**Caenorhabditis elegans** star formation and negative chemotaxis induced by infection with corynebacteria

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**INTRODUCTION**

*Caenorhabditis elegans* is a nematode with global distribution that lives by eating bacteria. Based on advantageous properties such as short life span, transparency, genetic tractability and ease of culture using an *Escherichia coli* diet. In its natural habitat, compost and rotting plant material, this nematode lives on bacteria. However, *C. elegans* is a predator of bacteria, but can also be infected by nematopathogenic coryneform bacteria such *Microbacterium* and *Leucobacter* species, which display intriguing and diverse modes of pathogenicity. Depending on the nematode pathogen, aggregates of worms, termed worm-stars, can be formed, or severe rectal swelling, so-called Dar formation, can be induced. Using the human and animal pathogens *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* as well as the non-pathogenic species *Corynebacterium glutamicum*, we show that these coryneform bacteria can also induce star formation slowly in worms, as well as a severe tail-swelling phenotype. While *C. glutamicum* had a significant, but minor influence on survival of *C. elegans*, nematodes were killed after infection with *C. diphtheriae* and *C. ulcerans*. The two pathogenic species were avoided by the nematodes and induced aversive learning in *C. elegans*.

*Abbreviation:* Dar, deformed anal region.
from contaminated lab cultures of the nematode (Hodgkin et al., 2000). Infection of *C. elegans* with this coryneform worm pathogen results in a distinctive phenotype, a deformed anal region (Dar). *C. elegans* mutants with altered response to *M. nematophilum* infection were isolated from screens for a bacterially unswollen (Bus) phenotype. The majority of these mutants exhibited little or no rectal infection when challenged with the pathogen and are probably not colonized by the bacteria (Gravato-Nobre et al., 2005; Yook & Hodgkin, 2007). Manifestation of infection depends on colonization of the rectum of the nematode and involves tight adherence of the bacteria to rectal and anal regions. Infected animals become mildly constipated and display reduced growth and fertility (Akimkina et al., 2006). Later, natural *C. elegans* populations, which showed Dar formation due to infection with *Leuconostoc* strains, were identified. One bacterium isolated from an aviary in Japan caused symptoms similar to *M. nematophilum* infection, while two strains isolated from worms from rotting banana trunks in Cape Verde exerted complementary virulence on *C. elegans*. In contrast to the former isolates, these strains, designated Verde1 and Verde2, were highly virulent and lethal to the nematodes (Hodgkin et al., 2013). A hallmark of Verde1 infection in liquid culture is the formation of aggregates of nematodes sticking together at their tails, termed worm-stars. In contrast, Verde2 infection results in distorted internal organs and vacuole formation within the worms, as well as a Dar phenotype (Hodgkin et al., 2013). While these coryneform strains cause interesting disease phenotypes in *C. elegans*, they are genetically intractable and little is known about their biology. It is therefore convenient to study these phenotypes using a well-characterized bacterial genus such as *Corynebacterium*. The inclusion of human-pathogenic coryneform strains in the *C. elegans* model also enhances the potential clinical relevance of these disease phenotypes.

In this study, we provide data on the species-specific colonization of *C. elegans*, localization of *Corynebacterium* infection and chemotactic behaviour of the nematodes towards different corynebacteria. Three species were tested for this purpose: *C. diphtheriae*, an important pathogen and the type strain of the genus; *Corynebacterium ulcerans*, a commensal corynebacterium isolated from a wide variety of domestic and wild animals, which shows zoonotic transmission to humans and is increasingly recognized as emerging pathogen; and *Corynebacterium glutamicum*, a non-pathogenic member of the genus, applied as a biotechnology workhorse (Becker & Wittmann, 2012; Burkovski, 2015) and isolated originally from a soil sample (Kinoshita et al., 1957).

