meso-diaminopimelate (Park *et al.*, 1998). The mutant showed a slight attenuation in lung, liver, spleen, and kidney. During growth, *E. coli* breaks down over one-third of its cell wall with each generation and re-utilises the tripeptide for synthesis of murein for the biosynthesis of the murein sacculus, the scaffolding structure of the bacterial cell wall. However, the principal pathway for uptake and re-utilization of this tripeptide is indirect and the MppA pathway does not appear to play a key role in recycling (Jacobs *et al.*, 1994; Park, 1993). This might suggest that a reduction in uptake/recycling through the MppA pathway would not have a significant effect on murein biosynthesis. However, as shown in other studies, MppA negatively regulates the expression of *marA* and hence MarA-dependent multiple antibiotic resistance genes in a signal transduction pathway (Li and Park, 1999). For the *Salmonella enterica* serovar Typhimurium, it has been demonstrated that the *mar* locus plays an important role in the interaction with porcine lung macrophages, and is involved in adherence to human gut cells, and invasion of and persistence in internal organs of chicken (Randall and Woodward, 2001). Thus, pre-

sumably *mppA* mediates effects on APEC virulence indirectly, through signal transduction pathways affecting other virulence loci.

An additional STM mutant (M19A01) showed disrupted *narK*, a gene encoding a membrane protein involved in nitrite transport (Clegg *et al.*, 2002). Moderate attenuation was found in liver (~20-fold), and heart (~30-fold). The observed attenuation could be the result of either loss of nitrate respiration and nitrate as an electron acceptor required for growth under anaerobic conditions (Rowe *et al.*, 1994) or the defect in NarK may limit utilization of nitrate as a nitrogen source. Also identified was a gene encoding a putative protein 100% identical to an acriflavine resistance protein (M19B12). This mutant showed slight attenuation in lung, heart, and spleen but not in liver and kidney.

5.2.5.5 The ability to synthesize certain metabolic enzymes may be required for pathogenesis of colisepticemia

The ability to adapt to the host environment is a key component of bacterial pathogenicity. Indeed, in this thesis two genes were identified, whose products are involved in metabolic pathways and nutrient uptake following this negative in vivo selection. Mutant M04C10 showed slight to high attenuation in liver, spleen, kidney, and heart and was slightly reduced in growth in vitro. The disrupted gene *tktA* encodes a transketolase catalyzing the reversible transfer of a ketol group between several donor and acceptor substrates. *TktA* has also recently been identified in APEC strain MT512 by genomic subtraction (Schouler *et al.*, 2004). This key enzyme is a link between glycolysis and the pentose phosphate pathway. *E. coli* contains two transketolase isozymes encoded by the *tktA* and *tktB* genes, with *tktA* encoding the major enzymatic activity in *E. coli*. Due to its wide substrate specificity this enzyme is involved in the catabolism of pentose sugars, the formation of D-ribose 5-phosphate and in the provision of D-erythrose 4-phosphate, a precursor of aromatic amino acids, aromatic vitamins and of pyridoxine (Sprenger, 1995). Since 4-(phosphohydroxy)-L-threonine and 1-deoxy-D-xylulose 5-phosphate are believed to be direct precursors of vitamin B6 in *E. coli*, and transketolase catalyses the formation of each precursor, vitamin B6 may be important for in vivo survival of APEC (Sakai *et al.*, 2003).

A further mutant M14G11 affecting the synthesis of pyrimidine and indirectly of vitamin B1 (vitamin B1 harbors a pyrimidine ring) is interrupted in the pyrimidine regulation gene *carP* (Tab. 2), a finding further pointing towards a crucial role of yet another vitamin, vitamin B1, for in vivo survival of APEC. Similar findings concerning the importance of vitamins were found in SCOTS-analyses with a highly virulent APEC strain (Dozois *et al.*, 2003). A gene encoding a putative dethibiotin synthase was highly expressed in vivo during APEC infection. The authors suggested that biotin limitation may be an innate host response against the bacterium as the biotin-binding protein avidin is generally induced in avian tissues after *E. coli* infection.

5.2.5.6 Identification of a putative new pathogenicity island

One of the selected mutants, M00C03, is disrupted in a gene with similarity to *r3* encoding a β-cystathionase. This enzyme converts cystathione into homocysteine (Brown *et al.*, 1990). In a previous study it was reported that a cell-free β-cystathionase preparation of *Bordetella avium* was highly toxic against a variety of eukaryotic cell lines including embryonic bovine tracheal cells and osteogenic cells (Gentry-Weeks *et al.*, 1993). M00C03 was sligthly out-competed by its parental strain in lung, liver, and spleen but not in heart and kidney. Another mutant, M13H03, was shown to be disrupted in a gene encoding a putative phosphosugar isomerase. It demonstrated slight attenuation in liver, spleen, and kidney and moderate attenuation in the lung. In mutant M11E02, the transposon inserted into a gene with significant similarity to *malX* (synonym *r2*) which encodes the maltose- and glucose-specific component IIa of a phosphoenolpyruvate-dependent phosphortransferase system. This mutant was shown to be moderately attenuated in lung, heart, and kidney.

