Troisième partie :

Présentation des résultats

1. Etude #1 :

Etudes sur l'anticorps monoclonal 2F3

Cette étude est basée sur les trois manuscrits suivants :

-2F3 Monoclonal Antibody Recognizes the O26 O-Antigen Moiety of the Lipopolysaccharide of Enterohemorrhagic *Escherichia coli* Strain 4276.
I. M. Szalo, B. Taminiau, F. Goffaux, V. Pirson, J. McCappin, H. J. Ball, and J. G. Mainil.
Clin. Diagn. Lab. Immunol. 2004, **11** : 532–537

- Genetic analysis of the rfb gene cluster from a non-AEEC O26 strain and comparison with the rfb locus from an AEEC O26 strain.
I.M. Szalo, F. Goffaux, B. China, B. Taminiau, V. Pirson, G. Daube, J.G. Mainil.

- Relationship between the 2F3 character and the O antigen: study by random insertional mutagenesis in a wild type O26 attaching and effacing *Escherichia coli* strain
I.M. Szalo, B. Taminiau, F. Goffaux, V. Pirson, J.G. Mainil.

1.1. Préambule

Les souches AEEC occupent une place important dans la pathologie digestive chez les bovins, ainsi que dans le domaine de la santé publique. Même si chez les bovins adultes les souches EHEC, dont les plus connues appartiennent aux sérotypes O157:H7 et O157:H-, ne provoquent aucun trouble, elles sont importantes en santé publique, car elles sont la cause des infections graves chez l'homme. Chez les veaux, les souches EPEC et EHEC sont associées à de la diarrhée entre 2 et 8 semaines avec un pic à 4-5 semaines, bien que des veaux nouveau-nés ou plus âgés puissent aussi être affectés (China *et al.*, 1998). La majorité des souches EHEC associées aux troubles cliniques chez les veaux appartiennent aux sérotypes O5:H-, O26:H11, O111:H- et O118:H16. Quant aux souches EPEC, elles ont été moins étudiées, mais celles qui l'ont été appartiennent surtout au sérotype O26:H11.

L'anticorps monoclonal 2F3 (Kerr *et al.*, 1999) a été dérivé contre des extraits de protéines de membrane externe et fimbriaires de la souche EHEC bovine 4276 du sérogroupe O26. Cet anticorps utilisé dans un test ELISA sandwich reconnaît les souches EPEC et EHEC bovines et humaines du sérogroupe O26 et la moitié de celles du sérogroupe O111. De plus, une protéine d'un poids moléculaire avoisinant 21 kDa est reconnue en western blotting sur des extraits protéique de membrane externe et fimbriaires de la souche 4276 d'origine. Le fait que : i) peu soit connu à ce jour sur les facteurs impliqués dans l'adhérence initiale des souches AEEC bovines ; ii) l'anticorps 2F3 est spécifique pour certaines souches AEEC ; et iii) la taille de la protéine reconnue en western blotting est similaire à celle de la sous-unité majeur de fimbriae de type IV, nous ont convaincu d'identifier l'antigène reconnu par l'anticorps 2F3 et d'en cloner les déterminants génétiques, dans l'espoir d'isoler et caractériser une adhésine spécifique.

Les travaux de cette étude impliquent la réalisation d'une banque génétique en cosmide à partir de la souche EHEC bovine 4276, le screening de cette banque dans un test ELISA avec l'anticorps monoclonal 2F3, afin d'identifier un clone exprimant à sa surface l'antigène reconnu par cet anticorps et d'obtenir des informations sur la base génétique de l'antigène reconnu par l'anticorps monoclonal 2F3 (article #1). Durant ces travaux, des souches AEEC O111 positives dans le test ELISA avec l'anticorps monoclonal 2F3 dans l'étude initial (Kerr *et al.*, 1999), se sont révélées contaminées avec des souches AEEC O26 (Ball, communication personnelle). Pour cette raison il a été décidé de vérifier la spécificité

de l'anticorps monoclonal 2F3 pour les souches AEEC des sérogroupes O26 et O111 (article #1).

Etant donné les résultats obtenus durant ces premiers travaux et afin de comprendre la spécificité de l'anticorps monoclonal 2F3 pour l'antigène O26 des souches AEEC, il a été par la suite décidé : i) de comparer la séquence génétique de l'opéron codant pour l'antigène O d'une souche AEEC de sérogroupe O26 avec celle de l'opéron codant pour l'antigène O d'une souche non-AEEC de sérogroupe O26 (article #2) ; ainsi que ii) de recréer, par mutagenèse aléatoire à l'aide du transposon mini-Tn*10* dans une souche AEEC sauvage de sérogroupe O26 une mutation provoquant la perte de la reconnaissance par l'anticorps 2F3 (article #3).

En conclusion, l'anticorps monoclonal 2F3 reconnaît le polysaccharide formant l'antigène O26 du lipopolysaccharide (LPS) des souches AEEC, quel que soit leur hôte, et pas l'antigène O26 des souches non-AEEC. Bien que ces deux types de souches puissent être distingués, la base de cette différence de reconnaissance n'a pas été identifiée. Elle ne vient apparemment pas des différences de séquences entre les loci *rfb* des souches AEEC et non AEEC.

