Caenorhabditis elegans as a simple model to study phenotypic and genetic virulence determinants of extraintestinal pathogenic Escherichia coli

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***Caenorhabditis elegans* as a simple model to study phenotypic and genetic virulence determinants of extraintestinal pathogenic *Escherichia coli* strains**

Caenorhabditis elegans* as a simple model to study phenotypic and genotypic determinants of pathogenicity of extraintestinal pathogenic *Escherichia coli

Virulence of extraintestinal pathogenic *Escherichia coli* strains in *Caenorhabditis elegans*

Virulence of extraintestinal pathogenic *Escherichia coli* strains in *Caenorhabditis elegans* and mice

Comparison of the virulence of extraintestinal pathogenic *Escherichia coli* in *Caenorhabditis elegans* and mice

Abstract

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains cause diseases by invading normally sterile niches within host body, e.g., urinary tract, blood and cerebrospinal fluid. Infections due to ExPEC strains, in particular urinary tract infections, are causing considerable morbidity and important health-care costs. The goal of our study is to evaluate whether the nematode *Caenorhabditis elegans* can be used as a model to study phenotypic and genetic virulence determinants of ExPEC strains. For this purpose, we used a collection of 31 *E. coli* strains isolated during acute extra-intestinal infections or from the feces of healthy individuals. For all strains, the phylogeny, the presence of ExPEC virulence factors, the resistance to biologically relevant stressors (i.e., bile, human serum and lysozyme), the motility, the growth rate, the virulence in *C. elegans* and in murine septicaemia model has been established. The results show that there is a strong link between virulence in *C. elegans* and certain phenotypic and genetic virulence predictors of ExPEC strains determinable *in vitro*. Furthermore, there is an excellent correlation between virulence of different ExPEC strains in *C. elegans* and in the murine models. The virulence of ExPEC strains, which is expected to be a continuous trait resulting from the interaction of variable number and types of virulence factors in different strains, is even better revealed and more easily analysed in *C. elegans* than in the murine model. Therefore, our results show that *C. elegans* can be used as a model to study virulence determinants of ExPEC strains

Introduction

Most *Escherichia coli* strains are harmless commensals of the gastrointestinal tract of humans and warm-blooded animals, however, some strains are able to cause intestinal and extraintestinal diseases **((16)).** *E. coli* strains that cause gastrointestinal diseases are rarely found in the fecal flora of the healthy hosts and are, in general, not capable of causing disease outside of the intestinal tract. Extraintestinal pathogenic *E. coli* (ExPEC) strains are normally found in host intestinal tract, sometimes in very high frequency, without causing enteric disease. ExPEC strains can cause diseases by invading normally sterile niches within host body, e.g., urinary tract, blood and cerebrospinal fluid. ExPEC strains are traditionally classified as pyelonephritogenic, uropathogenic, sepsis-associated and neonatal meningitis-associated pathotypes, on the basis of the syndrome they have been associated with. ExPEC strains can cause disease at all host ages, but the incidence of serious infections increases with age, due to the anatomical and physiological changes with aging and to the impairment of immune functions. Commensal strains can also cause extraintestinal infections, but only when aggravating factors are present, e.g., host immunodeficiency, obstruction of biliary tract, presence of foreign body like urinary catheter. Among different extraintestinal infections due to *E. coli* strains, urinary tract infections (UTI) are, by their frequency and potential severity, causing considerable morbidity and important health-care costs **(24)**.

ExPEC strains are distinct from commensal and intraintestinal pathogenic *E. coli* strains by their specific virulence factors. ExPEC virulence factors include adhesins (e.g., P and I fimbriae), iron-acquisition systems (e.g. aerobactin), host defense-avoidance systems (e.g., capsule, lipopolysaccharide), and toxins (e.g., hemolysin, cytotoxic necrotizing factor 1) **(15), (14)**. Most of ExPEC virulence factors are encoded by genes that are clustered on pathogenicity islands (PAI), i.e., big genomic or plasmidic regions, up to 200 kb in length **(11)**. PAIs seem to be acquired via horizontal gene transfer, as suggested by their G + C content that is usually different from that of the rest of the genome. They are often associated with tRNA loci, which may represent target sites for the integration in the chromosome. As PAIs are flanked by direct repeats or insertion sequence elements, they have tendency to delete with high frequencies and can undergo duplications and amplifications. Consequently, PAIs are present in different combination, with varying overlap among different PAIs with respect to virulence factors content, and with different frequency among *E. coli* strains responsible for extraintestinal infections. However, in spite of the formidable promiscuity of horizontal gene transfer in bacteria, ExPEC PAIs are found predominantly in strains belonging to *E. coli* phylogenetic

group B2 and, to lesser extent, to group D**(21)**. Their presence is extremely rare in phylogenetic groups A and B1 to which belong vast majority of commensal and intraintestinal pathogenic *E. coli* strains. Such link between ExPEC virulence factors and phylogeny could correspond to the necessity of having virulence determinants into the right genetic background for the emergence of a virulent clone.

The goal of our study is to evaluate whether *Caenorhabditis elegans* can be used as a model to study different phenotypic and genetic virulence determinants of ExPEC strains. *C. elegans* is a simple and economic invertebrate animal model that is increasingly used as model host for the study of mechanisms of microbial pathogenesis in recent years **(20)**. *C. elegans* is a free-living nematode, normally found in the soil and rotting vegetation worldwide. Use of hermaphrodite, self-fertilizing *C. elegans* enables maintenance of homozygous mutant stocks. *C. elegans* requires very cheap and simple growth conditions, i.e. they grow on a layer of bacteria in an agar plate. When worms eat bacteria, they are crushed by the grinder situated in the pharynx and then pass into the intestine. However, a small fraction of bacteria pass through the grinder intact and enter the intestine. Once in the gut, some pathogenic bacteria are capable of proliferation and killing the nematode by an infectious process, while other bacteria kill through the use of toxins. A rapidly growing number of human, plant and animal microbial pathogens, both gram-negative and gram-positive have been shown to injure and kill *C. elegans* **(20)**. In many cases, microbial genes known to be important for virulence in mammalian models were shown to be required for virulence in nematodes. Furthermore, *C. elegans* as a model system for pathogenesis provides opportunity to simultaneously explore the molecular genetic determinants of both pathogen virulence and host defense because it possesses a complex inducible innate immune system, involving multiple signaling pathways, as well as many antimicrobial proteins and peptides.

