

## GENETICALLY ENGINEERED ENTEROPATHOGENIC *ESCHERICHIA COLI* STRAIN PROTECTS RABBITS AGAINST COLIBACILLOSIS.

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### ABSTRACT

Enteropathogenic *E. coli* (EPEC) is a well-established cause of serious diarrhea in young children and in different animal species like rabbits (REPEC). EPEC strains induced a specific « attaching-effacing » lesion, characterized by a destruction of the enterocyte brush border and an intimate bacterial attachment. Bacterial effectors are coded in the locus of enterocyte effacement (LEE). Inactivation of every gene of the LEE leads to a decrease of virulence, although bacteria could still colonize the intestinal tract. The aim of this study was to generate a vaccinal REPEC strain to protect rabbits in breeding units. We inactivated by allelic exchanges two genes of the LEE, coding proteins injected into the host cell by a type III secretion system: the gene coding EspB, a protein forming a pore in the host cell membrane and the gene coding Tir, a protein injected in the host cell surface and representing the receptor of another bacterial protein, Intimin. Tir-intimin interaction allows intimate attachment of bacteria to cells. In the first part of our study we showed that the vaccinal strain, E22 $\Delta$ Tir/EspB, was completely safe : it did not induce diarrhea nor any histological intestinal lesions but it was still able to colonize the intestinal tract. We then showed that E22 $\Delta$ Tir/EspB was able to protect rabbits against an early (7 days post vaccination) and a late (28 days post vaccination) virulent challenges with the parental strain E22. In addition, the vaccinal strain blocked the shedding of the virulent strain, decreasing the risk of bacterial transmission between rabbits. Antibodies against LPS O103, the bacterial adhesin AF/R2 and the Intimin were detected as soon as 7 days post E22 $\Delta$ Tir/EspB inoculation. Anti-AF/R2 Abs could blocked bacterial adhesion *in vitro*. These results indicated that E22 $\Delta$ Tir/EspB is a good vaccine candidate to protect weaned rabbits against REPEC infections in fattening breedings, efficient with a single inoculation dose.

**Key words:** EPEC O103, *Oryctolagus cuniculus*, Vaccine, Anti AF/R2 Abs.

### INTRODUCTION

Enteropathogenic *E. coli* (EPEC) strains belonging to serotype O103:K-:H2 and to rhamnose-negative biotypes are responsible for considerable economical losses in industrial fattening rabbit farm in Western Europe consecutively to severe diarrhea in weaned rabbits (*Oryctolagus cuniculus*). These strains have been shown to be

epidemiologically dominant in field conditions (BLANCO *et al.*, 1996 ; CAMGUILHEM and MILON, 1989).

One of the best ways to prevent the symptoms induced by EPEC infection would be to have an efficient vaccine.

Different vaccination studies done in our laboratory using the rabbit model gave encouraging results. During the first vaccine trials, rabbits inoculated with high doses of formaldehyde-killed whole bacteria were protected against a challenge (CAMGUILHEM and MILON, 1990). However, the heaviness of the protocol and the high doses to be used lead to the search of another strategy. In addition, we demonstrated that protection against EPEC O103 infections could also be induced by oral administration of live *E. coli*. The weakly pathogenic strain C6 (O128, LEE and AF/R2 positive) protects at least partially weaned rabbits against REPEC O103 and induces local IgA anti-LPS responses (MILON *et al.*, 1992).

With the discovery of the LEE (Locus of Enterocyte Effacement) and the better understanding of EPEC virulence mechanisms, we decided to construct a live attenuated strain. Our goal was to obtain a mutant strain against REPEC that could be efficient (i.e non-pathogenic), protective (i.e immunogenic) and easy to handle in rabbit breeding units.

Several studies have demonstrated that EPEC *eae*, *tir*, *espA* or *espB* null mutants presented a decrease in their virulence *in vitro* with the disappearance of the attaching-effacing (A/E) lesions but also *in vivo* with a reduction of the incidence of diarrhea (ABE *et al.*, 1998 ; MARCHÈS *et al.*, 2000 ; NOUGAYREDE *et al.*, 1999; TACKET *et al.*, 2000 ) but none of them were tested for their protective capacity. Considering the virulence of the different LEE mutants, we chose to inactivate two genes *espB* and *tir*, involved in the A/E lesion whose products are translocated by the bacteria into the target cell. In addition, the double null mutant should still produced potential immunogenic bacterial proteins like EspA, the intimin and AF/R2 (FIEDERLING *et al.*, 1997; JERSE and KAPER, 1991 ; KNUTTON *et al.*, 1998 ) allowing the bacterial colonization and the induction of a specific immune response.

