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Résumé

ÉTUDE DES FACTEURS DE VIRULENCE DES COLIBACILLES ENTÉROPATHOGÈNES DANS LE MODÈLE DU SOURICEAU NOUVEAU-NÉ. — La souche de *E. coli* entéropathogène B41 inoculée par voie orale à des souriceaux nouveau-nés provoque une diarrhée mortelle. Ce modèle a été utilisé pour étudier les facteurs de virulence de souches entéropathogènes d'origines bovine et porcine. Des souches possédant différents facteurs de virulence déterminés par des plasmides ont été obtenues, soit par perte spontanée de ces facteurs à partir de la souche d'origine, soit par transfert de plasmides à différents colibacilles.

Un clone B41A sélectionné à partir de la souche B41 ne produit pas l'antigène K99 mais produit encore la toxine thermostable. Ce clone B41A est presque aussi pathogène que la souche B41 d'origine puisqu'il provoque la mort de pratiquement tous les animaux en moins de 48 h après l'inoculation de 10^3 bactéries. Des plasmides ont été transférés de la souche B41 à *E. coli* C600, à des mutants résistants à l'acide nalidixique de souches de sérotypes O8, O9, O101 et à un colibacille isolé de l'intestin d'un souriceau. Les clones transconjugants K99$^+$Ent$^+$ se sont révélés peu ou pas pathogènes. Deux souches d'origine porcine K88$^+$Ent$^+$ sont faiblement pathogènes pour les souriceaux. La perte du caractère K88 ne réduit pas leur virulence. Par contre, la perte à la fois des caractères K88 et Ent les rend avirulentes. La souche de *E. coli* K12 qui a acquis le caractère K88, éventuellement avec d'autres caractères à déterminisme plasmidique, mais non la capacité de produire la toxine thermostable, n'est pas pathogène. Ainsi les facteurs K99 et K88 ne doivent pas être essentiels à la pathogénicité au moins pour le souriceau.

D'autres facteurs doivent être impliqués dans la virulence de la souche B41A. Ces résultats sont discutés du point de vue de la pathogénicité pour la souris et pour les hôtes naturellement infectés par les colibacilles entéropathogènes.

Enterotoxigenic *E. coli* (ETEC) are able to provoke diarrhea followed by dehydration and death in newborn humans, pigs, calves and lambs.

Plasmid-determined colonization factors and toxins are the most frequently implicated factors in the determination of virulence of ETEC strains. Colonization factors CFAI and CFAII (Evans *et al.*, 1975 ; Evans and Evans, 1978) for strains of human origin, K88 (Stirm *et al.*, 1967 ; Jones and Rutter, 1972) for strains of porcine origin and K99 (Orskov *et al.*, 1975) for strains of

bovine, lamb and sometimes porcine origin are determined antigenically and morphologically as pili-like structures. It is thought that these structures provoke better adhesion of bacteria to intestinal epithelium and are responsible for the greater proliferation of these bacteria, which produce in addition one or two toxins (heat-labile (LT) or/and heat-stable (ST)) (Smith and Halls, 1967a ; Smith and Linggood, 1971a ; Smith and Linggood, 1972 ; Gyles *et al.*, 1974).

As these virulence factors are plasmid-determined it is possible to obtain strains with a different combination of these factors by plasmid transfer or elimination. Thus, the particular role of K88 antigen in the proliferation of *E. coli* strains in K88 sensitive pig intestines has been proved after experimental infection of pigs with different *E. coli* strains, both possessing and not possessing K88 antigen or the ability to produce toxin (Smith and Linggood, 1971b). The role of CFAI in producing disease has also been demonstrated after inoculation of volunteers with a derivative of H10407 strain which did not produce CFAI antigen and ST toxin (Satterwhite *et al.*, 1978).

Diarrheal disease is difficult to reproduce in the calf and can be obtained only by inoculating very young animals with 10^{10} or 10^{11} bacteria (Smith and Halls, 1967b ; Bellamy and Acres, 1979), or by using axenic animals (Gouet *et al.*, 1978). Smith and Huggins (1978) studied proliferation in the alimentary tract of piglets, calves and lambs of different *E. coli* bearing different plasmid-determined virulence factors. They showed that a non-pathogenic strain (though not *E. coli* K12) can be made enteropathogenic by implanting K88 or K99 and Ent plasmids, but that the presence of K antigen, and perhaps of O antigen, could be important for colonization of the intestine. So, the exact role of identified virulence factors of ETEC strains has still not been completely elucidated. These experiments on natural hosts are costly and obviously limited in number. A small animal model would be useful to study some parameters of ETEC virulence, the protection conferred by vaccination or to assess the efficacy of treatments.

