


Horizontally Acquired Cellulases Assist the Expansion of Dietary Range in *Pristionchus* Nematodes

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Associate editor: Ilya Ruvinsky

Abstract

Horizontal gene transfer (HGT) enables the acquisition of novel traits via non-Mendelian inheritance of genetic material. HGT plays a prominent role in the evolution of prokaryotes, whereas in animals, HGT is rare and its functional significance is often uncertain. Here, we investigate horizontally acquired cellulase genes in the free-living nematode model organism *Pristionchus pacificus*. We show that these cellulase genes 1) are likely of eukaryotic origin, 2) are expressed, 3) have protein products that are secreted and functional, and 4) result in endo-cellulase activity. Using CRISPR/Cas9, we generated an octuple cellulase mutant, which lacks all eight cellulase genes and cellulase activity altogether. Nonetheless, this cellulase-null mutant is viable and therefore allows a detailed analysis of a gene family that was horizontally acquired. We show that the octuple cellulase mutant has associated fitness costs with reduced fecundity and slower developmental speed. Furthermore, by using various *Escherichia coli* K-12 strains as a model for cellulosic biofilms, we demonstrate that cellulases facilitate the procurement of nutrients from bacterial biofilms. Together, our analysis of cellulases in *Pristionchus* provides comprehensive evidence from biochemistry, genetics, and phylogeny, which supports the integration of horizontally acquired genes into the complex life history strategy of this soil nematode.

Key words: *Pristionchus*, horizontal gene transfer, cellulase, biofilms, cellulose, gene duplication, animal evolution, microbe–animal interactions.

Introduction

Horizontal gene transfer (HGT) is common in prokaryotes and of substantial evolutionary significance in both bacteria and archaea (Koonin et al. 2001; Timmis et al. 2004). In contrast, HGT in eukaryotes was long thought to be rare, but new evidence indicates that HGT occurs more frequently than it was previously presumed and it might play a role in eukaryotic evolution (Dunning Hotopp et al. 2007; Dunning Hotopp 2011; Nakabachi 2015; Husnik and McCutcheon 2018). For example, multiple HGT events have been identified in arthropods and nematodes. Recent studies in centipedes (Undheim and Jenner 2021), whiteflies (Xia et al. 2021), ticks (Hayes et al. 2020), and mealybugs (Husnik et al. 2013) provide strong evidence that horizontally acquired genes can provide recipients with adaptive traits in diverse biological processes. Nematodes have also horizontally acquired genes encoding a variety of enzymes, but the evolutionary significance was so far mostly restricted to the facilitation of plant parasitism (Baldwin et al. 2004; Danchin et al. 2010), with the exception of HGT events identified in animal parasitic nematodes

(Ioannidis et al. 2013; Wu et al. 2013). However, plant cell wall degrading enzymes of bacterial origins are the hallmark of HGT in nematodes, which have been identified in genomes of many different plant parasites for degrading plant cell walls (Danchin et al. 2010).

Pristionchus are free-living omnivorous nematodes that became important model organisms in evolutionary biology and are also known to have acquired cellulase genes horizontally (Sommer et al. 1996; Herrmann et al. 2006; Dieterich et al. 2008). However, *Pristionchus* species have a distinct ecology compared with plant-parasitic nematodes or herbivorous insects (fig. 1A). They are soil nematodes that are most reliably found in association with scarab beetles (Herrmann et al. 2006). On adult beetles, these nematodes are arrested in a stress-resistant stage called “dauer larvae,” and are primarily found in the intersegmental folds of the beetle cuticle. When beetles die and carcasses are decomposed by microbes, *Pristionchus* nematodes recover from the dauer stage and start feeding on the bacteria for reproduction (Meyer et al. 2017; Renahan et al. 2021). Previous studies revealed

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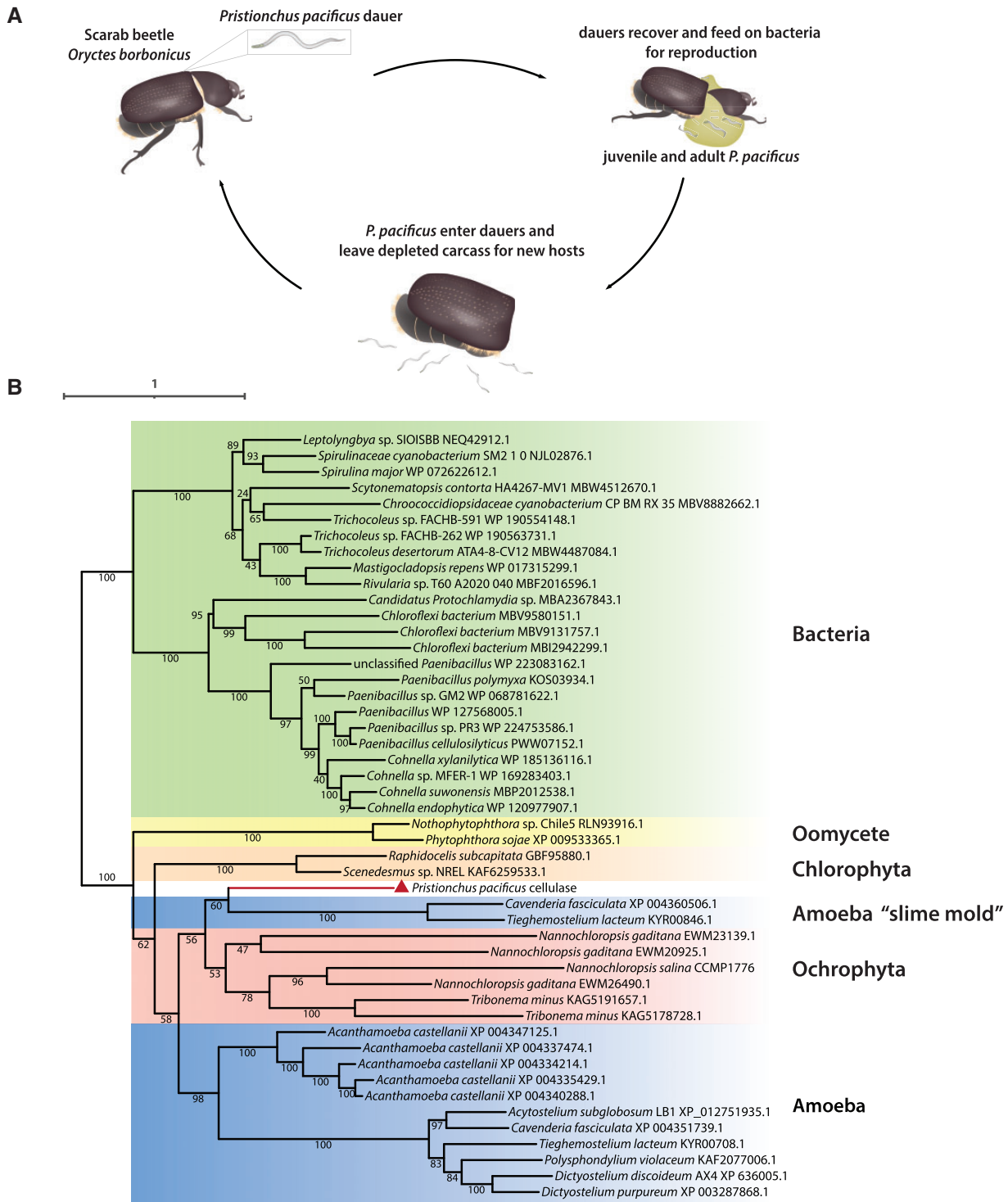


