# Mimicry of a G Protein Mutation by Pertussis Toxin Expression in Transgenic *Caenorhabditis elegans*

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Pathogens produce virulence factors that interact directly with host molecules, but in many cases the host targets are unknown. The genetic and molecular identification of these orphan targets is often not feasible with mammalian experimental models. However, a substantial number of known targets are molecules and pathways that are conserved among eukaryotes, and therefore the use of nonmammalian model hosts to identify orphan targets may prove useful. To demonstrate the feasibility of this approach, we transformed the nematode *Caenorhabditis elegans* with a gene encoding the catalytic subunit of pertussis toxin (PTX), which in mammals inactivates $G_{o/i}\alpha$ proteins. Expression of PTX in *C. elegans* produced phenotypes almost identical to those of a null mutation in the nematode gene encoding $G_{o/i}\alpha$. Furthermore, PTX suppressed the phenotype of a constitutively active form of nematode $G_{o/i}\alpha$ protein. These results indicate that PTX is functional in nematodes and acts specifically on the *C. elegans* homologue of the mammalian target.

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The biochemical function and host target of many microbial virulence factors remain unknown. Genetic screening using randomly mutagenized organisms is a powerful tool for identifying components of a pathway, but this use of genetics is not feasible for most experimental animals or mammalian cell culture systems used for infectious disease. An alternative strategy for identifying host targets of pathogens is to use a highly tractable genetic model organism, such as the nematode *Caenorhabditis elegans*.

The advantages of *C. elegans* as a model organism have been described in detail (6, 22, 30). The microscopic animals are grown at low cost on Petri plates, with a generation time of 3 days and broods numbering about 200 progeny. The cellular anatomy of wild-type *C. elegans* is essentially invariant and has been described completely. Because the nematodes are transparent, internal tissues of live specimens can be observed by microscopy and the animals can be recovered for further analysis. *C. elegans* grows as either a self-fertilizing hermaphrodite or in crosses between males and hermaphrodites, facilitating classical genetic analysis and manipulation. The genome of *C. elegans* has been sequenced (3). Nematodes can be stably transformed by exogenous DNA (18), reverse genetics can be used to create mutations in a specific gene (20), and the functions of many genes can be blocked by RNA interference (7, 9). Transcription throughout the genome can be analyzed using microarrays (21).

These advantages have led a number of researchers to develop experimental systems that use *C. elegans* to investigate bacterial pathogenesis. A slow-killing infection of *C. elegans* by *Pseudomonas aeruginosa* has been described earlier (26, 27); in addition, there are two distinct ways in which *P. aeruginosa* kills *C. elegans* rapidly with small-molecule toxins (5, 17). For one mode of toxin-mediated *Pseudomonas* killing, a mutation in

*C. elegans* that confers strong resistance has been identified (5), suggesting that genetic analysis of the nematode will be useful for understanding the host-pathogen interaction. Two research groups recently reported that *Salmonella enterica* serovar Typhimurium colonizes the nematode gut and eventually kills the animals (1, 15). Infection of *C. elegans* by a bacterium that is apparently a natural parasite of nematodes but is not known to be a pathogen of mammals also has been described previously (12).

In the present work, we describe a strategy that allows *C. elegans* to be used to search for host targets regardless of whether intact bacteria or their secretions have any effect on the nematodes. In this method, *C. elegans* is transformed with a bacterial virulence gene so that the nematodes themselves manufacture the virulence protein in their cytoplasm. A phenotype resulting from expression of the bacterial gene suggests that the virulence factor has a target that is conserved between mammals and nematodes. In these cases, host factors in the target pathway can be identified by screening *C. elegans* for mutations that alter the phenotype produced by the virulence gene.

Pertussis toxin (PTX) is well characterized biochemically and has a known target (8). PTX is an ADP-ribosylating enzyme that acts on a subset of heterotrimeric G proteins; the toxin covalently modifies $G_{o/i}\alpha$ subunits and thereby inactivates their signalling. We show that when PTX expression is induced in transgenic *C. elegans*, phenotypes appear that are almost identical to those of mutations in the nematode gene encoding $G_{o/i}\alpha$, indicating that the toxin interacts in vivo with its predicted nematode target.

## MATERIALS AND METHODS

**Nematode strains and culture.** *C. elegans* strains, genotypes, and sources are listed in Table 1; a standard nomenclature has previously been described (13, 22). Nematodes were grown at room temperature (approximately 22°C) on NGM agar seeded with *Escherichia coli* strain OP50 (30).