ETEC strains K99$^+$ST$^+$ of bovine origin, orally inoculated into infant mice, produce infectious diarrhea and rapid death of the animals (Duchet-Suchaux, 1980). To assess what parameters are implicated in the virulence of ETEC strains in the infant mouse model, the pathogenicity of strains with different plasmid coded virulence factors was tested. These strains were

Table 1. — Bacterial strains

Bacterial strains	Serotypes	Origin	Nalidixic acid resistant mutant[a]
Enterotoxigenic E. coli			
B41	O101:K?, K99	bovine reference strain (Smith, 1971)[b]	B41 Nal^r
P2200	O149:K91, K88ac	from a six days-old diarrheic piglet[c]	...
P5148	O149:K91, K88ac	from a four days-old diarrheic piglet[c]	...
Non enterotoxigenic E. coli			
H510a	O101 :K?:H33	reference *E. coli* strain (Ørskov *et al.*, 1977)[d]	H510 Nal^r
K14a	O9:K28:H$^-$	reference *E. coli* strain (Ørskov *et al.*, 1977)[d]	K14 Nal^r
G3404-41	O8:K8:H4	reference *E. coli* strain (Ørskov *et al.*, 1977)[d]	3404 Nal^r
C13	ND	from intestinal content of infant mouse[e]	C13 Nal^r
K12	O$^-$:K	CIP 54117 (Institut Pasteur, Paris)	K12 Nal^r
C600 PK1046	O$^-$:K$^-$	chromosomic rifampicin resistant strain[f]	...
Reference strains for plasmids			
V517	...	(Macrina *et al.*, 1978)[g]	...
C600 RP4	...	(Datta *et al.*, 1971)[f]	...

a : spontaneous mutants to nalidixic acid (40 μg/ml) obtained in our laboratory ;
b : provided by Ph. Gouet, INRA, Theix, France ;
c : provided, isolated and serotyped by L. Renault, Athis-Mons, France ;
d : provided by L. Renault ;
e : isolated in our laboratory ;
f : provided by J.S. Julliot, INRA, Versailles, France ;
g : provided by F. Casse-Delbart, INRA, Versailles, France.

obtained either after spontaneous loss of plasmid-determined characteristics from original ($K99^+$ or $K88^+$ and ST^+) strains or by plasmid transfer to different *E. coli*.

Materials and Methods

Bacterial strains

Bacterial strains and serotypes are listed in table 1.

Characterization of strains

Biochemical metabolic profiles were determined by the API system. Resistance to antibiotics was evidenced by using disks (BioMérieux) on Mueller Hinton Agar. Colicinogeny was detected by the agar-overlay method of Fredericq (1948) using *E. coli* K12 as the indicator strain. Haemolysin production was tested by culturing on sheep blood agar.

Presence of K99 or K88 antigens was identified by slide agglutination after culture at 37 °C, on Minca medium for K99 antigen (Guinée *et al.*, 1977) or on Trypticase Soy Agar (TSA) for K88 antigen. Specific antisera were obtained in the rabbit by multiple intravenous injections of $K99^+$ or $K88^+$ strains grown at 37 °C. These were absorbed with the same strain grown at 18 °C (K88 and K99 antigens are not expressed at 18 °C). Absence of K99 antigen in a derived clone from the strain B41 was confirmed by failure to absorb K99 antibodies of the B41 antiserum : this antiserum was absorbed with the strain B41 or the derived clone in the same way. Absorptions were carried out twice with one volume of serum and one volume of freshly prepared bacteria grown at 37 °C on Minca agar and on TSA. These sera were used to test agglutinability of $K99^+$ strains.

ST toxin production was determined by the suckling mouse assay (Dean *et al.*, 1972) by oral inoculation (Stavric and Jeffrey, 1977). ST toxin is the only *E. coli* toxin active upon the suckling mouse intestine, LT toxin is not ; for this reason, we did not direct our attention forward any particular study of LT toxin of porcine strains.

Plasmid-determined phenotypic characteristics were recorded as proposed by Novick *et al.* (1976). The Ent phenotypic trait is here mentioned only for ST toxin production.

Selection of clones that do not produce K99 antigen

Selection from the strain B41 of clones that do not produce K99 antigen was made by obtaining isolated clones on Minca agar medium with 0.2 ml of K99 antiserum spread on the surface. On this medium $K99^+$ clones were more opaque than clones that do not produce K99 antigen.

Selection of clones that do not produce K88 antigen

Clones that did not produce K88 antigen were selected as Raf^- colonies by growth on Drigalski medium where lactose is replaced by raffinose.

Matings

Matings were performed by mixing 0.02 ml of 24 h static cultures (10 ml Trypticase soy broth, 37 °C) of donor and recipient strains in the same medium and culturing them for 18 h at 37 °C.

For matings with $K88^+$ strains, selection of Raf^+ transconjugants was made on Drigalski medium where lactose is replaced by raffinose. After matings with the strain B41, numeration of the total number of bacteria was made on TSA ; numeration of the recipient bacteria was made on TSA with nalidixic acid (40 µg/ml) or on TSA with rifampicin (16 µg/ml) ; numeration of recipient bacteria which had acquired tetracycline resistance was made on TSA with nalidixic acid (40 µg/ml) and tetracycline (16 µg/ml) or on TSA with rifampicin (32 µg/ml) and tetracycline (16 µg/ml).

