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Paralysis and killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli* requires the bacterial tryptophanase gene

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Summary

Pathogenic *Escherichia coli*, including enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and enterotoxigenic *E. coli* (ETEC) are major causes of food and water-borne disease. We have developed a genetically tractable model of pathogenic *E. coli* virulence based on our observation that these bacteria paralyse and kill the nematode *Caenorhabditis elegans*. Paralysis and killing of *C. elegans* by EPEC did not require direct contact, suggesting that a secreted toxin mediates the effect. Virulence against *C. elegans* required tryptophan and bacterial tryptophanase, the enzyme catalysing the production of indole and other molecules from tryptophan. Thus, lack of tryptophan in growth media or deletion of tryptophanase gene failed to paralyse or kill *C. elegans*. While known tryptophan metabolites failed to complement an EPEC tryptophanase mutant when presented extracellularly, complementation was achieved with the enzyme itself expressed either within the pathogen or within a cocultured K12 strains. Thus, an unknown metabolite of tryptophanase, derived from EPEC or from commensal non-pathogenic strains, appears to directly or indirectly regulate toxin production within EPEC. EPEC strains containing mutations in the locus of enterocyte effacement (LEE), a pathogenicity island required for virulence in humans, also displayed attenuated capacity to paralyse and kill nematodes. Furthermore, tryptophanase activity was required for full activation of the LEE1 promoter, and for efficient formation of actin-filled membranous protrusions (attaching and effacing lesions) that form on the surface of mammalian epithelial cells following attachment and which depends on LEE genes. Finally, several *C. elegans* genes, including *hif-1* and *egl-9*, rendered *C. elegans* less susceptible to EPEC when mutated, suggesting their involvement in mediating toxin effects. Other genes including *sek-1, mek-1, mev-1, pgp-1,3* and *vhl-1,* rendered *C. elegans* more susceptible to EPEC effects when mutated, suggesting their involvement in protecting the worms. Moreover we have found that *C. elegans* genes controlling lifespan (*daf-2, age-1* and *daf-16*), also mediate susceptibility to EPEC. Together, these data suggest that this *C. elegans*/EPEC system will be valuable in elucidating novel factors relevant to human disease that regulate virulence in the pathogen or susceptibility to infection in the host.

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Introduction

Pathogenic *Escherichia coli*, including enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and enterotoxigenic *E. coli* (ETEC) are gastrointestinal pathogens transmitted via contaminated food and water (Nataro and Kaper, 1998; Clarke *et al*., 2002). EPEC induces symptoms which range from a subclinical infection to fulminating diarrhoea, vomiting and fever, and is a major contributor to mortality and morbidity in developing nations (Clarke *et al*., 2002). EPEC disease usually occurs in infants less than 2 years old, and is associated with significant mortality due to dehydration (Nataro and Kaper, 1998). EHEC has become endemic in cattle in western nations and contaminates food and water supplies. EHEC expresses Shiga toxins and can cause haemorrhagic colitis and haemolytic–uremic syndrome in both adults and children (Riley *et al*., 1983). EPEC and EHEC both form hallmark lesions on host intestinal epithelial cells (called attaching and effacing (A/E) lesions; (Knutton *et al*., 1989), characterized by disruption of microvilli and formation of an actin-filled mem-

branous pedestal beneath the attached bacterium. A/E lesions and progression of disease require a 35 kb for EPEC (Elliott *et al*., 1998) or 43 kb for EHEC (Perna *et al*., 1998; Elliott *et al*., 1999; Perna *et al*., 2001) pathogenicity island known as the locus of enterocyte effacement [LEE; Perna *et al*., (1998)]. EIEC and ETEC do not contain the LEE but cause diarrhoea by different mechanisms.

While much is known about how A/E lesions form, much less information is available on how genes outside the LEE contribute to disease. EHEC for example contains ~1.34 Mb (Perna *et al*., 2001) of DNA not present in the laboratory strain *E. coli* K-12. For EHEC, this represents some 1300 gains and 600 losses, relative to K-12. Of the gains, few have been evaluated for their role in pathogenesis. The motivation for this study was to develop a genetic system to identify novel bacterial genes required for virulence and novel host genes required for susceptibility to infection. Towards this end, we have developed a system based on the toxic interaction of EPEC or EHEC with the nematode *Caenorhabditis elegans*. *C. elegans* is a small, free-living soil nematode that feeds primarily on bacteria (Wood, 1988). In the laboratory, the *E. coli* strain OP50 is commonly used as a food source (Brenner, 1974). Because *C. elegans* is genetically tractable, it has been used to study a variety of biological processes including, for example, ageing, apoptosis and chemosensation (Bargmann, 1997; Metzstein *et al*., 1998; Kenyon, 2001). Recently, several bacterial pathogens including *Pseudomonas aeruginosa* and *Salmonella typhimurium* have been evaluated for their ability to kill *C. elegans.* (Darby *et al*., 1999; Tan *et al*., 1999; Aballay *et al*., 2000; Garsin *et al*., 2001; Gan *et al*., 2002; Jansen *et al*., 2002). Such models have proven advantageous because they are amenable to high-throughput screens for mutants in both the bacterium and the worm. Moreover, because 36% of *C. elegans* proteins have human homologues (*C. elegans* Sequencing Consortium, 1998), there exists a high likelihood of identifying *C. elegans* targets of pathogenesis that are also found in humans. In this regard, Tan *et al*. (1999) found *P. aeruginosa* mutants that were attenuated for killing of *C. elegans,* also were attenuated in plant and mouse models of infection, highlighting the utility of the *C. elegans* model in identifying conserved mechanisms of bacterial virulence.

To better understand the virulence mechanisms by which EPEC and EHEC cause disease, and to identify host targets of such mechanisms, we developed a model of EPEC and EHEC pathogenesis in *C. elegans*. To our knowledge no other invertebrate genetic system is available to study mechanisms of pathogenic *E. coli* virulence, or mechanisms of host susceptibility. We report here that coculture of EPEC or EHEC with *C. elegans* causes paralysis and subsequently death of the nematode. Paralysis and death do not require direct contact with EPEC or EHEC, suggesting that a secreted toxin mediates virulence. Other bacterial elements also appear to be required for virulence in nematodes. Tryptophan and the activity of the bacterial tryptophanase gene together with at least two genes located within the LEE pathogenicity island that are required for virulence in humans, all appear necessary for paralysis and killing of nematodes, and for the efficient formation of attaching and effacing lesions. Finally we define *C. elegans* genes which appear to mediate both susceptibility to and protection from the secreted EPEC toxin. Together, these data provide evidence for novel virulence mechanisms in the bacterium and the host, which may have relevance to human pathogenesis.

Results

Enteropathogenic E. coli and *EHEC grown on ECD agar paralyse and kill* C. elegans

To define conditions under which EPEC and EHEC were pathogenic to *C. elegans*, three different commercial agar growth media were assessed. Luria–Bertani (LB) plates and nematode growth medium agar (NGM) plates spotted with EPEC (see *Experimental procedures* and *Supplementary information*) produced no detectable effects on *C. elegans*. However, when wild-type *C. elegans* were added to ECD plates previously spotted with EPEC, some of the worms became paralysed, showing cessation of locomotion, and subsequently suspension of pharyngeal pumping and egg laying. Under these conditions, some of the worms were observed to move to the edge of the plate, crawl up the side and exit, suggesting that EPEC produced a chemo-repellant substance. This property created substantial scoring problems and complicated detailed analysis of effects of EPEC on wild-type *C. elegans*. As a result, subsequent experiments were carried out with *rol-6(e187)* (Cox *et al*., 1980), a *C. elegans* strain which rolls in a circle and has a compromised ability to undergo straight line motion. When *C. elegans rol-6* were added to ECD agar plates previously spotted with EPEC, more than 95% worms became paralysed within 30 min (Fig. 1A). The paralysing effect was not due to the agar alone because addition of *C. elegans* to plates without bacteria produced no adverse effects even with prolonged incubation (up to 40 h, the longest time tested; not shown). Moreover, the paralysing effect was only apparent when the bacteria were cultured on plates; no paralysis was observed when *C. elegans* were cocultured with the bacteria in ECD broth. Paralysis was not particular to the *rol-6* strain. Wild-type *C. elegans* (N2) remaining on the plates were paralysed with similar kinetics to *rol-6* (data not shown). When *C. elegans rol-6* worms were added to ECD plates spotted with other pathogenic *E. coli* strains, including enterohaemorrhagic *E. coli* (EHEC O157:H7),

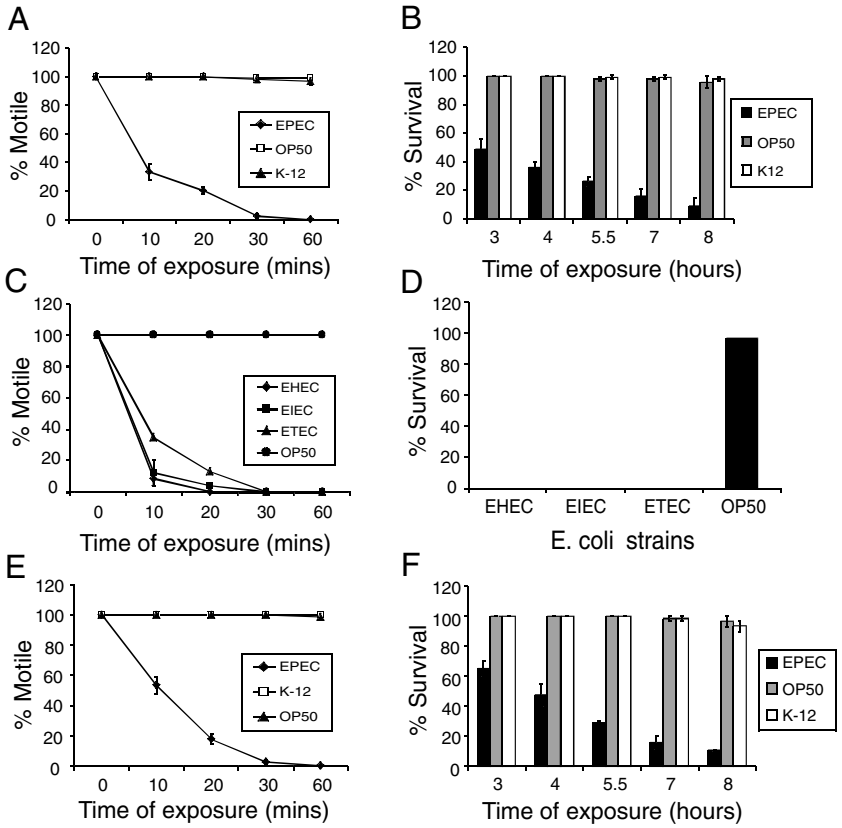

Fig. 1. Paralysis and killing of *C. elegans rol-6* by pathogenic *E. coli*.
A. Paralysis by EPEC grown on ECD plates. EPEC paralysed more than 95% of *C. elegans* worms within 30 min.
B. Killing of *C. elegans* by EPEC grown on ECD agar plates. Worms on ECD plates were exposed to EPEC for between 3.0 and 8.0 h, and then allowed to recover on NGM agar plates seeded previously with OP-50. Worms were judged to be dead if no motility was evident after 24 h on NGM plates. Note that after 8.0 h of exposure to EPEC, about 90% of *C. elegans* failed to recover.
C. Paralysis by other pathogenic *E. coli* strains on ECD plates. Paralysis occurred faster in EHEC and EIEC (10 min) compared with ETEC (30 min).
D. Killing by pathogenic *E. coli* strains. After 8 h exposure to various pathogenic *E. coli*., none of the *C. elegans* worms survived.
E–F. LB plates containing tryptophan also paralysed (E) and killed (F) *C. elegans*. Note that in all cases the normal *C. elegans* laboratory food source, OP50, and *E. coli* K12 failed to paralyse or kill worms.

enterotoxigenic *E. coli* (ETEC strain C921b-1) and enteroinvasive *E. coli* (EIEC strain 4R), paralysis occurred within 30 min (Fig. 1C). The paralysing effects to pathogenic *E. coli* strains were specific; neither *E. coli* OP50, the common laboratory food source for *C. elegans,* nor commensal *E. coli* K12 (strain MG1655), caused paralysis (Fig. 1A and C).

