Genetic host determinants associated with the adhesion of E. coli with fimbriae F4 in swine

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SUMMARY

The ability to colonize the intestine is a common feature of pathogenic, as well as of non-pathogenic bacteria. After adhesion of enterotoxigenic *Escherichia coli* (ETEC) to the surface of the small intestine by means of fimbriae, ETEC bacteria produce toxins that cause a secretory diarrhea. *E. coli* diarrhea represents the first source of mortality in post-weaning pigs. The bacteria expressing fimbriae F4 (K88) frequently colonize the intestine of neonatal and weaned pigs. The susceptibility to *E. coli* F4ac adhesion is dominantly inherited in the host. Susceptibility is conferred by specific receptors on the brush borders of enterocytes in the small intestine. In homozygous resistant pigs, no adhesion of F4ac fimbriae is observed. Three antigenic variants were identified: F4ab, F4ac and F4ad. In most cases, the variant F4ac was isolated from piglets affected by ETEC diarrhea.

We mapped the locus for the receptor F4acR (*F4acR*) to the band q41 of the porcine chromosome 13. The multipoint linkage analysis of 202 pigs with 17 microsatellites significantly localized *F4acR* inside of the interval *Sw*207-S0283, spanning 1.6 centimorgan. *F4acR* is localized in this interval near S0075. No significant linkage disequilibrium between any allele of the microsatellites around *F4acR* and the alleles of the *F4acR* locus was found. We examined four genes as candidates for the inheritance of *F4acR*: the transferrin receptor gene (*TFRC*) and three genes members of the galactosyltransferase family (*B3GnT5*, *B4GALT4* and *B3GALT3*). *TFRC* is involved in iron acquisition and the other three genes code for enzymes transferring the addition of galactose to various substrates. Galactosyl residue present in glycoprotein of brush borders and possibly other residues are necessary in the F4ac adhesion. Comparison between the sequences from resistant and homozygous susceptible pigs did not reveal any causative single nucleotide polymorphism in the four genes. We found two silent mutations at the positions 295 (C/T) and 313 (T/C) in *B3GALT3*. Using the somatic cell hybrid panel *B3GnT5* and *B3GALT3* were assigned to the chromosomal region 13q23 → q41. After mapping *F4acR*, the gene *B3GALT3* was excluded as a candidate due to its position on the chromosome. In the northern blot analyses for *TFRC*, *B3GnT5* and *B4GALT4* on RNA extracted from resistant and susceptible porcine intestine scrapings, the cDNA probes did not produce any signal, although these genes were isolated from a small intestine cDNA library.
We observed six phenotypes for the three fimbrial variants F4ab, F4ac and F4ad. The missing phenotypes F4abR⁻ / F4acR⁺ / F4adR⁺ and F4abR⁻ / F4acR⁺ / F4adR⁻ indicated that pigs susceptible to F4ac were always susceptible to F4ab. Furthermore, two types of adhesion for F4ab and F4ad bacteria were observed: a weak and a strong adhesion. The weak adhesion was characterized by enterocytes with few adhering bacteria. The weak receptor F4abR designated F4abR⁻ was present only in pigs devoid of the receptor F4acR (F4abR⁺/F4acR⁻). In contrast, in pigs with the phenotype F4abR⁺ / F4acR⁻, F4ab bacteria adhered to the majority of enterocytes. F4abR⁻ constitutes a frequently observed phenotype whose inheritance must be clarified by mating parents resistant to F4ac. Strong adhesion of F4ab and F4ac bacteria is influenced by the same receptor. The number of F4ad bacteria that adhered to enterocytes was very variable in the adhesion test. Aside from the fully resistant and susceptible phenotypes, some pigs belonged to an intermediate or weak phenotype with some to many enterocytes devoid of bacteria. Moreover, expression of F4adR was independent of age. Eight-week-old pigs as well as adult pigs showed adhesion to F4ad. Observed ratios of susceptible to resistant offspring in 25 litters from informative families indicated a dominant inheritance of F4adR, although the number of susceptible pigs was smaller than expected. We hypothesized that the microscopical adhesion test does not identify all pigs susceptible to F4ad. No linkage was observed between F4adR and the microsatellites on chromosomes 4, 11 and 13.

Marker assisted selection of pigs resistant to F4ac is still not feasible, but offspring phenotyping and haplotype determination of microsatellites close to the receptor locus allow to deduce the F4acR genotype in parents.
ZUSAMMENFASSUNG


Wir kartierten den Locus für den Rezeptor F4acR (F4acR) auf Band q41 des Chromosoms 13. Die Mehrpunkt-Kopplungsanalyse mit 17 Mikrosatelliten in einer Familie von 202 Schweinen zeigte, dass F-4acR mit grosser Sicherheit innerhalb des 1.6 Centimorgan Intervalls Sw207-S0283 liegt. Der Locus F-4acR liegt nahe dem im Intervall liegenden Mikrosatelliten S0075. Kein Allel der Mikrosatelliten um F-4acR herum war in einem signifikanten Kopplungungleichgewicht mit den Allelen des F4acR Locus. Vier Gene als Kandidaten für die Vererbung von F4acR wurden untersucht: das Transferrinrezeptorgen (TFRC) und drei Gene, die zur Familie der Galaktosyltransferasen (B3GnT5, B4GALT4 und B3GALT3) gehören. TFRC ist für die Aufnahme von Eisen verantwortlich und die drei anderen Gene kodieren Enzyme, die Galaktose auf verschiedene Substrate übertragen. Galaktosylreste in Glycoproteinen vom Bürstensaum und möglicherweise zusätzliche Residuen sind für die F4ac Adhäsion nötig. Der Vergleich der Sequenzen dieser vier Gene bei anfälligen und resistenten Schweine ergab keinen informativen Nukleotid-Polymorphismus. An den Positionen 295 (C/T), beziehungsweise 313 (T/C) in B3GALT3, wurde eine stille Mutation gefunden. B3GnT5 und B3GALT3 wurden mittels somatischen Hybridzelllinien der Chromosomenregion 13q23 → q41 zugewiesen. Nach der Feinkartierung von F-4acR kann das Gen B3GALT3 aufgrund seiner Position auf dem Chromosom als


La colonisation de l’intestin est une aptitude commune aux bactéries pathogènes et non pathogènes. Les *Escherichia coli* entérotoxinogènes (ETEC) s’attachent par les fimbriae à la muqueuse intestinale et produisent des toxines responsables de la diarrhée aqueuse. La diarrhée causée par *E. coli* représente la première cause de mortalité chez les porcs en post-sevrage. Les bactéries exprimant les fimbriae F4 (K88) colonisent fréquemment l’intestin des porcelets nouveau-nés et sevrés. La susceptibilité de l’hôte aux bactéries F4ac est contrôlée par un allèle autosomal dominant conduisant à la formation de récepteurs spécifiques sur les microvillosités (bordures en brosse) des cellules de l’intestin grêle. Aucune adhésion des fimbriae F4ac n’est observable chez des porcs homozygotes résistants. F4ab, F4ac et F4ad sont les trois variantes antigènes connues. Dans la plupart des cas de diarrhée colibacillaire, on a isolé la variante F4ac des porcelets.

Nous avons cartographié le locus pour le récepteur F4acR (F4acR) sur la bande q41 du chromosome 13 porcin. L’analyse de liaison multipoint de 202 porcs, typés avec 17 microsatellites, a permis de localiser significativement F4acR dans l’intervalle Sw207-S0283, à une distance respective de 1.6 centimorgan. Le locus F4acR est proche du microsatellite S0075 situé dans cet intervalle. En outre, aucun allèle des microsatellites proches de F4acR n’est en déséquilibre de liaison avec les allèles du locus F4acR. Nous avons examiné quatre gènes comme candidats pour la transmission de F4acR : le gène récepteur de la transferrine (TFRC) et trois gènes appartenant à la famille des galactosyl-transférases (*B3GnT5, B4GALT4* et *B3GALT3*). TFRC est impliqué dans l’absorption du fer et les trois autres gènes codent pour des enzymes qui catalysent le transfert de galactose sur divers substrats. Le résidu galactosyle, présent dans les glycoprotéines des bordures en brosse, ainsi que peut-être d’autres résidus, sont nécessaires pour l’adhérence au récepteur F4ac. La comparaison des séquences chez des animaux homozygotes susceptibles et résistants n’a pas mis en évidence de polymorphisme informatif dans les quatre gènes. Nous avons séquencé deux mutations silencieuses dans le gène *B3GALT3* aux positions 295 (T/C) et 313 (T/C). Les deux gènes *B3GnT5* et *B3GALT3* ont été assignés à la région chromosomique 13q23 → q41 à l’aide d’une collection d’hybrides de cellules somatiques. La cartographie de F4acR a permis d’exclure le gène *B3GALT3* comme candidat sur la base de sa position sur le
chromosome. Dans les analyses par northern blot des ARN de TFRC, B3GnT5 et B4GALT4 extraits de raclures intestinales de porcs résistants et susceptibles, les sondes d’ADN complémentaire n’ont montré aucun signal, bien que ces gènes aient été isolés à partir d’une banque d’ADN complémentaire de l’intestin grêle.

Nous avons observé six phénotypes pour les trois variantes F4ab, F4ac et F4ad. Les deux phénotypes F4abR⁻ / F4acR⁺ / F4adR⁺ et F4abR⁻ / F4acR⁻ / F4adR⁻, non identifiés, indiquent que les porcs susceptibles à F4ac sont également susceptibles à F4ab. En outre, les bactéries F4ab et F4ad montrent deux types d’adhérence : une adhésion faible et une forte. L’adhésion faible se traduit par un petit nombre de cellules intestinales portant des bactéries. Le récepteur faible F4ab désigné F4abR⁻ est uniquement identifié chez les porcs dépourvus du récepteur F4ac (F4abR⁺ / F4acR⁻) et, à l’inverse, les bactéries F4ab adhèrent la majorité des entérocytes chez les animaux présentant le phénotype F4abR⁺ / F4acR⁺. F4abR⁻ représente un phénotype fréquemment observé dont l’hérédité doit être clarifiée en accouplant des parents résistants à F4ac. La forte adhésion des bactéries F4ab et F4ac est influencée par le même récepteur. Le nombre de bactéries F4ad adhérentes aux entérocytes dans le test d’adhérence a montré une grande variabilité. A part les phénotypes entièrement résistants et susceptibles, certains porcs avaient un phénotype intermédiaire ou faible, caractérisé par des entérocytes adhérents et non adhérents. De plus, l’expression de F4adR est indépendante de l’âge. Les porcs de huit semaines et les adultes pouvaient exhiber une adhérence aux bactéries F4ad. Le ratio, observé dans 25 portées de descendants susceptibles et résistants issus de familles informatives, indique que la transmission de F4adR est dominante, bien que le nombre d’animaux susceptibles était plus petit qu’attendu. Il est possible que le test d’adhérence ne permette pas d’identifier tous les porcs susceptibles à F4ad. F4adR ne présente aucune liaison avec les marqueurs sur les chromosomes 4, 11 et 13.

En conclusion, la sélection assistée par marqueurs de porcs résistants à F4ac n’est actuellement pas réalisable, mais il est possible de déduire le génotype F4acR des parents grâce au phénotypage des descendants et à la détermination de l’haplotype des microsatellites situés près du locus du récepteur.
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ABBREVIATIONS

A, C, G, T  adenine, cytosine, guanine, thymine
bp  base pair
°C  degree Celsius
cDNA  complementary DNA produced from an RNA template
Ci  Curie
Cl⁻  chloride anion
cM  centimorgan
cpm  counts per minute
ddH₂O  double distilled H₂O or ultrapure H₂O
DEPC  diethyl pyrocarbonate
df  degree of freedom
DNA  deoxyribonucleic acid
dNTP(s)  deoxyribonucleotide(s) (= deoxynucleoside triphosphate solutions)
dATP  2'-deoxyadenosine 5'-triphosphate
dCTP  2'-deoxycytidine 5'-triphosphate
dGTP  2'-deoxyguanosine 5'-triphosphate
dTTP  2'-deoxythymidine 5'-triphosphate
E. coli  Escherichia coli (bacteria)
EDTA  ethylenediaminetetraacetic acid
ETEC  enterotoxigenic Escherichia coli
FISH  fluorescence in situ hybridization
F4  fimbrial antigen (former K88) F4 (F4ab, F4ac, F4ad)
g  gram
×g  acceleration of gravity (average)
h  hour
H₂O  water (see ddH₂O)
HSA  Homo sapiens chromosome
IPTG  isopropyl ß-D-thiogalactopyranoside
kb  kilobase
kDa  kiloDalton (protein molecular weight)
M  molarity (= mol/liter)
µg  microgram
min  minute
ml  milliliter
MOPS  3-(N-morpholino)propanesulfonic acid
µl  microliter
mRNA  messenger RNA
NaAc  sodium acetate
NaCl  sodium chloride
NaOH  sodium hydroxide
P  probability
PBS  phosphate buffered saline
PCR  polymerase chain reaction
pfu  plaque forming units
RFLP  restriction fragment length polymorphism
RNA  ribonucleic acid
RNase(s)  ribonuclease(s)
RNasin  RNases inhibitor
rpm  revolutions per minute
rRNA  ribosomal RNA
RT  reverse transcription
SD  standard deviation
SDS  sodium dodecyl sulfate
s  second (time)
SSC  saline-sodium citrate buffer
SSC  Sus scrofa chromosome

E. coli  Escherichia coli (bacteria)
EDTA  ethylenediaminetetraacetic acid
ETEC  enterotoxigenic Escherichia coli
FISH  fluorescence in situ hybridization
F4  fimbrial antigen (former K88) F4 (F4ab, F4ac, F4ad)
g  gram
×g  acceleration of gravity (average)
h  hour
H₂O  water (see ddH₂O)
HSA  Homo sapiens chromosome
IPTG  isopropyl ß-D-thiogalactopyranoside
kb  kilobase
kDa  kiloDalton (protein molecular weight)
M  molarity (= mol/liter)
µg  microgram
min  minute
ml  milliliter
MOPS  3-(N-morpholino)propanesulfonic acid
µl  microliter
mRNA  messenger RNA
NaAc  sodium acetate
NaCl  sodium chloride
NaOH  sodium hydroxide
P  probability
PBS  phosphate buffered saline
PCR  polymerase chain reaction
pfu  plaque forming units
RFLP  restriction fragment length polymorphism
RNA  ribonucleic acid
RNase(s)  ribonuclease(s)
RNasin  RNases inhibitor
rpm  revolutions per minute
rRNA  ribosomal RNA
RT  reverse transcription
SD  standard deviation
SDS  sodium dodecyl sulfate
s  second (time)
SSC  saline-sodium citrate buffer
SSC  Sus scrofa chromosome
<table>
<thead>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TDT</td>
<td>transmission disequilibrium test</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA buffer</td>
</tr>
<tr>
<td>T&lt;sub&gt;a&lt;/sub&gt;</td>
<td>annealing temperature</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>5' UTR</td>
<td>region at the 5' end of a transcript (preceding the initiation codon) that is not translated into a protein</td>
</tr>
<tr>
<td>3' UTR</td>
<td>region at the 3' end of a transcript (following the stop codon) that is not translated into a protein</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
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<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl β-D-galactopyranoside</td>
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I. INTRODUCTION

1.1 Diarrhea

The intestinal tract is colonized by a rich microbial flora. Over 400 bacterial species normally inhabit the intestinal tract and form a part of the ecosystem. The stomach and proximal intestine contain few microorganisms due to the presence of gastric acid. Otherwise, large bacterial populations survive in the large intestine and by a microbial fermentation process, digest cellulose in herbivores. E. coli that are able to induce enteric disease have been classified into categories with diarrheagenic properties explained by their virulence factors, interaction with the intestinal mucosa, and epidemiology. These categories of diarrheagenic E. coli are: enterotoxigenic E. coli (ETEC), enteroaggregative E. coli (EAEC), enteropathogenic E. coli (EPEC), enterohemorrhagic E. coli (EHEC), enteroinvasive E. coli (EIEC) and diffusely adherent E. coli (DAEC) (Nataro and Kaper 1998). Infection of animals or humans with such E. coli may have diarrhea as a consequence. Not only E. coli but also many viruses (rotavirus, calicivirus, astrovirus, adenovirus, etc.) and parasites (protozoa, nematodes) have been causally associated with diarrhea. All diarrheagenic E. coli follow four steps during infection: colonization of intestinal mucosa, evasion of host defenses, multiplication and host damage. The ability to colonize specific mucosal sites is a common feature of diarrheagenic E. coli, whereas nonpathogenic strains proliferate in the contents of the lower digestive tract. Colonizing mechanisms vary in strains: ETEC strains adhere to the surface of the small intestinal mucosa by means of fimbriae; EAEC enhance mucus secretion from the mucosa, with trapping of the bacteria in a biofilm; EPEC cause the dissolution of the microvilli structure and bind closely to the epithelial membrane via the protein intimin; EHEC attachment cause hemorrhage and edema of intestine after microvilli effacement; EIEC strains invade epithelium cells and spread from cell to cell; and DAEC mediate the formation of finger-like projections from the intestinal cell which wrap the bacterium (Nataro and Kaper 1998).