Plasmid analysis

Identification of plasmid DNA was effected by the rapid method devised by Eckhardt (1978) with some modifications concerning volumes of mixtures (F. Casse-Delbart, personal communication). Fourty microlitres of lysozyme mixture, 40 µl of SDS mixture and 100 µl of overlay mixture were used. Electrophoresis was carried out with a vertical slab gel apparatus (gel dimensions $18 \times 14 \times 2.7$ mm) (Pharmacia, type GE 4, Bois-d'Arcy, France), and two slabs with ten slots ($10 \times 10 \times 2.7$ mm). We used 0.7 or 0.8 % gel agarose (Sigma type II medium EEO) in electrophoresis buffer (89 mM tris-base, 2.5 mM disodium EDTA and 89 mM boric acid). DNA migration was carried out for 60 min at 14 mA and for 225 min at 70 mA (about 11 cm of migration). Gels were stained for 20 min with ethidium bromide (0.4 µg/ml) and photographed under UV light with a short-wave

transilluminator type C61, a polaroid type 665 film and a yellow filter.

The molecular weights of the plasmids were estimated by calculating them from a regression linear curve obtained with standard plasmids.

Pathogenicity for infant mice

Infant OF1 mice (Iffa-Credo, 69210 L'Arbresle, France), born of at least nine animals for each experiment, were randomly redistributed, eight per mother, 18 h before inoculation. At inoculation they were 18-48 h old and weighed about 2 g. For inoculation, ETEC strains of porcine origin and Raf$^+$ transconjugant clones were grown on TSA for 18-24 h at 37 °C. All other strains were grown in the same conditions on Minca agar medium (Guinée *et al.*, 1977). Bacteria were suspended in saline solution in order to inoculate infant mice orally with 10^3 or 10^5 bacteria by means of calibrated platinum loop. Mortality was recorded each day for six days.

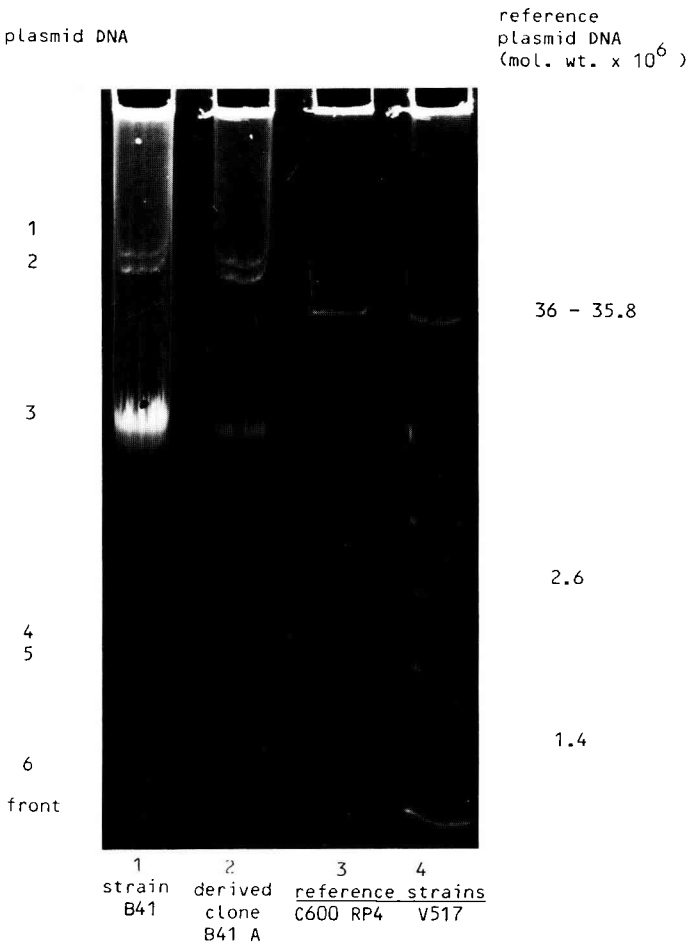

Fig. 1. — Agarose gel (0.7) electrophoresis of plasmid DNA of the strain B41 (line 1) and of a derived clone B41A (line 2) that did not produce K99 antigen. Six plasmid DNA bands can be seen on the two cases. Reference strains were C600 RP4 (line 3) (mol. wt. 36×10^6 daltons) and V517 (mol. wt. 35.8, 4.8, 3.7-3.4, 2.6, 2.0, 1.8 and 1.1×10^6 daltons).

Numeration of bacteria in intestines

Independent experiments were carried out for count of bacteria in intestines. Five days after inoculation, the infant mice were killed with chloroform and intestines were removed. They were homogenized and diluted as necessary, then plated on Drigalski medium for numeration of enterobacteria and on TSA with rifampicin (32 μg/ml) or nalidixic acid (40 μg/ml) for numeration of bacteria from the inoculated strain.

