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A DNA polymorphism influencing α(1,2)fucosyltransferase activity of the pig FUT1 enzyme determines susceptibility of small intestinal epithelium to *Escherichia coli* F18 adhesion

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Abstract The α(1,2)fucosyltransferases (FUT1 and FUT2) contribute to the formation of blood group antigen structures, which are present on cell membranes and in secretions. In the present study we demonstrate that both *FUT1* and *FUT2* are expressed in the pig small intestine. *FUT1* polymorphisms influence adhesion of F18 fimbriated *Escherichia coli* (ECF18) to intestinal mucosa, and *FUT2* is associated with expression of erythrocyte antigen 0. The *FUT1* polymorphisms result in amino acid substitutions at positions 103 (Ala→Thr) and 286 (Arg→Glu). Tightly controlled expression of the *FUT2* gene results in either an abundance or an absence of mRNA in small intestinal mucosa. ECF18-resistant animals were shown to be homozygous for threonine at amino acid 103 of the FUT1 enzyme. Susceptibility to ECF18 adhesion appeared to be solely dependent on the activity of FUT1 in intestinal epithelia. In intestinal mucosae of ECF18-resistant pigs which expressed *FUT1* but not *FUT2* RNA, the levels of α(1,2)fucosyltransferase activity were significantly lower (28- to 45-fold, $P<0.001$) than in susceptible pigs. Moreover, lysates of CHO cells transfected with *FUT1* constructs encoding threonine at amino acid position 103 also showed significantly reduced enzyme activity compared with constructs encoding alanine at this position. Our genetic and enzymatic studies support the hypothesis that the FUT1 enzyme, and particularly the amino acid at position 103, is likely important in the synthesis of a structure that enables adhesion of ECF18 bacteria to small intestinal mucosa.

Keywords FUT1 · FUT2 · *Escherichia coli* F18 · Blood group · Pig · Functional polymorphism

The nucleotide sequences reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the following accession numbers: *FUT1* and *FUT2* cDNAs, AF136896 and AF136895, respectively; *FUT2* upstream genomic sequence harboring the 5′ untranslated region, AF136897; *FUT1* and *FUT2* genomic sequences, U70883 and U70881

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Introduction

Adhesion of F18 fimbriated *Escherichia coli* (ECF18) to small intestinal brush border membranes causes edema disease and post-weaning diarrhea in piglets. These diseases are of considerable economic significance in the pig breeding industry, and most breeding companies would prefer to eliminate genetically susceptible pigs from their herds, due to their negative effect on health status. The gene controlling expression of the ECF18 receptor (*ECF18R*) is closely linked to the blood group inhibitor locus *S* on pig chromosome (Chr) 6. We have proposed two α(1,2)fucosyltransferase genes (*FUT1* and *FUT2*), localized on Chr 6q11, as candidate genes for the *ECF18R* and the inhibitor *S* loci (Meijerink et al. 1997).

Table 1 Comparison of the nomenclature, genetic basis, alleles, and phenotypes of pig equivalents to the human blood group *H*, the *SE*, and the *ABO* loci (in *parentheses*)

Locus in pig (locus in human)	Gene/enzyme	Alleles	Phenotype in pig (phenotype in human)
ECF18R, *H*[a]	α(1,2)fucosyltransferase FUT1	*B*, *b*; *G*, *A*	*Escherichia coli* F18 adhesion/no adhesion
(*H*)	(FUT1)	(*H*, *h*)	(H^+, H– on erythrocytes)
S[b]	α(1,2)fucosyltransferase FUT2	*S*, *s*	0^+, 0^- in plasma and on erythrocytes
(*Se*)	(FUT2)	(*SE*, *se*)	(H^+, H– in plasma and secretory tissue)
EAA[c]	α(1,3)*N*-acetylgalactosaminyltransferase[d]	*A*, "–"	A (or A^W), "–"
(ABO)		(*A*, *B*, *O*)	A^+, B^+, O

[a] The pig equivalent to the human blood group *H* locus is thought to be identical to the ECF18 receptor locus (*ECF18R*). Alleles *B* and *b* represent susceptibility and resistance to ECF18 adhesion, respectively. The *FUT1 M307* alleles *G* and *A* are assumed to be their genetic basis (Meijerink et al. 1997). No *FUT1*-dependent phenotype has been described for pig blood plasma and erythrocytes

[b] The *S* locus is determined from the *FUT2* gene, with alleles *S* and *s* assumed to be equivalent to *SE* and *se* in humans. The pig 0 antigen is identical to the H antigen produced from the human FUT2 enzyme and is detectable on erythrocytes (Sako et al. 1990)

[c] The pig erythrocyte antigen A (*EAA*) locus is the homologue of the human *ABO* locus. The *EAA* locus has two alleles, allele *A* being the equivalent to the human *A* allele, leading to either antigen A or weak antigen A (A^W) phenotypes. The "–" allele is a nonfunctional allele in pigs, as the *O* allele is in humans