FIG. 1. The necromenic nematode *Pristionchus pacificus* acquired cellulases horizontally from eukaryotic origin. (A) An illustration of the *P. pacificus* life cycle in the context of the adult scarab beetles. On La Réunion island, *P. pacificus* is best known for its association with the rhinoceros beetle *Oryctes borbonicus*. *P. pacificus* dauer larvae (a stress-resistant nonfeeding stage) are found on the adult living beetles. Nematodes reside in the intersegmental folds of the cuticle. After beetles die, nematodes recover from the dauer stage and feed on bacteria and other microbes decomposing the carcass. When bacteria are depleted, *P. pacificus* nematodes enter the dauer stage again and disperse for new hosts. Note that the illustrated beetles and nematodes do not reflect their relative sizes. (B) A maximum likelihood tree of cellulase genes showing that the cellulase in *P. pacificus* (highlighted with a red triangle) is most closely related to cellulases from two slime mold species. The amino acid sequence of *P. pacificus* cellulase domain was used to blast against the NCBI database. The tree was reconstructed with a total of 48 sequences using the LG + F + R5 substitution model and the bootstrap values are shown for each clade.

multiple cellulases in the model *Pristionchus pacificus* and other *Pristionchus* species. Those genes were horizontally acquired in the common ancestor of *Pristionchus* and a closely related genus *Micoletzkyia*, which was independent from those acquisitions in plant-parasitic nematodes (Mayer et al. 2011). Carboxymethyl cellulose (CMC) assays showed cellulase activity in the supernatants of several *Pristionchus* species cultures (Dieterich et al. 2008). These findings demonstrated that the products of these genes retained their cellulolytic activity after HGT and suggested that they might provide adaptive traits to *Pristionchus* and their relatives (Dieterich et al. 2008; Mayer et al. 2011; Schuster and Sommer 2012). However, the functional role of cellulases in free-living species remained unclear as well as the evolutionary significance of HGT. Like in many other examples of microbe–animal HGT, functional investigations had been missing for *P. pacificus* cellulase genes.

Species in the genus *Pristionchus* have been established as model organisms for integrative evolutionary biology. Previous studies have elucidated important phenomena of evolutionary and ecological significance, including developmental plasticity of feeding structures (Bento et al. 2010; Ragsdale et al. 2013; Susoy et al. 2015; Kieninger et al. 2016; Susoy et al. 2016; Sieriebriennikov et al. 2020), predatory behavior, and self-recognition (Wilecki et al. 2015; Lightfoot et al. 2019; Akduman et al. 2020). To date, 51 *Pristionchus* species have been collected and identified and are accessible as living cultures (Kanzaki et al. 2021). Multiple genome sequences are available in these species, which represent several million years of divergent evolution (Rota-Stabelli et al. 2013; Prabh et al. 2018). Also, in *P. pacificus*, genome-editing tools have been well-established that allow the knockout of individual genes with high precision (Witte et al. 2015; Han et al. 2020). Together, with deep taxon sampling and genome-editing tools, the *Pristionchus* system provides a unique opportunity to study HGT in animal evolution.

Here, we systematically investigated horizontally acquired cellulases in the *Pristionchus* genus, particularly in *P. pacificus*. We demonstrated that a single cellulase gene was horizontally acquired in the ancestor of *Pristionchus* and preceded gene duplication. Most importantly, using CRISPR/Cas9 genome-editing tools, we generated a *P. pacificus* cellulase-null mutant in which all eight cellulase genes were sequentially knocked out. Using the octuple mutant, we demonstrated that cellulases can enhance the foraging ability of *P. pacificus* on bacterial cellulosic biofilms. Our findings suggest horizontally acquired cellulases assist the expansion of nutrient acquisition of *P. pacificus* and related free-living nematodes.

Results

Cellulase Genes Diversified in the *Pristionchus* Lineage from a Single Horizontal Transfer Event of Eukaryotic Origin

To elucidate the possible origin of *P. pacificus* cellulase genes, we first built a phylogeny using homologs of *P. pacificus* cellulases from the NCBI database. We found that the *P. pacificus* cellulases are embedded in a cluster of cellulases from amoeba and algae with the most closely

related genes being from the two slime molds *Cavenderia fasciculata* and *Tieghemostelium lacteum* (note that these two genera were previously named *Dictyostelium*; fig. 1B). This result suggests that the *Pristionchus* cellulases are of eukaryotic origin. In contrast, in most plant-parasitic nematodes, cellulases were acquired from bacteria (Danchin et al. 2010; supplementary fig. S1, Supplementary Material online), with the exception of cellulases in the pine wood nematodes *Bursaphelenchus* spp. and the foliar plant-parasitic nematode *Aphelenchoides besseyi*, which are likely of fungal origin (Rödelsperger and Dieterich 2015; supplementary fig. S2, Supplementary Material online). Thus, the novel phylogenetic reconstruction confirms earlier conclusions that were based on the limited whole-genome sequences available at that time (Mayer et al. 2011).

Using the chromosome-level *P. pacificus* genome with its improved annotation (Rödelsperger et al. 2017, 2019; Athanasouli et al. 2020), we found a total of eight cellulase genes in the reference strain PS312 (thereafter referred to as *Ppa-cel*). All of the cellulases contain a catalytic domain that belongs to the glycoside hydrolase family 5 (GHF5; fig. 2A). We matched the eight genes found in this study to the previously described seven genes (Schuster and Sommer 2012) and determined that the discrepancy in the gene number is due to two newly identified genes, which we named *Ppa-cel-5.1* and *Ppa-cel-5.2*. Their amino acid sequences are relatively similar and they are in direct physical proximity, which indicates a gene duplication. In addition to the GHF5 domain, we found two more domain types, a carbohydrate-binding module CBM49 in *Ppa-cel-2* and *Ppa-cel-3*, which is known for enhancing cellulase enzymatic activity (Shoseyov et al. 2006) and a peptidase M10 domain in *Ppa-cel-4*, which has unclear function. *Pristionchus* species have four juvenile stages (J1–J4) and an adult stage, and we wondered if these cellulases are expressed. A published RNA-seq data set indicated that all *Ppa-cel* genes are expressed under standard growth conditions at all stages (plates with *Escherichia coli* OP50 as food source and on Nematode Growth Medium [NGM]; fig. 2B; Baskaran et al. 2015).

To explore the evolutionary dynamics of the cellulase genes in *Pristionchus*, we identified the *Ppa-cel* orthologs in the available genomes of seven *Pristionchus* species and the closely related species *Micoletzkyia japonica* (Prabh et al. 2018). Using Orthofinder (Emms and Kelly 2015), we found multiple cellulase genes in each species and all these genes are clustered in a single orthogroup. We constructed a phylogenetic tree of these genes (supplementary fig. S3, Supplementary Material online) and based on sequence similarities classified them into six clusters (fig. 2C). For example, *P. pacificus cel-2* and *cel-3* are both represented in the “*cel-2*” cluster, and *cel-5.1* and *cel-5.2* in the “*cel-5*” cluster (fig. 2C). Notably, the *cel-2* cluster is highly conserved with at least one ortholog of *Ppa-cel-2* being detected in all species except *P. maxplancki* (fig. 2C). By contrast, other cellulase genes are evolving more rapidly with larger copy number variations across the *Pristionchus* lineage. Note that many of the cellulase genes of the more basal *Pristionchus* species remain ungrouped due to their limited sequence similarities.