Results

1. Bovine ETEC strain B41

1.1. *Clone that does not produce K99 antigen*

One clone derived from the strain B41 (B41A) possessed the same phenotypic traits as the original strain, especially as it produced ST toxin and was resistant to streptomycin, tetracycline and sulfamide. However, this clone could neither be agglutinated by a serum anti-K99 nor could it absorb anti-K99 antibodies of a serum obtained with the strain B41.

Plasmid analysis of this clone evidenced the same number of DNA bands and migrated at the same levels as the original strain (fig. 1). This result was confirmed by several experiments. Approximative molecular weights were estimated at 75, 57, 3, 2.1, 1.8 and 1.1×10^6 daltons.

This clone B41A is very pathogenic for infant mice, and death of 21 animals out of 24 occurred as for the strain B41, 48 h after inoculation (fig. 2). Appearance of mortality was only delayed by about 20 h. We never obtained $K99^+$ revertant from this strain B41A after *in vitro* or *in vivo* multiplication.

1.2. E. coli *clones issued from mating with the strain B41 (table 2, fig. 3).*

Plasmid were transferred from the strain B41 to *E. coli* C600 PK1046 and to three *E. coli* strains with O serotypes O101, O8 and O9. These O serotypes are often found for ETEC strains $K99^+$ of bovine origin. Plasmids were also transferred to an *E. coli* isolated from infant mouse intestine. This last strain was expected to be a good colonizing strain.

Every clone which gained tetracycline resistance also gained at least streptomycin and sulfamide resistances as well as the plasmid of 57×10^6 daltons. Every clone which gained K99 and Ent phenotypic traits also gained the plasmid of 75×10^6 daltons (table 2, fig. 3).

Neither parental strains nor transferred clones which acquired only the streptomycin, tetracycline and sulfamide resistances provoked the death of more than one animal (table 3). Death of one animal in 23 or 24 has also sometimes been observed by us in groups of non-inoculated mice.

Transferred clones having gained K99 and Ent characteristics were much less pathogenic than the strain B41 which produced death of nearly all animals two days after inoculation (table 3 and fig. 2). H510 Nal^r and 3404 Nal^r clones which acquired at least the K99 and Ent characteristics, dit not provoke the death of more than one animal in 23 or 24 (table 3). K14 Nal^r and C13 Nal^r clones, which acquired at least the K99 and Ent characteristics, provoked the death of only some animal (20 % and 7 % respectively) (table 3).

Every clone tested from $K99^+Ent^+$ inoculated strains, isolated after being plated for numeration experiments in intestines, kept its ability to produce K99 antigen and ST toxin *in vitro*. An exception was the strain C13 Nal^r $K99^+$ Ent^+ for which three clones out of 35 lost these characteristics.

Comparison of levels of inoculated bacteria with total number of lactose-positive enterobacteria in the intestines was made five days after inoculation (table 3).

The strain C600 PK1046 was not found in intestines five days after inoculation. The same strain, after having acquired the Sm^rTc^r and Su^r characteristics, was found at a level of about 4 $\log_{10}$ bacteria in one intestine only. This same strain, after having also acquired the K99 and Ent phenotypic traits, was found at levels of about 6 $\log_{10}$ and 7 $\log_{10}$ respectively in one intestine in two experiments out of three. Hence the strain K12 C600 is probably not a good colonizing strain in the intestine of holoxenic animals. The derived clone C600 $K99^+Ent^+$ is perhaps better maintained in intestines, since it has been found at high levels in two intestines out of 24.

Strain H510 Nal^r, both with and without $Sm^rTc^rSu^r$ and K99 and Ent characteristics, was found at about 0.5 to 1 $\log_{10}$ less than total lactose-positive enterobacteria. Strain 3404 Nal^r was maintained at the same amount as the total amount of enterobacteria, but this strain, when it acquired $Sm^rTc^rSu^r$ and K99 and Ent characteristics was found in number about 1000

times less. Strain C13 Nal^r and the transconjugant clone K99$^+$ Ent$^+$ were found at high levels, but for an unknown reason, C13 Nal^r strain did not grow on Drigalski medium as the original strain C13 had done.

1.3. *B41 Nal^r mutant*

Comparison was made between virulence of the strain B41 and a derived clone B41 Nal^r. This was done to assess the influence that the

Table 2. — Plasmid content and phenotypic characteristics of the ETEC donor strain B41, of recipient strains and of transconjugant clones having acquired after mating at least tetracycline resistance