C. elegans has been used to evaluate virulence of *E. coli* intraintestinal pathogenic strains **(2)**, but the virulence of ExPEC strains have never been tested in this model organism. For this study, we used a collection of *E. coli* natural isolates composed of commensal and ExPEC strains. Our results indicated that *C. elegans* can successfully be used as a model organism for ExPEC pathogenesis. We think that the use of such a simple and economic animal model can greatly facilitate the study of the contribution of different virulence factors, their combinations and the strain genetic background to the *in vivo* virulence of ExPEC strains. The obtained results can then, in a targeted way, be validated in costly mammal model hosts.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains isolated from extraintestinal infections (21 strains) and from feces

of healthy humans (10 strains) used in this study, as well as their relevant genotypes, are listed in Table S1. *E. coli* 536 High Pathogenicity Island (HPI) and *rfe* (synonym for *wecA*) mutant strains were constructed using the PCR-based method as described by Datsenko and Wanner [**(6)**; For more details see ONLINE SUPPLEMENTARY MATERIAL]. *E. coli* 536 PAI III mutant strain was kindly provided by U. Dobrindt and J. Hacker (Institut für Molekulare Infektionsbiologie, Universität Würzburg, Germany). In addition, *E.coli* laboratory OP50 (standard food for *C. elegans*) and MG1655 strains were also used. Saturated overnight cultures of *E. coli* strains were obtained by growing cells in Luria–Bertani (LB) medium at 37 °C with shaking, supplemented with appropriate antibiotics when needed [chloramphenicol (30µg/ml), ampicilin (100µg/ml)].

Measurement of the growth rate of the *E. coli* strains in nematode growth medium (NGM). Saturated overnight cultures were diluted in $MgSO_4$ 10^{-2} mol/L, and about 10^7 cells were added to liquid NGM **(13)** in 96 wells plaque. Growth was followed by measuring the optical density at 600 nm, every 5 minutes during 24h, using IEMS spectrophotometer (Perkin Elmer) at 25°C with shaking. The generation time (min) was calculated as time necessary for a two-fold increase of optical density during growth phase fitting an exponential curve. Each assay was repeated in, at least, three independent experiments.

Measurement of the motility of the *E. coli* strains in NGM. Saturated overnight cultures were diluted in $MgSO_4$ 10^{-2} mol/L, and about 30 cells were added to 20 ml of melted NGM soft agar (4.5 g/l agarose) in 9-cm-diameter plates. Solidified plates were incubated for 36 h at 25°C, then the surface of, at least, 10 colonies was measured with Elix software (Microvision Instruments) and the mean surface calculated. For each strain, in each assay, the motility was normalized using values obtained for *E. coli* MG1655 strain. Each assay was repeated in, at least, three independent experiments.

Measurement of human serum, bovine bile and lysozyme/lactoferrin resistance of the *E. coli* strains. Saturated overnight cultures were diluted in $MgSO_4$ 10^{-2} mol/L and the sensitivity of cells to human serum, bovine bile and lysozyme/lactoferrin was measured as follows: (i) About 10^5 cells were added to 1ml of 75% human serum (**Institut Jacques Boy S.A.**) diluted in LB. After 1h of incubation at 37°C without shaking, the number of live bacteria was estimated by plating on LB agar. Serum resistance was estimated as follows: log [CFU/ml (t_{1h}) / CFU/ml (t_0)]. (ii) About 10^3 cells were added to 5ml of 20% bovine bile **(Merck)** diluted in LB. After 17h of incubation at 37°C with shaking, the number of live bacteria was estimated by plating on LB agar. Bovine bile resistance was estimated as follows: log [CFU/ml (t_{17h})/ CFU/ml (t_0)]. (iii) About 10^3 cells were added to 200µl of 1% bacto-

peptone supplemented with 0.05mg/ml of egg lysozyme **(Sigma)** and 1 mg/ml of lactoferrine from bovine colostrums **(Sigma)** (lactoferrin was used in order to enhance sensitivity to lysozyme (8)). Lysozyme is involved in the bacteriolytic activity of human serum **(26)**. After 17h of incubation at 25°C with shaking, the number of live bacteria was estimated by plating on LB agar. Lysozyme/lactoferrin resistance was estimated as follows: log [CFU/ml (t_{17h})/ CFU/ml (t_0)]. Each assay was repeated in, at least, three independent experiments.

***C. elegans* - killing assay.** The *C. elegans fer-15* conditional sterile mutant (kindly provided by J. J. Ewbank, CIML, Marseille, France) was maintained at 15°C to have fertile worms, and at 25°C to have sterile population after synchronization, as previously described **(13)**. Plates for killing assay were prepared as follows. 100 µl of the saturated overnight bacterial cultures were spread on 9-cm-diameter plates containing NGM agar. The NGM plates were incubated at 37°C for 24h, and then allowed to equilibrate to room temperature for 30 to 60 min before being seeded with worms. In each assay, at least 60 L4-stage synchronized nematodes were added per plate, and each assay was carried out at least tree times. The plates were incubated at 25°C and live and dead worms were scored every 24 h. During the first six to ten days, live worms were transferred to a new NGM plate with fresh bacterial lawn every 48h. A worm was considered dead when it failed to respond to plate tapping or gentle touch with a platinum wire. Worms that died as a result of getting stuck to the wall of the plate were excluded from the analysis.

Partie pour Erick: Murine septicaemia model. The virulence of *E. coli* strains that have not previously been assayed in mouse septicaemia model was performed as described previously (**ref**). Pathogen-free female of the outbred white Swiss mice lineage (Rj: Swiss-IOPS Orl) (6 to 8 weeks old, 25 to 30 g) were purchased from the Centre d'Elevage R. Janvier (Le Genest, Saint Isle, France). The *E. coli* strains were grown in Trypticase soy broth medium (pH 7.3, osmolarity = 271 mOsm/l) (AES Laboratories) with shaking at 37°C to a density of 10^9 bacteria per ml. The bacterial count was determined by measuring the optical density at 530 nm. A 2-ml sample of the culture was then centrifuged at 2,500 × g for 10 min, washed twice with Ringer solution, and resuspended in Ringer solution. These suspensions were used to inoculate the mice subcutaneously in the abdomen or intraperitoneally. Ten mice were inoculated with each strain, and number of dead mice during 7 days was noted.

Statistical analysis. A factorial analysis of correspondence (FAC) was used to describe associations among different sets of data. FAC uses a covariance matrix based on χ^2 distances **(10)**. The computation

determines a plane defined by two principal axes of the analysis; the first axis F1, accounts for most of the variance, and the second axis F2, orthogonal to F1, accounts for the largest part of the variance that is not accounted by F1.

FAC was conducted with SPAD.N software (Cisia, Saint Mandé, France) from a two- way table which had 31 rows, one for each *E. coli* strain and 34 columns corresponding to the 34 variables: 4 *E. coli* phylogenetic groups (A, B1, B2 and D), 17 virulence factors (named as in supplementary table S1), 5 serotypes (O2, O6, O18, O81, other O-groups), pathogen / commensal origin, motility, serum resistance, bile resistance, lysozyme/lactoferrin resistance, growth rate, fast / slow worm killer and mouse lethality (Figure 2). For each column, each strain was coded as a binary code: present =1, absent = 0.