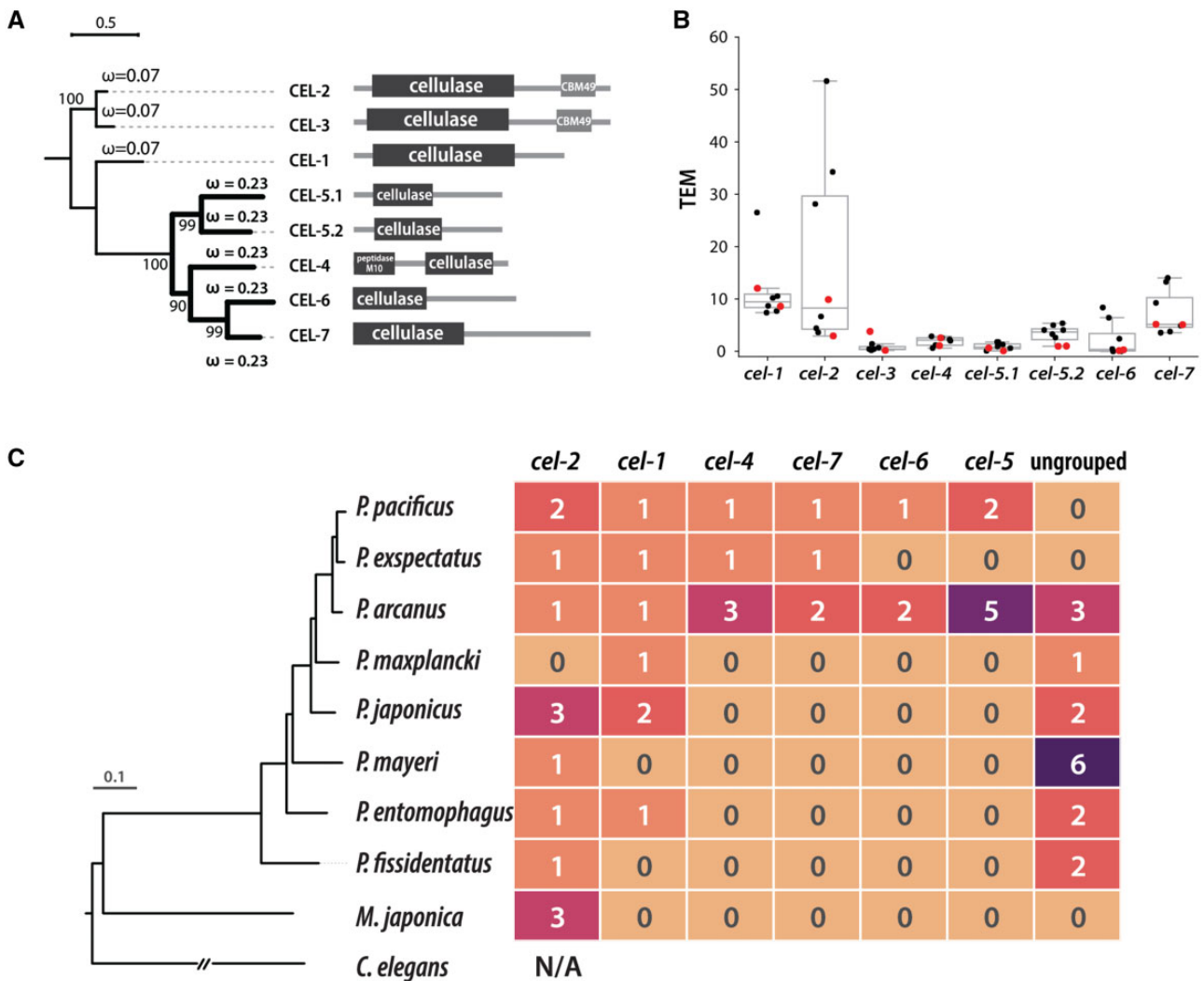


Fig. 2. Cellulase genes diversified in the *Pristionchus* lineage from a single horizontal transfer event. (A) Gene tree and domain compositions of eight cellulases in the model organism *Pristionchus pacificus* (wild-type strain PS312). The bootstrap value is 100 for all branches. The omega (ω) values are determined by branch models in PAML, which assumes independent ω values between *cel-1* to *cel-3* and *cel-4* to *cel-7*. (B) Expression levels of cellulase genes in *P. pacificus* from a stage-specific RNA-seq data set. Total transcripts per million (TPM) from all stages (second to fourth juvenile and young adult) are shown. Red dots indicate the TPM in the young adult stage. (C) Horizontally acquired cellulase in eight *Pristionchus* species with available whole-genome sequence drafts. (Left) A phylogenetic tree of the *Pristionchus* species, the closely related species *Micoletzky japonica* and *Caenorhabditis elegans* based on 1:1 orthologous genes from all species. (Right) Using a cellulase phylogenetic tree from the above species (supplementary fig. S3B, Supplementary Material online), cellulases are classified into six clusters with copy numbers of cellulase in each cluster being presented. A warmer color indicates a larger number of genes. Note that “ungrouped” cellulase genes are diverged sequences and do not support clustering. *Caenorhabditis elegans* does not have cellulase genes (shown as N/A).

However, among the three most closely related species, *P. expectatus*, *P. arcanus*, and *P. pacificus* (Kanzaki et al. 2021), most cellulase orthologs can be found (*cel-1* and *cel-4* to *cel-7*). Together, these results suggest that the *Pristionchus* cellulase genes result from a single acquisition and subsequent gene duplication events.

We next estimated the ratio (ω) of nonsynonymous (dN) to synonymous substitution (dS) values in cellulase duplicates. The branch containing the *cel-4*, *cel-5*, *cel-6*, and *cel-7* genes showed an increased substitution rate compared with the branch containing the genes *cel-1*, *cel-2*, and *cel-3* ($P < 0.0001$; fig. 2A; supplementary table S1, Supplementary Material online). However, ω values are smaller than 1 in both branches, suggesting that those genes are subject to purifying

selection. Using the site model, we identified a small subset of amino acids in the cellulase domain that are under positive selection (supplementary table S2, Supplementary Material online). Together, these analyses strongly indicate that, first, *Pristionchus* cellulases are of eukaryotic origin and, second, the HGT event was followed by a series of gene duplications and diversification with the *cel-2* lineage being most highly conserved.

Cellulases Are Secreted and Expressed in Multiple Tissue Types

Previous studies used a color-based CMC assay to show that the secretions of *Pristionchus* and its close relatives contain cellulolytic activity, whereas the secretion of *Caenorhabditis elegans*,

which does not have any cellulase genes, does not contain cellulolytic activity (Dieterich et al. 2008; Mayer et al. 2011; Schuster and Sommer 2012). First, we confirmed that cellulolytic activity is restricted to the secreted fraction by using a highly sensitive fluorescence-based assay (Enzchek) that allows quantification of total cellulase activity. We incubated *P. pacificus* in M9 buffer and collected supernatant as well as soluble and insoluble protein fractions from nematode tissue (fig. 3A). We detected the highest relative activity in the supernatant, whereas only a minor (five times less) amount of activity was observed in soluble proteins from crushed tissues (fig. 3A).

Next, we sought to corroborate this finding by examining whether cellulases are expressed in secretory tissues. To do this, we generated a transcriptional reporter line for *Ppa-cel-2*. *Ppa-cel-2* was selected because it contains a CBM49 domain and was shown to be highly conserved across *Pristionchus* species. We found strong expression in the pharyngeal gland cells, similar to the expression of the independently acquired cellulases in plant-parasitic nematodes (fig. 3B; Smant et al. 1998; Kikuchi et al. 2004; Ledger et al. 2006). Unexpectedly, however, we observed an expansion of *Ppa-cel-2*-expression in two additional tissues under certain conditions. These tissues were the excretory system (including the excretory gland cells and canals; Sundaram and Buechner 2016), and amphid and phasmid neuron sheath cells (fig. 3B). Specifically, such expansion was observed in older and more crowded nematode cultures and we hypothesized that it was triggered by the diminishing availability of food. Thus, we quantified the change of the expression patterns over time (fig. 3C). Four days after inoculation, when the first generation of nematodes were young adults and in the presence of bacterial food, over 90% of the individuals showed expression exclusively in pharyngeal gland cells (fig. 3C; 74–100 animals were examined for each treatment). However, in older cultures, more individuals expressed *Ppa-cel-2* in the excretory system, and amphid and phasmid neuron sheath cells (Day 7 vs. Day 4, $P = 0.038$; Fisher–Freeman–Halton test). Nine days after the inoculation and thereafter, when the bacterial lawn was totally depleted, over 60% of the individuals showed *Ppa-cel-2* expression in the excretory system, and amphid and phasmid neuron sheath cells in addition to the pharyngeal gland cells (Day 9 vs. Day 7, $P < 0.0001$; Day 11 vs. Day 9, $P = 0.0017$; Fisher–Freeman–Halton test). Also, when adult nematodes were under starvation conditions, we found a significant expansion in the expression patterns compared with nonstarved nematodes of the same age ($P < 0.0001$, Fisher–Freeman–Halton test; supplementary fig. S4, Supplementary Material online). Together, these findings indicate that *Ppa-cel-2* expression is sensitive to environmental conditions and shows a dynamic and consistent response in multiple unrelated excretory tissues. However, how cellulase genes in other *Pristionchus* species respond to different environmental cues remains currently unknown as transgenic animals can only be generated in *P. pacificus*.

