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Short communication

Effects of single and multiple pathogenicity island deletions on uropathogenic *Escherichia coli* strain 536 intrinsic extra-intestinal virulence

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ABSTRACT

Background: Escherichia coli strain 536 is a uropathogenic strain harboring 7 pathogenicity islands (PAIs). Whether or not these PAIs additively contribute to extra-intestinal virulence is unknown.
Methods: We tested 7 single and several multiple-PAI deletion mutants in a mouse septicemia model by monitoring mouse survival.
Results: E. coli 536 mutants in which PAIs II or III were deleted showed a significant decrease in virulence compared to the wild type (WT). All other single-PAI deletion mutants were as lethal to mice as was the WT. The mutant in which all seven PAIs were deleted showed milder virulence than the mutants in which PAI III or PAIs III and IV were deleted. The mutant in which PAIs II, III, IV, V, and VII were deleted tended to be less virulent than the mutant with deletion of PAI III only. All together, these results indicate a rough additive effect of PAIs in extra-intestinal virulence.
Conclusion: All PAIs of *E. coli* 536 do not play the same role in extra-intestinal virulence estimated in a mouse septicemia model and PAIs cooperate in an additive manner to achieve extra-intestinal virulence.

Introduction

Because *E. coli* is a versatile species containing both highly virulent and commensal strains, it is of importance to identify factors that are responsible for pathogenicity. The search for virulence factors (VFs) can be achieved in several ways. Firstly, it is possible to perform correlation studies between the presence of specific traits and a virulent phenotype. For example, epidemiologic studies have shown that hemolysin- and cytotoxic necrotizing factor-encoding genes *hly* and *cnf* were both associated with uro-virulence (Caprioli et al., 1987). These genes were found in 58.2% and 37.4% of uropathogenic *E. coli* (UPEC) strains, respectively, vs. 3% and 1% of fecal strains. Correlation studies allow analysis of numerous traits simultaneously but can be biased by both pathogen and host population inhomogeneity. Secondly, hypothetical VF deletion mutants can be experimentally tested using different infection models. To continue with the same example, it has been shown that *cnf1*-deficient isogenic mutants of UPEC strain CP9 were less virulent than their wild-type counterparts in a mouse urinary tract infection (UTI) model (Rippere-Lampe et al., 2001). The advantage of this approach is that the model is well controlled and reproducible. However, only a limited number of factors are usually tested. Therefore, a third alternative has been developed that combines the advantages of the first two. It consists in analyzing correlation between the presence of specific traits and a virulent phenotype tested in an animal infection model. Consistent with this approach, a mouse septicemia model has been developed that allows experimental measurement of intrinsic extra-intestinal virulence (Picard et al., 1999; Johnson et al., 2000). In this model, the bacteria to be tested are directly injected subcutaneously in the mice. Although it by-passes very important steps of the natural infection process, it has robustly and extensively been used to study extra-intestinal virulence. Using this model, it has been suggested that virulence was the result of the additive effect of VFs. The more VFs a strain has, the more virulent it seems to be (Picard et al., 1999, 2001; Johnson et al., 2000). This additive effect of VFs has not been experimentally assessed.

E. coli strain 536 was isolated from the urine of a German woman suffering from pyelonephritis (Hacker et al., 1983). Its genome has completely been sequenced (Brzuszkiewicz et al., 2006). It has become one of the model strains for *E. coli* extra-intestinal virulence. Seven pathogenicity islands (PAIs) have been described for this strain (Brzuszkiewicz et al., 2006). Here, we use single- and multiple-PAI deletion mutants of uropathogenic strain 536 to test the hypothesis of an additive contribution of VFs to extra-intestinal virulence.

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Materials and methods

Bacterial strains

E. coli strain 536 is an extra-intestinal pathogenic (ExPEC) strain isolated from a urinary tract infection (Hacker et al., 1983). It belongs to the B2 phylogenetic group (Jaureguy et al., 2008).

Throughout the manuscript, PAI deletion mutants of *E. coli* strain 536 are named by the symbol "Δ" followed by the numbers of the deleted PAIs. We used Arabic numbers instead of Roman ones (classically used to number PAIs in the literature) to ease readability. For example, Δ23457 is the mutant in which PAIs II, III, IV, V, and VII have been deleted.

Single PAI-deleted mutants

Construction of single- and multiple-PAI deletion mutants will be described in detail elsewhere (Diard et al., unpublished) and is summarized hereafter.