Influence of different factors on the hazard ratio (ratio between mortality rates) was tested using a Cox proportional hazard (CPH) model **(5), (27)**. The hazard ratio in survival analysis is a summary of the difference between two survival curves, representing the change in the risk of death compared to control, according to an explanatory variable (Phenotype/Genotype), over the period of follow-up. The model uses a computable algorithm to summarise the relative risks of death across all time intervals into a single figure (β).

Numerical values for the (multiplicative) effect of each covariate can be calculated as exp(β) after estimation of β**.** For figures 1, 3 and 4, 95% confidence intervals were calculated based on the estimated standard deviation of the z-distributed β, using a robust jackknife estimate of the variance and grouping observations with respect to bacterial strains, where necessary. In the text, hypothesis testing based on P-values (predictors of pathogenicity and effect of deletion mutations) followed the Bonferroni-Holm procedure **(12)** for repeated significance testing for the pooled CPH anaylses.

The proportional hazards regression model we use here assumes that the relative risk of death between the different groups is constant at each interval of time. This has been tested for using a chi-square test statistic for the pearson moment-correlation between the scaled Schoenfeld residuals and the log-transformed time **(27)**. In those cases where significant non-proportionality was found, a graphical representation showed that the effect of non-proportionality did not alter the overall conclusions. All survival analyses were performed using R (http://www.r-project.org/) version 2.2.1.

RESULTS

Characterization of *E. coli* natural isolates. The pathogenicity of *E. coli* natural isolates in *C. elegans* host model was evaluated by measuring the survival of worms fed on pure cultures of these strains. The results show that different strains have very different virulence (Fig. 1; for raw survival

curves see Fig S1). In order to identify variables that could explain such strain-dependent pathogenicity, *E. coli* isolates were genotypically and phenotypically characterized. For genetic characterization, the phylogenetic group, the presence of PAIs and of ExPEC virulence factors were determined (Table S1). The phenotypes under investigation were: the growth rate in NGM at 25°C, motility, resistance to bovine bile, human serum and lysozyme/lactoferrin, as well as the pathogenicity of the strains in the murine septicaemia model (Table 1). All phenotypic data were binarized for further analysis (Table 1).

Predictors of pathogenicity of *E. coli* natural isolates in the *C. elegans*. In order to identify associations between different variables and pathogenic potential of *E. coli* natural isolates in the *C. elegans*, factorial analysis of correspondence (FAC) was conducted using all data available for the studied strains (Fig. 2). The projections of the variables on the plane F1/F2 that accounts for 44.76 % of the total variance distinguish two main groups of variables by the positive and negative values of the first factor (F1). The first group (positive values of F1 and F2) encompasses the mouse lethality; the pathogenic origin of the strains; the "fast worm killer" character; the phylogenetic group B2; O2, O6 and O18 serotypes; the resistances to serum, bile and lysozyme/lactoferrin; the motility; fast growth rate, and the most of the virulence factors. The second group (negative values of F1 and positive values of F2) encompasses the commensal origin of the strains; the absence of mouse lethality; the "slow worm killer" character; the phylogenetic groups A and B1; and O81 serotype. A third group of variables that is distinguished by the negative values of F2, encompasses the phylogenetic group D, aerobactin, pap GII genes and presence of PAI I.

Therefore, FAC retrieved two distinct groups of *E. coli* natural isolates: (i) strains isolated during acute extra-intestinal infections that belong to B2 phylogenetic group, and that are highly virulent for humans and mouse, and (ii) strains isolated from the feces of healthy individuals that belong to A and B1 phylogenetic groups, and that are less virulent for humans and mouse. The character "fast worm killer" is unambiguously projected in the first group whereas the character "slow worm killer" is projected in the second one.

In order to confirm or reject hypotheses concerning connections between pathogenicity in *C. elegans*, *in vitro* phenotypes, presence of PAIs and virulence in murine septicaemia model indicated by FAC, we performed a Cox proportional hazard (CPH) analysis. Using CPH analysis, it is possible to take into account the entirety of survival data (including censored observations), instead of relying only on single values, e.g. mean survival. With regard to presence of PAI II, PAI III, and HPI, the CPH analysis reveals that PAI II ($p=6.2 \times 10^{-3}$), PAI III ($p=2.2 \times 10^{-2}$), are significantly associated with higher *C. elegans* mortality, while HPI is not ($p=2.2 \times 10^{-1}$) (Figure 3.a). Concerning *in vitro*

phenotypes, CPH analysis indicates that resistance to lysozyme/lactoferrin ($p=1.1 \times 10^{-5}$) and to human serum ($p=3.4 \times 10^{-2}$) are significantly correlated with an increase in mortality of *C. elegans*, while resistance to bovine bile, the growth rate in NGM and motility are not (Figure 3.b). Finally, CPH analysis indicates that pathogenicity of different *E. coli* strains in the *C. elegans* and in the mouse model is significantly correlated ($p=2.1 \times 10^{-4}$) (Figure 3.c).

Pathogenicity of 536 ExPEC strain mutants in *C. elegans*. Three *E. coli* 536 mutants were used to experimentally verify connections between different variables and pathogenicity in *C. elegans* as identified by CPH analysis. The first two mutant strains have PAI III and HPI deleted, respectively. PAI III was chosen because CPH analysis showed that it is significantly associated with higher *C. elegans* mortality, while HPI was chosen as a negative control. The third mutant, which has the *rfe* gene deleted, was used to test the link between resistance to lysozyme/lactoferrin and to human serum, and pathogenicity in *C. elegans*. *rfe* codes for the UDP-GlcNAc:undecaprenylphosphate GlcNAc-1-phosphate transferase involved in the biosynthesis of the Enterobacterial Common Antigen (ECA) and of the lipopolysaccharide (LPS) O-antigen **(reviewed in (23))**. We have chosen to inactivate this gene because it was previously reported that the inactivation of *rfe* gene decreases the resistance to bile of *Salmonella enterica* **(22)**, as well as the virulence of *S. enterica* during oral and intraperitoneal infection in mice **(22)**. The inactivation of *rfe* in 536 strain attenuates its resistance to lysozyme/lactoferrin, human serum and bovine bile (Table 1). A CPH analysis, with regard to presence of PAI III, HPI, *rfe* and *C. elegans* mortality, reveals that inactivation of PAI III ($p=2.6 \times 10^{-11}$) and *rfe* gene ($p=2.6 \times 10^{-17}$) significantly attenuates pathogenicity in *C. elegans*, while inactivation of HPI does not (Figure 4).