ETEC strain is defined as elaborating at least one member of two groups of enterotoxins: a heat-stable (ST) and a heat-labile enterotoxin (LT). The action of enterotoxins is responsible for the diarrhea. After binding to the host cell membranes,
the toxin is endocytosed and translocated through the cell. LT stimulates Cl⁻ (chloride) secretion from secretory crypt cells and inhibits NaCl absorption by villous tip cells. The increased luminal ion content draws water passively through the paracellular pathway, resulting in secretory diarrhea. ETEC affect both humans and animals. In humans, ETEC are associated with weanling diarrhea among children and traveler’s diarrhea. Fecal contamination of water and food is the principal reason for the high incidence of infection. In pigs, ETEC can express five types of fimbriae: F4 (K88), F5 (K99), F6 (987P), F41 and F18. Of these fimbriae types, F4 and F18 are more frequently associated with disease in weaned pigs. F18 bacteria cause diarrhea and/or edema disease in older and/or weaned pigs, whereas F4 cause diarrhea in neonatal as well as in weaned pigs (Moon et al. 1999). About 40% of swine E. coli isolates carry genes for ETEC and among ETEC, 80% bear F4 and 10% F18 fimbriae (Moon et al. 1999; Kwon et al. 2002). The mortality is higher (increase from 2% to 7%) and the average daily gains are poorer in herds following an outbreak of post-weaning E. coli diarrhea (Amezcua et al. 2002). E. coli diarrhea represents the first source of mortality (39%) in post-weaning pigs, followed by edema disease (26%) (Conzelman 2000). During the suckling period only 8% of piglets die of infectious diseases, whereas the major loss is due to crush (44%).

Fimbrial antigens allow E. coli to adhere to the intestinal mucosal surface despite peristalsis and competition for nutrients with the indigenous flora of the gut. Fimbriae confer the species specificity of the pathogen. ETEC strains expressing F5 fimbriae are pathogen for calves, lambs and pigs, whereas ETEC with F4 fimbriae are able to cause diarrhea only in pigs. Fimbriae are hairlike protein appendages; fimbriae F4 are flexible and very thin with a diameter of 2-3 nm and a length of 0.5-2 μm (Figure 1.1). Several hundreds of fimbriae are peritrichously arranged on the bacterium’s surface (De Graaf and Mooi 1986). The adhesive potential of F4 and F5 is determined by the adhesin being located along the length of the structure. Adhesins are carbohydrate-binding proteins which recognize receptors on microvillous membranes of absorptive host cells (Moon 1997).
Figure 1.1 *E. coli* bacterium with fimbriae F4 Transmission electron micrograph taken after platinum/carbon treatment. The single cell is surrounded by numerous slender, curled fimbriae. (Courtesy P. Wild and E. M. Schraner, Laboratory for electron microscopy, Institutes of Veterinary Anatomy and Virology, University of Zurich).
1.2  **F4 *E. coli***

Three antigenic variants were identified for F4 fimbriae: F4ab, F4ac and F4ad (Orskov *et al.* 1964; Guinée and Jansen 1979). Ten genes, *faeA* to *faeJ* located on a plasmid, are involved in the biosynthesis of F4 fimbriae (Van den Broeck *et al.* 2000). Antigenic diversity is the result of mutational changes in the *faeG* gene; hence differences between F4ab and F4ac serotypes are confined to the *faeG* gene (Bakker *et al.* 1992). A dramatic decrease in adhesive capacity of the F4 fimbriae results when the amino acid residue phenylalanine near position 150 at the carboxyl-terminal end of the *faeG* gene is replaced by a serine (Bakker *et al.* 1992). Apparently each variant shares antigen a, and expresses one of the three specific antigens b, c or d. Wilson and Hohmann (1974) demonstrated that antigens “b” and “c” were associated with the adhesion, whereas this association did not apply to “a” antigen (d antigen was unknown in 1974).

The role of the antigen F4 is essential in the mechanism of pathogenicity by interacting with brush borders of enterocytes in the small intestine (Jones and Rutter 1972). In 1975, Sellwood *et al.* observed some pigs which were more readily colonized by *E. coli* F4. These pigs were adhesive or susceptible, while the non-adhesive or resistant pigs showed no adhesion of F4 bacteria to the enterocytes. These results, confirmed by animal inoculation (Rutter *et al.* 1975), were the first indication of a genetic basis for resistance to enteric disease. The ability of pigs to bind F4 fimbriated bacteria is inherited as a dominant trait (Sellwood 1979; Gibbons *et al.* 1977). No adhesion of F4 fimbriae was observed in pigs which inherited both recessive alleles from parents.

Bijlsma *et al.* (1982) identified five patterns of adherence to porcine jejunal brush borders: these pig phenotypes were designated A (binds F4ab, F4ac and F4ad), B (F4ab and F4ac), C (F4ab and F4ad), D (F4ad) and E (binds no F4 variant). Baker *et al.* (1997) identified a sixth phenotype: the phenotype F (binds F4ab only) (Table 1.1). Therefore, it seems that the “a” antigen does not influence the adhesion properties of F4 variants proved by the existence of six phenotypes and particularly, by the binding of only one F4 variant in phenotypes D and F. Although three F4 variants were identified, only the F4ac variant was isolated in most cases from diarrheic piglets (Westerman *et al.* 1988; Choi and Chae 1999).
INTRODUCTION

Prior to adhesion of the bacteria to receptors, pathogenic *E. coli* have to pass through host defense mechanisms. The mucus layer, secreted by goblet cells, forms a protective barrier composed of highly glycosylated proteins called mucins. Mucus-secreting cells are widely distributed in the gastrointestinal and respiratory tracts. The multiple receptors contained in the mucus compete with enterocyte receptors sites. If pathogenic bacteria become attached to the glycolipids or glycoproteins contained in the mucus, they will be removed by fluid and peristalsis of the gut. Likewise, the normal intestinal microflora (e.g. lactobacilli and bifidobacteria) exerts a barrier against attachment and colonization of the gut epithelium by pathogenic bacteria. Some normal inhabitant species like *Bifidobacteria pseudolongum* are able to recognize receptors such as F4 *E. coli* and can bind them (Meng *et al.* 1998). Therefore, normal microflora prevents attachment by pathogenic bacteria by a mechanism of saturation of the adhesion receptors (Bernet *et al.* 1993). Hoskins (1984) showed that bacteria degrade mucins by extracellular glycosidases to penetrate the mucus and to access the brush border of the epithelial cells. In the case of F4 fimbriae, bacteria were present contiguous to the brush borders along villi, but not in the crypts (De Graaf and Mooi 1986). The highest amount of receptor for F4 fimbriae was collected from the mid-small intestine respectively, jejunum, and specific receptors were not apparent in the caecum or lower bowel (Chandler *et al.* 1994).

1.3 Prevention of *E. coli* diarrhea

The large amounts of antibiotics and their prophylactic use as feed additive in farm animals have resulted in a rising bacterial resistance. All F4 positive isolates were resistant to at least two distinct antimicrobial classes in a study by Amezcua *et al.* (2002). Recent strains like the serotype O149-K91:F4ac (1998-2001) have acquired a gene for STa (heat Stable Toxin a) and resistant percentages to antibiotics were higher compared to old strains isolated from 1974 to 1985 (Noamani *et al.* 2002). In a recent study on the antimicrobial resistance from different animal species, isolates from swine presented significantly more resistance than those from other animal species (Lanz *et al.* 2003). Therefore, antibiotics as feed additive in animal nutrition were prohibited in Sweden (1986), Switzerland (1999) and proscription is planned for 2006 in the European Union.
Classical approaches to prevent infection aim to avoid host-pathogen contact by management (hygiene, controlled temperature and ventilation, decreased density in pens, reduced mixing of pigs, etc.), food changes (decreased level of protein and increased level of fiber to 6%, food limitation, zinc oxide in starter ration, etc.). Immunization by the use of antibodies against ETEC fimbrial antigens administered orally to piglets have offered potential prophylactic and therapeutic values (Marquardt et al. 1997), but large amounts of antibodies derived from serum and colostrum are expensive to obtain (Kuhlman et al. 1998). A cheaper source of antibody is provided by vaccinating laying hens. Chicken egg yolk antibodies were evaluated for the treatment of porcine diarrhea and have been shown to provide protection against ETEC infection (Marquardt et al. 1999). On the other hand, the effectiveness of egg yolk antibodies, probiotics (e.g. bacteria Lactobacillus) and acidifiers were evaluated by Friendship et al. (2002), their series of trials indicate that these treatments were not efficient to control post-weaning E. coli diarrhea.

The most efficient means to control disease is the selection of naturally resistant animals. The feasibility of breeding for disease resistance can be illustrated by the E. coli F18 causing diarrhea and/or edema disease in post-weaning pigs. A polymorphism in the FUT1 gene (\(\alpha 1,2\) fucosyltransferase 1) is associated with the adhesion of F18 E. coli (Meijerink et al. 2000). Since the introduction of this molecular test in Switzerland in 1996, the percentage of Large White resistant pigs (33%) has tripled in five years and homozygous susceptible pigs (44% in 1996) were reduced to 18% (Vögeli et al. 2002). In a few years, marker assisted selection (MAS) will eliminate F18 susceptible Large White pigs. If breeding for resistance to E. coli F4 becomes applicable, the immunity component should be considered in regard to a breeding strategy. Virtually no transfer of immunoglobulin occurs across the placenta in the pig (cited in Sellwood et al. 1975). Piglets born to susceptible sows are protected by the F4 antibodies present in colostrum. Resistant sows devoid of receptors do not produce F4 antibodies in their colostrum and piglets are not passively immune. Thus, the selective advantage of resistant genotype is reduced, as the sensitive sows become immune. Sellwood (1979) showed that resistant sows mated with homozygous sensitive boars give the highest incidence (32%) of diarrhea in offspring, while there is no diarrhea imputed to F4 E. coli observed in offspring from fully resistant parents or in susceptible offspring of susceptible sows.
I.4 Biochemical composition of F4 receptors

Gibbons et al. (1975) inhibited the haemagglutination of fimbriated E. coli F4 to guinea-pig erythrocytes by some glycoproteins. His assays suggested that sugar residues could be involved. Since then, blocking studies and binding assays have been performed to elucidate the biochemical composition of the F4 receptor. Specific receptors to F4 E. coli fimbriae were isolated from mucus (Metcalfe et al. 1991; Willemsen and De Graaf 1992; Blomberg et al. 1993) and from brush borders (Erickson et al. 1992, 1994; Willemsen and De Graaf 1992; Grange and Mouricout 1996; Jin and Zhao 2000) of epithelial cells in the porcine small intestine.

I.4.1 Mucus receptors

In the mucus fraction, three proteins with molecular weights of 25, 35 and 60 kDa showing a high adhesion for F4 positive E. coli cells were detected. Differences in binding properties were noticed: F4ad fimbriae exhibited a rather weak binding to mucus proteins and F4ab/F4ac bound with a high adhesion to the mucus proteins. The age of the pigs influenced the detection of the receptors. One-week-old and 35-days-old post-weaning piglets contained F4 receptors in their mucus while these receptors were hardly detectable in 6-month-old pigs (Willemsen and De Graaf 1992). The mucus from 35-days-old unweaned piglets contained 16 times more F4 receptor than mucus from newborn piglets (Conway et al. 1990).

I.4.2 Brush border receptors

Billey et al. (1998) proposed three criteria for candidate receptors of porcine brush border for F4 fimbriae: the receptor must display specificity for the particular F4 fimbrial adhesin variant, the receptor must be detectable exclusively in brush borders phenotyped as adhesive for that particular F4 fimbrial adhesin variant, and the receptor must be expressed in multiple pigs of the same adhesive phenotype. Three candidate receptors satisfying these criteria were isolated: intestinal mucin-type sialoglycoproteins termed IMTGPs (Erickson et al. 1992, 1994), an intestinal transferrin or GP74 (Grange and Mouricout 1996), and an intestinal glycosphingolipid or IGLad (Grange et al. 1999) (Table 1.1). The presence of these receptors in the brush border fraction was shown to be independent of age while mucus receptors decrease with age (Willemsen and De Graaf 1992).
1) The IMTGPs-1 (210 to 230 kDa) and IMTGPs-2 (240 to 300 kDa) bind F4ab and F4ac (Francis et al. 1998; Erickson et al. 1992, 1994), but not F4ad and these receptors have been found in brush borders from pigs exhibiting the phenotypes A and B, but not in phenotypes C, D or E (Billey et al. 1998). Its presence in phenotype F was not determined. IMTGPs contain sialic acid and β-linked galactosyl residues. Treatment of IMTGPs with neuraminidase indicates that β-linked galactose residues are essential in recognition of receptors by the F4ac fimbriae (Grange et al. 1998).

2) A 74 kDa intestinal transferrin protein (GP74) binding F4ab, but not F4ac or F4ad, was detected in brush border exhibiting the phenotype A, but not in the phenotype E. Its presence in other phenotypes has not been determined. GP74 belongs to the transferrin family and differs from serum transferrin. Unlike intestinal transferrin GP74, the porcine serum transferrin (pSTF) from all pig phenotypes bound F4ab fimbriae. Thus the pSTF is not correlated with the susceptibility to F4ab bacteria (Grange and Mouricout 1996). Since mucosal (or intestinal) transferrin was found intimately entrapped on membranes, Grange and Mouricout (1996) hypothesized that a F4ab fimbriae-transferrin-cell transferrin receptor complex might allow the bacteria to adhere to specific sites of the mucosa.

3) The third receptor, IGLad, is found in pigs with the phenotype A or D, but not in C, B and E phenotypes (Billey et al. 1998; Grange et al. 1999). IGLad binds F4ad, but not F4ab or F4ac. The removal of terminal β-linked galactose residues from IGLad decreases the recognition of IGLad by the F4ad fimbriae (Grange et al. 1999).

In general F4ab and F4ac adhesins bind preferentially to glycoproteins, while F4ad appears to preferentially attach to glycolipids (Erickson et al. 1992, 1994; Granges et al. 1996; Billey et al. 1998). Furthermore, preincubation of brush borders of defined phenotype with F4 antigen allows some conclusions regarding the relationships between the receptors for distinct F4 variants. As expected, incubation of F4 antigen homologous to the receptor site blocks the adhesion of bacteria with the same F4 variant. Experiments were performed also with heterogeneous variants of the F4 antigen. Brush borders of phenotype A incubated with purified F4ad antigen does not interfere with the adhesion of F4ab or F4ac bacteria. F4ab and F4ac antigen completely blocks the adhesion of F4ad bacteria. Likewise, F4ab antigen blocks the adhesion of F4ac bacteria.
in brush borders of phenotypes A and B, and reversely (Bijlsma et al. 1982; Erickson et al. 1997). Thus, these experiments showed that F4ab and F4ac receptors differ from the F4ad receptor.

It has been postulated that a β-D-galactosyl residue present in glycoprotein of brush borders is necessary for the binding of F4 adhesin (Gibbons et al. 1975; Sellwood 1980b). Not only does galactose seem to play a role in the interaction with brush border receptors, but N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine and D-galactosamine may also be involved (Gibbons et al. 1975; Anderson et al. 1980; Sellwood 1980b; Payne et al. 1993; Grange et al. 1999, 2002). Moreover, the protein component in the receptor glycoprotein was shown to be implicated in adhesion. Complete loss of receptor activity after exposure to pronase or trypsin was observed in binding experiments (Sellwood 1980b; Staley and Wilson 1983; Laux et al. 1986).

1.5 Receptor model

Erickson et al. (1997) proposed a model with three receptors to account for the observed phenotypes: receptor bcd is adhesive to all three F4 fimbrial variants, receptor bc is specific for F4ab and F4ac fimbriae, and receptor d for F4ad fimbriae (Table 1.1). Billey et al. (1998) suggested five phenotypes: phenotype A pigs would express bcd and bc receptors, phenotype B pigs only the bc receptor, phenotype D pigs the d receptor, and phenotype E pigs none of the cited receptors. In the model, phenotype C is incompletely defined and phenotype F is not mentioned. Billey suggested that the binding of F4ab bacteria may be an artefact and, in fact, phenotype C pigs are phenotype D expressing only receptor d.

The 210- and 240-kDa glycoproteins are expressed in both phenotypes A and B and thus the IMTGPs may represent the bc receptor (Billey et al. 1998). The d receptor could be represented by IGLad.
Table 1.1 Summary (1.4.2) of the receptors detected in porcine brush borders and the corresponding phenotype. Model with three receptors (1.5).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Receptor for F4 variant</th>
<th>Biochemical nature of brush border receptors</th>
<th>Three receptor model</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ab ac ad</td>
<td>ab ac ad</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+ + +</td>
<td>GP74* IMTGPs<em>4 IGLad</em></td>
<td>bcd, bc</td>
</tr>
<tr>
<td>B</td>
<td>+ + -</td>
<td>nd2 IMTGPs*4 ne</td>
<td>bc</td>
</tr>
<tr>
<td>C</td>
<td>+ - +</td>
<td>nd ne3 ne</td>
<td>d</td>
</tr>
<tr>
<td>D</td>
<td>- - +</td>
<td>nd ne IGLad*</td>
<td>d</td>
</tr>
<tr>
<td>E</td>
<td>- - -</td>
<td>ne ne ne</td>
<td>ne</td>
</tr>
<tr>
<td>F</td>
<td>+ - -</td>
<td>nd nd nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

*+* = adhesion of the bacteria or presence of receptor  
*-* = non-adhesion  
*Phenotype designations A-F according to Bijlsma et al. (1982) and Baker et al. (1997)  
*nd = not determined  
*ne = not expressed  
*IMTGPs bind also F4ab fimbriae  
*Erickson et al. (1997)

1.6 Methods for F4 phenotype determination

Sellwood et al. (1975) developed a microscopic *in vitro* technique to test adhesion of F4 positive *E. coli* to brush borders from pig jejunal cells. Entire enterocytes (Wilson and Hohmann 1974) or isolated brush border vesicles (Sellwood et al. 1974) were used for microscopic assessment. Alternatively Girardeau (1980) and Van den Broeck (1999) used intestinal villi. Enzyme immunoassays to detect receptor activity using intestinal brush borders have been developed (Chandler et al. 1986; Valpotic et al. 1989a) and gave about 95% correlation with the classical adhesion test. With the absence of a reliable non-invasive test for phenotyping live pigs, alternative methods were tried. Atroshi et al. (1983a, 1983b) used sow milk as source of material for adhesion studies. Milk is known to contain large quantities of membrane material. The milk fat globule agglutination test gave the same results as the classical *in vitro* adhesion test. The authors proposed that the milk fat globule membrane contains similar receptor sites to those of the intestinal brush border. The fat globules may bind bacteria alternatively through their surface immunoglobulins. Another *in vivo* method was the typing of adhesiveness in faeces. Valpotic et al. (1989a, 1989b, 1992) used faeces as a source of brush border in an enzyme immunoassay. The sensitivity of the assay was low in adult...
(6 months old) compared with neonatal and weaned pigs, indicating that fecal samples from adults contained less receptor material.