**Construction of PTX expression vector.** The *ptx* gene, encoding the catalytic subunit S1 of PTX, was amplified from plasmid pPTX42 (16) by PCR using primers 5'-CGGGATCCATGGACGATCCTCCCGCCACCGTATACC and 5'-GGGGTACCCTAGAACGAATACGCGATGCTTTCGTAG. The PCR prod-

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TABLE 1. *C. elegans* strains[a]

| Strain | Genotype | Source |
|--------|----------|--------|
| N2 | Wild type | CGC |
| CB251 | *unc-36(e251)* | CGC |
| CB363 | *goa-1(n363)* | CGC |
| XA1413 | *unc-36(e251); qaEx1410[unc-36(+) hsp16-2::ptx]* | This study |
| XA1433 | *unc-36(e251); qaIs1400[unc-36(+) hsp16-2::ptx]* | This study |
| PS1493 | *dpy-20(e1362); syIs9[dpy-20(+) goa-1(Q205L)]* | P. Sternberg |

[a] CGC, *Caenorhabditis* Genetics Center, University of Minnesota, St. Paul. *Ex*, extrachromosomal transgenic array. *Is*, chromosomally integrated transgenic array.

uct lacked all nucleotides encoding the cleavable signal sequence except an ATG to encode an initial methionine. Restriction sites for *Bam*HI and *Kpn*I were included in the primers, and the product was cloned using those enzymes in the *C. elegans* expression vector pPD49.78 (18). The insert in the resulting plasmid, pCBD14, was verified by DNA sequencing.

**Transformation of nematodes.** Standard methods of transformation were used (18). Parent strain CB251 has an uncoordinated (Unc) phenotype because of a mutation in the gene *unc-36* (2, 22). Transformation with R1p16, a plasmid containing the wild-type *unc-36* gene, restores CB251 to wild-type locomotion (11). A DNA solution containing 50 ng of R1p16 per μl and 50 ng of pCBD14 per μl was injected into the gonad of parental worms, and F1 progeny that were non-Unc were propagated. Strains, including XA1413, were established when the array was transmitted to F2 and subsequent generations. Presence of the *ptx* gene was confirmed by PCR.

**Integration of *ptx*-carrying array.** In strain XA1413 the transgenic array that includes *ptx* is transmitted to approximately 16% of progeny. L4 stage XA1413 animals were gamma irradiated at a dose of 4,500 rads. F1 progeny were transferred to individual plates, and their broods were screened for an array transmission frequency of about 75%, indicating heterozygous integration into a chromosome. From these heterozygotes, numerous F2 progeny were picked, allowed to self-fertilize, and screened for 100% transmission, indicating homozygosity of the integrated array. A line thus established was outcrossed six times to *unc-36* mutants to remove secondary mutations induced by radiation, and the presence of *ptx* was confirmed by PCR and Southern hybridization (data not shown). The resulting strain, XA1433, was used for all reported experiments.

**Immunoblotting.** Strains N2 and XA1433 were grown to near saturation on duplicate 10-cm-diameter plates of NGM agar and *E. coli* OP50. One plate of each strain was heat shocked at 33°C for 2 h while the second plate was kept at room temperature. The worms were collected in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and washed once in TE, and then they were washed twice more in TE to which was added 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, and 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) detergent. Samples were brought to 80 μl of densely packed worms, 20 μl of 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added, and the samples were boiled for 10 min. Each lane of a 12.5% gel was loaded with 20 μl. Purified PTX subunit S1 (List Biological Laboratories) was used as a positive control. After electrophoresis and transfer to a membrane, protein was detected using a monoclonal antibody to S1 (gift of R. Rappuoli) diluted 1:500. Donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) conjugated to horseradish peroxidase was diluted 1:50,000. Signal was detected by chemiluminescence (ECL+; Amersham).

**Heat shock assays.** Nematodes were washed from a stock plate into M9 buffer (30) and aliquoted to two microfuge tubes. One tube was incubated in a water bath at 33°C for 20 min, while the control tube was kept at room temperature. Nematodes were pipetted to fresh NGM plates seeded with *E. coli* OP50. Locomotion was observed and photographed beginning 2 h after heat shock. Measurement of the pharyngeal pumping rate was performed as described previously (10) in the interval 4 to 8 h after heat shock.