DISCUSSION

The goal of our study is to evaluate whether *C. elegans* can be used as a model to study phenotypic and genetic virulence determinants of ExPEC strains. For this purpose, we used the collection of 31 *E. coli* strains isolated during acute extra-intestinal infections or from the feces of healthy individuals (Table S1). For all strains, we established the phylogeny, the presence of ExPEC virulence factors, *in vitro* resistance to biologically relevant stressors (i.e., bile, human serum and lyzosyme), the motility, the growth rate, the virulence in *C. elegans* and in murine septicaemia model (Table 1). Subsequently, in order to identify associations between all these different variables, we conducted factorial analysis of correspondence (FAC). FAC indicated that there is link between presence of various ExPEC virulence factors, B2 phylogenetic group and virulence in murine septicaemia model (Fig 2), which was

previously observed **(21)**. However, the association of these traits with the motility, the resistance to human serum, bile and lysozyme/lactoferrin, as well as the virulence in *C. elegans* is observed for the first time (Fig 2).

As motility has been described as an important feature of UTI causing strains **(28)**, it is not surprising that most of the studied ExPEC strains are motile (Table 1). The immobility of the most of strains isolated from the feces (Table 1) could be an adaptation to the commensal style of life, as *E. coli* flagellin can induce a pro-inflammatory response in the mammalian enterocytes in vivo **(3)**. Therefore, the *E. coli* strains that do not present flagellin on the cell surface could be favoured in the populations of intestinal bacteria. The association of ExPEC strains with the resistance to the human serum, to lysozyme/lactoferrin and to bile, with the virulence in *C. elegans* and in murine septicaemia model reflects the importance of the capacity of ExPEC cells to protect membrane integrity against host defences. Thus, many of the associations between different characters suggested by FAC are corroborated by what is already known from the scientific literature concerning *E. coli* species. The question remains which of those of those characters identified by FAC as linked with ExPEC strains are specifically involved in virulence in the *C. elegans* model. We tried to answer to this question using Cox proportional hazard (CPH) analysis.

Identification of ExPEC phenotypic and genetic determinants relevant for the virulence in *C. elegans* model. *C. elegans* appears to be killed by ExPEC strains in an infectious process as previously observed for other bacterial strains **(20)** (data not shown). This process consists of an accumulation of the pathogen inside the intestine of the worm causing distension of the gut and death of the animal. In our attempt to untangle this intrinsically multifactorial phenomenon of virulence, we decided to examine possibly relevant phenotypes on one hand, and known genetic virulence determinant on the other hand.

However, not all of the inspected phenotypic traits showed correlation with increased mortality (Fig 3): It seems that motility has no importance in the *C. elegans* model, this is corroborated by the observation that there is also no correlation between virulence in *C. elegans* and the capacity of *E. coli* strains to form biofilm, while there is a good correlation between motility and biofilm formation (data not shown). It should be noted that growth rate does not predict virulence in *C. elegans,* implying that worm mortality is not a simple indicator of overall bacterial fitness.

The capacity of bacteria to protect membrane integrity is important in *C. elegans* because the latter produces many molecules with antibacterial activity, e.g., lysozymes, lectins, lipases, aspartic and cysteine proteases, saposin-like proteins and several antibacterial peptides (reviewed in **(20)**). Among genes known to be required for the virulence of different bacterial species in *C. elegans*, many code for

membrane constituents, e.g., *Pseudomonas aeruginosa aefA* **(25)** gene, *S. enterica rfaL* **(1),** *ompR* gene **(18)** and *wze* homologue gene of *Serratia marcescens* **(17)**. Consequently, resistance to human serum and to lysozyme/lactoferrin of *E. coli* strains play a role in the capacity to kill nematodes (Fig 3). The non-correlation between worm-mortality and bile resistance suggests that the worm digestive tract mimics conditions of extraintestinal infections rather than those within a mammal intestine.

. We have experimentally verified connections between bacterial resistance and pathogenicity in *C. elegans* by inactivating *rfe* in ExPEC strains 536 (Fig 4, Table 1) and F11 as well as the commensal IAI1 strain (data not shown). *rfe* is required for LPS biosynthesis **(23)** and its deletion causes the "rough" phenotype, which has been shown to be associated with attenuation of resistance to human serum **(14).** All three *rfe* mutants have significantly diminished capacity to kill *C. elegans*, and heavily impaired resistance to human serum, bile and lysozyme/lactoferrin.

The PAIs under investigation differ both in size and in content, and include unique, PAI-specific functions as well as redundant ones. This was reflected by our finding that while PAI II and PAI III are clearly associated with higher *C. elegans* mortality, HPI is not or far less so. Our experiments do confirm these correlations; we deleted PAI III from 536 strain and observed that the mutant has indeed reduced virulence in *C. elegans*, while the deletion of HPI has no effect. Among known genes carried by PAI III, two categories could play a role in virulence in *C. elegans*, those coding for adhesins, and those coding for siderophores, as it was observed for the *pilA* gene encoding a putative pilus structural protein in *Burkholderia pseudomallei* **(9)** and the ferrisiderophore receptor (y3343) of *S. marcescens* **(17)**. The deletion of PAI III in 536 strain attenuated resistance to lysozyme (to a lesser extent than that of *rfe* mutant), which probably contributes to the attenuation of the virulence of PAI III mutant in *C. elegans* (Table 1). The lack of the effect of HPI deletion on virulence in *C. elegans* may be explained by redundancy between functions encoded by HPI and PAI III, i.e., both PAIs carry genes involved in the iron uptake,**(4)** ,**(7)**.

Concluding remarks. We have established a strong link between virulence in the *C. elegans* and certain phenotypic and genetic predictors of ExPEC strains' pathogenicity determinable *in vitro*. Excellent correlation between virulence of different ExPEC strains in the *C. elegans* and in the murine septicaemia model further confirms that *C. elegans* can be used as a model to study virulence determinants of ExPEC strains (Figure 3.c). Furthermore, the virulence of ExPEC strains, which is expected to be a continuous trait resulting from the interaction of variable number and types of virulence factors in different strains, is better revealed and more easily analysed in *C. elegans* than in the frequently used murine septicaemia model (Fig 1, Table 1). Therefore, *C. elegans* can be used to identify functions of many genes with unknown functions present on ExPEC PAIs, as well as new

virulence factors elsewhere on the chromosomes of ExPEC strains. The obtained results can then, in a targeted way, be validated in mammal model hosts.