1.7 Genetic mapping of F4

Selection of candidate genes was based on the previously known localization of $F_{4abR}$ and $F_{4acR}$ loci on the porcine chromosome 13. According to Guérin et al. (1993) and Edfors-Lilja et al. (1995) both loci $F_{4abR}$ and $F_{4acR}$ are tightly linked to the transferrin locus ($TF$), mapped to chromosome 13 by in situ hybridization (ISH) (Chowdhary et al. 1993). The localization of the $F_{4adR}$ locus is unknown, and Peelman (1999) excluded chromosome 13.

1.8 Candidate genes

Localization of four candidate genes on human chromosome 3 (HSA3) and porcine chromosome 13 (SSC13) is shown in the comparative map (Figure 3.10). Their position as well as their possible function of the genes in the pathogenesis is explained below.

1.8.1 TFRC

$TFRC$ (transferrin receptor) was chosen as the first candidate gene for F4 E. coli adhesion in pig. $TFRC$ is involved in iron acquisition. Efforts by bacteria to obtain iron from their host underline the importance of iron in pathogenesis. Bacteria and serum transferrin may form a complex and bind TFRC. Grange and Mouricout (1996) detected a 74kDa intestinal transferrin protein (GP74) in the brush border binding F4ab fimbriae, but not F4ac or F4ad. Parker et al. (2001) reported that canine and feline paroviruses bind to the feline and human transferrin receptors and use these receptors to enter and infect the cells.

Transferrin receptor (TFRC) is a disulphide-linked transmembrane dimer composed of two identical glycoprotein subunits each with a molecular weight of 90,000 kDa. The complete primary structure has been deduced from the cDNA sequence (Schneider et al. 1984). The human $TFRC$ contains 19 exons distributed over 31 kb (McClelland et al. 1984). Each monomer consists of a polypeptide chain containing 760 amino acid residues. The N-terminal domain of the protein, comprising the first 61 amino acids, is located within the cytoplasm of the cell. The next 28 residues (62 to 89) span the lipid bilayer of the cell membrane. The long C-terminal domain
(residues 90 to 760), containing the transferrin-binding site, is located on the extracellular side of the plasma membrane (Welch 1992). TFRC is a type II transmembrane protein, whose function is the cellular uptake of iron. The transferrin (TF) which is the iron-carrier interacts with its specific receptor (TFRC). Once transferrin has bound its receptor on the cell membrane, the entire complex transferrin-iron-receptor becomes internalized within the cell by a process of endocytosis and the iron is released into the cell.

1.8.2 B3GnT5

*B3GnT5* (UDP-GlcNAc: betaGal beta-1,3-N-acetylglcosaminyltransferase 5) encodes an enzyme that transfers N-acetylglcosamine to substrates. This enzyme is essential for the expression of Lewis X epitopes (Togayachi *et al.* 2001). The bacterium *Helicobacter pylori* causing gastric and duodenal ulcers is able to mimic the Lewis human blood group antigens expressed by the human gastric epithelium. Particularly the Lewis X expressed by *H. pylori* promotes adhesion to the human gastric epithelial cells (Appelmelk *et al.* 2000). Additionally, *H. pylori* Lewis antigens undergo phase variation. The required glycosyltransferase genes (*α2-fucosyltransferase, β3-galactosyltransferase, β3-N-acetyl-D-glucosaminyltransferase*) to produce antigens are either expressed or not expressed in the phase variation. Thus, the genes “expressed or not” combinations contribute to the high variability in the antigen phenotypes. The phase variation might allow antigen mimicry and adaptation to the host. *H. pylori* strains isolated from ulcer patients express an increased number of Lewis antigens compared with strains from dyspeptic patients (Zhenga *et al.* 2000). Lewis antigens are associated with the disease and are therefore a factor specific to adhesion of *H. pylori*. Moreover, N-acetylglcosamine inhibits binding and colonization by F17-positive enterotoxigenic *E. coli* of intestinal microvilli (Girardeau 1980). F17 *E. coli* strains cause diarrhea or septicemia in calves and nephropathy in lambs (Bertin *et al.* 1996). The F17-G adhesin at the tips of F17 fimbriae is the essential fimbrial adhesion factor for the disease. Buts *et al.* (2003) characterized the carbohydrate-binding site structure of the fimbrial adhesin F17-G and showed that the binding site of the lectin domain interacts laterally with N-acetylglcosamine residues-containing receptors.
1.8.3 B4GALT4

B4GALT4 (UDP-Gal: betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4) is one of seven enzymes of the beta 1,4-galactosyltransferase family which catalyze the reaction involving galactose and acetylglucosamine for the production of galactose beta 1,4-acetylglucosamine. Galactose is transferred to similar acceptor sugars (N-acetylglucosamine, glucose and xylose). The biological specificity in vivo of the beta 1,4-galactosyltransferase enzymes is still unknown. The encoded membrane-bound glycoproteins may be components of plasma membranes where they may function in intercellular recognition and/or adhesion. As explained previously (1.4.2), the minimum determinant decisive for the binding of F4 fimbriae has not been identified. A rather wide variety of sugar structures (galactose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine and D-galactosamine) may be involved in the formation of the host receptor.

1.8.4 B3GALT3

The gene B3GALT3 (UDP-Gal: betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 3) is a member of the beta 1,3-galactosyltransferase gene family. This family encodes membrane-bound glycoproteins with diverse enzymatic functions using different donor substrates (galactose and N-acetylglucosamine) and different acceptor sugars (N-acetylglucosamine, galactose, N-acetylgalactosamine). Of the beta 1,3-galactosyltransferase family only B3GALT3 does not use N-acetylglucosamine as an acceptor sugar, but the enzyme catalyzes the addition of N-acetylglucosamine to the substrate P(k) to form the P antigen which is one of the three antigens in the human P blood group system. Individuals with the erythrocyte phenotypes P(k) are not able to synthesize the P antigen identified as the cellular receptor for Parvo-B19 virus and some P-fimbriated E. coli causing urinary tract infections (Hellberg et al. 2002). P-fimbriated E. coli mimic carbohydrate receptors or the P antigen which is expressed on erythrocytes and also in the urinary tract. Four homozygous mutations in B3GALT3 render the enzyme nonfunctional resulting in the P(k) phenotype. The homologous gene B3GALT3 could explain both phenotypes of F4 E. coli in pigs. The nonfunctional B3GALT3 enzyme would not be able to add the terminal sugars recognized by the F4 bacteria. Thus this truncated version of the receptor would be the resistant phenotype
and the complete galactosylation by the functional enzyme would result in the susceptible phenotype.

1.9 Objectives of the study

The aims of this study were the following:
- to map the locus $F4acR$ on the porcine chromosome 13 by linkage analysis using available microsatellites and the $F4acR$ phenotypes of informative families.
- to isolate the porcine $TFRC$ as first candidate gene for the susceptibility to $E. coli$ F4ac and examine single nucleotide polymorphism(s).
- if $TFRC$ does not contain any polymorphism(s) associated with the susceptibility, to analyse other candidate genes according to their map position in regard to the receptor locus F4ac and their possible functional role in receptor formation.
- to determine the inheritance of the weak receptors for F4ab and F4ad by producing and testing informative families.
II. MATERIAL AND METHODS

II.1 Pigs

A Large White family and a Large White/Landrace family were bred for four generations at the Department of Farm Animals, Faculty of Veterinary Medicine, University of Zurich. The establishment of families started in 1998 by slaughtering of boars used in artificial insemination. Frozen semen of three boars negative to adhesion of F4ac bacteria was kept for matings. Phenotyping of progeny and the dominance of susceptibility reported by Sellwood (1979) allowed deducing the genotype of parents.

A random sample of 31 Large White and seven Landrace fattening pigs from the Swiss Performance Testing Station Sempach were collected as a representative sample of the Swiss porcine population. The F4 phenotype of the parents was unknown but their DNA was available.

II.1.1 Breeding strategy

Heterozygous susceptible (Ss) × resistant (ss) crosses were selected for a segregation study of the receptor F4acR. The same mating (Dd × dd) scheme was applied for the inheritance of the receptor F4adR because the receptor F4adR may be dominantly inherited. In phenotypes with receptor F4abR/F4acR the receptor F4abRw may also be present and go undetected. Therefore litters resistant to F4ac were produced to investigate the transmission of the weak F4abRw.

II.1.2 Pigs for segregation study

II.1.2.1 F4acR locus

For the linkage analysis of the receptor locus for fimbriae F4ac, 202 pigs in total were available. They consisted of eight parents (three boars and five sows) and 18 F1 (seven boars and 11 sows) which produced in 17 litters 176 F2 pigs. A total of 191 pigs were examined in the microscopic adhesion test: they comprised 173 F2 pigs slaughtered at eight weeks of age, three F2 fattening pigs, nine F1 parents and six parents. The remaining 11 pigs were kept for further production of piglets.
II.1.2.2  **F4adR locus**

A total of 136 pigs from 11 litters were bred for analysis of F4adR. Ten litters originated from $Dd \times dd$ crosses and one from a $Dd \times Dd$ cross. A total of 119 pigs were examined in the microscopic adhesion test: 110 $F_2$ pigs slaughtered at eight weeks of age, six $F_1$ parents and three parents. Seventeen parents were not analysed in adhesion test.

II.1.3  **Pigs for distribution of the F4 phenotypes**

A total of 534 pigs slaughtered (February 2000 to March 2003) at eight weeks of age and 64 pigs (38 fattening pigs from Sempach and 26 parents) slaughtered at more than eight weeks of age were phenotyped in the microscopic adhesion test with F4ab, F4ac and F4ad bacteria.

II.1.4  **Typing by the microscopic adhesion test**

The phenotype (susceptible or resistant) was established by means of a microscopic enterocyte adhesion test, using three variants of F4 *E. coli* obtained from the Central Veterinary Laboratory, Weybridge, Surrey GB. They carried the designations E68 I (O141: K85ab: F4ab), G4 (O45: K ‘E65’: F4ac) and Guinée (O8: K87: F4ad) (Thorns *et al.* 1987). On the day of the test, the bacteria were grown in tryptic soy broth to early exponential phase. Eight-week-old pigs (live weight about 20 kg) were separated from the sow and were not fed one day before slaughter. Within 30 min after death, a segment of 20 cm length was removed from the center part of the jejunum free of contents, opened longitudinally and immersed in cold (+ 4 °C) phosphate-buffered saline (PBS) pH 6.8 containing EDTA 3.72 gl$^{-1}$. Epithelial cells were removed by scraping the mucosal surface with a microscopic slide and collected in PBS containing 2% formaldehyde. Gross particles were allowed to settle, and the supernatant was cleansed twice by centrifuging 10 min at 200 × g, resuspended in 10 ml PBS and vortexed. The final suspension was diluted in PBS containing 2% mannose to approximately $10^5$ to $10^6$ cells per ml. A 1 ml aliquot of enterocyte suspension was mixed with 1 ml of undiluted broth culture in wells of a 6 well microtitre plate and statically incubated for 30 min at 37 °C. Following incubation, 20 µl were placed on a slide, covered with a cover-slip and examined by light microscopy (Figure 2.1). Twenty well-separated and intact enterocytes (Figures 2.1 and 2.2) were scored routinely.
However, in cases with less than 20% of cells with more than five adhering bacteria, another 20 cells were examined. A pig was classified as susceptible to F4ab or to F4ad *E. coli* if at least one (2.5%) out of the 40 cells bound more than five bacteria on the brush border. For F4ac this value was set higher: a pig was classified as susceptible if more than 15% of the cells bound more than five bacteria. The different threshold values were based on F4abR and F4acR negative control matings, as well as receptor distribution. No resistant mating was generated for F4adR. The threshold value for F4ad was fixed on receptor distribution and observed ratios in segregation.

**Figure 2.1 Adhesion test with F4ad bacteria** Two enterocytes of the same pig tested positive for adhesion of F4ad *E. coli*. The presence of one enterocyte with many adhering bacteria (A) and another enterocyte free of bacteria (B) illustrates the weak adhesion often observed for the receptor F4adR.

**Figure 2.2 Adhesion test with F4ac bacteria** Only intact and isolated enterocytes were scored. Arrow indicates the brush border without bacteria.

**Figure 2.3 Adhesion test with F4ac bacteria** Two enterocytes show adhesion of F4ac bacteria. More than five F4ac bacteria adhered to the brush borders of the enterocytes (arrow).
II.2 DNA methods

II.2.1 DNA extraction
DNA was extracted using a rapid lysis method. A volume of 0.5 ml lysis buffer was added to 0.6 ml blood in a 1.5 ml microtube and was mixed and centrifuged at 13,000 × g for 30 s. From the supernatant 0.8 ml were removed and the pellet was resuspended by vigorous vortexing in 1 ml lysis buffer. The step centrifugation-resuspension was repeated three times. Then, the pellet was resuspended in 0.4 ml PCR buffer. Tubes were incubated at 54 °C for 2 h with 40 μl proteinase K (20 mg/ml), followed by an incubation at 95 °C for 10 min to inactivate the enzymes.

II.2.2 Quantification of DNA
DNA yield was determined by measuring its absorbance (A) at 260 and 280 nm with a spectrophotometer. The $A_{260}/A_{280}$ ratio provides an estimate of the purity of DNA with respect to contaminants that absorb UV, such as protein. A pure DNA has a ratio of 1.8-2.0. If $A_{260} = 1$, then the DNA concentration is approximately 50 μg/ml. The following formula was used for calculating the concentration: DNA sample in μg/ml = 50 μg/ml × $A_{260} \times$ dilution factor.

The concentrations of small amounts of DNA were quantified by UV visualization of samples spotted on 0.8% agarose plates containing ethidium bromide. Concentration was estimated by comparing the brightness of λ-DNA standard dilutions (5, 10, 25, 50, 75 and 100 ng/μl) with that of unknown samples.

II.2.3 Polymerase chain reaction
Standard polymerase chain reactions (PCR) were performed in a final reaction volume of 25 μl containing 10-200 ng porcine DNA, 1 × PCR buffer, 200 μM dNTPs, 0.4 μM forward and reverse primers and 2.5 U of Taq polymerase (5 U/μl). The PCR profile was as follows: denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50-68 °C for 30 s and elongation at 72 °C for 30 s, terminated by an elongation at 72 °C for 7 min. An amount of 1.25 U Taq polymerase and 30 cycles only were applied for reactions amplifying a single microsatellite marker.
II.2.4 Primers

II.2.4.1 Primer design

Primers were designed using the following restrictions: primers should be 18-22 nucleotides in length, with a G-C content of 40-60%, G or C at 3' end. The annealing temperature (range 55-70 °C) was generally 5 °C lower than the estimated melting temperature. The annealing temperature was determined by the following formula: \[ 4(G + C) + 2(A + T) \] - 5 °C.

Primers were basically designed applying the program primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and changed depending on specific requirements.

**Table 2.1 Sequence (5' → 3') of primers and their annealing temperature (T_a)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5' → 3'</th>
<th>T_a (°C)</th>
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<tr>
<td><strong>TFRC</strong></td>
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<tr>
<td>TFRC-hsa-f3</td>
<td>-CTCCAGAGCTGCTGCAGAAAAGC-</td>
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<tr>
<td>TFRC-hsa-r4</td>
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<tr>
<td>T5-r10</td>
<td>-CACCATGATGAACCGGACC-</td>
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</table>
**B3GALT3**

T3-f13 - CTCCCCCACTACAACGTGAT - 58
T3-r12 - TGGGGCAAAATTTCAGTTACC - 58
T3-sch-f3 - CGTCTTACCCAAAAGCTAGAGA - 61
T3-sch-r4 - CCTCTGAACGGACAAGACTGT - 61
T3-f5 - TAGGACAGCGAGCTGAAAGG - 60
T3-hsa-r6 - TGAACCTGCCAAMAAGTGATG - 60
T3-hsa-f1 - GCTTGGGTGGGATATAGG - 55
T3-hsa-r2 - TGGGGCAAAACTCAGTTACC - 55

**B4GALT4**

T4-hsa-f1 - AGGATGAAACTGGGACTGC - 50
T4-hsa-r2 - AGTGCTGTTCCTGCAACC - 50
T4-f3 - GCGCCAAAATCTCTGAAATGGGCTA - 68
T4-r4 - TGGTCAACCCCATCTGTTCCAGACAG - 68
T4-r6 - CAGGCACAGGTCCACATCATGGAA - 65
T4-f11 - ACCTGGTGCTGAGAATGAC - 60
T4-r10 - TCGTGCCCTTGGCTCTTCTT - 60

**β-actin**

B-actin3 - ATCAACCATCGGCAACGAG - 54
B-actin4 - GTGATCTCTTCTGCAATCC - 54

**Anchored reverse transcription primer**

TVX - T(17)VX - V = A/C/G; X = A/C/G/T

**Vectors** (pBluescript, pGEM-T easy)

T3 - AATTAACCCTCACTAAAGGG - 50
T7 - TAATACGACTCTATAGGG - 50
SP6 - ATTAGGTGACACTATAG - 50

**Table 2.2 Microsatellite names, chromosomal localization, fluorescent dye, annealing temperature and microsatellites used in multiplex amplification.** For microsatellite marker analyses, primers were provided either by the U.S. Pig Genome Coordination Program, or synthesized by Microsynth (Balgach, CH). Sequence information of markers is available on the internet address (http://www.marc.usda.gov/genome/genome.html). Multiplex amplification for up to five markers was developed. Forward primer dyes for labeling are Fam (blue), Tet (green) and Hex (yellow). Annealing temperature (T_a) is indicated for each multiplex. A volume of 0.5 µl of amplified products were diluted in 3.5 µl Genescan-350 TAMRA
size standard/loading buffer/formamide (v/v 1:1:5). Results were evaluated with an ABI 672 Genescan program and Genotyper software (version 2.1, Applied Biosystems, Perkin-Elmer Corp., Foster City, CA; USA).