In this study, we describe the construction of a Tir/EspB null mutant of rabbit enteropathogenic *Escherichia coli* strain E22 (O103 :K- :H2) (BOULLIER *et al.* 2003). We show its safety, its ability to induce a specific humoral response and its efficacy in protecting rabbits against colibacillosis, after a single inoculation dose.

## MATERIAL AND METHODS

### Experimental infection of rabbits

Thirty two-day-old New Zealand white weaned rabbits were used for experimental infections. Rabbits were housed in cages of three animals and fed daily with antibiotic-free commercial feed supplemented with a coccidiostatic agent (Robenidine). Water was ad libitum. Rabbits were divided in 5 groups of 11 or 12 animals.

For the safety test, 3 groups of rabbits were respectively inoculated per os with E22, E22 $\Delta$ Tir/EspB and BM21 ( $2 \times 10^7$  CFU each, in 2ml of PBS, corresponding to  $10^3$  LD50 for the wild type (w-t) strain E22). Animals were monitored for 28 days, weighed twice a week and checked daily for loss of appetite, diarrhea, dehydration and mortality. For the late protection assay, the 2 groups of rabbits inoculated with BM21 and E22 $\Delta$ Tir/EspB were orally infected with E22 ( $2 \times 10^4$  CFU) at day 28, a dose corresponding to 1 LD50. Clinical monitoring was performed as previously described for 28 more days. Animals were sacrificed by intravenous injection of ketamine and sodium-barbital in accordance to the European council directive 86/609.

For the early protection assay, 2 other groups of rabbits were inoculated with E22 $\Delta$ Tir/EspB and BM21 ( $2 \times 10^7$  CFU each) respectively and orally challenged 7 days later with E22 ( $2 \times 10^4$  CFU). Animals were kept for 28 days and clinically monitored as described. For every group of rabbits, fecal shedding of *E. coli* was determined twice a week by dilution of fecal samples on MacConkey agar. After selective enumeration, screening of the inoculated strains was done on a set of randomly selected isolates (6 per samples) using the following markers ; for E22: O103 (slide agglutination) rhamnose negative (growth on phenol red agar plus 1% rhamnose) and kanamycin and chloramphenicol sensitive (growth on Luria agar containing the appropriate antibiotics); for E22 $\Delta$ Tir/EspB: O103 rhamnose negative and kanamycin and chloramphenicol resistant and for BM21: rhamnose positive nalidixic acid resistant. Each result was given in CFU/g of sampled feces.

## RESULTS AND DISCUSSION

### **Clinical safety of E22 $\Delta$ Tir/EspB strain**

In order to test the safety of the mutant strain E22 $\Delta$ Tir/EspB, three groups of 12 weaned rabbits (32 days old) were inoculated with  $2 \times 10^7$  CFU of non-pathogenic strain BM21, wild type strain E22 or E22 $\Delta$ Tir/EspB strain, respectively. A clinical follow-up was done daily and rabbits were weighed twice a week during 28 days. E22 infected rabbits died within 10 days post-infection after severe diarrhea episodes (Fig. 1A). In contrast, all of the rabbits infected with the mutant strain survived, and their weight gain curve was similar to that of BM21-inoculated rabbits (Fig. 1B).