To assess whether the paralysed *C. elegans* exposed to EPEC and other strains were still viable, we moved the paralysed worms to NGM plates seeded with OP50**.** Worms were then scored for movement 22–24 h later. The results indicate that the degree of killing of *C. elegans* was directly proportional to the amount of time that worms were initially exposed to EPEC. To determine the minimum length of exposure required to kill the worms, *C. elegans* were cocultured with EPEC for 3.0–8.0 h before testing for recovery (Fig. 1B). Recovery of motility in all EPEC-exposed animals severely decreased with time. After 8.0 h of exposure, about 90% of worms were judged to have died (Fig. 1B), and none survived when exposed to EPEC for 14 h or longer (not shown). In one experiment, *C. elegans* worms exposed to EPEC for 8.0 h were left on recovery plates for 72 h and were still found to be non-motile even after being prodded with a wire pick or shaken (data not shown), suggesting that the worms were indeed dead and not in a prolonged state of paralysis. Similar results were obtained when worms were exposed for 8 h to EHEC O157:H7, ETEC and EIEC strains (Fig. 1D) though some differences were observed in the rates of paralysis and killing. For this and other reasons, we chose to focus on EPEC for subsequent analysis.

Tryptophan in the growth media is required for paralysis and killing

To define the components of ECD agar required for EPEC to paralyse and kill *C. elegans,* assays were performed on plates containing the components of ECD as specified by the manufacturer (recapitulated ECD agar; see *Supplementary information*) or on plates lacking a particular component. EPEC grown on recapitulated ECD agar caused paralysis and killing to the same extent as on premixed ECD agar (data not shown). Dissection of the media components indicated that the presence of both potassium phosphate buffer and added L-tryptophan were required for killing. Different lots of premixed ECD media showed some variance in the degree of paralysis and killing. Because L-tryptophan is heat-labile, we reasoned that the apparent variance between lots might in part be due to differences in the tryptophan concentration or preparation. In this regard, we found that after autoclaving and cooling, addition of tryptophan to a final concentration of

0.1% (4.89 mM) to commercial, premixed ECD agar permitted consistent paralysis and killing results, though addition of tryptophan at concentrations as low as 500 μM was equally effective. Subsequent experiments requiring tryptophan addition were performed using this protocol. Identical results were obtained when L-tryptophan was added to LB plates. As seen in (Fig. 1E and F), EPEC seeded on LB plates containing tryptophan also caused paralysis and killing of *C. elegans*. Using LB plates, we determined that the minimal concentration of additional tryptophan required for complete paralysis and killing was 1.22 mM (compared with 4.8 mM in non-supplemented ECD plates) with an IC50 of ~500 μM.

A diffusible non-gaseous toxin mediates paralysis and killing of C. elegans *by EPEC*

Burkholderia pseudomallei (Gan *et al*., 2002) and *P. aeruginosa* PA01 (Darby *et al*., 1999) cause virulence in *C. elegans* without direct contact with the worms. The rapid kinetics of *C. elegans* paralysis and killing upon plating with EPEC suggested that the bacteria might secrete a diffusible toxin rather than colonize the worm. To test this possibility, we performed filter assays as described by Gan *et al*. (2002; see *Experimental procedures*). EPEC was added to 0.22 μM nitrocellulose (NC) filters placed atop ECD agar plates, and allowed to grow overnight. The filters preclude direct contact of the bacteria with the plate, but allow diffusion of soluble factors. Following overnight incubation, filters containing EPEC were removed, and *C. elegans* added to the plates. Under these conditions, EPEC still caused paralysis (Fig. 2A) and killing (Fig. 2B) of *C. elegans,* at rates comparable to those observed with direct contact. Thus, EPEC is capable of mediating paralysis and killing of *C. elegans* by secreted diffusible toxin(s) without direct contact.

Pseudomonas aeruginosa PA01 kills *C. elegans* without direct contact by secreting cyanide gas (Gallagher and Manoil, 2001). Measurement of cyanide production (Gallagher and Manoil, 2001) indicated that OP50, K12, wild-type EPEC, EPEC*ΔtnaA* and the other mutant EPEC strains do produce cyanide (Fig. 2E), but at levels which are not toxic to *C. elegans* (Fig. 2E). To determine if another gas was responsible for pathogenesis, we modified the protocol described by Gallagher and Manoil (2001; see *Experimental procedures*). Briefly, EPEC strains cultured overnight on two 5.0 cm ECD plates were placed opened in a 15 cm Petri dish together with a third opened 5.0 cm ECD plate containing 30 *rol-6* worms and no bacteria, and the Petri dish lid sealed tightly with para-

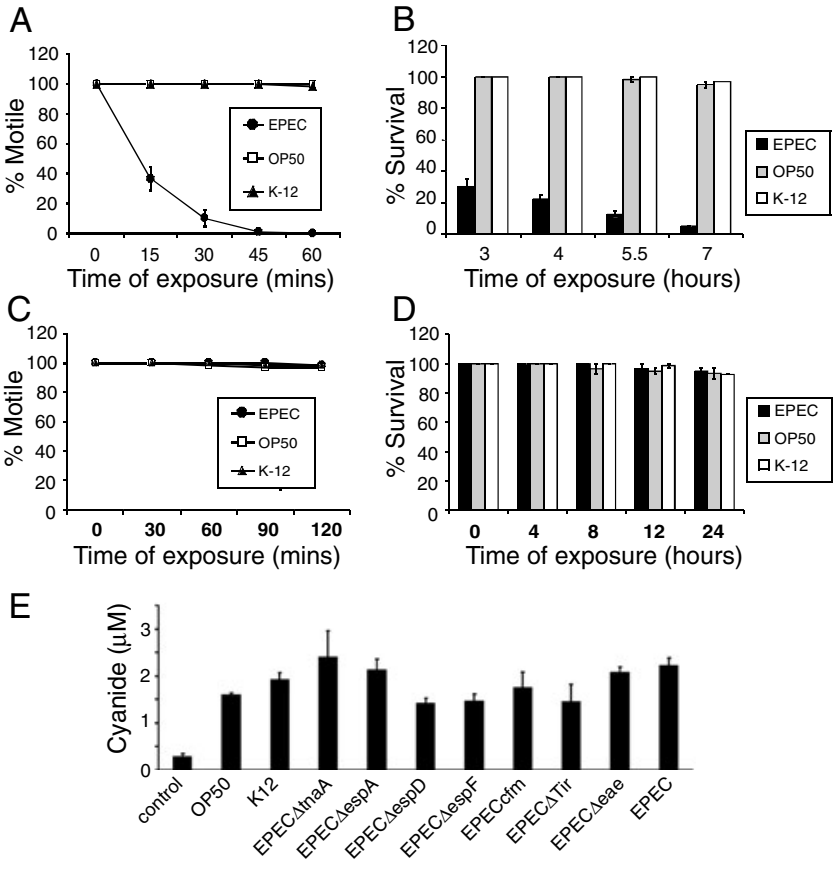

Fig. 2. Paralysis and killing of *C. elegans* by EPEC is mediated by a diffusible toxin, which is not likely a gas. EPEC was grown overnight on 0.22 μM filters placed on 5.0 cm ECD plates. After filters were removed, 30 worms were placed on the plates and paralysis and killing was assessed. No major difference in kinetics of paralysis (A) or extent of killing (B) was seen in these assays compared with experiments in which contact occurred (e.g. Fig. 1A and B).
C–E. EPEC does not secrete a gas to kill *C. elegans*. Worms placed on uncovered ECD plates without bacteria were exposed to EPEC, OP50 and K12-seeded plates in sealed 15 cm Petri dishes. Under these conditions, a negligible number of the worms became paralysed (C) or were killed (D) even with 24 h exposure (longest time tested). (E) Cyanide production by various bacteria strains.
All bacteria tested produce cyanide in amounts not sufficient to paralyse or kill *C. elegans*.

film. Parafilm acts as a barrier for H_2O vapour, but not to O_2 and CO_2. Thus, wrapping the plate in parafilm might be expected to concentrate some gases. After exposure of up to 24 h (the longest time tested) over 95% of the worms survived (Fig, 2C and D), indicating that the toxic factor(s) produced by EPEC that contribute to pathogenesis in *C. elegans* are likely not gaseous in nature.

The EPEC tryptophanase gene is required for paralysis and killing

Together, these data raise the possibility that tryptophan or a metabolite is required for secretion of a soluble toxin that paralyses and kills *C. elegans*. The bacterial gene t*naA* encodes tryptophanase, which catalyses the conversion of tryptophan into pyruvate, ammonia and indole (Snell, 1975). To determine whether tryptophan metabolism mediates paralysis and killing, we constructed a insertional mutant in EPEC *tnaA* (Datsenko and Wanner, 2000), called EPEC*ΔtnaA*. Inactivation of *tnaA* was confirmed by measurement of tryptophanase activity using Kovacs reagent (Kawamura-Sato *et al.*, 1999) which detects indole (data not shown) or by changes in absorbance of S-O-nitrophenyl-L-cysteine (SOPC), an artificial tryptophanase substrate [Fig. 3A; Gish and Yanofsky, (1993)]. The EPEC*ΔtnaA* mutant was fully attenuated for paralysis (Fig. 3B) and killing (Fig. 3C) of *C. elegans*, while complementation of *tnaA in trans* via plasmid JD50, restored virulence to wild-type levels (Fig. 3B and C). Control ECD plates containing antibiotics or IPTG alone, the inducer for exogenous tryptophanase expression, demonstrated that these additives did not affect *C. elegans* motility or survival (data not shown). Together these data indicate that one or more by-products of tryptophan metabolism mediate the virulent effects of EPEC on *C. elegans*.