Δ3: this strain was kindly provided by U. Dobrindt (Middendorf et al., 2004). It results from the spontaneous deletion of PAI III of wild-type uropathogenic strain 536 (536WT).

Δ1, Δ2, Δ4, Δ5, Δ6 and Δ7: each single PAI deletion mutant was generated using the λ Red recombinase gene inactivation method (Datsenko and Wanner, 2000). Flipase recognition target (FRT)-flanked kanamycin or chloramphenicol cassettes were generated by PCR using pKD4 or pKD3, respectively, as a template and primers each bearing 20 bases of homology with one end of the PAI to be deleted. Since tRNA deletions have been shown to result in a decreased fitness (Ritter et al., 1995; Dobrindt et al., 2002b), the primers were designed so that the tRNA flanking the PAIs were left untouched. After the deletion of the desired PAI, the antibiotic resistance cassette was removed using flipase (Flp) encoding helper plasmid pCP20 as described (Datsenko and Wanner, 2000).

Multiple PAI-deleted mutants

Δ34, Δ345 and Δ2345: Δ3 mutant was used to successively remove PAIs IV, V and II using the λ Red recombinase method as for single PAI deletion mutants. After the deletion of one PAI, the antibiotic resistance cassette was removed using Flp before proceeding to the next PAI deletion.

Δ23457, Δ234567 and Δ1234567: successive FRT genomic scars generated by the previous steps, limited the possibility of additional PAI deletion using the FRT method. This was due to aberrant recombination events at FRT scar sites rather than at the desired PAI (Diard et al., unpublished). A new strategy was therefore used to create the last three deletion mutants. It is very similar to the method described by Datsenko and Wanner (2000) but it uses the *loxP*-Cre system from budding yeast (Guldener et al., 1996; Gueldener et al., 2002) instead of the FRT-Flp system. A DNA segment containing a *loxP*-flanked chloramphenicol cassette was used as a template to generate *loxP*-flanked PCR products with primers each bearing 20 bases of homology with one end of the PAI to be deleted (Diard et al., unpublished). After the deletion of one PAI with the help of λ Red recombinase, the antibiotic resistance cassette was removed using a Cre protein-encoding plasmid before proceeding to the next PAI deletion. This method was used to successively remove PAI VII, VI and I from Δ2345.

WT_{eng} strain

Because multiple transformation, recombination and selection cycles could result in mutation accumulation, the *E. coli* 536 wild-type strain was simultaneously processed through all the same steps as Δ1234567 with distilled water instead of the recombinant PCR products. The resulting strain is called WT_{eng} (for wild-type engineered strain 536).

Competitive culture between Δ1234567 and WT_{eng} was performed in Luria Bertani (LB) broth in a chemostat for 96 generations. Δ1234567 showed no fitness decrease compared to ΔWT_{eng} (Diard et al., unpublished). Furthermore, growth curves of all single- and multiple-PAI deletion mutants were determined in LB broth and in minimum glucose medium. No growth difference was observed between any of the mutants (Diard et al., unpublished).

Septicemia mouse model

This model assesses the intrinsic extra-intestinal virulence of a strain and has already been described (Picard et al., 1999). It is basically described hereafter. OF1 14–16 g outbred females were obtained from Charles River® (L'Arbresle, France). Log-phase bacteria grown in LB broth were washed twice in 0.9% sodium chloride. Mice were challenged subcutaneously with 0.2 ml of sodium chloride buffer containing between 10^5 and 10^8 bacteria. Mice had free access to food and water. Time to death was recorded during the following 7 days. Experiments with single PAI deletion mutants were repeated twice, with 5 mice in each group. Experiments with multiple-PAI deletion mutants were repeated three times with 5 mice in each group. In addition, injections with WT_{eng}, Δ1234567 and Δ3 were routinely used as controls in the survival experiments. Because no death ever occurred after the third day with any of the inocula, survival curves in the Results section only represent the first 72 h of the experiments.

Animal experiments were performed in compliance with the recommendations of the French Ministry of Agriculture and approved by the French Veterinary Services (accreditation A 75-18-05).

Statistical analysis

Kaplan–Meier estimates of mouse survival were performed using StatA 8.0® software (StatA corporation™, TX, USA). Survival differences were estimated by Log-Rank test. Survival differences were considered significant if p was lower than 0.05.

Results

Dose effect of the inocula

High inocula (10^8 bacteria) of each of the mutants (including Δ1234567), of WT_{eng} or of WT *E. coli* strain 536 killed all the mice in less than 30 h (data not shown). No difference was observed in survival of mice injected with 10^8 WT_{eng} bacteria or with 10^8 WT 536. As a comparison, injection of 10^8 K-12 MG1655 bacteria killed no mice in 7 days in the same septicemia model (Johnson et al., 2006, and see Fig. 2).