1.2. Article #1

2F3 Monoclonal Antibody Recognizes the O26 O-Antigen Moiety of the Lipopolysaccharide of Enterohemorrhagic *Escherichia coli* Strain 4276.

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2F3 Monoclonal Antibody Recognizes the O26 O-Antigen Moiety of the Lipopolysaccharide of Enterohemorrhagic *Escherichia coli* Strain 4276

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Enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) organisms are groups of pathogenic strains whose infections are characterized by a typical lesion of enterocyte attachment and effacement. They are involved in enteric diseases both in humans and in animals, and EHEC strains can be responsible for hemolytic uremic syndrome in humans. Previously, it was shown that the 2F3 monoclonal antibody (MAb) is specific for the O26 EHEC and EPEC strains (P. Kerr, H. Ball, B. China, J. Mainil, D. Finlay, D. Pollock, I. Wilson, and D. Mackie, Clin. Diagn. Lab. Immunol. 6:610–614, 1999). As these groups of bacteria play an important role in pathology, the aim of this paper was to characterize the antigen recognized by the 2F3 MAb and its genetic determinant. A genomic locus containing the entire O-antigen gene cluster and half of the colanic acid gene cluster from an O26 EHEC strain was shown to be sufficient for the production of the antigen recognized by the 2F3 MAb in an *E. coli* DH5α strain. By transposon mutagenesis performed on the recombinant plasmid, all 2F3 enzyme-linked immunosorbent assay-negative mutants had their transposons inserted into the O-antigen gene cluster. The O-antigen gene cluster was also cloned from an O26 EHEC strain into the *E. coli* DH5α strain, which then produced a positive result with the 2F3 MAb. Further analysis of the type of lipopolysaccharides (smooth or rough) produced by the clones and mutants and of the O antigen of the 2F3-positive clones confirmed that the epitope recognized by the 2F3 MAb is located on the O antigen in the O26 EHEC and EPEC strains and that its genetic determinant is located inside the O-antigen gene cluster.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are an important class of pathogens for humans and animals. The term EHEC denotes *E. coli* strains that cause attaching and effacing lesions on epithelial cells, express Shiga toxin-encoding genes, possess a 60-MDa virulence plasmid, and produce hemorrhagic colitis and/or hemolytic uremic syndrome (17, 18).

Besides those of the O157:H7 and O157:H$^-$ serotypes, EHEC strains of serogroup O26 are the most common strains found in human infections (4, 17). In addition, O26 EHEC strains are an important cause of enteritis in calves (12). One of the most important steps in the pathogenic mechanism of enteric bacteria is the initial adhesion of the bacteria to the intestinal wall. Several stages of colonization have been recognized in attaching and effacing *E. coli* pathogenesis, some of which await characterization. Some level of host specificity is shown by EHEC strains, but the role in host specificity of the recognized virulence factors, such as Shiga toxin 1 (Stx1) and/or Stx2 and the intimin protein (a protein that is encoded by the *eae* gene and necessary for the production of the attaching and effacing lesion), is unknown (8, 19). The bovine host specificity shown by O26 EHEC and enteropathogenic *E. coli* (EPEC) strains could be based on the production of a colonization factor, such as an adhesin, that is specific for cattle; such a colonization factor has been demonstrated to be the basis of the host specificity of classical human EPEC strains. Such an adhesin is likely to be a specific immunogen exposed at the bacterial cell surface (3, 13, 15, 17, 23, 24).

A monoclonal antibody (MAb), 2F3, was derived against a surface antigen extracted from the bovine O26 EHEC strain 4276; it tested positive in an enzyme-linked immunosorbent assay (ELISA) with all of the O26 and half of the O111 bovine and human EHEC and EPEC strains (9). Recent reexamination of six of the O111 EHEC and EPEC strains that tested positive revealed a contamination by O26 EHEC or EPEC strains. Purification of the strains in the mixed culture demonstrated that the O111 strains were 2F3 negative and the O26 strains were 2F3 positive (J. McCappin and H. Ball, unpublished data).

Since the 2F3 MAb is specific for the O26 EHEC and EPEC strains, it may not only represent a diagnostic tool of these confirmed pathogens but also recognize a factor involved in the production of the attaching and effacing lesion. In view of these results, this study was initiated (i) to characterize the antigen recognized by the 2F3 MAb and its genetic determinant and (ii) to reexamine the specificity of the 2F3 MAb within the O26 serogroup.