We next determined whether tryptophan or its known metabolites indole, ammonia and pyruvate, directly or indirectly affect *C. elegans*. L-Tryptophan, indole, ammonia, or pyruvate was added in a range of concentrations to ECD plates. None of these chemicals added alone or in any combination produced any detectable effect on worm motility or survival (data not shown). Thus, these data suggest that known metabolites of tryptophan do not by themselves paralyse and kill *C. elegans* but instead require additional elements within EPEC. To test this possibility, we next assessed whether known metabolites of tryptophan would complement EPEC*ΔtnaA* mutants. We found that EPEC*ΔtnaA* strains grown on ECD plates containing various concentrations of indole, pyruvate or

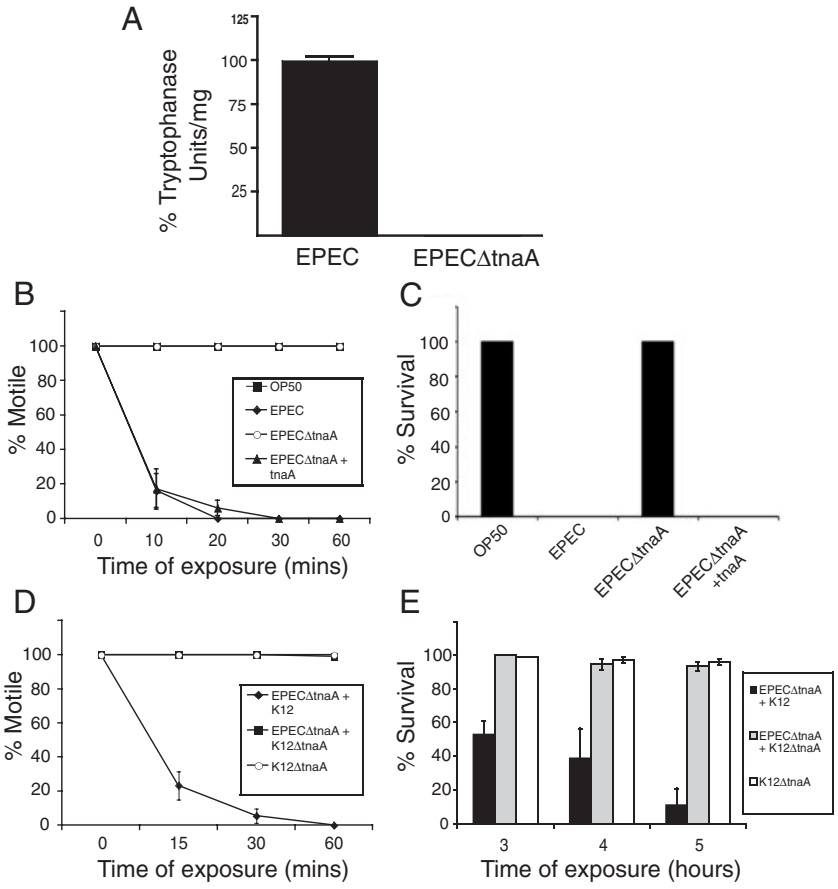

Fig. 3. The EPEC tryptophanase gene is required for paralysis and killing of *C. elegans*.
A. EPEC, but not EPEC*ΔtnaA*, displays tryptophanase activity. Data shown are from EPEC or EPEC*ΔtnaA* cultured on ECD plates. Units are expressed as a percentage of EPEC tryptophanase activity (0.1614 units/mg).
B. Paralysis of *C. elegans* occurs upon exposure to EPEC and EPEC*ΔtnaA* complemented with a plasmid expressing *tnaA*, but not upon exposure to the EPEC*ΔtnaA* mutant or to OP50.
C. EPEC*ΔtnaA* does not kill *C. elegans*. Paralysis (D) and killing (E) of *C. elegans* also occur upon exposure of worms to a mixture of EPEC*ΔtnaA* and K12 grown together on ECD plates. However, mixture of EPEC*ΔtnaA* with K12*ΔtnaA* failed to induce killing. Individual bacterial strains had no effect on *C. elegans* (D, E and Fig. 1A and B above).

ammonia (up to 10 mM each, the highest concentrations tested) individually or in various combinations did not paralyse or kill worms (data not shown).

To test the possibility that other unrecognized metabolites produced by tryptophanase or induced upon deletion of *tnaA* may mediate paralysis and killing, we assessed whether the addition of K12 or K12Δ*tnaA* would complement EPECΔ*tnaA* mutants and cause paralysis and killing of *C. elegans*. To do this, we mixed overnight cultures of EPECΔ*tnaA*, together with cultures of K12 or K12Δ*tnaA* in OD_{600} ratios of 150:1 respectively. After culturing the bacteria overnight on ECD plates, worms were added. As seen in Fig. 3D and E, EPECΔ*tnaA* grown together with K12 paralysed and killed worms. Notably, EPECΔ*tnaA* grown together with K12Δ*tnaA* did not cause paralysis or death of *C. elegans.* As expected, none of the individual bacteria strains caused paralysis or death (Fig. 1, Fig. 3B–E). Identical results were obtained with strains grown on LB plates supplemented with tryptophan (data not shown), but no paralysis or killing was evident on LB plates lacking tryptophan (data not shown). Together these data suggest that (i) there exist other factors produced by tryptophanase activity that directly or indirectly regulate production of factors toxic to *C. elegans* within EPEC; and (ii) that these inducing factors may be produced by commensal strains and affect EPEC.

Mutation of LEE locus genes within EPEC attenuates paralysis and killing of C. elegans

The LEE pathogenicity island has been associated with virulence and with formation of attaching and effacing lesions (Deng *et al*., 2004). EPEC has been shown to utilize a type III secretion system to facilitate transport of virulence factors into mammalian cells. Some of the genes within the LEE pathogenicity locus encode proteins that form a Type III secretion pore along with pore-associated proteins including EspA and EspD (Deng *et al*., 2004). Other genes within the locus encode translocated virulence factors [e.g. Tir; Kenny *et al*., 1997a). To test the possibility that the secreted toxin which mediates *C. elegans* killing might similarly require the LEE-encoded secretion system, we tested several EPEC mutants for their capacity to paralyse and kill *C. elegans* (Fig. 4A and B). EPEC virulence factor mutants segregated into two categories. The *espA*, *espD* and *espF* mutants displayed attenuated paralysis and killing of *C. elegans*, whereas *eae*, *tir* and *espC* did not (Fig. 4A and B). Failure of *espA*, *espD* and *espF* to paralyse and kill *C. elegans* could not be attributed to a loss of tryptophanase activity in these strains (Fig. 4C), although some differences between these mutants and wild-type EPEC were evident. Together these data indicate that the products encoded by the *espA*, *espD* and *espF* genes within the LEE locus are all required for virulence in *C. elegans*, possibly by facilitating the secretion of a toxin. These data also suggest that EPEC factors required for virulence in mice and presumably humans are also required for virulence in *C. elegans.*

Effects of indole and tryptophan on LEE1 promoter activity

Given the requirement for LEE genes in the toxic effects of EPEC on *C. elegans*, we next tested whether L-tryptophan or its metabolites might act in part by affecting transcription of genes within the LEE pathogenicity island. The LEE is comprised of five major operons (Elliott *et al*., 1999; Mellies *et al*., 1999; Sanchez-SanMartin *et al*., 2001). LEE1 encodes the LEE transcriptional regulator, Ler, which activates expression of LEE2, 3, 4 and 5 (Friedberg *et al*., 1999; Mellies *et al*., 1999). To test whether the addition of L-tryptophan or its metabolites regulated expression of LEE1, *lacZ* transcriptional fusions to the LEE1 promoter were transferred into EPEC or its isogenic EPECΔ*tnaA* mutant. To assess whether indole regulates LEE expression, EPEC or EPECΔ*tnaA* containing the LEE1-*LacZ* transcriptional fusion were grown on ECD plates containing 50 μM or 500 μM indole or the carrier 0.1% DMSO. The colonies were then collected by scraping and β-galactosidase activity was measured. As seen in Fig. 4D, LEE1 expression in EPECΔ*tnaA* strains was threefold lower than in wild-type EPEC, and indole present in the plate fully complemented the effect of deletion of *tnaA* strain on LEE1 expression. Together these data indicate that tryptophan acting via its metabolite indole can directly or indirectly regulate efficient transcriptional activity of the LEE1 operon.

Tryptophanase regulates formation of actin pedestals

To determine whether tryptophanase affected EPEC virulence in mammalian systems, we next measured the capacity of EPECΔ*tnaA* to form actin-filled membranous protrusions (pedestals), characteristic features of attaching and effacing lesions which also depend on LEE genes (McDaniel and Kaper, 1997). To do this, 3T3 cells were infected for 6 h with EPEC (Fig. 5A), or with EPECΔ*tnaA* (Fig. 5B and C), or with EPECΔ*tnaA* + *ptnaA* (Fig. 5D). The cells were then fixed and stained with DAPI to recognize bacterium, FITC-phalloidin to recognize actin, α-Tir pAb to recognize the translocated bacterial virulence factor Tir, and α-phosphotyrosine mAb 4G10 which recognizes phosphorylated Tir and likely the other phosphorylated pedestal proteins. Pedestals are visualized as intense actin staining (e.g. Fig. 5A, second panel and green in merged images) apposed to attached bacteria (e.g. Fig. 5A, first panel and blue in merged images), and occur in clusters on the surface of the infected cell. Phos-

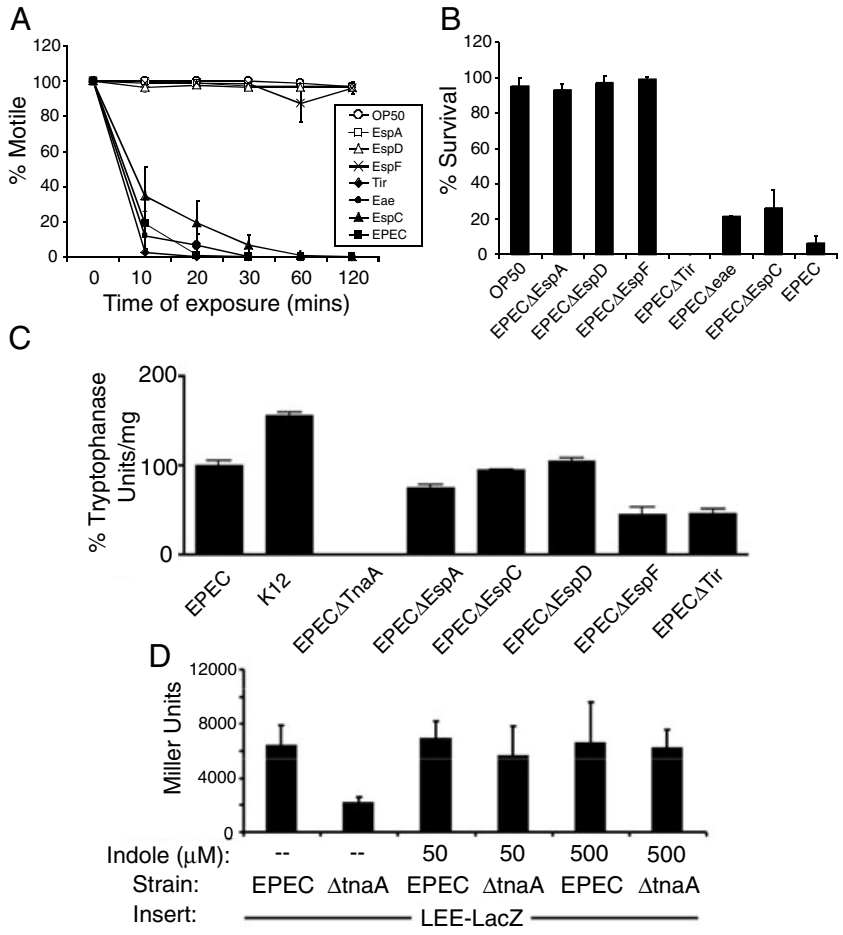

Fig. 4. Effects of LEE genes on paralysis and killing of *C. elegans*.
A. Paralysis of *C. elegans* by EPEC virulence factor deletion mutants. EPEC *espA*, *espD* and *espF* mutants are attenuated for paralysis of *C. elegans*, but *eae*-, *tir*- and *espC*- are not.
B. Killing of *C. elegans* by EPEC virulence factor deletion mutants after 7 h exposure. Note that EPEC *espA*, *espD* and *espF* mutants are attenuated for killing and *eae* and *espC* mutants are slightly attenuated.
C. EPEC and all LEE mutants have measurable tryptophanase activity when grown on ECD plates, indicating that failure of *espA, espD* and *espF* to paralyse and kill cannot be attributed to loss of tryptophanase activity. Data shown are expressed as a percentage of EPEC tryptophanase activity (0.1614 units/mg).
D. Effects of tryptophanase activity on LEE1 promoter. Miller assays performed on wild-type or EPEC*ΔtnaA* strains containing *lacZ* transcriptional fusions to the EPEC LEE1 promoter grown on ECD plates supplemented with 50 μM or 500 μM indole, or without any supplement. Note that EPEC*ΔtnaA* strains exhibit approximately threefold lower levels of LEE1 promoter activity than wild-type EPEC, and that addition of indole to the plates complemented loss of tryptophanase. The differences between EPEC*ΔtnaA* strain lacking indole and all other conditions are statistically significant ($P < 0.001$).