When 10^5 bacteria of strain WT_{eng} were injected, all the mice survived (data not shown). Intermediate inocula of 10^6 bacteria gave mouse survivals that were identical to inocula of 10^7 bacteria. Using these inocula (between 10^6 and 10^7) it was possible to see differences in virulence between the various deletion mutants. Therefore, all of the survival experiments described thereafter were performed with intermediate inocula of 10^7 bacteria.

Single deletion of PAIs differently affects extra-intestinal virulence of E. coli strain 536

Mouse survival curves obtained when 10^7 bacteria of Δ1, Δ4, Δ5, Δ6 or Δ7 mutants were injected into mice were not statistically different from survival obtained when WT_{eng} was injected (data not shown). All these strains killed 100% of the mice in less

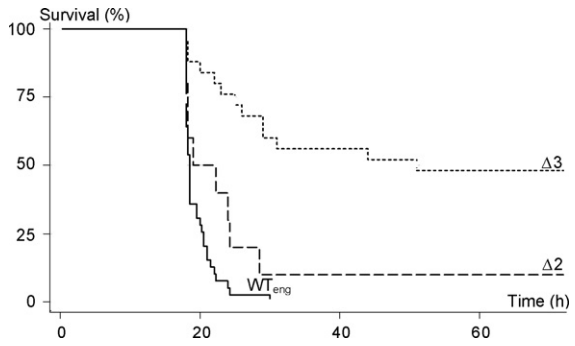

Fig. 1. Kaplan–Meier survival curves of mice injected with wild-type *E. coli* strain 536 or with two single-PAI deletion mutants of strain 536. Mice were injected subcutaneously with 10^7 bacteria. WT_{eng}: the mice were injected with wild-type engineered strain 536 (see Materials and methods). Δ2 and Δ3: mice were injected with PAI II and PAI III deletion mutants, respectively. Since no death occurred after the third day, only the first 72 h are shown.

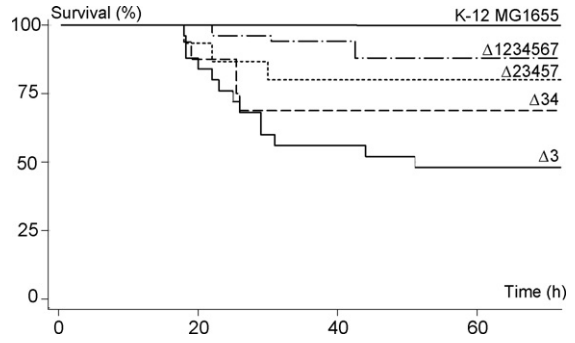

Fig. 2. Kaplan–Meier survival curves of mice injected with several *E. coli* strain 536 PAI deletion mutants. Mice were injected subcutaneously with 10^7 bacteria. The numbers after the Δ letter indicate the PAIs that were deleted in the *E. coli* strain 536 injected into the mice. MG1655: as a comparison, survival curve of mice injected with 10^8 bacteria of the K-12 MG1655 strain is represented. Since no death occurred after the third day, only the first 72 h are shown.

than 30 h. Conversely, Δ2 and Δ3 appeared to be significantly less virulent than WT_{eng} (Fig. 1), with respective 72-h survival of $10.0 \pm 9.5\%$, $48.0 \pm 10.0\%$ and 0% ($p = 0.039$ for comparison between Δ2 and WT_{eng}, $p < 0.00001$ for comparison between Δ3 and WT_{eng}, and $p < 0.02$ for comparison between Δ2 and Δ3). This indicates that not all PAIs have the same importance in *E. coli* 536 extra-intestinal virulence. PAIs II and III alone seem to play a greater role than any of the other PAI alone. Furthermore, PAIs I, IV, V, VI, and VII, individually, are not necessary for virulence as estimated in the mouse septicemia model.

