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Comparative molecular approaches to identify host determinants mediating adhesion of *E. coli* F4 strains in pigs

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To all the pigs.

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Contents

Notation

Abbreviations

Abbreviation	Description
A,T,G,C	Adenine, thymine, guanine, cytosine
ADP	Adenosine diphosphate
BAC	Bacterial artificial chromosome
bp	Base pair
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary Deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
ddH_2O	Double destilled water
del	Deletion of a nucleotide
delins	Deletion and insertion of a nucleotide
df	Degree of freedom
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EMBL	European Molecular Biology Laboratory
EST	Expressed sequence tag
ETEC	Enterotoxigenic *E. Coli*
F18	Fimbriae of type F18
F4ab, F4ac, F4ac	Fimbriae of type F4 with ab, ac or ad antigens
FA	Formaldehyde agarose
FaeG	Major subunit protein of F4 fimbriae
HSA	Homo sapiens, human

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Abbreviation	Description
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ins	Insertion of a nucleotide
IQR	Interquartile range
LT-I, LT-II	Heat-labile enterotoxin I and II
NEH	Nordic experimental herd
PCR	Polymerase chain reaction
r_s	Spearman correlation
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-PCR
SDS-page	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEH	Swiss experimental herd
SNP	Single nucleotide polymorphism
SPS	Swiss performing station
SSC	Sus scrofa, swine
STa, STb	Heat-stabile enterotoxin a and b
UTR	Untranslated region of a RNA

Units

Symbol	Meaning
dimensions	
k	10^3
m	10^{-3}
µ	10^{-6}
n	10^{-9}
p	10^{-12}
g	gram
g	m s^{-2}, gravity constant
cM	centi Morgan
kDa	kilo Dalton
LOD score	log_{10} of the odds
M	mol $litre^{-1}$
Mb	10^6 base pairs
mol	6×10^{23} molecules

Summary

The ability to colonise the intestine is a common feature of pathogenic and non-pathogenic bacteria. Enterotoxigenic *Escherichia (E.) coli* (ETEC) can adhere to the brush borders of small intestine enterocytes by means of fimbriae. ETEC produce toxins that cause a secretory diarrhoea. *E. coli* diarrhoea is the most important source of mortality in newborn and weaned pigs, and causes high losses in the pig industry. Susceptibility is conferred by specific receptors on the brush borders of enterocytes. Three *E. coli* variants carrying F4 fimbriae are known: F4ab, F4ac and F4ad. In most cases, the variant F4ac is isolated in pigs affected by ETEC diarrhoea. The receptor gene for F4ab and F4ac (*F4bcR*) is inherited as an autosomal dominant trait and was mapped to porcine chromosome 13.

A multipoint linkage analysis of eight microsatellites and one single nucleotide polymorphism (SNP) localised the *F4bcR* inside the interval *SW207* – [*MUC4-8227, MUC4gt*] – *S0075*. Genotyping and phenotyping data of 331 pigs from the Swiss experimental herd (SEH), 143 pigs from the Swiss performing station (SPS) and 236 pigs from the Nordic experimental herd were used. *F4bcR* was strongly associated with the g.8227C>G polymorphism in *MUC4*. Only 3 of 331 SEH pigs (1%) and 6 of 78 SPS offspring (8%) were discordant between this polymorphism and the phenotype. The *MUC4* g.7947A>G polymorphism was in complete linkage disequilibrium with the g.8227C>G polymorphism in 180 analysed pigs and, therefore, was equally reliable for prediction of susceptibility to *E. coli* F4ab/F4ac infection. We examined further four genes as candidates for the *F4bcR*: *TNK2, ST6GAL1, CLDN1* and *C3orf21*.

Comparison between the sequences in *TNK2* from two resistant and two homozygous susceptible pigs revealed 112 sequence variants. In further 180 pigs, three of these SNPs were genotyped, but their haplotypes did not coincide with the phenotype in 8.5% of these pigs. None of these three SNPs and the two SNPs in *MUC4* are located in the regulatory regions and change amino acids. Therefore, none of them are strong candidates. No sequence variants were identified in the other three candidate genes.

The number of *E. coli* F4ad that adhered to enterocytes varied considerably in the adhesion test within and between litters of repeated matings. External factors may particularly affect the adhesion strength of F4ad bacteria. Aside from the fully resistant and susceptible adhesion phenotypes, some pigs belonged to an intermediate phenotype with some to many enterocytes devoid of bacteria. Parent pigs with a known phenotype were divided into a resistant, an intermediate and

a highly susceptible class. Adhesion strengths of 1166 offspring from the resulting six mating combinations were recorded. Offspring from highly susceptible parents showed clearly more adhesive enterocytes than offspring from resistant or weak adhesive parents. We therefore conclude that the F4ad adhesion is genetically influenced by one or several receptors.

The *MUC4* g.8227C>G polymorphism, segregating with susceptibility and resistance to *E. coli* F4ab/F4ac, can be used in selection programs, although the underlying genetic variation remains unknown. The current progress in pig genome sequencing will make it possible to find new markers that allow to finally identifying the causal mutation for *F4bcR*.

Zusammenfassung

Die Besiedlung des Darms durch Bakterien ist eine gemeinsame Eigenschaft von nicht-pathogenen und pathogenen Bakterien. Von den pathogenen Bakterien haften enterotoxigene *Escherichia coli* (*E. coli*) mit ihren Fimbrien an spezifische Rezeptoren auf den Bürstensäumen von Enterozyten im Dünndarm und produzieren Toxine, die bei Ferkeln eine Durchfallerkrankung verursachen. *E. coli* Diarrhö ist die wichtigste Abgangsursache bei neugeborenen Ferkeln und Absetzferkeln und führt zu hohen Verlusten in der Schweineproduktion.

Von den drei bekannten *E. coli* Varianten mit F4 Fimbrien F4ab, F4ac und F4ad wird die F4ac Variante am häufigsten als Verursacherin von *E. coli* Durchfall gefunden. Das Rezeptor-Gen für die *E. coli* F4ab und F4ac Anfälligkeit (*F4bcR*) wird autosomal dominant vererbt und wurde auf dem Schweinechromosom 13 kartiert.

Mit einer Kopplungs-Analyse von acht Mikrosatelliten und einem einfachen Sequenzpolymorphismus (SNP) wurde der Bereich für den *F4bcR* auf das Intervall *SW207* – [*MUC4-8227, MUC4gt*] – *S0075* eingegrenzt. Dazu wurden Daten der Phänotypen und Genotypen von insgesamt 331 Schweinen aus unserer Versuchsherde (SEH), 143 Schweinen aus der Schweizer Schweinepopulation (SPS) und 236 Schweinen der Nordischen Versuchsherde verwendet. *F4bcR* war eng gekoppelt mit dem g.8227C>G Polymorphismus im Kandidatengen *MUC4*. In nur 3 von 331 SEH Schweinen (1%) und 6 von 78 SPS Nachkommen (8%) stimmte der F4ab/F4ac Phänotyp nicht mit dem Polymorphismus überein. Der *MUC4* g.7947A>C Polymorphismus war in 180 analysierten SEH und SPS Schweinen in einem kompletten Kopplungsungleichgewicht mit dem g.8227C>G Polymorphismus und war ebenso aussagekräftig für die Diagnose der Empfänglichkeit auf eine *E. coli* F4ab/F4ac Infektion.

Weitere vier Kandidatengene wurden untersucht: *TNK2*, *ST6GAL1*, *CLDN1* und *C3orf21*. Beim Vergleich der *TNK2* Sequenzen von zwei resistenten und zwei homozygot empfänglichen Tieren wurden 112 Sequenzvarianten gefunden. Drei dieser SNPs wurden in 180 SEH und SPS Schweinen untersucht, ihr Haplotyp stimmte jedoch in 8.5% der Fälle nicht mit dem F4ab/F4ac Phänotyp überein. Diese drei SNPs und die zwei SNPs in *MUC4* liegen in Intronsequenzen und sind deshalb keine bedeutenden Kandidaten für *F4bcR*. Gleiches gilt für die Sequenzen der anderen drei Kandidatengene, in welchen keine Sequenzvarianten gefunden wurden.

Bei der Untersuchung der *E. coli* F4ad Adhäsion zeigte sich, dass die Anzahl F4ad an den Bürstensäumen beträchtlich variierte, sowohl innerhalb der Würfe, als auch zwischen den Würfen wiederholter Paarungen. Externe Faktoren beein-

flussen vermutlich die Adhäsionsstärke der *E. coli* F4ad. Neben einem vollständig resistenten und empfänglichen Adhäsionsphänotyp wurde bei einigen Schweinen ein intermediärer Phänotyp gefunden mit einzelnen bis vielen Enterozyten ohne anhaftende Bakterien. Elterntiere mit bekanntem F4ad Phänotyp wurden in eine resistente, eine schwach empfängliche und eine hoch empfängliche Klasse eingeteilt. Der Vergleich der Adhäsionsstärke von 1166 Nachkommen aus den sechs möglichen Paarungskombinationen zeigte, dass Nachkommen hoch empfänglicher Eltern klar mehr Enterozyten mit anhaftenden Bakterien haben als Nachkommen resistenter oder schwach empfänglicher Eltern. Wir schliessend deshalb daraus, dass die *E. coli* F4ad Adhäsion genetisch beeinflusst wird durch einen oder mehrere Rezeptoren.

Der *MUC4* g.8227C>G Polymorphismus segregiert mit der Empfänglichkeit und Resistenz auf *E. coli* F4ab/F4ac und kann für die Selektion verwendet werden, obwohl die zugrunde liegende genetische Ursache unbekannt bleibt. Der gegenwärtige Fortschritt bei der Sequenzierung des Schweinegenoms wird es ermöglichen, neue Marker zu entwickeln und helfen, die kausale Mutation für *F4bcR* zu finden.

1. Introduction

1.1. *Escherichia coli* diarrhoea

Bacteria predominate the normal microflora in the intestinal tract of humans and animals and usually do not cause diseases. However, certain pathogenic strains of bacteria are able to colonize the intestinal tract of pigs and cause diarrhoea. Pathogenic *Escherichia coli* belong to the diarrhoeagenic bacteria and lead to high losses for pig industry. In a study from Denmark from the 1970s, about 12% of total losses of piglets was attributed to diarrhoea (Nielsen *et al.*, 1974). A study from Ontario, CA, reported an increase of mortality from 2% to 7% due to postweaning diarrhoea (Amezcua *et al.*, 2002).

These *E. coli* causing diarrhoea belong to the family Enterobacteriaceae within the gram-negative bacteria and are classified to six different categories: Enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (reviewed by Nataro and Kaper, 1998).

ETEC can express one or more of the plasmid coded fimbrial type F4 (K88), F5 (K99), F6 (987P), F18 and F41. In pigs, *E. coli* F4 cause diarrhoea in newborn and weaned pigs. *E. coli* F4 and F18 are most prevalent (section 1.2). In contrast, in humans, the fimbrial types F2 (CFAI) and F3 (CFAII) are the most prevalent types. Three variants of *E. coli* F4 fimbriae have been found, F4ab, F4ac and F4ad (Guinée and Jansen, 1979), whereas antigen factor a always occurs with one of the three other antigens b, c or d (Wilson and Hohmann, 1974).

The fimbriae on the surface of the bacteria are a precondition for successful colonisation of the small intestine. Additionally, bacteria must penetrate the mucus barrier to be able to adhere to specific surface structures on the brush borders of epithelial cells. The adhesion to these receptors on the brush borders occurs by the mean of fimbriae (Jones and Rutter, 1972). Finally, bacteria release enterotoxins that are taken up into the intestinal cells and that induce diarrhoea.

1.1.1. F4 fimbriae

F4 fimbriae are composed of single copies of the minor subunit FaeC at the tip of the fimbrium, and of about 100 copies of the major subunit FaeG forming the fimbrium (pilus). Other *fae* genes are also involved in fimbrial biosynthesis, but

do not affect fimbrial binding activity (reviewed by Van den Broeck *et al.*, 2000). One bacterium contains 100 to 300 of these filaments, which have length of 0.1 to 1 µm and a diameter of 2 to 4 nm. The molecular weight of the major subunit has been estimated between 23.5 and 26 kDa (Mooi and De Graaf, 1979). Later, the molecular weight of the FaeG proteins P14190 (F4ac), P14191 (F4ab) and P02970 (F4ad), based on amino acid composition was calculated between 27.4 and 27.6 kDa (Proteomics server on `http://www.expasy.ch`).

Removing the minor subunits FaeC of the F4 fimbriae by urea treatment did not affect adhesion properties. Therefore, it was proposed that the fimbrial binding site resides within FaeG (Bakker *et al.*, 1992b). Different regions in FaeG have been identified that determine the specificity of the three *E. coli* F4 variants. A domain determining the specificity of all three F4 variants was found in the hypervariable region between amino acids 163 and 174 of FaeG. Additionally, amino acids 136, 216, 81 and 105 were identified for F4ab specificity, and amino acid 147 for F4ac specificity (Bakker *et al.*, 1992a). An earlier study suggested that the region between amino acids 125 and 163 was responsible for determining the F4 binding specificity (Jacobs *et al.*, 1987). A recent study has confirmed the importance of this region and suggested that amino acid 152 was particularly important for F4ac and F4ad adhesion to brush borders (Zhang *et al.*, 2009). Another study determined the binding of monoclonal antibodies against F4ac to the amino acids between 64 and 107 of FaeG (Sun *et al.*, 2000).

1.1.2. F4 enterotoxins

Enterotoxigenic *E. coli* produce plasmid-regulated enterotoxins that are classified according to their thermal stability as heat-labile (LT-I, LT-II) or heat-stable (STa, STb) enterotoxins. Most *E. coli* strains with F4 fimbriae isolated in Switzerland in the 1990s produced LT(-I) and STb (Bertschinger, 1995; Bertschinger and Fairbrother, 1999), and few F4 strains produced STa (Sarrazin *et al.*, 2000).

STb is mainly produced by porcine ETEC and is the most frequently detected enterotoxin in *E. coli* isolates from pigs with diarrhoea. Casey *et al.* (1998) transformed a non enterotoxigenic F41 strain with STb and used this strain to infect pigs. They did not observe a significant contribution of STb to F4 diarrhoea in neonatal pigs. In another study, an F4 strain expressing LT was found to cause diarrhoea in all gnotobiotic piglets, whereas STb did not cause clinical symptoms in all infected piglets (Zhang *et al.*, 2006b). It has been suggested that LT promotes *in vitro* adhesion of ETEC to porcine intestinal cells (Johnson *et al.*, 2009).

The heat-labile enterotoxin

There are two known subtypes of the heat-labile enterotoxin (LT). LT-I is found in isolates that are toxic to humans and animals, and LT-II is mainly found in isolates

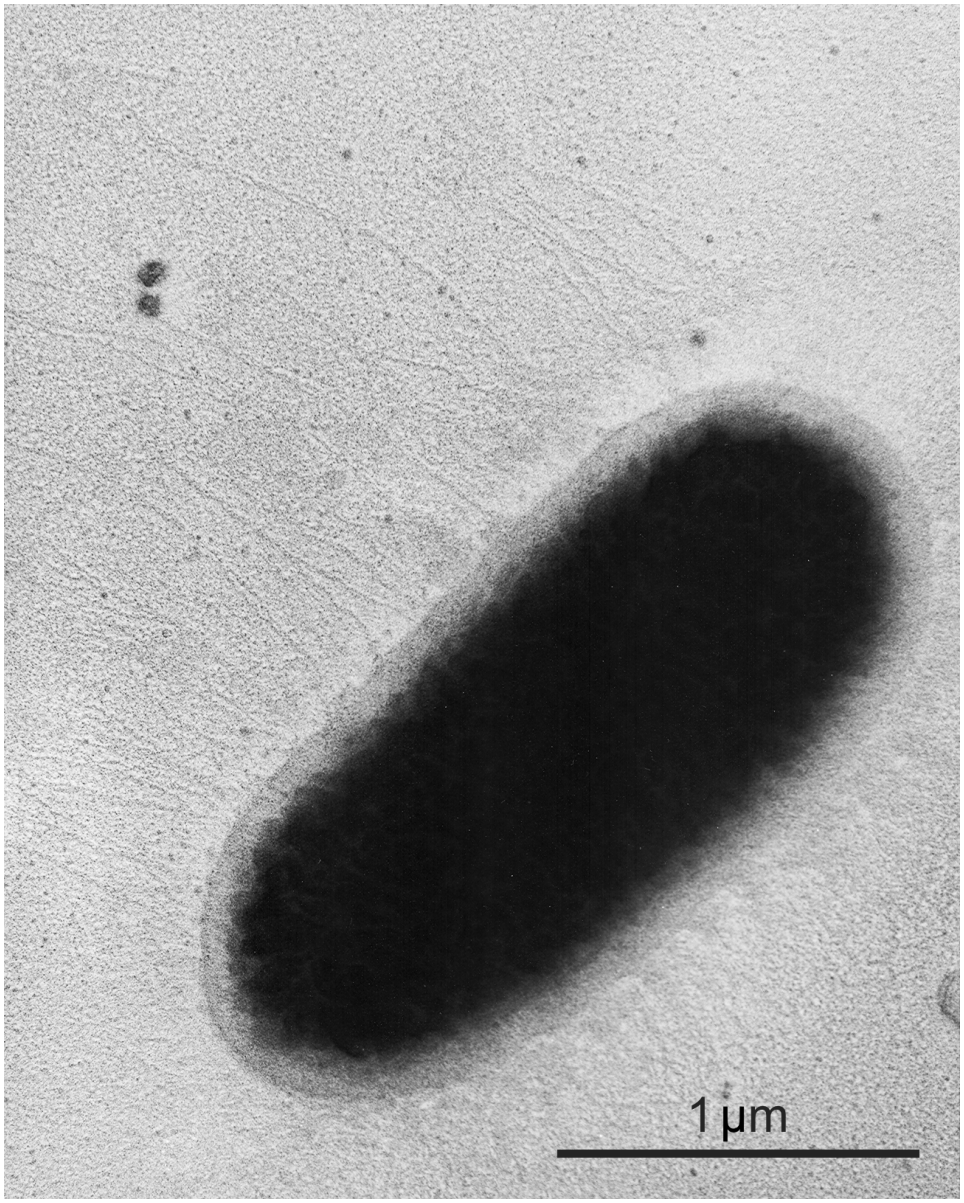

Figure 1.1.: *E. coli* bacterium with F4 fimbriae. Transmission electron micrograph after rotation shadowing with 5 nm carbon-platinum (Peter Wild, Laboratory for Electron Microscopy, Institutes of Veterinary Anatomy and Virology, University of Zurich).

that are toxic only to animals. Two variants of LT-II have been identified (LT-IIa and LT-IIb) but so far, neither has been reported to induce disease in humans or animals (reviewed by Sanchez and Holmgren, 2005).

The LT-I toxin is composed of one 28 kDa A subunit and 5 identical 11.5 kDa B subunits, which forms a pentagon around the A subunit. LT adheres to ganglioside GM_1 receptors or other receptors with a terminal galactose. After binding to the host cell membrane, the toxin is endocytosed and transported to the cytosol, where the A1 peptide of the subunit A catalyses the ADP ribosylation of the $G_{s\alpha}$ component of ADP. This ribosylation permanently activates the adenylyl cyclase, leading to increased levels of cAMP. The cAMP activates the cAMP dependent protein kinase, which activates chloride channels. An increased secretion of chloride ions and a reduced uptake of NaCl lead to an increased luminal ion concentration and draws water from the cells into the lumen.

The LT is similar to the cholera toxin (CT) expressed by *Vibrio cholerae*, regarding the amino acid sequence (80% identity), the 3d-structure and the intracellular pathway. However, diarrhoea in humans caused by CT is more severe and of longer duration than is the diarrhoea caused by LT-I. The reason for this could be a more efficient secretion of the toxin in *Vibrio cholerae*. Additionally, experiments with CT show that the enteric nervous system could be involved (reviewed by Sanchez and Holmgren, 2005).

The heat-stable enterotoxin

In contrast to the large oligomeric LT, the heat-stable enterotoxin exists in a 2 kDa STa and a 5 kDa STb subtype. The two predominantly plasmid encoded monomers differ in structure and mechanism. STa activates the transmembrane protein guanylate cyclase C on intestinal epithelial cells and leads to intracellular increase of cGMP level. The high cGMP level leads to increased Cl^- secretion into the lumen and reduced uptake of Na^+. Subsequently, water flows into the lumen, leading to diarrhoea (Sears and Kaper, 1996).

Details of the function of STb and its molecular characteristics are even less clear. STb seems not to affect cAMP or cGMP levels, but seems to stimulate non Cl^- secretion (reviewed by Dubreuil, 2008).

1.2. Prevalence of *E. coli* F4 and F18 diarrhoea

Enterotoxigenic *E. coli* are the most important cause of diarrhoea in newborn and weaned pigs and are dominated by F4 and F18 fimbriated *E. coli* (Frydendahl, 2002; Moon *et al.*, 1999). The prevalence of F4 and F18 in piglets with diarrhoea varies with geographic regions. Of 175 fimbriated *E. coli* strains isolated of faeces or faecal swab from diarrhoeic piglets in South Dakota, USA, 65% expressed F4

fimbriae and 34% expressed F18 fimbriae (Zhang, 2007). In 113 isolates of pigs dying from diarrhoea or oedema disease in Switzerland, F4 fimbriae and genes for F18 fimbriae were both found in about 50% of the strains (Sarrazin *et al.*, 2000). Of 4221 pigs dying from *E. coli* infection in Saxony, Germany between 1963 and 1990, almost all isolates carried the F4 variant (Wittig and Fabricius, 1992).

In Eastern European countries, F18 seems to be more prevalent than F4. Of 101 pigs from 20 farms in Slovakia, 19% of the isolates from these pigs with postweaning diarrhoea contained F4 genes and 35% contained F18 genes (Vu Khac *et al.*, 2007). In Polish piglets suffering from *E. coli* disease, 62% of the 21 strains from pigs with post weaning diarrhoea and 82% of the 19 strains from pigs with oedema disease carried F18 genes, and 22% of 41 strains carried F4ac fimbriae (Osek *et al.*, 1999). In a study from Cuba, no F4 strains were found in 36 strains of pigs with diarrhoea, but 61% carried F18 fimbriae and 8% carried F6 fimbriae (Blanco *et al.*, 2006).

1.2.1. *E. coli* F4 prevalence

Among the three *E. coli* F4 fimbrial variants F4ab, F4ac and F4ad, the F4ac variant is the most prevalent strain isolated from pigs with diarrhoea. More than 98% of the *E. coli* F4 isolates from 237 Czech and 184 Slovakian pigs with diarrhoea carried genes for F4ac fimbriae (Alexa *et al.*, 2001; Holoda *et al.*, 2005). In a US study, all 415 F4 positive isolates of *E. coli* proved to be of the F4ac variant (Westerman *et al.*, 1988). In another study, only 44 of 812 *E. coli* isolates from diarrhoeic pigs from Korea carried F4 genes. Of the F4 strains, 96% were carried F4ac genes and none carried F4ad genes (Choi and Chae, 1999).

As F4ac is the most prevalent F4 variant isolated from pigs with diarrhoea, it seems that *E. coli* F4ab and F4ad are not relevant for diarrhoea. However, a Chinese study found F4ad genes in 32 of 36 isolates of Chinese piglets suffering from diarrhoea caused by F4 fimbriae. Remarkably, in an earlier period with diarrhoeic pigs from a different region, all of the F4 strains carried F4ac genes (Wang *et al.*, 2006).

1.2.2. *E. coli* F4ac susceptibility among breeds

Susceptibility to *E. coli* F4ac varies among breeds. In several studies, 47% to 79% of Landrace (47%, >60%, 79%), Large White (49%, 53%, 56%, >60%), Yorkshire (48%, 75%) and Piétrain (>60%) breed pigs were susceptible. Between 0% and 46% of pigs were susceptible among Chester White (15%, 36%), Duroc (33%, 40%), Hampshire (<20%, 22%, 46%), Meishan (0%) and Songliao Black (10%) breeds, and among the cross breeds Meishan x Minzu (27%), Meishan x Fengjing (24%) and Yorkshire x Landrace (41%) (Baker *et al.*, 1997; Edfors-Lilja *et al.*, 1986; Engel *et al.*, 1998; Gautschi and Schwörer, 1988; Li *et al.*, 2007; Michaels *et al.*, 1994; Rapacz and Hasler-Rapacz, 1986). However, one has to consider that the threshold

in phenotyping for susceptible pigs varied among these studies. In some studies, adhesion of at least one bacterium to more than two of 10 to 60 classified brush borders was sufficient for susceptibility (Engel, 1998; Engel *et al.*, 1998), while in other studies more than two (Baker *et al.*, 1997) or five (Li *et al.*, 2007) bacteria adhering to more than 10% of 20 enterocytes were necessary for a susceptible rating.

1.3. F4 receptors in pigs

1.3.1. Determination of the F4 phenotype

Three methods for the preparation of enterocyte surfaces have been reported for adhesion tests: (1) Brush border membrane vesicles prepared from enterocytes (Baker *et al.*, 1997; Bijlsma *et al.*, 1982; Sellwood *et al.*, 1975), (2) entire isolated enterocytes (Edfors-Lilja *et al.*, 1986; Hu *et al.*, 1993; Python *et al.*, 2002; Rapacz and Hasler-Rapacz, 1986; Vögeli *et al.*, 1996) and (3) villous brush borders (Cox and Houvenaghel, 1993; Rasschaert *et al.*, 2007). Bacterial adhesion to these structures was detected either by light microscopy or by phase contrast microscopy. Chandler *et al.* (1986) developed an ELISA with immobilised F4 fimbriae that were exposed to brush border membranes and subsequently detected by brush border antibodies.

The microscopic adhesion test used in our lab was first described by Vögeli *et al.* (1996) and based on Sellwood *et al.* (1975), who developed an *in vitro* test with *E. coli* F4ab and F4ac to distinguish between adherence and non-adherence of *E. coli* F4 to intestinal brush borders. Artificial *E. coli* F4ac infection of piglets showed that adhesion to brush borders is linked to neonatal diarrhoea of pigs, and non-adhesion is linked to resistance to *E. coli* F4 (Rutter *et al.*, 1975). It is accepted that adhesion of *E. coli* F4 is a prerequisite for diarrhoea. Nevertheless, in a study with gnotobiotic pigs, 8 of 17 pigs were F4 adhesion positive, but did not develop diarrhoea (Francis *et al.*, 1998).