Pristionchus pacificus Proteins Have Endo-Cellulase Activity

Next, we sought to determine the biochemical properties of the cellulases of *P. pacificus*. Cellulolytic enzymes can act as

endo- or exo-cellulases to break down the chain of cellulose into the disaccharide cellobiose (Dashtban et al. 2010). Cellobiose can be further digested to glucose by the enzyme β -glucosidase (fig. 3D). However, only a few organisms contain a complete set of cellulolytic enzymes (Béguin and Aubert 1994).

Endo- or exo-cellulases have a substrate-dependent enzymatic activity (Dashtban et al. 2010). We performed the “reducing sugar method” using the cellulose-derivative CMC as substrate. If endo-cellulase activity is present in *P. pacificus* secretions, the product would contain cellobiose or glucose, or both, whereas exo-cellulases cannot efficiently digest CMC. Using liquid chromatography–mass spectrometry (LC-MS), no traces of glucose or cellobiose were found in the secretion of *P. pacificus* alone (fig. 3D, row 4). When the secretion was incubated with CMC, cellobiose was detected, but not glucose (fig. 3D, row 5). β -glucosidase is ubiquitous in animals (Ketudat Cairns and Esen 2010; Watanabe and Tokuda 2010). Indeed, we found a β -glucosidase-encoding gene expressed in *P. pacificus* (gene id: PPA40324; El Paco annotation V3). However, the expression of PPA40324 is enriched in the intestinal region but not in the anterior body region that contains the secretory gland cells (Rödelsperger et al. 2021). Together, these results confirm that *P. pacificus* produces and secretes endo-cellulases and can break down cellulose into simple sugars.

Pristionchus pacificus Cellulase Paralogs Are Functional

Gene duplication can occur after HGT events (Husnik and McCutcheon 2018) and duplicates may degenerate during evolution. Given the different domain compositions in the *P. pacificus* cellulase genes, we wondered if all cellulase copies are functional. To test this, we first expressed *Ppa-cel-2*, *Ppa-cel-4*, *Ppa-cel-5.1*, and *Ppa-cel-6* in *E. coli* to analyze whether they had enzymatic activities (fig. 4A). Among these genes, only *Ppa-cel-2* contains a CBM49 domain. Using a CMC diffusion assay, *E. coli* lysates of all expressed cellulase proteins showed a halo indicating that they are enzymatically active (fig. 4B). Thus, among the expressed proteins, the presence of the CBM49 domain did not seem to be necessary for the function of cellulase in this assay.

Second, we used the CRISPR/cas9 genome-editing system to generate *P. pacificus* knockout mutants of *Ppa-cel-1*, *Ppa-cel-2*, or *Ppa-cel-3*. When we tested these single mutants for cellulase activity, we found gene-specific reduction of cellulase activities. Specifically, the *Ppa-cel-2* mutant showed a loss of more than 80% of total activity (fig. 4C). By contrast, *Ppa-cel-1* mutants only lost 60% activity and mutations in the lowly expressed *Ppa-cel-3* resulted in a minimal activity loss. Thus, all three genes tested by CRISPR/Cas9-induced knockouts contribute to the observed cellulase activity of *P. pacificus*, but individual genes contribute differently to the total cellulase activity. As the regulation of cellulase gene expression is influenced by the environment, that is different bacterial food sources (supplementary fig. S5, Supplementary Material online), further genetic approaches must involve the inactivation of all genes.

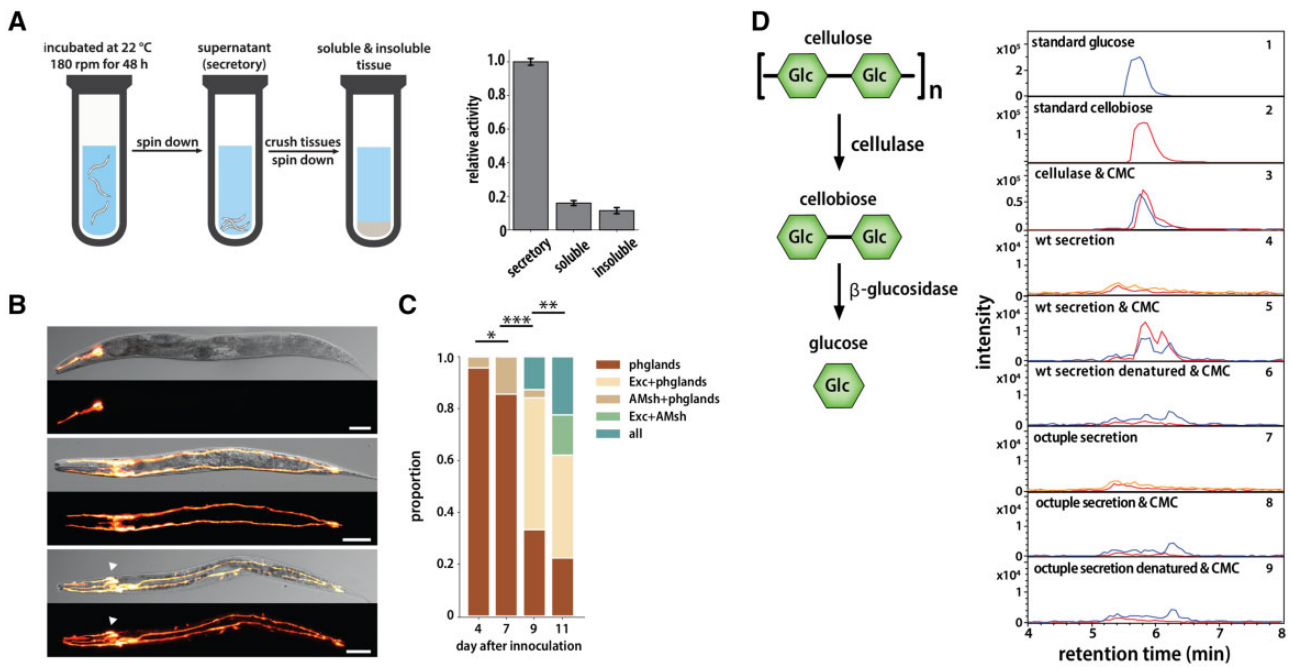


FIG. 3. *Pristionchus pacificus* cellulases are expressed and have endo-cellulase activity. (A) Comparison of cellulase activities between secretion and whole tissue using Enzchek assays. Error bars indicate mean \pm SEM. (B) Expression patterns of the *Ppa-cel-2* promoter driving TurboRFP. Images of overlaid RFP and DIC, and RFP alone are shown. Images from top to bottom show expressions in pharyngeal gland cells, the excretory system (including excretory gland cells and canals) and amphid neuron and phasmid neuron sheath cells. The white arrowheads in the bottom two images indicate the expression in the amphid neuron sheath cells. Scale bar = 50 μ m. (C) The *Ppa-cel-2* expression dynamics due to starvation of the culture. Phglands = pharyngeal gland cells, Exc = the excretory system, AMsh = amphid neuron sheath cells. Seventy-four to 100 nematodes were examined for each treatment. *, **, and *** indicate $P < 0.05$, $P < 0.001$, and $P < 0.0001$ from Fisher–Freeman–Halton tests, respectively. (D) Biochemical characterization of *P. pacificus* cellulase. (Left) A simplified illustration of how cellulolytic enzymes digest cellulose. (Right) Biochemical characterization of *P. pacificus* cellulase using LC-MS. The reducing sugar method was conducted using CMC as substrate to detect endo-cellulase activity. Cellulases from *Aspergillus niger* were used as positive control.