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<thead>
<tr>
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<th>Fluorescent dye</th>
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### II.2.5 PCR-RFLP of *B3GALT3*

A 403 bp fragment of genomic DNA was amplified with the primers T3-f13/r12 using the standard PCR (II.2.3) protocol. An amount of 10 µl PCR products was digested
overnight with 5 U enzymes AluI at 37 °C in a final reaction volume of 20 μl and fragments were separated on a 1.5% agarose gel.

11.2.6 Mapping of B3GnT5 and B3GALT3 using the somatic cell hybrid panel
Assignment of B3GnT5 and B3GALT3 to porcine chromosome 13 was done using the INRA somatic cell hybrid panel (Yerle et al. 1996). This panel is made up of 19 pig × Chinese hamster hybrids (clones 1 to 19) and eight pig × mouse hybrids (clones 20 to 27). PCR reactions were performed on genomic DNA extracted from each of 27 hybrid cells of the panel. Porcine primers were designed in the 3' untranslated region of the gene. The annealing temperature was optimized using a gradient PCR. Porcine genomic DNA was taken as a positive control, rodent and mouse genomic DNA as negative controls. The amplified products were separated on a 2% agarose gel and stained with ethidium bromide. The results were submitted to the INRA web page (http://www.toulouse.inra.fr/lgc/pig/pcr/pcr.htm).

Amplification of a 262 bp fragment for B3GnT5 was obtained with the primers T5-sch-fl/r2. After an initial denaturation of 5 min at 95 °C, a 35-cycle program (denaturation for 45 s at 95 °C, annealing for 45 s at 58 °C, elongation for 45 s at 72 °C) followed by a final elongation for 7 min at 72 °C, was performed. Primers T3-sch-f3/r4 amplified a 400 bp fragment for B3GALT3 under the same conditions, except that the annealing temperature was 61 °C and that the elongation time was 1 min.

11.2.7 Sequencing
DNA from purified PCR product or plasmid clones were sequenced on the automated ABI Prism 377 DNA Sequencer (PE Applied Biosystems) with the BigDye Terminator chemistry, based on the technology of dideoxynucleotide chain termination (Sanger et al. 1977). The amount (ng) of DNA required for the sequencing was calculated as the 1/20 of the length (bp) of the PCR product. For plasmid DNA, between 250 and 300 ng of template was used. The sequencing reaction was carried out in a final reaction volume of 10 μl: 2 μl BigDye Terminator, 2 μl ha/f/BigDye, 0.32 μM primer and DNA. The profile of the reaction was 5 min denaturation at 95 °C, followed by 30 cycles of 30 s at 95 °C, 15 s at 50 °C, and 4 min at 60 °C. Samples were precipitated with 10 μl NaAc (pH 5.2), 80 μl millipore H2O and 200 μl absolute ethanol at a final concentration of 66.67%. Tubes were vortexed, put at least 5 min at -80 °C, centrifuged at 15,000 rpm
at 4 °C for 5 min. The ethanol solution was carefully aspirated with a Pasteur pipette. The pellets were washed in 200 μl ethanol 70%, centrifuged at 4 °C for 5 min, the supernatant was removed and the pellet was dried in a vacuum centrifuge. The pellet was resuspended in 1.5 μl formamide/loading buffer (v/v 5:1). Samples were denatured for 5 min at 95 °C before loading to a gel of 4.5% acrylamide/bis (29:1)/6 M urea/1 × TBE. The gel was run on an ABI Prism 377 DNA Sequencer (PE Applied Biosystems) for 12 h. Sequences were analyzed with the GCG sequence analysis software package version 10.1 (Genetics Computer Group, Madison, WI, USA).

11.2.8 SNaPshot

The SNaPshot ddNTP primer extension kit (Applied Biosystems) allows detecting single nucleotide polymorphisms (SNPs) by amplification of one single base after the primer. This method was used in single-plex reactions for checking a putative polymorphism at the base 1,752 present in the RT-PCR product of one pig (TFRC: Genbank accession number AF416763). The PCR fragment was gel-purified (QIAEX, Qiagen, Basel, CH) and added to the SNaPshot mix with the primer TFRC-mut1752 for single extension reaction. Unincorporated ddNTPs were removed by calf intestinal phosphatase (CIP) treatment and the samples were run on an ABI Prism 377 DNA Sequencer for 1 h. Data was analyzed with the ABI 672 Genescan program. The results are represented by a single peak, whose dye color corresponds to a nucleotide (green for A, red for T, blue for G and yellow for C).

11.2.9 Genome-wide scan for F4adR locus

A genome-wide scan was carried out to find any linkage of microsatellites (MS) with the receptor locus for F4ad bacteria. MS spread at an average genetic distance of 20-30 cM on the 18 autosomes were used in 33 Large White pigs (27 F1 pigs and six parents). Microsatellite markers were chosen from the U.S. Pig Genome Coordination Program. Standard PCR (11.2.3), fragment length analysis (11.2.4; Table 2.2) and linkage analysis (11.4.1) were performed.
II.2.10 Screening of a porcine small intestine cDNA library

II.2.10.1 Cross-species PCR and probe amplification
To isolate phages with the gene of interest, cross-species PCR was performed to get a porcine fragment which was subsequently used as a probe to screen the cDNA library. Primers were designed on conserved sequences of homologous genes between species (human and mouse). The porcine fragment amplified from the small intestine cDNA library was isolated from the agarose gel with the QIAEX II Gel Extraction Kit or with the GENE CLEAN II Kit (Qbiogene, Basel, CH), sequenced and compared with the BLAST program to the web page http://www.ncbi.nlm.nih.gov/BLAST/, for homology with the human sequence.

II.2.10.2 Preparation of bacteria
The XL1-Blue MRF’ (Stratagene, Amsterdam, NL) cells were transfected with phages from the small intestine cDNA library (Meijerink 1999) constructed using the ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene). The SOLR cells (Stratagene) were used for the in vivo excision with ExAssist Helper Phage (Stratagene). Bacterial glycerol stock of XL1-Blue MRF’ and SOLR were streaked onto LB agar plates (82 mm Ø) containing 12.5 µg/ml tetracycline and 50 µg/ml kanamycin, respectively. Plates were incubated overnight at 37 °C, then sealed with parafilm and stored at 4 °C. These plates served for one month as stock for bacterial culture. One single colony was inoculated in a falcon tube containing 15 ml LB medium, 0.2% maltose and 10 mM MgSO$_4$. The tube was shaked at 250 rpm at 37 °C for 3-4 h until the medium appeared milky. At an OD$_{600}$ of approximately 0.5, the suspension was centrifuged at 2,000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 10 mM MgSO$_4$ solution to an OD$_{600}$ of 0.5.

II.2.10.3 Titering, plating of phage and bacterial mixture
The phage lysate was serially diluted in SM buffer to derive the number of viable lambda phage particles present in the original ZAP cDNA library. After primary library titering, around 50,000 pfu were plated for the screening onto each 20 pre-warmed NZY agar large plates (137 mm Ø) containing 12.5 µg/ml tetracycline. A volume of 600 µl of fresh XL1-Blue MRF’ bacteria mixture and a maximum of 300 µl bacteriophage
solution were incubated 15 min at 37 °C in the shaker at 100 rpm. A volume 6.5 ml NZY top agar, melted and cooled at 48 °C, were gently mixed with each aliquot of infected bacteria and spread onto NZY agar plates. These plates were inverted and incubated overnight at 37 °C for 8 h. To prevent the NZY top agar from sticking to the Duralon membrane, the plates were chilled for 2 h at 4 °C.

II.2.10.4 Phage DNA transfer to membranes

A Duralon-UV membrane (Stratagene) was placed on each NZY agar plate for 2 min to transfer phage DNA from plaques. Three holes were stamped out of the membranes and the agar to orient the replicate. After lifting the membranes they were denatured (2 min, 1.5 M NaCl/0.5 M NaOH), neutralized (5 min, 1.5 M NaCl/0.5 M Tris-HCl pH 8.0) and rinsed (30 s, 2 x SSC/0.2 M Tris-HCl pH 7.5). Membranes were baked for 2 h at 80 °C to cross-link the phage DNA.

II.2.10.5 Hybridization

II.2.10.5.1 Labelling and incorporation test

The gel-purified template was labelled with the Prime-It II Random Primer Labelling Kit (Stratagene). An amount of 30 ng DNA probe was denatured for 5 min at 95 °C using 10 µl random primer solution. After adding 10 µl 5 x nucleotide buffer dATP, 1 µl Klenow enzyme and 5 µl [α-32p]-dATP (10 mCi/ml) with 14.3 days half-life, the mix was incubated for 20 min at 38 °C. The reaction was stopped by giving 2 µl stop mix and filled to 100 µl with TE.

To test the incorporation of radioactive nucleotide in the probe, 1 µl solution was placed on a 25 mm Ø glass microfibre filter (Whatman) for determination of counts per minute (cpm) with a handheld Geiger counter. Non-incorporated nucleotides were rinsed from the filter with 12.5 ml of 5% trichloroacetic acid (TCA) under vacuum. A second measurement should approximately amount to 50% of the first measurement which indicates an efficient incorporation.

II.2.10.5.2 Hybridization solution

The hybridization solution was composed of 50% dextran sulfate in formamide (10 g dextran sulfate per 50 ml formamide), 5 x SSC, 1% (w/v) SDS and 5 x Denhardt’s
solution. About 0.75 ml hybridization solution per membrane of 137 mm ø was required, 0.6 ml with membrane of 82 mm ø. Half of the hybridization solution, preheated to 42 °C, was taken for pre-hybridization of the membranes. A pre-hybridization step reduces the background. Salmon sperm DNA was denatured by boiling for 5 min at 95 °C and added to warmed fresh pre-hybridization solution to a final concentration of 100 μg per ml. After at least 2 h of pre-hybridization, most of the solution was removed. The 100 μl of radiolabelled probe were mixed with the remaining hybridization solution and incubated with the membranes in a petri dish by constant shaking for 16-20 h at 40 °C.

11.2.10.6 Post-hybridization washing and exposure of membranes
Membranes were washed once in 1 x SSC/1% SDS for 5 min at room temperature to remove parts of the hybridization solution. In general, membranes were washed for 10 min. In high stringency conditions either the SSC concentration (1 x SSC/0.1% SDS, 0.1 x SSC/0.1% SDS) was decreased, or the temperature of the solution was increased (up to 65 °C). Between each step, the radioactivity was measured and the membranes were washed until background was 5-15 cpm. After washing, membranes were enveloped in saran wrap and exposed to X-ray films in cassettes at -80 °C for 6-20 h.

11.2.10.7 Positive clones and in vivo excision
With the large end of a sterile pasteur pipette, the area containing the positive plaque was cut out and put into 1 ml SM buffer supplemented with 20 μl chloroform, incubated at room temperature for 15 min and vortexed. One round of screening is insufficient to obtain a single phage clone. Therefore a second or even a third screening was done for isolating a single phage clone.

The Uni-ZAP XR vector allows efficient in vivo excision of the pBluescript II(-) phagemid. A volume of 200 μl XL1-MRF’ cells (OD₆₀₀ = 1), 1 μl of ExAssist helper phage and 100 μl of phage stock (>1 x 10⁵ pfu) were incubated for 15 min at 37 °C, then 3 ml of LB medium were added and the mixture was incubated with shaking for 3 h at 37 °C. The tubes were heated to 70 °C for 20 min, centrifuged at 1,000 x g for 15 min and the supernatant was decanted into new sterile tubes. Ten μl of supernatant and 100 μl of the excised pBluescript phagemid solution supplemented with 200 μl of freshly SOLR cells (OD₆₀₀ = 1) were incubated at 37 °C for 15 min. Then 50 μl of the cell
MATERIAL AND METHODS

A mixture was plated onto LB agar plates containing 50 μg/ml ampicillin and incubated overnight at 37 °C. The following day a single colony was isolated for growing in 8 ml LB medium containing 50 μg/ml ampicillin. The mixture was shaked overnight at 250 rpm at 37 °C. Then the plasmid DNA was extracted using the NucleoSpin system (Macherey-Nagel, Oensingen, CH). Control for presence of the insert was done by digestion with EcoRI and XhoI restriction enzymes. Plasmid DNA was sequenced using T7 and T3 vector primers.

II.2.11 Cloning into pGEM-T easy vector system and transformation

Products from RT-PCR and PCR were gel-purified or used directly from the reaction (amplified DNA was cleaned in microcon-PCR filter, Millipore) and cloned in the pGEM-T easy vector (Promega, Wallisellen, CH). Taq polymerase generates PCR fragments with sticky ends (A-tail) complementary to the 3'-T overhangs in the multiple cloning site of the vector. The 10 μl-ligation reaction (5 μl 2× rapid ligation buffer T4, 25 ng pGEM-T easy vector, 3 U of T4 DNA ligase and 3.5 μl PCR product of an insert:vector ratio of 3:1) was incubated overnight at 4 °C.

The transformation was performed in a 14 ml falcon tube containing 5 μl ligation reaction and 100 μl competent cells (XL10-Gold Kan) (Stratagene). The mix was stored on ice for 30 min, incubated 30 s at 42 °C and returned on ice for 2 min. A volume of 450 μl SOC medium was added and the reaction was incubated at 37 °C for 90 min with shaking (100 rpm). For colour screening X-Gal and IPTG were plated on LB/ampicillin (50 μg/μl) agar plates. Fifty and 150 μl of each transformation culture were plated on LB/ampicillin/IPTG/X-Gal plates and incubated at 37 °C for 16 h. White colonies contain the plasmid with the insert, while blue colonies have no insert. A positive colony was grown overnight in 8 ml LB medium supplemented with 50 μg/ml ampicillin with shaking (200 rpm) for plasmid DNA extraction with the NucleoSpin system (Macherey-Nagel). Plasmids were digested with EcoRI (2 h at 37 °C) for size determination of insert. Plasmid DNA was sequenced using T7 and SP6 vector primers.
II.3 RNA methods

II.3.1 Total RNA isolation
Total RNA was extracted from 0.4-0.6 g of small intestinal mucosa, liver, fat and brain tissue using the RNeasy Maxi Kit (Qiagen, Basel, CH). Samples were first lysed and homogenized under highly denaturing conditions (4 M guanidine isothiocyanate containing 25 mM sodium citrate pH 7.0 and 0.5% sarcosyl and β-mercaptoethanol) which immediately inactivate RNases to ensure isolation of intact RNA. One volume of 70% ethanol was added to provide appropriate binding conditions and the samples were then applied to an RNeasy column where the total RNA bound and contaminants were efficiently washed away. High-quality RNA was then eluted in 0.8 ml RNase-free water.

II.3.2 Quantification of RNA
The concentration of RNA after extraction from tissue was measured in the spectrophotometer. The same formula as DNA (II.2.2) was applied for RNA, but 40 μg/ml corresponds to $A_{260} = 1$. Pure RNA exhibits an $A_{260}/A_{280}$ ratio between 1.8 and 2.1.

II.3.3 Gel electrophoresis of RNA
Three μg of RNA diluted in 3.5 μl DEPC H2O were denatured for 10 min at 60 °C in 11.5 μl of 10 x MOPS/formaldehyde/formamide (1.5:2.5:7.5), and cooled on ice. The RNA was mixed with 2 μl RNA loading dye and run on a 1.25% formaldehyde-agarose gel in 1 x MOPS buffer. 28S and 18S rRNAs bands were visible after staining the gel in methylene blue (0.02% (w/v) in 10 mM Tris HCl ph 7.5) and destained in ddH2O overnight at 4 °C.

II.3.4 Reverse transcription
Reverse transcription was carried out in a total volume of 25 μl. An amount of 2.5 μg RNA and 4 μM of either TVX primer or a specific reverse primer, made up to 10 μl with DEPC H2O, was denatured at 70 °C for 5 min and then cooled down to 25 °C in 10 min. Then the following substances were added: 5 x RT buffer (Promega), 250 μM dNTPs, 2.5 μl of DEPC H2O, 4 mM of Na-pyrophosphate, 40 U RNASin and 30 U AMV
(avian myeloblastosis virus) reverse transcriptase. The mixture was incubated for 45 min at 42 °C, 10 min at 50 °C then at 55 °C, and finally for 10 min at 72 °C. For standard amplification 5 μl of transcript solution was taken.