### METHODS

**Strains and growth conditions.** Strains used in this study are listed in Table 1. *C. diphtheriae* and *C. ulcerans* were grown in heart infusion (HI) broth at 37 °C, *C. glutamicum* was grown in brain heart infusion broth at 30 °C and *Escherichia coli* OP50 was grown in Luria broth at 37 °C (Sambrook et al., 1989). If appropriate, kanamycin 50 μg ml⁻¹ or chloramphenicol 25 μg ml⁻¹ was added. *C. elegans* N2, used as the WT strain, and the respective mutant strains were maintained and propagated on *E. coli* OP50 as described (Brenner, 1974).

**Infection of *C. elegans***. *C. elegans* N2 were maintained on agar plates inoculated with *E. coli* strain OP50 for 3–7 days until the worms become starved, indicated by clumping behaviour (de Bon & Bargmann, 1998). Subsequently, the nematodes were infected with different *Corynebacterium* strains transformed with pXMJ19mcherry, as well as *E. coli* OP50. Infection of 20 L4 stage larval worms was carried out with 20 μl of each bacteria strain (from an overnight culture) on NGM plates at 21 °C for 24 h. Worms were then transferred to plates with 20 μl of unlabelled *E. coli* OP50 for a further 24 h, to allow the gut to clear of fluorescent organisms and cell debris. Worms were assessed each day following infection. Transfer back to *E. coli* OP50 is essential following infection, as corynebacteria are not a preferred prey source for *C. elegans*, and continued feeding results in the nematodes attempting to leave the culture plates. Nematodes were paralysed with

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5–10 μl of 0.6% 2-phenoxy-2-propanol (Sigma; Wormbook.org) or 20 mM sodium azide, mounted onto agar pads and photographed using a Leica DMR fluorescence microscope.

To investigate worm-star formation, worms were exposed to bacterial cells in M9 buffer based on methods adapted from Hodgkin et al. (2013). Briefly, adult worms were washed three times in M9 buffer and aliquots of 100 μl were placed into 500 μl of M9 buffer in each well of a 24-well microplate. Aliquots of 0.05 ml, 0.1 ml and 0.2 ml of bacterial strains grown in 20 ml HI medium overnight were placed in each well. Worm-star formation was initially observed 2 days post-incubation at 21 °C. The interaction between nematodes and bacteria was inspected with light microscopy (Nikon C-DSD 230). Worm aggregates were picked out of liquid onto an agar plate and photographed as described above.

**Nematode killing assay.** *C. elegans* N2 was maintained on *E. coli* OP50 for 6–7 days until the worms became starved, as described above (de Bono & Bargmann, 1998). Infection of L4 stage larval worms was carried out with 20 μl of each bacterial strain (from an overnight culture) on NGM plates at 21 °C for 24 h. Worms were assessed each day following infection, and the dead nematodes were counted and removed every 24 h. For each strain, approximately 60 nematodes were used and the assays were performed three times. Kaplan–Meier survival analysis was used and the curves were compared with the logrank test. The statistical analysis was performed with Prism 5.0 (GraphPad), with P-values of less than 0.05.

**Chemotactic behaviour.** Chemotaxis and learning behaviour was analysed as described previously (Zhang et al., 2005). The chemotactic behaviour of the nematodes towards different corynebacteria was determined based on a choice index with −1.0 representing a complete preference for *C. glutamicum* or *E. coli*, the control bacterium used, an index of 1.0 representing complete preference for the test bacterium and an index of 0 representing an equal distribution.

Two bacterial suspensions (20 μl, OD<sub>600</sub>=1.0) were spotted onto NGM plates for 24 h at 37 °C. Adult worms were washed twice in small drops of M9 buffer containing 25 μg ml<sup>−1</sup> nalidixic acid to kill adhering *E. coli* from the feeding plate. Twenty animals were placed near the centre of the plate at equal distance to the two spots of bacteria. For training, a suspension of 200 μl of the test bacteria was spread on a plate and 50 μl of OP50 suspension was used to place a small lawn on the side. Plates were incubated at 37 °C for 24 h before

![Fig. 1. Induction of worm-star formation by corynebacteria. Aggregates of worms were observed after incubation for about 2–4 days in liquid medium in the presence of *C. glutamicum* ATCC13032 (a–c), *C. diphtheriae* CDC-E8392 (d–f), *C. ulcerans* 809 (g–i) and BR-AD22 (j–l).](image-url)
use. The nematodes were allowed to move freely on the plate, counted after 1, 2 and 24 h for the training assay and counted after 24 h for the index choice measurement. Analysis by *t*-test was performed with Prism 5.0 (GraphPad), with *P*-values of less than 0.05.