[d] The *A* allele of humans and pigs encodes an α(1,3)*N*-acetyl-galactosaminyltransferase. The *B* allele of the human *ABO* locus encodes an α(1,3)galactosyltransferase and is not found in the pig (Sako et al. 1990; Yamamoto et al. 1990)

The epistatic interaction between the inhibitor locus (*S*) and the erythrocyte antigen A locus (*EAA*) determines the phenotypic appearance of the pig blood group A0 system (Rasmusen 1964). At the inhibitor locus *S* the alleles S^S and S^s symbolize the presence and the absence of antigen 0 on erythrocytes, respectively (Table 1). The antigen 0 structure, equivalent to the H antigen in humans, serves as a precursor for antigen A formation by an *N*-acetylgalactosaminyltransferase (blood group A transferase). This enzyme is encoded by a gene at the *EAA* locus on pig Chr 1, which we have previously characterized (Ellegren et al. 1994; unpublished data). The *EAA* locus possesses two alleles: allele *A* is dominant over allele "–." In combination with expression of the inhibitor locus S^S allele, only the EAA^A allele leads to antigen A expression (*EAA* phenotype A). When the dominant S^S allele is not expressed (S^{ss}), only a weak agglutination reaction is detected determining a weak *EAA* phenotype (A^W). Genotypes that lack the EAA^A allele ($EAA^{-\,-}$) do not express A antigen in either case.

The pig blood group A0 system corresponds to the human blood group ABO system. In humans, the H antigen is the precursor of the A and B antigens. Expression of H antigen in the erythrocyte precursor cells is controlled by the *H* locus, whereas expression in secretory tissues depends on the *Secretor* (*Se*) locus (Oriol et al. 1981; Watkins 1980). In the pig, however, erythrocyte antigens A and 0 are derived from secretory tissues or cells that release antigens into the plasma (Bell 1983). Therefore, the pig inhibitor locus *S* is suggested to be the equivalent of the *Secretor* locus.

The pig plasma 0 antigen has been chemically identified as a glycolipid with a terminal α(1,2)-fucosylated galactopyranoside [Fucα(1,2)Gal] (Hanagata et al. 1990). A pig α(1,2)fucosyltransferase exhibiting α(1,2) fucosylation of glycolipid and glycoprotein acceptors has been purified from submaxillary gland mucin (Beyer et al. 1980). More recently, Thurin and Blaszczyk-Thurin (1995) identified this enzyme as the homologue of the human Secretor enzyme. We previously reported the cloning and mapping of the corresponding pig gene (*FUT2*) and of pig *FUT1*, which is homologous to the human *H* gene (Meijerink et al. 1997). In that study, we found a polymorphism in the pig *FUT1* gene, with either a guanine (G) or an adenine (A) residue at nucleotide 307 ($M307^G$ and $M307^A$, respectively). At the corresponding amino acid position 103 of the FUT1 peptide product, an alanine is conserved in human, mouse, rat, and rabbit (respective GenBank accession numbers M35531, U90553, AB015637, and X80226). This finding suggests a functional significance for the alanine at this position, which is present in the pig FUT1 $M307^G$ variant. In a family comprising 221 progeny, the *M307* polymorphism was shown to be highly correlated with the inhibitor locus *S* and *ECF18R* genotypes (with *ECF18R* alleles *B* and *b* representing susceptibility and resistance to adhesion, respectively) (Meijerink et al. 1997). By linkage analysis, we estimated the *FUT1* polymorphism to be less than 1 centimorgan (lod score 50.6) from the *S* and *ECF18R* loci. Therefore *FUT1* is regarded as a good candidate gene for controlling expression of the receptor for ECF18 bacteria. Functional interaction of fucosylated blood group

structures with pathogens has been shown in humans, where the *FUT2* gene is involved in the synthesis of Lewis b blood group antigens, which function as receptors for *Helicobacter pylori* (Borén et al. 1993). Distinguishing between the pig *FUT1* and *FUT2* genes and resolving their relationship with the ECF18R and 0 antigen phenotypes is necessary to enable phenotypic or molecular genetic polymorphisms in these genes to be used as selection markers.

King and Kelly (1991) investigated the developmental timing of appearance and the localization of fucosylated antigen 0 structures in the neonatal pig intestine. Their findings suggest phenotypic manifestations of *FUT1* and *FUT2* gene activity. Weak fucosylation of glycoconjugates in goblet cells is detected in the first postnatal week, preceding detection of the 0 antigen on erythrocytes. On absorptive intestinal cells, these antigenic structures are first detected in postnatal week 5. When animals are restricted to an early milk diet, however, the 0 antigen is expressed on absorptive cells as early as 3 weeks postpartum. Like the gradual onset of antigen 0 expression on absorptive cells, the appearance of receptors for ECF18 in piglets is also reported to increase during the first 3 weeks of the neonatal period (Nagy et al. 1992). Therefore, we tested for the presence of *FUT1* and *FUT2* transcripts in small intestinal mucosae of ECF18-resistant and susceptible animals. In the same tissue samples, we also measured total α(1,2)fucosyltransferase activity. The inhibitory effect of *FUT1* mutations on fucosyltransferase activity was determined by expressing the cloned *FUT1* coding sequence (CDS) in eukaryotic cell cultures.