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MATERIALS AND METHODS

Bacterial strains and media. The O26 EHEC strain 4276, isolated from a calf with a case of enteritis in Northern Ireland, was used to characterize the genetic

TABLE 1. *E. coli* strains used in this study and their 2F3 ELISA results

Strain	Pathotype	Serotype[b]	Test result[a]			Source
			2F3[c]	eae[d]	O26[e]	
HS	K-12	O9:H4	−	−	−	Human
E2348	EPEC	O127	−	+	−	Human
H511	EHEC	O102	−	+	−	Human
193	EHEC	O26	+	+	+	Cattle
4276	EHEC	O26	+	+	+	Cattle
357S89	EHEC	O26:H11	+	+	+	Cattle
379S89	EHEC	O26:H11	+	+	+	Cattle
631KH91	EHEC	O26	+	+	+	Cattle
122	EHEC	O26	+	+	+	Cattle
63	EHEC	O26	+	+	+	Cattle
A39	EHEC	O26	+	+	+	Cattle
A14	EHEC	O26	+	+	+	Cattle
3953	EPEC	O26	+	+	+	Cattle
C15333	EPEC	O26	+	+	+	Cattle
333KH91	EPEC	O26	+	+	+	Cattle
334KH91	EPEC	O26	+	+	+	Cattle
351KH91	EPEC	O26	+	+	+	Cattle
352KH91	EPEC	O26	+	+	+	Cattle
TC3108	EHEC	O26	+	+	+	Cattle
TC3109	EHEC	O26	+	+	+	Cattle
TC3117	EHEC	O26	+	+	+	Cattle
TC3180	EHEC	O26	+	+	+	Cattle
TC3269	EHEC	O26	+	+	+	Cattle
TC3273	EHEC	O26	+	+	+	Cattle
TC3302	EHEC	O26	+	+	+	Cattle
TC3305	EHEC	O26	+	+	+	Cattle
TC3375	EHEC	O26	+	+	+	Cattle
TC3380	EHEC	O26	+	+	+	Cattle
TC3629	EHEC	O26	+	+	+	Cattle
TC3630	EHEC	O26	+	+	+	Cattle
TC3631	EHEC	O26	+	+	+	Cattle
TC3632	EHEC	O26	+	+	+	Cattle
TC3656	EHEC	O26	+	+	+	Cattle
TC3657	EHEC	O26	+	+	+	Cattle
TC3748	EHEC	O26	+	+	+	Cattle
TC6169	EHEC	O26:H11	+	+	+	Cattle
TC659	EPEC	O26:HNM	+	+	+	Cattle
TC1988	EPEC	O26:HNM	+	+	+	Cattle
TC3145	EPEC	O26	+	+	+	Cattle
TC3486	EPEC	O26	+	+	+	Cattle
TC3848	EPEC	O26:H11w	+	+	+	Cattle
TC4004	EPEC	O26	+	+	+	Cattle
TC4219	EPEC	O26	+	+	+	Cattle
TC4221	EPEC	O26	+	+	+	Cattle
EH182	EHEC	O26:H11	+	+	+	Human
EH193	EHEC	O26:H11	+	+	+	Human
EH196	EHEC	O26	+	+	+	Human
EH284	EHEC	O26	+	+	+	Human
EH296	EHEC	O26:H11	+	+	+	Human
EH298	EHEC	O26	+	+	+	Human
EH322	EHEC	O26	+	+	+	Human
EH324	EHEC	O26	+	+	+	Human
EH31	EHEC	O26:H11	+	+	+	Human
TC5710	EHEC	O26:H11	+	+	+	Human
TC5711	EHEC	O26:H11	+	+	+	Human
TC6168	EHEC	O26:H11	+	+	+	Human
H19	EHEC	O26:H11	+	+	+	Human
TC6165	EPEC	O26:H11	+	+	+	Human
TC6166	EPEC	O26:HN	+	+	+	Human
TC6167	EPEC	O26:HN	+	+	+	Human
C3888	EPEC	O26:H-	+	+	+	Human
C4115	EPEC	O26:H-	+	+	+	Human
02/113	EHEC	O26	+	+	+	Human
99/109	EHEC	O26	+	+	+	Human
03/178	EPEC	O26	+	+	+	Human
03/151	EHEC	O26	+	+	+	Human
03/139	EHEC	O26	+	+	+	Human
99/145	EHEC	O26	+	+	+	Human
99/147	EHEC	O26	+	+	+	Human
00/054	EPEC	O26	+	+	+	Human
00/106	EPEC	O26	+	+	+	Human
00/113	EPEC	O26	+	+	+	Human
02/145	EPEC	O26	+	+	+	Human
02/057	EPEC	O26	+	+	+	Human
00/103	Non-EPEC	O26	−	−	+	Human
00/130	Non-EPEC	O26	−	−	+	Human
03/023	Non-EPEC	O26	−	−	+	Human
C4071	Non-EPEC	O26:H32	−	−	+	Human
T652	Non-EPEC	O26:H28	−	−	+	Pig
739	Non-EPEC	O26	−	−	+	Poultry
T282	Non-EPEC	O26:H11	−	−	+	Cattle

[a] +, positive result; −, negative result.
[b] According to the laboratory of origin.
[c] 2F3 MAb ELISA.
[d] PCR multiplex assay (5).
[e] Anti-O26 *E. coli* immune serum.