**RESULTS**

**Worm-star formation by Corynebacterium infection**

An obvious and striking symptom of surface colonization of *C. elegans* by certain pathogenic bacteria is the formation of aggregates of worms sticking together at their tails. This phenomenon of worm-star formation was described, until now, only for *Leucobacter* Verdel infection of nematodes. Worms trapped in the stars usually cannot escape, and die within 24 h. The bacteria then use the worm carcasses as a nutrient source for growth. Interestingly, when *C. elegans* was brought into contact with the investigated corynebacteria, all species including the *C. ulcerans* strains isolated from a human (809) and animal (BR-AD22) source were able to induce worm-star formation (Fig. 1). Aggregates of worms were observed after incubation for about 2–4 days in liquid culture in the presence of *C. glutamicum* ATCC13032 (Fig. 1a–c), *C. diphtheriae* CDC-E8392 (Fig. 1d–f), *C. ulcerans* 809 (Fig. 1g–i) and BR-AD22 (Fig. 1j–l). Compared to *Leucobacter* Verdel infection, star formation was markedly slower and tail-to-tail attachment due to corynebacterial colonization was weaker, since worms were able to escape the stars at least some of the time. The stars observed were formed by adult and larval state nematodes (Fig. 1b, f, i, k). No differences in respect to the period of time for or the extent of worm-star formation were observed. In all cases, nematodes started to break and lyse after prolonged incubation and stars dissolved due to the destruction of the worms (Fig. 1c, l). Worm-stars were not observed during cultivation with *E. coli* OP50 (data not shown).

**Dar induction by corynebacteria**

Compared to worm-star formation, the induction of tail swelling (Dar phenotype), previously described as a result of *M. nematophilum* and *Leucobacter* Verdel infections, is a more subtle symptom. In this case, adherence of bacteria to the rectal and post-anal cuticle of *C. elegans* induces local swelling of the underlying hypodermal tissue resulting in significant morphological changes (Hodgkin et al., 2000). Corynebacterial biofilms are already visible by light microscopy (Fig. 2b–d). However, the molecular background of colonization is unknown and needs further investigation, e.g. scanning electron microscopy approaches, fluorescence microscopy of biofilm formation and mutant analyses.

As in the case of worm-star formation, all *Corynebacterium* species tested induced Dars approximately after 2 days of infection of worms cultivated on NGM plates or in liquid medium (Fig. 2). However, significant species-specific differences were observed. Dar formation in liquid medium was most pronounced in the case of *C. ulcerans* BR-AD22 and 809 (Fig. 2d, e), followed by *C. glutamicum* ATCC 13032 (Fig. 2b) and *C. diphtheriae* CDC-E8392 (Fig. 2c). The highest rates were observed upon infection with *C. ulcerans* BR-AD22, with 5–43 % of worms showing Dars, followed by *C. ulcerans* 809 with 10–40 %, *C. glutamicum* ATCC 13032 with 6–26 % and *C. diphtheriae* CDC-E8392 with 3–4 % of nematodes showing rectal swelling after 72 h exposure. The fluctuating values for Dar formation in the four independent biological replicates carried out might indicate an influence of worm development on susceptibility and colonization. Furthermore, Dar formation was more abundant on solid
Colonization of *C. elegans* N2