**II.3.5 Amplification of cDNA ends**

The oligo-capping rapid amplification of cDNA ends (RACE) (Invitrogen, Basel, CH) and the RNA ligase-mediated methods allow obtaining full-length 5' or 3' ends of a cDNA. This technique was used for the determination of the sequence of the missing 5' ends of the *B4GALT4* gene. After treating total RNA with 10 U of calf intestinal phosphatase (10 U/μl) to remove the 5' phosphates and with 0.5 U tobacco acid (0.5 U/μl) pyrophosphate to remove the 5' cap structure from full-length mRNA, an oligo was ligated to the 5' end of the mRNA. The ligated mRNA was reverse-transcribed using AMV (II.3.4) to create first-strand cDNA. To obtain 5' ends, the first-strand cDNA was amplified with the reverse gene-specific primer (T4-r6) and the GeneRacer 5' primer (homologous to the GeneRacer RNA oligo, Invitrogen). The primers T4-f3/r4 (447 bp, Tm = 68 °C) were used as control for successfully reverse transcription of the gene.

**II.3.6 Northern blot**

Twenty μg of total RNA per sample of three genotypes for the receptor F4acR (SS, Ss and ss) were extracted from the small intestine mucosa. Total RNA was transferred on Duralon-UV nylon membrane by the passive capillary transfer method (Southern 1975) for at least 16 h. After blotting, the gel was stained with methylene blue (II.3.3) to estimate the transfer efficiency by assessment of the intensity of the bands. The membrane was baked 2 h at 80 °C. Information on the expression of human genes in different tissues was taken from the website (http://bioinfo.weizmann.ac.il/bioinfo.html). Brain RNA was chosen as positive control for expression of porcine genes (*TFRC, B3GnT5* and *B4GALT4*), negative control tissues were liver for *B4GALT4*, adrenal gland for *TFRC* and fat tissue for *B3GnT5*. The gene specific probes were amplified with the primers TFRC-f19/r20 (209 bp, Tm = 58 °C), T4-f11/r10 (301 bp, Tm = 60 °C) (*B4GALT4*) and T5-f11/r10 (286 bp, Tm = 60 °C) (*B3GnT5*). The β-actin (U07786) generated by RT-PCR was used as control to quantify the blotted RNA. Probe labelling and hybridization was carried out at a temperature of
MATERIAL AND METHODS

45-50 °C (II.2.10.5.1; II.2.10.5.2). For RNA membranes, low stringency conditions (2 × SSC/0.5% SDS or 2 × SSC/0.1% SDS; at room temperature or at 45 °C) were done until background decreased to 10-20 cpm. Membranes were exposed in X-ray films (Kodak, X-Omat AR) in cassettes at -80 °C.

To stripe signals, the membrane was immersed in 0.1 × SSC/1% SDS at 70 °C for 45 min. The membrane was rehybridized with the β-actin probe.

II.4 Statistics

II.4.1 Linkage analysis

Pairwise linkage analysis and calculation of recombination fractions were performed with the CRI-MAP version 2.4 program (Green et al. 1990). Multipoint analysis was performed by sequential insertion of loci with option ALL. Option FLIPS was used to determine the statistical support of the obtained order. To establish the genetic map the option FIXED was used and the recombination fractions were converted into genetic distances using the Kosambi mapping function.

II.4.2 Linkage and transmission disequilibrium test

The linkage disequilibrium method was used to test for linkage disequilibrium between the specific microsatellite alleles and the alleles of $F_{4acR}$. In case of linkage disequilibrium, two alleles at two distinctive closely linked loci are found together in a population at a greater frequency than that predicted simply by the product of their individual allele frequencies.

The transmission disequilibrium test (TDT) is a modified form of the haplotype relative risk (HRR) method (Falk and Rubinstein 1987). The TDT tests for linkage in the presence of linkage disequilibrium (LD) and eliminates stratification effects (intrapopulation heterogeneity, cross effect, segregation distortion at the marker locus) completely. The TDT considers only heterozygous parents for markers and phenotypes, and evaluates the frequency with which the (supposed) diseased allele ($M$) or its alternate ($M^-$) is transmitted to affected offspring (Spielman et al. 1993). After filling the Table 2.3, the chi-square ($\chi^2$) with a one degree of freedom (df) is calculated with the following formula for the TDT: $\chi^2 = (b - c)^2 / (b + c)$. 
Table 2.3 Transmission disequilibrium test (TDT) Distribution of the marker allele from heterozygous parents transmitted (M) and not transmitted (M~) to an adhesive offspring.

<table>
<thead>
<tr>
<th>Transmitted Allele</th>
<th>Non-transmitted allele</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>M~</td>
</tr>
<tr>
<td>M</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>M~</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>Total</td>
<td>a + c</td>
<td>b + d</td>
</tr>
</tbody>
</table>

n = number of alleles of adhesive offspring  
a, b, c, d = number of alleles

II.4.3 Statistical analysis of adhesion test

The statistical software SYSTAT version 10 was used for performing a two sample t-test, an analysis of variance with the General Linear Model (GLM) and for calculating the correlation. The observed mean percentages (P) in adhesion test were transformed by the inverse sine of the root of P/100 to approximate a normal distribution.

The effect of day on the number of adhered bacteria to the cells in a given adhesive phenotype (A, B, C, D and F) was tested with model I. The day effect was included into quarter (four quarter for one year), because few data were available in some phenotypes. The effect of day was tested with the following model I:

\[ Y_i = \mu + \text{quarter}_i + e_i, \]

where \( Y_i \) = the dependent variable; \( \mu \) = overall mean, \( \text{quarter}_i \) = effect of day test (i = 1 for day 0-91, 2 for day 92-182, etc.), and \( e_i \) = residual error.

The effects of quarter, phenotype (A, B, C, D and F) on the observed results of one receptor type (F4abR, F4acR and F4adR) in adhesion test were measured with the following model II:

\[ Y_i = \mu + \text{phenotype}_i + \text{quarter}_i + \text{phenotype}_i \text{quarter}_i + e_i, \]
where $Y_i$ = the dependent variable; $\mu$ = overall mean; phenotype$_i$ = effect of phenotype ($i = A, B, C, D$ and $F$); quarter$_i$ = effect of day test ($i = 1$ for day 0-91 to $i = 13$ for day 1092-1183); phenotype$_i$ _quarter$_i$ = effect of interaction between phenotype and quarter; and $e_i$ = residual error.
III. RESULTS

III.1 Adhesion test

III.1.1 The F4 phenotypes

The phenotypes of pigs tested with the three fimbrial variants F4ab, F4ac and F4ad are summarized in the Table 3.1. The mean percentages of adhering bacteria were calculated for 534 eight-week-old pigs. Pigs susceptible to F4ab, F4ac and F4ad (phenotype A) showed a strong adhesion: 81 ± 17.9%, 79 ± 17.2% and 58 ± 33.7%, respectively. Likewise, F4ab (76 ± 20.7%) and F4ac (75 ± 19.8%) bacteria bound strongly in phenotype B. Contrary to phenotypes A and B, few enterocytes (24 ± 17.6% and 21 ± 23.9%, respectively) bound more than five F4ab bacteria in phenotypes C and F. The receptor F4abR in these two phenotypes, in lack of receptor F4acR, is said to be weak. We therefore designate the receptor (F4abR+ / F4acR-) as weak receptor (F4abRw). In phenotype D the adhesion of F4ad bacteria to the cells is weak, as only 35 ± 29.1% of enterocytes bound bacteria. High standard deviations were obtained for F4adR in phenotypes A, C and D: ± 33.7%, ± 38.8% and ± 29.1%, respectively. Finally, in pigs resistant to one (phenotypes B and C), two (phenotypes D and F) or three fimbrial variants (phenotype E), exceptional adhesion of bacteria was observed: range from 0 to 0.9 ± 2.7%.

Of a total of 64 fattening and breeding pigs slaughtered at more than eight weeks of age 41 showed phenotype A, nine phenotype B, five phenotype C, three phenotype D, six phenotype E and none expressed phenotype F.
Table 3.1 Receptor phenotypes for the three F4 fimbral variants in eight-week-old pigs. Mean percentages (± standard deviation) of enterocytes with more than five adhering bacteria per enterocyte.

<table>
<thead>
<tr>
<th>F4 phenotype ( ^3 )</th>
<th>8-week-old pigs</th>
<th>Percent of enterocytes binding &gt; 5 bacteria of variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>ab ac ad Number % F4ab(^1 ) F4ac(^2 ) F4ad(^3 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A + + + 128 24 81 (± 17.9) 79 (± 17.2) 58 (± 33.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B + + - 66 12 76 (± 20.7) 75 (± 19.8) 0.1 (± 0.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C + - + 130 24 24 (± 17.6) 0.9 (± 2.7) 53 (± 38.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D - - + 69 13 0.1 (± 0.3) 0 (± 0) 35 (± 29.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E - - - 111 21 0.05 (± 0.2) 0.01 (± 0.1) 0.1 (± 0.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F + - - 30 6 21 (± 23.9) 0.2 (± 0.6) 0.2 (± 0.4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total 534 100

\(^* + ^* = \) adhesion of the bacteria or presence of receptor
\(^* - ^* = \) non-adhesion
\(^1 \) Threshold value ≥ 2.5% enterocytes (1 of 40) with > 5 adhering bacteria
\(^2 \) Threshold value > 15% enterocytes (3 of 20) with > 5 adhering bacteria
\(^3 \) Phenotypes designations A-F according to Bijlsma et al. (1982) and Baker et al. (1997)

Figure 3.1 shows only phenotypes for both variants F4ab and F4ac, independently from F4ad phenotype. The phenotypes A and B in Table 3.1 correspond to the phenotype F4ab\(^+ / \) F4ac\(^+ \) in Figure 3.1 and the phenotypes C and F to F4ab\(^+ / \) F4ac\(^- \) or the weak receptor F4ab\(^w \). Distribution of the 135 pigs susceptible to F4ab and/or F4ac bacteria (Figure 3.1) has allowed fixing the threshold values for the susceptible pigs indicated in Table 3.1. Figure 3.1 confirmed that adhesion of F4ab and F4ac bacteria is strong in the presence of the receptor type (F4ab\(^+ / \) F4ac\(^+ \)): between 70 and 100% of the enterocytes bound more than five bacteria in the majority of the pigs. Moreover, the number of pigs exhibiting a strong adhesion for the receptor F4ab\(^+ \) (hatched boxes) was highly correlated (\( r = 0.85 \)) with the number of pigs carrying the receptor F4ac\(^+ \) (black boxes). In the case of the pigs being F4ab\(^+ / \) F4ac\(^- \), only 10-30% of the enterocytes bound more than five F4ab bacteria.
Figure 3.1 Histogram of the number of pigs (n = 135) related to the percentage of enterocytes binding more than five bacteria. F4abR<sup>−</sup> is the weak receptor for F4abR detected in the phenotype F4abR<sup>−</sup>/F4acR<sup>−</sup> symbolized by white boxes, F4abR<sup>+</sup> (hatched boxes) and F4acR<sup>+</sup> (black boxes) are detected in the phenotype F4abR<sup>−</sup>/F4acR<sup>−</sup>.

Of a total of 534 pigs 327 were susceptible to F4ad bacteria (Figure 3.2). Pigs displaying 100% of enterocytes are as many as pigs with 10% of enterocytes binding more than five F4ad bacteria. A threshold value for susceptible pigs to F4ad is therefore difficult to define. A threshold value of 15% like F4ac would lead to too many pigs resistant to F4ad fimbriae if we hypothesized a dominant inheritance for the receptor F4adR. Therefore, a pig was classified as susceptible to F4ad *E. coli* if at least one (2.5%) out of the 40 cells bound more than five bacteria on the brush border.
Results

Figure 3.2 Histogram of number of pigs (n = 327) related to the percentage of enterocytes binding more than five F4ad bacteria.

III.1.2 Two-sample t test

Converted adhesion percentages for the same receptor (e.g. F4adR) were grouped by phenotype (in two groups: e.g. A and C) and the means of adhesion were compared in the two groups. The two-sample t test was performed to observe if the adhesion was different in the phenotype for the same receptor. For each group (or phenotype), three graphical displays are combined: a box plot displaying the sample median, quartiles, and outliers (if any), a normal curve calculated using the sample mean and standard deviation, and a dit plot displaying each observation (Figures 3.3 to 3.6). In each table the number of observations (n), the mean of adhesion and the standard deviation (SD) are given for a phenotype. The separate variance t, degree of freedom (df), the probability (P-value), the difference in means and the endpoints for the confidence interval (CI) of the mean are below the table.

The adhesion of F4ab bacteria in phenotype A differed significantly (P = 0.00) from the adhesion in phenotype C (Table 3.2 and Figure 3.3). Pigs of the phenotype A (F4abR+/F4acR+/F4adR-) had a higher adhesion (mean 1.188) than those of phenotype C (F4abR+/F4acR+/F4adR-) (mean 0.487). Between the phenotypes A and F, B and C or F, the means of adhesion were significantly different as well (data not
shown). For A and B like C and F the difference in means for the F4ab bacteria adhesion was not significant (data not shown).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>128</td>
<td>1.188</td>
<td>0.268</td>
</tr>
<tr>
<td>C</td>
<td>130</td>
<td>0.487</td>
<td>0.215</td>
</tr>
</tbody>
</table>

SD = standard deviation
Separate variance t = 23.118, df = 243, P = 0.00
Difference in means = 0.701, 95% CI = 0.641 to 0.760

The adhesion strength of F4ac bacteria was not significant (P = 0.245) between phenotypes A and B (Table 3.3 and Figure 3.4). The F4ac-receptor in phenotypes A and
B displays high adhesion strength, 1.130 and 1.086, respectively. Additionally, the normal curves of both phenotypes fit well with the number of observations, while it is not the case for the adhesion of F4ab (Figure 3.3) and F4ad (Figures 3.5 and 3.6).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>128</td>
<td>1.130</td>
<td>0.236</td>
</tr>
<tr>
<td>B</td>
<td>66</td>
<td>1.086</td>
<td>0.251</td>
</tr>
</tbody>
</table>

SD = standard deviation
Separate variance t = 1.168; df = 124.4; P = 0.245
Difference in means = 0.043; 95% CI = -0.030 to 0.117

Two-sample t tests of F4adR adhesion strength, which is expressed in phenotypes A, C and D, showed high standard deviations (SD) of 0.425 in phenotype A,
of 0.517 in C (Table 3.4 and Figure 3.5) and of 0.345 in phenotype D (Table 3.5). Phenotype A did not differ \( (P = 0.554) \) from phenotype C (Table 3.4 and Figure 3.5), while the F4adR adhesion strength in phenotype A (mean 0.896) was significantly \( (P = 0.000) \) superior than in phenotype D (mean 0.610) (Table 3.5 and Figure 3.6). Similarly enterocytes bound more F4ad bacteria in phenotype C than in phenotype D (results not shown).

### Table 3.4
Two-sample t-test of the converted percentage of enterocytes of pigs binding more than five F4ad bacteria to the total amount of enterocytes examined. Comparison between phenotypes A and C.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>128</td>
<td>0.896</td>
<td>0.425</td>
</tr>
<tr>
<td>C</td>
<td>130</td>
<td>0.861</td>
<td>0.517</td>
</tr>
</tbody>
</table>

SD = standard deviation
Separate variance \( t = 0.593; \) df = 248.2; \( P = 0.554 \)
Difference in means = 0.035; 95% CI = -0.081 to 0.151

### Figure 3.5
Graphical distribution of the converted percentage of enterocytes of pigs binding more than five F4ad bacteria to the total amount of enterocytes examined (y axis) and of the number of pigs (x axis) of phenotypes A and C.
The length of the two boxes shows the range of 50% of the values and the vertical lines outgoing from the boxes indicate the range of the remaining values. The horizontal line in the box marks the median.
Table 3.5 Two-sample t test of the converted percentage of enterocytes of pigs binding more than five F4ad bacteria to the total amount of enterocytes examined. Comparison between phenotypes A and D.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>128</td>
<td>0.896</td>
<td>0.425</td>
</tr>
<tr>
<td>D</td>
<td>69</td>
<td>0.610</td>
<td>0.345</td>
</tr>
</tbody>
</table>

SD = standard deviation
Separate variance t = 5.106; df = 165.3; P = 0.000
Difference in means = 0.286; 95% CI = 0.175 to 0.397

III.1.3 Correlation of adhesion strength (AS) between different receptor phenotypes

The adhesion strength (AS) is defined as the converted percentage of enterocytes for a pig binding more than five bacteria to the total amount of enterocytes examined. The correlation of AS between F4ab and F4ac of phenotype A (n = 128) were r = 0.46,
between F4ab and F4ad \( r = 0.08 \) and between F4ac and F4ad \( r = 0.07 \). In phenotype B (\( n = 66 \)) the correlation between F4ab and F4ac was \( r = 0.66 \). Correlation between F4ab and F4ad of phenotype C (\( n = 130 \)) was \( r = 0.50 \). The correlations between F4ab and F4ac in phenotype A (\( r = 0.46 \)) and B (\( r = 0.66 \)) were lower than the correlation between the distribution of F4ab and of F4ac (\( r = 0.85 \)) (Figure 3.1). The first correlation was lower because it was calculated between receptor types considering the combined adhesion strength for each pig. The correlation calculated from the distribution of the pigs was higher due to the fact that both receptor types were considered independently from the individual pig.