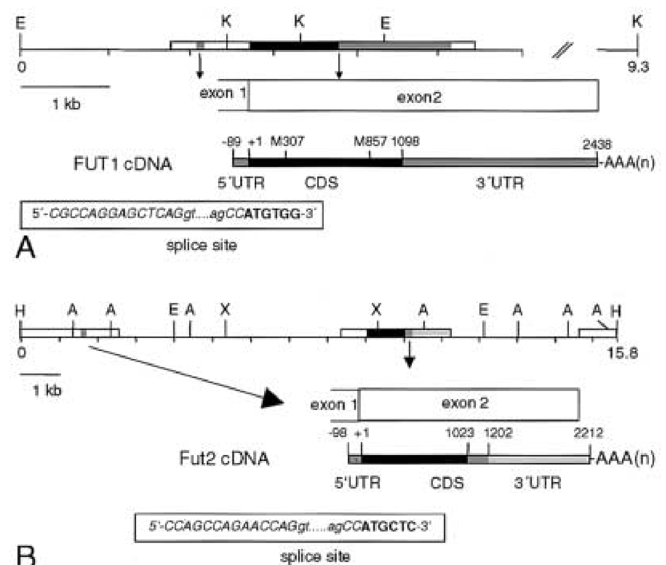

Fig. 1A,B Restriction map of the *FUT1* and *FUT2* genomic DNA and positions of coding and untranslated regions of their respective cDNAs. *Boxed segments* were sequenced from genomic and cDNA clones. Restriction sites were derived from sequence analysis and Southern blot analysis of restriction digests. Coding sequences (CDS) are *solid*, untranslated regions (UTRs) are in *gray*. The intron-exon boundaries are indicated; coding sequences are printed in *uppercase italics*, and intron sequences are printed in *lowercase italics*. **A** The restriction map of the *FUT1* gene includes the *Ksp*I (K) and *Eco*RI (*E*) restriction sites. Exon 1 presumably starts at position –559 and includes 87 bp of the 5′UTR. Exon 2 contains 2 bp of the 5′UTR, the CDS, and the 3′UTR (GenBank AF136896). Allelic variants (guanine to adenine transitions) were detected on positions 307 and 857 of the *FUT1* CDS. **B** The restriction map of the *FUT2* gene includes the *Eco*RI (*E*), *Xho*I (X), *Hind*III (H), and *ApaI* (A) restriction sites. *FUT2* exon 1 contains 96 bp of the *FUT2* 5′UTR, and its genomic sequence (GenBank AF136897) is localized approximately 7 kb upstream of the CDS. Exon 2 contains 2 bp of the 5′UTR, the CDS, and the 3′UTR. Two different 3′UTRs were found, 180 and 1190 bp in length (GenBank AF136895)

Materials and methods

Animals and tissue preparation

Blood and tissue samples were collected from a total of 56 pigs at 5 months of age. These consisted of 35 progeny from 12 Large White and Landrace matings, plus 21 unrelated pigs: 6 Large White and 15 Duroc. Shortly after slaughtering, two adjacent pieces (10 cm) of mid-jejunum were taken from each pig. For RNA analysis and determination of fucosyltransferase activity, one of the jejunal sections was gently rinsed with PBS to remove excessive mucus, and the mucosal tissue layer was scraped off and quickly frozen in liquid nitrogen. The second piece of jejunum was used to determine ECF18 adhesion to enterocytes by means of an in vitro microscopic adhesion assay. The animals were typed for blood group systems S and EAA with a hemolytic test using goat anti-0 serum and pig anti-A serum, respectively, and the 12 loci of the halothane linkage group were typed as outlined by Vögeli and co-workers (1996).

Isolation of cDNA clones

Total RNA was isolated from small intestinal mucosal scrapings of a Large White pig expressing the blood group antigen 0, using the guanidinium thiocyanate method followed by cesium chloride gradient centrifugation (Chirgwin et al. 1979). A λ-ZAP cDNA library (Stratagene, La Jolla, Calif.) was constructed from poly(A)$^+$ RNA (2.5 μg), which was isolated using an mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). Replica membranes with 10^6 total plaques were hybridized to [α-^{32}P]dATP-labeled (Prime-It II, Stratagene) *FUT1* (bp 69 to 489) and *FUT2* (bp –74 to 461) specific genomic PCR products (Fig. 1). Plasmids were excised from isolated λ phages, purified with an alkaline lysis method, and sequenced according to the standard protocols for the ABI 373A automated sequencer (Perkin-Elmer Cetus, Norwalk, Conn.).