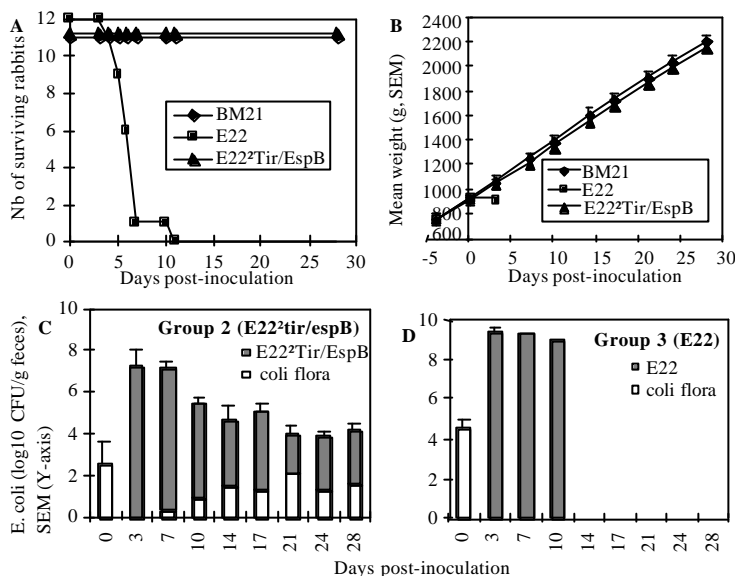
### **Early intestinal colonization by E22 $\Delta$ Tir/EspB**

In order to ensure that the absence of pathogenicity was not consecutive to an impaired colonization of the intestinal tract by E22 $\Delta$ Tir/EspB, fecal bacteria were counted. The results showed that intestinal colonization occurred in E22 and E22 $\Delta$ Tir/EspB inoculated rabbits with similar kinetics. We detected both strains in feces as soon as 3 days post infection (Fig. 1C-D). In addition, the E22 $\Delta$ Tir/EspB strain persisted at low level in the intestinal tract of infected rabbits for at least 28 days (Fig. 1C).

### **“Early” protection brought by E22 $\Delta$ Tir/EspB**

We first tested the precocity of the protection brought by E22 $\Delta$ Tir/EspB. Two groups of 32 days old weaned rabbits were inoculated with BM21 ( $2 \times 10^7$  CFU) and E22 $\Delta$ Tir/EspB

( $2 \times 10^7$  CFU) respectively and challenged with E22 ( $2 \times 10^4$  CFU) 7 days later. The challenged dose of  $2 \times 10^4$  CFU corresponds to an extremely severe EPEC epidemic. Indeed, when an EPEC epidemic occurs in breeding units, the morbidity rate is around 80% and the mortality rate around 40%. In our experiment, 73% of the rabbits inoculated with the control strain BM21 and challenged with  $2 \times 10^4$  CFU of E22 died during the experimentation. In contrast, all the rabbits inoculated with E22 $\Delta$ Tir/EspB survived the virulent challenge (Fig. 2A). In addition, E22 $\Delta$ Tir/EspB inoculated animals had a weight gain curve similar to that of BM21-infected control rabbits (mean of 45g per day) (Fig. 2B).



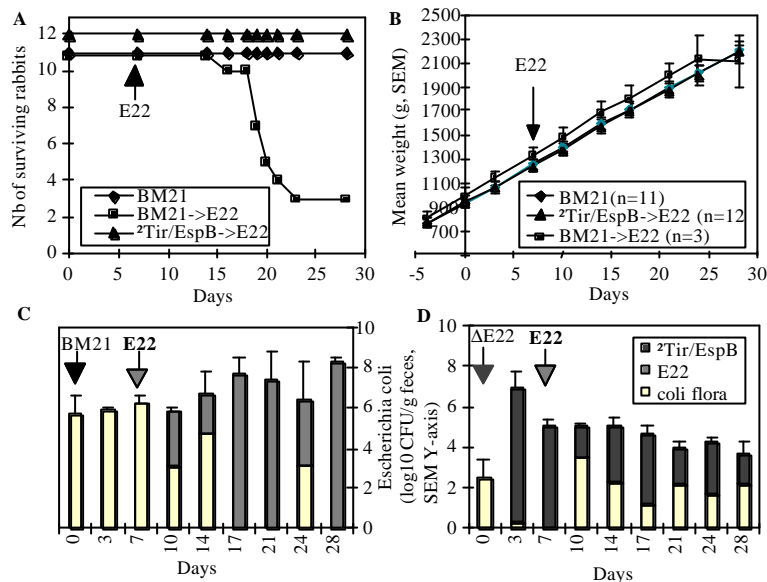
**Figure 1: Mortality, morbidity and intestinal colonization induced by E22 $\Delta$ tir/EspB strain.**

We further determined whether the mutant strain could prevent intestinal colonization by the virulent strain. Fecal samples were analyzed twice a week as before. In BM21 inoculated rabbits, E22 was found in the feces as soon as 3 days post-challenge (Fig. 2C). In E22 $\Delta$ Tir/EspB inoculated animals, the mutant strain was found in the feces 3 days post-inoculation and was still present at low level at the time of the virulent challenge (Fig. 2D). After infection with E22, E22 $\Delta$ Tir/EspB remained in the feces and no E22 bacteria could be detected in the feces of these rabbits.