	Plasmid DNA bands[a] (approx. mol. wt × 10^6 daltons)						Phenotypic characteristics		
	75	57	4	2.1	1.8	1.1	K99	Ent	A[b]
Donor strain									
B41	+	+	+	+	+	+	+	+	R
Recipient strain									
C600 PK1046	−	−	−	−	−	−	−	−	S
Transconjugant clones									
B41 × C600 PK1046									
(1)	−	+	+	+	+	−	−	−	R
(2)	+	+	+	−	+	−	+	+	R
Recipient strain									
H510 Nal^r	−	−	−	−	−	−	−	−	S
Transconjugant clones									
B41 × H510 Nal^r									
(5)	−	+	−	−	−	−	−	−	R
(13)	+	+	−	+	−	−	+	+	R
Recipient strain									
K14 Nal^r	−	−	−	−	−	−	−	−	S
Transconjugant clones									
B41 × K14 Nal^r									
(9)	−	+	+	+	+	−	−	−	R
(23)	+	+	−	+	−	+	+	+	R
Recipient strain									
3404 Nal^r	−	−	−	−	−	−	−	−	S
Transconjugant clones									
B41 × 3404 Nal^r									
(1)	−	+	−	−	−	−	−	−	R
(13)	+	+	−	−	−	−	+	+	R
Recipient strain									
C13 Nal^r	−	−	−	−	−	−	−	−	S
Transconjugant clone									
B41 × C13 Nal^r									
(4)	+	−	−	−	−	−	+	+	S

a : presence (+) or absence (−) of plasmid DNA bands in agarose gel electrophoresis.
b : resistance (R) or sensitivity (S) to streptomycin, tetracycline and sulfamide.

Table 3. — Pathogenicity and persistance in intestine of some non ETEC *E. coli* strains and of the same strains having acquired by plasmid transfer from the strain B41, at least resistances to streptomycin, tetracycline and sulfamide and for the ability to produce K99 antigen and ST toxin.

	Pathogenicity for infant mice[a]	Persistance in intestine[b]			
		total number of enterobacteriacae		number of inoculated *E. coli*	
Recipient strain					
C600 PK1046	1/24 (4.2)	7.5±0.1	(0)	...	(8)
C600 PK1046	...	7.6±0.1	(0)	...	(8)
Transconjugant clones					
B41 × C600 PK1046					
(1)	0/24 (0)	7.7±0.2	(0)	3.9	(7)
(2)	0/48 (0)	5.8±0.9	(2)	...	(8)
(2)	...	7.5±0.2	(0)	6.3	(7)
(2)	...	8.4±0.3	(0)	7.2	(7)
Recipient strain					
H510 Nal^r	0/16 (0)	7.6±0.2	(0)	6.5±0.2	(0)
Transconjugant clones					
B41 × H510 Nal^r					
(5)	0/48 (0)	ND[c]		ND	
(13)	1/23 (4.3)	6.3±0.8	(0)	5.5±0.8	(0)
(13)	...	7.2±0.3	(0)	6.7±0.5	(0)
Recipient strain					
K14 Nal^r	0/24 (0)	6.4±0.2	(0)	6.5±0.3	(0)
Transconjugant clones					
B41 × K14 Nal^r					
(9)	0/24 (0)	ND		ND	
(23)	10/48 (20.8)	5.6±0.8	(2)	5.5±0.9	(0)
Recipient strain					
3404 Nal^r	1/24 (4.2)	6.2±0.3	(0)	6.4±0.2	(0)
Transconjugant clones					
B41 × 3404 Nal^r					
(1)	0/24 (0)	ND		ND	
(13)	0/24 (0)	7.2±0.4	(0)	4.2±0.4	(1)
Recipient strain					
C13 Nal^r	0/24 (0)	6.9±0.6	(6)	7.4±0.6	(0)
Transconjugant clone					
B41 × C13 Nal^r					
(4)	7/93 (7.5)	6.4	(7)	6.7±0.6	(0)
(4)	...	7.4	(7)	7.6±0.4	(0)

a : number of dead infant mice six days after inoculation with 10^5 *E. coli* / number of inoculated infant mice ; percentage between brackets.
b : for each independant experiment, eight infant mice were slaughtered five days after oral inoculation of 10^5 bacteria ; mean ± standard error in $\log_{10}$ (number of bacteria per intestine) ; between brackets, number of animals with less bacteria than limit of detection and excluded from mean.
c : non determinated.

nalidixic-resistant mutation may have on pathogenicity because recipient strains are Nal^r mutants.

With this B41 Nal^r clone, death of the animals occurred later, and only 19 animals (versus 23 for the parental strain) were recorded six days after inoculation (fig. 2).

2. Porcine ETEC strains

K88 antigen was lost or transferred from the two porcine ETEC strains with some other phenotypic characteristics (table 4). The two porcine ETEC strains K88$^+$ Ent$^+$ were less pathogenic for infant mice than the strain B41 (table 5 to compare with fig. 2).

Derived strains having spontaneously lost only the capacity to produce K88 antigen with their raffinose fermentation capability, but which still produced ST toxin, were approximately as pathogenic as the original strains. One P5148 derived clone which also lost its ability to produce ST toxin as well as tetracycline resistance, dit not provoke death of the animals.