Bijlsma *et al.* (1982) identified five patterns of *E. coli* F4 adhesion to brush borders of enterocytes. The adhesion phenotypes were confirmed (Rapacz and Hasler-Rapacz, 1986) and a sixth phenotype F was reported by Baker *et al.* (1997) (Table 1.1). In few cases, pigs with additional adhesion patterns were found: Li *et al.* (2007) reported 3 of 366 pigs with phenotype H ($F4ab^-/F4ac^+/F4ad^+$), and 3 other pigs with phenotype G ($F4ab^-/F4ac^+/F4ad^-$), assuming 10% of enterocytes with more than five adhering bacteria as the threshold for susceptibility. Additionally, Bonneau *et al.* (1990) reported phenotype G in 5 of 149 pigs, but gave no further details of the methods and results.

Table 1.1.: *E. coli* F4 phenotypes A through F according to Bijlsma *et al.* (1982) and Baker *et al.* (1997). Bacterial adhesion to enterocytes is marked with +, whereas no adhesion is marked with −.

E. coli	Phenotype					
variant	A	B	C	D	E	F
F4ab	+	+	+	−	−	+
F4ac	+	+	−	−	−	−
F4ad	+	−	+	+	−	−

1.3.2. F4ab/F4ac inheritance

The adhesion phenotype controlled by the F4ac receptor gene is inherited as an autosomal dominant trait (Gibbons *et al.*, 1977; Sellwood *et al.*, 1974). Some studies have indicated several distinct but linked genes for F4ab and F4ac receptor (*F4abR, F4acR*) (Edfors-Lilja *et al.*, 1995; Guérin *et al.*, 1993), but other studies have indicated a common receptor gene for F4ab and F4ac adhesion (*F4bcR*) (Bijlsma and Bouw, 1987; Jørgensen *et al.*, 2003; Python *et al.*, 2002). In recent Asian studies, two separate loci have been indicated (Li *et al.*, 2007; Peng *et al.*, 2007).

Besides the strong and unambiguous adhesion or non adhesion of F4ab and F4ac, a weak *E. coli* F4ac adhesion with only 3 to 4 bacteria per brush border has also been reported. The impact on diarrhoea does not seem very high, since oral *E. coli* F4ac infection of a litter with weak-adhesive piglets did not lead to diarrhoea in any of the 8 piglets (Sellwood, 1980, 1984). Several studies have mentioned a weak F4ab or a weak F4ac adhesion, but the inheritance and relevance of the weak adhesion remain unclear (Baker *et al.*, 1997; Bijlsma and Bouw, 1987; Li *et al.*, 2007; Michaels *et al.*, 1994; Python *et al.*, 2002, 2005). Bijlsma and Bouw (1987) propose that a weak adhesion arises from the influence of epistatic genes on the receptor expression or from modification of the receptor expression.

1.3.3. F4ad inheritance

The mode of inheritance of the F4ad receptor (*F4adR*) is not yet clear. According to the adhesion phenotypes, Bijlsma and Bouw (1987) have suggested a dominant receptor locus for *F4adR* that is independent from *F4bcR*. However, the inheritance of the receptor does not seem to be persistent, as adhesive enterocytes have been found in offspring of parents that had been phenotyped as resistant. Python *et al.* (2005) also suggest a dominant inheritance of *F4adR*, as the fraction of susceptible offspring did not deviate from the Mendelian inheritance pattern expected of a dominant receptor gene. Hu *et al.* (1993) proposed an age related expression of a low affinity F4ad receptor (*F4adL*) in phenotype D, which is not found in pigs

older than 16 weeks of age, and a permanent high affinity receptor (*F4adH*) in phenotype A.

1.3.4. Genetic mapping of *F4bcR*

Guérin *et al.* (1993) reported a linkage of the *E. coli* F4ab and F4ac receptor loci with the transferrin locus on swine chromosome 13 (SSC13), which was confirmed by Edfors-Lilja *et al.* (1995). Our group mapped the locus for *F4bcR* to the interval between *S0068* and *SW1030* on SSC13q41 (Python *et al.*, 2002) and later refined it to the region between *SW207* and *SW225* (Python, 2003; Python *et al.*, 2005). The position of *F4bcR* was confirmed by Jørgensen *et al.* (2003).

The same group identified polymorphisms in the Mucin 4 gene (*MUC4*, DQ848681) that can be used as markers for F4ac susceptibility and resistance (Jørgensen *et al.*, 2004).

Several studies have analysed polymorphisms in *MUC4* and the association with the F4ac adhesion phenotype. Rasschaert *et al.* (2007) genotyped the *MUC4* DQ848681:g.8227C>G polymorphism of 63 pigs. With a threshold of five adhering bacteria to 5x50 µm brush borders, 19 (30%) F4ac susceptible pigs were resistant according to the g.8227C>G genotype. As there could be differences in the phenotype classification in their method compared to other methods, these numbers are difficult to interpret. Further, in a Chinese study, the g.8227G allele coincided with susceptibility in 92% of Landrace breed pigs (n=84), 95% of Large White (n=149) and 92% of Songliao Black (n=77) breed pigs (Li *et al.*, 2008).

Peng *et al.* (2007) analysed the intronic polymorphisms DQ848681:g.15581G>A and g.15672G>A of 748 White Duroc x Erhualian cross breed pigs. The F4ac adhesive phenotype coincided with the g.15581A allele in 95% of the pigs, and the resistant phenotype coincided with the g.15581GG genotype in 84% of the pigs. In three other studies, researchers from the same group investigated one informative polymorphism in *TFRC* (Wang *et al.*, 2007), three polymorphisms in *MUC13* (Zhang *et al.*, 2008), and three polymorphisms in *SLC12A8*, *MYLK* and *KPNA1* (Huang *et al.*, 2008). In all three studies, haplotypes were associated with F4ab and F4ac phenotypes, but the polymorphisms were not causative.

Further, analysis of cDNA in *TFRC*, *B3GNT5*, *B4GALT4* and *B3GALNT1* (former *B3GALT3*) mapping to the interval between *SW207* and *SW1030* did not reveal polymorphisms that were associated completely with F4ac susceptibility and resistance (Python, 2003; Python *et al.*, 2005). In a recent study, Ren *et al.* (2009) defined a small region on SSC13, spanning from the genes *DLG1* (HSA3:198.3 mb) to *KPNA1* (HSA3:123.6 mb), as region that harbours the *F4acR*.

Comparative mapping of humans and pigs showed homologies between human chromosome 3 (HSA3) and SSC13 with a few chromosomal rearrangements (Meyers *et al.*, 2005; Sun *et al.*, 1999; Van Poucke *et al.*, 1999, 2001, 2003; Vingborg *et al.*, 2009). The comparative map of SSC13 and HSA3 is shown in Figure 1.2.

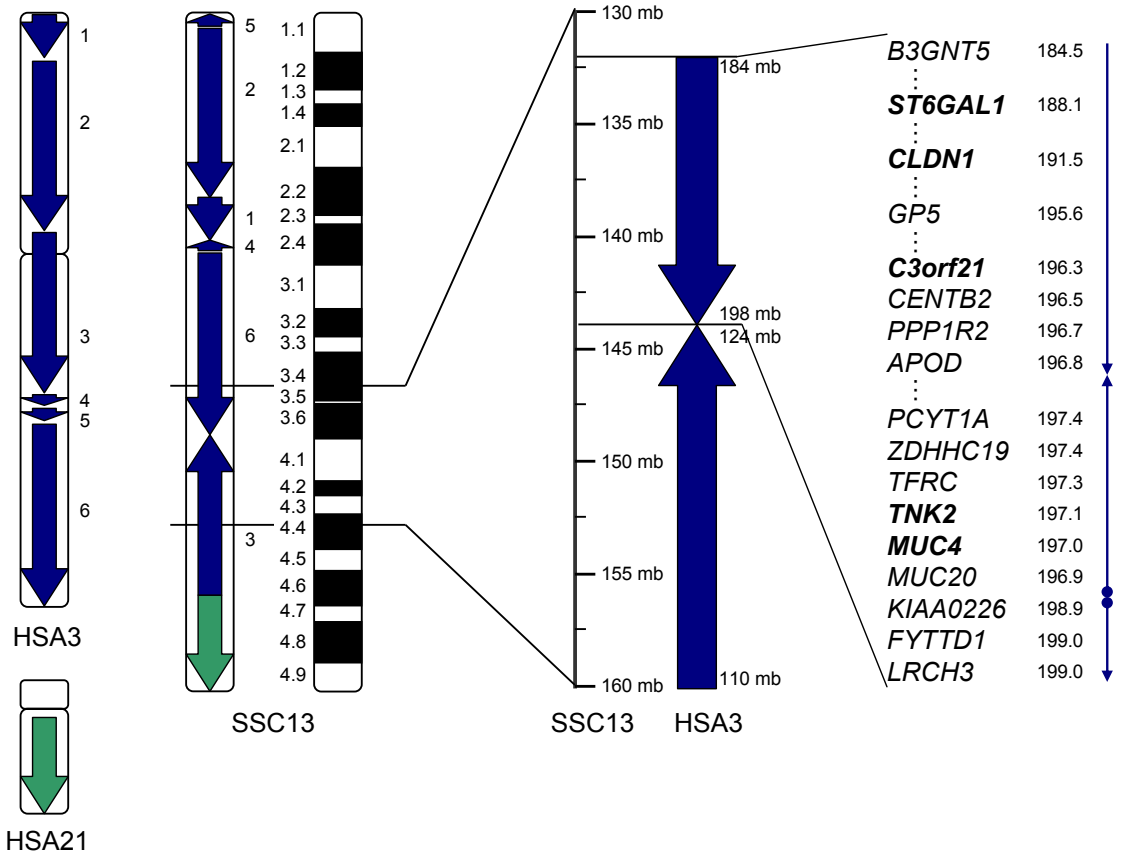

Figure 1.2.: Comparative map of porcine chromosome 13 (SSC13) and human chromosome 3 and 21 (HSA3 and HSA21) as determined by Meyers *et al.* (2005) is shown on the left. The number of the chromosomal segment and the direction of arrow are based on orthology to the human chromosome. The physical position of the mentioned genes on SSC13 are derived from the BAC fingerprint contig map (Humphray *et al.*, 2007), and reveals an inversion as shown to the right.

1.4. Intestinal host receptor proteins

Mucin type proteins and lipids are present on almost every epithelial tissue and are important for defence against pathogens from the exterior. Mucins are optimal targets for microbial attachment because they contain a variety of oligosaccharide structures providing binding sites for bacteria. All three *E. coli* F4 fimbrial variants appear to recognise glycoproteins or glycolipids on the surface of intestinal epithelial cells. In particular, they bind to terminal N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) that are 1,3β- or 1,4β-linked to galactose of glycoproteins (Galβ1,3GalNAc and Galβ1,3/4GlcNAc) (Anderson *et al.*, 1980; Seignole *et al.*, 1994). The terminal linkage of Gal to the GalNAc seems to enhance F4 binding (Grange *et al.*, 2002).

As receptors for F4ab and F4ac fimbriae, two intestinal brush border proteins of 210 to 230 kDa and 240 to 300 kDa were identified by Western blot of SDS-PAGE. These proteins were found only in adhesion positive pigs (Billey *et al.*, 1998; Erickson *et al.*, 1992). Pigs expressing these proteins in brush borders developed clinical symptoms for diarrhoea after infection with F4ab and F4ac strains (Francis *et al.*, 1998).

An intestinal transferrin of about 74 kDa (GP74) was identified as receptor protein for specific F4ab adhesion. The GP74 protein isolated from brush borders specifically bound to F4ab fimbriae, but not to F4ac or F4ad fimbriae (Grange and Mouricout, 1996). Another study identified a galactosylceramide receptor in mucus specific for F4ab adhesion (Blomberg *et al.*, 1993b). Further, adhesion of fimbriae to purified isolated mucus scrapings revealed glycoproteins of 40 to 42 kDa as receptors for F4ab adhesion (Metcalfe *et al.*, 1991), and glycoproteins of 26 kDa and 41 kDa (Fang *et al.*, 2000), and of 80 kDa (Jin *et al.*, 2000) as receptors for F4ac adhesion.

Specific binding of F4ad fimbriae to isolated intestinal brush borders of F4ad adhesive pigs identified a neutral glycosphingolipid with a terminal β-linked Gal as the receptor. This lipid was found in phenotypes A and D, but not in phenotype C (Grange *et al.*, 1999). The mechanism of interaction between the fimbriae and the host receptor has not yet been elucidated and the biochemical structure of the receptor remains unknown.

1.5. Measures against F4 diarrhoea

Different strategies can be pursued to reduce *E. coli* diarrhoea. One possibility is breeding for resistance by marker assisted selection. Other possibilities are to increase immunity by vaccination of the pigs, by medication or by feeding measures.

1.5.1. Antibiotics

In the past decades, the wide use of antibiotics as growth promoter in animal production led to tolerance and resistance of pathogens against antibiotics and is compromising reliable antibiotic therapy in humans and animals (Jensen *et al.*, 2006; Lanz *et al.*, 2003; Uemura *et al.*, 2003). As a consequence, preventive administration of antibiotics is now prohibited for most animals in many countries around the world. However, according to Danmap, antibiotic consumption in pigs was still increasing in Denmark in 2007 (Emborg and Hammerum, 2008).

1.5.2. Immunisation

During pregnancy, embryos do not receive immune protection by immunoglobulins from the sow, as there is no placental transfer of macromolecules in pigs. An effective protection against neonatal infection was achieved by passive immunisation of piglets with colostrum and milk from an *E. coli* F4 susceptible sow, but not from a resistant sow (Sellwood, 1979, 1982). Furthermore, this passive immunisation is only given by sows that developed a systemic immune defence. In practice, pregnant sows are usually vaccinated by injection. Commercial available vaccines often contain inactivated or living F4 fimbriae and LT to temporarily protect the newborn piglets by antibodies in the milk. However, when maternal immunity is lost at weaning, an *E. coli* F4 infection will lead to post-weaning diarrhoea.

Administration of F4 fimbriae

One strategy to prevent post-weaning diarrhoea in pigs due to *E. coli* F4 infection would be vaccination with an inactivated or a live F4 vaccine. Few antigens have been described that would induce a specific immune response. Oral administration of purified F4ac fimbriae to 5 and 14 weeks old pigs resulted in specific IgG and IgA immune response and provided pigs with complete protection against *E. coli* F4 infection (Van den Broeck *et al.*, 1999a). Oral administration of purified F4 fimbriae/human serum albumin (HSA) conjugates also induced a systemic and mucosal (IgG, IgA) immune response against HSA. The immune response was improved by coadministration of CT and was followed by reduced excretion of *E. coli* F4 in faeces (Verdonck *et al.*, 2005).

Only a weak IgG immune response was found when seven-week-old pigs intradermally primed with expression-optimised FaeG and LT-Ib were challenged intragastrically with *E. coli* F4. Nevertheless, *E. coli* F4 excretion in faeces of F4 susceptible pigs was reduced more efficiently compared to the controls (Melkebeek *et al.*, 2007). The systemic immune system was also primed in pigs without the F4 receptor by intramuscular administration of low dose F4 antigen (Van den Broeck *et al.*, 2002). However, oral administration of non-replicating antigens can lead to reduced spe-

cific immune response and to oral tolerance, especially when administered at high doses (Van den Broeck *et al.*, 2002; Van der Stede *et al.*, 2002).

Furthermore, an orally administered soluble antigen may be deactivated by acids, bile and enzymes on its way through the stomach. Antigens administrated in a solid form proved to be more resistant to denaturation. The encapsulated as well as the soluble form of F4 fimbriae could not completely prevent *E. coli* F4 colonisation. However, a significant reduction of *E. coli* F4 excretion was reported in piglets vaccinated with encapsulated F4 fimbriae (Snoeck *et al.*, 2003).

Administration of transgenically expressed FaeG and LT-I

Transgenic expression of the fimbrial subunit FaeG and the enterotoxin LT-I has been reported for alfalfa, tobacco, barley grain, tomato, soybean leaves, maize, lettuce and ginseng (reviewed by Floss *et al.*, 2007). FaeG produced in alfalfa and intragastrically administered to newly weaned pigs resulted in increased immune reaction against F4, but only in a slightly reduced excretion of *E. coli* F4 in faeces after challenging pigs with *E. coli* F4 (Joensuu *et al.*, 2006). In another study, mice were immunised with FaeG produced in tobacco and barley. The mouse FaeG antiserum could inhibit the *in vitro* attachment of *E. coli* F4ac (Joensuu *et al.*, 2006; Verdonck *et al.*, 2004a) and F4ad (Liang *et al.*, 2006) to receptors on porcine intestinal enterocytes. In recent studies, monoclonal antibodies and bacteriophages have been discussed as candidates for prevention and therapy of F4 diarrhoea (Harmsen *et al.*, 2005; Jamalludeen *et al.*, 2007).

Administration of egg yolk immunoglobulin

A distinctive reduction of diarrhoeic symptoms was found in 40-day-old pigs following treatment with chitosan-alginate encapsulated anti F4 egg yolk immunoglobulins (IgY), whereas non encapsulated IgY did not reduce symptoms as effectively (Li *et al.*, 2009). In field trials, two weeks old pigs fed with spray-dried IgY were protected against F4 diarrhoea (Marquardt *et al.*, 1999). IgY may be used prophylactically, but not therapeutically, as *E. coli* F4 did not adhere *in vitro* to intestinal mucus that was incubated with IgY, and as IgY could not remove previously bound F4 bacteria (Jin *et al.*, 1998).

1.5.3. Feed measures

Dietary addition of probiotic bacteria seems to have an effect on F4 adhesion in small intestine. *In vitro* experiments with *Lactobacillus* strains reduced the amount of *E. coli* F4 in the small intestine and the *E. coli* F4 adhesion to the small intestine (Blomberg *et al.*, 1993a; Roselli *et al.*, 2007).

Furthermore, a temporary protection against *E. coli* infection was suggested upon administration of porcine spray-dried plasma to approximately three-week-old weaned pigs (Bosi *et al.*, 2004; Niewold *et al.*, 2007; Yi *et al.*, 2005) and of bromelain from pineapples to weaned pigs and fattening pigs (Chandler and Mynott, 1998; Mynott *et al.*, 1996). Lastly, non-starch polysaccharides (NSP) have been implicated as having an influence on pig diarrhoea (Hopwood *et al.*, 2004; Wellock *et al.*, 2008).

1.6. Candidate genes for *F4bcR*

Candidate genes for the *F4bcR* were selected according to their function and/or their position between *SW207* and *S0075* on SSC13. Comparative mapping in the human genome was used to search for and select genes, because most of the porcine genes currently remain unknown. As described in section 1.4, a glycoprotein like structure containing N-terminal GlcNAc or GalNAc on small intestinal cell surfaces may act as receptor for adhesion. Therefore, genes were selected that encode transferases or glycoprotein like structures that could be expressed in intestine.

1.6.1. *MUC4*

The Mucin 4 gene (*MUC4*) belongs to a family of 18 known mucins. In humans, these high-molecular weight glycoproteins are present on membranes on probably every mucosal epithelial tissue and are responsible for the viscous properties of the mucus layer (reviewed by Linden *et al.*, 2008). Mucins are important in the defence against pathogens in mucosal layers. They act as a barrier between the host and exterior environment and therefore act as infection site for many pathogens causing disease. The carbohydrate structures on mucins are determined by glycosyl transferases and vary between the mucins, tissue location and development stage. As described in section 1.4, mucin-like glycoproteins have been discussed as candidates for F4 adhesion in several studies (Erickson *et al.*, 1994; Francis *et al.*, 1998). Additionally, Jørgensen *et al.* (2004) determined several SNPs in *MUC4*, in which the g.8227C>G polymorphism was in high association with the F4ac phenotype.

Expression of *MUC4* in small intestine has been investigated in a few studies in humans. In one study, *MUC4* expression was found in human embryos (Zhang *et al.*, 2006a), but in another study, expression was not found in human small intestine by RT-PCR of the 3' end of *MUC4* (Moniaux *et al.*, 2000). In the gene expression repository of the NCBI website (GEO profile; `http://www.ncbi.nlm.nih.gov/geo`), only about 20 records of *MUC4* expression in small intestine were found. Records from small intestine were found for mice, humans and rats, but not for pigs.

Mucin 4 is an integral membrane glycoprotein and may play a role in regulating cellular adhesion (reviewed by Chaturvedi *et al.*, 2008). The human *MUC4* consists of 25 exons with a total genomic length of about 65 kb and is located on HSA3:196 959 −197 024 kb (ENSG00000145113, release code 54 of May 2009; `http://www.ensembl.org/Homo_sapiens/Info/Index`). Based on BLAST searches on BAC clones and BAC end sequences of the fingerprint contig from the Wellcome Trust Sanger Institute (release code 53 of March 2009; `http://pre.ensembl.org/Sus_scrofa_map/Info/Index`), the homologue sequence of *MUC4* maps to SSC13:143 100 kb and makes *MUC4* also a positional candidate gene. More than 10 splicing variants of *MUC4* are known. The focus of this thesis is splicing variant 1 (ENST00000308466).

1.6.2. *TNK2*

The tyrosine kinase non-receptor 2 gene (*TNK2*) encodes a tyrosine kinase that binds to CDC42 (cell division cycle 42 protein), a GTPase of the Rho-subfamily, and inhibits its GTPase activity. *TNK2* expression in the small intestine of mice, humans and rats has been reported on GEO profile (about 30 records). It is not clear how *TNK2* interferes with receptors in the small intestine. In humans, several transcript variants for this gene have been found, but only two variants are full length-transcripts. The transcript variant 1 (ENST00000333602) referred to in this thesis is the more frequently occurring full-length transcript. Compared to variant 2, it contains a different alternate exon 11 (ENSE00001317846), and lacks one alternate 3' exon between exon 13 and exon 14 (ENSE00001317558). The human *TNK2* (ENSG00000061938) has a genomic length of about 46 kb and is located on HSA3:197 075 −197 120 kb. The homologue position corresponds to SSC13:143 000 kb, proximal to *MUC4*, making *TNK2* a gene of interest.

1.6.3. *C3orf21*

The chromosome 3 open reading frame gene (*C3orf21*) encodes a hypothetical transmembrane protein, of unknown function in humans and animals. No records from intestine were found on GEO profile. According to the EST profile viewer on the UniGene server of NCBI (`http://www.ncbi.nlm.nih.gov/unigene`), the gene is expressed in almost all tissues, including the intestine. ESTs similar to *C3orf21* have been found in tissue of Danish pigs (personal communication Claus Jørgensen). The human *C3orf21* (ENSG00000173950) has a genomic length of 202 kb, contains 4 exons and is located on HSA3:196 270 −196 473 kb. Of the three known transcript variants, this thesis refers to variant 1 (ENST00000310380). The homologue position of *C3orf21* corresponds to SSC13:141 100 kb.

1.6.4. *CLDN1*

The claudin 1 gene (*CLDN1*) encodes a protein with four transmembrane domains that are part of the tight junctions. This protein is expressed in almost all epithelial and endothelial cells in humans and most animals. Tight junctions allow the regulation of the molecular transport from cell to cell, prevent the intercellular passage of molecules and hold the cells together. According to the EST profile of *CLDN1*, its RNA is expressed in most human tissues including the small intestine. According to the GEO profile, small intestinal expression has been reported mainly for the mouse (30 records). Several studies have investigated *CLDN1* in mouse or human cancer cell lines, but there have been no reports for *CLDN1* in pigs. Human *CLDN1* consists of 4 exons with a total genomic length of 17 kb and is located on HSA3:191 506 −191 523 kb (ENSG00000163347). The homologue sequence in swine is located on SSC13:137 300 kb. One transcript variant (ENST00000295522) of *CLDN1* is known.

1.6.5. *ST6GAL1*

The ST6 β-galactosamide α-2,6-sialyltransferase 1 gene (*ST6GAL1*) encodes a membrane protein that transfers sialic acids to terminal β-D-galactosyl residues of glycoproteins or lactose. The protein exists in several isovariants and seems to be involved in the generation of cell surface carbohydrate determinants, in lymphozyte proliferation and activation and in embryonic development. According to the GEO profile, *ST6GAL1* is expressed in several tissues in humans, but not in the small intestine. In mouse and rat small intestine, *ST6GAL1* expression has been reported. The human *ST6GAL1* (ENSG00000073849) has a genomic length of 148 kb on HSA3:188 131 −188 279 kb and contains eight exons. Annotation in this thesis is referred to the first of the two transcript variants (ENST00000169298) of the gene. The homologue sequence of *ST6GAL1* maps to SSC13:133 900 kb. According to position and transferase activity, *ST6GAL1* was selected as a candidate gene.

1.7. Objectives of the study

The aims of this study were the following:

- to fine-map the *F4bcR* locus on chromosome 13 by linkage analysis using markers and F4ac phenotypes of informative families,
- to isolate the porcine *MUC4* and *TNK2* as candidate genes for the susceptibility to *E. coli* F4ac,
- to identify new markers that are associated with the F4ac phenotype to refine the *F4bcR* region,
- to develop a diagnostic test applicable to commercial herds to distinguish between *E. coli* F4ac susceptible and resistant pigs,
- to determine the inheritance of the receptor for F4ad by producing and testing informative families.

2. Materials and Methods

2.1. Pigs

Large White and Landrace purebred pigs and Large White/ Landrace crossbred pigs were bred at the Faculty of Veterinary Medicine, Vetsuisse Faculty, University of Zurich for several generations. In 1998, the *E. coli* F4 adhesion test was established and pigs were bred to determine an inheritance pattern for resistance and susceptibility to *E. coli* F4ac. The entire pig material consisted of about 1900 slaughtered pigs from 188 litters. Since the beginning of the year 2000, adhesion of all three F4 variants have been routinely determined in the pigs in this Swiss experimental herd (SEH).