Comparison of cDNA and genomic sequence

To analyze the genomic organization of the *FUT1* and *FUT2* genes, sequences derived from cDNA clones of the small intestine library were compared with genomic sequences. DNA of ECF18-susceptible and -resistant animals was analyzed. The *FUT1* and *FUT2* genomic sequences were determined from subcloned *Ksp*I and *Bam*HI restriction digest products of isolated cosmids (Meijerink et al. 1997). The sequences flanking the *FUT1* 5′UTR were revealed by sequencing directly from cosmid DNA. The combination of sequence data with Southern blot analysis of an EcoRI/*Ksp*I digest of a *FUT1* cosmid clone revealed the relative positions of *Eco*RI and *Ksp*I recognition

sites. The genomic sequences of the *FUT2* untranslated regions (UTRs) and of bordering regions were obtained by sequencing a subcloned *Hin*dIII restriction fragment of cosmid DNA. A restriction enzyme map of the *Hin*dIII clone was constructed after analysis of the described sequence data, using restriction digests with *Eco*RI, *Xho*I, *Apa*I, and the combined enzymes *Hin*dIII/*Eco*RI, *Hin*dIII/*Xho*I, *Apa*I/*Eco*RI, *Apa*I/*Xho*I, and *Eco*RI/*Xho*I electrophoresed on agarose gels (gel images not shown).

Northern blot analysis

Total RNA was obtained using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). For 51 animals of various *ECF18R* and *S* genotypes, *FUT1* and *FUT2* gene expression in small intestinal mucosa was analyzed by blotting 15 µg of total RNA onto Hybond N membranes after electrophoresis on an agarose gel (1.2% agarose, 0.66 M formaldehyde and 1×MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0). Five samples of small intestinal mucosa were tested for expression of *FUT1* and *FUT2* by Northern analysis of 1 µg poly(A)$^+$ RNA (Oligotex mRNA Mini Kit, Qiagen, Hilden, Germany). Similarly, mandibular salivary gland and cerebral tissues of a pig expressing *FUT2* in the intestine were tested for tissue-specific expression of *FUT2*. Membranes were incubated overnight (45 °C) with [α-^{32}P]dATP-labeled *FUT1* and *FUT2* PCR products (bp 69–489 and bp 1–461 of the CDS, respectively) in a solution containing 50% formamide, 10% dextran sulfate, 5×standard sodium citrate (SSC), 1% SDS, and 0.1 mg/ml salmon sperm DNA. Membranes were washed for 30 min in a solution of 1×SSC and 0.1% SDS at 65 °C, followed by a 10-min wash in a solution of 0.1×SSC and 0.1% SDS at 65 °C, and exposed to X-Omat AR X-ray film (Kodak, Rochester, N.Y.). *FUT1* and *FUT2* probes were removed by washing for 10 min in 0.1×SSC, 1% SDS at 95 °C, and the membranes were subsequently hybridized with an [α-^{32}P]dATP-labeled pig *β-actin* cDNA fragment (bp 220–587 of the CDS; GenBank accession number U07786) as a control for RNA amount and transfer efficiency. To compare different blots, control samples were included.

α(1,2)fucosyltransferase activity assay

Intestinal scrapings, kept at –80 °C, were ground in liquid nitrogen. Two volumes of 15 mM sodium phosphate (pH 6.1)/1% Triton X-100 were added, and the mixture was put on ice for 10 min. The lysates were sonicated for 15 s with a Branson microtip Sonifier B-12 at 30 W and cleared by centrifugation for 2 min at 15,000 *g* at 4 °C. Lysates of intestinal scrapings (12.5 µl) were tested for fucosyltransferase activity in a 50-µl volume of assay mixture containing 50 mM sodium phosphate buffer pH 6.1, 0.1 mM GDP-fucose, 1.5 µM GDP[^{14}C]-fucose (0.74 kBq), 5 mM ATP, and 25 mM phenyl-*β*-D-galactopyranoside (phenyl-*β*-D-Gal) as acceptor. To inhibit possible fucosidase activity, 10 mM L-fucose was included (Rajan et al. 1989). Reactions were stopped after 2 h when residual increases of product levels were negligible. Utilization of enzyme substrate did not exceed 15% under these conditions. Reactions were stopped with 0.5 ml H_2O, and reaction products were separated from unincorporated GDP[^{14}C]-fucose on Sep-Pak Plus C-18 columns (Waters Corporation, Milford, Mass.). Total fucose-^{14}C transfer was measured in a *β*-counter using 10 ml IrgaSafe Plus scintillation fluid. The total protein amount in lysates was determined using the Bio-Rad Protein Assay with bovine serum albumin fraction V as a standard. Transferase levels of GDP-fucose in pmol/mg protein were calculated by multiplying the GDP-fucose amount by the incorporated percentage of GDP[^{14}C]-fucose, and were corrected for the protein amount in the cell lysate.

Cell lysates (25 µl) of transfected CHO cells were incubated for 1 h at 37 °C in a 50-µl volume of assay mixture containing 50 mM sodium phosphate buffer, pH 6.1, 0.1 mM GDP-fucose, 1.5 µM GDP[^{14}C]-fucose (0.74 kBq), 5 mM ATP, and 5 mM of phenyl *β*-D-Gal as acceptor.