**Expression of *ptx* in constitutive *goa-1* background.** Hermaphrodites of strain PS1493, carrying the constitutively active *goa-1* construct *syIs9* (19), were mated either by XA1433 males carrying *ptx* or by wild-type control males. PS1493 worms have a dominant sluggish phenotype that prevents distinguishing their cross-progeny from self-progeny directly. Therefore, virgin (L4 stage) hermaphrodite progeny were separated from their male siblings; the next day, when the hermaphrodites had matured into adults, they were heat shocked, picked to individual plates, examined, and photographed. Three days later the plates were scored for segregation of the background mutations in the parent strains used to create the transgenic lines, i.e., *unc-36* from XA1433 and *dpy-20* from PS1493

(19). Only cross-progeny segregated the markers; data from self-progeny were disregarded.

## RESULTS

**The *C. elegans* genome contains a single putative target of PTX.** PTX attaches an ADP-ribose moiety to a cysteine residue four amino acids from the carboxyl terminus of target $G_{o/i}\alpha$ proteins (28). The genome of the nematode *C. elegans* has been sequenced (3), and analysis showed that there are 20 predicted Gα proteins (14). Examination of the predicted amino acid sequences indicated that only one, encoded by the gene *goa-1*, is in the $G_{o/i}\alpha$ class; it is also the only *C. elegans* Gα in which cysteine is the fourth amino acid from the carboxyl terminus. GOA-1 protein is expressed in virtually all neurons, many muscles, and some miscellaneous cells of the nematode (19, 23).

Mutations in *goa-1* have been characterized previously (19, 23). Animals with the deletion allele *goa-1(n363)* move hyperactively, with an increase in their body flexion, but their feeding (by contraction of pharyngeal muscles) is slowed (23). Nematodes with a constitutively active *goa-1* mutation have an opposite locomotion phenotype, moving sluggishly with little flexion (19). Thus, $G_{o/i}\alpha$ in *C. elegans* is a modulator of several neuromuscular functions.

**Nematodes transformed with the *ptx* gene express PTX.** We predicted that if PTX functions normally in *C. elegans*, it would inactivate GOA-1 protein, thereby producing phenotypes similar to those of the deletion mutation *goa-1(n363)*. If PTX affects only GOA-1, expression would not be expected to be lethal, because *goa-1* deletion mutants are viable. However, the possibility existed that PTX expression would have pleiotropic effects, possibly leading to lethality. We therefore used an inducible expression system. The *ptx* gene was cloned downstream of nematode promoter *hsp16-2*, which is induced by heat shock and, like *goa-1*, functions in neurons and muscles (18, 25). The construct, pCBD14, encodes a protein identical to the mature form (i.e., after signal sequence cleavage) of the PTX catalytic subunit S1, except for the addition of a methionine residue at the amino terminus.

We chose as a transformation marker the *unc-36* gene, which encodes a predicted calcium channel (22). *unc-36* mutants are healthy but severely uncoordinated (Unc phenotype), and their locomotion defect is readily distinguished from the hyperactivity of *goa-1* mutants. *unc-36* mutants transformed with the wild-type *unc-36* gene are restored to normal locomotion and are indistinguishable from the wild type. Transgenic nematodes were generated by injecting a DNA mixture of pCBD14 and an *unc-36(+)* clone into the gonads of parental *unc-36* mutants. F1 progeny that inherited the *unc-36(+)* marker were easily identified because they had wild-type locomotion in a background of Unc animals. Transgenic lines were established in cases in which the marker was inherited by subsequent generations. Eight independent lines were generated, and the presence of the *ptx* gene was confirmed in each by PCR (data not shown). Preliminary characterization indicated that the phenotype expected after heat shock, hyperactive locomotion, was present (data not shown).