### III.1.4 Effects of the quarter, phenotype and phenotype-quarter interaction on adhesion strength

Analysis of the variance was performed with two models as described in II.4.3. In model I, the influence of quarter on AS was determined in each phenotype (Table 3.6). Model II involved additionally the phenotype effect and the interaction between phenotype and quarter effect (Table 3.7). The date (grouped in quarter) of the test has no influence on the amount of F4ac adhering bacteria in both phenotypes A and B, whereas the variance due to the quarter was significant in phenotypes A, C and F for the receptor F4abR. The impact of the quarter on the adhesion of F4ad bacteria is significant in phenotypes A and D (Table 3.6).

**Table 3.6** Effect of quarter on the adhesion strength of receptors F4abR, F4acR and F4adR in each phenotype. Model I was used for statistical analysis. Probability values (P-values) are indicated for each receptor type.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>n</th>
<th>Periods</th>
<th>F4abR</th>
<th>F4acR</th>
<th>F4adR</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>128</td>
<td>6</td>
<td>0.00</td>
<td>NS</td>
<td>0.04</td>
</tr>
<tr>
<td>B</td>
<td>66</td>
<td>5</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>130</td>
<td>11</td>
<td>0.02</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>69</td>
<td>11</td>
<td></td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>30</td>
<td>9</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS = non-significant, \( n = \) number of observations

In Table 3.7 (model II), the effects of phenotype, quarter, and phenotype-quarter interaction did not modify significantly the results of F4ad adhesion. This could be due to the huge standard deviation in adhesion strength of F4ad bacteria. For F4acR the quarter is the only effect to be significant, whereas in model I the quarter influence was
not significant in phenotypes A and B. Furthermore, the adhesion to the receptor F4abR was affected by all three factors (Table 3.7).

Table 3.7 Effects of phenotype, quarter and phenotype-quarter interaction on the adhesion strength of receptors F4abR, F4acR and F4adR. Model II was used for statistical analysis. Probability values (P-values) are indicated for each receptor type.

<table>
<thead>
<tr>
<th>Effect</th>
<th>F4abR</th>
<th>F4acR</th>
<th>F4adR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>0.00</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Quarter</td>
<td>0.01</td>
<td>0.00</td>
<td>NS</td>
</tr>
<tr>
<td>Phenotype_quarter</td>
<td>0.00</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>n</td>
<td>354</td>
<td>194</td>
<td>327</td>
</tr>
</tbody>
</table>

NS = non-significant, n = number of observations

The least square means observed for AS (vertical axis) of the three fimbrial variants (F4ab, F4ac and F4ad) in regard to their adhesive phenotype (horizontal axis) are summarized in Figure 3.7. The number of observations in each phenotype was the same as in Table 3.6. The graph to the left illustrates the four adhesive phenotypes of the receptor F4abR: the weak adhesion (F4abRw) in C and F, and the strong adhesion in A and B. F4acR is also a high adhesion strength receptor in A and B (graph in the middle). The last receptor F4adR (graph to the right) is characterized by a less strong adhesion in A and C than F4acR and F4abR (in A and B), and a weak adhesion in D.

Figure 3.7 Least square means for adhesion strength (AS) of F4ab, F4ac and F4ad grouped by phenotype. Vertical bars represent mean values ± standard deviations of AS. AS is defined as the converted percentage of enterocytes for a pig binding more than five bacteria to the total amount of enterocytes examined.
Figure 3.8 shows the scatterplot of adhesion strength depending on test date (quarter) for the three fimbrial variants in each phenotype. An increase of strength was observed in the phenotypes A (n = 128) and B (n = 66) for F4abR and F4acR for a period of six quarters (18 months). On the other hand, F4adR tends to decline slightly in A, C (n = 130) and D (n = 69) like F4abR in C and F (n = 30) during the whole period (36 months). Observations in phenotypes A and B broke off after six quarters only because matings resistant to F4ac were produced to examine the inheritance of the weak receptor F4abRw.
Figure 3.8 Bivariate scatterplot with quarter as x variable and the least square mean of adhesion strength for each receptor as y variable for phenotypes A, B, C, D, E, F. The regression line indicates the tendency of the adhesion strength in course of time (quarters).
III.2 Mapping of receptor locus \textit{F4acR}

Pairwise lod scores (Z) and recombination fractions (\( \theta \)) of \textit{F4acR} and 17 chromosome 13 marker loci are presented in Table 3.8. All markers were significantly linked (Z = 8.6 to 43.6) with \textit{F4acR}. Two-point analysis provided a recombination fraction of 0.00 between \textit{S0075} and \textit{F4acR}. Small recombination fractions of 0.01 were obtained with \textit{S0283}, \textit{Sw1030}, \textit{Sw1876}, \textit{Sw225} and \textit{Sw2459}. Other marker loci had recombination fractions between 0.02 and 0.11.

<table>
<thead>
<tr>
<th>Marker</th>
<th>( \theta )</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S0075}</td>
<td>0.00</td>
<td>33.7</td>
</tr>
<tr>
<td>\textit{S0283}</td>
<td>0.01</td>
<td>43.2</td>
</tr>
<tr>
<td>\textit{Sw1030}</td>
<td>0.01</td>
<td>34.6</td>
</tr>
<tr>
<td>\textit{Sw1876}</td>
<td>0.01</td>
<td>25.6</td>
</tr>
<tr>
<td>\textit{Sw225}</td>
<td>0.01</td>
<td>25.0</td>
</tr>
<tr>
<td>\textit{Sw2459}</td>
<td>0.01</td>
<td>29.1</td>
</tr>
<tr>
<td>\textit{Sw1386}</td>
<td>0.02</td>
<td>25.8</td>
</tr>
<tr>
<td>\textit{S0068}</td>
<td>0.03</td>
<td>28.0</td>
</tr>
<tr>
<td>\textit{Sw2007}</td>
<td>0.03</td>
<td>8.6</td>
</tr>
<tr>
<td>\textit{Swr1627}</td>
<td>0.03</td>
<td>15.1</td>
</tr>
<tr>
<td>\textit{Sw698}</td>
<td>0.03</td>
<td>43.6</td>
</tr>
<tr>
<td>\textit{Sw398}</td>
<td>0.04</td>
<td>39.6</td>
</tr>
<tr>
<td>\textit{Sw520}</td>
<td>0.04</td>
<td>33.8</td>
</tr>
<tr>
<td>\textit{TF}</td>
<td>0.04</td>
<td>11.7</td>
</tr>
<tr>
<td>\textit{Sw207}</td>
<td>0.05</td>
<td>21.2</td>
</tr>
<tr>
<td>\textit{Swr1306}</td>
<td>0.06</td>
<td>13.0</td>
</tr>
<tr>
<td>\textit{S0222}</td>
<td>0.11</td>
<td>17.9</td>
</tr>
</tbody>
</table>

The 17-point analysis allows ordering the locus \textit{F4acR} and the 16 marker loci (Figure 3.9). The marker \textit{Sw1386} could not be precisely mapped and, consequently, was not incorporated into the genetic map. Our map is compared to the sex averaged Nordic 2 map of Marklund \textit{et al}. (1996). The differences in log likelihood (\( \Delta \log L \)) against the inversion of adjacent loci are given to the right of the map. \textit{F4acR} lies inside of the interval \textit{Sw207-S0283}, spanning 7.2 Kosambi cM according to our analysis or 1.6 cM in
accordance with Rohrer et al. (1994, 1996). S0075 is the single marker with a recombination fraction of zero with F4acR. Nevertheless some pigs were recombinant for S0075 (Table 3.9) because the Maximum Likelihood analysis maximizes the probability that the observed data fits the model. The alternative order Sw225-S0075 is weakly significant ($Z = 1.9$). But the order Sw207-(F4acR, S0075)-Sw225-S0283 is the most probable.

To get a more detailed map, we typed our pigs with the additional microsatellites Swr2189 and S0293. But both genetic markers were not informative and consequently, could not be used for the linkage analysis.

Figure 3.9 Assignment of the F4acR (K88AC in Nordic 2) gene on porcine chromosome 13. Comparison between Nordic 2 map (Marklund et al. 1996) and the map constructed based on our findings. Sex-averaged map distances are given in Kosambi cM and common loci in both maps are connected by a line. Differences in log likelihood ($\Delta \log L$) against the inversion of adjacent loci are shown to the right of the map. The order of adjacent loci supported by odds of at least 1000:1 is significant. Marker Sw1386 could not be mapped precisely. The interval of its localization is indicated by a vertical bar.
Table 3.9 contains six recombinant offspring with the genotype of eight microsatellites and F4acR. These recombinants were derived from families used for linkage analysis of F4acR. The nine genotypes are shown on the left half of Table 3.9. The most probable haplotype of parents is based on several litters as well as a three generation pedigree. The most probable paternal (from ♂, haplotype 1 or 2) and maternal haplotype (from ♀, haplotype 3 or 4) transmitted to offspring (F1) is shown on the right half of Table 3.9. The significant order S0068-Sw207-Sw225-S0283-Sw698-Sw1030 is based on our 17-point analysis which assigns F4acR inside the interval Sw207-S0283 (Figure 3.9). Swr1627 is localized in the order Sw225-S0283-Swr1627-Sw698 according to our linkage analysis and Rohrer et al. (1996). Marker loci S0222, Swr1306, Sw2459, Sw520, Sw398, Sw1386 and TF were omitted in Table 3.9 due to their long distance to the receptor locus F4acR. Both microsatellites Sw1876 and Sw2007 were not included because they were homozygous in most parents and thus not informative. Recombinant pigs provide information for order of S0075 and F4acR in the interval Sw207-S0283. Recombinant pig 757 indicates that F4acR should be localized in the range S0075-Sw1030. Pig 757 has inherited the paternal haplotype 2 and the maternal haplotype 3. A crossing over occurred in the maternal haplotype 3: alleles of S0068 and Sw207 originate from maternal haplotype 4 associated with the resistant allele (s) for F4acR and the other alleles correspond to maternal haplotype 3. As pig 757 was identified as susceptible to F4ac bacteria in the adhesion test, the locus for the receptor F4acR should be localized in the range S0075-Sw1030 (bold) of maternal haplotype 3 which is associated with the susceptible allele (S). Resistant offspring 795 suggest same localization of F4acR in microsatellite block S0075-Sw1030 (bold) from maternal haplotype 4 associated with the resistant allele (s). Microsatellite block S0068 to Sw225 (bold) should contain F4acR in pig 836. The maternal haplotype 3 (microsatellites S0068 to Sw225, and Sw1030) associated with the susceptible allele (S) was transmitted to the offspring 836 identified as susceptible to F4ac bacteria and alleles of S0283 to Sw698 from phase 4 were recombinant in the maternal haplotype 3. Offspring 606 and 607 were recombinant for S0283 in the maternal haplotype 3, but this crossing over is not informative because the mother 198 is homozygous resistant (ss). Both offspring 606 and 607 show informative recombination for haplotypes 1 and 2 from the heterozygous susceptible (Ss) father. Pig 607 that was identified susceptible in
the adhesion test received the paternal haplotype 2 (*S0068* to *S0075*, and *Sw698* to *Sw1030*) associated with the susceptible allele. A recombination occurred for alleles of *Sw225* to *Swrl627* from the paternal haplotype 1 (associated with the resistant allele for *F4acR*) in haplotype 2 of pig 607. Therefore microsatellite block *S0068-S0075* (bold) contains the locus for the receptor *F4acR* in pig 607. Pig 606 is a double recombinant because two crossing overs happened in the paternal haplotype 1: the first locus to recombinate is the microsatellite *Sw698* from haplotype 2 and the second one is the block *S0068-S0075* from the same haplotype. The rest of alleles (microsatellites *Sw225*, *S0283* and *Swrl627*) of resistant pig 606 belongs to the paternal haplotype 1 associated with the resistant allele (s). Thus pig 606 indicates that block *Sw225-Swrl627* encloses *F4acR*.

Therefore informative recombinant offspring allows us to refine the order of *F4acR* compared to the linkage analysis (Figure 3.9). In particular, recombinant pigs 606 and 607 argue for a localization of *F4acR* between *S0075* and *Sw225*. Thus the proposed order is *Sw207-S0075-F4acR-Sw225-S0283*. 

Table 3.9 The occurrence of crossing overs in informative offspring allows predicting the most plausible localization (bold) of the receptor locus $F_{4acR}$ in the linkage group $S0068$-$Sw207$-$S0075$-$F_{4acR}$-$Sw225$-$S0283$-$Swr1627$-$Sw698$-$Sw1030$. $F_{4acR}$ is localized in the interval $Sw207$-$S0283$ according to our linkage analysis.

<table>
<thead>
<tr>
<th>Parents and offspring</th>
<th>Genotypes</th>
<th>Haplotypes of parents and probable haplotypes of offspring for order $S0068$-$Sw207$-$S0075$-$F_{4acR}$-$Sw225$-$S0283$-$Swr1627$-$Sw698$-$Sw1030$ (phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S0068$</td>
<td>$Sw207$</td>
<td>$S0075$</td>
</tr>
</tbody>
</table>

- $\delta$ = father  
- $\varphi$ = mother  
- $F_{1}$ = offspring  
- $\uparrow$ = recombinant  
- $\updownarrow$ = most probable recombination  
- Bold alleles indicate the range of localization of $F_{4acR}$ based on the recombinant offspring
Physical mapping of the four candidate genes (TFRC, B3GnT5, B4GALT4 and B3GALT3) on human chromosome 3 (HSA3) and on porcine chromosome 13 (SSC13) is shown in Figure 3.10. Information on the mapping of the four genes to human chromosome 3 was obtained from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi. TFRC was mapped with the IMpRH (INRA-Minnesota Porcine Radiation Hybrid) panel, containing 118 hybrid clones (Yerle et al. 1998), on the porcine genetic map by Van Poucke et al. (2001). TFRC was closely linked to marker Swr2189 (not shown) in the Radiation Hybrid map (Van Poucke et al. 2001). B4GALT4 was mapped by FISH (fluorescence in situ hybridization) to band 13q41—q42 (Van Poucke et al. 2001). We assigned both genes B3GALT3 and B3GnT5 by means of the somatic cell hybrid panel (Yerle et al. 1996) to the band 13q23—q41 (III.7.8).

Furthermore seven genetic markers used for linkage analysis were physically mapped to SSC13: Sw207 and S0075 to band 13q41 (Jorgensen et al. 2002), Sw225 to 13q44 (Jorgensen et al. 2002), Sw1386 to 13q46—q47 and S0283 to 13q41 by Lopez-Corrales et al. (1999), TF (Chowdhary et al. 1993) and Sw2459 to band 13q31 (Rohrer et al. 1996).

The previously comparative data between SSC13 and HSA3 (Sun et al. 1999; Van Poucke et al. 1999; Pinton et al. 2000) is compiled in this modified map of Van Poucke et al. (2001) in Figure 3.10. According to our multipoint linkage analysis (Figure 3.9), the gene F4acR is close to S0283 (0.01 recombination fraction) and S0075 microsatellites, which were physically mapped to band 13q41. The 13q41 region contains a rearrangement breakpoint between blocks 3 and 4.
III.3 Linkage disequilibrium

Highest chi-square values with one degree of freedom (df) were obtained for linkage disequilibrium between microsatellites $S0075$ and $Sw225$, and $F4acR$ (2.25 and 2.27, respectively). Levels of both markers were not significant ($P = 0.13$). Eight adhesive pigs issued from heterozygous parents for $Sw225$, and 22 pigs for $S0075$ were available to calculate if the allele 106 for $Sw225$ and the allele 136 for $S0075$ were associated with the allele $F4acR^+$. 

Figure 3.10 Comparative map between human chromosome 3 (HSA3) and porcine chromosome 13 (SSC13). The localization of the four candidate genes is shown on HSA3 and SSC13 (except TFRC). Seven marker loci used for linkage analysis of $F4acR$ were physically mapped by FISH on SSC13. (Comparative map modified from Van Poucke et al. 2001)
III.4  *F4abRw* inheritance

Offspring from parents resistant to F4ac were slaughtered to investigate the inheritance of the weak *F4abRw* (Table 3.10). Ratios of susceptible to resistant offspring in 23 litters did not allow to clarify the transmission of the weak receptor *F4abRw*. *F4abRw* phenotype was determined only for two sows (249 and 9391, both show no adhesion to F4ab bacteria). Two matings were repeated: 194 × 195 and 214 × 198. The first mating gave an identical ratio, the second one (214 × 198) gave an opposite ratio (ten susceptible to one resistant and two susceptible to nine resistant). If a dominant inheritance of the weak receptor *F4abRw* is assumed, parents of matings 194 × 280, 194 × 195 and 214 × 250 should be homozygous resistant (bb) because all their offspring were resistant to F4ab bacteria in the adhesion test. Boar 194 mated to 249 (bb) had four susceptible and five resistant offspring; therefore, he is likely heterozygous susceptible (Bb). However, other matings show inconsistencies. For example, 194 (Bb) × 280 (bb) gave ten resistant offspring, 214 (bb) × 249 (bb) seven susceptible and four resistant offspring. Conversely, data of pigs segregating for *F4abRw* as a recessive trait also give contradictory results. Parents with susceptible offspring must have at least one susceptible recessive allele (b). Thus pigs 194 (first seven matings), 195 (mated to 194, 193 and 197), 280 and 214 (214 × 280) should have at least one b allele. But matings 214 (Bb) × 195 (Bb) and 194 (Bb) × 280 (Bb) had no susceptible offspring (bb).
Table 3.10 Numbers of pigs show the susceptible (F4abR\(^+\)) or
the resistant phenotype (F4abR\(^-\)) in 23 litters segregating for the
weak receptor F4abR\(^w\). Offspring were produced from matings
resistant to F4ac.