photyrosine staining (third panel) and Tir (fourth panel) colocalize at the tip of the actin pedestal directly apposed to the bacterium (red in merged images; Swimm *et al*., 2004). Notably, EPEC formed actin pedestals on nearly every cell (Fig. 5A; Fig. S1). Like EPEC, EPEC*ΔtnaA* appeared to attach normally to cells (Fig. 5B and C). However, in about 50% of cells, actin bundles beneath the EPEC*ΔtnaA* appeared short and disorganized compared with wild-type EPEC (compare Fig. 5A and B; second column). In these cells Tir and phosphotyrosine staining appeared disorganized beneath EPEC*ΔtnaA* compared with wild-type EPEC (compare Fig. 5A and B; third and fourth panels). In the remaining 50% of cells infected with EPEC*ΔtnaA*, no actin or phosphotyrosine staining was evident despite the presence of Tir (Fig. 5C). The aberrant pedestal formation by EPEC*ΔtnaA* was directly attributable to tryptophanase activity, because actin pedestals induced upon infection with EPEC*ΔtnaA* + *tnaA* resembled those seen with EPEC (Fig. 5D). Addition of indole at concentrations as high as 500 μM did not facilitate normal pedestal formation upon infection with EPEC*ΔtnaA* (not shown); higher concentrations proved deleterious to mammalian cells.

To test whether the effects of EPEC*ΔtnaA* on pedestal formation could be complemented by commensal strains, cells were infected with or with EPEC*ΔtnaA* + K12 (Fig. 5E), or with EPEC*ΔtnaA* + K12*ΔtnaA* (Fig. 5F), or with K12 alone (Fig. 5G), and stained as in Fig. 5A–D. Notably, aberrant pedestal formation by EPEC*ΔtnaA* could be complemented by K12 but not by K12*ΔtnaA* (Fig. 5E and F; Fig. S1). Pedestals failed to form when cells were in infected with either K12 (Fig. 5G) or K12*ΔtnaA* (not shown). Together, these data suggest that tryptophanase activity within EPEC or within cocultured commensal strains regulates expression of LEE factors required for efficient formation of actin pedestals, and are consistent with an effect of tryptophanase on LEE1 activity (Fig. 4D).

Caenorhabditis elegans *genes which mediate toxic effects or confer protection*

To identify *C. elegans* genes that mediate toxic effects or confer protection from EPEC virulence, we carried out paralysis and killing assays on a set of *C. elegans* strains containing mutations in genes which coordinately control

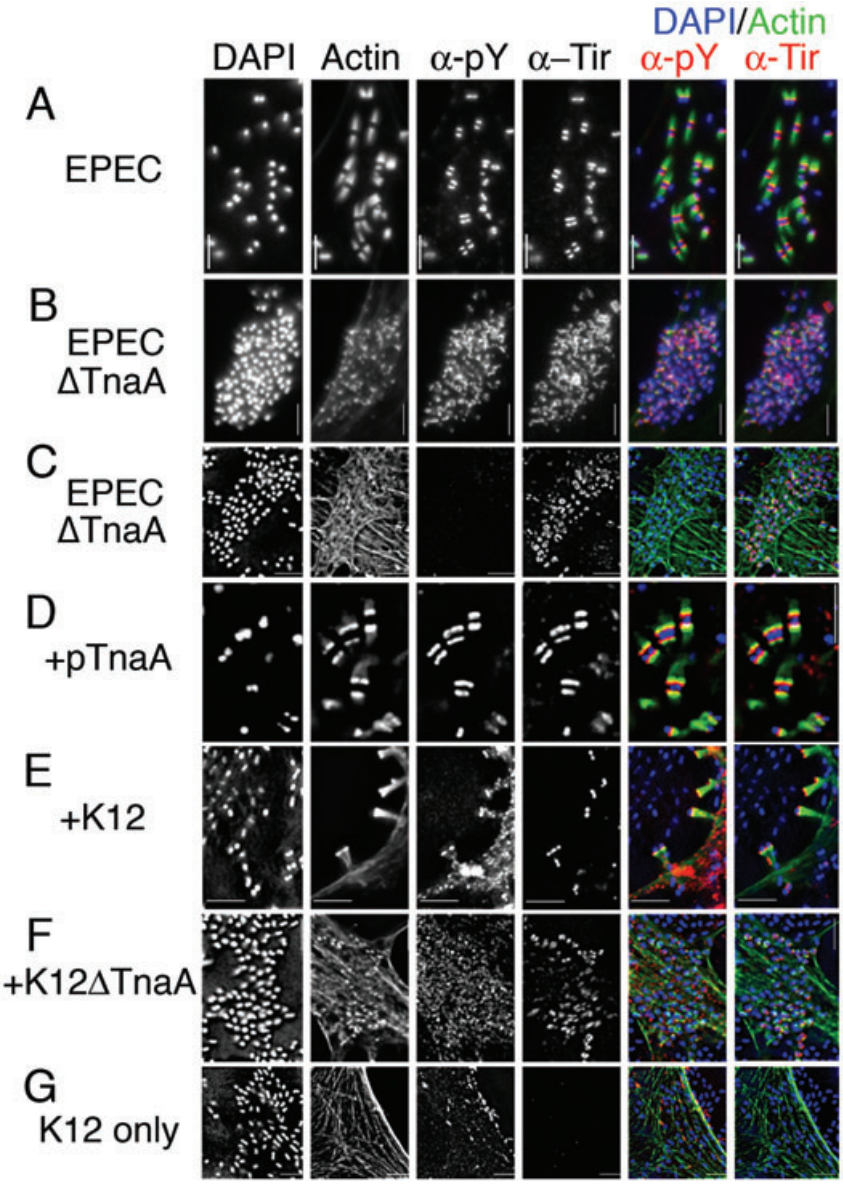

Fig. 5. Effects of tryptophanase activity on EPEC pedestal formation. 3T3 cells were infected with EPEC (A), EPEC*ΔtnaA* (B, C), EPEC*ΔtnaA* + p*tnaA* (D), EPEC*ΔtnaA* + *K12* (150:1) (E), EPEC*ΔtnaA* + *K12ΔtnaA* (150:1) (F), or K12 alone (G), fixed and stained with DAPI to recognize bacteria (first column), FITC-phalloidin to recognize actin (second column), α-phosphotyrosine mAb 4G10 conjugated to Cy3 (third column), and α-Tir-Cy5 (fourth column). In merged images (fifth and sixth columns), actin is pseudocoloured green, the bacterium blue, phosphotyrosine red (in the fifth column), and Tir red (in the sixth column). Note that infection with EPEC (A) results in long pedestals, and Tir and phosphotyrosine staining at the tip of the pedestal apposed to the bacterium. Infection with EPEC*ΔtnaA* (B, C) results in small disorganized actin bundles beneath the bacterium in some cells (B) and none in others (C). Though Tir and phosphotyrosine were still evident, the staining pattern is likewise disorganized compared with EPEC (A-C; third and fourth columns). Note also that the defect in actin pedestals could be restored by exogenous expression of tnaA within EPEC (D) or by coincubation with K12 (E), but not with K12*ΔtnaA* (F). K12 by itself produce no effects on actin (G), but did adhere to the cover slip and to some cells. The effect on pedestal formation was quantified in 100 cells (Fig. S1). Scale bars represent 5 μm.

innate immunity (e.g. *sek-1 and mek-1*; Kim *et al*., 2002, 2004), responses to ageing and stress (*daf-2, age-1*, *daf-16* and *mev-1*; Kenyon *et al*., 1993; Ogg *et al*., 1997; Ishii *et al*., 1998), hypoxia (*daf-2, hif-1, vhl-1 and egl-9*; Epstein and Gleadle *et al*., 2001; Scott *et al*., 2002), toxins or toxic metals (e.g. *mek-1*, *mev-1, pgp-1:pgp-3*; Ishii *et al*., 1990; Broeks and Janssen, 1995; Koga *et al*., 2000) and apoptosis (*ced-3* and *ced-4;* Aballay and Ausubel, 2001), or which additionally have been implicated in infections caused by other pathogenic bacteria (e.g. *ced-3*, *ced-4, bre-5, srf-3* and *mev-1;* Mahajan-Miklos *et al*., 1999; Aballay and Ausubel, 2001; Griffitts *et al*., 2001; Cipollo *et al*., 2004).

Several genes implicated in responses to other bacterial pathogens had no effect on EPEC virulence. Thus, *ced-3* and *ced-4* gene products are involved in apoptosis, and mutations in these genes were found to partially protect worms from killing by *S. typhimurium* (Aballay and Ausubel, 2001). However, *ced-3(n717)* and *ced-4(n1162)* provided no such protection from EPEC virulence suggesting that apoptotic pathways do not mediate the response to EPEC (Table 1). *C. elegans bre-5(ye27)* mutants are resistant to the *Bacillus thuringiensis* toxin Cry5B (Griffitts *et al*., 2001), and *srf-3(yj10)* mutants are resistant to infection by *Microbacterium nematophilum* and to binding of biofilm produced by *Yersinia psuedotuberculosis* and *Yersinia pestis* (Cipollo *et al*., 2004). However, neither of these mutants showed resistance to paralysis nor killing induced by EPEC (Table 1). Thus, mechanisms by which EPEC causes paralysis and death

Table 1. *C. elegans* mutants which have similar response as wild type to EPEC.

Worm mutant	Description/function	Role in other bacterial systems	Paralysis[a]	Killing[b]
N2	Wild-type worm used as standard	Control for motile mutants	++[c]	++[d]
rol-6(*e187*) roller	Alternative to N2 to aid in scoring due to motility defect	Control for-non-motile mutants	++[c]	++[d]
ced-3(n717) cell death abnormality	Protease required for programmed cell death	Resistance to *Salmonella typhimurium*	++[c]	++[d]
ced-4(n1162) cell death abnormality	Protease required for programmed cell death	Resistance to *Salmonella typhimurium*	++[c]	++[d]
bre-5(ye27) *Bacillus thuringiensis* toxin resistance	Transferase predicted to transfer galactose into proteins or lipids; also required for Cry5B toxicity.	Resistance to *Bacillus thuringiensis*	++[c]	++[d]
srf-3(yj10) surface antigen abnormal	Nucleotide sugar transporter. *srf-3* may have altered glyconjugates which prevent adhesion of the bacteria.	Resistance to *Mycobacterium nematophilum* and to binding of *Yersinia pseudotuberculosis* and *Yersinia pestis biofilms*	++[c]	++[d]

a. Paralysis assays were performed every 5 min for 30 min.
b. Killing assays were performed after 3-h onwards exposure.
c. >85% paralysis at the 15 min mark.
d. Less than 5% recovery after 6 h exposure

in *C. elegans* are different from those used by several other bacterial pathogens.