basis of the antigen recognized by the 2F3 MAb (*2f3* antigen). This strain was characterized to be *eae* and stx_1 positive and to produce a complete lipopolysaccharide (LPS) with an O antigen belonging to the O26 serotype (9). The DH5α K-12 derivative *E. coli* strain was used as the host strain for the cloning and mutagenesis assays. It possesses the O-antigen (*rfb*) gene cluster for the production of an O16-type O antigen but produces an LPS without the O antigen and rough-type colonies on solid medium as a consequence of two independent mutations in the *rfb* gene cluster. The first mutation (the *rfb-50* mutation) consists of an IS5 insertion at the 3′ end of the *rfb* gene cluster and is representative of most K-12 derivatives. The second mutation (the *rfb-51* mutation) consists of a deletion at the 5′ end of the *rfb* gene cluster (11).

A collection of 70 O26 EPEC or EHEC strains, 7 O26 non-EPEC and non-EHEC strains, and 3 *E. coli* strains belonging to other serogroups were used to reexamine the specificity of the 2F3 MAb. The strains were isolated from humans or animals, either with diarrhea disease or hemolytic uremic syndrome or without clinical signs, in Europe and North America (Table 1).

Bacteria were grown overnight at 37°C in Luria broth (LB) or Luria agar (LB with 1.5% [wt/vol] agar [Oxoid]). Media were supplemented with ampicillin at 100 μg/ml or kanamycin at 50 μg/ml (Sigma) when needed.

Construction of a DNA genomic library. The cosmid library was obtained from the O26 *E. coli* strain 4276 (9) by using the Expand cloning kit (Roche, Mannheim, Germany). Briefly, total genomic DNA, extracted with an AquaPure genomic DNA purification kit (Bio-Rad), was partially digested by serial dilution at 37°C with EcoRV (Invitrogen) in order to generate blunt-end fragments compatible with the linearized vector. Following the inactivation of the enzyme at 65°C for 15 min, fragments approximately 10, 20, and 30 kb in length were inserted into three pHC79 cosmid derivative vectors with a cloning capacity of 7.0 to 16.5, 16.5 to 25.0, and 25.0 to 36.0 kb, respectively. The constructs were packaged into a lambda bacteriophage and then transfected into a DH5α *E. coli* magnesium culture, according to the manufacturer's instructions. Transformants were selected on LB plates containing ampicillin, and the *2f3* antigen-positive clones were identified by an ELISA.

In vitro mutagenesis. Independent mutations in the recombinant plasmid from the 2F3 ELISA-positive clone were performed by an in vitro insertion strategy using the EZ::TN <KAN-2> insertion kit (Epicentre). The kit uses the benefit of the Tn*5* transposition system. The transposon containing the kanamycin resistance gene is randomly inserted into the target DNA under the control of transposase that is separately added to the reaction mix. After the inactivation of

TABLE 2. Sequences of the primers used in this study

Primer name	Primer sequence	Reference
gnd	5′-CACTGCCATACCGACGACGCCGATCTGTTGCTTG-3′	2
galF	5′-ATTGGTAGCTGTAAGCCAAGGGCGGTAGCGT-3′	2
Kan2 FP	5′-ACCTACAACAAAGCTCTCATCAACC-3′	Epicentre
Kan2 RP	5′-GCAATGTAACATCAGAGATTTTGAG-3′	Epicentre
M13 FP	5′-GTAAAACGACGGCCAG-3′	Invitrogen
M13 RP	5′-CAGGAAACAGCTATGAC-3′	Invitrogen
wbuA FP	5′-CTCTGATTATACAGAAGCA-3′	7
wbuA RP	5′-AGTCGTAATATGAGCTTTTC-3′	7

the transposase, the transposon can no longer move from its insertion site. The target DNA was the cosmid DNA purified from the 2F3 ELISA-positive clone with the plasmid midi kit (QIAGEN, Westburg, Germany). After a 2-h insertion reaction at 37°C, the reaction mix (the target DNA, the EZ::TN <KAN-2> transposon, and EZ::TN transposase in corresponding buffer) was incubated for 10 min at 70°C to inactivate the transposase and dialyzed for 60 min against distilled water. From the mix, 0.04 μg of DNA was electroporated at 2,250 V, 40 μF, and 132 Ω into *E. coli* DH5α electrocompetent cells (Invitrogen). After incubation for 45 min at 37°C, the transformants were plated onto Luria agar plates containing kanamycin for selection.

ELISAs. An ELISA with the 2F3 MAb was used to screen the cosmid and mutant libraries for 2F3-positive and 2F3-negative clones, respectively, and all the strains from the collection. An ELISA with the anti-O26 *E. coli* agglutinating serum (Bio-Rad) was used to serotype the 2F3 ELISA-positives clones. Assays were performed with 96-well microtiter plates (Greiner) as previously described (9).