In total, pigs between 37 and 98 days of age (mean age 65±10.1 days) and 151 older pigs were phenotyped with the three F4 variants until the end of November 2008. Of the total of 166 litters, 32 matings were done more than one time: 24 matings were done twice, 5 three times, 2 four times and 1 mating six times. The repeated matings produced 749 offspring from 77 litters.

Of 215 SEH pigs slaughtered between September 2004 and August 2005, intestinal samples free of contents and intestinal samples with contents were taken for phenotyping. Pigs were from 27 litters from 21 matings. Four matings were done two times, and one mating three times. Two intestinal samples free of contents and two with contents were taken from 59 of these 215 pigs. These pigs were from 12 litters. Two piglets from each of five SEH litters were selected for biopsies.

The 331 SEH pigs used for linkage analysis of the *E. coli* F4ab/F4ac receptor locus consisted of 14 founders (6 dams and 8 sires), 17 F_1 parents and 300 offspring from F4ac resistant x heterozygous susceptible matings. The phenotype was unknown in 7 founders, 1 F_1 parent, 1 offspring, and 3 pigs used for further production of piglets. This material comprised the pigs used in the studies of Python *et al.* (2002, 2005) and Python (2003).

The 236 pigs of the Nordic experimental herd (NEH) consisted of 10 purebred founders, 26 F_1 pigs and 200 F_2 offspring of a European Wild boar x Swedish Yorkshire cross and are described by Edfors-Lilja *et al.* (1995).

As a representative sample of the Swiss porcine population, 78 pigs from 38 litters with 65 parent pigs of Landrace and Large White breed were randomly selected at the Swiss Performing Station Sempach (SPS). In addition, 193 boars of Large White, Landrace, Piétrain x Duroc, Duroc and Piétrain breeds used for

artificial insemination in Switzerland in 2005 were used for genotyping the *MUC4* g.8227C>G polymorphism.

The pigs selected for DNA sequencing were from the SEH and consisted of two F4ac resistant pigs from two resistant x heterozygous susceptible litters (pig/lab no. 542/411, 741/1170), one homozygous susceptible male (534B/5064) and one homozygous susceptible female parent animal (179B/1450).

Tissue for RNA analysis was taken from the liver of a resistant (599/497) and a homozygous susceptible (633/748) piglet, and from intestinal scrapings of a resistant (601/499) and a homozygous susceptible (641/756) piglet. These four piglets were also from the SEH.

For analysis of sequence variants and of haplotypes of *TNK2* and *MUC4*, 180 pigs were taken. This group of pigs contained the 78 SPS offspring, the 48 SEH pigs of four resistant x heterozygous susceptible litters and 56 additional SEH pigs that showed a recombination in the interval between *SW207* and *SW398* (section 3.2).

2.2. Determination of the phenotype

2.2.1. Preparation of bacterial strains

The *E. coli* F4 strains E68I (O141:K85ab:F4ab), G4 (O45:KE65:F4ac) and Guinée (O8:K87:F4ad) (Thorns *et al.*, 1987) were obtained from the Veterinary Laboratories Agency Weybridge, Surrey, GB, grown on Columbia sheep blood agar plates and then stored at 4 °C. Every second month, material from five colonies was plated on a fresh blood agar plate. The expression of F4 fimbriae of each subculture was confirmed by slide agglutination of confluent growth with polyvalent F4 antiserum.

After three transfers on blood agar plates, the cultures were renewed from frozen stock. For frozen stock, bacteria of confluent growth on blood agar plates were harvested and frozen at −70°C in 0.5 ml trypticase soy broth (TSB) containing 10% glycerine. Each batch of frozen stock suspensions was confirmed by slide agglutination with OK antisera. The antisera were produced at the Institute of Veterinary Bacteriology, University of Zurich.

Confluent growth was picked on the blood agar plates and grown at 37 °C for 24 h in TSB in test-tubes one day before use. Shortly before use, 1 ml of bacterial culture was diluted 1:10 in prewarmed TSB and incubated at 37 °C for 90 min to achieve maximum growth rate of the bacteria.

2.2.2. Sampling of intestinal tissues

Piglets were slaughtered routinely at the age of about two months or at about 20 kg live weight. In most cases, pigs were weaned and food was withheld for one day before slaughter. Exceptionally, they were weaned earlier. About 30 min after the

death of the piglets, 10 to 20 cm of a jejunal segment without contents was taken between 3.5 and 7.5 m distal the Arteria mesenterica cranialis.

A segment free of contents and a segment with contents was taken in the same jejunal region from 215 pigs. Two adjoining segments with contents and two adjoining segments without contents were taken from the same part of the small intestine from 59 pigs. These samples were made anonymous prior to purification of enterocytes. All segments were opened longitudinally, placed in wide-necked bottles containing 80 ml 4 ℃ EDTA buffer and stored at 4 ℃ until further processing.

Two biopsies of each of 10 pigs were taken to determine the influence of age on adhesion of *E. coli* F4ad. A first biopsy was taken at the age of four to six weeks and a second at the age of about six months. Pigs underwent surgery under general anaesthesia at the Veterinary Clinic of the Vetsuisse Faculty, University of Zurich. A part of the small intestine free of contents was surgically excised. The anatomical position of the intestinal segment could not be determined precisely.

2.2.3. Purification of enterocytes

The microscopic adhesion test was performed according to Sellwood *et al.* (1975) with modifications of Vögeli *et al.* (1996) and Python *et al.* (2002). Superficial layer of the intestinal segment was scraped off the surface with a microscopic slide and collected in 50 ml centrifuge tubes that contained 30 ml PBS-formaldehyde. The suspension was stirred vigorously with forceps for 1 min and stored at 4 ℃ for 15 min to sediment large cell fragments. The supernatant was decanted and stored at 4 ℃ for 20 min again for sedimentation of the cells. Subsequently, the supernatant was centrifuged at 200 *g* for 10 min. The pellet was carefully resuspended in 10 ml PBS and centrifuged again. The enterocytes were resuspended in 5 ml mannose buffer and diluted to a concentration of 10^5 to 10^6 cells/ml.

2.2.4. Microscopic adhesion test

One millilitre of resuspended enterocytes were incubated in 6-well macroplates at 37 ℃ for 30 min with 1 ml freshly grown culture from each of the three *E. coli* F4 strains. Subsequently, 20 well-separated and intact enterocytes were scored for each sample under a light microscope with a 400 x magnification. An enterocyte was classified as adhesive if more than five bacteria adhered to the brush border (Figure 2.1). Twenty additional enterocytes were scored if adhesion of more than five bacteria was observed in >0% to 30% of the scored enterocytes. Pigs with more than 15% of *E. coli* F4ac adhesive enterocytes, and pigs with more than 2.5% of F4ab and F4ad adhesive enterocytes were considered to be susceptible.

The same person performed sampling of jejunum, purification and classification of the enterocytes. As far as possible, a second person repeated the scoring of samples with ambiguous phenotypes.

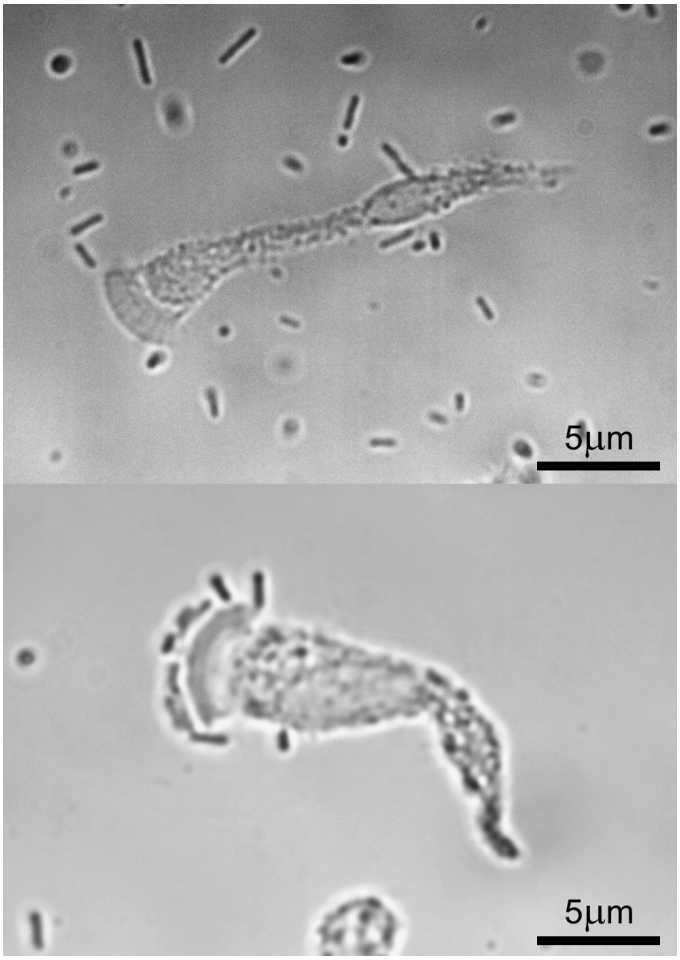

Figure 2.1.: Determination of receptor phenotype in the microscopic adhesion test after preparation of enterocytes. Cell without adhesion (top) and cell with multiple *E. coli* F4ac adhering to the brush border (bottom).

2.2.5. Enterocytes for adhesion control

Beginning in July 2006, adhesive and non adhesive enterocytes were kept for further use as positive and negative controls for F4 adhesion. After scoring, the remaining enterocytes in mannose buffer and the cells of the second decantation were pooled, supplemented with 10 ml of PBS and stored at 4 ℃ for 5 min for sedimentation of large cell fragments. After a centrifugation step at 200 *g* for 10 min, the pellet was resuspended in 5 ml DMSO-Hanks-medium (Bosi *et al.*, 2004). Aliquots of the suspension were frozen in cryotubes at −70℃.

Control cells were included in each test series. Before use, the cells were thawed at room temperature, diluted in 10 ml PBS-formaldehyde and centrifuged. The pellet was washed in PBS, centrifuged, resuspended in 1 ml mannose buffer and was ready for incubation with bacterial strains.

2.3. DNA methods

2.3.1. DNA extraction from blood

Blood was collected in Vacuette tubes or Venosafe tubes containing EDTA and stored at 4 ℃ or at −20 ℃ until processing. DNA extraction from blood samples was done using a lysis method described in Vögeli *et al.* (1994). In brief, 600 µl blood was mixed with 500 µl lysis buffer, left at room temperature for 15 to 30 min, and centrifuged at 13 000 *g* for 30 s. The pellet was resuspended in 1 ml lysis buffer and shaken vigorously or vortexed. After 15 min at room temperature, the mixture was centrifuged for 25 s. Resuspension and centrifugation were repeated two more times. The pellet was resuspended in 200 to 400 µl PCR turbo buffer, and 20 to 40 µl proteinase K (20 mg/ml) were added to the suspension. After incubation at 54 ℃ for 2 h and deactivation of the proteinase K at 95 ℃ for 10 min, samples were stored at −20 ℃ until further use.

Alternatively, a so-called quick and dirty method was used for DNA extraction from blood. First, 100 µl blood were mixed with 1 ml ddH_2O, vortexed and centrifuged at 13 000 *g* for 2 min. The pellet was suspended in 1 ml 0.9% NaCl and centrifuged. After adding 400 µl 0.2 M NaOH to the pellet, the suspension was incubated at 95 ℃ for 10 min. Subsequently, the solution was neutralised by adding 400 µl 0.2 M Tris-HCl (pH7.5) and centrifuged. The final the supernatant contained about 25 µg/µl DNA.

DNA concentration was estimated using 0.8% agarose plates with etidium bromide (EtBr). λ-phage DNA at concentrations of 5, 10, 25, 50, 75, 100 and 500 ng/µl was compared to the genomic DNA under UV-light after drying the DNA on the plate at room temperature.

2.3.2. Polymerase chain reaction

Primer design

Primers for PCR and sequencing were based on porcine DNA or RNA sequences or on homologous and conserved DNA sequences of human, cow, rat and chicken published on the EMBL/GeneBank databases. Primer sequences were 18 to 22 bp in length with a GC content of 40 to 60% and preferably with one or two G/C clamps at the 3' end. The primers were basically designed with the web-based software PRIMER3 VERSION 0.4 (Rozen and Skaletsky 2000; `http://fokker.wi.mit.edu`) and NETPRIMER (Premier Biosoft International, Palo Alto, USA; `http://www.premierbiosoft.com/netprimer/index.html`). Primer sequences, fragment lengths, names and positions within EMBL sequences are shown in Table 2.1.

Standard PCR

PCR was generally performed in 25 μl reaction volumes containing 50 to 250 ng DNA, 200 µM of each dNTP, standard PCR Buffer (10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% Gelatin), 0.4 µM of each the forward and reverse primer, up to 0.5 mM additional $MgCl_2$ and 1.5 U Taq DNA polymerase or 0.75 U Taq DNA Jumpstart polymerase. Amplification was carried out in 200 µl single tubes, 8-tube strips or 96-well plates on a PTC100 (MJ Research, Bioconcept, Allschwil) or a Robocycler (Stratagene, Agilent Technologies, Basel). After an initial denaturation at 95 °C for 5 min, the samples were cycled 30 to 38 times as follows: denaturation at 95 °C for 30 s, annealing at 55 to 65 °C for 30 s, extension at 72 °C for 30 to 40 s. For PCR fragments longer than 1 kb, extension time was set to 1 min. Finally, samples were extended at 72 °C for 7 min. For greater reaction volumes, the cycling times were extended.

PCR for the *MUC4* DQ848681:g.8227C>G polymorphism was done according to Jørgensen *et al.* (2004): After initial denaturation at 95 °C for 5 min, touchdown PCR was performed for the first 10 cycles by lowering the annealing temperature 1 °C per cycle, starting at 60 °C for 15 s. The other 19 cycles were performed with 50 °C annealing temperature for 45 s. Denaturation at 95 °C was for 15 s and extension at 72 °C for 1 min.

2.3.3. Agarose gel electrophoresis

PCR products were run on 1 to 1.5% agarose gels in 0.5 x TBE buffer containing 100 µg/l EtBr at 75 to 100 mA for 0.5 to 1 h. A 6 x DNA loading dye was added to the PCR products prior to loading. Depending on the size of the fragments, a 50 bp or 100 bp size standard was used. DNA was visualised under UV-light.

Table 2.1.: Primers for PCR and sequencing are given with the DNA (or cDNA) fragment size and the position within the EMBL sequences.

Accession no.	Gene symbol			
Sequence position (bp)	Primer name	Primer F (5' → 3')	Primer R (5' → 3')	Fragment size (bp)
DQ848681	*MUC4*			
6138–6887	MUC4-6138-F/6887-R	GTTACTGGCCTCGACTCTCC	AGGTTGTACCCTTGGCATTC	749
	MUC4-6668-R		TTCAACCTCCTAATACCCAGTGC	
7741–8429	MUC4-7741-F/8429-R	GGTCCTACGCCTTGTTTCTC	GTCCCCATCCATCTCTCTGT	688
	MUC4-8202-R		CCTTCATGGGGTTGTTGTAATA	
8012–8378	MUC4-8012-F/8378-R	CACTCTGCCGTTCTCTTTCC	GTGCCTTGGGTGAGAGGTTA	367
FN393558	*TNK2*			
<0–1649	TNK2e2j-F/3j-R	GTGGGACCAGGTGGGTACAG	GAAGCAAAGATGTCCTAGGTCCA	1666
	TNK2e2-3-Fv2	CTTAAGGCAGGGTCACCTGTTA		
	TNK2e2-3-Fb	TCTAGAGGTCCTAGTGTTCCTTGG		
	TNK2e2-3-Rb		CCGACTCTCTAGGCTAAAAGCAG	
1413–3254	TNK2e3-4-F/R	ATCCGACAGGGCACTTGTAGAG	AAGGTGCTCTGAGAGTGATGAGG	1842
	TNK2e3-4-Fb	GCCAGAAGAAGCCCTCAGACTA		
	TNK2e3-4-Rb		GCCAAAGGTCAAGGGACAGAAG	
3076–4607	TNK2e4j-F/4-5-R	TGGAAATTTAGCAAAGCCAGGA	CGAGTGCATGGCGTTGACCT	1532
	TNK2e4-5-Fb2	GCAGTAAAGCACACAGTTCTTGA		
	TNK2e4-5-Rb		ATCAAAATAGTGGCTTCCCTTGA	
	TNK2e4-5-Rc		CAGGGATGGAACTGGAGCCACAGC	
4515–5409	TNK2e5-6-F/R	GTGAGTGTGGCTGTGAAGTGC	TGTTCCTGCATGACGTAGTGG	895
5274–7260	TNK2e6-7-F/R	AGTCCAAGCGCTTTATTCACC	GACAGGGACACCAACAGCTAA	1987
	TNK2e6-7-Fb	GCTTATGGAGGTTCCCAGGCTA		
	TNK2e6-7-Rb		GGTGGAACCAAGAGAAATGAACT	
	TNK2e6-7-Fc	ACCATCCTGCTGAAATGACCCAA		
	TNK2e6-7-Fc2	GGAAGCCACTTTGATTGTTCTC		
	TNK2e6-7-Rc		TGACCGTACATCCTAGATTAGAAG	
7065–7846	TNK2e7-8-Fv2/8-9-R	TGAAGACACGCACCTTCTCC	ATCTGGATGTGCAGCTTGTCC	782
7503–8054	TNK2e9j-F/R	GAAGAGCTGGGTGCTCTGCTT	AGCGGCAGCTGCATACTTGA	552
8155–8723	TNK2e10j-F/R	CAGCATGGGAACCTACTCTGATC	TGGAAGCCTGTTTCCTAGTCCA	569
8824–9354	TNK2e11j-F/R	CAACCTGCGATCAGTTCGTTC	GGCGGATACAGCTGCCTCTC	531
9341–9867	TNK2e11Aj-F/R	CAGCTGTATCCGCCGAAGTG	ACCTTCCAGGAGGAGAGGAATG	527
9830–10603	TNK2e11-12-F/R	GCCCCCTCTCACTTCTCATTCC	GGCTCAGCCTCTTCAAGTCAC	774
10552–11436	TNK2e12b-Fv2/R	CCTGTGAGTGAAGACCAAGACC	CATGGTCTTCCCAGGTGAGC	885
	TNK2e12b-Rv2		CCCCATCTCCTCCTCACTTGA	
11157–11774	TNK2e12c-Fv2/12-Rv3	CAGCTCTCTTGTCCCCTTGC	CTGTTGTTGGTGGAGAAGTTGG	617
11558–12324	TNK2e12-14-Fv2/R	GCACCCACTACTACCTGCTACC	AGGTTCCAGTCGAACATCTCC	767
12255–13853	TNK2e14-15-Fv2/15j-R	AGCTATTTGGGTTGGGTCTGC	GTTAGGCCACAAGTCACATTCTGT	1499
	TNK2e14-15-Fb	CAGAAGTGGTTAGAGGAGTGAATGA		
	TNK2e14-15-Rb		TGGCTAACTTCAGTGATGGTCTC	
FN392681	*C3orf21*			
1–229	CORF21e4-F/R	GCACACATTCTGGCAGTTC	CGATCATGGTGAAGAAGTCC	229
FM205928	*CLDN1*			(cDNA)
1–908	CLDN1e1-F/4-R	GCCCCAGTGGAGGATTTAC	TGCTTATGCCAACATAAAGAGA	908
	CLDN1e2-F	GTGCCTTGATGGTAATTGG		
	CLDN1e2-R		TCTGCACCTCATCATCTTCC	
FN392680	*ST6GAL1*			(cDNA)
1–1220	ST6GAL1e4b-F/8b-R	GGACAGAGTGGTTTCCTTGAACA	CTTTCCCAAGCAGGTAGATGTC	1220
	ST6GAL1e4-F/4-R	CTGGTCTTTCTCCTGTTTGC	GGAAGAGCTGTCCTTGTTCC	263
	ST6GAL1e8-F/8-R	ATGATGACGCTGTGTGACCA	CAGTGAATGGTCCGGAAGCCAG	228

2.3.4. Genescan analysis

Microsatellite polymorphisms of labelled PCR products were visualised by genescan analysis. A mixture of 0.5 µl PCR product, 2.5 µl formamide and 0.5 µl genescan 350 size standard was denatured at 95 °C for 5 min, and 1 to 2 µl was loaded on a 4.5% polyacrylamide gel. The samples were run on an ABI Prism 377 DNA sequencer and analysed with the software GENOTYPER 2.1 (Applied Biosystems, Rotkreuz). Table 2.2 shows the markers used for genescan analysis with accession number, labelling dye, annealing temperature, size range and reference.

2.3.5. PCR restriction

PCR products were digested in a total volume of 25 µl using 1 to 2 U restriction enzyme at appropriate temperature for 2 to 16 h according to the manufacturer's instructions. Positions of the polymorphisms in *TNK2* and *MUC4*, primer combinations, sequence variants, restriction enzymes and the fragment sizes are shown in Table 2.3. Restricted samples were run on agarose gels containing EtBr, to visualise and identify the restriction fragment length polymorphisms (RFLPs).

2.3.6. PCR purification and sequencing

PCR products used for sequencing were purified and concentrated using Montage PCR centrifugal filter devices as described in the manufacturer's protocol. The PCR product was applied to the filter and supplemented with ddH_2O to 400 µl. After centrifugation at 1000 *g* for 15 min, 21 µl ddH_2O was added to the filter. After 5 min at room temperature, the filter was placed upright on an empty tube and centrifuged at 1000 *g* for 2 min. Concentration of DNA was estimated by comparing 1 µl purified PCR product to different concentrations of λ-phage DNA on an agarose plate with EtBr. The templates were diluted to the concentration that was required and sent for sequencing (Microsynth, Balgach).

For sequencing on the ABI Prism 377 DNA sequencer, sequencing PCR was prepared as followed: 2 µl BigDye sequencing mix, 2 µl halfBD, 3.2 pmol primer and 1 µl DNA template were completed with ddH_2O to a total volume of 10 µl. The sequencing reaction was started with an initial denaturation at 95 °C for 5 min and followed by 35 cycles at 95 °C for 20 s, 50°C for 10 s and 72 °C for 4 min.

The sequenced product was precipitated by adding 10 µl 3 M Sodium acetate, 80 µl ddH_2O and 200 µl ice-cold 100% ethanol (EtOH) to the sequenced PCR solution, vortexing and storing at −20 °C for at least 20 min. The mix was centrifuged at 13 000 *g* at 4 °C for 30 min. The pellet was washed twice by adding 200 µl 70% ice-cold EtOH and centrifuging at 4 °C for 5 min. The pellet was air-dried or vacuum-dried, and the sequencing product was denatured in 1.5 µl formamide loading dye

Table 2.2.: Microsatellite markers used for genescan analysis shown with the accession number, fluorescent labelling at the 5' end of the forward primer and annealing temperature of the microsatellite, as well as size range of the PCR product. References: a) Fredholm *et al.* (1993), b) Winterø and Fredholm (1995), c) Robic *et al.* (1994), d) Davies *et al.* (1994), e) Rohrer *et al.* (1994), f) Alexander *et al.* (1996), g) Wang *et al.* (2001), h) Joller *et al.* (2009), i) Fahrenkrug *et al.* (2005).

Microsatellite marker	Accession no.	Modification at 5' end	Annealing temp. (℃)	Size range (bp)	Ref.
S0068	M97244	TET	62	211–260	a
S0075	AF044970	FAM/ HEX	62	134–162	b
S0222	L30151	FAM	55	178–202	c
S0283	X79925	FAM	62	132–148	d
SW207	AF235238	FAM	58	170–188	e
SW225	AF235243	TET	55	94–118	e
SW398	AF235289	FAM	55	166–192	e
SW520	AF235317	FAM	62	102–124	e
SW698	AF235339	TET/ FAM	58	194–224	e
SW1030	AF235172	FAM	58	137–145	e
SW1876	AF253726	FAM	65	204–258	f
SW1901	AF253734	FAM	58	107–129	f
SW2007	AF253772	TET	60	137–145	f
SWR1627	AF253889	FAM	60	158–168	f
KS502	AF305933	FAM	60	163–183	g
HSA125gt	FM877810	HEX	59	200–240	h
MUC4gt	FM877809	FAM	59	210–250	h
UMNp884	AY285570	HEX	62	138–138	i
UMNp894	AY285577	FAM	54	208–210	i
UMNp1062	AY285217	HEX	58	260–275	i
UMNp1197	AY285320	FAM	62	246–256	i
UMNp1226	AY285345	FAM	60	165–175	i
UMNp1239	AY285356	HEX	60	140–150	i
UMNp1298	AY285397	FAM	60	250–265	i
UMNp1320	AY285414	FAM	65	130–130	i
UMNp1341	AY285430	FAM	60	140–160	i

Table 2.3.: Six selected restriction fragment length polymorphisms in *TNK2* and *MUC4*. Position of the single nucleotide polymorphisms (SNPs) in the sequence and the nucleotide changing the restriction pattern of the enzyme are given.

Accession no.	Gene symbol		Restriction	Length of digested
Position of SNP (bp)	Primer combination	SNP	enzyme	fragments (bp)
FN393558	*TNK2*			
7075	TNK2e6-7-Fc2/R	C	*Taq*I	625
		A		189, 436
7717	TNK2e9j-F/R	T	*BseD*I	9, 19, 24, 43, 45, 88, 142, 181
		C		9, 19, 24, 43, 45, 73, 88, 108, 142
11142	TNK2e12b-Fv2/Rv2	A	*Alu*I	18, 27, 97, 134, 214, 261
		G		17, 18, 27, 97, 117, 214, 261
DQ848681	*MUC4*			
6242	MUC4-6138-F/6887-R	G	*HpyF3*I	99, 261, 383
		A		99, 102, 261, 281
7947	MUC4-7741-F/8202-R	G	*Hin1*II	7, 35, 42, 147, 231
		A		7, 35, 42, 102, 129, 147
8227	MUC4-8012-F/8378-R	C	*Xba*I	367
		G		154, 213

by incubating at 95 °C for 5 min. Finally, 1 µl was loaded on a 4.5% polyacrylamide gel and run for appropriate time.