Global additive effect of PAIs on extra-intestinal virulence of 536

Fig. 2 shows the survival curves of the mice that were injected with 10^7 bacteria of strain 536 Δ3, Δ34, Δ23457, and Δ1234567 mutants or with 10^8 bacteria of the K-12 MG1655 strain. Δ1234567 virulence was very low but significantly higher than that of MG1655. Survival was $88.0 \pm 4.6\%$ for mice injected with Δ1234567 vs. $100 \pm 0\%$ for mice injected with MG1655 ($p = 0.01$). Conversely, Δ1234567 showed a significantly lower virulence than Δ3 and Δ34. Survival was $48.0 \pm 10.0\%$ for mice injected with Δ3 ($p < 0.0001$ when compared to Δ1234567), and $68.8 \pm 11.6\%$ for mice injected with Δ34 ($p < 0.05$ compared to Δ1234567). Survival of mice injected with Δ23457 was higher than survival of mice injected with Δ3 ($80.0 \pm 10.3\%$ of survival with Δ23457; $p = 0.06$ vs. survival of mice that were injected with Δ3). Survival of mice injected with Δ345, Δ2345 or Δ234567 was not statistically different from survival of mice injected with Δ23457. Therefore, survival curves obtained with these mutants have been omitted in Fig. 2 to keep it more easily readable.

These results show that PAIs additively contribute to extra-intestinal virulence. The more PAIs are deleted, the less virulent is the strain.

Discussion

We have investigated the effect of single- and multiple-PAI deletions on *E. coli* strain 536 extra-intestinal virulence. The first important result is that high inocula (10^8 bacteria) of any of the mutants killed all the mice in this model (albeit less quickly in the case of Δ1234567 (Diard et al., unpublished data and this work)). For comparison, equivalent inocula of a non-pathogenic strain of phylogenetic group A killed no mice in the same model (Picard et al., 1999; Johnson et al., 2006). These observations confirm that not all of the VFs are included on the PAIs and that new VFs probably remain to be discovered among strain 536 genes, the functions of which are still unknown.

Even though not all *E. coli* 536 extra-intestinal VFs are carried by the 7 PAIs, PAIs do participate in an additive manner to extra-intestinal virulence. Our work is an experimental demonstration of a cooperation of PAIs in virulence. As it was suggested by previous works based on correlation studies using animal models (Picard et al., 1999, 2001; Johnson et al., 2000), the more PAIs are present in the cell, the more virulent it is.

In order to facilitate the comprehension of the discussion below, a summary of the content of PAIs of *E. coli* strain 536 is included in Table 1.

PAI IV (HPI, encoding the yersiniabactin siderophore) was identified on the basis that it was necessary for *Yersinia pestis* virulence (Carniel et al., 1996). In our model, HPI deletion did not decrease extra-intestinal virulence of *E. coli* 536. Yet, it was shown that HPI directly participated in extra-intestinal virulence in the same septicemia model of two highly virulent uropathogenic strains belonging to the B2 phylogenetic group, IAI51 and IAI52 (Schubert et al., 2002). Mice lethality was dramatically decreased in isogenic mutants of IAI51 and IAI52 in which HPI had been inactivated. Therefore, HPI is crucial for IAI51 and IAI52 virulence but is only one of the many contributors to extra-intestinal virulence of strain 536. Interestingly, IAI51 and IAI52 both bear a O92 serotype and have

Table 1
Description of PAI content of *E. coli* strain 536.

PAI	tRNA gene	Size (kbp)	Encoded proteins (gene)
I	*selC*	76	Alpha hemolysin, F17-fimbriae, CS12-fimbriae
II	*leuX*	102	Alpha hemolysin, P-fimbriae (*pap*), hemagglutinin
III	*thrW*	68	S-Fimbriae, salmochelin (*iroN*), heme receptor, toxin, hemoglobin protease, Ag 43
IV = HPI	*asnT*	30	Yersiniabactin siderophore
V	*pheV*	80	Pilus P (*pix*), Ag43, capsule antigen (kps_{K15})
VI	*asnW*	54	Polypeptide-polyketide synthase (*pks*)
VII	*serU*	20	Shufflon, histone-like protein

PAIs are named by their usual Roman number. tRNA gene: tRNA gene next to which the PAI is inserted. HPI: high-pathogenicity island.

an unusual MLST pattern (Olivier Clermont and Erick Denamur, unpublished data), as they do not belong to any of the 9 recently described B2 subgroups (Le Gall et al., 2007). Taken together, these data show the interplay between VFs and the genetic backgrounds (Escobar-Paramo et al., 2004).

One possibility to explain the low importance of *E. coli* 536 yersiniabactin in our septicemia model could be the redundancy of iron scavenger molecules in this uropathogenic strain. At least three other siderophores are encoded on PAI III (salmochelin encoded by the *iro* genes) (Dobrindt et al., 2001), and elsewhere on the genome (enterochelin encoded by the *ent* genes and hemin uptake system encoded by the *chu* operon) (Brzuszkiewicz et al., 2006). Since loss of PAI III but not of HPI results in a decreased virulence in our model, we can hypothesize that *iroN* seems to play a specific role in strain 536 blood stream infections. This is corroborated by the relatively high proportion of septicemia strains harboring *iroN* (Ananias and Yano, 2008), and by its role in new born meningitis in which the septicemic phase has been shown to be critical (Negre et al., 2004; Peigne et al., 2009).