In *C. elegans*, exogenous transforming DNA assembles into an extrachromosomal tandem array that is unstable mitotically
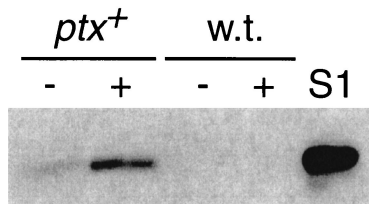
FIG. 1. Expression of PTX in transgenic *C. elegans*. Immunoblot showing the catalytic subunit of PTX in nematodes without (−) or with (+) heat shock. *ptx*+, nematode strain XA1433 carrying the *ptx* gene under a heat shock promoter. w.t., wild-type nematode strain N2. S1, 500 pg of purified catalytic subunit S1. Brightness and contrast were adjusted using Adobe Photoshop.

and inherited by less than 100% of progeny; the frequency of transmission varies widely between strains (18). Although strains with unstable arrays can be maintained indefinitely by propagating animals carrying the marker, any given nematode can be a genetic mosaic that has the array in only some cells, and this variability hampers analysis. Arrays can be stabilized, however, by using gamma irradiation to integrate them into the *C. elegans* genome, after which they are 100% heritable and contained in every cell of the nematode (18). We integrated the *ptx* array of strain XA1413, outcrossed the nematodes multiple times to remove secondary mutations induced by radiation, and confirmed the presence of *ptx* by PCR and Southern hybridization (data not shown). All subsequent analyses were performed using the resulting strain, XA1433.

Without heat shock, nematodes carrying the *ptx* gene expressed the toxin at a level barely detectable by immunoblotting (Fig. 1), but no phenotypes were observed. Heat shocking of transgenic nematodes greatly increased the expression of PTX (Fig. 1), indicating that the heat shock promoter functions as expected.

*C. elegans* **expressing PTX mimics G$_{o/i}\alpha$ mutants.** Heat shocked XA1433 animals exhibited dramatic phenotypes. The body flexion of the nematodes was greatly increased compared to that of the wild type, which was observable in both the animals themselves and the tracks they left in a lawn of bacteria (Fig. 2, compare B to A). This phenotype strikingly resembles that of *goa-1* loss-of-function mutants (Fig. 2C). Wild-type controls did not display this phenotype after heat shock (Fig. 2A), nor did transgenic animals carrying the *unc-36*(+)

transformation marker but not *ptx* (data not shown). One difference between PTX-expressing worms and *goa-1* mutants was observed. Although both have greatly increased body flexion, *goa-1* mutants roam widely across a lawn of bacteria, whereas PTX-expressing worms move back and forth in one spot, thus obliterating their own tracks (Fig. 2B).

To quantify the effects of PTX expression we measured pumping, the rate of pharyngeal contraction by which *C. elegans* feeds on bacteria. The pumping rate of a *goa-1* deletion mutant is strongly reduced from that of the wild type (23). As shown in Fig. 3, XA1433 transgenic animals also had a markedly reduced pumping rate after heat shock induction of PTX. The transgenic strain pumped identically to the wild type in the absence of heat shock, and heat shock alone did not affect the pumping of either wild-type nematodes or the *goa-1* deletion mutants.

**Expression of PTX suppresses a constitutively active G$_{o/i}\alpha$ mutation.** The availability of *C. elegans* expressing a constitutively active form of GOA-1 provided a further test of the specificity of PTX activity. The transgene *syIs9* contains a glutamine-to-leucine mutation that locks GOA-1 protein in the GTP-bound conformation, making it constitutively active (19). Nematodes carrying this construct move sluggishly, with little body flexion (Fig. 4A), a phenotype opposite to that of loss-of-function mutants. We hypothesized that if PTX modifies GOA-1 in *C. elegans*, it will inactivate the constitutive protein and therefore suppress the sluggish phenotype. We mated hermaphrodites carrying *syIs9* to either XA1433 males carrying the *ptx* transgene or wild-type control males. When progeny were heat shocked, animals heterozygous for both *syIs9* and *ptx* were hyperactive, moved with increased body flexion, and were virtually identical to PTX-expressing worms that lacked *syIs9* (compare Fig. 4C to 2B). Control progeny, which were heterozygous for *syIs9* alone, retained the sluggish, low-flexion phenotype (Fig. 4B).