Sequencing data were analysed and assembled using the software Chromas Pro 1.33 (Technelysium Pty Ltd, Tewantin, AUS). The software BioEdit 7 (Hall, 1999) was used to compare and align sequences.

2.4. RNA methods

Liver tissue for RNA extraction was taken immediately after the slaughter of pigs. Intestinal scrapings were removed with glass slides from an intestinal segment free of contents that was rinsed with ddH_2O or PBS. Liver tissue and scrapings were wrapped in aluminium foil or put in 1.5 ml tubes and frozen in liquid nitrogen. Samples were stored at −70 °C until RNA extraction.

2.4.1. RNA extraction

Total RNA from intestinal scrapings and from liver was extracted with an RNeasy Midi kit according to the protocol for animal tissues (version June 2001). The tissue was deep frozen in liquid nitrogen and ground with a pestle in a mortar. Of the ground material, 0.2 to 0.4 g was homogenised by vortex with 4 ml RLT buffer in a 15 ml centrifuge tube. The homogenate was centrifuged at 4800 *g* for 10 min. The supernatant was transferred to a new tube and homogenised in 1 volume of

70% EtOH by shaking vigorously. The solution was transferred to an RNeasy Midi column and centrifuged at 4800 *g* for 5 min. After centrifugation, 2 ml RW1 buffer was added to the column followed again by centrifugation. The remaining DNA was removed by adding 20 µl DNase to the column. After incubation at room temperature for 15 min, 2 ml RW1 buffer was added to the column and the unit was left at room temperature for 5 min. The column was again centrifuged at 4800 *g* for 5 min and the flow-through was discarded.

To wash the column, 2.5 ml RPE buffer was added to the column and the unit was centrifuged at 4800 *g* for 2 min. Again, RPE buffer was added, followed by centrifugation for 5 min. The column was placed on a new tube and 150 µl RNase free water was added to the column. After incubation for 1 min, RNA was eluted by centrifugation of the column at 4800 *g* for 3 min. The last step was repeated to increase the yield of RNA.

2.4.2. RNA quantification

The relative amount and quality of total RNA were estimated according to Qiagen's instructions. A 5 x RNA loading buffer was added to 8 µl RNA. The samples were incubated at 65 °C for 5 min, chilled on ice and loaded on a 1.2% formaldehyde agarose gel with EtBr. The gel was run in 1 x FA gel running buffer at 50 mA. The relative amount was estimated by comparing the intensity of the bands of the samples under UV-light. The quality of the RNA was estimated with the ratio of the 18S:28S RNA. High RNA quality should be present in a ratio of 1:1.8 for 18S:28S RNA.

2.4.3. Reverse transcription-PCR

The Reverse Transcription System was used for reverse transcription-PCR (RT-PCR) of total RNA according to the manufacturer's protocol (version June 2006). RT-PCR was performed in a 20 µl reaction volume containing 3 to 5 µl total RNA, 1 mM of each dNTP, reverse transcription buffer (10 mM Tris-HCl pH9.0, 50 mM KCl, 0.1% Triton X-100), 5 mM $MgCl_2$, 5 µM Oligo$(dT)_{15}$ primer, 20 U recombinant RNasin and 15 U AMV reverse transcriptase. The first strand synthesis was carried out in 200 µl PCR tubes or strips on PTC100 at 42 °C for 40 min. Denaturation at 95 °C for 5 min inactivated the reverse transcriptase. First strand cDNA was visualised under UV-light on agarose gel containing EtBr.

2.4.4. PCR and sequencing

The RT-PCR reaction volume was diluted with ddH_2O to 100 µl. Thereof, 3 to 5 µl was used for the specific PCR reaction with primers designed from exon sequences. PCR was performed in 100 µl reaction volumes analogous to subsection 2.3.2 but

with 0.5 µM of each the forward and reverse primer and 1.25 to 2.5 U Taq JumpStart DNA polymerase. After an initial denaturation at 95 °C for 5 min, the samples were cycled 35 to 40 times as follows: denaturation at 95 °C for 45 s, annealing at 55 to 65 °C for 45 s, extension at 72 °C for 70 s. Finally, samples were extended at 72 °C for 7 min. Purification and sequencing of cDNA was performed as described in subsection 2.3.6.

2.5. Computational methods

2.5.1. Linkage analysis

The software CRIMAP 2.4 (Green *et al.*, 1990) was recompiled and used on a HP/UX B.11.23 U Itanium processor based system at the ETH with standard settings for twopoint and multipoint linkage analyses. The significance level for linkage was set to a LOD score value above three. The option `build` was used to create a map based on a few highly informative markers, whose order was known from literature. Subsequently, the option `flips` was used to determine or confirm the most probable and statistically significant order(s). Finally, the option `chrompic` was used to find recombination and double recombination events.

2.5.2. Statistics of F4ad adhesion

Bacterial adhesion to enterocytes of most pigs was either 0% or 100%, and obviously, this adhesion strength is not distributed normally. A normal distribution would be difficult to achieve by transformation of the data. Therefore, for statistical analysis of the F4 adhesion distribution, tests were used that do not require normal distribution. Differences in F4ad adhesion strength between litters of repeated matings were compared using the Kruskal-Wallis one-way analysis of variance.

The Kruskal-Wallis test is based on ranks and does not require normal distribution. Differences in F4ad adhesion strength were also tested with the Kolmogorov-Smirnov test. This test compares the cumulative distribution of two independent datasets and tests whether the values fit the same distribution.

For sample sizes of more than five, this test behaves similar to Chi-square distribution with $n - 1$ degrees of freedom (df). Statistical analysis and descriptive statistics were performed with the software SYSTAT 11 (Systat Software GmbH, Erkrath, D).

2.5.3. *In silico* mapping

Gene and microsatellite sequences were used for BLAST searches of the pig genome sequence being generated by the Swine Genome Sequencing Consortium (Schook

et al. 2005; `http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_scrofa`). The physical position of the BAC clones comprising the BLASTed sequence was derived from the BAC fingerprint contig map (Humphray *et al.* 2007; `http://pre.ensembl.org/Sus_scrofa_map/Info/Index`).

3. Results

3.1. *E. coli* F4 adhesion to enterocytes

3.1.1. Phenotypes of the experimental herd pigs

The *E. coli* F4 phenotypes of 1569 pigs of the Swiss experimental herd (SEH) were determined with the microscopic adhesion test. The phenotypes according to Bijlsma *et al.* (1982) and Baker *et al.* (1997) are shown in Table 3.1. The 594 pigs of phenotypes A and B showed a strong adhesion, with more than 85% of F4ab and F4ac adhesive enterocytes. The 247 pigs of phenotypes C and F were adhesion negative for F4ac but adhesion positive for F4ab and showed a low adhesion strength, with about 20% of F4ab adhesive enterocytes. Ninety-five of these 247 pigs (42%) had <15% of adhesive enterocytes and only 12 pigs (5%) had >60% adhesive enterocytes.

The 799 F4ad adhesion positive pigs of phenotypes A, C and D had about 60% F4ad adhesive enterocytes in phenotypes A and C, and about 40% adhesive enterocytes in phenotype D. Standard deviation (SD) of F4ab and F4ac adhesion strength was at most ±23%, but it rose up to ±37% in the F4ad adhesion positive pigs.

The Spearman correlation (r_s) was r_s=0.878 between F4ac and F4ab adhesion, r_s=0.218 between F4ac and F4ad adhesion and r_s=0.363 between F4ab and F4ad adhesion. The scatterplot matrix of Figure 3.1 compares the F4 adhesion strengths, and the bar charts illustrate the accumulation of F4ab, F4ac and F4ad adhesion strength around 0% and around 100% of adhesive enterocytes.

F4ac adhesion to enterocytes was unambiguous in most pigs (Figure 3.2). More than 95% of the 1569 pigs had either >60% or ≤15% of F4ac adhesive enterocytes. Only 56 pigs had between >15% and ≤60% of adhesive enterocytes. F4ab adhesion to enterocytes was less clear, but still 90% of the pigs had >60% or ≤15% of adhesive enterocytes. With the current threshold for susceptibility of 2.5% of adhesive enterocytes, the percentage of unambiguously adhesive F4ab enterocytes was 82%.

F4ad adhesion to enterocytes was even less clear: 84% of pigs had >60% or ≤15% of adhesive enterocytes, and only 72% of pigs had >60% or ≤2.5% of adhesive enterocytes.

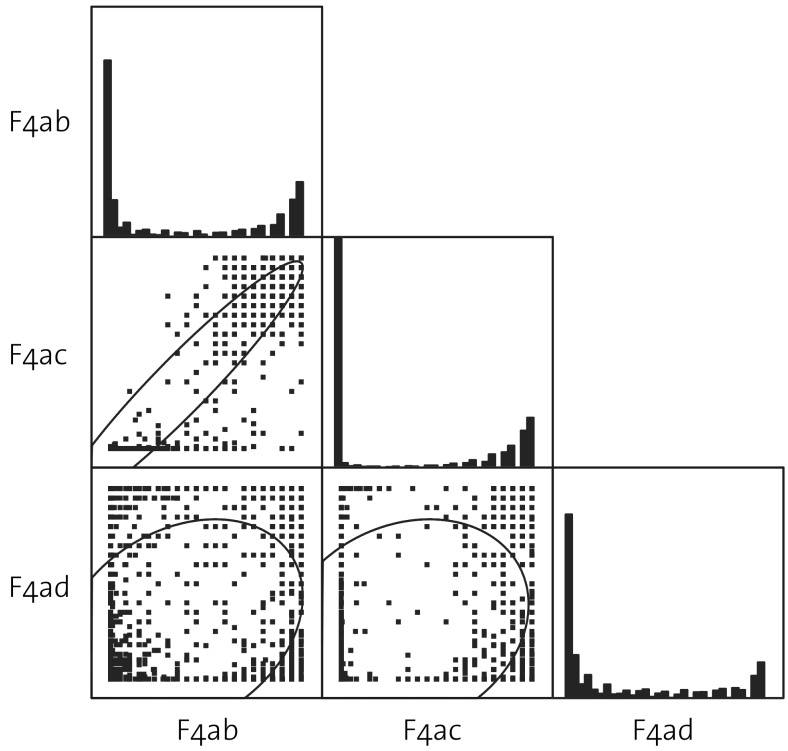

Figure 3.1.: Scatterplot matrix of F4ab, F4ac and F4ad adhesion of 1569 SEH pigs. The scatterplots compare the adhesion strengths of two F4 variants on a scale from 0% to 100%. Adhesion strength of one variant is shown on the vertical axis (with increasing % from bottom to top) and adhesion strength of the other variant is shown on the horizontal axis (with increasing % from left to right). The ellipse marks the 95% confidence interval. The bar charts show the distribution of F4 adhesion strength with number of pigs (vertical axis) and percentage of adhesive cells (horizontal axis).

Table 3.1.: *E. coli* F4 phenotypes of 1569 SEH pigs according to Bijlsma *et al.* (1982) and Baker *et al.* (1997). The phenotypes are explained in Table 1.1. Mean percentage ±standard deviation (SD) of adhesive enterocytes are given. An enterocyte was classified as adhesive if more than five bacteria were bound to the brush border. Pigs with more than 15% of F4ac adhesive enterocytes, and pigs with more than 2.5% of F4ab and F4ad adhesive enterocytes were considered to be susceptible.

E. coli	Phenotype and mean adhesion strength ±SD (%)					
variant	A	B	C	D	E	F
F4ab	89 ±14	85 ±17	23 ±18	0 ±1	0 ±0	22 ±23
F4ac	87 ±16	85 ±17	1 ±3	0 ±0	0 ±0	1 ±3
F4ad	60 ±36	0 ±0	58 ±37	42 ±31	0 ±0	1 ±1
Total no.	367	227	197	235	493	50
Percentage	23	14	13	15	31	3

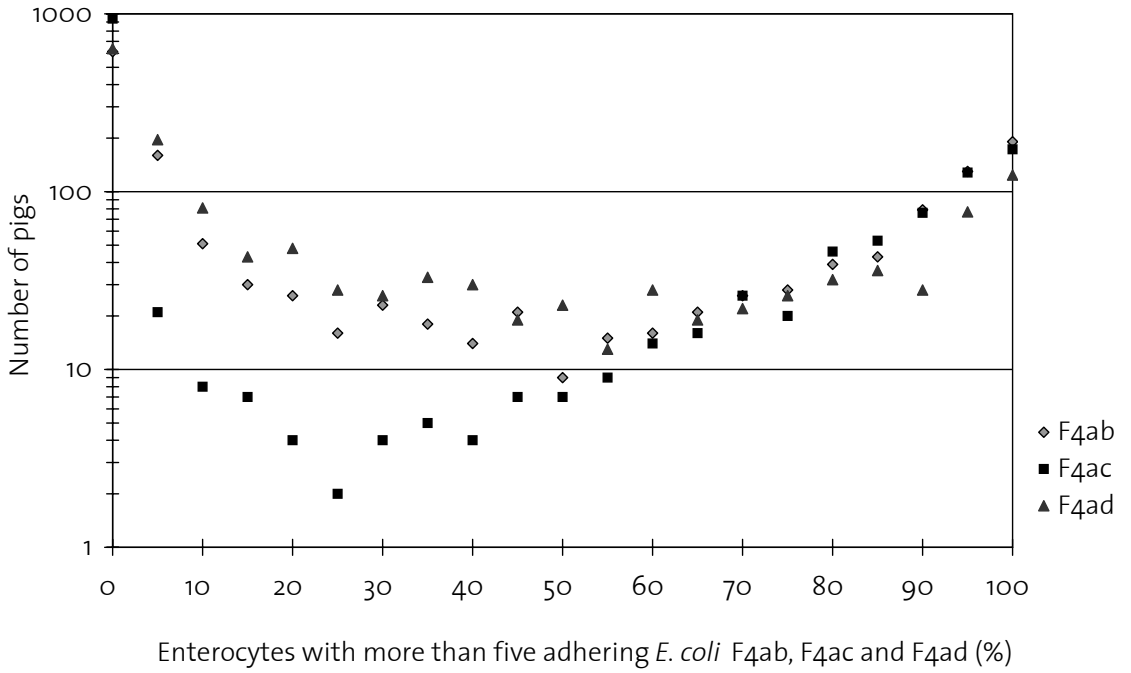

Figure 3.2.: Percentage of adhesive enterocytes of 1569 pigs phenotyped for F4ab, F4ac and F4ad adhesion. Number of pigs is shown in the vertical axis and percentage of adhesive enterocytes in the horizontal axis.

3.1.2. Phenotypes of the Swiss performing station pigs

Of the 78 offspring of 38 litters from the Swiss performing station (SPS), 64 pigs (83%) were of phenotypes A or B with more than 70% of F4ab and F4ac adhesive enterocytes (Table 3.2). The mean F4ad adhesion strength to enterocytes of the 41 pigs of phenotype A was 55%, but it was only 27% to enterocytes of the 5 pigs of phenotype D. Phenotype C was found in one pig but phenotype F was not found in any pigs. Ninety percent of the 61 Landrace breed pigs and 53% of the 17 Large White breed pigs were F4ab and F4ac adhesion positive (Table 3.5 on page 50).

The SD for the F4ab and F4ac adhesion strengths was greater in SPS pigs than in SEH pigs, but SD was smaller for the F4ad adhesion strength in SPS pigs. The Spearman correlation was r_s=0.799 between F4ab and F4ac adhesion, r_s=0.223 between F4ad and F4ab adhesion, and r_s=0.226 between F4ad and F4ac adhesion. Figure 3.3 shows the correlation between each of the pairwise comparisons and shows the distribution of the adhesion strengths.

Bacterial adhesion to enterocytes was less clear in SPS pigs compared to SEH pigs: 21% of the SPS pigs had between 15% and 60% of F4ab or F4ac adhesive enterocytes, and even 29% of the pigs had between 15% and 60% of F4ad adhesive enterocytes.

Table 3.2.: *E. coli* F4 phenotypes of 78 SPS pigs according to Bijlsma *et al.* (1982) and Baker *et al.* (1997). Mean percentage ±SD of adhesive enterocytes are given. An enterocyte was classified as adhesive if more than five bacteria were bound to the brush border. Pigs with more than 15% of F4ac adhesive enterocytes, and pigs with more than 2.5% of F4ab and F4ad adhesive enterocytes were considered to be susceptible.

E. coli	Phenotype and mean adhesion strength ±SD (%)					
variant	A	B	C	D	E	F
F4ab	72 ±27	81 ±20	18	0 ±0	0 ±0	-
F4ac	75 ±22	77 ±23	0	0 ±0	0 ±0	-
F4ad	55 ±28	0 ±0	55	27 ±22	0 ±0	-
Total no.	41	23	1	5	8	0
Percentage	53	30	1	6	10	0

3.2. F4ab/F4ac receptor position on SSC13

Genotyping data of two new microsatellite markers (*MUC4gt* and *HSA125gt*) and of six known microsatellite markers (*SW207, S0283, S0075, SW1876, SW225, SW1030*)

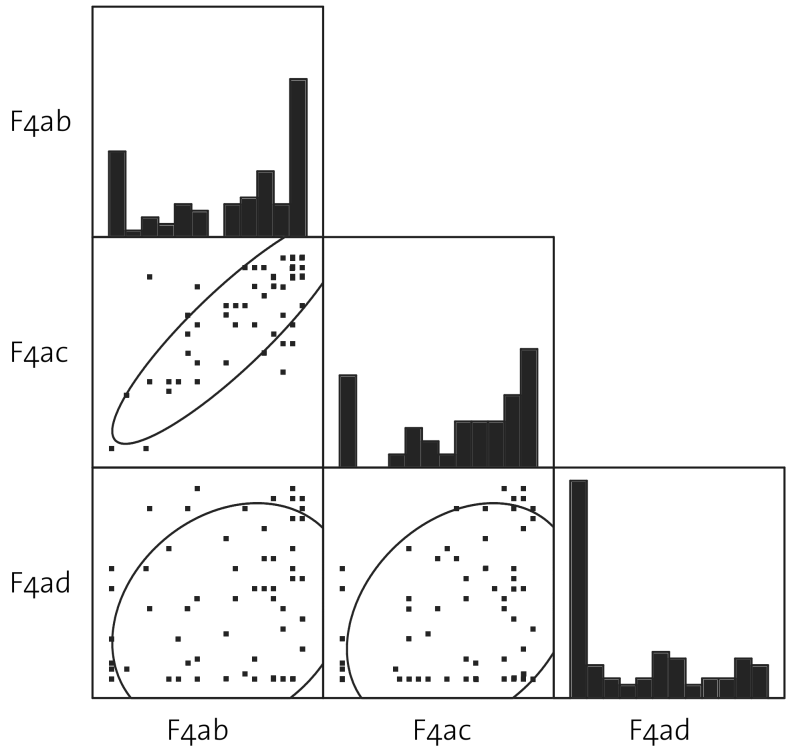

Figure 3.3.: Scatterplot matrix of F4ab, F4ac and F4ad adhesion of 78 SPS pigs. The scatterplots compare the adhesion strengths of two F4 variants on a scale from 0% to 100%. Adhesion strength of one variant is shown on the vertical axis (with increasing % from bottom to top) and adhesion strength of the other variant is shown on the horizontal axis (with increasing % from left to right). The ellipse marks the 95% confidence interval. The bar charts show the distribution of F4 adhesion strength with number of pigs (vertical axis) and percentage of adhesive cells (horizontal axis).

in the interval *SW207* – *SW398* and of the *MUC4* g.8227C>G polymorphism (*MUC4-8227*) were used to generate a linkage map. The F4ac receptor locus (*F4acR*) of offspring from informative matings and of parents with a phenotype confirmed by progeny was coded with the genotype. *F4acR* of pigs with unconfirmed F4ac genotype was coded with the phenotype.

A total of 331 SEH pigs, 143 SPS pigs and 236 pigs from the Nordic experimental herd (NEH) were used. The linkage map of the combined data was built with the software CRIMAP 2.4 and is shown in Figure 3.4. A LOD score of more than 3 supported the order and was considered to be significant. For each marker interval, the differences in log_{10} likelihood (Δ log L) between the order as shown and the order with the two markers inverted, as well as the recombination fractions between the markers, are shown to the right. Microsatellites *S0222* and *SW398* were included in order to assist in the orientation of the linkage map. Due to software limitations, these were excluded from analysis. *F4bcR* could be assigned with significance to the interval

$$SW207 - [MUC4\text{-}8227,\ MUC4gt] - S0075.$$

The most likely order was *SW207* – (*F4bcR* – [*MUC4-8227* – *MUC4gt*] – *S0283* – *HSA125gt*) – *S0075* – *SW1876* – *SW225* – *SW1030* (log L -201.9). If *S0283* was omitted from the analysis, the most likely position for *F4bcR* shifted to [*MUC4-8227* – *MUC4gt*] – *S0075*. However, a clear position of *F4bcR* within the parentheses could not be established with a significant LOD score. Using the `chrompic` option of CRIMAP revealed 54 SEH pigs with a chromosomal recombination between *SW207* – *SW398*, based on the origin of grandparental chromosomes. Nine of these pigs were double recombinant in one microsatellite marker, i.e., the microsatellite was received from grandfather, while the adjacent markers were received from grandmother, or vice versa.

Further microsatellites were analysed in the Swiss pigs, but not in the Nordic pigs and therefore were not included in the linkage analysis. In the Swiss pigs, the option `build` of CRIMAP mapped the marker *S0068* close to *SW207* and the markers *SW2007* and *SWR1627* close to *SW1876*. The marker *SW698* was mapped close to *SW1030*, and the marker *SW520* was mapped between *SW1030* and *SW398*. Additional microsatellite markers were tested in four SEH litters with a total of 52 pigs. The three microsatellites *UMNp894*, *UMNp1239* and *UMNp1320* were not polymorphic in these litters. The six microsatellites *UMNp1062*, *UMNp1226*, *UMNp1298*, *UMNp1341*, *UMNp1197* and *UMNp884* showed only two alleles in the same four litters and were not considered for further analysis. The microsatellite *KS502* was not further considered as the alleles were not obviously linked to the F4ac phenotype.

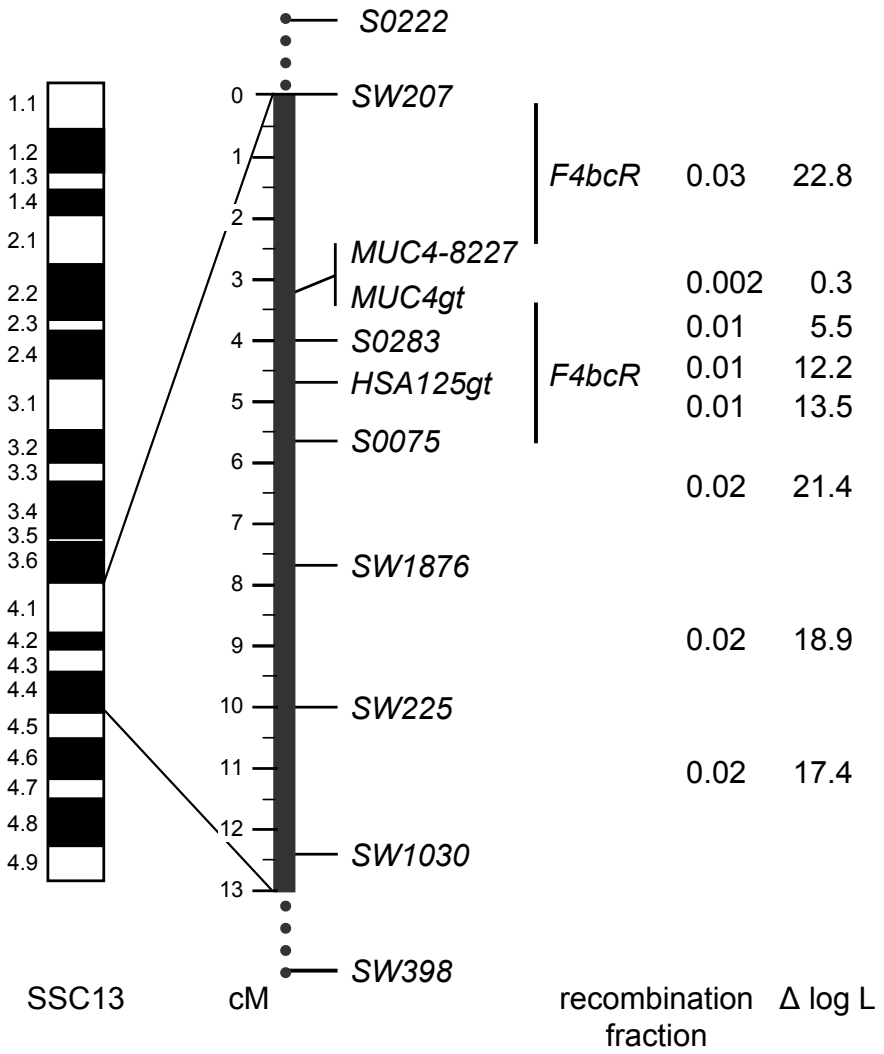

Figure 3.4.: Assignment of the receptor gene for *E. coli* F4ab/F4ac adhesion (*F4bcR*) on porcine chromosome 13 (SSC13). Data of Jørgensen *et al.* (2003) and Python *et al.* (2002, 2005) supplied by Nordic.2 map (Marklund *et al.*, 1996) and USDA MARC map (Rohrer *et al.*, 1996). The order of loci is supported by a LOD score of more than 3. Sex-averaged map distances are given in Kosambi cM. Recombination frequency of adjacent loci and differences in log_{10} likelihood (Δ log L) against the inversion of adjacent loci are shown to the right.

3.2.1. Physical mapping of markers

The microsatellite sequences were used to BLAST search pig genome sequences being generated by the Swine Genome Sequencing Consortium. The physical order of the BAC clones on the fingerprint contig from the Wellcome Trust Sanger Institute, which contains the marker sequences, was in agreement with the order of the markers on the linkage map. Table 3.3 shows the order of the BAC clones containing the marker, the BLAST search sequence and the physical position of the clones on SSC13.

Table 3.3.: Mapping by BLAST searches of microsatellite sequences to BAC clones being sequenced within the Swine Genome Sequencing Consortium pig genome project. The order of the clones corresponds to their physical order on SSC13, as indicated by the start position (based on release code 53 of March 2009; `http://pre.ensembl.org/Sus_scrofa_map/Info/Index`). Sequence identity was at least 90%. As statistical value the *e*-value is given.