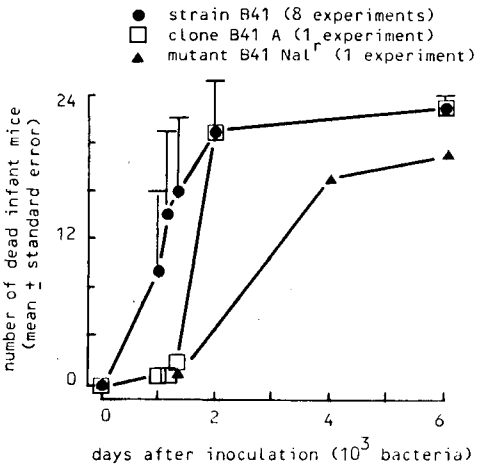

Fig. 2. — Comparison of virulence of the strain B41 with a derived clone B41A that does not produce K99 antigen and with a nalidixic mutant of the strain B41 (B41 Nal^r). Virulence was measured by mortality induced in infant mice inoculated when 18-48 h old with 10^3 bacteria.

Table 4. — Phenotypic characteristics of parental ETEC porcine strains of derived clones wich have lost at least the raffinose fermentation ability and of some transconjugant *E. coli* K12 clones which acquired at least the raffinose fermentation ability.

Strain	Phenotypic characteristics[a]								Conclusions	
	Raf	K88	Ent	Hly	Col	Tc	Su	Sm	co-transfert	co-elimination
Mating experiments										
Donor strain P2200	+	+	+	+	+	R	R	R	...	...
Recipient strain K12 Nal^r	−	−	−	−	−	S	S	S	...	...
Transconjugant clones P2200 × K12 Nal^r										
clone A	+	+	−	−	−	S	R	R	Raf K88 Su^r Sm^r	...
clone B	+	+	−	+	−	S	S	R	Raf K88 Hly$^+$ Sm^r	...
clone C	+	+	−	−	−	R	R	R	Raf K88 Tc^r Su^r Sm^r	...
Derived clones Raf$^-$										
Parental strain P2200	+	+	+	+	+	R	R	R	...	...
Derived clone P2200 Raf$^-$	−	−	+	+	+	R	R	R	...	Raf K88
Parental strain P5148	+	+	+	+	+	R	R	R	...	...
Derived clones										
P5148 Raf$^-$ (A)	−	−	+	+	+	R	R	R	...	Raf K88
P5148 Raf$^-$ (B)	−	−	−	+	+	S	R	R	...	Raf K88 Ent Tc^r

a : presence (+) or absence (−) of phenotypic characteristic ; sensibility (S) or resistance (R) to antibiotics.

Discussion

We have found that a clone B41A (K99⁻ Ent⁺) derived from the strain B41 is still very pathogenic and that some transconjugant *E. coli* clones K99⁺Ent⁺ are few or non-pathogenic. Hence the K99 antigen does not seem implicated to a great extent in virulence for infant mice. The same conclusion is also true for K88 antigen, though ETEC K88⁺ porcine strains studied are much less pathogenic for infant mice than the strain B41.

The strain B41A apparently possesses the same plasmid content as the original B41 strain. In the former strain it is possible that K99 antigen is not expressed but, alternatively, genes involved in K99 expression could be deleted without significantly affecting the global molecular weight of the plasmid DNA. It is known that the smallest cloned DNA expressing K99 antigen is a 4.5×10^6 daltons DNA (Van Embden *et al.*, 1980).

Why do the K99⁺ Ent⁺ transferred *E. coli* clones, three of which belong to the major O serotypes of ETEC strains, not provoke rapid death of infant mice as the strain B41 does ? For one strain to be pathogenic, it must be a good colonizing strain. Strain C600 PK1046 is not a good-colonizing strain because it does not persist in the intestine. However, the K99⁺ Ent⁺ transconjugant clone is perhaps slightly better maintained in the intestine. Increased survival of an *E. coli* K12 which carries an Ent plasmid in the calf intestine has been reported (Falkow *et al.*, 1976). Other K99⁺ Ent⁺ transconjugant clones were found in number above 10^4 bacteria in infant mice intestines, five days after inoculation. Presence of K99 and Ent characteristics does not necessarily favour persistance of a given *E. coli* strain. The strains K14 Nal^r and C13 Nal^r their transconjugant K99⁺ Ent⁺ clones (which are moreover the only clones that are midly pathogenic for the infant mouse) constitute the major part of the enterobacteria population found five days after inoculation.

Excepting *E. coli* C600, recipient strains are nalidixic-acid mutants. Selection of such mutants may probably change some properties of the original strain. We found that the strain C13 Nal^r lost its ability to grow on Drigalski medium,

Table 5. — Pathogenicity for infant mice of two ETEC strains of porcine origin, of some derived clones having lost at least K88 antigen (selectioned as Raf⁻) and of *E. coli* K12 K88+ transconjugant clones.