<table>
<thead>
<tr>
<th>Parents</th>
<th>Offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boar</td>
<td>Sow</td>
</tr>
<tr>
<td>194 x</td>
<td>195</td>
</tr>
<tr>
<td>194 x</td>
<td>195</td>
</tr>
<tr>
<td>194 x</td>
<td>198</td>
</tr>
<tr>
<td>194 x</td>
<td>249</td>
</tr>
<tr>
<td>194 x</td>
<td>215</td>
</tr>
<tr>
<td>194 x</td>
<td>207</td>
</tr>
<tr>
<td>194 x</td>
<td>9391</td>
</tr>
<tr>
<td>194 x</td>
<td>280</td>
</tr>
<tr>
<td>193 x</td>
<td>195</td>
</tr>
<tr>
<td>193 x</td>
<td>246</td>
</tr>
<tr>
<td>193 x</td>
<td>207</td>
</tr>
<tr>
<td>193 x</td>
<td>215</td>
</tr>
<tr>
<td>193 x</td>
<td>198</td>
</tr>
<tr>
<td>214 x</td>
<td>195</td>
</tr>
<tr>
<td>214 x</td>
<td>249</td>
</tr>
<tr>
<td>214 x</td>
<td>198</td>
</tr>
<tr>
<td>214 x</td>
<td>198</td>
</tr>
<tr>
<td>214 x</td>
<td>280</td>
</tr>
<tr>
<td>214 x</td>
<td>207</td>
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<tr>
<td>214 x</td>
<td>215</td>
</tr>
<tr>
<td>214 x</td>
<td>250</td>
</tr>
<tr>
<td>197 x</td>
<td>195</td>
</tr>
<tr>
<td>197 x</td>
<td>215</td>
</tr>
</tbody>
</table>

\(^1\)Threshold value ≥ 2.5% enterocytes (1 of 40) with > 5 adhering
bacteria

III.5  F4adR inheritance

Observed ratios of susceptible to resistant offspring in 25 litters confirmed the dominant
inheritance (DD and Dd for susceptible pigs to F4ad bacteria and only carriers of two
alleles, i.e. dd, are resistant) of the receptor F4adR. The observed ratios in 123 pigs (Dd
× dd matings) and in 129 pigs (Dd × Dd matings) did not deviate significantly from the
expected ratios: 1:1 ratio (χ\(^2\) = 0.08; P = 0.78) and 3:1 ratio (χ\(^2\) = 2.71; P = 0.10; 1 df),
respectively. For significance at the 0.05 level, chi-square should be greater than or equal to 3.84.

III.6 F4adR linkage analysis

Three Large White litters (27 F1 pigs and six parents) used for the genome-wide scan lacked informative genotypes. The parents were previously supposed homozygous resistant × heterozygous. Two years later, after slaughter, all parents were found to be heterozygous susceptible. Such breeding scheme is not suitable to show or to exclude linkage. A larger sample (136 pigs) consisting of 11 litters (1 Dd × dd and one Dd × Dd) was used for chromosomes 4 and 11, and the same families as for F4acR were used for chromosome 13 (II.1.2.1) linkage analysis. No linkage was found between F4adR and the microsatellite markers on chromosomes 4, 11 and 13.

III.7 Candidate genes

III.7.1 TFRC

Amplification of a probe (1,223 bp) for the transferrin receptor (human TFRC: M11507) was performed with TFRC-hsa-f3/r4 primers using as template λ-phage cDNA extracted from the small intestine library because TFRC is made up of 19 exons. After isolation and sequencing of clones containing the gene, the complete coding sequence of the porcine TFRC gene (2,413 bp) was known and submitted to the GenBank (http://www.ncbi.nlm.nih.gov) under the accession number AF416763. The porcine coding sequence of TFRC is 2,307 bp in length coding for 769 amino acids. Thirty-five bp belong to 5' UTR and 71 bp to 3' UTR. Comparison between the sequences of three pigs each resistant (ss) and susceptible (SS) to F4ac bacteria did not reveal any informative single nucleotide polymorphisms (SNPs). The whole coding sequence of each pig was cloned in eight overlapping clones for sequencing. One to six clones per pig showed SNPs. Most SNPs were found in only one clone. Few SNPs were in several pigs and were not associated with resistance and susceptibility.

III.7.2 B3GnT5

To obtain a probe for the screening of B3GnT5 porcine genomic DNA was amplified, as the gene is contained in a single exon according to the human sequence AF368169
(Henion et al. 2001). The cDNA library was screened with PCR products (397 bp) of primers T5-hsa-f1/r2. Genomic DNA was amplified by two successive standard PCRs (II.2.3) with following modifications: 1.2 μM primers and 40 s for the extension. Genomic DNA sequencing of both F4ac phenotypes showed no polymorphism in B3GnT5. We deposited the porcine coding sequence of the B3GnT5 gene under the accession number AY253338. Porcine B3GnT5 gene is 3037 bp in length: 558 bp for 5' UTR, 1,134 bp for the coding sequence or 378 amino acids and 1,345 bp for 5' UTR.

III.7.3 B4GALT4

The coding sequence of B4GALT4 is arranged in six exons (Schwientek et al. 1998). λ-phage cDNA extracted from the small intestine library was used for the amplification of a 119 bp fragment with T4-hsa-f1/r2 (human B4GALT4: BC004523). The PCR product was re-amplified with 45 s for the annealing and extension. No informative polymorphism was found in the gene B4GALT4 after sequencing of the RT-PCR product. Only a deletion of approximately 60 bp was found in B4GALT4 of a pig after reverse transcription, but a second RT-PCR of the same pig showed that it was an artefact. We submitted the partial porcine coding sequence of the B4GALT4 gene under the accession number AY253339. The porcine B4GALT4 gene is 1,732 bp in length. About 100 bp were missing at the 5' end of the 935 bp coding sequence. The 3' UTR is 797 bp in length.

III.7.4 B3GALT3

The porcine primer T3-f5 was designed based on the sequence obtained from the human primers T3-hsa-f1/r2 (human B3GALT3: AF154848). A probe of 615 bp was generated by T3-f5 and the human reverse primer T3-hsa-r6. Genomic porcine DNA was taken, as B3GALT3 is contained in a single exon (Hennet et al. 1998). The porcine coding sequence of the B3GALT3 gene was deposited under the accession number AY253340. Porcine B3GALT3 gene is 1,843 bp in length: 34 bp for 5' UTR, 996 bp for the coding sequence or 332 amino acids and 813 bp for 5' UTR. Two SNPs in the coding sequence at positions 295 (C/T) and 313 (T/C) were detected after sequencing of genomic DNA. These two SNPs did not affect the amino acid sequence.
III.7.5 Identity with human sequence

Identity between human and porcine genes for the coding sequence is 85.3% (TFRC), 84.2% (B3GnT5), 85.4% (B4GALT4) and 87.3% (B3GALT3); for the amino acid sequence 78.5% (TFRC), 89.1% (B3GnT5), 90.9% (B4GALT4) and 95.8% (B3GALT3).

III.7.6 Nucleotide 1752 at TFRC as SNP artefact

A part of genomic DNA in TFRC was sequenced for the design of primers around the putative mutation 1752 in the SNaPshot method (II.2.8). The genomic structure of the transferrin receptor is made up of 19 exons (McClelland et al. 1984). Exon 17 should contain the mutation at position 1752. The amplification of genomic DNA by the primer TFRC-f7 (exon 15) and TFRC-r8 (exon 17) generated a product of about 3,500 bp. The intron p between exon 16 and 17 is about 3,000 bp in length. The end of intron p was sequenced with the reverse primer TFRC-r8 to design a forward primer in intron p. A forward primer (TFRC-mut1752) ending one base before the mutation was designed. The SNaPshot procedure could begin by the genomic amplification with TFRC-intron-p-f5 and TFRC-r8.

Genomic DNA of pig 175 was used for SNaPshot analysis and revealed a T base. The plasmid DNA of pig 175 (after RT-PCR and cloning) showed a C base whereas other clones displayed a T base (Figure 3.11). Thus the base C at position 1752 of TFRC (AF416763) in the plasmid clone of pig 175 is probably due to an artefact caused by Taq polymerase incorporations (during reverse transcription, PCR, cloning or sequencing). Therefore RT-PCR has generated polymorphisms, which revealed artefacts after sequencing of genomic DNA.

Figure 3.11 Artefact revealed by SNaPshot at nucleotide 1752 of porcine TFRC. The T base in genomic DNA (A) is represented by a red single peak (*) and a C base by a black peak (**) is detected in plasmid DNA (B) of the same pig.
III.7.7 Two polymorphisms in B3GALT3

Two silent mutations at positions 295 (C/T) and 313 (T/C) in the coding sequence (Genbank accession number AY253340) were found in B3GALT3. The mutation 313C (AG ↓ CT) offers a cutting site for the enzyme Alul. Three fragments could be generated by Alul PCR-RFLP (restriction fragment length polymorphism) (Figure 3.12). Sequencing and digestion revealed three genotypes: homozygous 295T/T-313C/C (two fragments of 166 and 237 bp), heterozygous 295C/T-313T/C (166, 237 and 403 bp) and homozygous 295C/C-313T/T (403 bp). On a set of ten non-related pigs, eight (gel not shown) were homozygous 295T/T-313C/C, indicating that this allele is the wild type. F4acR genotypes do not correlate with the polymorphisms of B3GALT3. F4acR genotype Ss occurs in the three genotypes of B3GALT3 and F4acR genotype ss in two genotypes.

![Figure 3.12 Alul PCR-RFLP of B3GALT3](image)

Figure 3.12 Alul PCR-RFLP of B3GALT3. Genomic DNA was amplified with the primers T3-f13/r12, digested with Alul and fragments were separated on 1.5% agarose gel. Lanes 1 and 2 show homozygous pigs (at base 295C/C-313T/T); lanes 3, 4 and 6 homozygous pigs (295T/T-313C/C) and lanes 5 and 7 heterozygous pigs (295C/T-313T/C). F4acR genotypes are indicated for each pig: lanes 1, 2, 4 and 7 show heterozygous susceptible (Ss) pigs, and lanes 3, 5 and 6 resistant pigs (ss).
III.7.8 Chromosomal assignment of $B3GnT5$ and $B3GALT3$

Statistical evaluation of the PCR results for chromosome probabilities, region probabilities and correlation was performed according to Chevalet et al. (1997) at http://www.toulouse.inra.fr/lgc/pig/pcr/pcr.htm. The genes $B3GnT5$ (Figure 3.13) and $B3GALT3$ (Figure 3.14) were mapped to band q23 $\rightarrow$ q41 (half way) of porcine chromosome 13 with a high probability ($P = 0.80$, error risk $< 0.005$) and with a maximal correlation.

![Figure 3.13 PCR analysis of the INRA cell hybrid panel for assignment of porcine $B3GnT5$. The expected fragment size (262 bp, primers T5-sch-f1/r2) is visible in the rodent-pig hybrid cell lines 6, 12 and 22. Positive control from porcine genomic DNA (P) and negative controls from mouse (M), hamster (H) and water (W) were used. M indicates the 100 bp DNA size marker.](image)
III.7.9 Northern blot analysis

Hybridization of a specific probe (TFRC, B3GnT5, and B4GALT4) failed and is not shown. The successful hybridization of the housekeeping gene beta-actin with total RNA of three different genotypes indicates that the method was working. Northern blot analysis is possibly not enough sensitive to detect expression of these three genes.
IV. DISCUSSION

IV.1 Phenotypes

In the 534 pigs of the experimental herd slaughtered at eight weeks of age (Table 3.1), all six phenotypes described by Bijlsma et al. (1982) inclusive the phenotype F (Baker et al. 1997) were observed. Therefore, our in vitro adhesion test was enough sensitive to detect the weak adhesion phenotype of F4ab bacteria in the phenotypes C and F as well as the weak adhesion of F4ad bacteria in phenotype D.

When the 38 fattening pigs from the testing station were considered in view of F4 phenotype distribution in the Swiss pig population, only 18% of the pigs were resistant (phenotypes D and E) to F4ab and F4ac bacteria and the double receptor phenotypes A and B were detected in the majority of the fattening pigs (79%). In the study of Gautschi and Schwörer (1988), approximately half of the Swiss Landrace and Large White pigs were susceptible to F4ab and to F4ac bacteria (F4ad adhesion was not analysed). This higher proportion of susceptible pigs in our study could be due either to the small random sample and therefore these pigs would be not representative of the Swiss pig population, or the phenotyping of Gautschi and Schwörer can not be compared with ours. From these data it appears that the proportion of pigs susceptible to E. coli F4 has increased in the Swiss pig population during last years.

Seventy-one percent among pigs of four breeds (Chester White, Duroc, Hampshire and Yorkshire) were represented by A (43%) and E (28%) phenotypes (Baker et al. 1997). Chinese Meishan was reported uniformly resistant to F4ac E. coli and the susceptible allele exists at a low frequency (8%) in Minzhu breed (Michaels et al. 1994). Thus the allele of susceptibility is persistent among breeds except in Chinese breeds. This persistence could be explained by the passive immunisation of the piglets during the first weeks of life. In fact offspring born to susceptible sows are protected by antibodies present in colostrum, whereas resistant sows mated with homozygous sensitive boars give the highest incidence of diarrhea in offspring (Sellwood 1979). Another explanation of the high representation of phenotype A could be due to a relation between the performance traits and F4 susceptibility, but results of studies are contradictory. For susceptible pigs from birth to 25 kg, Gautschi and Schwörer (1988)
reported a later start in the fattening program: two days for Landrace and three days for Large White. Loci in the chromosomal region of F-4acR had effects on daily gain during the fattening period according to Edfors-Lilja et al. (1986), while Gautschi and Schwörer (1988) did not discover any significant difference. Gibbons et al. (1977) failed to show a difference between resistant and susceptible pigs in various production parameters.

IV.2  **Weak adhesion**

**IV.2.1 F4ab receptor**

Interestingly, enterocyte brush borders of the pigs of phenotypes C and F bound less bacteria of the F4ab variant than in phenotypes A and B. This difference in F4ab adhesion was mentioned by Baker et al. (1997). When the receptor F4acR was missing as in phenotypes C and F, enterocytes bound less F4ab bacteria; in presence of the receptor F4acR, the adhesion of F4ab bacteria to enterocytes is strong. Therefore we designated the receptor F4abR in phenotypes C (F4abR⁺ / F4acR⁻ / F4adR⁺) and F (F4abR⁺/F4acR⁻/F4adR⁻) as weak receptor (F4abR⁺⁻).

**IV.2.2 F4ad receptor(s)**

Three adult pigs showed the phenotype D, in contradiction with the hypothesis of Hu et al. (1993) (III.1.1). Hu et al. observed two types of adhesion for F4ad E. coli: a high adhesion receptor in phenotype A (F4abr⁺ / F4acr⁺ / F4adr⁺) expressed during entire life and a low adhesion receptor in phenotype D (F4abr⁻ / F4acr⁻ / F4adr⁻), whose expression is terminated at 16 weeks of age. Thus the age of the pigs did not influence the phenotype D in our study. The strong adhesion to the receptor F4adR in phenotype A and the weak adhesion in phenotype D support the observations of two types of receptor. The study of Hu et al. (1993) was in agreement with the conclusions of Rapacz and Hasler-Rapacz (1986) who suggested phenotype A corresponds to phenotype IV and phenotype D corresponds to phenotype II. Hu et al. (1993) and Rapacz and Hasler-Rapacz (1986) did not identify any pigs with the phenotype C (F4abR⁻ / F4acR⁻ / F4adR⁻). Therefore we cannot compare the adhesion of F4ad in phenotype C. In our study, F4ad adhesion in phenotype C appears to be more like the adhesion seen in phenotype A than in D (Table 3.1).
High standard deviations between 29% and 39% in the adhesion strength of F4adR were observed compared to the other receptors (Table 3.1). Many litters tested in the adhesion test showed no uniform phenotypes with F4ad bacteria e.g. pigs from a same litter and tested the same day could exhibit three phenotypes of F4adR: a "fully resistant" phenotype with all enterocytes without bacteria, a "fully susceptible" phenotype with all enterocytes binding more than five bacteria, and an intermediate susceptible/resistant phenotype characterized by adhesive and non-adhesive enterocytes. This intermediate phenotype was named mixed adhesive phenotype by Rapacz and Hasler-Rapacz (1986) and was also detected by Hu et al. (1993). Two maxima at very low and high adhesion strength are visible from the distribution of the 327 pigs susceptible to F4ad bacteria (Figure 3.2), while the distribution of F4acR positive pigs (Figure 3.1) accumulates in the range of 70 to 100% of enterocytes binding more than five bacteria. Hypothetically, external factors affect particularly the results with F4ad bacteria. Although the same procedure was applied for the three bacterial variants in the adhesion test, highly variable strength of adhesion of F4ad fimbriae was observed. Therefore, the strong and the weak receptor observed for F4ad must not represent two distinct phenotypes or receptors like for F4abR (strong and weak).

The non-significant deviation of ratios is in favour of a dominant inheritance of F4adR against a recessive one (III.5). However, the observed percentages of pigs devoid of the receptor F4adR were higher than expected: 52% of observed resistant pigs (expected 50%) from Dd x dd matings and 35.7% (expected 25%) in pigs from heterozygous parents. The high percentage of resistant pigs could be due to the different kind of receptor; F4abR and F4acR are rather glycoproteins and F4adR seems to be a glycolipid (Billey et al. 1998; Erickson et al. 1992, 1994; Grange et al. 1996).