Micro-satellite marker	Search sequence no.	BAC clone name	Clone sequence no.	*e*-value	SSC13 start position of BAC clone (bp)
S0222	L30151	CH242-165E13	CU856425	4×10^{-95}	53 285 853
SW207	AF235238	CH242-42J12	CU861632	2×10^{-108}	132 271 767
MUC4gt	FM877809	CH242-240L11	CU468995	5×10^{-89}	142 999 145
S0283	X79925	CH242-89F13	CU466522	3×10^{-121}	144 341 579
HSA125gt	FM877810[a]				145 100 000
S0075	AF044970[b]	CH242-220A1	CU466991	2×10^{-70}	145 838 211
SW1876	AF253726	CH242-275M20	CU633693	4×10^{-130}	149 656 892
SW225	AF235243	CH242-81P9	CU928545	3×10^{-86}	158 364 572
SW225	AF235243	CH242-165L7	CU467096	3×10^{-86}	158 461 324
SW1030	AF235172	CH242-246E8	CU915590	4×10^{-89}	185 715 153
SW698	AF235339	CH242-170C13	CU928595	1×10^{-98}	187 105 963
SW398	AF235289	CH242-267N6	CU633688	1×10^{-64}	196 654 176

[a] *HSA125gt* was mapped to homologous regions on HSA3, as no sequenced pig clone was available in that region as of June 2009, and its position in SSC13 was estimated using the BAC contig.

[b] The sequence part containing the marker sequence was used for blasting.

A clone map in the region of *MUC4* was created by BLAST search of human gene sequences to pig sequences. The porcine gene order on the BAC fingerprint contig revealed a rearrangement compared to the human genome: The human part HSA3 196.9–198.9 Mb spanning from *MUC20* to *MFI2* comprising *MUC4* seems to be

inverted in SSC13 141.2–143.2 Mb.

3.3. Sequencing of candidate genes

3.3.1. *MUC4*

Genomic DNA of *MUC4* was amplified using the primers described in Table 2.1 (page 23). The DQ848681:g.6138–6887 fragment spanning exon 6 (157 bp) and flanking sequences of intron 5 (231 bp) and intron 6 (362 bp) was sequenced from 25 pigs. The sequence g.7741–8429 containing part of intron 7 was sequenced from 19 pigs. The sequence variants are described in subsection 3.4.1. The accordance of the sequence variants with the F4ac phenotype is described in section 3.5 and section 3.6.

3.3.2. *TNK2*

Genomic DNA of *TNK2* of two F4ac susceptible and two resistant animals was sequenced using the *TNK2* primers in Table 2.1. A sequence length of about 14 kb was obtained, spanning from exon 2 to exon 15. The complete coding sequence of 3114 bp and most introns were obtained. The sequences of pigs with lab numbers 411, 1170 and 5064 are stored in the EMBL database under the accession numbers FN393558, FN393559 and FN393560. The sequence of pig 1450 was identical to the sequence of pig 5064. Intron 9 and intron 10 of the four sequences contain a gap of unknown length, and a part of intron 4 contains some unknown nucleotides due to the slightly superimposed sequence. The sequence variants and the accordance with the phenotypes are described in subsection 3.4.2 and section 3.6.

3.3.3. *C3orf21*

Genomic DNA was amplified using primers C3orf21e4-F/e4-R derived from a porcine EST (CN164007) and human *C3orf21*. The 229 bp sequence corresponds to the 5' part of human exon 4 and was identical in two F4ac susceptible and two resistant pigs. The sequence is stored in the EMBL database under the accession number FN392681. DNA amplification of other exons was not successful.

3.3.4. *CLDN1*

A 908 bp cDNA fragment of *CLDN1* was reverse transcribed from liver and from intestinal scrapings using primers CLDN1e1-F/e4-R. Compared with the human sequence, the sequenced cDNAs contain 143 bp (of 223 bp) of exon 1, exons 2 to 4 and 352 bp of 3' UTR, and were identical in the four sequenced pigs. The sequence

is stored under the accession number FM205928. The primers based on a porcine mRNA sequence (AJ318102) and a porcine EST (BF079579).

3.3.5. *ST6GAL1*

A 1220 bp cDNA fragment of *ST6GAL1* was reverse transcribed from RNA of the liver and of the intestinal scrapings each of an F4ac resistant and a homozygous susceptible pig. The sequences were obtained with the primers ST6Gal1e4b-F/e8b-R. All four cDNA sequences were identical and are stored under the accession number FN392680. The sequence contains most coding sequence of the gene: 565 bp (of 607 bp) of exon 4, exon 5 to 7, and 239 bp (of 243 bp) of exon 8.

3.4. Sequence variants in *MUC4* and *TNK2*

3.4.1. Sequence variants in *MUC4*

Eleven single nucleotide polymorphisms (SNPs) and one deletion were found in *MUC4* DQ848681:g.6138–6887 by sequence analysis of 25 pigs. Two SNPs were found in g.7741–8429 by sequence analysis of 19 pigs (Table 3.8 on page 53). Of these sequence variants, the g.6242G>A *HpyF3*I, g.7947A>G *Hin1*II and g.8227 C>G *Xba*I polymorphisms were determined in 180 pigs consisting of 78 SPS offspring, 48 SEH pigs of four resistant x heterozygous susceptible litters and in 56 additional SEH pigs (Table 3.7 on page 52). Figure 3.5 and Figure 3.6 show examples of the RFLP of PCR products from a heterozygous susceptible, a susceptible and a resistant pig. PCR and restriction was performed according to Table 2.3 (page 26).

The three SNPs determined by enzyme restriction were selected according to the haplotype information of 10 NEH pigs and 10 Swiss pigs that was provided by the Danish group of Claus Jørgensen. The genotype of the SNPs that we analysed coincided with the F4ac phenotype in all 20 pigs. Three additional SNPs coincided with the F4ac phenotype in the 20 pigs: g.6308G>T, g.6317G>A and g.6321G>C.

3.4.2. Sequence variants in *TNK2*

A total of 104 SNPs, 2 di-nucleotide polymorphisms (DNPs), 9 insertions and 7 deletions were found in *TNK2* sequences of four sequenced SEH pigs (Table 3.4 on page 44ff). Of the totally 122 sequence variants, 104 were found in introns and 18 in exons. Amino acid changes can be expected in four exon SNPs, all of them in exon 12. The FN393558:g.10622C>A mutation alters p.Pro543His, g.11008G>A alters p.Val673Met, g.11585G>A alters p.Arg865His and g.11684G>C alters p.Ser989Thr. The other 14 exon polymorphisms do not lead to any change in

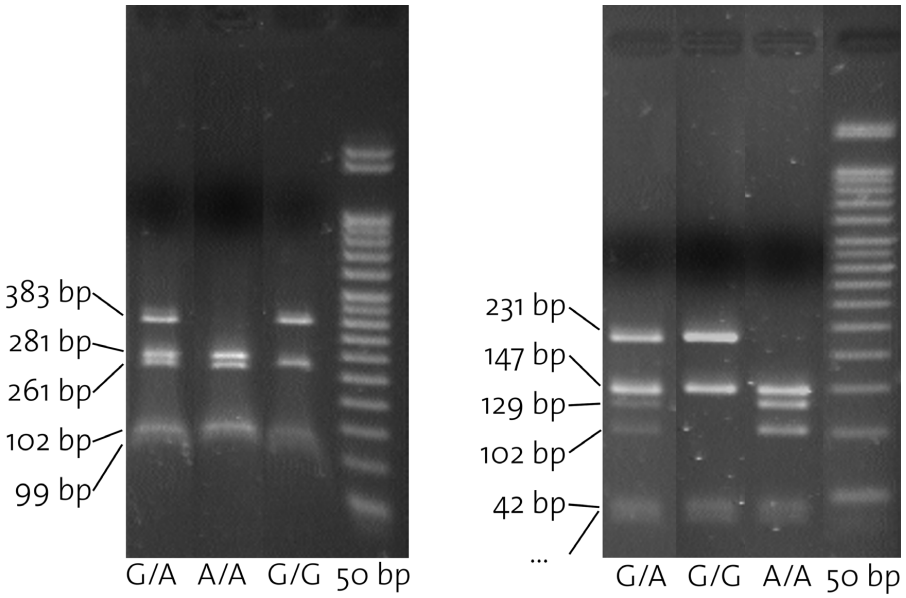

Figure 3.5.: Left: Restriction pattern of *HpyF3*I digestion of *MUC4* DQ848681: g.6138–6887 PCR products to determine the g.6242G>A polymorphism. The g.6242G>A substitution inserts a restriction site at 102 bp of the PCR product in addition to the existing restriction sites at 383 bp and 637 bp. Right: Restriction pattern of *Hin1*II digestion of *MUC4* g.7741–8202 PCR products to determine the g.7947A>G polymorphism. The g.7947A>G substitution removes the 206 bp restriction site, leaving the existing restriction sites at 35 bp, 77 bp, 308 bp and 455 bp of the PCR product. The digested samples were run with a 50 bp ladder on a 3% agarose gel.

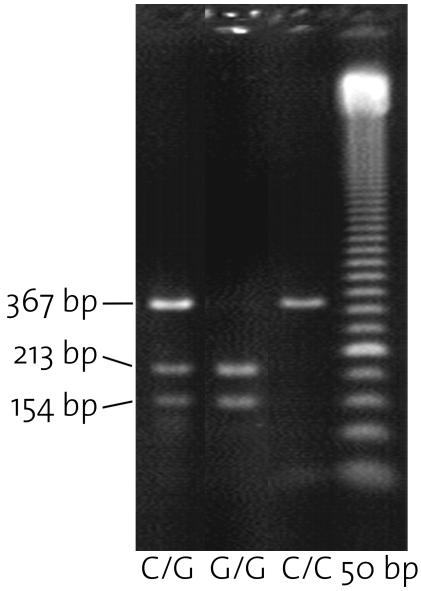

Figure 3.6.: Restriction pattern of *Xba*I digestion of *MUC4* g.8012–8378 PCR products to determine the g.8227C>G polymorphism shown with a 50 bp ladder. The g.8227C>G substitution inserts a restriction site at 213 bp in the PCR product.

amino acids. Most of the deviating genotypes (79%) occurred in the F4ac resistant pig 1170: 81 SNPs, 1 DNP, 8 insertions and 7 deletions, whereas 25 SNPs and one DNP occurred in more than one pig.

Three SNPs were selected for further genotyping: the FN393558:g.7075C>A *Taq*I polymorphism of exon 7, the g.7717C>T *BseD*I polymorphism of intron 8 (Figure 3.7) and the g.11142G>A *Alu*I polymorphism of exon 12 (Figure 3.8). These SNPs were in agreement with the F4ac genotype in the four sequenced pigs. Additional haplotype information of 10 phenotyped NEH pigs and of 6 SPS pigs, provided by Claus Jørgensen, supported the selection of the SNPs. The three SNPs were determined in 180 SEH and SPS pigs (Table 3.7 on page 52).

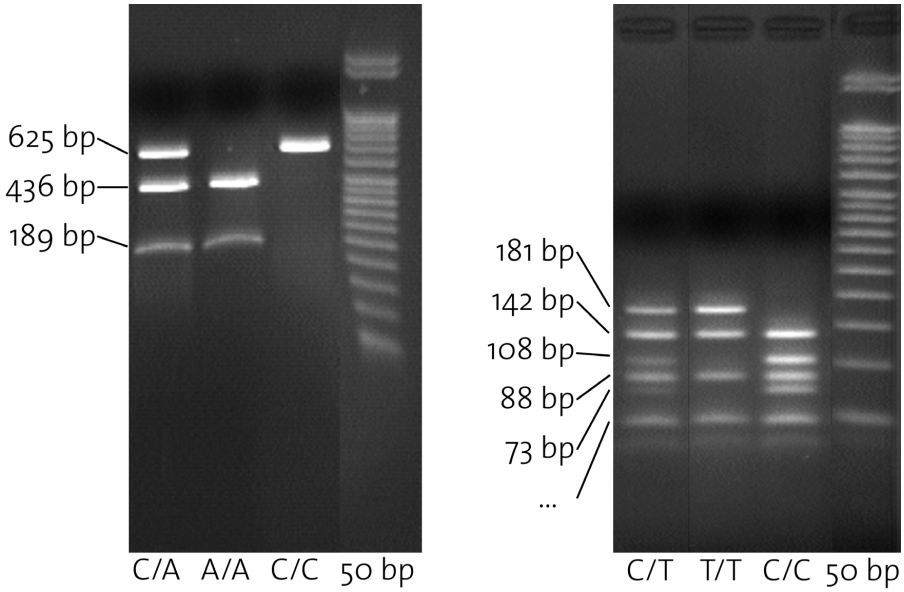

Figure 3.7.: Left: Restriction pattern of *Taq*I digestion of TNK2e6-7-Fc2/R PCR products to determine the FN393558:g.7075C>A polymorphism. The g.7075C>A substitution inserts a restriction site at 436 bp of the PCR product. Right: Restriction pattern of *BseD*I digestion of TNK2e9j-F/R PCR products to determine the g.7717C>T polymorphism. The g.7717C>T substitution removes the 215 bp restriction site, leaving the existing restriction sites at 88 bp, 107 bp, 288 bp, 430 bp, 431 bp, 455 bp, 464 bp and 507 bp of the PCR product.

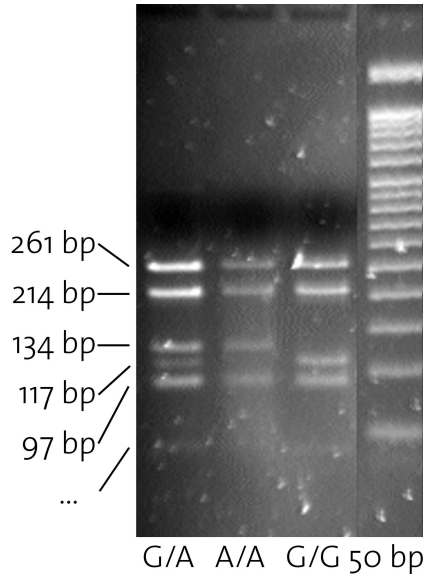

Figure 3.8.: Restriction pattern of *Alu*I digestion of TNK2e12b-Fv2/Rv2 PCR products to determine the FN393558:g.11142G>A polymorphism. The g.11142G>A substitution inserts an additional restriction site at 592 bp to the existing restriction sites at 214 bp, 475 bp, 609 bp and 706 bp of the PCR product.

Table 3.4.: Sequence variants in the genomic sequence of *TNK2* of two F4ac resistant (lab no. 411 and 1170) and two homozygous susceptible pigs (lab no. 1450 and 5064). Sequence variants and positions are given on the basis of the sequence FN393558 of pig 411 and noted as `1>2`. The genomic information is giving for human.

Sequence position (bp)	Genomic information	Sequence variant	Haplotype of pigs F4ac resistant 411	1170	susceptible 1450	5064
233	Exon 2 165 bp	`T>C`	1 1	2 2	1 1	1 1
245	Exon 2 177 bp	`G>T`	1 1	2 2	1 1	1 1

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Table 3.4 – continued from previous page

Sequence position (bp)	Genomic information	Sequence variant	Haplotype of pigs F4ac resistant 411	1170	susceptible 1450	5064
430	Intron 2 181 bp	G>C	1 1	2 2	1 1	1 1
454	Intron 2 205 bp	C>T	1 1	2 2	1 1	1 1
541	Intron 2 292 bp	G>A	1 1	2 2	1 1	1 1
616	Intron 2 367 bp	C>A	1 1	2 2	1 1	1 1
773	Intron 2 524 bp	T>C	1 1	2 2	1 1	1 1
812	Intron 2 563 bp	T>C	1 1	2 2	1 1	1 1
889	Intron 2 640 bp	A>G	1 1	2 2	1 1	1 1
1011	Intron 2 762 bp	T>C	1 1	2 2	1 1	1 1
1148	Intron 2 899 bp	C>G	1 1	2 2	1 1	1 1
1149_1150	Intron 2 901 bp	insCCC	1 1	2 2	1 1	1 1
1224	Intron 2 975 bp	C>T	1 1	2 2	1 1	1 1
1281	Exon 3 47 bp	C>T	1 1	2 2	1 1	1 1
1317	Intron 3 12 bp	G>A	1 1	2 2	1 1	1 1
1411	Intron 3 106 bp	G>T	1 1	2 2	1 1	1 1
1442	Intron 3 137 bp	A>G	1 1	2 2	1 1	1 1
1668	Intron 3 563 bp	T>C	1 1	2 2	1 1	1 1
1944	Intron 3 639 bp	G>A	1 1	2 2	1 1	1 1
2245	Intron 3 940 bp	A>G	1 1	2 2	1 1	1 1
2272	Intron 3 967 bp	delA	1 1	2 2	1 1	1 1
2313	Intron 3 1008 bp	C>T	1 1	2 2	1 1	1 1
2343	Intron 3 1038 bp	C>T	1 1	2 2	1 1	1 1
2356	Intron 3 1051 bp	G>T	1 1	2 2	1 1	1 1
2364	Intron 3 1059 bp	C>G	1 1	2 2	1 1	1 1
2379	Intron 3 1074 bp	T>G	1 1	2 2	1 1	1 1
2447_2448	Intron 3 1142 bp	insAGGGGGGG	1 1	2 2	1 1	1 1
2470_2471	Intron 3 1165 bp	insCT	1 1	2 2	1 1	1 1
2833	Intron 3 1528 bp	A>G	1 1	2 2	1 1	1 1
3049_3050	Intron 3 1744 bp	insG	1 1	2 2	1 1	1 1

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Table 3.4 – continued from previous page

Sequence position (bp)	Genomic information	Sequence variant	Haplotype of pigs F4ac resistant 411	1170	susceptible 1450	5064
3108	Intron 3 1803 bp	G>A	1 1	2 2	1 1	1 1
3164	Intron 3 1859 bp	G>A	1 1	2 2	1 1	1 1
3450	Intron 4 33 bp	G>A	1 1	2 2	1 1	1 1
3581	Intron 4 164 bp	A>G	1 1	2 2	1 1	1 1
3652	Intron 4 235 bp	G>C	1 1	2 2	1 1	1 1
3712_3940	partly superimposed					
3801	Intron 4 384 bp	delG	1 1	2 2	1 1	1 1
3882_3883	Intron 4 467 bp	ins300 bp	1 1	2 2	1 1	1 1
4189	Intron 4 774 bp	T>C	1 1	2 2	1 1	1 1
4217	Intron 4 802 bp	T>C	1 1	2 2	1 1	1 1
4406	Intron 4 991 bp	T>C	1 1	2 2	1 1	1 1
4778	Intron 5 110 bp	G>A	1 1	2 2	1 1	1 1
4850	Intron 5 182 bp	C>G	1 1	2 2	1 1	1 1
4860	Intron 5 192 bp	C>T	1 1	2 2	1 1	1 1
4905	Intron 5 237 bp	G>A	1 1	2 2	1 1	1 1
5038	Intron 5 370 bp	G>T	1 1	2 2	1 1	1 1
5081	Intron 5 413 bp	A>G	1 1	2 2	1 1	1 1
5085	Intron 5 417 bp	A>G	1 1	2 2	1 1	1 1
5107	Intron 5 439 bp	C>G	1 1	2 2	1 1	1 1
5451	Intron 6 20 bp	A>G	1 1	2 2	1 1	1 1
5498	Intron 6 67 bp	C>T	1 1	2 2	1 1	1 1
5718	Intron 6 287 bp	G>A	1 1	2 2	1 1	1 1
5738	Intron 6 307 bp	A>T	1 1	2 2	1 1	1 1
5842	Intron 6 411 bp	G>A	1 1	2 2	1 1	1 1
5997	Intron 6 566 bp	A>G	1 1	2 2	1 1	1 1
6102_6105	Intron 6 671 bp	delTGTT	1 1	2 2	1 1	1 1
6224	Intron 6 793 bp	C>T	1 1	2 2	1 1	1 1
6295	Intron 6 864 bp	T>A	1 1	2 2	1 1	1 1

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Table 3.4 – continued from previous page

Sequence position (bp)	Genomic information	Sequence variant	Haplotype of pigs F4ac resistant 411	1170	susceptible 1450	5064
6421	Intron 6 990 bp	A>C	1 1	2 2	1 1	1 1
6532	Intron 6 1101 bp	A>G	1 1	2 2	1 1	1 1
6620	Intron 6 1189 bp	T>C	1 1	2 2	1 1	1 1
6632	Intron 6 1201 bp	T>G	1 1	2 2	1 1	1 1
6649	Intron 6 1218 bp	A>G	1 1	2 2	2 2	2 2
6686_6687	Intron 6 1256 bp	insT	1 1	2 2	2 2	2 2
6687_6688	Intron 6 1256 bp	delinsCC	1 1	2 2	2 2	2 2
6844_6846	Intron 6 1414 bp	delCCT	1 1	2 2	1 1	1 1
6888_6889	Intron 6 1458 bp	delTT	1 1	2 2	1 1	1 1
6971	Intron 6 1541 bp	C>A	1 1	2 2	2 2	2 2
7075	Exon 7 28 bp	C>A	1 1	1 1	2 2	2 2
7138	Exon 7 91 bp	T>C	1 1	2 2	2 2	2 2
7187	Intron 7 13 bp	G>T	1 1	2 2	1 1	1 1
7194	Intron 7 20 bp	delG	1 1	2 2	1 1	1 1
7267	Intron 7 93 bp	G>A	1 1	2 2	1 1	2 2
7339	Intron 7 165 bp	A>G	1 1	2 2	1 1	2 2
7371	Intron 7 197 bp	T>C	1 1	2 2	1 1	2 2
7717	Intron 8 19 bp	C>T	1 1	1 1	2 2	2 2
7850	Exon 9 69 bp	T>C	1 1	2 2	1 1	1 1
7853	Exon 9 72 bp	C>T	1 1	2 2	1 1	1 1
7900	Intron 9 24 bp	C>G	1 1	2 2	2 2	2 2
7910	Intron 9 34 bp	G>A	1 1	2 2	1 1	1 1
7916	Intron 9 40 bp	C>T	1 1	2 2	1 1	1 1
7934	Intron 9 58 bp	G>A	1 1	2 2	1 1	1 1
7980	Intron 9 104 bp	C>G	1 1	2 2	1 1	1 1
7986_7987	Intron 9 110 bp	delinsTG	1 1	2 2	1 1	1 1
8023	Intron 9 147 bp	A>G	1 1	2 2	1 1	1 1
8055_8154	not sequenced part, inserted 100 Ns					

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Table 3.4 – continued from previous page

Sequence position (bp)	Genomic information	Sequence variant	Haplotype of pigs F4ac resistant 411	1170	susceptible 1450	5064
8384	Exon 10 70 bp	A>G	1 1	2 2	2 2	2 2
8724_8823	not sequenced part, inserted 100 Ns					
8888	Intron 10 1337 bp	C>T	1 1	2 2	1 1	1 1
8998	Intron 10 1447 bp	G>A	1 1	1 1	2 2	2 2
9010	Intron 10 1459 bp	G>A	1 1	2 2	2 2	2 2
9042_9043	Intron 10 1491 bp	insAC	1 1	2 2	1 1	1 1
9272	Intron 11 103 bp	T>C	1 1	2 2	1 1	1 1
9374	Intron 11 205 bp	G>C	1 1	1 1	2 2	2 2
9392	Intron 11 223 bp	G>C	1 1	2 2	1 1	1 1
9417	Intron 11 248 bp	delC	1 1	2 2	1 1	1 1
9461	Intron 11 292 bp	C>T	1 1	2 2	1 1	1 1
9829	Intron 11 660 bp	C>T	1 1	2 2	1 1	1 1
10007	Intron 11 838 bp	C>T	1 1	2 2	1 1	1 1
10023	Intron 11 854 bp	G>A	1 1	2 2	1 1	1 1
10123_10124	Intron 11 954 bp	insTA	1 1	2 2	1 1	1 1
10159	Intron 11 990 bp	A>G	1 1	2 2	2 2	2 2
10331	Intron 11 1162 bp	G>A	1 1	1 1	2 2	2 2
10396	Intron 11 1227 bp	G>A	1 1	1 1	2 2	2 2
10520	Intron 11 1351 bp	A>G	1 1	2 2	1 1	1 1
10622	Exon 12 85 bp	C>A[a]	1 1	2 2	2 2	2 2
10725	Exon 12 188 bp	A>G	1 1	2 2	1 1	1 1
10731	Exon 12 194 bp	G>A	1 1	2 2	1 1	1 1
11008	Exon 12 471 bp	G>A[b]	1 1	2 2	1 1	1 1
11142	Exon 12 605 bp	G>A	1 1	1 1	2 2	2 2
11585	Exon 12 1048 bp	G>A[c]	1 1	2 2	1 1	1 1
11684	Exon 12 1147 bp	G>C[d]	1 1	2 2	1 1	1 1

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[a] missense SNP: p.Pro543His
[b] missense SNP: p.Val673Met
[c] missense SNP: p.Arg865His
[d] missense SNP: p.Ser989Thr

Table 3.4 – concluded from previous page

Sequence position (bp)	Genomic information	Sequence variant	Haplotype of pigs F4ac resistant 411	1170	susceptible 1450	5064
12205	Intron 13 237 bp	T>C	1 1	2 2	2 2	2 2
12622	Intron 14 247 bp	T>C	1 1	2 2	1 1	1 1
12630	Intron 14 255 bp	T>A	1 1	2 2	1 1	1 1
12649	Intron 14 274 bp	G>C	1 1	1 1	2 2	2 2
12699	Intron 14 324 bp	C>T	1 1	2 2	2 2	2 2
12723	Intron 14 348 bp	T>C	1 1	1 1	2 2	2 2
12750_12751	Intron 14 375 bp	insTATA	1 1	2 2	1 1	1 1
13009	Intron 14 634 bp	G>A	1 1	2 2	1 1	1 1
13136	Intron 14 761 bp	C>T	1 1	2 2	2 2	2 2
13342	Intron 14 967 bp	C>G	1 1	2 2	1 1	1 1
13593	Exon 15 UTR 248 bp	C>A	1 1	2 2	1 1	1 1
13714	Exon 15 UTR 369 bp	C>A	1 1	2 2	1 1	1 1
13764	Exon 15 UTR 419 bp	G>A	1 1	2 2	1 1	1 1

3.5. *MUC4* g.8227C>G polymorphism as a marker for F4ac susceptibility and resistance

Swiss experimental herd pigs

The 331 SEH pigs used for linkage analysis (section 3.2) were genotyped for the *MUC4* g.8227C>G polymorphism. The F4ab/F4ac phenotype could be determined in 329 pigs, and in 325 of these pigs, the phenotype coincided with the *MUC4* g.8827C>G genotype. In 4 of the 329 phenotyped pigs (1%), the F4ab/F4ac phenotype did not coincide with the g.8227C>G polymorphism. Two pigs were phenotyped as resistant and the genotype was heterozygous g.8227CG. Two other pigs were phenotyped as susceptible, but the genotype was homozygous g.8227CC associated with resistance (bold type printed numbers in Table 3.7). Two discordant SEH pigs, one of haplotype combination A (lab no. 1663) and one of combination D (lab no. 3584), were full-sibs of a repeated mating. They shared a resistant allele of the paternal grandfather with the maternal grandfather of a third discordant pig of haplotype combination A (lab no. 6229). The fourth discordant SEH pig (lab no. 9524) was not related to the others. Additionally, haplotype analysis in the interval between *SW207* and *SW1030* did not support the phenotyping results of these six pigs. Independent of the position of the F4ab/F4ac phenotype in the interval between *SW207* and *S0075*, a recombination or a double recombination occurred next to *F4bcR*.