A *P. pacificus* Octuple Cellulase Mutant Lacks Any Cellulase Activity

We attempted to engineer a *P. pacificus* cellulase-free mutant by sequentially knocking out all eight cellulase genes (fig. 5A). In each step, we sequenced mutant candidates in the selected gene, but only considered mutants with frameshift mutations for further CRISPR gene knockout of the next gene to be inactivated. Surprisingly, the resulting octuple mutant is viable and can be cultured on standard *E. coli* OP50 conditions (fig. 5B). No cellulase activity from the secretion can be detected in this cellulase-null mutant using LC-MS (fig. 3D, row 7–9). In the following, we will refer to this mutant as “octuple mutant.” The viability of this mutant strain allows testing the function of cellulase activity at the biochemical and organismal levels.

Cellulases Might Assist *P. pacificus* to Exploit Cellulose from the Environment

To study the role of the *P. pacificus* cellulases at the organismal level, we compared the growth of the cellulase octuple mutant with wild-type animals on different food sources. First, we asked if *P. pacificus* could use cellulose from the environment as an additional nutrient to increase its fitness (Sun et al. 2020). We grew wild-type *P. pacificus* and the octuple mutant on nutrient-limited agar plates with the standard food source *E. coli* OP50 and supplemented with CMC

(fig. 6A and B; supplementary table S3, Supplementary Material online). If *P. pacificus* is able to use CMC, a larger brood size might be observed. A two-way ANOVA test indicated that the two *P. pacificus* strains (wild type vs. octuple mutant) affected brood size ($P = 0.0057$). Indeed, on CMC-supplemented plates, wild-type animals showed a large brood size compared with the octuple mutant (fig. 6B; $P = 0.016$, one-tailed Mann–Whitney rank test). In contrast, no significant difference was observed between wild type and the octuple mutant on plates not supplemented with CMC (fig. 6B; $P = 0.247$, one-tailed Mann–Whitney rank test). Thus, cellulases can potentially help *P. pacificus* utilize environmental cellulose.

Cellulases Increase Nematode Fitness on Bacterial Cellulosic Biofilms

Cellulose is a component in some bacterial biofilms (Hall-Stoodley et al. 2004) and thus, a potential target for soil nematodes. It has been suggested that the resilient nature of biofilms is a defense strategy from the predation of other organisms (Seiler et al. 2017; Chan et al. 2021). The *E. coli* OP50 strain is not known to produce cellulosic biofilms under standard laboratory conditions for nematode maintenance. Therefore, we used *E. coli* K-12 strains, which are well-studied and known to form cellulosic biofilm (Serra et al. 2013), to test if the *P. pacificus* cellulases can help disrupt

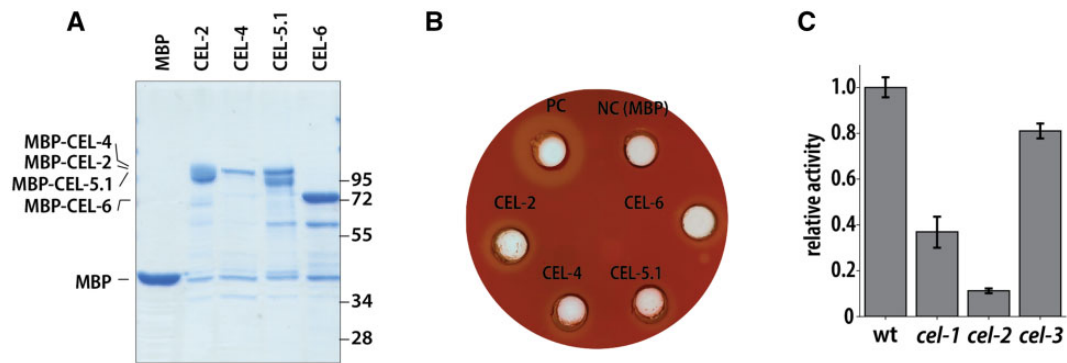


FIG. 4. *Pristionchus pacificus* cellulases are functionally active. (A) Expression of recombinant cellulase proteins from *P. pacificus* in *Escherichia coli*. *Pristionchus pacificus* cellulases were tagged with the MBP and purified proteins were analyzed on a 10% SDS gel. (B) The *E. coli* cell lysates were tested for cellulase enzymatic activity using a diffusion assay with a 0.5% CMC-supplement agar plate. Cellulases from *Aspergillus niger* were used as positive control and the lysate of MBP alone was used as negative control. (C) Quantifications of cellulase activities in the secretions of *P. pacificus* *cel-1*, *cel-2*, and *cel-3* single mutants using Enzchek assays. Mutations in all three genes cause a reduction in cellulase activity at the organismal level. Error bars indicate mean \pm SEM.

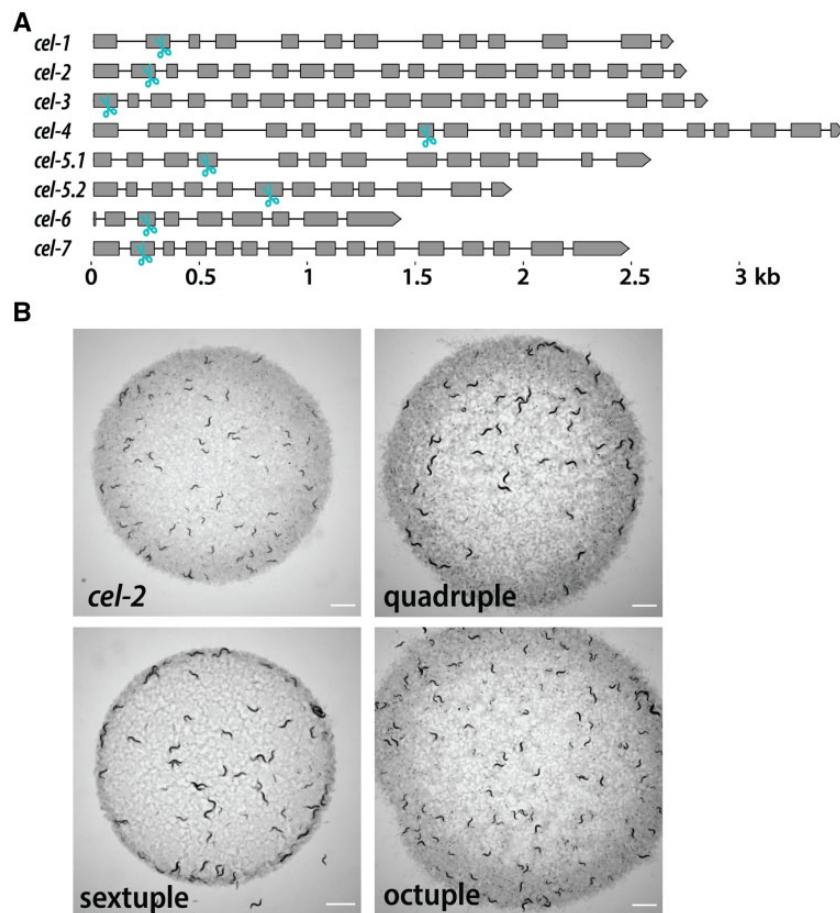


FIG. 5. A *Pristionchus pacificus* octuple cellulase mutant lacks cellulase activity. (A) An illustration of cellulase gene structures in *P. pacificus* and CRISPR/Cas 9 knockout sites. (B) Brightfield images of *P. pacificus* *cel-2*, quadruple, sextuple, and octuple mutants on the *Escherichia coli* OP50 bacterial lawn. Scale bar = 1,000 μ m.