These genes have so far only been described in the uropathogenic *E. coli* (UPEC) strain CFT073 (Guyer *et al.*, 1998), but not in any other bacterial species. Interestingly, in CFT073 these genes are localized on a pathogenicity island inserted near the tRNA gene *metV*. It is well known that UPEC and APEC, both causing extraintestinal diseases, share identical virulence genes (Welch *et al.*, 2002). In order to identify a similar pathogenicity island as described for CFT073, we utilized primer pairs R2 plus R3, R1 plus R2, and R1

plus f447 (Tab. 2), and indeed we were able to amplify the right junction of a *metV* genomic island in IMT5155 as well. This genomic island also harbored insertion sequences *r14* and *r15* identified in CFT073, and ORFs c3406 – R3 were found in identical order as in CFT073 (Fig. 15). As all three newly identified STM mutants M00C03, M11E02, and M13H03 showed attenuation by in vivo competition assay, we predict a role of this *metV* genomic island in APEC pathogenesis. However, further studies are required to characterize this island and to determine whether the term "pathogenicity island" is justified.

5.2.5.7 Other mutants

Furthermore, two putative oxidoreductases were identified, one GTP-binding protein, one putative polysaccharide hydrolase, and one putative regulator to be involved in in vivo virulence. In addition, three *orfs* are predicted to encode proteins with a high degree of similarity to proteins of unknown function, and a further two mutants had disruptions in genes which have no similarity to any known sequences in the databases (Tab. 3). Thus, it is a major future challenge to elucidate the functions of these genes during APEC pathogenesis.

5.2.6 Conclusion

In this study, Signature-tagged transposon mutagenesis (STM), a large-scale simultaneous screening method (Hensel *et al.*, 1995), was established and used to identify genes required for in vivo survival of the highly virulent APEC strain IMT5155 (O2:H5) in the chicken, the natural host organism of this important pathogen. Due to the literature, this is the first time STM has been used to analyse *E. coli* infections of the respiratory tract of chickens. Prior studies with *E. coli* strains have used STM analyses only for urinary and gastrointestinal tract infections of mammals (Bahrani-Mougeot *et al.*, 2002; Martindale *et al.*, 2000).
After two rounds of screening and individual infection studies, 1.8% of the total pool of mutants was confirmed to be attenuated for survival in internal organs. A total of 28 genes/*orfs* were identified, mutation of which led to in vivo-attenuation, including both previously known as well as novel factors critical for avian pathogenic *E. coli* (APEC)

infections in chickens. These results confirmed the importance of LPS, capsule, and iron acquisition systems as colisepticemia-associated virulence determinants in APEC. Two mutants were identified with interruptions in genes directly or indirectly involved in the synthesis of vitamins. Furthermore, various genes were identified known to be common virulence determinants for bacteria in general, some of which are described for APEC for the first time in this study. However, several virulence associated factors already described for APEC were not targeted by this STM approach (Dho-Moulin and Fairbrother, 1999; Ewers *et al.*, 2003; La Ragione and Woodward, 2002). One possible reason for this could be the fact, that STM does not represent an exhaustive search for virulence associated genes in APEC. Virulence genes critical for disease, especially toxins such as the APEC vacuolating autotransporter toxin (Parreira and Gyles, 2003), may not appreciably affect survival of the bacterium in the host and thus would not be regularly selected by STM. The method may also not identify mutants with insertions in genes encoding secreted products such as the temperature-sensitive hemagglutinin (Tsh) of APEC which could theoretically be *trans*-complemented by other bacteria in the mixed population of mutant pools (Dozois *et al.*, 2000). Perhaps not surprisingly, genes of the *fim*-cluster encoding F1 fimbriae in APEC strains were also not identified. It remains controversial whether F1 fimbriae contribute to the adhesion of APEC to epithelial cells of trachea and lung, since *fim*-mutants show conflicting results in in vivo tests (Arne *et al.*, 2000; Marc *et al.*, 1998; Mellata *et al.*, 2003a). Results presented here contrast those from an STM study of *E. coli* urinary tract infections, where eight *fim*-mutants were selected in the UPEC strain CFT073 (Bahrani-Mougeot *et al.*, 2002). The authors confirmed the potential role of type 1 fimbriae for the pathogenesis of urinary tract infection by isolating six different *fim* genes, demonstrating the attenuation of these mutants in a murine infection model. UPEC strains also express pyelonephritis-associated fimbriae that have been shown to be critical for urinary tract infections (Welch *et al.*, 2002). IMT5155 does not possess the *pap*-operon, as these genes could not be deteced either by by PCR or hybridization analyses. In general, the high virulence of IMT5155 questions an essential role for P-fimbriae in the APEC infection process. Beyond LPS and capsule genes, other genes encoding serum resistance factors in APEC such as outer membrane protein A, transfer protein T or increased serum survival protein,

were also not identified in this screen, although some of these factors have recently been shown to be expressed in vivo during APEC infection (Dozois *et al.*, 2003). The failure to detect the above mentioned factors for serum resistance could be due to the limitations of STM described above. Another reason could be the differing inoculation route of bacteria in the cited work. While here intra-tracheal infections were elaborated, the intra-air sac inoculation is the predominant route of infection in experimental chicken infection studies with APEC, circumventing physiological borders. This suggests an influence of the route of infection and expression of virulence associated genes in different host compartments.
Future studies in our laboratory should help clarify these questions. In addition, functional analyses of the disrupted genes are needed to elucidate the mechanisms by which they exert their effects as well as complementation and construction of defined deletion mutations to confirm the observed attenuation. This and future studies will hopefully lead to a more comprehensive understanding of APEC virulence in the chicken.