Strains	Pathogenicity for infant mice[a]	
	10^3 bacteria	10^5 bacteria
Mating experiments		
Donor strain		
P2200	1/48 (2.1)	9/72 (12.5)
Transconjugant clones		
P2200 × K12 Nal^r		
clone A	0/24 (0)	0/48 (0)
clone B	ND[b]	0/24 (0)
clone C	ND	0/24 (0)
Derived clones Raf⁻		
Parental strain		
P2200	1/48 (2.1)	9/72 (12.5)
Derived clone		
P2200 Raf⁻	3/64 (4.7)	12/96 (12.5)
Parental strain		
P5148	3/23 (13.0)	9/47 (19.1)
Derived clones		
P5148 Raf⁻ (A)	0/24 (0)	21/48 (43.7)
P5148 Raf⁻ (B)	ND	0/24

a : number of dead infant mice six days after inoculation with 10^5 *E. coli* / number of inoculated infant mice ; percentage between brackets.
b : ND, non determined.

unlike the strain C13. However, the strain C13 Nal^r is still able to persist in intestines. In competition experiments with *E. coli* K12 in germ-free mice, chromosomal nalidixic acid mutants have a better colonizing ability than chromosomal streptomycin and rifampicin resistant mutants (Onderdonk *et al.*, 1981). A similar nalidixic acid mutant obtained from the strain B41 (B41 Nal^r) has been found to be slightly less pathogenic than the parental strain, but is still able to provoke death of the majority of mice. Thus, use of nalidixic acid mutants as recipient strains probably cannot entirely explain the mild or non-pathogenicity of $K99^+Ent^+$ transconjugants.

Smith and Huggins (1978) found that O and K antigens could be important factors for the colonizing ability of *E. coli* strains in the calf intestine. *In vitro* K99 production appears to be related to the O antigen which is carried by the host strain, and it seems to be independent of the absence or presence of various K polysaccharide antigens (De Graaf *et al.*, 1980). It has also been found that O101 strains (like our strain H510) produced more K99 antigen than strains with O8 antigen (like the strain 3404) and O9 (like the strain K14 which was the only one which became mildly pathogenic after having acquired K99 and Ent characteristics). It is possible, however, that *in vivo,* regulation of K99 expression would be different.

The possibility that K99 and Ent characteristics could be lost after *in vivo* multiplication has been considered. From our results, at least the large majority of bacteria inoculated kept their ability to produce K99 antigen and ST toxin *in vitro* after *in vivo* multiplication.

None of the transconjuguant clones received

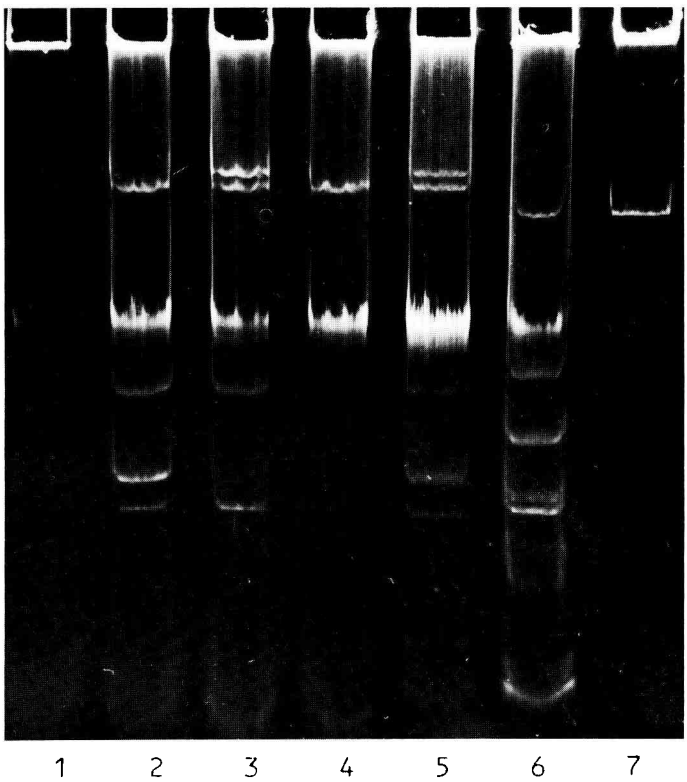

Fig. 3. — Agarose gel (0.8 %) electrophoresis of plasmid DNA of the strain C600 PK1046 (free of plasmid) (line 1), of two clones which acquired after mating with the strain B41 resistance to streptomycin, tetracycline and sulfamides (lines 2 and 4), and of one clone which acquired with the three resistances to antibiotics, the capacity to produce K99 antigen and ST toxin (line 3). For reference strains, see legend fig. 1.

all DNA bands. Hence it may not be excluded that other properties coded by small plasmids could contribute to pathogenicity.

For the strain B41, we found repetitively six DNA bands, by this soft lysis method of plasmid analysis. These DNA bands could be transferred independently of one another with only cotransfer with tetracycline resistance (even though this tetracycline resistance was lost. later by the clone B41 × C13 Nal^r (4)). This suggests that they were plasmid covalently closed circular DNA bands and not other forms derived from one another.

In the strain B41, production of K99 antigen and ST toxin have never been reported as being coded by the same plasmid nor the three resistances to antibiotics, coded by another plasmid, and the four small DNA bands have never been described (So *et al.*, 1976 ; Meyers *et al.*, 1976 ; Van Embden *et al.*, 1980). Recombination events of DNA have probably occurred in this strain possibly by transposition of genes. It is known that ST toxin has been found to be coded by a transposon (So *et al.*, 1979), as well as antibiotic resistances. For instance, transposition of ampicillin resistance to a ST + LT plasmid has been described (So *et al.*, 1978).