the integrity of biofilms and enable more efficient motility and foraging. First, we measured the motility between *P. pacificus* wild type and the octuple mutant in the following *E. coli* K-12 strains: 1) an *E. coli* K-12 “natural form” containing the full architecture of biofilm (referred as cellulose +; strain AR3110); 2) a mutant strain with a single-nucleotide

polymorphism in the *bsc* operon that cannot produce cellulose (referred as cellulose –; strain AR182) and, 3) a mutant that cannot produce cellulose and curli fibers, and lacks biofilm structures altogether (referred as cellulose - and curli –; strain AR182; Serra et al. 2013). It has been shown that the resilient nature of some biofilms can hinder the mobility of

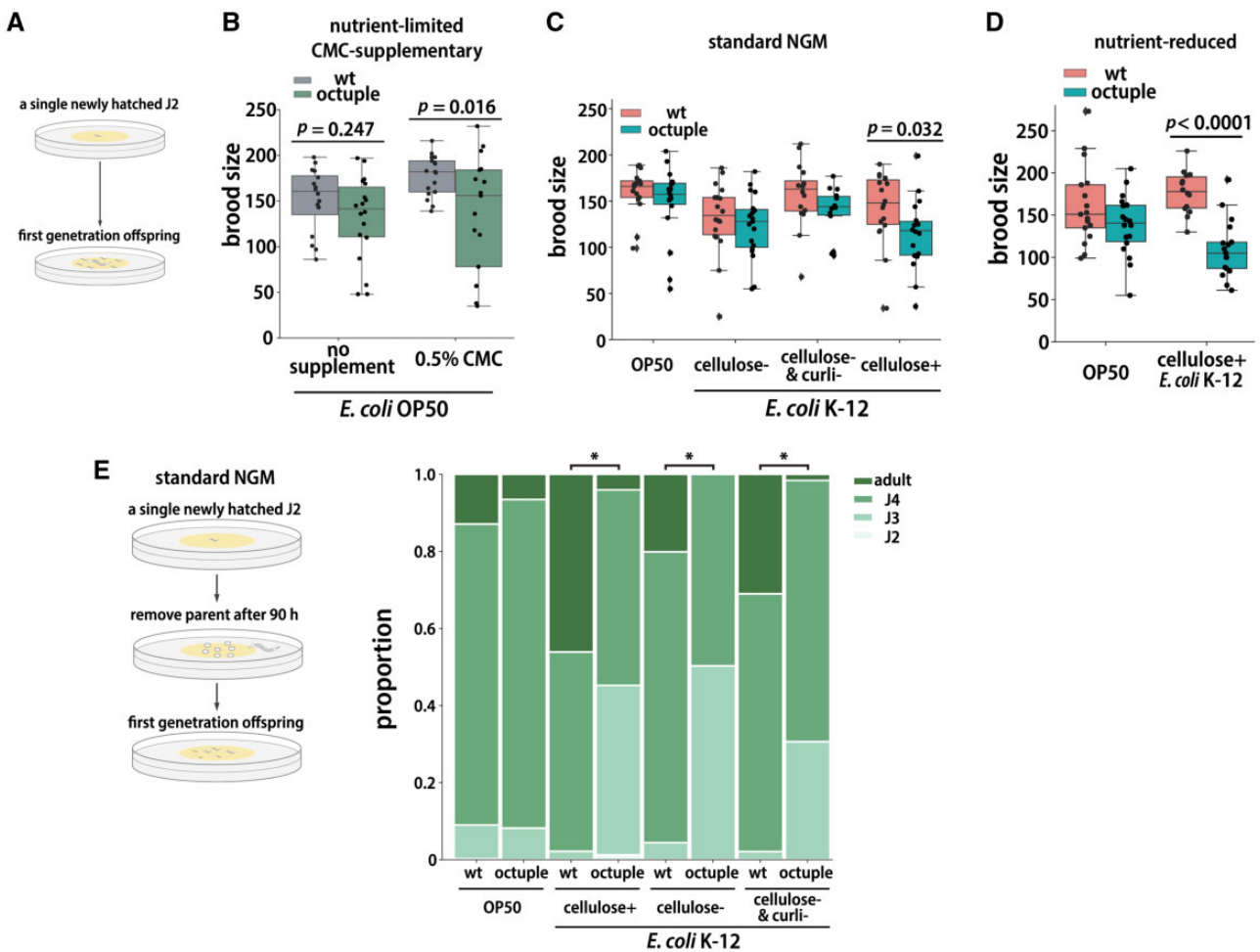


FIG. 6. Cellulases of *Pristionchus pacificus* increase nematode fitness on bacterial cellulosic biofilms. (A) An illustration of assays for testing brood size in *P. pacificus*. (B) Brood size of *P. pacificus* wild type (wt) and octuple mutant (oct) using *Escherichia coli* OP50 as the food source under nutrient-limited conditions supplemented with 0.5% CMC (one-tailed Mann–Whitney rank test). (C) Brood size of *P. pacificus* wild type (wt) and octuple mutant (oct) on different *E. coli* K-12 strains under the standard NGM condition (one-tailed *t*-test). (D) Brood size of *P. pacificus* wild type (wt) and octuple mutant (oct) under nutrient-reduced condition, showing an enhanced reproductive potential of wild-type animals on *E. coli* K-12 cellulose + (one-tailed *t*-test). (E) (Left) Assays for measuring developmental speed. (Right) Under the standard NGM condition, *P. pacificus* wild type (wt) and octuple mutant (oct) have a similar developmental speed on *E. coli* OP50, whereas the wild type develops faster than the octuple mutant on *E. coli* K-12 strains. The developmental speed of offspring from multiple hermaphrodites was determined (PERMANOVA; examined plates between 14 and 28). * indicates $P < 0.05$. All experiments were performed in duplicate. Nutrient conditions are summarized in [supplementary table S3, Supplementary Material](#) online.

C. elegans (Chan et al. 2021). However, contrary to our expectation, there were no observable differences in motility between *P. pacificus* wild type and the octuple mutant on any *E. coli* strain ([supplementary fig. S6, Supplementary Material](#) online).

To test if cellulases can help *P. pacificus* obtain additional nutrients from bacterial biofilms, we quantified *P. pacificus* fitness under different growth conditions. Under standard NGM conditions, a two-way ANOVA test showed that both *P. pacificus* strains ($P = 0.005$) and bacterial strains ($P = 0.002$) had a significant effect on brood size, but no interaction between the two factors was observed. Specifically, when grown on the *E. coli* K-12 cellulose + strain, *P. pacificus* wild-type animals showed an increase in brood size relative to the octuple mutant ($P = 0.032$, one-tailed *t*-test; [fig. 6C](#)). In contrast, on *E. coli* OP50 and K-12 cellulose– and cellulose– & curli– strains, the lack of cellulases did not affect the

reproductive potential of *P. pacificus*. However, we hypothesized that the cellulolytic ability would not provide excessive benefits through the degradation of cellulose as long as far more accessible carbon and other energy sources are available on standard laboratory NGM growth plates. To explore this possibility further, we generated a nutrient-reduced condition, in which peptone, cholesterol, and salts were limited or completely excluded from the agar plates ([supplementary table S3, Supplementary Material](#) online). Indeed, under these restrictive conditions, a two-way ANOVA test showed that the *P. pacificus* strains had a significant effect ($P < 0.001$) and there was an interaction between *P. pacificus* strains and bacterial strains ($P = 0.018$). Specifically, wild-type *P. pacificus* produced an increased brood size with a larger effect size on *E. coli* K-12 cellulose + strains compared with standard NGM conditions. We observed an average brood size of 177 in the wild type versus 110 in the octuple mutant ($P < 0.0001$,

one-tailed *t*-test; fig. 6C). Thus, cellulases increase the reproductive potential of *P. pacificus* in the presence of environmental cellulose and bacterial cellulosic biofilms.