Table 1 **Phenotypique characterization of *E. coli* wild type and mutant strains.**

Strain	Worm Mean Lifespan (days)	Resistance to Serum (S.E.M)*	Binarized Résistance to Serum ‡	Resistance to Lysozyme (S.E.M)*	Binarized Resistance to Lysozyme ‡	Resistance to Bovine Bile (S.E.M)*	Binarized Resistance to Bile bovine ‡	Motility (S.E.M)*	Binarized Motility ‡	Generation time (min) (S.E.M)*	Binarized generation time ‡	Mice killed†	Binarized Mice killing phenotype
MG1655	8.56	-5.00 (0.00)	0	0.28 (0.38)	0	-0.01 (1.87)	0	1.00 (0.00)	1	123.7 (21.8)	0	0	MNK
OP50	11.76	-5.00 (0.00)	0	-0.99 (0.32)	0	-3.00 (0.00)	0	0.04 (0.01)	0	116.8 (30.5)	1	0	MNK
F11	5.94	-0.19 (0.32)	1	3.57 (0.65)	1	6.21 (0.02)	1	1.97 (0.75)	1	93.2 (1.5)	1	9	MK
F63	5.72	-0.27 (0.31)	1	4.07 (0.37)	1	3.79 (0.29)	1	1.07 (0.33)	1	118.8 (5.6)	0	9	MK
RS218	6.06	-0.26 (0.30)	1	4.01 (0.27)	1	3.93 (1.11)	1	1.31 (0.52)	1	117.5 (2.0)	0	10	MK
ED1A	8.32	-0.94 (0.23)	1	0.14 (0.39)	0	-0.50 (1.54)	0	0.04 (0.00)	0	130.0 (8.8)	0	0	MNK
CFT073	7.78	-0.10 (0.05)	1	4.01 (0.50)	1	-3.00 (0.00)	0	3.34 (0.60)	1	128.4 (5.1)	0	9	MK
J96	6.13	-1.28 (0.06)	0	4.06 (0.36)	1	5.19 (0.67)	1	4.08 (0.36)	1	137.7 (3.4)	0	0	MNK
IAI1	5.68	0.01 (0.04)	1	0.37 (0.48)	0	6.60 (0.15)	1	0.04 (0.00)	0	104.5 (1.1)	1	0	MNK
IAI2	9.13	-1.79 (1.60)	0	0.71 (0.43)	0	-0.86 (2.14)	0	0.04 (0.00)	0	179.3 (38.9)	0	0	MNK
IAI4	8.68	-4.00 (1.00)	0	1.17 (0.38)	0	1.13 (2.08)	1	0.04 (0.01)	0	116.5 (11.4)	1	0	MNK
IAI12	7.60	-0.30 (0.17)	1	1.88 (0.09)	0	6.59 (0.06)	1	0.11 (0.05)	0	111.8 (3.9)	1	0	MNK
IAI13	10.13	-1.07 (0.14)	0	1.25 (0.31)	0	-3.00 (0.00)	0	0.04 (0.01)	0	140.5 (3.5)	0	0	MNK
IAI15	9.01	-5.00 (0.00)	0	-1.37 (0.42)	0	-3.00 (0.00)	0	0.73 (0.33)	1	135.9 (18.3)	0	0	MNK
IAI19	5.25	-0.66 (0.08)	1	3.28 (0.83)	1	-3.00 (0.00)	0	3.51 (0.60)	1	132.4 (6.6)	0	0	MNK
IAI21	8.08	-5.00 (0.00)	0	-1.66 (0.57)	0	-2.23 (0.77)	0	0.06 (0.02)	0	124.5 (3.9)	0	0	MNK
IAI39	7.33	-4.23 (0.77)	0	2.38 (0.20)	0	-3.00 (0.00)	0	0.03 (0.01)	0	142.4 (8.4)	0	10	MK
IAI44	9.67	-4.23 (0.77)	0	1.83 (0.13)	0	0.57 (0.36)	1	0.43 (0.06)	1	134.2 (7.5)	0	1	MNK
IAI48	8.70	-0.43 (0.22)	1	0.83 (0.47)	0	1.21 (2.12)	1	0.07 (0.00)	0	152.9 (7.9)	0	0	MNK
IAI49	7.73	-0.18 (0.06)	1	3.94 (0.37)	1	-3.00 (0.00)	0	2.45 (0.41)	1	116.8 (3.5)	1	10	MK
IAI51	6.08	-2.86 (1.12)	0	3.41 (0.30)	1	-3.00 (0.00)	0	0.04 (0.00)	0	113.0 (2.4)	1	10	MK
IAI52	5.99	-3.04 (1.07)	0	3.07 (0.98)	1	-3.00 (0.00)	0	0.04 (0.00)	0	111.8 (1.8)	1	10	MK
IAI60	9.87	-2.90 (1.08)	0	2.05 (0.11)	0	4.65 (1.09)	1	1.03 (0.39)	1	100.9 (1.7)	1	1	MNK
IAI64	7.56	-4.33 (0.67)	0	2.33 (0.99)	0	-0.67 (1.39)	0	0.96 (0.37)	1	112.4 (8.5)	1	10	MK
IAI72	7.05	-0.98 (0.09)	1	3.88 (0.54)	1	6.58 (0.37)	1	0.04 (0.00)	0	120.2 (3.4)	0	10	MK
IAI73	6.48	-0.44 (0.09)	1	3.96 (0.43)	1	2.85 (0.23)	1	3.01 (0.46)	1	236.3 (7.0)	0	10	MK
IAI74	5.42	-5.00 (0.00)	0	3.34 (0.81)	1	-3.00 (0.00)	0	1.27 (0.27)	1	107.7 (36.8)	1	10	MK
ColF6c	7.49	-0.94 (0.31)	1	2.78 (0.56)	1	6.66 (0.13)	1	0.04 (0.01)	0	114.3 (5.4)	1	10	MK
ECOR64	6.27	-0.18 (0.11)	1	4.10 (0.33)	1	-3.00 (0.00)	0	0.03 (0.01)	0	123.8 (4.3)	0	10	MK
EC7372	6.92	-0.27 (0.12)	1	1.47 (0.31)	0	5.24 (0.53)	1	1.33 (0.39)	1	101.4 (3.0)	1	10	MK
ECOR58	6.17	-0.02 (0.07)	1	2.34 (0.47)	0	6.81 (0.17)	1	0.40 (0.11)	1	105.0 (6.2)	1	10	MK
Ben13f	5.49	-0.24 (0.09)	1	2.66 (0.34)	1	6.27 (0.35)	1	0.03 (0.01)	0	110.6 (3.4)	1	10	MK
536	5.97	0.01 (0.01)	1	3.43 (0.41)	1	6.59 (0.06)	1	2.57 (0.32)	1	114.3 (0.6)	1	9	MK
536 Δ*rfe*	7.72	-5.00 (0.00)	0	1.10 (0.18)	0	-3.00 (0.00)	0	0.25 (0.09)	0	156.3 (4.3)	0	NA	NA
536 ΔPAI III	6.72	-0.17 (0.07)	1	2.15 (0.06)	0	6.58 (0.04)	1	2.09 (0.18)	1	125.3 (4.6)	0	NA	NA
536 ΔHPI	5.83	-0.01 (0.08)	1	3.40 (0.06)	1	5.50 (0.97)	1	1.40 (0.21)	1	126.8 (1.5)	0	NA	NA