Several genes have been found to confer on *C. elegans* increased sensitivity to bacterial pathogens or toxins when mutant. For example, *mev-1* encodes a protein that catalyses electron transport from succinate to ubiquinone (Ishii *et al*., 1998). *mev-1* mutant animals have decreased superoxide dismutase activity, decreased lifespan, are highly sensitive to methyl viologen (paraquat) treatment (Ishii *et al*., 1990) and to pathogenic effects of phenazine, a toxin produced by *P. aeruginosa* (PA14) (Mahajan-Miklos *et al*., 1999). *mev-1(kn1)* mutants showed increased sensitivity to EPEC indicating that *mev-1* may mediate protection from EPEC paralysis and killing (Fig. 6A and B).

Other *C. elegans* genes also mediate responses to bacterial toxins. For example, mutations in the P-glycoprotein (*pgp*) genes, which encode ATP-binding membrane transporters, result in increased sensitivity to toxins such as colchicines (Broeks *et al*., 1995). Because EPEC killing is mediated by diffusible toxins, we tested the effects of EPEC on *pgp-1(pk17); pgp-3(pk18)* mutant worms. We observed increased sensitivity to EPEC with this mutant compared with wild-type N2 (Fig. 6A and B), indicating that P-glycoprotein transporters mediate protection from EPEC virulence, possibly by removing EPEC toxins.

Kim *et al*. (2002, 2004) established a conserved role for the *C. elegans* PMK-1 p38 mitogen-activated protein (MAP) kinase (K) pathway in innate immunity protection by identifying three genes: *nsy-1* (Sagasti *et al*., 2001), a MAPKKK; *sek-1* (Tanaka-Hino *et al*., 2002), a MAPKK; and *pmk-1* (Berman *et al*., 2001), a MAPK required for *P. aeruginosa* (strain PA14) resistance. With EPEC, *sek-1(km4)* mutants were more susceptible than N2 to paralysis and killing induced by EPEC (Fig. 6A and B). We also assessed the role of *mek-1*, a member of the c-Jun N-terminal kinase (JNK) pathway required for resistance to heavy metal stress (Koga *et al*., 2000), and which mediates stress and innate immunity pathways (Kim *et al*., 2004). When exposed, *mek-1(ks54)* mutants were more susceptible to EPEC-induced paralysis and killing (Fig. 6A and B). These results indicate a role of the innate immunity genes in protection from EPEC virulence.

We next determined the role in EPEC virulence of a set of genes that mediate responses to hypoxic stress. *egl-9* participates in normal egg laying and has widespread expression in the muscles, gonads and neurons (Darby *et al*., 1999). *egl-9* encodes a dioxygenase that mediates the response to hypoxic stress in both *C. elegans* and in mammalian systems by prolyl hydroxylation of hypoxia-inducible factor (HIF) leading to its proteasomal destruction by the von Hippel-Lindau (VHL) protein, a ubiquitin ligase (Epstein *et al*., 2001). Mutations in *egl-9* also render *C. elegans* resistant to toxins, and to cyanide produced by *P. aeruginosa* (strain PA01) (Darby *et al*., 1999; Gallagher and Manoil, 2001). To determine whether paralysis and killing by EPEC depends on the hypoxia response pathway, we tested *C. elegans* strains containing mutations in the *egl-9* (*n586, n571, sa330 and sa307*), *hif-1(ia4)* and *vhl-1*(*ok161*). All *egl-9* and *hif-1* mutants displayed partial resistance to paralysis and killing by EPEC (Fig. 6C–F), whereas mutants in *vhl-1* show increased rate of paralysis and killing compared with wild-type N2 (Fig. 6E and F). To rule out the possibility that hypoxia mediates EPEC effects, we measured the susceptibility of *egl-9, hif-1, vhl-1* and *daf-2(e1370)* mutants to hypoxia induced by exposure to 0.5 M sodium azide for 1.5 h. *Egl-9* and *vhl-1* were as susceptible to hypoxia as N2, whereas *hif-1* was more susceptible and *daf-2* more

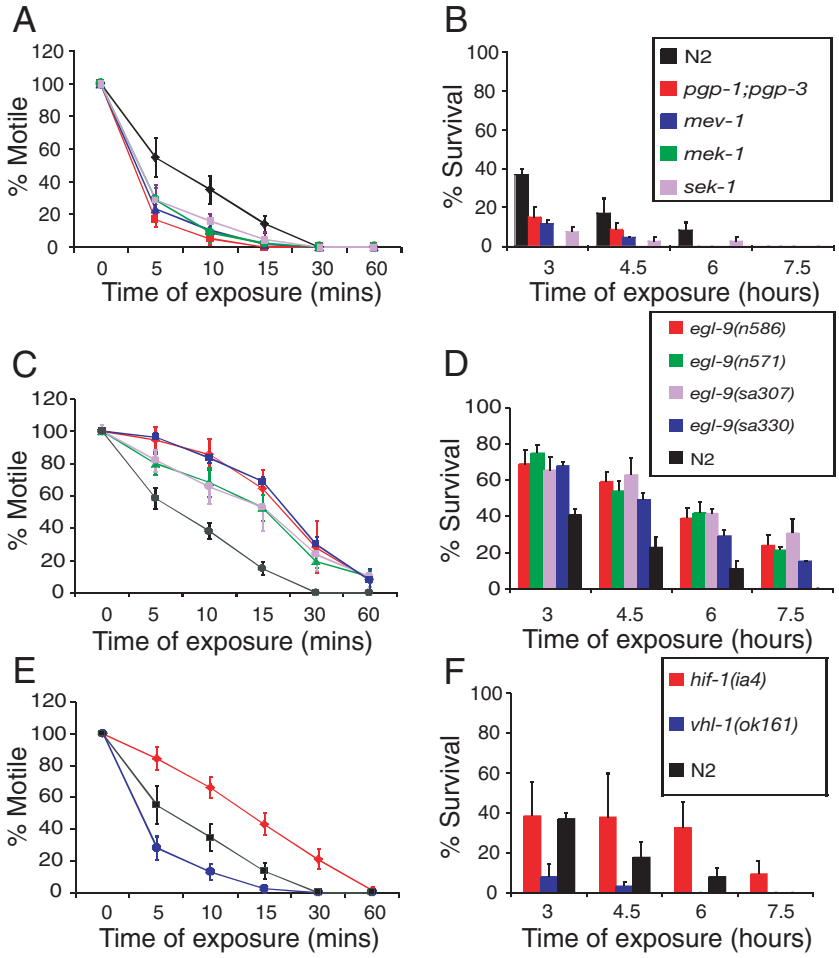

Fig. 6. EPEC induced paralysis and killing assays on *C. elegans* mutants.
A. Paralysis of *C. elegans* mutants by EPEC. *mev-1, pgp-1; pgp-3, sek-1 and mek-1* mutants display faster kinetics of paralysis compared with N2.
B. Killing of the mutants by EPEC. The mutants were also killed by EPEC, at a significantly faster rate. Note that *mek-1* did not survive at all even at the earliest time point of 3 h. (C-F) Paralysis of *C. elegans* by EPEC involves *egl-9* and *hif-1*, but not *vhl-1*.
C. *Egl-9* shows partial resistance to paralysis when exposed; all four *egl-9* alleles were significantly resistant to paralysis compared with N2.
D. Killing of *egl-9* alleles.
E and F. Paralysis and killing of *hif-1* and *vhl-1*. *hif-1* was also partially resistant to both paralysis (E) and killing (F) compared with N2, whereas *vhl-1* was more sensitive.

resistant compared with N2 (Fig. S2), as expected based on previous reports (Scott *et al*., 2002). Together, these data suggest that *egl-9* and *hif-1* mediate toxic effects of EPEC, whereas *vhl-1* participates in a protective capacity. Indeed, these data indicate a role for *egl-9, hif-1* and *vhl-1* in EPEC infection different from their described roles in hypoxia.

Finally we tested a set of genes which when mutated affect lifespan of *C. elegans. daf-2* encodes an insulin-like receptor that functions upstream of *age-1* a phosphatidylinositol 3-kinase (Morris *et al*., 1996). Temperature sensitive partial loss of function mutations in *daf-2(e1368 and e1370)*, for example, result in dauer formation at the restrictive temperature and a twofold increase in adult lifespan at the permissive temperature (Kenyon *et al*., 1993; Kimura *et al*., 1997). *daf-2* via *age-1* lead to the inhibition of *daf-16*, a forkhead transcription factor which regulates genes involved in, for example, toxin inactivation, response to hypoxia and stress, and responses to bacteria (Murphy and McCarroll, 2003). Null alleles of *daf-16* do not affect lifespan but antagonize the effects of *daf-2* on dauer larvae formation and lifespan (Kenyon *et al*., 1993; Ogg *et al*., 1997). *daf-2* and *age-1* mutants are also partially resistant to killing by *Enterococcus faecalis*, *Salmonella aureus* and *P. aeruginosa* (Garsin *et al*., 2003).

In response to EPEC, *daf-2(e1370), daf-2(e1368)* and *age-1(hx546)* mutants proved more resistant to paralysis compared with N2 (Fig. 7A). These mutants were also resistant to killing compared with N2, though the effects of *daf-2(e1370)* was most pronounced with approximately 80% of worms surviving exposure to EPEC at a time when nearly all N2 worms had succumbed (Fig. 7B). *daf-2(e1368)* was more susceptible to killing compared with *daf-2(e1370)* in agreement to previous reports that this allele is also more susceptible to killing by other bacterial pathogens (Garsin *et al*., 2003). The two null alleles *daf-16(m26)* and *daf-16(mgDf50)* showed partial resistance to paralysis but were susceptible to killing in response to EPEC (Fig. 7C and D). However, the double mutants *daf-16(m26); daf-2(e1370)* and to a lesser extent *daf-16(mg54); daf-2(e1370)* proved somewhat more resistant to EPEC-induced paralysis compared with N2 or the single *daf-16* mutants. Notably, these double mutants were as susceptible to killing as the *daf-16* single mutants and

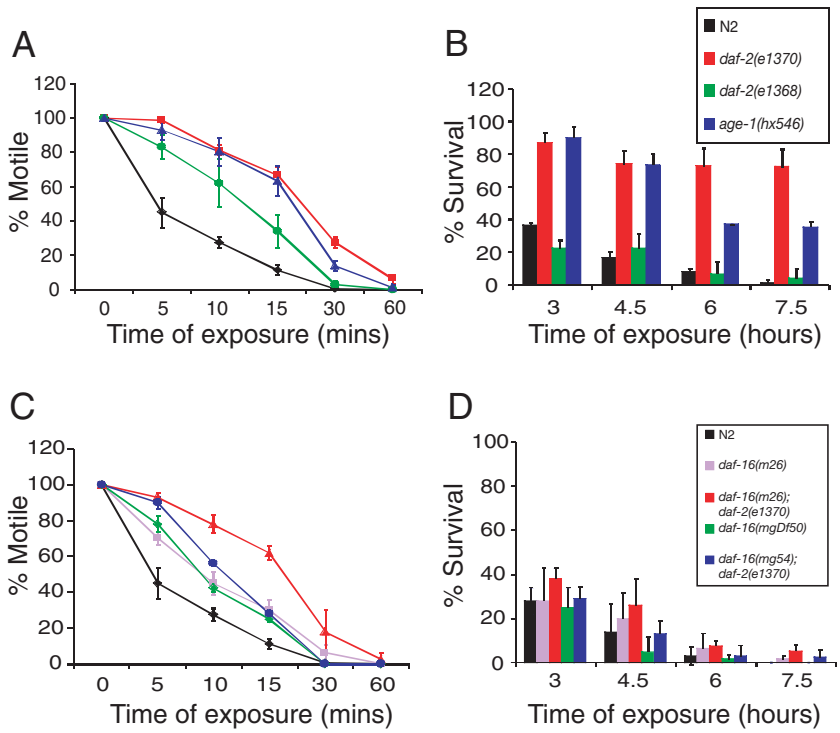

Fig. 7. EPEC induced paralysis and killing on daf-2 pathway mutants.
A. Paralysis of *daf-2* and *age-1* mutants. Both *daf-2*(*e1370)* and *age-1*(*hx546)* were very resistant while *daf-2*(*e1368)* was partially resistant.
B. Killing of the mutants by EPEC. Note that the *daf-2*(*e1368)* being a weaker allele was more susceptible to killing.
C and D. Paralysis and killing of the *daf-16* single and *daf-16; daf-2* double mutants.
C. The *daf-16* single mutants were partially resistant to paralysis. They could not however, suppress the *daf-2* effect of the double mutants during paralysis. Note that *daf-16*(*m26); daf-2*(*e1370)* is as resistant as *daf-2*(*e1370)*.
D. For killing assays, both the *daf-16* single and double mutants were very susceptible to killing indicating separation between paralysis and killing mechanisms.