## DISCUSSION

The ability to identify bacterial virulence factors outpaces the ability to understand their modes of action. The advent of complete genome sequencing promises to make the full catalog of virulence factors known, but methods are still required to identify their host targets and biochemical functions. Most
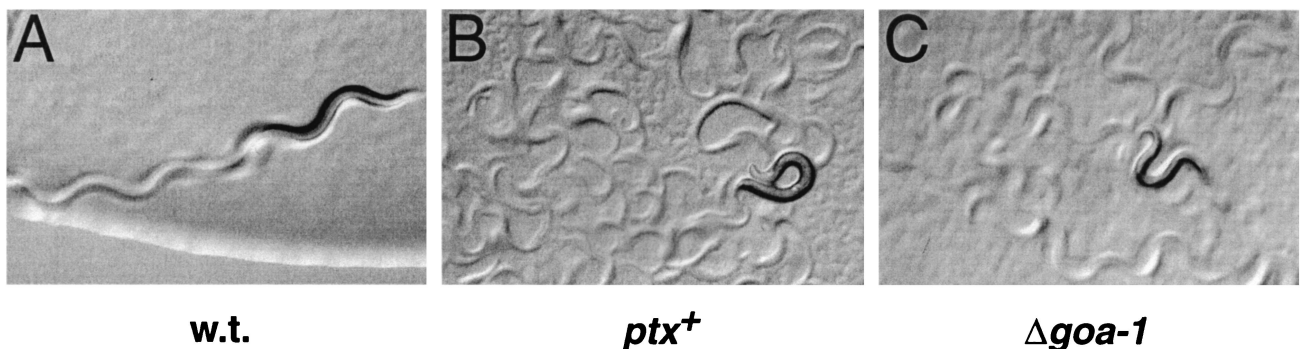


FIG. 2. Phenotypes of PTX expression. Adult nematodes were heat shocked, transferred to NGM agar seeded with *E. coli*, and photographed. (A) w.t., wild-type strain N2. (B) *ptx*+, strain XA1433, carrying a heat shock inducible *ptx* gene. (C) Δ*goa-1*, strain CB363, a *goa-1* deletion mutant. Color transparencies were digitally scanned, converted to grayscale, and adjusted slightly for brightness and contrast with Adobe Photoshop.
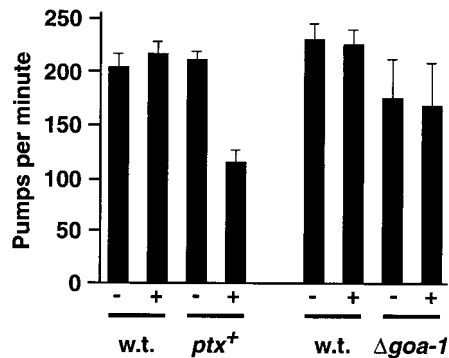
FIG. 3. Rates of pharyngeal pumping without (−) or with (+) heat shock treatment. w.t., wild-type strain N2. $ptx^+$, strain XA1433, carrying a heat shock inducible $ptx$ gene. $\Delta goa\text{-}1$, strain CB363 carrying a $goa\text{-}1$ deletion allele, $n363$. Each cluster of four shows assays on a separate day. Data are mean pumps per minute ± standard deviation for 12 animals.

experimental models for pathogenesis rely on live vertebrates or their cultured cells, and in these systems genetic manipulation of the host is often cumbersome and in some cases impossible.

Some researchers have turned to readily manipulated model organisms, such as *C. elegans*. Using these nematodes as host, experimental systems have been developed for the human pathogens *P. aeruginosa* (5, 17, 26, 27) and *S. enterica* serovar Typhimurium (1, 15) as well as for *Microbacterium nematophilum* (12), an apparently nematode-specific pathogen. These models require either that whole, live bacteria are pathogenic to *C. elegans* or that the bacteria produce a diffusible toxic substance.

Despite these successes, it seems unlikely that more than a few mammalian pathogens will affect *C. elegans* directly. Pathogenesis is a complex collection of sequential phenomena, including host recognition, adherence, virulence gene induction, virulence factor delivery, and evasion of host defenses. Bacteria that have evolved to perform these tasks in a mammal face a different set of challenges with a nematode. Importantly, *C. elegans* is a natural predator of bacteria, so that before a pathogen can recognize, adhere to, and colonize this host it

must avoid lysis and digestion by tissues highly specialized for those purposes.

In another sense, however, the differences between a nematode and a mammal are small. Both possess a collection of fundamental cellular processes that are highly conserved in eukaryotes despite huge evolutionary divergence of other characteristics. Translation of mRNA into protein, signal transduction through G proteins, and regulation of cytoskeletal structure are examples of such processes, and they are also examples of targets of bacterial virulence factors: diphtheria toxin and exotoxin A of *P. aeruginosa* inactivate elongation factor 2, an essential component of translation (29). PTX and cholera toxin modify heterotrimeric G proteins (8, 24). Yops produced by *Yersinia* spp. interact with the cytoskeleton (4).