The absorbance was measured at 450 nm with an ELISA plate reader (Bio-Rad). The strains 4276 and DH5α were used as positive and negative controls, respectively. The 2F3 ELISA-positive cosmid clone used in the mutagenesis assay was also used as a positive control to screen the mutant library. Optimum dilution of the reagents was obtained by titration. Readings three times higher than the average negative-control value were considered positive reactions.

LPS production and serotyping assays. The presence of a complete LPS (with the O antigen) or of an incomplete LPS (without the O antigen) was detected by staining distinct colonies by the crystal violet staining assay (22). The smooth colonies are not stained since the presence of the O antigen on the LPS prevents the dye from penetrating the bacterial cells, whereas the rough colonies, because of the absence of the O antigen on the LPS, take up the dye and stain blue.

Serotyping was performed on wild-type strains, clones, and mutants in an agglutination assay using *E. coli* antiserum from Laboratorio de Referencia de *E. coli* (LREC), Universidad de Santiago de Compostela, Lugo, Spain, for serogroups O26 and O16 and in an ELISA using *E. coli* antiserum (Bio-Rad) for serogroup O26. Briefly, the agglutination reaction was performed in microtiter plates with 50 μl of antiserum diluted 1/80 and 50 μl of boiled antigen suspension. Plates were examined for agglutination after overnight incubation at 37°C. O26 and O16 reference strains were used as positive and negative controls. *E. coli* strain DH5α was used as the negative control.

The anti-O26 O-antigen immune serum (Bio-Rad) was also used in the slide agglutination assay to detect the O26 O antigen among the collection of *E. coli* O26 strains.

A PCR assay using primers for a specific gene (*wbuA*) (Table 2) of the O26-antigen gene cluster was performed on mutants that did not produce any O antigen to confirm the presence of the O-antigen gene cluster.

DNA amplification. PCR amplifications were performed in a PCR Sprint thermocycler (Labsystems, Brussels, Belgium). A long-range PCR assay, using the Expand high-fidelity PCR system (Roche), was performed to amplify the O-antigen gene cluster or to obtain an estimation of the position of the insertion site of the transposons. To amplify the O-antigen gene cluster, primers specific for the *gnd* and *galF* genes (Table 2), two housekeeping genes bordering the O-antigen gene cluster, were used either on the genomic DNA from strain 4276 or on the cosmid DNA extracted from the 2F3 ELISA-positive clones from the cosmid library (2). The amplified DNA fragment from strain 4276 was subsequently cloned into DH5α *E. coli* by using the Expand cloning kit (Roche). To estimate the position of the insertion site of the transposons, the template DNA was the cosmid DNA extracted from the 2F3 ELISA-negative mutants. The primers used (Kan2 FP and Kan2 RP) were specific for the transposon (Table 2). After an initial denaturation stage of 5 min at 94°C, PCRs were carried out under the following conditions: denaturation at 94°C for 30 s, annealing at 64°C for 30 s, and extension at 68°C for 15 min for 17 cycles in a 50-μl reaction volume. A final extension stage at 68°C for 15 min was also performed.

Standard PCR assays were also performed using Taq DNA polymerase (Roche) to screen the rough mutants for O26-specific genes, as previously described (7). The primers used were specific for the *wbuA* gene (Table 2). The pathotypes of the strains from the collection were checked by PCR assay as described previously (5).

DNA sequencing. The cosmid DNA purified from the clones and mutants of interest and DNA fragments produced by long-range PCR assay and purified with a QiaQuick PCR purification kit (QIAGEN) were submitted for sequencing. Sequencing was performed by the dideoxynucleotide chain termination method (20). Automatic reading was performed by using an ALF DNA sequencer (Pharmacia) and primers fluorescently labeled with Cy5 from Pharmacia. Sequence analysis and comparisons were performed by using Gene Work 2.4 (Intelli-Genetics, Mountain View, Calif.) and the National Center for Biotechnology Information BLAST network server (1).

The M13 FP and M13 RP primers (Invitrogen) were used to sequence the extremities of the fragment inserted into the 2F3 ELISA-positive clone from the cosmid library. The Kan2 FP and Kan2 RP primers (Epicentre) were used to sequence the extremities bordering the transposon in the 2F3 ELISA-negative clones from the mutant library (Table 2).

Western blotting. Polyacrylamide gel electrophoresis was carried out on a whole-cell preparation from the wild-type O26 EHEC (4276) and EPEC (C15333) strains and from the clones obtained by restriction (IXD2) and long-range PCR (IIIC7) using NuPAGE 4 to 12% gradient gels under reducing conditions (Novex; Invitrogen) according to the manufacturer's instructions. Gels were stained with SimplyBlue SafeStain (Invitrogen) or transferred onto a nitrocellulose membrane by blotting at 30 V for 1 h. After the transfer and blocking stages, the membranes were probed either with the 2F3 MAb or with the anti-O26 immune serum (Bio-Rad) as primary antibody. Specific immunoglobulin peroxidase conjugate was used as a secondary antibody (Sigma). The peroxidase substrate was 4-chloro-1-naphthol (Sigma), and after 10 min of incubation, the membrane was washed with distilled water to stop any further reaction.