Pig FUT1 expression in Chinese hamster ovary cells

The pig *FUT1* coding regions were amplified from genomic DNA with *Pfu* polymerase (Stratagene) using primers positioned up- and downstream of the *FUT1* coding region (5′-agcctccagccccga**ggatcc**gctaatttcacttttc-3′ and 5′-ctgggttgaccga gg**gaattc**cagaaaggctaatggc-3′, T_A=61 °C) containing a *Bam*HI and an *Eco*RI restriction site (boldface). Genomic template DNA was genotyped to produce PCR products with a guanine residue at nucleotide position 307 (variant $M307^G$) or with a G→A transition at this position (variant $M307^A$). The amplified DNA was digested with *Bam*HI and *Eco*RI and directionally cloned into the corresponding sites of the eukaryotic expression vector pcDNA3.1 (Invitrogen, Groningen, The Netherlands). The recombinant pcDNA3.1 plasmids were sequenced to confirm identity to their genomic template.

A Chinese hamster ovary cell line (CHO) was grown in 3.5-cm wells using Ham's F12 culture medium (Life Technologies, Rockville, Md.) supplemented with penicillin/streptomycin (50 units/ml), glutamine (2 mM), and 5% fetal calf serum (FCS). For transient expression, 50% confluent cultures were transfected with 0.5, 1.0, 1.5, and 3 µg of either pcDNA3.1 or the recombinant pcDNA3.1 plasmids (purified with a Qiagen Endofree Plasmid Maxi Kit), using 20 µg LipofectAmine in 1 ml of OPTIMEM 1 transfection medium (Gibco BRL, Rockville, Md.). After 6 h of transfection, 1 ml of Ham's F12 medium containing 10% FCS was added. This medium was replaced by complete culture medium after an additional 14 h of culture. Cells were grown for a further 2 days, then lysed in 30 mM sodium phosphate (pH 6.1)/1% Triton X-100, and cell debris was spun down (10 min at 1000 *g*). Subsequently, supernatants were probed for α(1,2)fucosyltransferase activity. Each transfection was carried out in triplicate.

Statistics

Tetrachoric correlation (*r*) of genotypes and significance of association were calculated using a χ^2-test applied to a 2×2 table reflecting the genotype combinations. χ^2 was corrected for the degree of relationship between animals, using the correction factor (*w*) of Cotterman (1974).

The log-transformed fucosyltransferase activity data were analyzed with SYSTAT version 9.0 (SPSS, Chicago, Ill.). All data were tested for normal distribution by the Kolmogorow-Smirnow test. The general linear least-square model was used to estimate the means and the significance of defined categorical factors. Post hoc pairwise mean differences were estimated using the Bonferroni test.

Results

cDNA and genomic organization

For both the *FUT1* and *FUT2* gene, cDNA clones were found in the pig small intestine cDNA library (Fig. 1). The *FUT1* cDNA was 2527 bp in length, with the 5′UTR positioned 559 bp upstream from the CDS on genomic DNA. The 3′UTR and the CDS sequences were included in exon 2. Two *FUT2* cDNA variants differed in the length of their 3′UTRs (179 and 1189 bp), depending on the site of polyadenyla-

tion. Exon 1 included 96 bp of 5′UTR, and its genomic sequence was localized approximately 7 kb upstream of the CDS. The 3′UTR sequence was included in exon 2.

FUT1 and FUT2 mRNA expression

Northern blots of small intestinal samples probed with a *FUT1*-specific sequence revealed three different transcripts migrating between the 18S and 28S RNAs (Fig. 2A, lanes 1–5). The variation in the amount of *FUT1* RNA paralleled that of *β-actin* RNA in the sample, indicating that the absence of the ECF18 receptor was not due to altered *FUT1* expression.

In both small intestine and salivary gland (Fig. 2B, lanes 5, 6), the same two bands hybridized to the *FUT2* probe, supporting specific *FUT2* expression patterns in secretory tissues (Fig. 2B). In contrast to the *FUT1* gene, the *FUT2* gene was not expressed in all intestinal samples. Not all ECF18-susceptible animals ($ECF18R^{B\cdot}$) expressed *FUT2* in the intestine (Table 2, Fig. 2B, lanes 1, 2, and 4), and *FUT2* was not expressed in all $S^{S\cdot}$ animals (Fig. 2B, lanes 2 and 4). Nevertheless, all animals expressing abundant *FUT2* RNA were $S^{S\cdot}$, and S^{ss} animals were devoid of *FUT2* expression (Table 2; Fig. 2B, lane 5).

FUT1 and FUT2 activity in small intestinal scrapings

In progeny of the Large White and Landrace families, significant differences in total fucosyltransferase activity in small intestinal scrapings were found (Table 3), depending on *FUT1* genotype, FUT2 expression, and ECF18R status. The activity values of groups a and b, which expressed FUT2 (+), were significantly higher than the values of groups c, d, e, and f, which did not express FUT2 (–). For the pigs not expressing FUT2, the *FUT1 AA* genotype (*$ECF18R^{bb}$*, group f) led to significantly lower fucose transfer activities (P<0.001) when compared with *GG* and *AG* types (*$ECF18R^{B\cdot}$*, groups c, d, and e). The *GG* and *AG* genotypes (group c compared with d and e) were not significantly different.