Results obtained with porcine ETEC K88 strains suggest that the presence of K88 antigen is not a virulence factor necessary for the pathogenicity of ETEC K88 strains for infant mice. Also the K88$^-$ clone derived from the strain P5148 which lost its capacity to produce ST toxin was no longer pathogenic for infant mice. The same result was obtained with the K12 K88$^+$ transconjugant clones which acquired certain plasmid-determined characteristics (notably haemolysin for one clone) but did not acquire the ability to produce ST toxin. Hence ability to produce ST toxin is probably an essential factor affecting virulence. However, the clone P5148 K88$^-$ Ent$^-$ might have lost other plasmid-determined characteristics on the same plasmid or plasmids other than the one which codes for ST toxin.

Smith and Huggins (1978) found that implanting K88 or K99 and Ent plasmids in non-pathogenic *E. coli* strains (except *E. coli* K12) could favour their proliferation in the alimentary tract of piglets, calves and lambs, but other surface structures of the bacteria might also be important.

More generally, not all suspected enteropathogenic strains bear CFAI, CFAII, K88, 987P or K99 antigens. Other types of pili have been found in human enteropathogenic strains with hemagglutinating or adherent properties (Deneke *et al.*, 1979 ; Wevers *et al.*, 1980 ; Bergman *et al.*, 1981), and other adhesins could also exist (Cravioto *et al.*, 1979 ; Levine *et al.*, 1980 ; Bergman *et al.*, 1981).

From our results, we may hypothesize that the strains B41 and B41A possess, while K99$^+$Ent$^+$ transconjuguant clones do not possess, a factor involved in the ability of these strains to multiply in intestines above the normal rates that would produce mortality of nearly all animals. Concentrations of 10^8 or 10^9 bacteria per intestine are always found when an animal first appears sick (Duchet-Suchaux, personal communication). The same concentrations have been found for the strain B41A (unpublished data).

The strain B41 and other O101:K99$^+$ strains bear two adhesive antigens and only one can be transferred to *E. coli* K12 (Morris *et al.*, 1980). Our strain B41A might bear at least the other adhesin that the K99 antigen. Recently, what was probably a fimbrial adhesive antigen (F41), which could not be transferred with K99 antigen, was evidenced in a mutant of the strain B41 which, like our strain B41A, did not produce K99 antigen (Morris *et al.*, 1982 ; De Graaf and Roorda, 1982).

Apart from possible differences in the *in vivo* expression of virulence characteristics, our results suggest that K99 antigen alone is not the major virulence factor in the infant mouse model. Other virulence factors must be implicated, perhaps relevant to F41 structure, or other properties not yet determined.

Concerning comparison of virulence for mice with that for ETEC natural hosts it is interesting to note that the strain B41 K99$^-$F41$^+$ could still produce diarrhoea in piglets (Morris *et al.*, 1982). That leads to the question of the respective roles of K99 antigen or F41 structure in the virulence for natural hosts of the strain B41.

We think that the infant mouse model would facilitate the study of some virulence properties of ETEC strains and protection conferred by vaccination with some antigens. However, further studies are still needed to assess exactly what properties or structures of the bacteria are involved in their virulence in this model. Other studies on ETEC natural hosts are also needed to establish comparison.

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Summary

Enterotoxigenic *E. coli* (ETEC) strain B41 orally inoculated in infant mice at birth induces lethal diarrheoal disease. This model was used to study virulence factors of ETEC strains of bovine and porcine origins.

Strains possessing different plasmid-coded virulence factors were obtained either after spontaneous loss of these factors from the original strain or by plasmid transfer to different *E. coli.*

A clone B41A derived from the strain B41 did not produce K99 antigen, but still produced heat-stable (ST) toxin. This clone B41A was about as pathogenic as the strain B41 in that it caused death of nearly all animals less than 48 h after inoculation with 10^3 bacteria.

Plasmids were transferred from the strain B41 to *E. coli* C600, to nalidixic acid resistant mutants of strains of serotypes O8, O9, O101 and to an *E. coli* strain isolated from a suckling mouse intestine. $K99^+Ent^+$ transconjuguant clones were either not all or mildly pathogenic.

Two strains of porcine origin $K88^+Ent^+$ were mildly pathogenic for mice. Loss of factor K88 did not reduce virulence. In contrast, loss of both K88 and Ent characteristics suppressed virulence. Strain *E. coli* K12 that had acquired K88 virulence factor possibly with other plasmid-coded characteristics but not the ability to produce ST toxin was non-pathogenic.

Hence factors K99 and K88 may not be essential for inducing mortality in the infant mouse model. Other factors may be involved in virulence of the strain B41A. These results were discussed from the viewpoint of pathogenicity for mice and for ETEC natural hosts.

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