RESULTS

Cosmid library screening. A cosmid library of more than 2,000 clones was obtained from strain 4276. One of them (IXD2) was ELISA positive with the 2F3 MAb (Table 3). The length of the insert was estimated, in a 0.5% agarose electrophoresis gel, to be approximately 30 kb.

Both the M13 FP and M13 RP primers were used to sequence the two extremities of the insert. The sequence obtained with the M13 RP primer showed 95% identity (129 of 135 bp) with the *ugd* gene that codes for UDP-glucose 6-dehydrogenase and is found on existing genomic maps of both *E. coli* O157:H7 and *E. coli* K-12. The sequence obtained with the M13 FP primer showed 97% identity (216 of 222 bp) with the *wca*F gene, which codes for a putative acetyltransferase also found on existing genomic maps of both *E. coli* O157 and *E. coli* K-12.

TABLE 3. Typing the wild-type strains, the 2F3 ELISA-positives clones, and the mutants

Test[a]	Test result											
	O26 wild-type *E. coli* strain:		Clone 1[b] (IXD2)	Host strain (DH5α)	Mutant obtained from the clone IXD2					Clone 2[c] (IIIC7)	Control strain serotype:	
	4276	C15333			F5	F6	E4	H2	B1		O26	O16
2F3	+	+	+	−	−	−	−	−	−	+	−	−
LPS	+	+	+	−	−	−	−	−	−	+	+	+
O26	+	+	−	−	−	−	−	−	−	+	+	−
O26 ELISA	+	+	+	−	−	−	−	−	−	+	+	−
O16	−	−	−	−	−	−	−	−	−	−	−	+
wbuA	+	+	+	−	+	+	+	+	+	+	+	−

[a] 2F3, ELISA with the 2F3 MAb as the primary antibody; LPS, LPS production checked by the crystal violet staining method; O26 and O16, agglutination with polyclonal immune serum to O26 (from Bio-Rad and LREC) and O16 (from LREC); O26 ELISA, O26 ELISA with polyclonal immune serum to O26 (Bio-rad); *wbuA*, PCR for an O26-specific gene.
[b] Clone obtained by restriction of genomic DNA.
[c] Clone obtained by long-range PCR.

In vitro mutagenesis. In order to identify the genetic determinant of the antigen recognized by the 2F3 MAb, in vitro insertional mutagenesis was performed with the recombinant plasmid from the 2F3 ELISA-positive clone. More then 1,400 mutants were obtained. After this mutant library was screened by ELISA with the 2F3 MAb, more than 100 mutants were found to be negative.

The DNA sequences bordering the extremities of the transposon in the recombinant plasmid from five of these mutants (Table 3) were determined by using specific primers for the extremities of the transposon (Kan2 FP and Kan2 RP). The sequences obtained were compared with those from GenBank.

The sequences bordering the transposons from the H2, F5, and F6 mutants showed 99% identity with the *fnl-1* gene from *E. coli* O26:K60:H311b. These sequences also showed 80% identity with the *wbjB* gene from *Pasteurella multocida* and *Pseudomonas aeruginosa* and 76% (192 of 258 bp) identity with the *rfb* gene cluster of *Leptospira interrogans*. For the E4 mutant, the sequences obtained showed 99% (320 of 323 bp) identity with the *wbuA* gene from *E. coli* O26:K60:H311b.

The sequences obtained with the B1 mutant showed 95% (622 of 652 bp) identity with the *wbuB* gene from *E. coli* O26:K60:H311b. The site of the transposon insertions and the disrupted genes are presented in Fig. 1.

Presence of the O26 gene cluster in the 2F3 ELISA-positive clone. By long-range PCR using specific primers (gnd and galF) to amplify the O-antigen gene cluster, a fragment approximately 13 kb long was obtained. Further PCR assays using specific primers for the extremities of the transposons and for the *galF* and *gnd* genes showed that for all sequenced mutants, the transposons had been inserted inside the O-antigen gene cluster at different positions (Fig. 1).

For three of the mutants (F5, F6, and H2), the transposon was inserted at similar locations, approximately 5 kb from the *gnd* gene and approximately 8 kb from the *galF* gene. The distance between the sites of insertion of the transposons in the mutants F5 and F6 was 31 bp, and similarly, the sites of insertion of the transposons in the mutants F6 and H2 were 174 bp apart.

In the E4 mutant, the transposon was inserted approximately 6 kb from the *gnd* gene and 7 kb from the *galF* gene. In the B1 mutant, the transposon was inserted 0.8 kb from the *gnd* gene and approximately 12 kb from the *galF* gene.

Cloning the O-antigen gene cluster. The product of the long-range PCR using the galF and gnd primers from strain 4276 was cloned into the *E. coli* strain DH5α. This recombinant clone (IIIC7) tested positive in the ELISA with the 2F3 MAb and with the anti-O26 immune serum (Table 3).