Swiss performing station pigs

The 78 SPS pigs of 38 litters were genotyped for the *MUC4* g.8227C>G polymorphism (Table 3.5). Pigs were of Landrace and Large White breed and represented the Swiss porcine population. The F4ac phenotype coincided in 72 pigs (92%) with the G-allele for susceptibility and the g.8227CC genotype for resistance, while the results were discordant in six pigs (8%). Five pigs were phenotyped as resistant, whereas the genotype was g.8227CG, and one pig was phenotyped susceptible while the genotype was g.8227CC (bold type printed in Table 3.5).

Table 3.5.: *MUC4* g.8227C>G polymorphism and F4ac phenotypes of 78 SPS pigs. Number of pigs where the phenotype did not coincide with the g.8227C>G polymorphism are in bold type.

MUC4 g.8227 genotype	F4ac phenotype	No. of pigs Landrace	No. of pigs Large White
C/C	resistant	5	4
	susceptible	**1**	0
C/G	resistant	**3**	**2**
	susceptible	6	44
G/G	susceptible	2	11
Total no. of pigs		17	61
Pigs carrying G-allele		65%	93%

Boars for artificial insemination

The g.8227C>G polymorphism was determined in 193 boars used for artificial insemination in Switzerland in 2005. Considerable differences in the frequency of the g.8227G allele were found between breeds (Table 3.6). More than 70% of Landrace and Large White boars carried the G-allele, associated with susceptibility, whereas only 13% of Duroc boars and 4% of Piétrain x Duroc boars carried the G-allele. Of the five genotyped Piétrain boars 60%, carried the G-allele.

3.6. *MUC4* and *TNK2* haplotypes

A total of 13 different haplotype combinations were found in 78 SPS and 102 SEH pigs by analysis of three SNPs in *TNK2* and three SNPs in *MUC4* (Table 3.7). The eight haplotype combinations A to H were found in several pigs, and five were found only in one pig. The SNPs of haplotype combinations A, D and H, comprising

Table 3.6.: *MUC4* g.8227C>G polymorphism of 193 boars of five breeds used for artificial insemination in Switzerland in 2005.

MUC4 g.8227 genotype	No. of pigs				
	Landrace	Large White	Duroc	Piétrain x Duroc	Piétrain
C/C	7	30	14	27	2
C/G	13	61	2	1	1
G/G	6	27	0	0	2
Total no. of pigs	26	118	16	28	5
Pigs carrying G-allele	73%	75%	13%	4%	60%

about 85% of the analysed pigs, were of the same genotype as the g.8227C>G polymorphism. These data confirm the soundness of the g.8227C>G genotyping results. The two haplotype combinations A and D were found in 47 of 48 SEH pigs of four resistant x heterozygous matings. In one pig, the *TNK2* g.7075C>A polymorphism was different to the combination A (footnote *a* of Table 3.7).

A greater haplotype diversity was found in the 54 SEH pigs, with a recombination in the interval between *SW207* and *SW398*. Six haplotype combinations were found in these pigs: combinations A and D were found in 46 pigs, combination C in five pigs, and combinations B, E and footnote *g*, modifying combination C, in one pig. In each of the two haplotype combinations A and D, the haplotypes of two pigs did not correspond to the F4ac phenotype.

Eleven different haplotype combinations occurred in the 78 SPS offspring indicating that SPS pigs are genetically the most diverse of the pig samples. Again, the heterozygous haplotype combination D was most prevalent: it was found in 41 F4ac susceptible and 4 resistant pigs. Haplotype combinations E to H were found in 22 susceptible pigs and in one resistant pig. Haplotype combinations A and B were found in 9 pigs, and combination C, associated with the resistant phenotype, was found in one susceptible pig. Three of the haplotype combinations occurred only once and are mentioned in footnotes *b, c* and *e* of Table 3.7.

Two further haplotype combinations were determined in *MUC4* 6138–3887 by sequence analysis of 25 pigs (Table 3.8). The g.6242G>A, g.6317G>A, g.6321G>C, g.7947A>G and g.8227C>G polymorphisms coincided with the F4ac phenotype in 12 of 19 resistant pigs. The g.6308G>T polymorphism coincided with the phenotype even in 15 of 19 pigs.

Table 3.7.: *TNK2* and *MUC4* haplotype combinations of 180 pigs determined by RFLP. The position of the variation in genomic sequences and the genomic information for the human sequence are given. SNPs in the haplotypes are encoded as `1>2`. The F4ab/F4ac phenotype of the pigs is given as «R» for resistant and «S» for susceptible.

Position of the variation (bp)	Genomic information	SNP	Haplotype combinations							
			A	B	C	D	E	F	G	H
FN393558	*TNK2*									
7075	Exon 7 28 bp	`C>A`	1 1[a]	1 2	1 2	1 2	1 2	2 2	2 2[b]	2 2[c]
7717	Intron 7 19 bp	`C>T`	1 1	1 2	1 2	1 2	1 2	2 2	2 2	2 2
11142	Exon 12 605 bp	`G>A`	1 1	1 1	1 2[d]	1 2[ef]	2 2	1 2	2 2	2 2
DQ848681	*MUC4*									
6242	Intron 5 618 bp	`G>A`	1 1	1 1	1 1[g]	1 2	1 2	1 2	1 2	2 2
7947	Intron 7 114 bp	`A>G`	1 1	1 1	1 1	1 2	1 2	1 2	1 2	2 2
8227	Intron 7 396 bp	`C>G`	1 1	1 1	1 1	1 2	1 2	1 2	1 2	2 2
No. of pigs and F4ac phenotype	Four SEH litters	R	23							
		S				25				
	Selected SEH pigs	R	20	1	6	**2**				
		S	**2**			22	1			
	SPS pigs	R	5	4		**4**		**1**		
		S			**1**	41	2	2	5	13
	Total no.		50	5	7	94	3	3	5	13

[a] genotype of one R pig (lab no. 419): 1 2
[b] genotype of one pig (lab no. 3686): 1 2
[c] genotype of one pig (lab no. 949): 1 2
[d] not determined in one pig (lab no. 512)
[e] genotype of one S pig (lab no. 961): 1 1
[f] not determined in two pigs (lab no. 513 and 982)
[g] genotype of one R pig (lab no. 512): 1 2

Table 3.8.: Additional polymorphisms in *MUC4* g.6138–6887 of 25 pigs determined by sequencing. Pigs shown with their lab no. are already included in Table 3.7. Sequence variants shown in Table 3.7 are in bold type. The position of the variations in the genomic sequence of DQ848681 and the genomic information for the human sequence are given. SNPs in the five haplotypes are encoded as `1>2`. The F4ab/F4ac phenotype of the pigs is given as «R» for resistant and «S» for susceptible.

Sequence position (bp)	Genomic information	Sequence variant	Haplotype associated with resistance		susceptibility		
6242	Intron 5 618 bp	`G>A`	1 1	1 1	1 2	1 2	2 2
6308	Intron 5 684 bp	`G>T`	1 1	1 1	1 2	1 1	2 2
6317	Intron 5 693 bp	`G>A`	1 1	1 1	1 2	1 2	2 2
6321	Intron 5 797 bp	`G>C`	1 1	1 1	1 2	1 2	2 2
6609	Intron 6 84 bp	`T>A`	1 1	1 2	1 2	1 2	2 2
6616	Intron 6 91 bp	`G>T`	1 1	1 2	1 2	1 2	2 2
6634	Intron 6 109 bp	`A>C`	1 1	1 2	1 2	1 2	2 2
6675_6680	Intron 6 150 bp	`delGAACGT`	1 1	1 2	1 2	1 2	2 2
6690	Intron 6 165 bp	`A>T`	1 1	1 2[a]	1 2	1 2	2 2
6745	Intron 6 220 bp	`T>C`	1 1	1 2[a]	1 2	1 2	2 2
6770	Intron 6 245 bp	`G>T`	1 1	1 2[a]	1 2	1 2	2 2
6862	Intron 6 337 bp	`T>C`	1 1	n.d.[b]	1 2[c d]	1 2[d]	2 2
7947	Intron 7 114 bp	`A>G`	1 1	1 1	1 2	1 2	2 2
8227	Intron 7 396 bp	`C>G`	1 1	1 1[e]	1 2	1 2	2 2
Lab no. and F4ac phenotype	Four SEH litters	R	411	4827 4831 5063 1170			
	Selected SEH pigs	R		1141 1142 1183 1656 1657 1660	3584 9524		
		S	6229	1663			1450 5064
	SPS pigs	R		979	3673 3674	963 3652 3669	
		S	3656		3666		

[a] not determined in 1183 and 1660
[b] not determined
[c] not determined in 3584, 3666 and 9524
[d] determined by Claus Jørgensen
[e] determined by RFLP in six pigs (979, 1141, 1142, 1656, 4827, 5063)

3.7. F4ad susceptibility

3.7.1. F4ad adhesion of repeated matings

Thirty-two matings were repeated several times to produce 77 litters with a total of 749 offspring for phenotyping. The percentages of F4ad adhesive enterocytes of the pigs were compared within and between repeated litters. Six of the repeated matings were significantly different in the percentage of F4ad adhesive enterocytes between the repeated litters in the Kruskal-Wallis and the Kolmogorov-Smirnov test (Table 3.9 and Figure 3.9). These matings were of the following boar x sow combinations: 194B x 180B, 194B x 183B, 147*B x 156*B, 147*B x 239B, 214B x 215B and 381B x 190*B. The distributions of adhesion strength of these matings are shown in Figure 3.9, and indicate adhesion differences between repeated matings. The differences in adhesion strength of litters from the other 26 matings were either not significant or only in one test significant.

Adhesion strength of litter 90 from mating 194B x 180B was significantly and obviously different from the adhesion strengths of the litters 65, 97 and 107, but it was not significantly different from litters 51 and 77. Adhesion strength was also significantly different between litter 65 and the litters 77 and 97. Finally, litter 77 was also significantly different from litter 97. The mating 194B x 183B had significant differences only between litter 50 and 98.

Further, litters of mating 147*B x 156*B had obviously different distributions in adhesion strength: Litter 119 had seven non adhesive pigs and one pig with 8% adhesive enterocytes, whereas litter 132 had five pigs with 0% to 40% adhesive enterocytes and three pigs with 75% to 100% adhesive enterocytes. The adhesion differences in mating 214B x 215B were less obvious: Litter 81 had 11 pigs with 0% to 2.5% adhesive enterocytes, and two pigs with 100% adhesive enterocytes. On the other hand, litter 93 had 10 pigs with 0% to 8% adhesive enterocytes, one pig with 18% adhesive enterocytes and two pigs with 85% and 95% adhesive enterocytes.

3.7.2. Influence of intestinal contents on F4ad adhesion

Rating of two samples with and two samples without contents

Enterocytes were scored for F4ad adhesion of two adjoining intestinal segments without contents and of two adjoining intestinal segments with contents in a total of 59 pigs of 12 litters. Both intestinal segments free of contents of 50 pigs (85%) were classified to the same phenotype (Table 3.10) and adhesion strengths did highly correlate (r_s=0.887). Of these pigs, 27 pigs were adhesion negative or had less than 2.5% of adhesive enterocytes, and 23 pigs were adhesion positive. Nine pigs (15%) were rated adhesion negative in one sample, but were rated adhesion positive in the other sample. The percentage of adhesive enterocytes in these nine samples was between 3% and 50%.

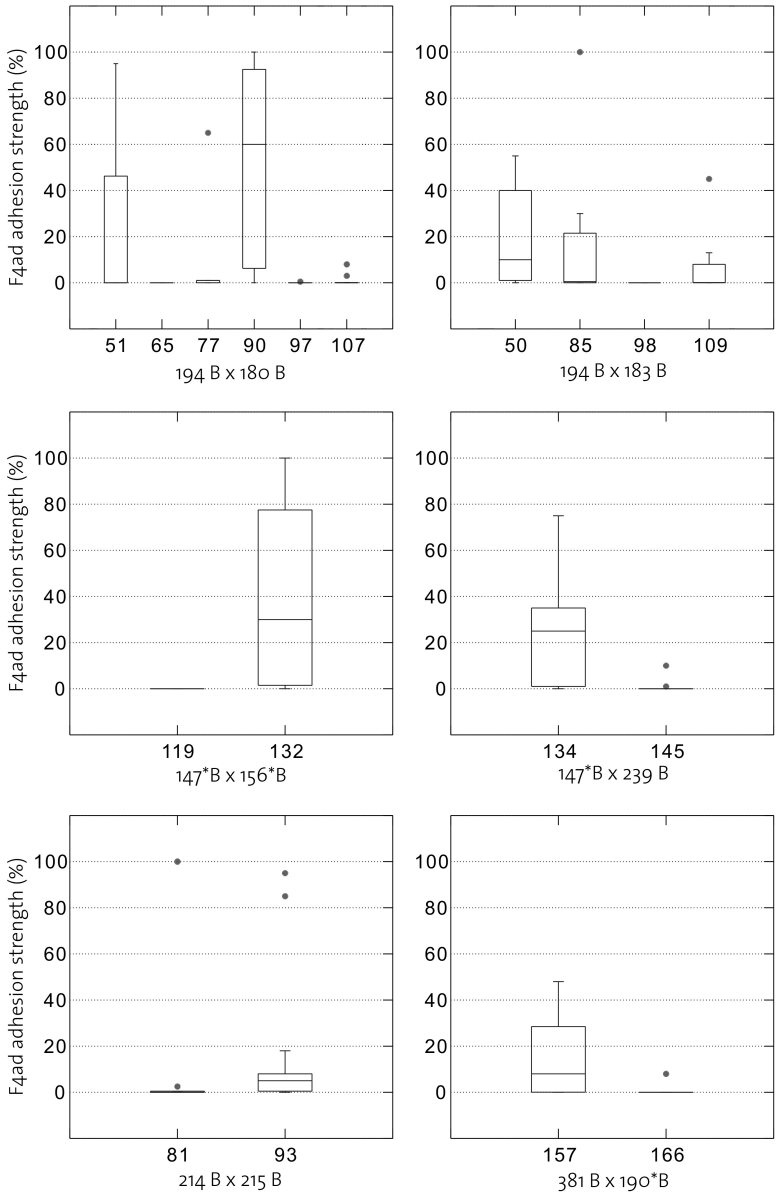

Figure 3.9.: F4ad adhesion strength of repeated matings with litter numbers are shown in boxplots. Boxes show the ranges of the central 50% of the values (interquartile range, IQR). The median is drawn as a horizontal line in the box. The end of the vertical line indicates the last value smaller than 1.5 xIQR. The outliers are drawn as •. Litters of the matings 147*B x 156*B, 147*B x 239B, 214B x 215B and 381B x 190*B are significantly different within the matings at the 5% level. Test statistics of the matings 194B x 180B and 194B x 183B are shown in Table 3.9.

Table 3.9.: Kruskal-Wallis and Kolmogorov-Smirnov significance values showing differences in the percentages of F4ad adhesive enterocytes between litters. Litters of 194B x 180B and 194B x 183B are shown with bold type printed values p≤0.05 considered as significant.

Matings with litters no.	Litter no. Kolmogorov-Smirnov probabilities					
194B x 180B	51	65	77	90	97	107
51	-	0.185	0.915	0.421	0.185	0.421
65		-	**0.028**	**0.000**	**0.000**	0.112
77			-	0.086	**0.028**	0.415
90				-	**0.000**	**0.003**
97					-	0.598
107						-
Kruskal-Wallis	**0.000** assuming Chi-square distribution with 5 df					
194B x 183B	50	85	98	109		
50	-	0.735	**0.022**	0.386		
85		-	0.189	0.999		
98			-	0.112		
109				-		
Kruskal-Wallis	**0.024** assuming Chi-square distribution with 3 df					

The ratings of the two intestinal segments with contents gave similar results: 51 pigs (86%) agreed in both ratings (r_s=0.839). Of these pigs, 28 were of the resistant phenotype and 23 were of the susceptible phenotype. Eight pigs were adhesion negative in one sample, whereas the other sample had between 5% and 70% of adhesive enterocytes. The ratings of four of these eight pigs were also dissenting in intestinal segments free of contents.

The ratings of all four segments were in agreement in 61% (36 of 59 pigs) of the pigs. Five pigs were rated resistant in both segments without contents but were rated susceptible in both segments with contents. An additional five pigs were rated susceptible in both segments free of contents while they were rated susceptible in both segments with contents. In 13 other pigs, the rating of adjoining segments did not coincide.

Table 3.10.: F4ad rating of two adjoining segments free of contents and of two adjoining segments with contents of 59 pigs with a threshold of 2.5% of adhesive enterocytes. «R, R» indicates resistant ratings in both segments, «S, S» indicates susceptible ratings in both segments, and «R, S» indicates a resistant rating in one segment and a susceptible rating in the other segment.

Rating of two segments	No. of pigs with contents			
free of contents	R, R	S, S	R, S	Total
R, R	20	5	2	27
S, S	5	16	2	23
R, S	3	2	4	9
Total	28	23	8	59

Rating of one sample with and one sample without contents

The influence of contents to adhesion strength was compared by phenotyping an intestinal sample with contents and a sample without contents for 215 pigs (including the 59 pigs mentioned above). No significant differences were found with Kolmogorov-Smirnov (p=0.594) and Mann-Whitney (p=0.325) by comparing adhesion strength of the segment with contents to the adhesion strength of the segment without contents of 215 pigs. Additionally, the differences of adhesion were not different from zero in the t-test (p=0.473, df=214, mean=0.011, SD=0.215) (Figure 3.10 right part). Adhesion strengths of segments with contents and segments without contents were compared (Figure 3.10, left part) and did highly correlate (r_s=0.700). More than 75% of the pigs (161 of 215 pigs) had an adhesion difference

between the two segments of ≤10%. In total, 90 pigs had no bacterial adhesion in both samples.

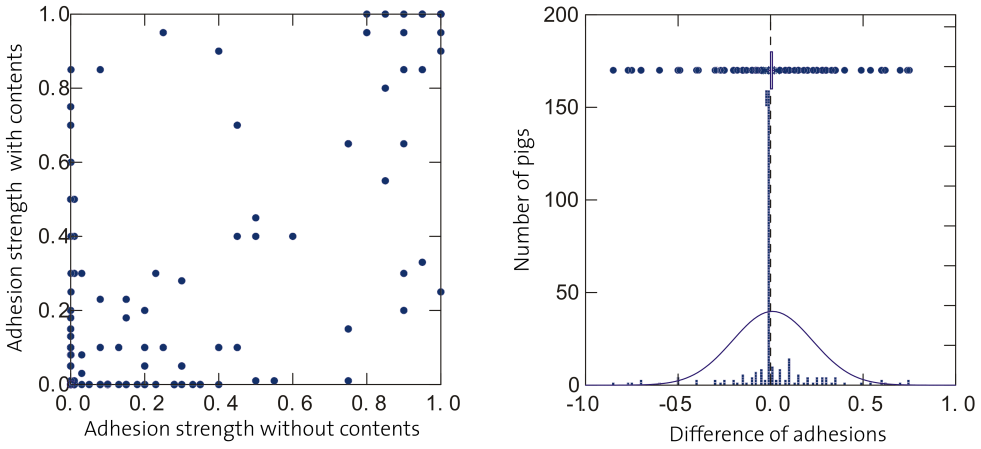

Figure 3.10.: An intestinal segment free of contents and a segment with contents were taken from each of 215 pigs, and the percentages of F4ad adhesive enterocytes (adhesion strength) were compared. Left: Scatterplot of adhesion strengths of intestinal segments with contents and intestinal segments without contents. Right: Distribution of differences in adhesion strengths of the two intestinal segments. A boxplot and the normal distribution curve are shown.

3.7.3. Threshold of 60% for F4ad susceptibility

The cumulative number of pigs rated as resistant depending on the threshold for F4ad susceptibility of 1569 phenotyped SEH pigs is shown in Figure 3.11. The gradient of pigs rated as resistant decreased until a threshold of 60%, and increased again with a threshold of >60%. The slowest increase of resistant pigs was determined at a threshold between 45% and 60% of adhesive enterocytes. The steepest slope of growth was between the threshold of 0% and 5%, and between the threshold of 95% and 100% of adhesive enterocytes. Of the total, 637 pigs (41%) had no adhesive enterocytes, 149 pigs had between >0% and 5% of adhesive enterocytes and 91 pigs had between >5% and 10% adhesive enterocytes. The adhesion strength of 2.5% was subject to high changes in susceptible pigs, and this questioned the actual threshold.

The number of adhering *E. coli* F4ad per enterocyte was counted for 154 SEH pigs and compared to the percentage of adhesive enterocytes (Figure 3.12). Pigs (n=110) with less than 60% adhesive enterocytes had less than a mean of 8 adhering bacteria per enterocyte except for two piglets with a mean of 9.6 and 10.3 bacteria,

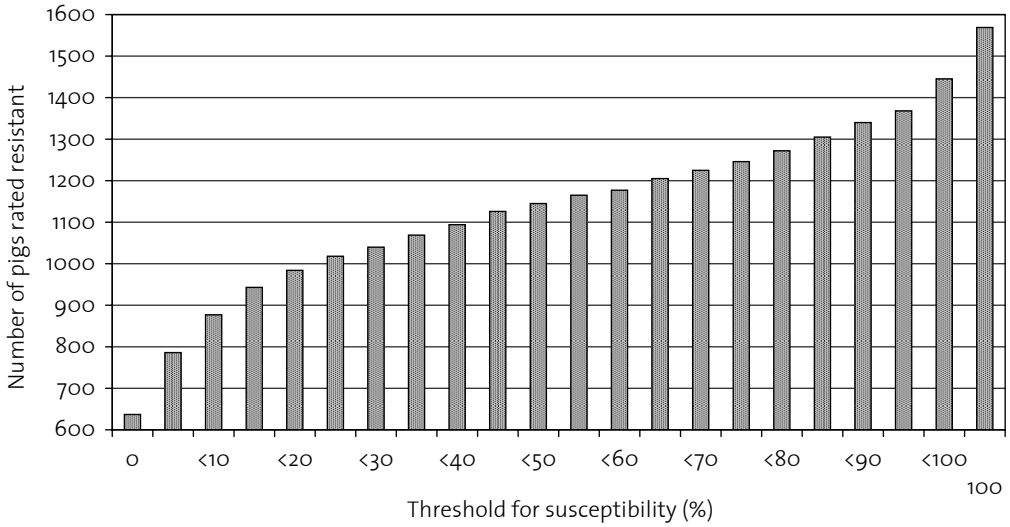

Figure 3.11.: The cumulative number of pigs rated as resistant depending on the threshold for susceptibility. In total, 1569 pigs from the experimental herd were phenotyped.

respectively. Pigs (n=44) with more than 60% adhesive enterocytes had at least 8 adhering bacteria per enterocyte except for one piglet with 7.6 bacteria. The mean number of bacteria per enterocyte was 2.4±2.6 for pigs with adhesion below 60% and 12.7±3.7 for pigs with more than 60% adhesive enterocytes. No pigs were found with adhesion between 53% and 64%.

3.7.4. Offspring of phenotyped parents

Subsequently, the threshold of 60% adhesive enterocytes was used to evaluate genetic influence on F4ad adhesion. This threshold was proposed by Hu *et al.* (1993) to distinguish the *F4adH* from the *F4adL*.

Parents with known phenotype were divided into three classes according to the threshold for high F4ad susceptibility of 60% adhesive enterocytes: pigs with 0% to 5% adhesive enterocytes were considered to be resistant (r), pigs with >5% to 60% adhesion to be low susceptible (l) and pigs with >60% adhesion to be high susceptible (h). Based on these adhesion classes, the 122 litters with a mean of 9.5 offspring were divided in one of the six possible mating combinations. The adhesion strengths of the 1166 pigs from these mating combinations are shown in boxplots in Figure 3.13. From resistant x resistant to high x high combinations, an increasing range of the central 50% of the values, shown the boxes, was observed. This increase was reflected in a rising percentage of adhesive enterocytes (Table 3.11).