N2 (Fig. 7C and D). These data suggest (i) that *daf-2* and *age-1* mediate EPEC toxicity; (ii) that *daf-16* confers protection by antagonizing the effects of *daf-2* on EPEC-induced killing. Moreover our observations with *daf-16(m26); daf-2(e1370)* double mutants indicate that EPEC-induced paralysis and killing may be separable processes. Taken together, toxic effects of EPEC appear to be mediated by the *C. elegans* genes *egl-9, hif-1, daf-2* and *age-1*, whereas protective effects are mediated by *mek-1, sek-1, pgp-1,3, mev-1, vhl-1* and *daf-16*. We could identify no role in EPEC virulence for *ced-3, ced-4*, *bre-5* or *srf-3*. These results are summarized in Fig. 8.

Discussion

Genetic systems model aspects of pathogen-induced human disease

The use of model genetic systems to decipher host-pathogen relationships has begun to yield important information about novel mechanisms of virulence in the pathogen as well as determinants of susceptibility in the host. *C. elegans* has been used as a model for infections caused by *P. aeruginosa* and *S. typhimurium*, among others (Darby *et al*., 1999; Tan *et al*., 1999; Aballay *et al*., 2000; Garsin *et al*., 2001; Gan *et al*., 2002; Jansen *et al*., 2002). Likewise, *Drosophila melanogaster* is now being used to study *Candida albicans* (Alarco *et al*. 2004) and *Plasmodium gallinaceum* (Schneider and Shahabuddin, 2000) among others. Our studies demonstrate the feasibility of using *C. elegans* to study pathogenic *E. coli* virulence.

Work on *C. elegans* and bacterial pathogens have led to the identification of conserved virulence mechanisms. For example, the *Pseudomonas* factors gacA and gacS have been implicated in virulence in *C. elegans* as well as in *Arabidopsis* and in a mouse burn model (Rahme *et al*., 2000). GacA and gacS comprise a two-component system which regulates virulence factor production including some factors identified as quorum-sensing molecules (Sperandio *et al*., 2002a). Thus, these data raise the possibility that molecules associated with intercellular signalling may be a general feature of pathogenesis (Rahme *et al*., 2000).

Our goal was to establish a model using a genetically tractable system to identify conserved EPEC virulence factors and host susceptibility mechanisms. In this regard, our data show that for virulence in *C. elegans*, EPEC requires EspA, EspD and EspF, LEE factors critical for infections in mice and therefore presumably in humans as well (Deng *et al*., 2004). Furthermore, our data point to a role of tryptophanase in regulating LEE expression and for pedestal formation in mammalian cells (Fig. 5), a process which depends on genes within the LEE (Kaper *et al*., 1997; McDaniel and Kaper, 1997). Although the secreted toxin causing paralysis and death in *C. elegans* requires tryptophanase activity and LEE-encoded EspA, EspD and EspF, it remains to be determined whether it is important for pathogenesis in mammalian systems.

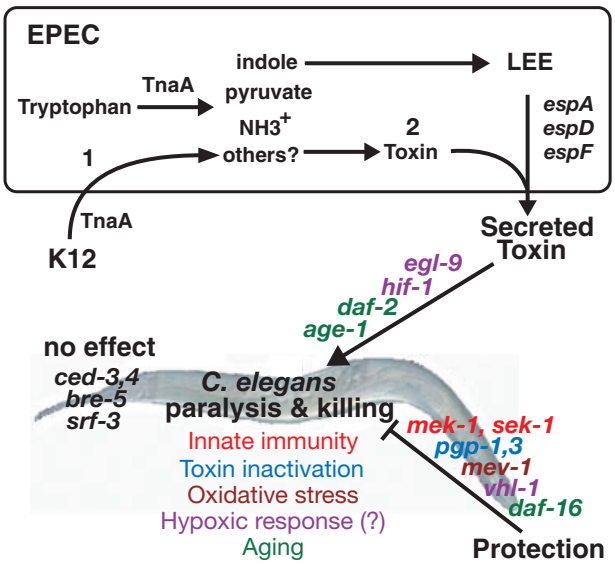

Fig. 8. Summary of effects of EPEC on *C. elegans*. EPEC tryptophanase (*tnaA*) catalyses production of indole and other metabolites, which directly or indirectly regulate production of a putative toxin that can paralyse and kill *C. elegans*. *TnaA* expressed on a plasmid, but not exogenous indole (or other known metabolites) restores the capacity of EPECΔ*tnaA* to paralyse and kill *C. elegans* and form actin pedestals in mammalian cells, as does mixing EPECΔ*tnaA* with K12 (but not K12Δ*tnaA*). However, *tnaA* appears to regulate either directly or indirectly, expression of LEE genes in an indole-dependent manner, and mutation of LEE genes (e.g. EspA, D and F) prevents paralysis and killing. Thus, LEE genes may facilitate secretion of the toxin. In *C. elegans*, mutations in *egl-9, hif-1*, *daf-2* and *age-1* confer resistance to paralysis and killing by EPEC, suggesting that these genes normally mediate toxin effects. By contrast, mutations in *mek-1*, *mev-1, pgp-1,3* and *vhl-1* render *C. elegans* more sensitive to effects of EPEC, suggesting that these genes mediate resistance to EPEC toxins. Mutation of *daf-16* antagonizes effects of *daf-2* on survival, though not paralysis, suggesting that paralysis and killing are separable processes, and providing evidence that genes controlling ageing in *C. elegans* also control susceptibility to EPEC.

Metabolic requirements for paralysis and killing of C. elegans

How does tryptophan metabolism result in production of a factor toxic to *C. elegans*? The observation that none of the known metabolites of tryptophanase activity (indole, pyruvate, or ammonium) alone or in combination restore the capacity of EPECΔ*tnaA* to paralyse and kill *C. elegans*, raises the possibility that a previously unrecognized metabolite of tryptophanase results in synthesis of a secreted toxin(s). Mass spectroscopy would be required to identify such putative metabolites. Alternatively, changing tryptophan catabolism by addition of tryptophan or deletion of tryptophanase may have complex metabolic consequences that permit production of a toxin, independent of known tryptophan metabolites. For paralysis and killing assays high tryptophan concentrations in the media are required. ECD and LB were originally designed to facilitate indole production measured with Kovac's reagent. These media stimulate synthesis of tryptophanase, and tryptone in LB and peptone in ECD supply high concentrations of tryptophan for conversion to indole. In our experiments, supplemental tryptophan is required presumably because tryptophan in the media is degraded during autoclaving below critical levels for reproducible paralysis and killing. On both LB and ECD media, bacterial growth likely occurs through amino acid catabolism. Pruss *et al*. (1994) have shown that in LB medium, *E. coli* degrades serine and then aspartate in preference to tryptophan, first metabolizing pyruvate derived from serine, then oxaloacetate from aspartate, and finally pyruvate from tryptophan. The catabolism of amino acids is complex and highly sensitive to media composition and growth state. As a result, alterations in tryptophan concentration by supplementing LB with tryptophan, by growth on ECD, or by deleting tryptophanase can alter bacterial catabolism and thereby indirectly affect metabolism in unpredictable ways. Such metabolic changes might in turn result in the production of a toxin independent of tryptophanase metabolites.

Intercellular signalling and regulation of virulence in C. elegans *and mammalian cells*

Our results show that factors derived from commensal or pathogenic strains may regulate the production, via intercellular signalling mechanisms, of toxins or other virulence factors in cocultured pathogenic strains, and raises questions about the origins of virulence regulation and the role of such signalling in infected organisms. Several groups have reported the isolation of bacterial mutants using *C. elegans* models which indicate a role for putative intercellular signalling or quorum-sensing factors (Rahme *et al*., 2000). Ausubel and colleagues (Mahajan-Miklos *et al*., 1999; Mahajan-Miklos *et al*., 2000) have suggested that the link between secreted factors and regulation of virulence derives from the ancient predator-prey relationship between *C. elegans* and bacteria. According to this view, bacterial colonies coordinating communal activity by secreted factors may have proved vulnerable to detection by *C. elegans*. Selection may have linked the control of communal behaviour via secreted factors to virulence mechanisms and colony defence. Thus, virulence mechanisms may have originally evolved to protect bacteria from *C. elegans*, but proved generally deleterious to a variety of organisms, including humans upon initial contact. Such a possibility does not rule out subsequent evolution of an avirulent relationship of bacteria with its mammalian host following colonization.

Intercellular signalling mechanisms could also facilitate colonization of new niches and competition with other bacterial species. In this regard, such signalling mecha-

nisms may prove critically important in the context of an EPEC infection in the intestinal tract. Finlay and coworkers have demonstrated that expression of EPEC virulence factors depends on pH, and have proposed that such regulation allows the bacterium to distinguish acidic conditions of the stomach from the more basic environment of the small intestine, the preferred site of infection (Kenny *et al*., 1997b). Kaper and colleagues have identified other quorum sensing factors (e.g. LuxS in *E. coli*) that directly regulate expression of genes in the LEE locus (Sperandio *et al*., 1999; Sperandio *et al*., 2001; Sperandio *et al*., 2002a). Recently, another autoinducer AI-3 has also been shown to regulate expression of LEE genes in EHEC and K-12 (Sperandio *et al*., 2003). Our results raise the additional possibility that the by-products of tryptophan metabolism produced by commensal strains or by EPEC might in turn regulate EPEC virulence. Among these products, indole has been identified as an intercellular signalling factor expressed at the onset of stationary phase in *E. coli* (Baca-DeLancey *et al*., 1999; Wang *et al*., 2001), and extracellular addition of indole activates expression of several *E. coli* genes (Wang *et al*., 2001). Indole is secreted by EPEC, and although it does not itself appear to cause paralysis or killing of *C. elegans,* it does appear to regulate, either directly or indirectly, expression of the LEE promoter (Fig. 4D). Our mixing experiments raise the possibility that indole, together with other unidentified factors derived from EPEC or from other commensal strains, regulates LEE expression and toxin production, and, in mammalian cells, formation of actin pedestals.