Light and fluorescence microscopy experiments were carried out in order to investigate morphological effects induced by colonization of *C. elegans* N2 by corynebacteria (Fig. 3). Worms were infected with the indicated mCherry-labelled bacteria for 48 h and colonization of different parts of the worms was analysed by fluorescence microscopy using identical settings for all samples. Strong colonization of all regions of the worm was observed for *C. diphtheriae* CDC-E8392 and *C. ulcerans* BR-AD22 (Fig. 3b, d), followed by *C. ulcerans* 809 (Fig. 3c), while worms colonized with *C. glutamicum* ATCC 13032 showed the weakest fluorescence signals (Fig. 3a). At 48 h post-infection, worms on all *Corynebacterium* strains began to exhibit distension of the gut, perhaps because of this, reduced movement around the plate. Many adult worms additionally exhibited a Dar phenotype. If larvae were present on the plate, they frequently also exhibited a Dar phenotype, but with less apparent distension of the gut and no obvious impairment of motility. In summary, at the 48 h time point, differences were discernible between the effects of the different bacterial strains, with *C. glutamicum* inducing milder symptoms than *C. diphtheriae*, and the two *C. ulcerans* strains inducing the most severe symptoms across all worm strains. Examination by microscopy at the 72 h time point revealed a large number of bacteria in the guts of the worms, and what appear to be bacteria adhering to the post-anal cuticle (data not shown).

Worms proliferated most successfully on *C. glutamicum*, and by 10 days post-infection there were more progeny and fewer Dauer worms on *C. glutamicum* plates than on the other bacterial strains, suggesting the worms were more successful in obtaining nutrition from *C. glutamicum* than from *C. diphtheriae* or *C. ulcerans* (data not shown).

In a subsequent assay, the worms infected for 48 h were placed on plates with unlabelled *E. coli* OP50 for a further 24 h to investigate persistence of colonization in the absence of external corynebacteria (Fig. 3f–h). In this experimental set-up, *C. glutamicum* was unable to persist within the worms (Fig. 3e), while colonization with *C. diphtheriae* CDC-E8392 was completely resistant to the possibility of new infection and uptake of OP50 (Fig. 3f). *C. ulcerans* strains 809 and BR-AD22 also persisted even in the presence of *E. coli*, although slightly less successfully than *C. diphtheriae* (Fig. 3g, h).

Survival of *C. elegans* in response to corynebacterial contact

As shown above, corynebacteria are able to colonize *C. elegans* and evoke morphological changes as well as altered movement behaviour. In order to study putative detrimental effects of *C. elegans* colonization, survival of nematodes in relation to bacterial contact was determined. As expected, *E. coli* OP50 had no detrimental effects on the worms. A significant, but minor influence of *C. glutamicum* ATCC13032 was observed, while *C. diphtheriae* CDC-E8392 as well as *C. ulcerans* 809 and BR-AD22 impaired survival of *C. elegans* dramatically. After 5 days post-infection, about 70% mortality was observed for worms infected with *C. diphtheriae*, and about 90% in the case of the two *C. ulcerans* strains (Fig. 4). These results correlated with the extent of colonization and persistence within the worms (Fig. 3).

Adult *C. elegans* worms normally lay eggs that hatch outside the parental body, but internal egg hatching, so-called ‘worm bagging’, was reported to be induced at a high frequency by exposure to pathogenic bacteria, e.g. virulent *E. coli* strains and *Enterococcus faecalis*, and can be regarded as a reliable population-wide stress reporter (Mosser et al., ...)
Fig. 5. Chemotactic behaviour of C. elegans. Nematodes were transferred to plates with combinations of C. glutamicum and pathogenic corynebacteria (a) or E. coli and different Corynebacterium species (b) as indicated. A choice index was calculated as described in Methods, with a value of −1.0 representing a complete preference for the control bacterium (C. glutamicum or E. coli), an index of 1.0 representing complete preference for the test bacterium and an index of 0 representing an equal distribution. For each strain, approximately 20 nematodes were used and the assays were performed in three independent experiments. A t-test analysis was performed with P-values of less than 0.05 (indicated by asterisks). Bars represent mean values ± SEM.
in the worm (Fig. 3).

on nematode survival, most likely due to its poor persistence strains, while no preference was observed compared to species and preferred the nematodes avoided the more detrimental pathogenic strains, the preference of nematodes for different species in order to avoid more pathogenic coryneform bacteria such as Microbacterium and physical protection for the small larvae under this condition (Chen & Caswell-Chen, 2003). Therefore, in addition to the killed worms, worm bagging was also scored as dead for the survival assay in this study.