O-antigen production and serotyping assays. In order to study the relationship between the O26 antigen and the 2F3 character, the production of the O antigen was examined by using the crystal violet staining assay (Table 3). The 2F3 ELISA-positive clones (IXD2 and IIIC7) and the wild-type 2F3 ELISA-positive strains (4276 and C15333) produce a smooth-type LPS (with the O antigen). The host strain (*E. coli* DH5α) and all 2F3 ELISA-negative mutants produce a rough-type LPS (without the O antigen). The antigenic specificity of the O antigen was confirmed by an agglutination assay and by an ELISA. The 2F3 ELISA-positive strains and clones tested positive with the O26 immune serum, but the IXD2 clone

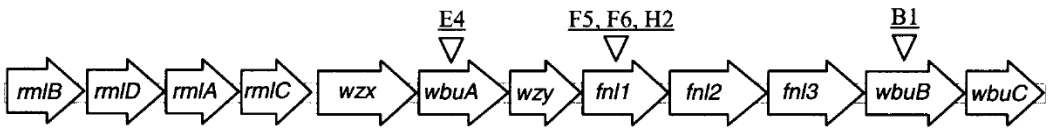

FIG. 1. Schematic representation of the insertions of the transposon EZ::TN <Kan-2> within the O26-antigen gene clusters in the mutants (E4, F5, F6, H2, and B1) obtained by insertional mutagenesis from the clone IXD2. The arrowheads target the mutated gene and not the actual insertion site.

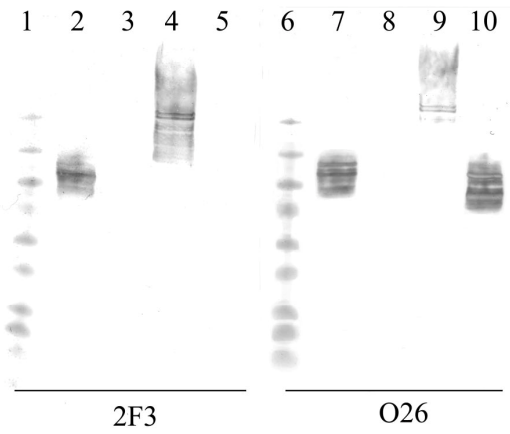

FIG. 2. Nitrocellulose immunoblot using 2F3 MAb and the anti-O26 immune serum (Bio-Rad) comparing whole-cell preparations of *E. coli* strains 4276 (lanes 2 and 7) (O26; *eae* and verotoxin positive), DH5α as a negative control (lanes 3 and 8), clone IXD2 (lanes 4 and 9), and T282 (O26; *eae* and verotoxin negative). Lanes 1 and 6 contain the benchmark prestained molecular weight markers (Invitrogen).

tested negative in the agglutination assay and with the O16 serum. Conversely, the 2F3 ELISA-negative strain and mutants gave negative results with both immune sera. Nevertheless, in a PCR using primers for a specific gene of the O26 O-antigen gene cluster (*wbuA*), positive results were obtained with all 2F3 ELISA-positive and -negative wild-type strains, clones, and mutants (Table 3).

Testing the specificity of the 2F3 MAb. The 2F3 MAb gave positive reactions in the ELISA with all 70 *eae*-positive *E. coli* strains belonging to the O26 serogroup in a slide agglutination assay (Table 1). Conversely, the seven *eae*-negative O26 *E. coli* and the three *eae*-positive or *eae*-negative non-O26 *E. coli* strains tested negative in the ELISA with the 2F3 MAb. One 2F3 ELISA-positive strain, one negative wild-type O26 strain, and two positive clones (IXD2 and IIIC7) gave almost identical results by Western blotting with the 2F3 MAb and the O26 immune serum (Fig. 2). The only difference was the absence of a reaction of the 2F3 ELISA-negative wild-type O26 strain with the 2F3 MAb. The molecular weights of the bands from the 2F3 ELISA-positive wild-type strains and the IXD2 clone were the only other noticeable differences (Fig. 2).

DISCUSSION

The fact that related EPEC and EHEC strains are pathogens for humans and animals is well recognized. There is a diversity of strains that are pathogens for humans and animals, and even inside the same serogroup, the strains found as pathogens in human diseases are often different from those found in animal diseases, which shows that they have host specificity. This host specificity demonstrates a need to classify the strains into different groups according to their properties and their pathogenicity factors. Similarly, it is a necessity for assays to provide the tools to differentiate these strains.

To obtain a better understanding of the specificity of the 2F3 MAb for the O26 EHEC and EPEC strains, the antigen recognized by the 2F3 MAb and its genetic determinant were characterized. In this study, a recombinant plasmid carrying the genetic basis of the antigen recognized by the 2F3 MAb was obtained. The length of the cloned chromosomal DNA fragment was estimated to be about 30 kb. According to the sequences of the extremities of this fragment, its position on the existing *E. coli* maps corresponds to two gene clusters, the complete O-antigen gene cluster (22) and half of the colanic acid gene cluster from open reading frame 9 to open reading frame 21 (21). Both of these clusters are involved in LPS biosynthesis pathways, and based on information available from existing genomic maps for *E. coli* K-12 and *E. coli* O157:H7, the length of the fragment could be 30.5 kb with 27 genes or 33.7 kb with 28 genes, respectively (1).