Brzuszkiewicz et al. (2006) have also tested single deletion of PAIs I–V in a mouse septicemia model and in a mouse ascending UTI model. In the mouse septicemia model, they found no difference between single PAI deletion mutants. This is probably because of the high inocula they used (10^8 bacteria). Only a PAI I and II double-deletion mutant showed reduced virulence in the mouse septicemia model. However, this mutant resulted from a spontaneous deletion that also involves the adjacent tRNAs *selC* and *leuX*, respectively. It has been shown that *selC* and *leuX* tRNA deletions have important metabolic consequences that directly influence the virulence of the mutant (Ritter et al., 1995; Dobrindt et al., 2002b). Therefore, it is difficult to draw definitive conclusions from these experiments. In the mouse ascending UTI model, they found that deletion of PAIs I, II or III resulted in decreased uro-virulence. Deletion of PAIs IV or V had no effect on uro-virulence. Since we have shown that deletion of PAI I had no impact on septicemia lethality, we can conclude that PAIs have different effects in different infection models. PAI I seems to be important in uro-virulence while PAI II and III are implicated both in uro-virulence and in blood stream infections. This might be because PAIs II and III contain both blood-interacting elements (hemolysin, hemagglutinin, hemoglobin protease) and adhesion proteins the role of which is determinant in uro-epithelial adhesion (Marre and Hacker, 1987; Dobrindt et al., 2002a; Wiles et al., 2008).

Deletion of *E. coli* 536 PAI V has been shown to result in a decreased uropathogenicity (Schneider et al., 2004). However, PAI V deletion mutants showed normal resistance to serum (Schneider et al., 2004). Consistently, in our model Δ5 showed the same septicemia lethality as WT_{eng}.

It was shown that PAI VI encoded a non-ribosomal peptide synthesis system and three polyketide synthases (Nougayrede et al., 2006). The activation of this machinery results in a cell cycle arrest at the G2/M transition of cells from several epithelial cell lines. This phenomenon is believed to be important in the regulation of *E. coli* intestinal commensalisms/toxicity balance (Hayashi, 2006).

PAI VII was identified on the basis of its presence in 3 virulent *E. coli* strains and its absence in the K-12 MG1655 genome. Its participation in virulence remains to be determined.

In our model, deletion of PAI VI or VII did not affect strain 536 extra-intestinal virulence.

Trying to summarize all these data would result in a complex network of pathogenetic possibilities. PAIs have an additive effect but their involvement in extra-intestinal virulence also depends on the genetic background in which they are and on the infection model used to test them. PAIs I and V are important for UTI (Schneider et al., 2004; Brzuszkiewicz et al., 2006), but not for blood stream infection ((Schneider et al., 2004) and our study). PAIs II and III seem to be important for both UTI and blood stream infection ((Brzuszkiewicz et al., 2006) and our study). PAI IV (HPI) is crucial for IAI51 and IAI52 virulence but not for strain 536 extra-intestinal virulence as assessed in a blood stream infection model (Schubert et al., 2002 and our study). Furthermore, it is not necessary for virulence of CFT073 as tested in a UTI model (Lloyd et al., 2009). PAI VI does not seem to be involved in extra-intestinal virulence as assessed in our blood stream infection model. Finally, PAI VII encodes potential VFs, the significance of which still needs to be confirmed in vivo. The fact that PAIs have different implications in different virulence models reinforces the idea that virulence is only a side-effect of the capacity of *E. coli* to adapt to many different non-pathologic environments (Le Gall et al., 2007).

Conclusion

All attempts to simplify commensal/pathogenic dichotomy in *E. coli* species to a set of genes, a set of PAIs, or a single phylogenetic process turned out to be vain. Large genome comparisons showed that outside of the core genome, no genes were specifically present or specifically absent in all commensal strains, or in all virulent strains (Touchon et al., 2009). Here we have shown that not all *E. coli* 536 PAIs play the same role in extra-intestinal virulence as defined by mouse septicemia lethality. Others have come to the same conclusions when studying other virulence models and/or other strains sharing some of the same PAIs. Taken together, these results indicate that virulence is a complex multigenic process resulting from numerous genetic combinations.

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