**Chemotactic behaviour of worms**

As indicated above, corynebacteria are able to infect *C. elegans* and induce morphological changes similar to those previously described for other nematode pathogens. Consequently, it would be beneficial for *C. elegans* to avoid these bacteria. As shown previously, *C. elegans* is able to avoid pathogenic *Pseudomonas aeruginosa* strains and shows aversive olfactory learning (Zhang et al., 2005). In order to investigate if *C. elegans* is able to distinguish different Corynebacterium species in order to avoid more pathogenic strains, the preference of nematodes for *C. glutamicum* ATCC13032 and *C. diphtheriae* CDC-E8392, *C. ulcerans* 809 or BR-AD22 was tested. It was found that the nematodes avoided the more detrimental pathogenic species and preferred the *C. glutamicum* ATCC13032 strain (Fig. 5a). A similar behaviour was found for the choice between *E. coli* and corynebacteria. Strain OP50 was clearly preferred when tested versus *C. diphtheriae* and *C. ulcerans* strains, while no preference was observed compared to *C. glutamicum* (Fig. 5b), which showed only a minor effect on nematode survival, most likely due to its poor persistence in the worm (Fig. 3).

Moreover, the worms showed learning behaviour. For this set of experiments, worms with different cultivation histories (OP50 grown, unstarved/starved, without and with previous contact to *C. diphtheriae*) were brought into contact with *C. diphtheriae* CDC-E8392. Worms that were previously in contact with pathogenic corynebacteria, i.e. trained specimens, avoided these at early time points of contact in contrast to untrained individuals. However, after 24 h, all worms avoided *C. diphtheriae* (Fig. 6). Taken together, the experiments suggest learning of nematodes to avoid tainted, detrimental food sources.

**DISCUSSION**

*C. elegans* lives on bacteria but also can be infected by nematopathogenic coryneform bacteria such as *Microbacterium* and *Leucothrix* species. The nematodes are a well-established infection model system for many pathogenic bacteria (Clark & Hodgkin, 2014) including *C. diphtheriae* (Ott et al., 2012; Broadway et al., 2013; Antunes et al., 2015; Santos et al., 2015). Interestingly, in this study, worm-star and Dar formation, morphological changes typical for nematopathogenic bacteria, were observed with both pathogenic and non-pathogenic corynebacteria. To our knowledge, this has never been observed before for other pathogenic bacteria like *Serratia* or *Pseudomonas* species. The grade of morphological changes did not correlate with survival, since *C. glutamicum* had only a minor effect compared to strong negative influences of *C. diphtheriae* and *C. ulcerans*. Interestingly, *C. elegans* is able to distinguish between *E. coli* or
C. glutamicum, and strongly detrimental bacteria such as C. diphtheriae and C. ulcerans. The feeding strain OP50 and C. glutamicum, which has only a minor effect on survival and is a poor gut colonizer, are preferred in comparison to C. diphtheriae and C. ulcerans, which showed strong negative influences on nematode survival. The worms showed learning behaviour as described previously for other bacterial species including Pseudomonas fluorescens and Serratia marcescens (Zhang et al., 2005). The molecular background of aversive learning with respect to pathogenic Corynebacterium species needs to be elucidated, and stimuli as well as signal pathways have still to be unravelled.

Interactions between coryneform bacteria and soil nematodes are likely to be ecologically important and diverse, in view of the abundance of these bacteria in soil. The described species are likely to be ecologically important and diverse, in view of the abundance of these bacteria in soil. The abovementioned species needs to be elucidated, and stimuli as well as signal pathways have still to be unravelled.

In conclusion, the interaction of C. elegans with bacterial pathogens at an organismal level.

**REFERENCES**


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