* Measured as described in Material and Method; S.E.M Standard Error of the Mean

† Number of dead mice after 7 days post-intraperitoneal inoculation; MK: Mice Killer; MNK: Mice Non Killer; NA: Not available

‡ Binarization was based on a graphic representation (histogram) of the data from the wild type natural isolates, exept for growth rate where an above-or-below-the-median criterion was used

Table 2: *E. coli* strains

Strain	Origin†	Type‡	Phylogenetic group	serotype	K1 antigen (neuC)	sfa/foc	iroN	aer (iucC)	papC	papG*	hly	cnf1	hra	fyuA	irp2	PAI I $_{CFT073}$	PAI II $_{J96}$	PAI III $_{536}$	HPI (PAI IV $_{536}$)
MG1655	Fs	Lab	A	O16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OP50	ND	Lab	A0	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F11	Ur	UTI	B2	O6:K15	-	+	+	-	+	III	+	+	+	+	+	-	+	+	+
F63	Ur	UTI	B2	O18:K1	+	+	+	-	+	III	+	+	+	+	+	-	+	+	+
RS218	CSF	MNN	B2	O18:K1	+	+	+	-	+	III	+	+	+	+	+	-	+	+	+
ED1A	Fs	C	B2	O81	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
CFT073	Bl	S	B2	O6:K2	-	+	+	+	+	II	+	-	-	+	+	+	-	+	+
J96	Ur	UTI	B2	O4:K6	-	+	+	-	+	I+III	+	+	+	+	+	-	+	+	+
IAI1	Fs	C	B1	O8w	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IAI2	Fs	C	B1	ONT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IAI4	Fs	C	A	ONT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IAI12	Fs	C	A	O148	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IAI13	Fs	C	A	O1	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
IAI15	Fs	C	A	ONT	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+
IAI19	Ur	UTI	A	O?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IAI21	Ur	UTI	B1	O?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IAI39	Ur	UTI	D	O7:K1	+	-	-	+	+	II	-	-	-	+	+	-	-	-	+
IAI44	Ur	UTI	A	O12	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+
IAI48	Bl	S	B2	O81	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
IAI49	Ur	UTI	B2	O8	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+
IAI51	Ur	UTI	B2	O?:K1	+	+	+	-	-	-	-	-	-	+	+	-	-	+	+
IAI52	Ur	UTI	B2	O?:K1	+	+	+	-	-	-	-	-	-	+	+	-	-	+	+
IAI60	Bl	S	B2	O2:K1	+	-	+	+	+	II	-	-	-	+	+	-	-	-	+
IAI64	Ur	UTI	B2	O83	-	+	+	-	+	III	+	+	+	+	+	-	+	+	+
IAI72	Ur	UTI	B2	O2	-	+	+	+	+	II	+	+	+	+	+	-	+	+	+
IAI73	Bl	S	B2	O6	-	+	+	-	+	III	+	+	+	+	+	-	+	+	+
IAI74	Ur	UTI	B2	O2:K1	+	+	+	-	+	III	+	+	+	+	+	-	+	+	+
ColF6c	Fs	C	B2	O2	-	-	-	-	+	II	+	+	+	+	+	-	+	-	+
ECOR64	Ur	UTI	B2	O75:K5	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+
EC7372	Ur	UTI	B2	O25	-	-	-	+	+	II	+	-	-	+	+	+	-	-	+
ECOR58	Fs	C	B1	O112	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-
Ben13f	Fs	C	B2	O6	-	+	+	-	+	III	-	-	+	+	+	-	-	+	+
536	Ur	UTI	B2	O6:K15	-	+	+	-	+	III	+	-	+	+	+	-	+	+	+

† Fs: Feces of healthy individual; Bl: Blood of scepticemic patient; Ur: Urine of urinary tract infected patient; CSF: Cerebro Spinal Fluid of new born patient with meningitis

‡ The type of a given strain was determined by his origin, except for OP50 and MG1655, the two laboratory (Lab) strains. C: Commensal; UTI: Urinary Tract Infectious; MNN: Meningitis associated; S: Scepticemia associated

* For papG, the presence of the three existing alleles is indicated by his number (I, II or III)

(+) presence ; (-) absence; PAI: Pathogenicity Island; HPI: High Pathogenicity Island

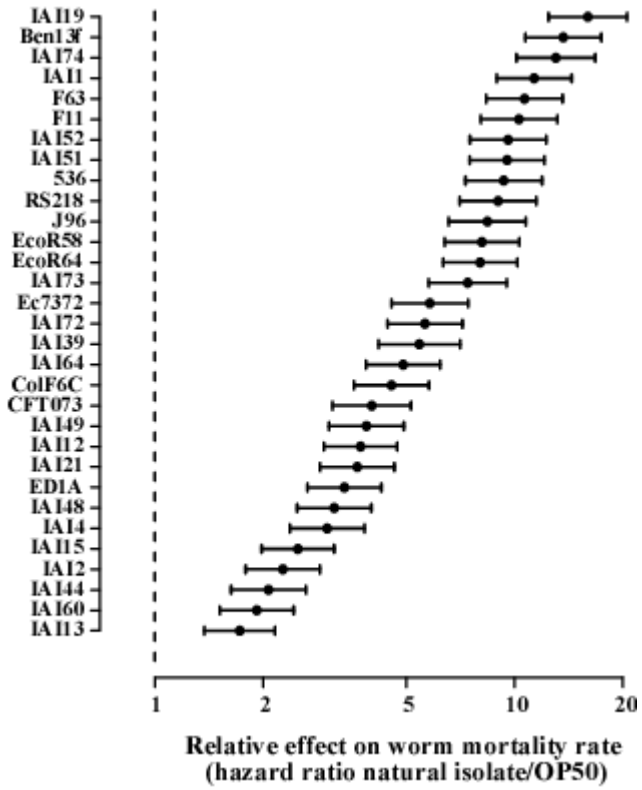


Figure 1: Cox proportional hazards survival analysis of *C. elegans* fed on different *E. coli* natural isolates. Values on the x-axis represent virulence of the *E. coli* strains, measured as the relative effect on worm mortality rate when grown on the natural isolate compared to the reference food strain OP50. Horizontal bars represent 95%-confidence intervals for the effect of each strain; the vertical dashed line corresponds to no effect ($\beta=0$ and thus $e^{\beta}=1$). Inspired by this representation, the boundary between the classes "slow killer" and "fast killer" were arbitrarily chosen to be between strains Ec7372 and IAI73.