### Late protection mediated by E22 $\Delta$ Tir/EspB

In order to evaluate the late protection mediated by the mutant strain, rabbits inoculated at day 0 with  $2 \times 10^7$  CFU of BM21 and E22 $\Delta$ Tir/EspB respectively were orally challenged with E22 ( $2 \times 10^4$  CFU) at day 28. Animals were kept for 21 more days and a clinical follow-up was done every day as before. Among the 11 BM21-infected rabbits, only 4 survived the virulent challenge (64% mortality), while all E22 $\Delta$ Tir/EspB-inoculated rabbits survived (Fig. 3A). In addition, their weight gain curve was regular and similar to that of

non-infected control rabbits (about 40g/day) (Fig. 3B). These results suggest that the mutant strain protects rabbits against E22 infection for a long period of time.

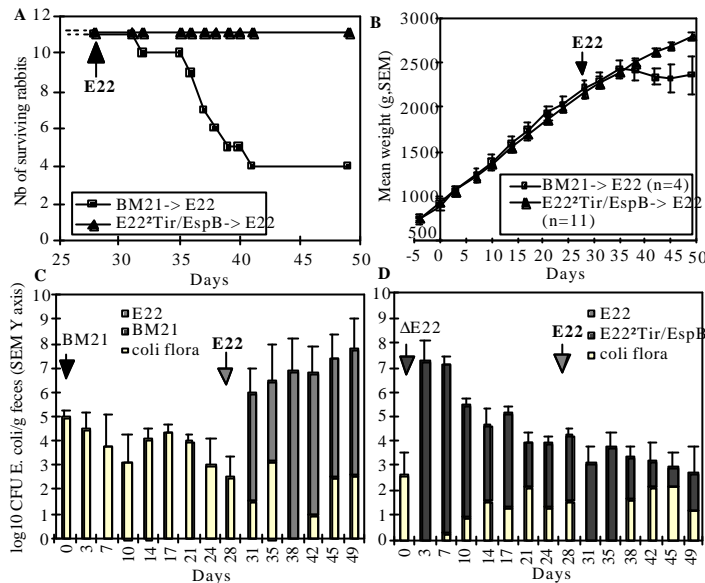


**Figure 2 : Protection test by E22Δtir/EspB strain against an early virulent challenge**

We then checked whether the mutant strain was still present in the intestinal tract of rabbits and was still able to prevent colonization by the virulent strain. At the time of the virulent challenge, E22ΔTir/EspB strain was still present in feces of inoculated animals ( $10^3$  CFU/g feces) (Fig. 3D) but the colonization level decreases during the whole experimentation (49 days). In addition, we could not detect E22 bacteria in the feces of E22ΔTir/EspB-inoculated animals after the virulent challenge while E22 colonized very rapidly the intestinal tract of BM21-inoculated rabbits, as soon as 3 days post-inoculation ( $10^5$  CFU/g feces 3 days post challenge) (Fig. 3C). These results indicate that E22ΔTir/EspB inoculation prevents late intestinal colonization by the virulent strain.

### CONCLUSION

The results obtained in this study suggest that the attenuated strain E22ΔTir/EspB is a good “vaccine” candidate to protect rabbits against REPEC infections. Indeed, it induces a specific immune response (BOULLIER *et al.* 2003) and brings a complete protection against the virulent homologous strain, used in conditions aimed at reproducing clinical features encountered in field O103 colibacillosis episodes. This strain protects rabbits after a single oral dose and thus could be easily handled in breeding units. In addition, the protection lasts the entire fattening period, including the early post-weaning period when animals are the most sensitive to EPEC infections (CAMGUILHEM and MILON, 1989). This strain also prevents excretion of the virulent strain by E22ΔTir/EspB-inoculated rabbits, which may decrease EPEC dissemination and animal contaminations.



**Figure 3 : Protection test by E22 $\Delta$ tir/EspB strain against a late virulent challenge**

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