The median of the adhesion strength of the high x high combination was 65%, whereas the median of the other mating combinations was 5% or below. The

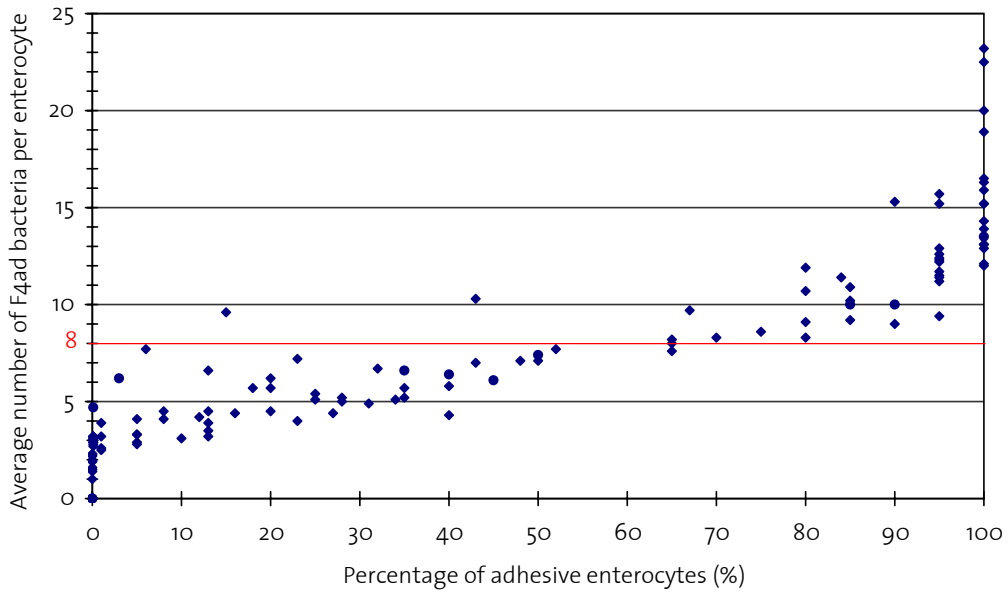

Figure 3.12.: The average number of bacteria adhering to enterocytes are compared to the percentage of adhesive enterocytes per pig. The 154 SEH pigs of 67±8 days of age of 17 litters were slaughtered between February and November 2007 and are shown as rhombi (r_s=0.955). Twelve additional fattening pigs or parent pigs are drawn as bullets.

adhesion strengths of pigs from the high x high and resistant x low combinations were significantly different from the other combinations (Table 3.11). The adhesion strength of the resistant x resistant combination was significantly different from all combinations but not from the low x low combination. In the other cases, lox x low, resistant x low and low x high combinations significantly different from the other three combinations, whereas they were not significantly different from each other. The adhesion of the lox x low, resistant x low and low x high combinations were not significantly different from each other, but from the other three combinations.

To eliminate the impact of repeated matings on the distribution of adhesion strengths, the mean adhesion strengths of offspring from matings were compared. The tendency between the mating combinations was comparable to the results shown in Figure 3.13.

Susceptible offspring of resistant x resistant matings and resistant offspring from susceptible x susceptible matings were also produced. Of the 137 offspring from 13 litters of resistant x resistant matings, 15 piglets from five different litters were highly susceptible. On the other hand, 28 offspring of 8 litters from high x high matings had an adhesion strength of $\leq$1%.

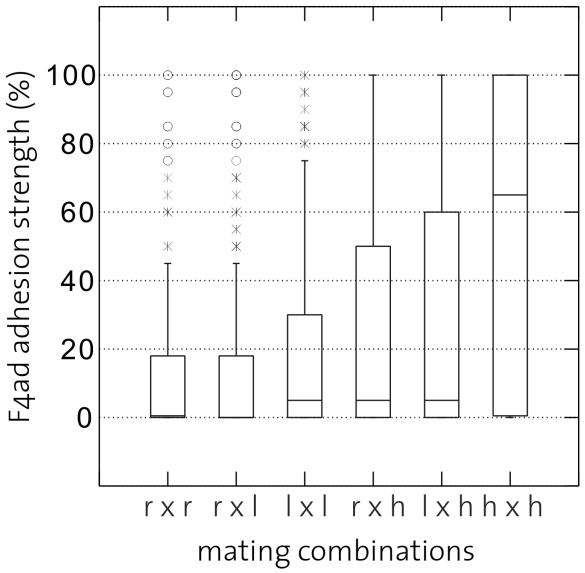

Figure 3.13.: Distributions of F4ad adhesion strengths of pigs of the six mating combinations according to a resistant ($\leq$ 5% adhesive enterocytes, r), low susceptible (between 5% and 60%, l) or high susceptible (>60%, h) classification of the parents. The box-plots show the ranges of the central 50% of the values (box), the outside values ($*$) and the far outside values ($\circ$).

Table 3.11.: Kruskal-Wallis one-way analysis of variance with the six mating combinations of the three adhesion classes as described in Figure 3.13. Significance was confirmed with Kolmogorov-Smirnov test. Number of pigs and number of litters with mean adhesion of each mating combination are given. Significant differences ($p<0.05$) between the combinations are bold type printed.

	Comparison of mating combinations probabilities for similarity					
	r x r	r x l	l x l	r x h	l x h	h x h
resistant x resistant	-	**0.012**	0.210	**0.024**	**0.000**	**0.000**
resistant x low		-	**0.005**	**0.000**	**0.000**	**0.000**
low x low			-	0.287	0.174	**0.000**
resistant x high				-	0.967	**0.000**
low x high					-	**0.000**
high x high						-
No. of pigs	137	309	134	203	285	98
No. of litters	13	33	15	20	31	10
Mean adhesion	16.2%	16.9%	20.6%	26.4%	27.9%	53.7%

3.7.5. Repeated intestinal biopsies

For two piglets from each of five litters, biopsies of the small intestine were taken at the age of about four weeks and about six months. F4ad adhesion of six pigs was in agreement between the two biopsies (Table 3.12). In four pigs (lab no. 542, 543, 1398, 1399), the enterocytes showed no adhesion or a weak adhesion at the first biopsy (0% and 20%), but a strong adhesion (73%, 75%, 95% and 100%) at the second biopsy. Cell quality of intestinal segments in the first biopsy was modest in most samples, while it was good in the second biopsy.

Table 3.12.: Adhesion strength of intestinal biopsies of 10 pigs taken at the age of four to six weeks, at about six months and, if pigs are already dead, at slaughter. The first two pigs of each group are the boar and the sow. *E. coli* F4ad adhesion strength is shown with the age of pigs when the intestine sample was taken. The F4ac genotypes verified by progeny or/and descent are given as «s» for resistant and «S» for susceptible. The genotypes determined by the g.8227C>G polymorphism are shown in *italics*.

	Genotype	Biopsy 1		Biopsy 2		Slaughter	
Pig/lab no.	F4ac	F4ad (%)	Age (d)	F4ad (%)	Age (d)	F4ad (%)	Age (d)
605B	ss						
619B	ss					0	643
2570/1400	ss	0	25	0	185		
2577/1401	ss	0	25	0	185		
381B	Ss					0	1253
928B	ss					0	1926
2554/1396	ss	0	26	0	202		
2557/1397	ss	0	26	0	202	0	341
381B	Ss					0	1253
190*B	ss						
2452/542	Ss	20	27	100	186		
2464/543	ss	0	27	95	186		
535*B	Ss					95[a]	613
614B	ss					100[b]	775
2559/1398	Ss	20	41	93	188		
2562/1399	Ss	0	41	75	188	18[c]	251
407B	SS						
391B	Ss						
2526/549	*Ss*	100	28	100	160	95[d]	211
2528/551	*Ss*	90	28	100	160		

[a]Segment free of contents taken 9 m distal the Arteria mesenterica cranialis. Adhesion strength was 3% at 5 m (with contents).

[b]Segment free of contents taken at 10 m. Adhesion strength was 15% at 5 m (with contents).

[c]Segment free of contents taken at 5 m. Adhesion strength was 0% at 0 m (with contents) and 58% at 10 m (without contents).

[d]With contents, position not clear. Pig was euthanised after chronic disease.

4. Discussion

4.1. *E. coli* F4 adhesion

4.1.1. Adhesion phenotypes

All five F4 adhesion phenotypes described by Bijlsma *et al.* (1982) and Baker *et al.* (1997) occurred in the 1569 pigs of more than 180 litters from the Swiss experimental herd (SEH) and in the 78 pigs from the Swiss performance station (SPS). Phenotypes A to E occurred in both herds, whereas phenotype F was found in SEH pigs but not in SPS pigs. In an earlier study with fewer SEH pigs, the same adhesion phenotypes were reported (Python, 2003; Python *et al.*, 2005). The two adhesion phenotypes G ($F4ab^-/F4ac^+/F4ad^-$) and H ($F4ab^-/F4ac^+/Fad^+$) reported in 3 of 366 Large White and Songliao Black breed pigs in a study of Li *et al.* (2007) were not found in our SEH and SPS pigs.

4.1.2. Strong F4ab/F4ac adhesion

Adhesion of F4ab and F4ac bacteria to enterocytes was unambiguous in most of the 1569 SEH pigs. Less than 10% of F4ab and less than 5% F4ac adhesion positive pigs had between 15% and 60% adhesive enterocytes (Figure 3.2 on page 33). As a result, mean adhesion strengths of pigs of phenotypes A and B were 85% or more (Table 3.1). The 20 pigs with 15% to 60% F4ac adhesive enterocytes were all suggested to be susceptible by haplotype analysis. Pigs with a high percentage of F4ac adhesion positive enterocytes also showed a high percentage of F4ab adhesion positive enterocytes (SEH pigs r_s=0.878, SPS pigs r_s=0.799).

We did not observe a pig in our herds that was F4ac adhesion positive but F4ab adhesion negative. These results support a single-locus model of a strong F4ab/F4ac receptor (*F4bcR*) inheritance. Nevertheless, inheritance of the F4ab and the F4ac receptor remains controversial. Some studies have suggested two or more distinct but linked genes for F4ab and F4ac receptors (Edfors-Lilja *et al.*, 1995; Guérin *et al.*, 1993; Li *et al.*, 2007; Peng *et al.*, 2007), while others have indicated a common receptor gene for F4ab and F4ac adhesion (Bijlsma and Bouw, 1987; Jørgensen *et al.*, 2003; Python *et al.*, 2002).

Strikingly, the mean adhesion strength of SPS pigs was slightly lower and had a greater standard deviation than in SEH pigs. It is not known if this is a result

of altered sample quality due to the delay between sample taking and enterocyte purification.

4.1.3. Weak F4ab adhesion

In addition to the strong F4ab/F4ac adhesion, we observed 247 SEH pigs (16%) that were F4ab adhesion positive (mean adhesion 23±18%) but F4ac adhesion negative (phenotypes C and F). However, 42% of these pigs had less than 15% adhesive enterocytes, and only 6% of the pigs had ≥60% adhesive enterocytes. These results support the existence of a receptor for weak F4ab adhesion. A weak F4ab adhesion in phenotypes C and F has been found earlier by our group (Python, 2003; Python *et al.*, 2005), but a weak F4 adhesion is being discussed since decennia.

Previously, Sellwood (1980) reported weak adhesive pigs with three to four F4ac bacteria per brush border. However, the weak adhesion seemed not to be sufficient for diarrhoea, as these pigs did not show clinical symptoms after oral infection with F4ac (Sellwood, 1984). Michaels *et al.* (1994) reported 20 of 85 Fengjing crossbred pigs as F4ac weak adherent, with one to six adherent bacteria per enterocyte; the other 65 pigs were resistant. Nevertheless, the authors could not explain the low adhesion. Explanations for the weak F4ab and F4ac phenotypes was discussed by Bijlsma and Bouw (1987) as an influence of epistatic genes or an inhibition or modification of receptor expression. Weak F4ab, F4ac and F4ad adhesion was reported by Baker *et al.* (1997), where adhesion was also dependent on the bacterial strains used. We did not note any obvious differences in adhesion when we compared the routinely used bacterial strain F4ad Guinée with the two strains F4ad Morris and Westerman in 12 piglets and 6 parent pigs.

4.1.4. F4ac adhesion differences between breeds

Our results revealed a high frequency of *E. coli* F4ab/F4ac susceptible pigs in commonly used pig breeds in Switzerland. The 78 SPS pigs of the representative sample of the Swiss porcine population were phenotyped as F4ac susceptible in 53% of the Landrace breed and in 90% of the Large White breed pigs. In an earlier report from Switzerland with 116 Landrace and 243 Large White breed pigs, about 50% of pigs from both breeds were phenotyped as F4ac susceptible (Gautschi and Schwörer, 1988). In a German study investigating occurrence of F4 receptors in different breeds, more than 60% of Landrace and Large White breed pigs were phenotyped as F4ac susceptible (Engel, 1998; Engel *et al.*, 1998). Similar to our results, Li *et al.* (2007) phenotyped 79% pigs of Landrace breed and 49% pigs of Large White breed from China as susceptible to F4ac. Differences in susceptibility within the same breed may result from different breeding strategies in the geographical regions and indirect selection due to traits close to the F4ac receptor gene. Differences may also result from different phenotyping methods.

The *MUC4* DQ848681:g.8227C>G *Xba*I polymorphism was used as an indicator of F4ac susceptibility to genotype 193 boars used for artificial insemination in 2005 in Switzerland (Table 3.6 on page 51). The g.8227G allele associated with susceptibility was found in 73% of Landrace breed pigs and in 75% of Large White breed pigs, but only in 13% of Duroc breed and 4% of Piétrain x Duroc crossbreed pigs. The high frequency of Landrace and Large White breed pigs with the G-allele corresponds to the findings of SPS pigs.

Differences in F4ac susceptibility between breeds have been shown in different studies. Baker *et al.* (1997) and Engel *et al.* (1998) reported 33% and 40% of the pigs of the Duroc breed that were susceptible to F4ac. Duroc may be responsible for the low percentage of susceptible pigs in the Piétrain x Duroc crossbreed, as Engel *et al.* (1998) reported that more than 60% of the Piétrain breed pigs were F4ac susceptible. The 60% of our Piétrain boars carrying the G-allele further support these results, but the number of five pigs is not high enough to statistically support this assumption.

A high percentage of resistant pigs has been reported for other pig breeds: Songliao Black had 90% resistant pigs (Li *et al.*, 2007), Chester White had 64% and 85% resistant pigs (Baker *et al.*, 1997; Rapacz and Hasler-Rapacz, 1986), the Meishan breed had 100% resistant pigs and Meishan x Minzu and Meishan x Fengjing crossbreeds had 73% and 76% resistant pigs (Michaels *et al.*, 1994). It is not clear whether the pigs in the studies by Li *et al.* (2007), Michaels *et al.* (1994) and Rapacz and Hasler-Rapacz (1986) are representative of the population, as the pigs were from a research herd.

4.2. F4ab/F4ac mapping on SSC13

In this thesis, *F4bcR* could be assigned with significance to the interval *SW207* – [*MUC4-8227, MUC4gt*] – *S0075* by linkage and the results were published in Joller *et al.* (2009). Data of two earlier studies (Jørgensen *et al.*, 2003; Python *et al.*, 2005) were combined to a data set of 710 pigs to refine the position for *F4bcR*. Pigs consisted of 10 purebred founders (F_0), 26 F_1 pigs and 200 F_2 offspring from a European Wild boar x Swedish Yorkshire cross of the Nordic experimental herd (NEH) as described by Edfors-Lilja *et al.* (1995). Our data comprised 143 SPS pigs, and 331 SEH pigs.

The most likely order was *SW207* – (*F4bcR* – [*MUC4-8227* – *MUC4gt*] – *S0283* – *HSA125gt*) – *S0075* – *SW1876* – *SW225* – *SW1030* (log L -201.9) (Figure 3.4 on page 37). If *S0283* was omitted from the analysis, the most likely position for *F4bcR* shifted to [*MUC4-8227* – *MUC4gt*] – *S0075*. However, a clear position of *F4bcR* within the parentheses could not be established with a significant LOD score. Nevertheless, the joint analysis presented here confirmed the mapping of *F4bcR* to the *SW207* – *S0075* interval. Earlier studies assigned the position of

F4bcR to *SW207 – SW225* by a 41-marker linkage analysis of the NEH (Jørgensen *et al.*, 2003) and a 17-marker linkage analysis of 200 pigs of the SEH (Python *et al.*, 2005). That range would cover a 9.9 cM region in the map presented here, which has now been reduced to a 5.7 cM region. In Joller *et al.* (2006), with slightly fewer genotyping data, the most probable position for *F4bcR* was assigned to the *SW207 – S0283* interval.

Further strong evidence for the current order is given by the locations of the markers on the physical map (Table 3.3 on page 38). The physical order of the marker sequences inferred from the BAC contig map (Humphray *et al.* (2007); `http://pre.ensembl.org/Sus_scrofa_map/Info/Index`) agrees with the order of the markers on the linkage map. On the fingerprint map, the region for *F4bcR* was narrowed down from 26 Mb to 14 Mb. The order is also supported by the Nordic.2 map (Marklund *et al.*, 1996) and USDA MARC map (Rohrer *et al.*, 1996).

The g.8227C>G polymorphism and the microsatellite marker *HSA125gt* could be inserted into the map. The microsatellite marker *MUC4gt* could not be assigned to a single position on the map. The two new markers *HSA125gt* and *MUC4gt* were highly informative and mapped close to *F4bcR*. Nevertheless, they did not allow us to narrow down the candidate region. Further refinement of the receptor position using the described family material and linkage analysis would be difficult due to the limited number of meioses surveyed, but analysis of haplotypes in the founder animals in the candidate region could prove successful. Although the method for phenotyping used by the Nordic group was slightly different from our method, the phenotypic results are comparable and can be used in a combined linkage analysis. As discussed in subsection 4.1.2, F4ac adhesion to brush borders was diagnosed unambiguously in most cases.

In addition, the assignment of *S0283* to the interval *SW207 – S0075*, as proposed by Jørgensen *et al.* (2003), was confirmed. In an earlier analysis with part of the SEH, *S0283* proposed to be distal to *S0075 – SW225*, due to a putative double recombination in one pig (pig/lab no. 836/1491 in Python *et al.*, 2005). However, *F4bcR* was outside of this chromosomal segment and was therefore not affected by these recombinations.

Further microsatellite markers were analysed in the Swiss pigs, but not in the Nordic pigs and the data therefore were not included in the joint linkage analysis. However, linkage of our data mapped marker *S0068* next to *SW207*, and the two markers *SW2007* and *SWR1627* next to *SW1876*. The marker *SW698* mapped next to *SW1030*, and *SW520* mapped between *SW1030* and *SW398*. The current order of the markers is given by the locations on the physical map inferred from the BAC contig map, and agrees with the order of the markers on the linkage map.

Additional microsatellite markers analysed in four SEH litters comprising 52 pigs could not narrow down the region for *F4bcR*. The six markers *UMNp1062, UMNp1226, UMNp1298, UMNp1341, UMNp1197* and *UMNp884* showed only two alleles in the four litters. The three markers *UMNp894*, *UMNp1239* and *UMNp1320*

were not polymorphic in the same litters. Comparing the F4ac phenotype to the marker alleles by eye, the markers were not obviously linked to F4ac adhesion or non-adhesion. Therefore, and due to lacking information about the physical position of the markers on SSC13, markers were not used for further linkage analysis. However, recent BLAST search on the genome data from the Swine Genome Sequencing Consortium on the Sanger webpage revealed the physical position of *UMNp884* in the interval between *S0283* and *S0075*, on clone CU861607.

With the current progress in swine genome sequencing, it will be possible to search for new microsatellites and for SNPs based on the swine genome sequences. In combination with our material, this will allow us to considerably refine the region for the *F4bcR* and finally to identify the causal mutation.

4.2.1. Sequence variants in candidate genes

MUC4

The intronic *MUC4* DQ848681:g.8227C>G polymorphism proved to be a good marker for *F4bcR*. Of 331 SEH pigs and 78 SPS pigs, we found only 4 SEH pigs (1%) and 6 SPS pigs (8%) that were discordant between the g.8227 genotype and the F4ac phenotype. The intronic g.7947A>G polymorphism was of the same genotype as g.8227C>G in 180 genotyped pigs consisting of 78 SPS offspring, 48 SEH pigs of four resistant x heterozygous susceptible litters and 56 additional SEH pigs (Table 3.7 on page 52). The intronic g.6262G>A polymorphism was discordant to the phenotype in one more pig than the g.7947A>G and the g.8227C>G polymorphisms (footnote *g* in Table 3.7). Therefore, the g.7947A>G and the g.8227C>G polymorphisms could be equally reliable for prediction of the F4ac genotype.

Sequencing of 25 pigs in the region g.6168–6887 revealed two additional SNPs with reliability equal to that of the g.8227C>G and g.7947A>G polymorphisms: the g.6317G>A and g.6321G>C polymorphisms in intron 5 (Table 3.8 on page 53). However, the g.6308G>T polymorphism showed the highest correspondence between genotype and F4ac phenotype of the 25 pigs. This polymorphism was in agreement with the phenotype in three more pigs (18 of 25) than the g.8227C>G polymorphism (15 of 25). Other intronic SNPs between g.6609–6862 were more discordant to the *F4acR* genotype.

Searching the *MUC4* gene for potential regions of micro-RNA sequences by special algorithms revealed the two regions g.5965–6065 and g.6260–6360 as potential regions for micro-RNA (Malik Yousef, personal communication). As the region g.6260–6360 contains several SNPs that are comparable with the g.8227C>G polymorphism, further investigation in this emerging area of research would be interesting.

TNK2

A total of 122 sequence variants were found in the genomic sequence of *TNK2* of two resistant and two homozygous susceptible pigs (Table 3.4 on page 44ff). The genotype of nine sequence variants coincided with the F4ac phenotype in the four sequenced pigs. Two of these sequence variants were SNPs in exon sequences (FN393558:g.7075C>A, g.11142G>A) and seven were SNPs in intron sequences (g.7717C>T; g.8998G>A, g.9374G>C, g.10331G>A, g.10396G>A, g.12649G>C, g.12723T>C). The two exon SNPs and the intron g.7717C>T polymorphism coincided with the F4ac phenotype in most of the 122 SEH and 78 SPS genotyped pigs (Table 3.7). The genotype of the g.11142G>A polymorphism did not coincide with the F4ac phenotype in 8.5% of the 122 SEH and 78 SPS pigs. The g.7717C>T and the g.7075C>A polymorphism genotyped in these pigs did not coincide with the phenotype in 11.1% and 11.7%. As the genotyped pigs represent a selection, the discordance values may not represent the values in the population.

When taking only the 78 SPS pigs, 5% of the pigs were discordant with the F4ac phenotype in the g.11142G>A polymorphism, 12% and 13%, respectively of the pigs were discordant in the g.7717C>T and the g.7075C>A polymorphism. However, the results show that none of the analysed polymorphisms in *TNK2* is causative for the *F4bcR*. By comparing the SNPs in our pigs to SNPs of the Nordic family, the two exon SNPs (g.7075C>A and g.11142G>A) and two intron SNPs (g.7717C>T; g.8998G>A) remained, whose genotypes coincided best with the phenotypes.

Eighteen of the sequence variants were found in exons and four of these (g.10622 C>A, g.11008G>A, g.11585G>A, g.11684G>C) led to changes in amino acids. The g.10622C>A mutation alters p.Pro543His, g.11008G>A alters p.Val673Met, g.11585G>A alters p.Arg865His and g.11684G>C alters p.Ser989Thr. In the four sequenced pigs, none of these four SNPs coincided with the F4ac phenotype, and the SNPs only occurred either in one susceptible or in one resistant pig. Furthermore, the relevance of these SNPs for expression of *TNK2* remains unclear in the pig, as we did not do any transcription experiments. It is remarkable that 79% of the polymorphisms occurred in the one pig with lab no. 1170 (FN393559). At least 40 of these sequence variants also occurred in sequenced pigs of the NEH, therefore these sequence variants are probably not artefacts.

ST6GAL1, *CLDN1* and *C3orf21*

No sequence variants were found in the partially sequenced cDNA and DNA of two F4ac resistant and two homozygous susceptible pigs in the three candidate genes *ST6GAL1* (cDNA of exon 4 to exon 8, FN392680), *CLDN1* (cDNA of exon 1 to exon 4, FM205928) and *C3orf21* (DNA of exon 4, FN392681). However, these genes cannot be excluded as candidate genes, as polymorphisms in intronic sequences may carry a mutation closely associated to the *F4bcR*.

4.2.2. Discordance between *F4bcR* and *MUC4* g.8227C>G

The results of the F4ac phenotyping did not correspond to the *MUC4* g.8227 genotypes in 8% (6 of 78) of the SPS pigs. In SEH pigs, the phenotype was discordant to the g.8227 genotype only in 1% (4 of 329) of the pigs. The small genetic diversity in SEH pigs compared to SPS pigs could be responsible for these differences. Furthermore, a longer storage of small intestine samples from SPS pigs prior to preparation may have led to reduced accuracy of F4 phenotyping results. Indeed, reduced adhesion strength of bacteria to enterocytes and a higher percentage of pigs with F4ac adhesion strength between 15% and 60% were observed in SPS pigs compared to SEH pigs. Despite these differences, the method and working process from slaughtering to phenotyping were established and reduced the risk for mistyping to a minimum. Additionally, adhesion positive and adhesion negative enterocytes were used as controls for bacterial adhesion to enterocytes.

Mistakes during the slaughtering and phenotyping process were very improbable in SPS pigs that showed discordance between the F4ac phenotype and the g.8227 genotype. The six discordant pigs were from five different litters and were slaughtered on four different days, together with other SPS pigs. Two SPS pigs (lab no. 3683 and 3684 in Table 3.8) of one litter slaughtered on two different days (A and day B) were phenotyped as F4ab/F4ac resistant, but genotyped as heterozygous g.8227CG. On day A with six pigs slaughtered, a second pig (lab no. 3656) of a second SPS litter was phenotyped as F4ab/F4ac susceptible, but genotyped as g.8227CC. Theoretically, a mix-up of the intestine samples is possible for these two discordant pigs. A mix-up of the blood was excluded due microsatellite analysis. However, on day B with 15 pigs slaughtered, a second pig (lab no. 3652) of a third SPS litter was phenotyped as F4ab/F4ac resistant, but genotyped as g.8227CG. At least on day B, a mix-up of intestine samples was not possible, on the two discordant pigs were of the same phenotype (resistant) and the same genotype (g.8227CG). Changing of samples during enterocyte preparation or phenotyping can be excluded in the two remaining pigs, which were discordant between F4ab/F4ac phenotype and g.8227C>G genotype, because they were slaughtered on two different days.