Furthermore, we found that nematode growth was retarded on some bacterial strains tested. To systematically test for developmental speed, newly hatched J2 larvae whose parents were raised on *E. coli* OP50 were placed onto cellulose-rich bacteria under standard NGM conditions (fig. 6E). We then tested the first-generation offspring of these animals for developmental rate. Although both *P. pacificus* wild type and the octuple mutant grew at a similar speed on *E. coli* OP50 (adjusted $P = 0.504$; PERMANOVA), wild-type animals grew substantially faster on *E. coli* K-12 cellulose + compared with the octuple mutant (adjusted $P = 0.028$; PERMANOVA; fig. 6E). On the *E. coli* K-12 cellulose—(adjusted $P = 0.028$; PERMANOVA) and cellulose—curli—diet (adjusted $P = 0.028$; PERMANOVA), we also observed a faster development in wild-type animals. Thus, our results show that the cellulolytic ability can potentially assist *P. pacificus* to use more carbon sources in the environment and access cellulosic biofilms, which can expand the potential food sources of *P. pacificus*. These findings are consistent with the idea that cellulosic biofilms may function as a bacterial survival strategy by decreasing the foraging behavior of nematodes. However, horizontally acquired cellulases enhance the ability of *P. pacificus* to feed on biofilm-producing bacteria.

The Lack of Cellulases Caused Global Metabolic Changes

The experiments described above indicate that cellulase activity can provide a nutritional advantage to *P. pacificus* when it encounters biofilms and cellulose in its environment. Thus, we finally wanted to determine the metabolic consequences of the overall nutritional limitations in the octuple mutant. Therefore, we conducted RNA-seq experiments and explored global gene expression changes on *E. coli* K-12 cellulose + under standard NGM conditions between the octuple mutant and wild-type animals. To explore the potential biological functions of gene expression changes, we tested for enriched gene ontology (GO) terms, which associate genes with biological processes. We adapted GO terms from *C. elegans* data sets (WormEnrichr; Chen et al. 2013; Kuleshov et al. 2016) and performed a Gene Set Enrichment Analysis (GSEA) with these GO terms (Subramanian et al. 2005). We found that when grown on *E. coli* K-12 cellulose +, 54 GO terms that were downregulated in the *P. pacificus* octuple mutant compared with the wild type (fig. 7A; supplementary file S1, Supplementary Material online; FDR-adjusted P value < 0.05) indicating potential changes in biological processes. Notably, GO terms for positive regulations of reproductive process, developmental growth, and oviposition were all downregulated in octuple mutant. Downregulation of genes in these biological processes correspond to reduced brood size and developmental speed as observed in the octuple mutant when grown on the *E. coli* K-12 cellulose + strain. In addition, several GO terms involved in feeding and locomotion behaviors were also downregulated. Importantly, none of these 54 GO terms

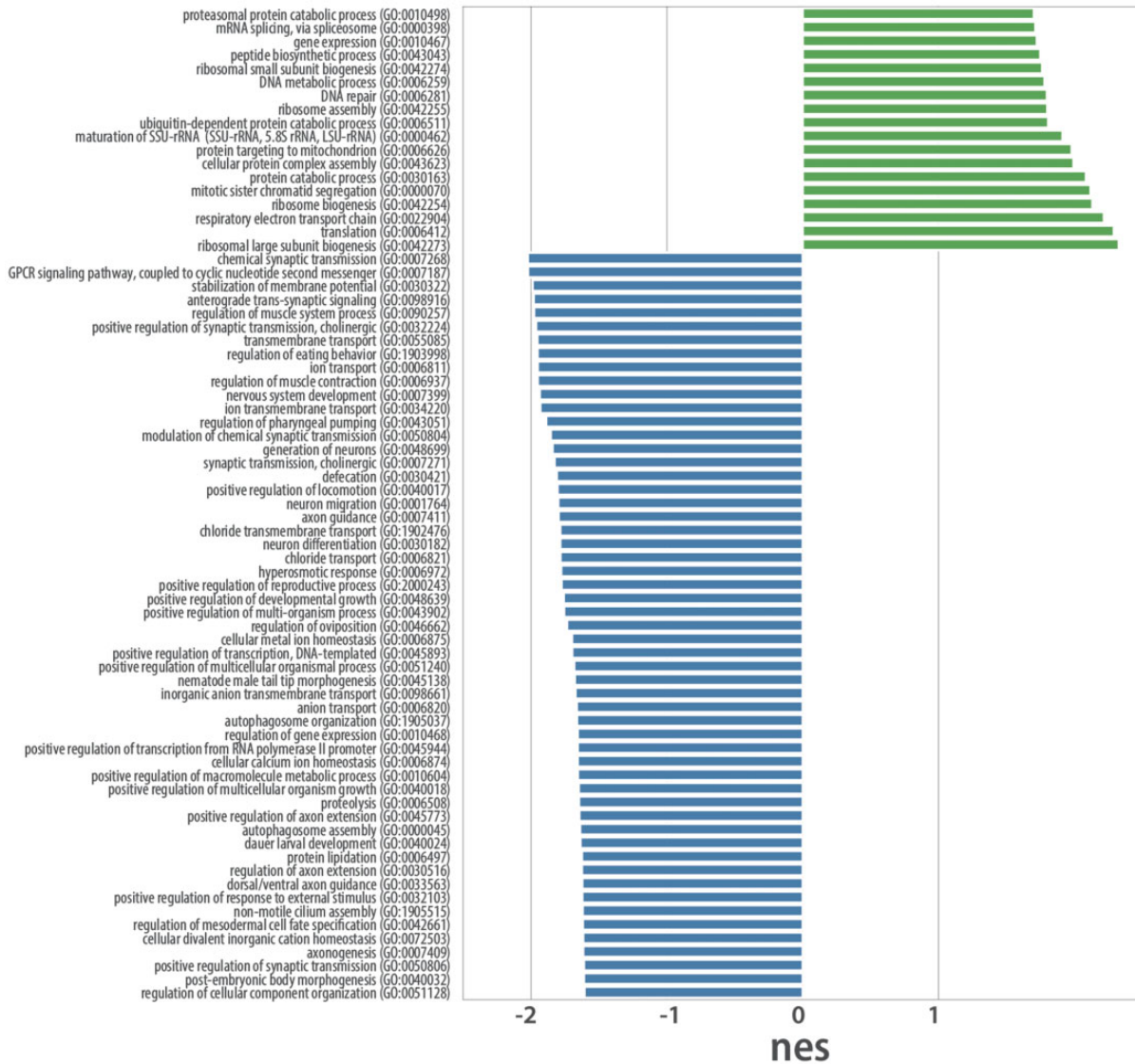
were differentially expressed between octuple mutant and wild-type animals on *E. coli* OP50 (fig. 7B and supplementary file S1, Supplementary Material online). These findings demonstrate that horizontally acquired cellulases significantly impact the metabolism of *P. pacificus* when interacting with cellulosic biofilms and support that these genes are fully integrated into the life history strategy of the nematode.