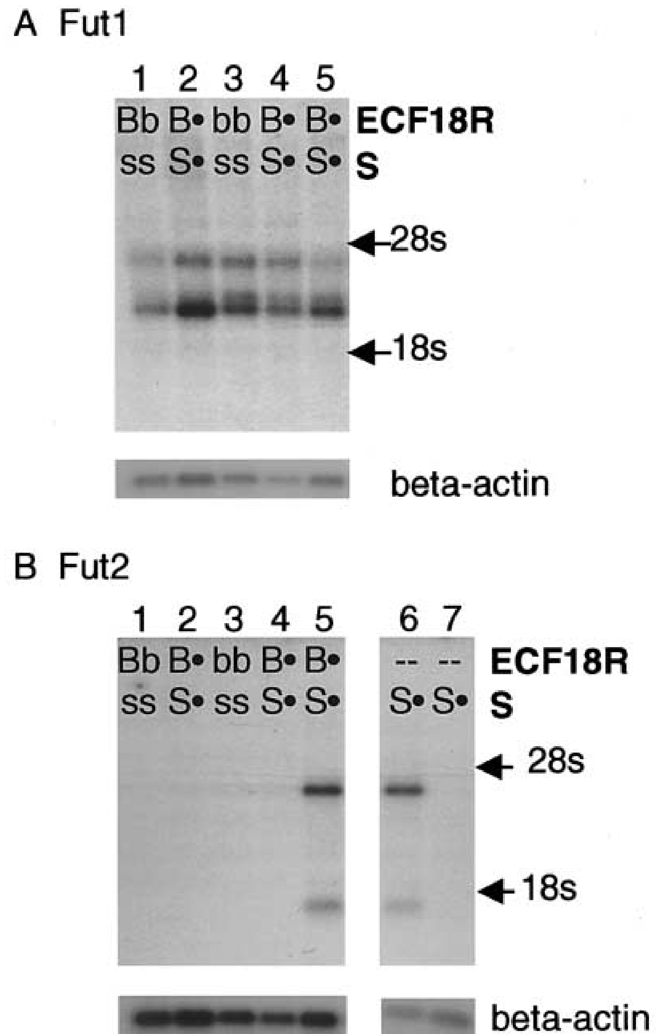

Fig. 2A,B Northern blots of poly(A)$^{+}$ RNA from intestinal tissue (*lanes 1–5*), salivary gland (*lane 6*), and cerebrum (*lane 7*). The same animals of various S and ECF18R phenotypes were tested on each blot. S phenotypes either possessed erythrocyte antigens 0 (S·) or lacked them (ss). ECF18R B· phenotypes indicate susceptibility to adhesion; bb phenotypes are resistant. Dots (·) symbolize the second allele for the respective genotypes, which could not be determined due to the dominance of *S* and *B* alleles. Blots were hybridized with the *FUT1* (**A**) and the *FUT2* (**B**) DNA probes as described in Materials and methods. All animals showed three *FUT1* transcripts. *FUT2* hybridization showed two transcripts expressed in the intestines and salivary glands of $S^{S\cdot}$ animals (*lanes B5* and *B6*), but the transcripts were not detected in the intestines of every animal of $S^{S\cdot}$ phenotype (*lanes B2* and *B4*). As a loading control, blots were also hybridized with *β-actin*. The positions of 18S and 28S ribosomal RNA are indicated by *arrows*

FUT1 activity in transfected CHO cells

Least-square analysis of the activity data was performed for a model containing the following categorical variables: FUT1 variants, amount of transfected DNA, and the influence of different transfection experiments. The amount of transfected DNA had no significant effect. However, the level of fucose transfer activity did differ significantly between independent transfection experiments, most probably due to different transfection efficiencies in various experiments. Therefore, this factor was taken into account in our least-square analysis. The least-square means for the FUT1 $M307^{G}$ and FUT1 $M307^{A}$ constructs were 2455±105 and 1710±105 pmol/mg protein, respectively. These values are significantly different (P<0.007).

Table 2 Tetrachoric correlation (r) and significance ($\chi^2\times w$) of association of *FUT1* and *FUT2* genotypes with *S* (allele *S* being dominant over *s*) and *ECF18R* (alleles *B* being dominant over *b*) loci in Large White and Landrace pigs

Genotype	*S* locus		r	χ^2	$\chi^2\times w$ [a]	*ECF18R*		r	χ^2	$\chi^2\times w$ [a]
	ss	*S·*				*bb*	*B·*			
$FUT1M307^{AA}$ [b]	7	–	0.71	20.4	8.16**	7	–	1.00	41	16.4***
$FUT1M307^{AG}$ [b]	5	12				–	17			
$FUT1M307^{GG}$ [b]	–	17				–	17			
$FUT2^{--}$ [c]	12	6	0.72	21.7	8.67**	7	11	0.51	10.8	4.3*
$FUT2^{+\cdot}$ [c]	–	23				–	23			

$*P(\chi^2\times w)<0.05$; $**P(\chi^2\times w)<0.005$; $***P(\chi^2\times w)<0.001$

[a] A weight factor (w=0.4) was applied to correct for the inclusion of related animals (Cottermann 1974)