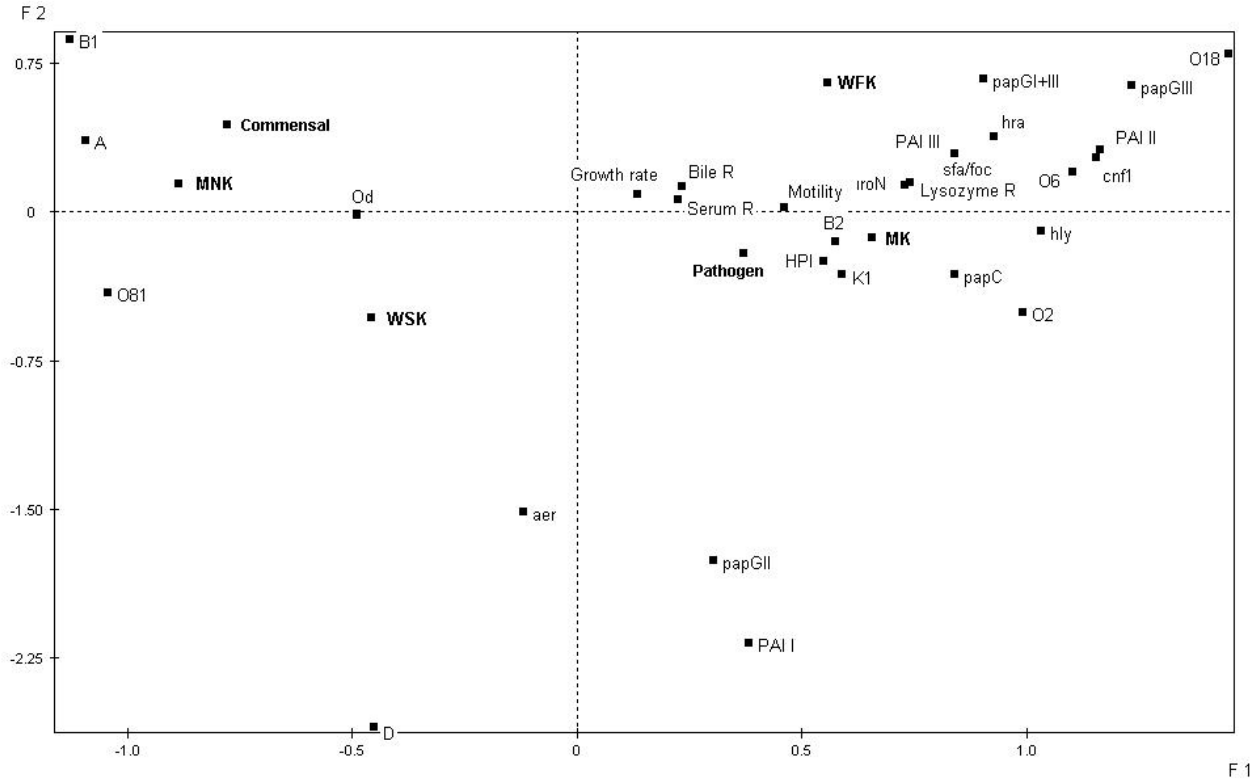


Figure 2. Projections of the 34 bacterial variables characterized in the 31 *E. coli* strains on the plane F1/F2 computed from the Factorial analyses of correspondence. The abreviations were as follows: A, B1, B2, and D, phylogenetic groups A, B1, B2 and D; O2, O6, O18, O81, Od, serotypes O2, O6, O18, O81, O divers types; Serum R, serum resistance; Bile R, bile resistance; Lysozyme R, lysozyme/lactoferrin resistance; MK, mouse killer; MNK, mouse non killer; FWK, fast worm killer; SWK, slow worm killer; the virulence factors are abreviated as in table S2. For clarity, *irp* and *fuyA* are projected on the same point that HPI and Commensal, Pathogen, MK, MNK, FWK and SWK are in bold types.

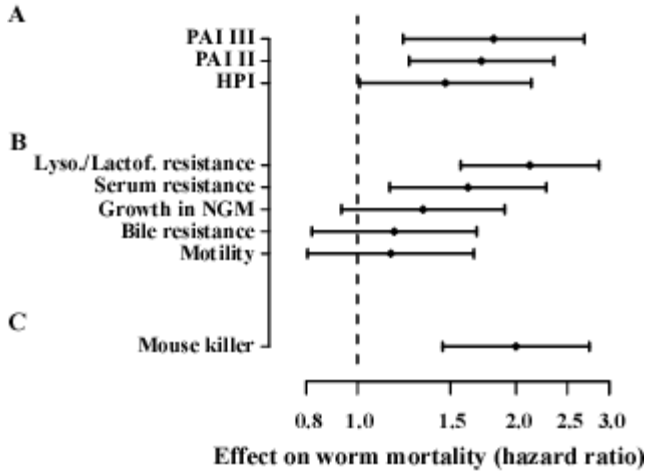


Figure 3. Cox proportional hazards survival analysis of *C. elegans* fed on different *E. coli* isolates with regard to different phenotypic and genetic variables. Values on the x-axis represent the effect of each trait when compared between the 2 classes for each variable (Table 1). Horizontal bars represent 95%-confidence intervals for the effect of each covariate; the vertical dashed line corresponds to no effect ($\beta=0$ and thus $e^{\beta}=1$). a) Presence of ExPEC pathogenicity islands PAI II and PAI III but not HPI (after adjustment for multiple comparisons) were associated with increased mortality of *C. elegans*. b) Of the 5 observed phenotypes, only resistance to lysozyme/lactoferrin and to human serum significantly correlate with pathogenicity. c) On the average, strains that kill mice double the mortality of *C. elegans*.

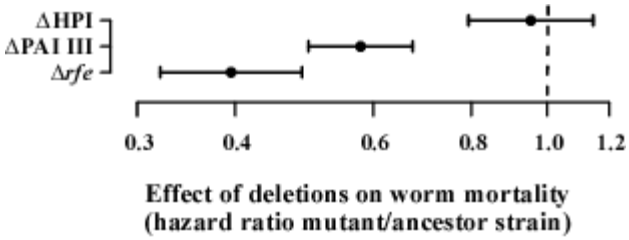


Figure 4. Cox proportional hazards survival analysis of *C. elegans* fed on different *E. coli* 536 mutant strains. Horizontal bars represent 95%-confidence intervals for the effect of each deletion compared to the ancestor strain; the vertical dashed line represents neutrality ($\beta=0$ and thus $e^{\beta}=1$). Δ*rfe* was the most attenuated strain (about 2.5 fold lower mortality), ΔPAI III was attenuated about 1.5 fold; deletion of HPI had no significant effect on worm mortality.