By long-range PCR assays, it was shown that the fragment from the recombinant plasmid contains the entire O-antigen gene cluster from EHEC strain 4276. Furthermore, the sequences obtained after a mutagenesis assay showed a high degree of identity with genes involved in LPS biosynthesis pathways. The *fnl-1* gene is one of the three genes that seem to be responsible for the synthesis of *N*-acetyl-L-fucosamine, one of the sugars composing the O26 O antigen (7). The *wbjB* gene from *Pasteurella multocida* and *Pseudomonas aeruginosa* and the *rfb* gene cluster of *L. interrogans* are also involved in the O-antigen biosynthesis pathway (6, 16). The *wbuA* and *wbuB* genes belong to the O-antigen gene cluster from the O26:K60:H311b *E. coli* strain and code for a putative rhamnosyl transferase and a putative L-fucosamine transferase, respectively (7).

Further analysis concerning the physical position of the transposons in the recombinant plasmid showed that all the 2F3 ELISA-negative mutants had inserted the transposon inside the O-antigen gene cluster. The F5, F6, and H2 mutants had inserted the transposon into the *fnl-1* gene, the E4 mutant inserted it into the *wbuA* gene, and the B1 mutant inserted it into the *wbuB* gene.

The relationship between the character of 2F3 and LPS was clarified by the crystal violet staining of the colonies. By cloning a 30-kb fragment containing the entire O-antigen gene cluster from an EHEC strain into a rough-type LPS strain which is 2F3 ELISA negative, a 2F3 ELISA-positive clone with a smooth-type LPS was obtained. After in vitro insertional mutagenesis from the 2F3 ELISA-positive clone, several 2F3 ELISA-negative mutants were obtained, and all mutants produced a rough-type LPS. These results demonstrated the positive linkage between the character of 2F3 and the production of a smooth-type LPS. Moreover, the LPS produced carries the O26 O antigen recognized in agglutination assays, ELISAs, and Western blot assays with the two 2F3 ELISA-positive clones obtained after genomic DNA digestion and long-range PCR. Only the clone obtained after genomic DNA digestion did not react with the anti-O26 immune serum in the agglutination assay. One explanation could be that the complementation of the two mutations within the O-antigen gene cluster of the host strain, DH5α, produces an O antigen that is recognized by the 2F3 MAb but by neither the anti-O26 immune serum nor the anti-O16 immune serum. Alternatively, the presence in this clone of the second half of the colanic acid gene cluster, which is involved in LPS biosynthesis, may affect the agglutination result. This hypothesis is supported by the observation that the

2F3 ELISA-positive clone obtained after PCR, which contains only the O-antigen gene cluster as the insert, is positive in the agglutination assay. So the presence of the disrupted colanic acid gene cluster may have resulted in the production of a hybrid LPS of a different size, as was observed by Western blotting, with interference in the agglutination assay.

In conclusion, the target of the 2F3 MAb is the O26 antigen from the EHEC and EPEC strains, and its genetic determinant is located inside the O-antigen gene cluster. The epitope recognized by the 2F3 MAb on the O26 O antigen has still not been identified. The O antigens are heterogeneous due to their sugar compositions, either monomers or polymers, and the linkage between them. The O26 O antigen contains three sugars: L-rhamnose, *N*-acetyl-L-fucosamine, and *N*-acetyl-D-glucosamine (14). The 2F3 MAb may recognize one of these sugars, a particular linkage, or a particular arrangement between them. Transposons in two of the 2F3 ELISA-negative mutants were inserted in genes involved in sugar transferase synthesis (*wbuA* and *wbuB*), resulting in the absence of the O antigen on the surfaces of these mutants. It is possible that these two genes have a role in the production of the O antigen by acting during the process of the assemblage of the O unit. On the other hand, the mutations in the *fnl-1* gene may indicate that the antigen recognized by the 2F3 MAb is its synthetic product (*N*-acetyl-L-fucosamine). These conclusions must be correlated with a possible polar effect of the transposon.

Another aspect of our findings is the presence of the antigen recognized by the 2F3 MAb on the O26 O antigen from the EIIEC and EPEC strains and not on the O26 O antigen from the non-EHEC and non-EPEC strains. This fact may be explained by a genetic alteration or modification of O-antigen biosynthesis after a lateral gene transfer or a lysogenic conversion followed by recombination (10). Because of the strong association between the *eae* gene and the antigen recognized by the 2F3 MAb among O26 EHEC and EPEC strains, it is possible that the strain acquired them in the same moment of evolution. Nevertheless, the specificity of the 2F3 MAb for the O26 EPEC and EHEC strains allows the identification of these pathogenic strains within the O26 serogroup. The 2F3 MAb can therefore be considered a pertinent epidemiological tool.

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