[b] The *FUT1* genotype consists of either two $M307^A$ alleles, two $M307^G$ alleles, or both an $M307^A$ and an $M307^G$ allele

[c] *FUT2* genotypes express RNA from one or both alleles ($FUT2^{+\cdot}$) or are devoid of expression ($FUT2^{--}$)

Table 3 Average GDP-fucose transfer activity (in pmol/mg protein) of intestinal mucosal extracts for specified groups of Large White and Landrace pigs. The analysis of least-square means was performed using log-transformed fucosyltransferase activities with the categorical variable group (a–f). The back-transformed least-square means (μ) and SE of the mean are indicated. The Bonferroni pairwise mean comparison procedure was used, and the pairwise comparison probability (P) is indicated. Groups a–f were defined according to genotypes for the *FUT1 M307* polymorphism with G for guanine and A for adenine, to phenotypes for the *FUT2* locus with either expression (+) or absence (–) of FUT2 transcripts, to the *S* locus with phenotypes either expressing erythrocyte antigen 0 (S·) or lacking it (ss), and to the *ECF18R* locus with susceptible (B·) and resistant phenotypes (bb)

FUT1/FUT2/S/ECF18R	μ±SE (pmol/mg protein)	P (μ1=μ2)
a GG/+/S·/B· (n=12)	16443±2011	P(a,b=c,d,e)<0.012
b AG/+/S·/B· (n=7)	10616±2602	
c GG/–/S·/B· (n=2)	2460±1676	P(c,d,e=f)<0.001
d AG/–/S·/B· (n=3)	2630±744	
e AG/–/ss/B· (n=5)	1675±453	
f AA/-/ss/bb (n=4[a])	58±26	P(f=a,b,c,d,e)<0.001

[a] The fifth animal (FUT1 activity=448 pmol/mg protein) was regarded as an outlier and has not been included in the analysis.

Discussion

In these studies, we showed that the *FUT1 M307* genotype and the presence of *FUT2* RNA are associated with α(1,2)fucosyltransferase activities of pig intestinal mucosal lysates. Northern blot analysis (Fig. 2) showed that *FUT1* RNA is present in similar amounts in the small intestines of pigs, independent of blood group inhibitor S and ECF18R status. However, a guanosine-to-adenosine nucleotide transition at position 307 of the pig *FUT1* CDS results in an alanine-to-threonine substitution at amino acid position 103. Alanine in this position is conserved in other species and may therefore be considered as the wild type.

Typing all animals for G→A transitions at nucleotide 307 (*M307*) of the CDS (Table 2) revealed that all seven homozygous $M307^{AA}$ animals were $ECF18R^{bb}$ and S^{ss}. However, whereas *FUT1* genotypes had 100% association with ECF18R phenotypes, correlation between $M307^A$ and S^s alleles was not absolute, since five animals of genotype $M307^{AG}$ also had S^{ss} genotypes. Therefore, there may be a causal influence of the *FUT1* mutation on the ECF18R but not on the blood group inhibitor S phenotype.

For the experimental animals presented in Table 3, it was possible to determine both alleles of the *ECF18R* genotype for all 5 $FUT1^{AA}$ ($ECF18R^{bb}$, group f) and for 9 $FUT1^{AG}$ ($ECF18R^{Bb}$, groups b, d, and e) animals, due to pedigree information. In these 14 pigs, $ECF18R^B$ and $ECF18R^b$ were associated 100% with the $M307^G$ and $M307^A$ alleles, respectively. The haplotypes $ECF18R^B/M307^A$ or $ECF18R^b/M307^G$ have not been detected. Consequently, the coefficient of linkage disequilibrium *ECF18R*(*B*,*b*)–*FUT1*(*G*,*A*) is of maximum value. The finding that the two categorical variables *ECF18R* and *FUT1* are identical is consistent with the assumption that they represent the same gene. This is further supported by our previously published genetic studies (Meijerink et al. 1997). As a direct result of the cosegregation of alleles at the *FUT1* and *ECF18R* loci, we found that in pigs not expressing FUT2, the average GDP-fucose activity in intestinal cell extracts was significantly lower in $FUT1M307^{AA}$ ($ECF18R^{bb}$) pigs than in $FUT1M307^{G\cdot}$ ($ECF18R^{B\cdot}$) pigs, in the presence of phenyl-β-D-Gal as acceptor (Table 3). A significant difference (30%, $P<0.007$) in enzyme activity was also observed when the FUT1 enzyme variants were expressed in CHO cells. Fucosyltransferase assays using other acceptors (paranitrophenyl-β-D-galactopyranoside, N-acetyllactosamine, and lacto-tetraose) showed a similar reduction for the FUT1 $M307^A$ construct. Furthermore, results obtained in transfection experiments using a pig liver endothelial cell line (PLECT) were consistent with the results found in CHO cells. Results both from experiments with mucosal scrapings and from the transfection experiments suggested that the alanine-to-threonine substitution at amino acid position 103 affected either the properties or the quantity of mature FUT1 enzyme. In comparison with the fucosyltransferase

activity data obtained with intestinal scrapings, we observed a relatively high residual activity for the FUT1 M307^{A} variant in the transfection experiment. This may be due to overexpression of the FUT1 protein in transient cell cultures, which does not mimic *FUT1* expression and processing in enterocytes.