Effects of EPEC on C. elegans *require secretion of soluble toxin(s) by an tryptophanase and LEE-dependent mechanism*

What is the nature of the toxin? Complementation of EPEC∆*tnaA* by K12 suggests the existence of at least two diffusible secreted factors, both of which depend on *tnaA*. We hypothesize that the production of the first ('1' in Fig. 8) regulates directly or indirectly the production of the second, which is toxic to *C. elegans* ('2' in Fig. 8). Further studies will be required to determine whether EPEC indeed produces the first factor, or whether this factor is required to activate EPEC virulence by endogenous K12 strains *in vivo*. Our results also suggest that the factor toxic to *C. elegans* is soluble, though apparently not gaseous, and requires a secretion system used to export other virulence factors. In particular, *espA* and *espD* are attenuated for paralysis and killing (Fig. 4A and B). EspA is thought to form the hollow filament through which effector molecules are translocated from bacterium to host cell (Ebel *et al*., 1998; Knutton *et al*., 1998; Shaw *et al*., 2001). EspB (Wolff *et al*., 1998) and EspD (Wachter *et al*., 1999) forms a pore in the host cell membrane which links EspA and the host cell cytoplasm (Warawa *et al*., 1999). Such a type III system may be required for toxin secretion.

Other LEE virulence factors associated with attachment, including Tir (Kenny *et al*., 1997a) and intimin, depend on the type III system for secretion (Jarvis *et al*., 1995; Stein *et al*., 1996), but do not appear to be toxic to *C. elegans* (Fig. 4). Likewise, other known toxins or secreted factors in EPEC or EHEC such as cyanide (Fig. 2E), EspC (Fig. 4), Shiga toxin and LifA (D. Kalman, G.M. Benian and J.M. Klapproth, unpubl. obs.), do not appear to participate in *C. elegans* paralysis and killing, though additional secreted factors not evaluated here have been identified [e.g. NleA and EspP (Gruenheid *et al*., 2004].

One identified secreted factor that does appear required for toxicity towards *C. elegans* is EspF. EspF disrupts intestinal barrier function and can induce host cell death by mechanisms involving disruption of mitochondrial function (Crane *et al*., 2001; McNamara *et al*., 2001; Nougayrede and Donnenberg, 2004). EspF has previously been regarded as acting intracellularly following secretion, and further experimental work will be required to determine whether it retains activity when provided extracellularly, or whether *C. elegans* mutants resistant to EPEC define targets of EspF. These data do not rule out the possibility that additional toxins exist. Indeed the observations that EIEC and ETEC (Fig. 1), which do not contain a LEE or EspF also paralyse and kill *C. elegans*, support the idea that common toxins or common regulatory mechanisms exist in many pathogenic *E. coli* strains. The observation that the toxic factor(s) can be secreted through a filter and kill without the bacterium present, suggests a means of purification and an activity assay, a strategy we are currently pursuing.

Caenorhabditis elegans *factors mediating susceptibility to and protection from EPEC*

Our data, together with those of others, also provide evidence that susceptibility of the host to different bacterial pathogens requires a variety of different, and not necessarily overlapping, mechanisms. Thus, susceptibility of *C. elegans* to *Salmonella* but not EPEC requires genes important for apoptosis such as *ced-3* and *ced-4* (Elliott *et al*., 1999). Moreover, although *egl-9* mutants appear resistant to both *Pseudomonas* and EPEC, the mechanisms appear different. *Pseudomonas* PA01 produces cyanide, which induces anoxia in *C. elegans*, and *egl-9 has* now been demonstrated to mediate downregulation of cellular responses to hypoxia by facilitating destruction of the anoxic response regulator HIF-1 by the VHL ubiquitin ligase (Epstein *et al*., 2001). Cyanide does not appear to play a role in EPEC virulence (Fig. 2E). Moreover, our data showing that *egl-9* and *hif-1* mediate toxicity

by EPEC, and *vhl-1* mediates protection suggests that the hypoxic response pathway defined by these genes does not participate in EPEC virulence, and that, in this context, these genes function differently. Indeed reports of others suggest that *egl-9* may have other activities that are independent of hypoxia (J. Hodgkin, pers. comm.), though it remains to be determined whether such activity is important for susceptibility to EPEC. In summary, our data provide evidence that genes known to participate in response to hypoxia act in a novel way in response to EPEC.

Caenorhabditis elegans *genes affecting lifespan also regulate susceptibility to EPEC*

Our experiments with mutants which affect *C. elegans* lifespan provide important information on the mechanisms by which *C. elegans* succumb to EPEC. *C. elegans* have the capacity to enter a dauer state or live longer, for example under harsh environmental conditions. This has been shown to require a means to overcome or inactivate environmental and metabolic toxins and stresses (e.g. reactive oxygen species), and attempts by bacteria to colonize among other factors. To do this, *C. elegans* upregulates a variety of genes including cellular stress response, antimicrobial and metabolic genes, and downregulate specific life-shortening genes. We have found that the capacity to live longer is directly correlated with the capacity to survive EPEC. Thus, mutations in *daf-2* and *age-1*, which facilitate longevity, also facilitate survival in response to EPEC. Similarly, mutations in *daf-16* antagonize the effects of *daf-2* on longevity (Kenyon *et al*., 1993) and survival in response to EPEC (Fig. 7). Likewise, mutations in *mev-1* shorten lifespan (Ishii *et al*., 1990), and increase susceptibility to EPEC paralysis and killing (Fig. 6A and B). Thus, the mechanism by which EPEC kills *C. elegans* appears to involve inactivation of factors that permit the worm to accommodate to environmental or other stresses associated with ageing. Such a mechanism is supported by our observations that *sek-1* and *mek-1* (Fig. 6A and B), which encode factors associated with innate immunity and cellular defences against bacterial toxins (Huffman *et al*., 2004), also mediate protection from EPEC.

It is also noteworthy that nearly all of the genes we have identified as mediating susceptibility to or protection from EPEC have a similar effect on paralysis and killing. However, the identification of *daf-16* as a gene that controls susceptibility to killing, but not paralysis (Fig. 7C and D), suggests that the two processes may be mediated by separable, non-overlapping mechanisms. In the long term, the system we have described here will permit identification of the components that connect pathways mediating, for example, innate immunity and hypoxic responses with ageing, and which govern mechanisms of susceptibility to or protection from paralysis and killing. The similarity of *C. elegans* and human genomes raises the possibility of identifying human homologues of such factors.

In summary, data presented here define a genetic system based on paralysis and killing of *C. elegans* by EPEC to investigate mechanisms of virulence in the bacterium, and mechanisms of susceptibility to virulence in the host. We have defined tryptophan and its metabolites, together with factors required for secretion of toxins and virulence factors important in human disease, as mediators of paralysis and killing of *C. elegans*. Moreover, we have defined indole as a putative intercellular signalling factor that can regulate LEE promoters. This observation, taken together with the observation that pedestal formation is markedly altered in EPECΔ*tnaA* (Fig. 5), suggests that this tryptophanase pathway may have relevance to pathogenesis in mammalian systems. We have also provided evidence that *C. elegans* ageing pathways mediate responses to EPEC.

Our data suggest a model in which the functional expression of an EPEC toxin depends directly or indirectly on tryptophan metabolites derived from EPEC or from commensal strains. We hypothesize that the putative toxin is secreted via a LEE-encoded type III secretion pore in a manner dependent on EspA and EspD. The toxin, possibly EspF, is diffusible and produces paralysis and subsequent lethality in *C. elegans* (Fig. 8). These data raise the possibility that screens for additional virulence mechanisms in the bacterium, or for determinants of susceptibility to paralysis and killing in *C. elegans* will provide important information on the aetiology of EPEC disease in humans.

Experimental procedures

Bacterial and worm strains

Enteropathogenic *E. coli* serotype O127:H6 strain E2348/69 (Levine *et al*., 1985), EHEC serotype O157:H7 strain EDL933 (Riley *et al*., 1983), ETEC strain C921b-1 and EIEC strain 4R were provided by June Scott. *E. coli* OP50 (Brenner, 1974) and *E. coli* K12 strain MG1665, a gift of Bernie Weiss (Emory University) were primarily used in this study. EPEC virulence gene mutants have been previously described: *espA*- strain UMD872 (Kenny *et al*., 1996), *espD*- strain UMD870 (Lai *et al*., 1997), *eae*- strain CVD206 and *cfm* (Donnenberg and Kaper, 1991), were provided by James Kaper (University of Maryland); *espF*-, the *tir* deletion mutant (Kenny *et al*., 1997a) and *espC*- strain MAS111 (Stein *et al*., 1996) were provided by Brett Finlay (University of British Columbia). K12Δ*tnaA* was provided by Philip Rather (Veterans Affairs Medical Center, Atlanta). *C. elegans* mutants were obtained from the *Caenorhabditis* Genetics Center (NIH National Center for Research Resources). The following alleles of each mutant which have already been described were used: *rol-6(e-187)*, *ced-3(n717)*, *ced-4(n1162)*, *bre-5(ye27)*, *srf-*

3(yj10), pgp-1(pk17); pgp-3(pk18), mev-1(kn1), mek-1(ks54), sek-1(km4), egl-9(n586), *egl-9(n571), egl-9(sa307), egl-9(sa330), hif-(ia4), vhl-1(ok161), daf-2(e1370), daf-2(e1368), age-1(hx546), daf-16(m26), daf-16(m26); daf-2(e1370), daf-16(mgDf50), daf-16(mg54); daf-2(e1370)* and wild-type Bristol strain N2 (Brenner, 1974). All *C. elegans* strains were maintained under standard culturing conditions with *E. coli* OP50 as food source (Sulston and Hodgkin, 1988).

Media

Fluorocult ECD Agar (EM Science, Gibbstown, NJ) used for most paralysis and killing assays was prepared by adding 5.31 g to 100 ml Milli-Q filtered water and autoclaving for a sterilizing time of 20 min. After immediate removal from the autoclave, the ECD agar was allowed to cool to 46°C, L-tryptophan (0.1 g/1 ml) (Sigma-Aldrich, St. Louis, MO) dissolved in 0.5 M HCl was added to a final concentration of 0.1% prior to pouring. ECD agar for media composition assays was made according to the manufacturer's specifications: 20 g l^{-1} peptone from casein, 5 g l^{-1} lactose, 5 g l^{-1} sodium chloride, 1.5 g l^{-1} bile salt mixture, 4 g l^{-1} *di*-potassium hydrogen phosphate, 1.5 g l^{-1} potassium dihydrogen phosphate, 15 g l^{-1} agar and 1 g l^{-1} tryptophan. Difco LB broth (Miller, 1972) was used to grow overnight liquid cultures of all bacteria. Fisher LB agar (Miller, 1972) (40 g l^{-1}) was also used for the paralysis and killing after supplementing with the tryptophan. NGM plates and 3XD broth were prepared as described (Lewis and Fleming, 1995). Where necessary, antibiotics were used at the following concentrations: chloramphenicol at 25 μg ml^{-1} and ampicillin at 100 μg ml^{-1}.