Supplementary material

Construction of E. coli 536 strain mutants. High Pathogenicity island (HPI) and *rfe* gene mutants of *E. coli* 536 strain were obtained using the PCR-based method of Datsenko and Wanner **((6))**. A temperature-sensitive plasmid pKD46, which carries the bacteriophage λ Red system (λ, β, and *exo* genes) under the control of an arabinose-inducible promoter, was introduced by electroporation into the *E. coli* 536 strain. The chloramphenicol acetyltransferase (*cat*) gene carried by plasmid pKD3 (REF) was amplified by PCR with primers bearing extensions of 40 nucleotides (Eurogentec, Seraing, Belgium) homologous to the initial and final portions of the target region. Transformation by electroporation of *E. coli* 536 strain (expressing bacteriophage λ Red functions) with the PCR product yielded deletion of the targeted chromosomal region fused to the *cat* gene. The *cat* gene was eliminated by using plasmid pCP20 expressing FLP recombinase (REF), which acts on the directly repeated FLP recognition target (FRT) sites flanking the resistance gene in plasmid pKD3.) Correct introduction and excision of the *cat* gene were controlled by PCR by using primers flanking the initial and final portions of the target region. All the primers used are shown in Table 2S.

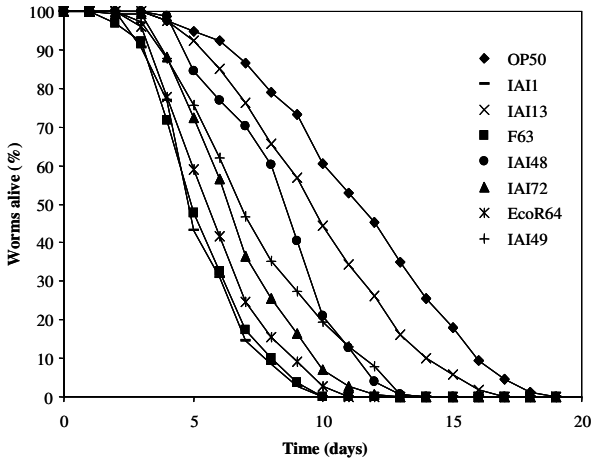
Table S2. Oligonucleotide primers

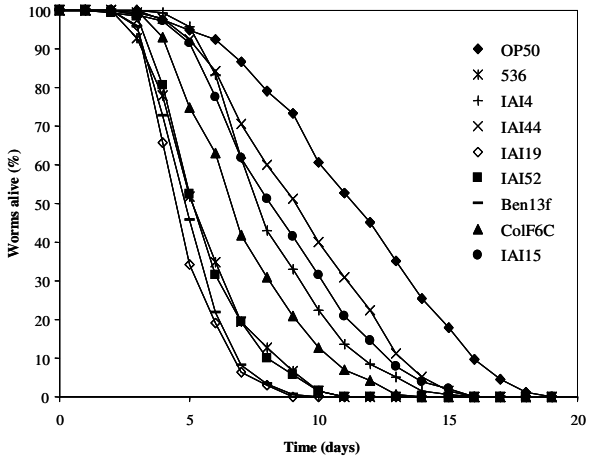
Primer designation	Primer sequence	Réference
rfe.P0	5'GTGAATTTACTGACAGTGAGTACTGATCTCATCAGTATTT**TGTGTAGGCTGGAGCTGCTTC**-3'	This study
rfe.P2	5'-CATAGAGGAAGAATGCTAGCAAAAAGAGCACCAGCATG**ACCATATGAATATCCTCCTTAG**-3'	This study
rfe.FR1	5'-GCGCTGGCGATGTTAGGAA-3'	This study
rfe.FR2	5'-GATGGTTGGTCGGCAGAAG-3'	This study
HPI.P0	5'-GCTGGCTATCCTGAAATACGGAACGCAGAACTAGCGCCGC**TGTGTAGGCTGGAGCTGCTT**-3'	**(19)**
HPI.P2	5'-GTACGGTAACGACGGTCGAACAGGTTATCGACATAGACGG**CATATGAATATCCTCCTTAG**-3'	**(19)**
HPI.FR1	5'-TGAGTCATCAGGAGCGCAAT-3'	**(19)**
HPI.FR2	5'-AAGGAGAGAAAGGAACGAGAAA-3'	

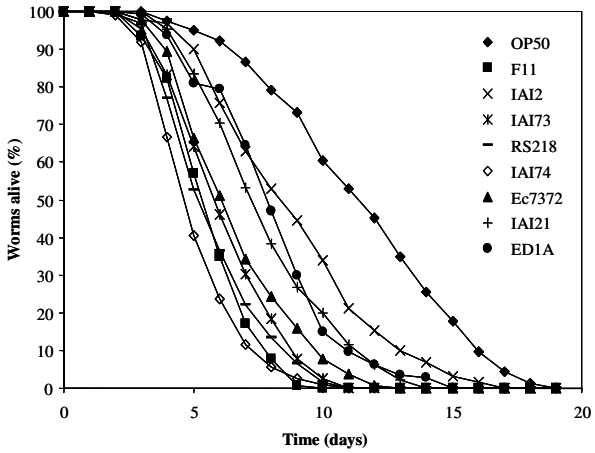
Oligonucleotide primers used for gene inactivation are designated by the suffixes P0 and P2; boldface characters in the primer sequences indicate the 20 nucleotides homologous to the *cat* gene sequence. The oligonucleotide primers used to control correct introduction and excision of the *cat* gene are designated by the suffixes FR1and FR2 and flank the target gene or region.

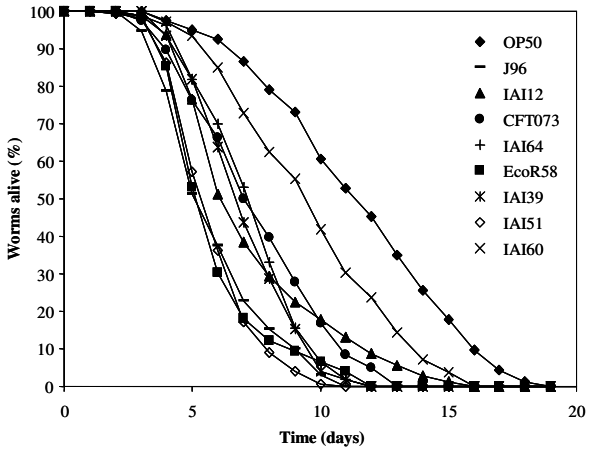
Genetic characterization of the E. coli natural isolates (partie pour Olivier)

Survival curves of *C. elegans* fed with different strains of *E. coli*









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