It is therefore expected that the targets of many orphan virulence factors also will be conserved molecules and pathways. In these cases, expression of the virulence factor in nematodes is likely to produce an observable phenotype, thus permitting the use of *C. elegans* genetics to identify and analyze host targets. To demonstrate the feasibility of this approach, we have made transgenic *C. elegans* containing a gene for the catalytic subunit S1 of PTX. Toxin expression was made inducible by use of a heat shock promoter. In the absence of the induction signal no phenotype was observed, despite a very low level of basal expression. Induction of PTX produced two dramatic phenotypes: a strong increase in body flexion during locomotion and a strong reduction in pharyngeal contraction rate. These phenotypes are essentially identical to those of mutant nematodes that lack GOA-1, the protein predicted to be the target of PTX. The simplest explanation for these results is that PTX functions in vivo in *C. elegans* as it does in mammals, i.e., by inactivating $G_{o/i}\alpha$ subunits, thus mimicking the loss-of-function mutation. Because intensive study of *C. elegans* has provided a wealth of strains and reagents, we were able to conduct an additional test for the activity of PTX in nematodes. Animals expressing a constitutively active form of GOA-1 move sluggishly with little body flexion, a phenotype opposite to that of animals that lack the protein altogether. We found that PTX expression completely suppressed this sluggish phenotype and again mimicked the phenotype of $G_{o/i}\alpha$ deletion.
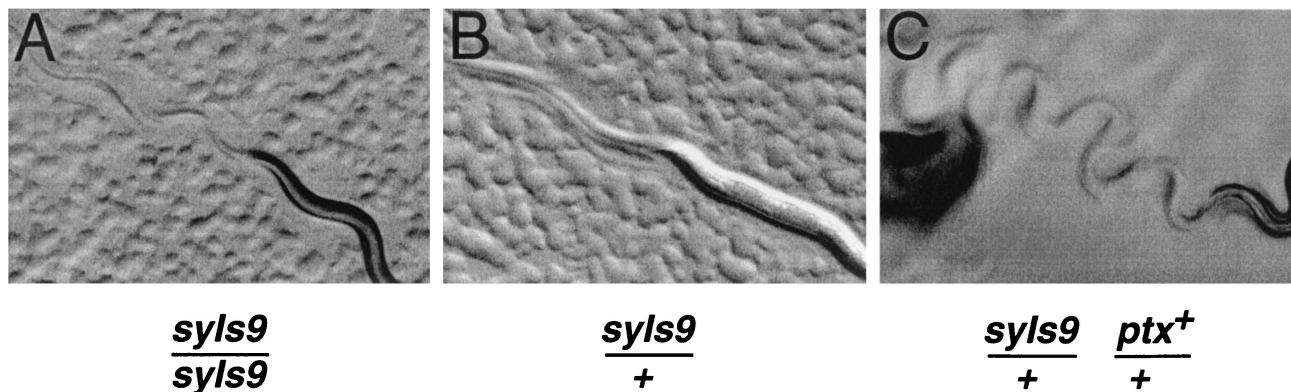


FIG. 4. Rescue of constitutive *goa-1* mutation by PTX expression. Nematodes were heat shocked, transferred to seeded NGM agar, and photographed. (A) Parental strain PS1493, carrying the constitutive *goa-1* transgene *syIs9*. (B) Progeny of PS1493 hermaphrodite and wild-type male. (C) Progeny of PS1493 and a *ptx* male. Genotypes of relevant integrated transgenes are indicated; marker genotypes are omitted for clarity. Images were processed as described in the legend to Fig. 2.

The most parsimonious interpretation of these results, taken as a whole, is that PTX finds the right target in *C. elegans*. Although the evolutionary distance between nematodes and mammals is huge, the $G_{o/i}\alpha$ protein is so sufficiently conserved that it functions as a PTX target in both organisms. This is despite the fact that live, virulent *Bordetella pertussis* cells, the source of PTX, have no effect on *C. elegans* (C. Darby, unpublished data). These experiments, therefore, establish the feasibility of expressing orphan virulence proteins in the nematode as part of a strategy to identify targets genetically, regardless of whether the bacterium in question is a nematode pathogen. When virulence factor expression produces a phenotype in *C. elegans*, nematodes can be screened for mutations that either abolish the phenotype or enhance it. Some of these mutations are expected to be in genes that encode either the direct target of the virulence factor or a component of a pathway that includes the target. Using well-developed techniques of *C. elegans* genetics, these genes can be identified and further characterized.

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