Several lines of evidence support the hypothesis that the G→A transition at nucleotide 307 in the *FUT1* gene is the cause of resistance to adhesion of F18 *E. coli* bacteria to enterocytes. These include the results of the previous linkage analysis (Meijerink et al. 1997), the FUT1 transfer activities measured in small intestinal scrapings, and the differences observed in fucose transfer between the two *FUT1* variants in transfected mammalian cells. The availability of a diagnostic test for the *M307* mutation will enable breeders to eliminate the *FUT1 M307^{G}* allele from their herds, thereby eliminating the major cause of edema-induced death and post-weaning diarrhea.

We found no polymorphisms in the *FUT2* CDS, but it was differentially expressed, resulting in either the absence or the presence of *FUT2* RNA in the intestinal mucosa. The specific DNA modification suppressing *FUT2* expression is predicted to be localized in a regulatory domain of the gene. Expression of *FUT2* RNA has a significant influence on fucose transferase activity. Nonetheless, *FUT2* RNA expression does not determine the ECF18R phenotype, as 11 of the 34 ECF18-susceptible animals (*ECF18R$^{B\cdot}$*) did not express detectable levels of *FUT2* RNA in small intestinal mucosal preparations (Table 2). A possible function of the *FUT2* gene in determining plasma antigen 0 and erythrocyte antigen 0 expression in the pig is proposed based on its homology to the human *Secretor* gene. All pigs expressing *FUT2* in intestinal mucosa (23) plus 15 additionally tested *FUT2*-expressing Duroc pigs were shown to present the 0 antigen on erythrocytes ($S^{S\cdot}$). However, six animals that failed to express *FUT2* in intestinal mucosa were also genotyped as $S^{S\cdot}$. This demonstrates that expression of *FUT2* in intestinal mucosa does not mirror precisely the presence of antigen 0 on erythrocytes. We expect that expression patterns of *FUT2* in other secretory tissues will more precisely reflect and determine erythrocyte antigen 0 appearance.

The origin of pig erythrocyte 0 antigens, which are first released in plasma and secondarily presented on erythrocytes, shows similarities to the expression of the human H type I antigen determined by the *Secretor* locus. In humans, the *Secretor* locus (*FUT2*) forms an epistatic system with the *Lewis* (*Le*) gene [α(1,3/4)fucosyltransferase, *FUT3*], and most of the H type I antigen (Fucα1,2Galα1,3GlcNAc) is transformed into the Lewis^{b} (Le^{b}) [Fucα1,2Galα1,3(Fucα1,4)GlcNAc] antigen. However, the plasma 0 antigen in pigs is of type I (Sako et al. 1990), but is not transformed into Lewis antigen. This can be explained by the preference of the pig Lewis enzyme for type II (Fucα1,2Galα1,4GlcNAc) substrates. We found the pig *FUT3* nucleotide sequence (unpublished data) to encode an amino acid motif that is conserved among fucosyltransferases using type II substrates (Costache et al. 1997). This was also shown by acceptor specificity studies of endogenous fucosyltransferases in a pig liver endothelial cell line (Sepp et al. 1997). Therefore, the hypothesis that the appearance of serum antigen 0 in pigs is the counterpart of the human *Secretor* phenotype cannot be rejected.

A second explanation can be proffered for the fact that the plasma 0 antigen in pigs is of type I but is not transformed into Lewis antigen. Another closely linked gene exhibiting α(1,2)fucosyltransferase activity possibly influences the blood group inhibitor S^{S} phenotype. Two arguments speak against *FUT1* being involved in pig plasma and erythrocytic 0 antigen of type I. The occurrence of five pigs of the S^{ss} phenotype (Table 2) possessing at least one *M307^{G}* allele, which is regarded as the functional allele, rules out a possible significance of the *FUT1 M307* mutation for plasma and erythrocyte antigen 0 levels. Furthermore, in vitro, pig FUT1 preferentially fucosylates type II chains and FUT2 preferentially acts on type I precursor (M. Sandrin, personal communication).

We also analyzed the sequence and expression of a previously reported pig *FUT2* paralogue, designated as *Sec1* in other mammalian species (Barreaud et al. 2000). We could not identify an acceptable start codon (Meijerink et al. 1997), nor could we obtain this sequence from our cDNA library. This sequence also failed to cross-hybridize with the labeled *FUT2* probe in lanes 1–4 and 7 of Fig. 2B. If *Sec1* were expressed in intestinal mucosa, cross-hybridization would be expected from its sequence homology to the *FUT2* gene. Although we cannot exclude the possibility that this pig *Sec1* homologue is expressed in other tissues, we assume it to be an inactive pseudogene.

Taken together, these studies clearly demonstrate that the FUT1 enzyme plays a critical role in the process of adhesion of ECF18 bacteria to the mucosa of the small intestine in pigs. This suggests that the *FUT1* gene is a potentially useful target to prevent edemic disease and post-weaning diarrhea in piglets.

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