Preliminary results of an SEH boar currently used for mating showed a recombination between the g.8227C>G polymorphism and *F4bcR*. The recombination was confirmed by phenotyping and genotyping of progeny that were produced with sows with a confirmed F4ab/F4ac phenotype.

The *MUC4* g.8227C>G polymorphism has been analysed in a few studies reporting a high accordance between this polymorphism and the F4ac phenotype. An accordance of 93% between the F4ac phenotype and the g.8227C>G polymorphism found in SPS pigs, was also reported in a Chinese study with 310 pigs (Li *et al.*, 2008). The F4ac phenotype was not in agreement with the g.8227 genotype in 8% of 84 Landrace breed pigs, in 5% of 149 Large White breed pigs and in 8% of 77 Songliao Black breed pigs. Surprisingly, all Songliao Black breed pigs were of

the g.8227CC genotype but some of them were phenotypically adhesive.

In a recent study, Rasschaert *et al.* (2007) determined that 19 of 63 pigs (30%) were phenotypically susceptible but of the g.8227CC genotype linked to resistance. They determined the number of F4ab and F4ac bacteria on a brush border length of 250 µm (Van den Broeck *et al.*, 1999b) and set the threshold for susceptibility at five adhering bacteria per 250 µm brush border. This threshold corresponds to five bacteria adhering to a total of 50 enterocytes and is far below our threshold of at least five bacteria per single enterocyte. Therefore, a too high percentage of pigs was probably rated susceptible in that study.

Another polymorphism in *MUC4* was analysed by Peng *et al.* (2007). They determined 77 of 748 pigs (10.3%) with an F4ab/F4ac phenotype not corresponding to the g.15581 genotype. Their threshold for susceptibility, based on two bacteria adhering to more than 10% of 20 scored brush borders, could have led again to false positive pigs compared to our phenotyping method.

Supported by these studies, our results clearly indicate that the *MUC4* g.8227C>G, the g.6242G>A and the g.7947A>G polymorphism are not causative for susceptibility and resistance to *E. coli* F4ab/F4ac.

4.3. Inheritance of F4ad susceptibility

4.3.1. Ambiguous F4ad adhesion

Adhesion of *E. coli* F4ab and F4ac bacteria to enterocytes was unambiguous in most of the 1569 SEH pigs of adhesion phenotypes A and B (subsection 4.1.2). On the other hand, *E. coli* F4ad were, on average, less adhesive to enterocytes. Adhesion strength of *E. coli* F4ad to enterocytes of SEH pigs was only around 60% and with a SD of >30% in phenotypes A and C, and only 42±31% in phenotype D (Table 3.1 on page 33). Over 28% of the SEH pigs had between >2.5% and 60% adhesive enterocytes, and still 16% SEH pigs had between >15% and 60% adhesive enterocytes (Figure 3.2). A weaker F4ad adhesion to enterocytes was reported as well by Cox and Houvenaghel (1993), who found fewer F4ad bacteria adhering to intestinal villous brush borders in 33 four to five weeks old pigs, compared to F4ab and F4ac adhesion. Rapacz and Hasler-Rapacz (1986) not only observed a lower number of F4ad bacteria per brush border, but also considerable differences in the number of bacteria per brush border. These results additionally support our findings.

In this study, we observed a wide range of F4ad adhesion from 0% to 100% not only in pigs within the litters, but also between litters of repeated matings. Six of the matings showed significantly different adhesion strengths between the repeated litters. For example, the mating 194B x 180B (Figure 3.9 on page 55) was performed six times and produced almost exclusively resistant phenotype offspring (40 of 41)

in four litters (litters 65, 77, 97, 107), but in two litters (litters 51 and 90), 4 of 13 (31%) and 7 of 11 pigs (64%) were of the adhesive phenotype (>60% of adhesive enterocytes). The mating 194B x 183B was performed four times and produced exclusively resistant offspring in one litter (litter 98). Several pigs with adhesion strength between 15% and 60% adhesive enterocytes were found in the other three litters, and in one litter (litter 85) a pig showed 100% of adhesive enterocytes. In the other four matings, almost all pigs of one litter were phenotyped F4ad resistant, while in the second litter, three pigs (litter 132 of mating 147*B x 156*B) and one pig (litter 134 of mating 147*B x 239B) were phenotyped highly susceptible. In all pigs of these six F4ac resistant x heterozygous susceptible matings, F4ac adhesion was unambiguous.

To explain these adhesion differences between litters, we tried to measure the influence of intestinal contents and of the examiner on the adhesion results. To investigate the influence of contents on the F4ad adhesion, we compared the bacterial adhesion to enterocytes from segments without contents and segments with contents. We often observed a better quality of enterocytes from intestinal segments without contents. Nevertheless, no statistically significant influence of intestinal contents on adhesion strength was found in 215 phenotyped pigs (two sided t-test of the adhesion differences, p=0.473, SD=0.215). Similar to our observations, a negative influence of intestinal contents on adhesion has been reported by Chandler *et al.* (1994). They found reduced F4ac binding activity in ELISA when scrapings were exposed to intestinal contents.

Two adjacent segments without contents and two adjacent segments with contents of 59 pigs were made anonymous prior to phenotyping (Table 3.10 on page 57) to measure the influence of the examiner. In 36 of 59 pigs (61%), all four ratings were in agreement, using a threshold for susceptibility of 2.5% of adhesive enterocytes. In 10 of 59 pigs (17%), only the two ratings of the adjacent segments were in agreement, but the ratings of the segments without contents and with contents were not. In the remaining 13 pigs (22%), not even the ratings of adjacent segments were in agreement. Despite the ambiguous ratings, mean adhesion strengths of segments without contents and with contents were not significantly different in the 59 pigs (Mann-Whitney p=0.825, Kolmogorov-Smirnov p=0.985). The low percentage of 61% for agreement for all four ratings showed that the F4ad rating is much more sensitive to the influence of the intestine and of the examiner than are F4ab and F4ac adhesion. For a better control of F4ad adhesion, we re-phenotyped conserved enterocytes with known phenotypes from earlier analysed adhesion positive and adhesion negative pigs. At this point, the ratings of the controls were always as they were expected. A more objective method for quantitative adhesion of F4 is described by Verdonck *et al.* (2004b) who measured adhesion of isolated F4 fimbriae to enterocytes using surface plasmon resonance.

4.3.2. Application of an alternative F4ad threshold

The variation in F4ad adhesion strength within and between litters questioned the actual threshold of 2.5%. Furthermore, the high number of pigs with F4ad adhesion strength around 2.5% made it difficult to classify pigs as resistant or susceptible. Therefore, we applied an alternative threshold for strong *E. coli* F4ad adhesion of 60% of adhesive enterocytes. The percentage of adhesive enterocytes of 154 SEH pigs was compared with the number of adhering bacteria per brush border (Figure 3.12 on page 60). The data formed two groups: a group of 110 pigs with <60% of adhesive enterocytes and less than eight bacteria per brush border, and a second group of 44 pigs with >60% of adhesive enterocytes and more than eight bacteria per brush border.

The threshold of 60% for F4ad adhesion corresponds with the threshold proposed by Hu *et al.* (1993). Based on segregation studies with 368 pigs, Hu *et al.* proposed a two receptor model consisting of a permanent high affinity receptor (*F4adH*) and a low affinity receptor (*F4adL*), whose expression is terminated by the age of 16 weeks. A threshold of eight bacteria per enterocyte divided the pigs into an F4adL phenotype with a mean of 4.95±1.32 bacteria per brush border and an F4adH phenotype with 11.89±1.71 bacteria per brush border.

These results are similar to our results: We counted a mean of 2.4±2.6 bacteria per brush border for the 44 pigs with less than 60% adhesion, and a mean of 12.7±3.7 bacteria for pigs with more than 60% adhesion. Further support for a threshold of 60% is shown in Figure 3.11 (page 59) with the cumulative number of pigs rated resistant, depending on the threshold. In this figure, the slowest gradient of the curve was between a threshold of 45% and 60%.

A threshold of 60% would considerably reduce the number of dissenting ratings of adjoining segments with or without contents. The ratings of all four segments would be in agreement in 83% (49 of 59) of the pigs, the ratings of at least the two adjoining segments would be in agreement in six pigs. Only of four pigs, the rating of adjoining segments would not coincide.

4.3.3. Genetic impact on F4ad susceptibility

According to the 60% threshold for susceptibility, phenotyped parent pigs were divided into three classes according their F4ad adhesion strength. The resistant (r) class contained pigs with <5% of adhesive enterocytes, the low susceptible (l) class contained pigs with 5% to 60% of adhesive enterocytes, and the high susceptible (h) class contained pigs with >60% of adhesive enterocytes. Phenotyping of 1166 SEH offspring of the resulting six mating combinations revealed a wide range for F4ad adhesion strength, but clearly showed a rising percentage of adhesive enterocytes from resistant x resistant (16.1%) to high x high matings (53.7%) (Figure 3.13 on page 61). Furthermore, offspring from high x high matings had a F4ad adhesion

strength significantly different from offspring of the other mating combinations. These results indicate that genetic factors influence the F4ad adhesion strength.

Nevertheless, 28 of 98 pigs from high x high matings had ≤2.5% F4ad adhesion, and assuming a threshold of 60%, even 46 pigs from nine high x high matings were phenotyped resistant. Additionally, 15 of 137 pigs from five resistant x resistant matings were highly susceptible.

In both cases, the susceptible offspring of parents with weak adhesion (≤10 bacteria per brush border), and the offspring with weak adhesion of susceptible parents have been reported by Rapacz and Hasler-Rapacz (1986). Bijlsma and Bouw (1987) also reported F4ad adhesion positive offspring from matings of resistant parents. Python *et al.* (2005) suggested a dominant inheritance of the *F4adR*. However, our results are difficult to explain with this model. On the contrary, the high proportion of resistant pigs from susceptible matings indicates a recessive expression of an F4ad receptor. As far as is known, receptors serving as adhesive factors for bacteria are expressed dominantly and a recessive expression seems rather improbable.

4.3.4. Age related expression of F4ad receptor

An explanation for susceptible offspring of resistant x resistant matings is given by Hu *et al.* (1993) with *F4adL* being expressed in pigs younger than 16 weeks. The age dependant receptor could explain the weak F4ad adhesion of 122 (of 137) SEH pigs from resistant x resistant matings. However, an F4adL phenotype could not explain the high adhesive F4ad phenotype of six pigs from resistant x resistant matings that were older than 100 days.

An age related F4ad expression questions the slaughter age of two months of the pigs. It is unknown how an expression of the receptor is age related. A decrease in susceptibility of pigs to *E. coli* F6 by age has been reported by Dean (1990). This decrease was associated with an increase in the F6 receptor in mucus of the intestinal lumen that inhibits adhesion and colonisation by F6 in older pigs. In contrast, Nagy *et al.* (1992) reported an increase in F5 and F6 susceptibility in pigs until the 21^{st} day.

Unexpected F4ad adhesion results were found in biopsies taken of 10 SEH pigs at two time points. Four pigs of two litters were adhesion negative or had only weakly adhesive enterocytes (0% and 20%) when resected at the age of four to six weeks, but the pigs had strongly adhesive enterocytes when resected at the age of six months (Table 3.12 on page 63). The other six pigs were either adhesion negative or adhesion positive in both biopsies. These adhesion results are in contrast to the assumption of a decreasing receptor by age proposed by Hu *et al.* (1993).

In the meantime, one pig (lab no. 1399) was slaughtered and adhesion strength was again confusing: a segment (with few intestinal contents) 0 m distal the Arteria mesenterica cranialis did not show F4ad adhesion, but the segment (free of contents) taken 5 m distal showed 18% adhesive enterocytes and the segment (free of

contents) taken 10 m distal the Arteria mesenterica cranialis showed 58% adhesive enterocytes. Notably, F4ab and F4ac adhesion was between 95% and 100% in all segments and the cells were of good and comparable quality.

4.3.5. Other influences on F4ad adhesion

The variation of F4ad adhesion strength in different small intestine segments free of contents within pigs was not expected. For the enterocyte adhesion test, a small jejunal segment without contents was taken between 3.5 and 7.5 m distal the Arteria mesenterica cranialis. This location has been reported by different publications to be accurate for F4 adhesion tests. Chandler *et al.* (1994) and Mynott *et al.* (1996) reported that the middle part of the small intestine showed most distinct F4ac adhesion after comparing adhesion of different small intestine segments by ELISA. The position of the most distinct adhesion was confirmed by Cox and Houvenaghel (1993), who compared bacterial adhesion to duodenal, jejunal and ileal segments of 33 pigs of four to five weeks of age. They found the highest number of bacteria of all three *E. coli* F4 variants to adhere to brush borders isolated from the cranial or caudal jejunum.

However, a position related expression of a *F4adL* would explain the confusing results of resected pigs, as position of the biopsies in the small intestine could not be determined exactly. Additionally, the F4ad adhesion results of many pigs could be explained with a position dependent *F4adL* expression.

In further studies, phenotyping results from offspring of pigs with phenotypes determined by biopsies could give more information about the inheritance of the F4ad receptor. Additional information about an age related expression of the F4ad receptor will be obtained by phenotyping pigs, one part of the litters slaughtered at the age of about four weeks, and the other part at the age of about six months. Further findings of a position related expression of the F4ad receptor could be obtained by phenotyping different positions of the small intestine from the same pigs.

4.4. Conclusions and perspectives

The joint linkage analysis of Swiss and Nordic data could refine the region for the *F4bcR* on SSC13 from 28 mb to 14 mb. However, use of these markers did not further narrow down the region for the *F4bcR* and, therefore, the causative mutation for the *F4bcR* remains unknown. A further refinement of the candidate region by linkage using these data would be difficult due to the low number of pigs with a recombination between *MUC4* and *F4bcR*, and limitations of the software program. Haplotype analysis of additional, unrelated resistant and homozygous susceptible founder pigs with confirmed F4 genotypes could prove more successful

to determine the flanking regions for the *F4bcR* and to find new sequence variants.

The analysed sequence variants of *MUC4* and *TNK2* were not completely linked to the phenotype, and none of these sequence variants is causative for the *F4bcR*. Nevertheless, the *MUC4* g.8227C>G and g.7947A>G polymorphisms were highly associated with the F4ac phenotype and can be used as diagnostic test for selection of F4ac resistant pigs.

Sequence information in the candidate region that is currently being generated, and next generation sequencing (Solid, Solexa) and genotyping methods (SNP-chip, Pyrosequencing) will make it possible to identify sequence variants much more efficiently in the future. More SNPs evenly distributed in the candidate region are necessary to better characterise the susceptible and resistant haplotypes.

The SEH boar showing a recombination between the *MUC4* g.8227C>G polymorphism and *F4bcR*, as well as recombinant offspring of this boar, will provide an important source to considerably refine the candidate region for *F4bcR*. Sequence analysis of this F4ac homozygous susceptible boar and closely related resistant pigs will allow us to exclude haplotypes that do not contain the causative mutation.

A genetic influence on *E. coli* F4ad adhesion in the small intestine of pigs was demonstrated in this thesis. However, other factors such as age of the pigs and sampling position within the small intestine seem to have an impact on adhesion strength. Intestinal contents could influence adhesion as well, although no statistically significant influence could be shown in the analysed material.

Analysis of existing data considering these factors and analysis of new data from offspring of resected pigs will provide more information about the influence of such different factors. Phenotyping data of resected pigs will further help to prove/disprove the two receptor model of a weak adhesive and a strong adhesive phenotype.

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Appendix

A. Appendix

Media and solutions

Product	Amount	Grade	Article no.	Producer	Supplier
DMSO-Hanks-medium					
according to Bosi *et al.* (2004)					
Hanks' Balanced Salt solution (with sodium bicarbonate)	80 ml	sterile-filtered	H9269	Sigma	Sigma-Aldrich, Buchs
Fetal calf serum	10 ml				
DMSO	30 ml	p.p.a., ACS	41640	Fluka	Sigma-Aldrich, Buchs
Glycerol	30 ml	≥98.0%	49780	Fluka	Sigma-Aldrich, Buchs
BSA	1 g	≥96.0%	A9647	Fluka	Sigma-Aldrich, Buchs
DNA loading dye 6 x					
Xylene Cyanol FF	0.25% (v/v)		95600	Fluka	Sigma-Aldrich, Buchs
Sucrose	40% (v/v)	≥99.0%	84100	Fluka	Sigma-Aldrich, Buchs
EDTA buffer					
NaCl	96 mM	puriss. p.a.	71380	Fluka	Sigma-Aldrich, Buchs
Na_2HPO_4	5.5 mM	puriss. p.a.	71640	Fluka	Sigma-Aldrich, Buchs
		puriss. p.a.	6589	Merck	Merck, Zug
KH_2PO_4	8 mM	≥99.5%	60219	Fluka	Sigma-Aldrich, Buchs
		≥99.5%	P3535	Sigma	Sigma-Aldrich, Buchs
KCl	1.5 mM	puriss. p.a.	60130	Merck	Merck, Zug
EDTA	10 mM	molecular biology	E5134	Sigma	Sigma-Aldrich, Buchs
adjust to pH6.8 with 1 M Na_2CO_3			71640	Sigma	Sigma-Aldrich, Buchs
autoclave					
Formaldehyde agarose gel buffer 10 x					
3-[N-Morpholino]propanesulfonic acid (MOPS)	200 mM		69949	Fluka	Sigma-Aldrich, Buchs
Sodium acetate (trihydrate)	50 mM	p.a.	106267	Merck	Merck, Zug
EDTA	10 mM	molecular biology	E5134	Sigma	Sigma-Aldrich, Buchs
adjust to pH7.0 with NaOH					

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Product	Amount	Grade	Article no.	Producer	Supplier
FA gel running buffer 1 x					
FA gel buffer 10 x	1 x				
Formaldehyde solution complete with RNase-free H_2O	246 mM	36%	47630	Fluka	Sigma-Aldrich, Buchs
Formamide loading dye					
Formamide	4 x	p.p.a., ACS	47670	Fluka	Sigma-Aldrich, Buchs
Loading buffer 5 x	1 x			AB	Applied Biosystems, Rotkreuz
Lysis buffer					
Sucrose	320 mM	≥99.0%	84100	Fluka	Sigma-Aldrich, Buchs
Tris-HCl pH7.5	10 mM				
$MgCl_2 \cdot 6H_2O$	5 mM	puriss. p.a.	63072	Fluka	Sigma-Aldrich, Buchs
Triton X-100	1%				
autoclave					
Mannose buffer 2%					
D(+)-Mannose	2%	≥99.0%	63582	Fluka	Sigma-Aldrich, Buchs
ad PBS immediately before use					
PBS – Phosphate buffered saline					
NaCl	145 mM	puriss. p.a.	71380	Fluka	Sigma-Aldrich, Buchs
Na_2HPO_4	9 mM	puriss. p.a.	71640	Fluka	Sigma-Aldrich, Buchs
		puriss. p.a.	6589	Merck	Merck, Zug
NaH_2PO_4	1.3 mM	reinst			Bender und Hobein, Zürich
pH7.6, autoclave					
PBS-formaldehyde					
formaldehyde solution complete with PBS	2% (v/v)	36%	47630	Fluka	Sigma-Aldrich, Buchs
PCR turbo buffer					
KCl	50 mM	puriss. p.a.	60130	Merck	Merck AG, Zug
Tris-HCl pH8.3	10 mM				
Gelatin from porcine skin	0.1 mg/ml	microbiology	48722	Fluka	Sigma-Aldrich, Buchs
Nonidet P-40	0.45% (v/v)		74385	Fluka	Sigma-Aldrich, Buchs
Tween 20	0.45% (v/v)		93773	Fluka	Sigma-Aldrich, Buchs
autoclave					
Polyacrylamide gel 4.5%					
Urea	18 ml	≥99.5%	02493	Sigma	Sigma-Aldrich, Buchs
TBE buffer 10 x	5 ml				
Acrylamide:bis-acrylamide (29:1) solution	7.5 ml	30%	1610121	Biorad	Biorad, Reinach
TEMED	15 ul	99%	0761	Amresco	Bioconcept, Allschwil
Ammonium persulfate 10% solution	350 ul	p.p.a., ACS	9915	Fluka	Sigma-Aldrich, Buchs

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Product	Amount	Grade	Article no.	Producer	Supplier
Proteinase K					
Proteinase K from Tritrachium album	20 mg/ml	≥30 U/mg	P6556	Sigma	Sigma-Aldrich, Buchs
ddH_2O ad 1 ml					
RNA loading buffer 5 x					
aqueous bromphenol blue solution	saturated		18030	Fluka	Sigma-Aldrich, Buchs
EDTA	4 mM	molecular biology	E5134	Sigma	Sigma-Aldrich, Buchs
Formaldehyde solution	886 mM	36%	47630	Fluka	Sigma-Aldrich, Buchs
Glycerol	2% (v/v)	≥98.0%	49780	Fluka	Sigma-Aldrich, Buchs
Formamide	31% (v/v)	p.p.a., ACS	47670	Fluka	Sigma-Aldrich, Buchs
FA gel buffer 10 x	4 x				
complete with RNase-free H_2O					
TBE Buffer 10 x					
Tris(hydroxymethyle) aminomethane	890 mM	for Microbiology	200923	Biosolve	Biosolve, Valkenswaard, NL
H_3BO_3	890 mM	puriss. p.a.	31146	Sigma	Sigma-Aldrich, Buchs
EDTA	20 mM	molecular biology	E5134	Sigma	Sigma-Aldrich, Buchs
pH8.3, autoclave					
Tris-HCl pH**7.5**					
Tris(hydroxymethyle) aminomethane	200 mM	for Microbiology	200923	Biosolve	Biosolve, Valkenswaard, NL
pH7.5, autoclave					

Chemicals

Product	Article no.	Producer	Supplier
λ-phage DNA 500 µg/ml	27-4118-07	GE	GE Healthcare, Glattbrugg
100 bp ladder	27-4007-01	GE	GE Healthcare, Glattbrugg
100 bp ladder directload	D3687	Sigma	Sigma-Aldrich, Buchs
2-mercaptoethanol	63689	Fluka	Sigma-Aldrich, Buchs
50 bp ladder	27-4005-01	GE	GE Healthcare, Glattbrugg
50 bp ladder directload	D3812	Sigma	Sigma-Aldrich, Buchs
Agarose low EEO	A5093	Sigma	Sigma-Aldrich, Buchs
*Alu*I, 10 U/ul	ER0011	Fermentas	Labforce, Nunningen
BigDye sequencing mix		AB	Applied Biosystems, Rotkreuz
*BseD*I, 10 U/ul	ER1081	Fermentas	Labforce, Nunningen
Columbia sheep blood agar	PB5008A	Oxoid	Oxoid, Basel
DNase, RNase-free	79254	Qiagen	Qiagen, Hombrechtikon
dNTPs, 100 mM	DNTP100	Sigma	Sigma-Aldrich, Buchs
dNTPs, 100 mM		GE	GE Healthcare, Glattbrugg
Ethanol (EtOH)	100983	Merck	Merck, Zug
Etidium bromide (EtBr)			
Formamide	47670	Fluka	Sigma-Aldrich, Buchs

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Product	Article no.	Producer	Supplier
Genescan 350 TAMRA or ROX size standard		AB	Applied Biosystems, Rotkreuz
HalfBD		Genetix	Genetix, München, D
*Hin1*II, 5 U/ul	ER1831	Fermentas	Labforce, Nunningen
*HpyF3*I, 10 U/ul	ER1881	Fermentas	Labforce, Nunningen
Reverse Transcription System	A3500	Promega	Promega, Dübendorf
RNeasy Midi kit	75144	Qiagen	Qiagen, Hombrechtikon
Sodium acetate	71190	Fluka	Sigma-Aldrich, Buchs
Taq DNA polymerase recombinant, 5 U/ul	D1806	Sigma	Sigma-Aldrich, Buchs
Taq JumpStart DNA polymerase, 2.5 U/ul	D9307	Sigma	Sigma-Aldrich, Buchs
*Taq*I, 10 U/ul		GE	GE Healthcare, Glattbrugg
Trypticase soy broth (TSB)	211768	Becton Dickinson	Chemie Brunschwig, Basel
*Xba*I, 10 U/ul	10674257001	Roche	Roche, Rotkreuz
*Xba*I, 10 U/ul	ER0681	Fermentas	Labforce, Nunningen

Labware

Product	Article no.	Producer	Supplier
6-well macroplates	657 102	Greiner	Huber und Co., Reinach
6-well macroplates	5530505	Orange	Milian SA, Meyrin
Blood tubes 10 ml with EDTA Vacuette	455036	Greiner	Greiner Bio-one, St. Gallen
Blood tubes 10 ml with EDTA Venosafe	VF-109SDK	Terumo	Cosanum AG, Schlieren
Centrifuge tubes 15 ml	FA-352096	Falcon	Milian SA, Meyrin
Centrifuge tubes 50 ml	91050	TPP	Omnilab AG, Mettmenstetten
Cover glass 18 x 18 mm		Menzel	Uni lab shop
Cryotubes 1.8 ml	363401	Nunc	Milian SA, Meyrin
Glass slide frosted ends 76 x 26 mm		Menzel	Uni lab shop
Micro tubes 1.5 ml	96.7514.2.01	Treff	Milian SA, Meyrin
Montage PCR centrifugal filter devices	UFC7PC250	Millipore	Milian SA, Meyrin
PCR 8-strip tube	3230-00	SSI	Bioconcept, Allschwil
PCR plates 96 wells	PCR-96-FLT-C	Axygen	Brunschwig, Basel
PCR plates 96 wells	ABI0600	ABI	Bioconcept, Allschwil
PCR plates 96 wells low profile	AB-0700	Thermo	Milian SA, Meyrin
PCR tubes 0.2 ml		SSI	Bioconcept, Allschwil
Tips 10 µl	T-300-L-R	Axygen	Brunschwig, Basel
Tips 30 µl	MX-7600	Matrix	Milian SA, Meyrin
Tips 200 µl	96.1701.4.02	Treff	Uni lab shop
Tips 1000 µl	96.1702.6.02	Treff	Uni lab shop
Wide-necked bottles PVC 100 ml	3851	Semadeni	Semadeni, Ostermundigen