Paralysis and killing assays

Three days prior to each assay, chunks of the *C. elegans* strain(s) were propagated at 20°C on NGM plates seeded with OP50 to allow for the growth of a sufficient number of young adult worms; 5.0 cm plates of ECD, LB or NGM were poured and 5 ml bacterial cultures in LB broth were started. Cultures were incubated overnight at 37°C without shaking to an OD_{600} of between 0.8 and 1.0. All the bacteria cultures used for each assay were from fresh overnight cultures. On the second day, the ECD, LB or NGM plates were spotted with the bacterial overnight cultures (30 μl per spot and three spots per plate) and were incubated at 37°C for at least 16 h. For assays involving wild type and other motile *C. elegans* mutants, 150 μl of the bacteria was spread on the 5.0 cm plates. On the third day, after equilibrating the plates to room-temperature, 30 *C. elegans* worms were transferred to each plate and kept at room temperature. For each assay, at least three replicates were performed to achieve statistically significant results. The number of worms fully paralysed per total number of worms was scored within the first 120 min after addition of worms to the plate. Worms were considered paralysed if they did not respond either to vibrations from tapping the plate or to prodding with a wire pick. For killing assays, worms were removed from the plates after 3 h onwards and transferred to NGM plates previously seeded with OP50 and kept at room temperature. After 24 h the number of motile (living) versus non-motile (dead) worms was recorded.

Contact assays

Assays to test whether contact between worms and bacteria were required for killing were performed as described (Gan *et al*., 2002). Briefly, 4.7 cm diameter NC filters (0.22 μM) were placed on the media in 5.0 cm plates and 600 μl of bacteria spotted onto the filters stepwise in 300 μl aliquots. After overnight incubation (16–18 h) at 37°C, the filters were lifted off of the plate along with the bacteria, and worms were then added to the plate. Paralysis and killing were scored as described above. To confirm that this procedure resulted in complete removal of bacteria, some plates with the filters lifted were cultured at 37°C overnight. Such plates were found to be free of bacterial colonies.

Cyanide assays

To measure cyanide production by EPEC strains we followed the protocol as described (Gallagher and Manoil, 2001). Briefly, various bacteria strains were cultured overnight to OD_{600} of 0.8–1.0. A total of 150 μl of each bacterium was spread onto 5.0 cm ECD plates, placed in 15 cm Petri dishes and cultured at 37°C for 24 h. The lids of the 5.0–cm plates were then removed and in the inverted lid, 1 ml of 4 M NaOH was added. The 15 cm Petri dishes containing the bacteria and NaOH were sealed tightly with parafilm and incubated for 4 h at room temperature. Afterwards, the NaOH was collected and diluted to 0.09 M with double distilled water. The cyanide in each sample for each strain was quantified by comparison with KCN standards made in 0.09 M NaOH: 210 μl sample aliquots were mixed with 700 μl aliquots of fresh 1:1 mixture of 0.1 M O-dinitrobenzene (Sigma) in ethylene-glycol monomethyl ether (Sigma) and 0.2 M P-nitrobenzaldehyde (Sigma) in ethylene-glycol monomethyl ether. After exactly 30 min incubation at room temperature, the OD_{578} was measured and values extrapolated to the standard curve.

To test whether paralysis and/or killing by EPEC was caused by any emitted gas, overnight cultures of EPEC grown on 5.0 cm ECD plates were placed uncovered in the 15 cm Petri dish together with uncovered ECD plates having 30 *rol-6* worms. The Petri dish was sealed with parafilm and kept at room temperature. Worms were checked for paralysis and killing as before.

Complementation assays

To determine if other unrecognized metabolites produced by tryptophanase may mediate paralysis and killing, overnight cultures of EPEC*ΔtnaA* were each mixed together with overnight cultures of K12 or K12*ΔtnaA* in OD_{600} ratios of 150:1, respectively, and 150 μl spread on each plate. After culturing the bacteria overnight, worms were added and checked for paralysis and killing. This experiment was also performed using LB plates with or without tryptophan.

Tryptophanase enzyme assays

Tryptophanase activity was determined by measuring the conversion of S-O-nitrophenyl-L-cysteine (SOPC) to O-

nitrothiophenolate (Suelter *et al.*, 1976). The assay procedure was based on that described by Stewart, 1985) and by Gish and Yanofsky (1993) but optimized for EPEC. Cells were grown to mid-log phase (optical density at 600 nm of 0.8–1.0) in ECD medium, washed in cold lysis buffer (100 mM potassium phosphate (pH 7.8) 50 μM pyridoxal phosphate, 10 mM β-mercaptoethanol and 2 mM EDTA) and sonicated for 60 s on ice. Following centrifugation (12 000 *g* for 2 min), protein concentrations in the supernatant were determined using a Bio-Rad Protein assay reagent according to the manufacturers specifications. Fifty microlitres of the supernatant were added to assay buffer (50 mM potassium phosphate (pH 7.8) 50 μM pyridoxal phosphate, 5 mM β-mercaptoethanol) and incubated at room temperature for 5 min. The reaction was started by addition of 500 μl of 0.66 mM SOPC. The absorbance at 470 nm was measured over the course of 3 min in a spectrophotometer, and the ΔA_{470} calculated when the reaction rate was linear. Using an extinction coefficient of 0.626 M^{-1} cm^{-1} for O-nitrothiophenolate, tryptophanase activity was calculated according to the Lambert-Beer equation as follows: (ΔA470 × 1.0 ml reaction volume)/{(0.626 μmol ml^{-1} × [time (min)] × [extract volume (ml)]} as described (Gish, 1993). One unit of tryptophanase activity is defined as 1 μmol of O-nitrothiophenoloate produced per minute at room temperature (~25°C). Activity was normalized to protein concentration. Tryptophanase activity shown in Fig. 3A is expressed as a per cent of tryptophanase activity measured for EPEC. All assays were performed in triplicate. Tryptophanase activity of EPEC and EPEC mutants grown on ECD plates was carried out essentially as described above except that instead of growing bacteria in liquid culture, bacteria were seeded on ECD plates overnight and then harvested by scraping.

*Construction of the EPEC*Δtna*A insertion mutant*

The EPEC strain E2348/69Δ*tnaA* insertion mutant, JMD21, was constructed by homologous recombination of a polymerase chain reaction (PCR) product as described (Datsenko and Wanner, 2000). Primers used to generate the PCR product were JD9 and JD10 and primers used to confirm the presence of the insertion in *tnaA* were primers c1 and c2 (Datsenko and Wanner, 2000) together with JD7 and JD8. To confirm that the colony picked was EPEC, primers 5′epec2349EcoR1, and 3′epec2349Hind, both gifts of Bettina Bommarius, were used to detect EPEC *tir*. The insert for the cloning of *tnaA* was generated by PCR using KOD HiFi Polymerase (Novagen, Madison, WI) on template DNA from EPEC E2348/69 whole-cell lysate (i.e. colony PCR). Primers used to generate the *tnaA* insert were JD18, and JD19. (All primers are listed in the Table S1). The PCR product was purified with the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA), digested with PstI and EcoRI, and purified again. Plasmid pKK223 (Amersham Pharmacia Biotech, Piscataway, NJ) was digested with PstI and EcoRI, and then purified. Ligation was performed with the Rapid DNA Ligation Kit (Roche Diagnostics GmbH, Penzberg, Germany) as prescribed by the manufacturer, and electroporated into DH5α using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA) at 25 μF, 2.5 kV and 200 Ω. Presence of the plasmid, named JD50, was confirmed by plasmid purification (QIAGEN), EcoRI-PstI double digest, and visualization on a 1% agarose gel. JD50 was then purified and electroporated into EPEC strain JMD21. The resulting strain was named JMD22.

Killing and paralysis for genetic complementation studies was performed as usual, except for the following modifications. For paralysis assays in which JMD22 was tested, ECD plates contained both chloramphenicol and ampicillin. For JMD22 killing assays, overnight cultures of JMD22 contained chloramphenicol, ampicillin, and 1 mM IPTG for induction of *tnaA* expression from JD50. ECD plates on which killing were performed also contained the two antibiotics and were spread with 1 mM IPTG. As controls, OP50 was spotted onto ECD plates spread with 1 mM IPTG.

Construction of lacZ *transcriptional fusion strains*

The insert containing the promoter for EPEC LEE1 was made using the primers JD37a, and JD37b with EPEC colonies as the source of template DNA for the PCR reaction. The expected product of 719 base pairs therefore contained approximately 392 bp upstream of the promoter, the promoter itself, and approximately 227 bp upstream of the transcriptional start point. This PCR product was agarose gel purified (QIAGEN) and digested with EcoRI and BamHI. The vector pGE593 (Eraso and Weinstock, 1992) was a gift from Tony Romeo, and was also digested with EcoRI and BamHI. The vector and insert were gel purified (QIAGEN), ligated, and transformed into DH5α (Sambrook and Russell, 2001). Plasmid containing the insert was prepared from DH5α clones and electroporated into JMD21 and EPEC E2348/69.

Measurements of LEE promoter activity

β-Galactosidase activity of LEE promoter constructs was assayed by using O-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma Aldrich) as substrate, essentially according to the method of Miller (Miller, 1972), with the exception that cells were not washed in M9 before being assayed. Miller assays from bacteria grown on plates were carried by first culturing bacterial strains containing the LEE1-LacZ reporter overnight in LB broth, and then spotting on to 5.0 cm ECD plates supplemented with 0.2% tryptophan and either 50 μM or 500 μM indole. Following overnight culture, bacteria was scraped into 1 ml of ECD media and quantified by measuring OD_{600}. After addition of ONPG and stopping the reaction, product formation was quantified spectrophotometrically in multiwell polypropylene blocks using a Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT) as described by Griffith and Wolf (Griffith and Wolf, 2002).

Cell culture, immunofluorescence analysis and microscopy

3T3 cells were grown on glass coverslips in DMEM containing serum and incubated for 6 h at 37°C with wild-type EPEC (strain 2389/69), EPECΔ*tnaA*, EPECΔ*tnaA* + *ptnaA*, EPECΔ*tnaA* + K12, EPECΔ*tnaA* + K12Δ*tnaA,* K12 or K12Δ*tnaA* at a multiplicity of infection (moi) of 10. For immunofluorescence analysis, cells were fixed in 2% formaldehyde and permeablized in Triton-X-100 as described (Swimm

et al., 2004). EPEC was recognized by staining with 4,6-diamidino-2-phenylindole (DAPI; 1 μg ml^{-1}; Sigma), and pedestals by staining with FITC-phalloidin (1 μg ml^{-1}; Molecular Probes). Before staining, some pAbs were preincubated with EPEC or EPECΔTir previously fixed in formaldehyde, and then centrifuged. This procedure removed serum contaminants that non-specifically bound EPEC. The primary antibodies and concentrations used in this study were as follows: α-Tir pAb (1:2000 for microscopy) from Jim Kaper, and α-phosphotyrosine mAb 4G10. Secondary antibodies were obtained from Jackson Immunochemicals. Images were acquired with a scientific-grade cooled charge-coupled device (Cool-Snap HQ with ORCA-ER chip) on a multiwavelength wide-field three-dimensional microscopy system (Intelligent Imaging Innovations) based on a Zeiss 200 M inverted microscope using a 63× N.C14 lens (Zeiss). Immunofluorescent samples were imaged at room temperature using a standard Sedat filter set (Chroma) in successive 0.25 μM focal planes through the samples, and out-of-focus light was removed with a constrained iterative deconvolution algorithm (Swedlow *et al*., 1997).

Statistical analysis

Error bars depict the standard deviation. Experiments were generally repeated a minimum of three times. At least 90 worms were assessed in each experimental condition. The significance of differences between the means of two variables was determined using the Student's *t*-test with unpaired values and a two-tailed hypothesis (GraphPad).

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Supplementary material

The following supplementary material is available for this article on line.

Fig. S1. Quantification of the percentage of cells with normal pedestals upon infection with EPEC, EPEC mutants, K12, or combinations of EPEC and K12.

Fig. S2. Hypoxic death induced by exposure to sodium azide.

Table S1. PCR primers used in this work.

Table S2. Composition and preparation of various media.