IV.2.3 F4ac receptor

Out of 299 offspring, which were all considered to be resistant to F4ac due to resistant parents, 14 pigs were adhesive. Among these 14 pigs, nine pigs from eight litters had few bacteria bound to enterocytes (between 1% and 13% of enterocytes with more than five bacteria) which is below the threshold value. The other five pigs from one litter bound the F4ac bacteria with a higher strength of adhesion (15% to 60%). The parentage and the phenotype of the parents were confirmed by microsatellite analysis.
and by phenotyping after the slaughtering. There is no explanation for the adhesion in these five pigs from only one litter and being the only outliers of 299 pigs.

The adhesion of F4ac in the nine pigs from resistant parents was probably an artefact and not a weak receptor as in F4abR, because compared to F4abR there were only few pigs and they did not follow a normal distribution. The inoculation of weakly adhesive piglets by Sellwood (1980a) using presumably the F4ac variant indicated that such piglets did not develop diarrhea (Sellwood 1984; Bijlsma and Bouw 1987). Thus, the weak adhesion is not relevant to the diarrhea, but is important for the definition of the phenotypes and the genotypes.

Only one pig with F4acR susceptible parents (SS × Ss genotype) was tested two times as resistant. This inconsistency could be due to the presence of protease in the intestine which destroys F4 receptor activity (Chandler et al. 1994).

**IV.3 Genetic transmission of F4 receptors**

**IV.3.1 F4abR and F4acR inheritance**

The monogenic inheritance of the receptor F4acR has been evident since the 1970’s (Sellwood et al. 1975, 1979; Rutter et al. 1975; Gibbons et al. 1977). However, the inheritance of F4abR is not as clear. Two theories explicate the transmission of the receptor F4abR: 1) the locus for F4abR is closely linked with the locus F4acR on chromosome 13 (Bonneau et al. 1990, Guérin et al. 1993), 2) one single locus codes for F4acR and for F4abR (Bijlsma and Bouw 1987). In our study six phenotypes for the three bacterial variants were observed. If we assume that one locus each would be responsible for the adhesion or non-adhesion of each of the three fimbrial variants, we would detect eight phenotypes. The missing phenotypes F4abR⁻ / F4acR⁻ / F4adR⁻ and F4abR⁺ / F4acR⁺ / F4adR⁺, indicate that pigs susceptible to F4ac are always susceptible to F4ab. We therefore conclude that the adhesion of F4ab and F4ac bacteria is influenced by the same receptor. Furthermore, many F4ab and F4ac fimbriae adhere to this receptor with a high adhesion in phenotypes A and B (both with F4abR⁺ / F4acR⁻), while the adhesion in phenotypes C and F (F4abR⁻ / F4acR⁺ or F4abR⁺⁺) is weak (Table 3.1; Figure 3.7). We assume that the strong adhesion of F4ab fimbriae in phenotypes A and B constitutes a phenotype distinct from the weak adhesion in C and F. In other breeds, pigs of phenotypes C and F were considered as recombinant, since very few of
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...them were identified (Bijlsma et al. 1982; Edfors-Lilja et al. 1986, 1995; Rapacz and Hasler-Rapacz 1986; Guérin et al. 1993; Peelman 1999). The second type of recombinant phenotype would be F4abR⁻/F4acR⁺ in the case of two closely linked loci for F4abR and F4acR (Bonneau et al. 1990; Guérin et al. 1993), but this phenotype was either not identified or very rare. No pig was detected with this phenotype among 368 pigs (Hu et al. 1993) as well as among 534 eight-week-old pigs and 64 adult pigs in our study. Bonneau et al. (1990) identified five pigs among 149 being F4abR⁻/F4acR⁺ and Rapacz and Hasler-Rapacz (1986) identified two pigs among 345.

In our study, the high percentage of pigs (24%) with the phenotype F4abRw is not in agreement with the hypothesis of two closely linked loci (θ = 0.02 according to Bonneau et al. 1990 and Guérin et al. 1993). Recombinant pigs (F4abR⁺/F4acR⁻ or F4abRw, F4abR⁻/F4acR⁺), which should be in the range of 2%, had a recombination fraction of clearly above 2% for F4abRw. Moreover, the second type of recombinant (F4abR⁻/F4acR⁺) was not observed at all. The genetic distance between both loci would depend only on the number of weak receptor phenotypes, and therefore, our results support a not linked locus or a non genetic factor causing F4abRw phenotype.

The experiment of Billey et al. (1998) gives additional argument that the receptor for F4ac binds F4ab bacteria as well. Both F4ab and F4ac fimbriae bind to the previously identified intestinal mucin-type sialoglycoproteins IMTG-1 and IMTG-2 (1.4.2). Both IMTG receptors bind F4ab and F4ac bacteria, but not F4ad and these receptors have been found in brush borders from pigs exhibiting the phenotypes A and B, but not in the phenotypes C, D and E (Billey et al. 1998). The binding of IMTG receptors was not determined in the phenotype F. Furthermore, the biological significance of the IMTG receptors was tested in neonatal gnotobiotic piglets (Francis et al. 1998). Among 31 piglets inoculated with either F4ab or F4ac bacteria, 14 developed severe diarrhea and 12 of them expressed IMTGPs. The remaining 17 piglets that failed to become severely ill, did not express IMTGPs. Therefore the strong correlation between the expression of intestinal IMTG receptors and piglet susceptibility to F4 E. coli (Francis et al. 1998) gives further evidence that the strong receptor for F4abR coincides with the presence of F4acR.
IV.3.2 *F4ab*R° inheritance

One locus coding for IMTGP receptors for F4ab and F4ac bacteria in phenotypes A and B, is insufficient to explain the weak adhesion of F4ab fimbriae in phenotypes C and F. The weak binding has been considered to arise from either the influence of epistatic genes on receptor expression or from post-expression inhibition or modification of the receptor sites (Bijlsma and Bouw 1987). Billey et al. (1998) postulated that the weak adhesive phenotype was an artefact because few of them were detected. In our study, the 160 pigs of phenotypes C and F (Table 3.1) reject this hypothesis. Francis et al. (1998) suggested the existence of another receptor besides IMTGP that can bind F4ac (and which may also bind F4ab and F4ad), because some pigs that did not become severely ill and did not express IMTGP, had brush borders that supported the adherence of large numbers of F4ab and F4ac *E. coli*. However, this receptor did not support colonization.

Litters resistant to F4ac were produced to investigate the transmission of the weak F4abR (F4abR°). However, 30 litters from F4ac resistant parents tested so far have not provided sufficient information to clarify the mode of inheritance of F4abR°.

IV.3.3 *F4ad*R inheritance

Our observations show that the receptor F4adR is likely dominantly inherited. Hence, the hypothesis of Bijlsma and Bouw (1987), who assumed a locus with two alleles (*D* for adhesion and *d* for non-adhesion), is confirmed by our phenotypic data (III.5). However, the genome-wide scan performed in 33 pigs did not allow finding markers linked with *F4adR*. Based on a larger pedigree no linkage was found between *F4adR* and microsatellites on chromosomes 4, 11 and 13, already rejected by Peelman (1999).

Bijlsma and Bouw (1987) proposed that the expression of the *D* locus is influenced by epistatic genes resulting in an intermediate susceptible/resistant phenotype or weak phenotype described in many studies (IV.2.2). The different composition and size of the receptor F4adR could explain this phenotype. IGLad (receptor for F4ad) which is a much smaller molecule than IMTGP (1.4.2), does not project through the surface of the intestine and may not be easily accessible to F4ad *E. coli* (Francis et al. 1999). Furthermore inoculation with F4ad *E. coli* of pigs expressing IGLad did not lead to severe diarrhea (Francis et al. 1999). The weak adhesion and mild
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Diarrhea agree with our observations, but our data suggest the inheritance of \( F4adR \) is independent of \( F4abR \) and \( F4acR \).

IV.4 Linkage analysis of \( F4acR \) locus

The multipoint linkage analysis assigns \( F4acR \) significantly to an interval within \( Sw207-S0283 \) on chromosome 13. Both microsatellites were mapped by FISH to band 13q41 (Jorgensen et al. 2002; Lopez-Corrales et al. 1999). Consequently, the gene controlling the susceptibility to \( E. coli \) F4ac is mapped to the region 13q41. One discrepancy between the genetic map (Figure 3.9) and the physical mapping of \( Sw225 \) is noted (Figure 3.10). Although \( Sw225 \) lies significantly inside the interval \( Sw207-S0283 \), \( Sw225 \) was mapped to 13q44 by Jorgensen et al. (2002).

Van Poucke et al. (2001) demonstrated that although there is an extended homology observed between human chromosome 3 (HSA3) and porcine chromosome 13 (SSC13) there is not complete conservation of gene order (Figure 3.10). For example, block 3 is located around the centromere in human 3p11 → q21 and inverted in a distal terminal position on SSC13 (13q41 → q46). Block 4 is located at the distal part of HSA3 adjacent to block 3 (3q21 → qter) and at the central part of SSC13 between blocks 1 and 3 (13q31 → q41). The SSC13q41 region of the comparative map contains genes belonging to blocks 3 and 4. To exploit the comparative mapping strategy for positional candidate cloning, human genes mapped to 3q13.3 → q21 and 3q27 → q29 should be considered. Van Poucke et al. (2001) defined the breakpoint limited to the genes TFRC (region 4) and ZNF148 (region 3), close to marker Swr2189. Thus, the marker Swr2189 constituted the breakpoint between two conserved groups (Van Poucke et al. 2001). Unfortunately, Swr2189 was not informative in our families. In conclusion we could map \( F4acR \) in a 1.6 cM interval with the breakpoint inside.

IV.5 Candidate genes for susceptibility to F4ac

No causative mutation was found after sequencing four candidate genes (TFRC, B3GnT5, B4GALT4 and B3GALT3) from pigs resistant and susceptible to F4ac bacteria. We detected two nucleotide polymorphisms in the B3GALT3 gene which were both silent mutations. According to our results, none of these four genes are responsible for the receptor \( F4acR \) genotype. These results were based on the whole coding sequence,
except approximately a 100 bp region of the 5' end of the \textit{B4GALT4} gene which was not analysed.

According to the comparative map, the genes \textit{TFRC}, \textit{B3GnT5} and \textit{B4GALT4} were good positional candidates. The \textit{B3GALT3} gene was mapped to the human band 3q25 (Figure 3.10). The corresponding porcine chromosomal segment for \textit{B3GALT3} should be band 13q33 \textendash{} 34. Thus the position of \textit{B3GALT3} is outside of band 13q41 which is the location of \textit{F4acR} determined by linkage analysis (IV.4).

\textbf{IV.6 Conclusions and perspectives}

Selection of pigs resistant to F4ac \textit{E. coli} by marker assisted selection is still not feasible for breeders, although the dominant monogenic inheritance of the receptor \textit{F4acR} is well established and the receptor locus is well mapped. Unfortunately, any allele of the microsatellites around the receptor locus was not in a significant genetic disequilibrium with the susceptible or resistant phenotype. Therefore, selection has to be done in each family separately due to association based on linkage. To select F4ac resistant pigs, offspring are slaughtered for phenotyping by an \textit{in vitro} adhesion test and both offspring and parents are typed with microsatellites. Offspring phenotyping and haplotype determination of microsatellites enable the deduction of the \textit{F4acR} genotype in live parents. Thus the information of phenotyped progeny is necessary to genotype \textit{F4acR} in live pigs.

The search for the genetic mechanism for resistance to \textit{E. coli} F4 and the development of a molecular test for breeders should focus on the F4ac variant, because this fimbrial variant was isolated from the majority of pigs having diarrhea. F4ab and F4ad \textit{E. coli} were less virulent than F4ac and were present in few isolates.

The biochemical/physiological candidate gene approach is difficult to apply because the minimal molecular structure required for the adhesion of F4ac bacteria to the receptor is not identified. With the precise localization of the receptor locus \textit{F4acR}, a positional cloning could be envisaged. The microsatellites proximal to the receptor locus could be used to screen a BAC library and the isolated clones could be employed to construct a clone contig across the region of the receptor locus. The contig represents an appropriate tool for isolating transcribed sequences.
The inheritance of the receptor F4abR is not yet clear. Additional litters have to be produced to clarify its inheritance. Despite ambiguous phenotypes determined by the adhesion test for the third receptor, F4adR is likely dominantly inherited.
V. LITERATURE


Sellwood R. (1979) *Escherichia coli* diarrhoea in pigs with or without the K88 receptor. *Veterinary Record* 105, 228-30.


VI. Media and solutions

### Agarose gel
- Agarose gel 0.8-2% (w/v)
- TBE 1×
- Agarose 0.8 to 2%
- Ethidium bromide 0.1 μg/ml

### Denhardt’s solution 100×
- BSA 2% (w/v)
- Polyvinylpyrrolidone 2% (w/v)
- Ficoll 2% (w/v)

### DEPC-treated H₂O
- DEPC-treated H₂O 0.5 ml/l

### DNA loading dye-Bromophenol blue
- Bromophenol blue 0.25% (w/v)
- D(+)-sucrose 40% (w/v)

### DNA loading dye-XCFF
- XCFF 0.25% (w/v)
- Orange G 0.26% (w/v)
- D(+)-sucrose 40% (w/v)

### Ethidium bromide plate
- Agarose 0.8% (w/v)
- TE 5×
- Ethidium bromide 0.1 μg/ml

### Hybridization solution
- Formamide 50% (v/v)
- Dextran sulfate (diluted in formamide) 10% (w/v)
- Denhardt’s solution 5×
- SDS 1% (w/v)
- SSC 5×
- Salmon sperm DNA denatured 0.1 mg/ml

### IPTG/X-Gal
- IPTG 2.5% (w/v) 50 μl
- X-Gal 2.5% (w/v) in dimethylformamide 50 μl

### LB-agar plate
- Agar 20 g/l in LB medium

### LB medium
- Bacto tryptone 10 g/l
- Yeast extract 5 g/l
- NaCl 10 g/l
- Adjusted pH to 7.0 with NaOH

### Lysis buffer (isolation of genomic DNA)
- D(+)-saccharose 0.32 M
- Tris-HCl pH 7.5 10 mM
- MgCl₂ 5 mM
- Triton X-100 0.94% (w/v)

### Methylene blue
- Methylene blue 0.02%
- Tris-HCl pH 7.5 10 mM

### MOPS 10×
- MOPS 83.7 g/l
- Sodium acetate trihydrate 13.6 g/l
- EDTA 3.7 g/l

### Natrium acetate pH 5.2
- NaAc × 3H₂O 408.24 g/l
- ddH₂O 500 ml
- Adjusted pH to 5.2 with 96% acetic acid

### NZY broth
- NaCl 5 g/l
- MgSO₄·7H₂O 2 g/l
- Yeast extract 5 g/l
- Casamino acids 10 g/l

### NZY top agar
- Agarose 7 g/l in NZY broth

### PBS 10×
- NaCl 5.61 g/l
- KCl 0.11 g/l
- Na₂HPO₄·2H₂O 0.795 g/l
- KH₂PO₄ 1.09 g/l
- EDTA 3.72 g/l
- Adjust pH to 6.8 with 0.5 M Na₂CO₃

### PCR buffer (isolation of genomic DNA)
- KCl 50 mM
- Tris HCl pH 8.3 10 mM
- Gelatin 0.1% (w/v)
- Nonidet 0.45% (w/v)
- Tween-20 0.45% (w/v)

### Polyacrylamide gel 4.5%
- Urea 18 g
- ddH₂O 23 ml
- TBE 10× 5 ml
- Acrylamide/bis (29:1), 30% 7.5 ml
- TEMED 15 μl
- APS 10% (w/v) 350 μl
RNA loading dye
Bromophenol blue 0.04%
EDTA 1 mM
Glycerol 61.5% (w/v)

SM buffer
NaCl 5.8 g/l
MgSO₄·7H₂O 2.0 g/l
Tris HCl pH 7.5 50 ml/l
2% (w/v) gelatine 5 ml/l

SOB medium
Bacto tryptone 20 g/l
Yeast extract 5 g/l
NaCl 0.5 g/l
250 mM KCl 10 ml/l
2 M MgCl₂ 5 ml/l
Adjusted pH to 7.0 with NaOH

SOC medium
1 M Glucose (filtrated) 20 ml
in SOB medium cooled to 60 °C

SSC 20×
3 M NaCl 175.3 g/l
0.3 M Sodium citrate pH 7.0 88.2 g/l

TBE 10×
Trizma base 107.8 g/l
Boric acid 55 g/l
EDTA 4.9 g/l
pH should be 8.3 without correction

TE 1× pH 7.5-8
Tris HCl pH 7.5 10 mM
EDTA 1 mM

Tris HCl
1 M Tris base 121.16 g/l
1 M Trizma HCl 157.6 g/l
Adjust to required pH mixing tris base and trizma HCl

X-Gal (2.5%)
5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside 100 mg
N,N-Dimethyl-formamide 4 ml
CURRICULUM VITAE

Name: Pascal Python
Date of birth: September 4, 1974
Place of birth: Fribourg
Nationality: Swiss

1990 – 1994 High school degree, Matura type B
1994 – 1999 Institute of Animal Sciences, Department of Agriculture and Food Sciences, Swiss Federal Institute of Technology Zurich
1998 Practical work experience at Swiss Federal Research Station for Animal Production of Posieux (RAP) and at « Service romand de vulgarisation agricole de Lausanne » (SRVA)
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1999 – 2003 PhD studies and assistant in the group of Prof. G. Stranzinger at the Institute of Animal Sciences, Breeding Biology, Swiss Federal Institute of Technology Zurich

PUBLICATION


ABSTRACT

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