Discussion

We present a comprehensive study to demonstrate the evolutionary and ecological significance of HGT in free-living *Pristionchus* nematodes. First, using biochemical and mutant analyses, we show that these cellulase genes are functional and increase the fitness of the animal recipient by enhancing the foraging ability on cellulosic biofilms. This result is primarily based on the analysis of a CRISPR-induced octuple mutant that eliminates all cellulase genes in *P. pacificus* and provides a novel ecological context for animal cellulases. Pre-existing knowledge in contrast was largely limited to plant invasion and digestion in nematodes and insects (Baldwin et al. 2004; Watanabe and Tokuda 2010). Second, we elucidate the evolutionary history of horizontally acquired genes in the *Pristionchus* lineage: a single cellulase gene from eukaryotic origin was horizontally acquired in the common ancestor of *Pristionchus* nematodes, has undergone duplication and likely functional diversification, the latter of which has still to be proven in future biochemical experiments. This evolutionary scenario is consistent with previous suggestions that HGT should be present in a lineage-specific pattern (Martin 2017). Third, our transcriptome analyses indicate that the lack of cellulase in *P. pacificus* can cause a global change in gene expressions on cellulosic biofilms. Besides nematodes, some beetle species are also found to have acquired cellulase genes through HGT, but the ecological functions were limited to the digestion of plant materials (McKenna et al. 2019). It is important to note that the potential transfer of cellulases to the nematode via its beetle hosts is unlikely as our previous work has not given any indication that the rhinoceros beetle *Oryctes borbonicus*, the scarab beetle with the most highly known *P. pacificus* infestation, contains cellulase genes itself (Meyer et al. 2016). Together, our findings add to several recent studies that have provided strong support for the importance of HGT in animal evolution in phylogenetically distinct taxa (Danchin et al. 2010; Moran and Jarvik 2010; Husnik et al. 2013; Wybouw et al. 2014; Hayes et al. 2020; Undheim and Jenner 2021; Xia et al. 2021). This study provides several conclusions that enhance our understanding of the complex tripartite interactions between beetles, nematodes, and bacteria.

The interactions between bacteria and nematodes are more complex than merely food and foragers. Biofilms are thought to be common in natural environments (Hall-Stoodley et al. 2004; Jefferson 2004; Danhorn and Fuqua 2007; Flemming and Wuerz 2019) and have been found to affect nematodes in a wide spectrum from pathogenesis to behavioral alteration (Darby et al. 2002; Nandi et al. 2016; Desai et al. 2019; Chan et al. 2021). A recent study demonstrated that *Pseudomonas aeruginosa* biofilms could impede

A Cellulase octuple mutant vs. wild type grown on *E. coli* K-12 cellulose + strain (FDR < 0.05)



B Cellulase octuple mutant vs. wild type grown on *E. coli* OP50 (FDR < 0.05)

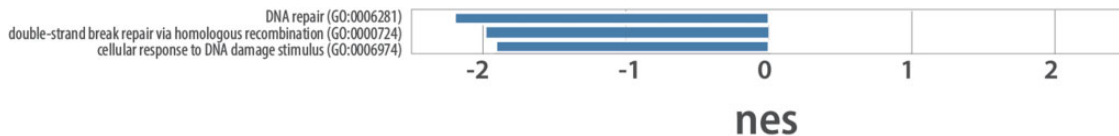


Fig. 7. The lack of cellulases caused global metabolic changes. RNA-seq experiments were performed as described in Materials and Methods. GSEAs were performed with GO terms between *Pristionchus pacificus* octuple (oct) and wild type (wt). Nematodes were grown on *Escherichia coli* K-12 cellulose + strain (A) and *E. coli* OP50 (B), respectively, and mixed stages of nematodes were used for sequencing. The values of normalized effect size (nes) of different expressed GO terms are presented using FDR-adjusted P value < 0.05. Each experimental condition contains three technical biological replicates. A complete list of GO terms is summarized in [supplementary file S1, Supplementary Material](#) online.

the locomotion in *C. elegans* in order to protect the part of the community, which is more susceptible to foraging by *C. elegans* (Chan et al. 2021). In our study, the transcriptomic data revealed the downregulation of several behavior-associated GO terms when *P. pacificus* lacking cellulolytic ability was grown on biofilms, including regulations of muscle system process, muscle contraction, pharyngeal pumping, and locomotion (fig. 7A and [supplementary file S1,](#)

[Supplementary Material](#) online). These gene expression changes could possibly explain why even under sufficient nutritional conditions, cellulase-free *P. pacificus* developed slower. Together, our results strongly suggest that horizontally acquired cellulases can alter the interaction between *P. pacificus* and cellulosic biofilms.

In addition, recent studies suggest that in nature, *Pristionchus* nematodes live in complex ecosystems with

competing nematode species (Herrmann et al. 2006; Meyer et al. 2017; Gong et al. 2021; Renahan and Sommer 2021). In this context, cellulases might provide *P. pacificus* more pronounced ecological advantages. *Pristionchus* nematodes exhibit phenotypic plasticity resulting in a predatory morph and a strict microbial feeding morph. A recent study showed that bacterial communities from insect carcasses occur in a rapid cycle from boom to depletion (Renahan et al. 2021) and *Pristionchus* likely encounter cellulosic biofilms. The faster development and a higher reproductive potential provided by cellulases might assist *Pristionchus* nematodes to quickly establish on the bacterial community and suppress other nematodes through predation (Quach and Chalasani 2021).

Finally, the cellulolytic ability might provide *P. pacificus* additional benefits by bringing additional resources into the ecosystem, which is termed as “niche construction” (Lotka 1922; Peacock 2011). Through secreted cellulases, *P. pacificus* might be able to “engineer” the local microbial community. Specifically, simple sugars released from *Pristionchus*’ degradation of cellulose from biofilms or the environment could benefit certain bacterial species and thereby influence the bacterial composition of the ecological niche. This could further influence the structure of the local bacterial community and thereby enhance the mutualism between bacteria and nematodes in the insect decaying ecosystem. These examples show sophisticated interactions of a tripartite ecosystem consisting of nematodes, beetles, and microbes. Together, our study provides new insights into HGT in animal evolution.

Materials and Methods

Nematode Maintenance

Pristionchus pacificus strains were maintained on the standard NGM agar with *E. coli* OP50 at 20 °C following the standard protocols (Stiernagle 2006). Standard 6-cm plates were used for maintenance or assays, if not further specified. *Pristionchus* strains are listed in [supplementary table S4, Supplementary Material](#) online.

Bacterial Strains Maintenance

Escherichia coli OP50 was provided by the *Caenorhabditis* Genetics Center. *Escherichia coli* K-12 strains AR3110, AR198, and AR182 were provided by Dr Regine Hengge from Humboldt-Universität zu Berlin (Serra et al. 2013). All bacterial strains were maintained on LB plates. A single colony of the given bacterial strain was inoculated into the LB broth and incubated overnight at 37 °C. The LB culture was then used for seeding the bacterial lawn on NGM plates. Specific NGM conditions for reproductive assays are summarized in [supplementary table S3, Supplementary Material](#) online.

Construction of Cellulase Gene Phylogenetic Trees

To identify the cellulase genes in the *P. pacificus* genome, we predicted PFAM domains in all translated El Paco v1 gene predictions using HMMER v. 3.1b2 (Finn et al. 2016; Rödelsperger et al. 2017). The search identified seven genes with hits for the cellulase (PF00150.12) domain. Next, we compared their predicted exon–intron structure to the

alignment of previously published RNA-seq data in IGV (Robinson et al. 2011; Sinha et al. 2014; Serobyian et al. 2016). Based on the RNA-seq coverage, we manually split one of the predictions (UMM-S60-23.68-mRNA-1, or *Ppa-cel-5*) into two (*Ppa-cel-5.1* and *Ppa-cel-5.2*). Then, we established the correspondence between the El Paco v1 predictions and the previously published genes named *Ppa-cel-1* to *Ppa-cel-7* using reciprocal Basic Local Alignment Search Tool (BLAST; Schuster and Sommer 2012).

We searched for potential homologs of the *Ppa-CEL-2* catalytic domain using the BLASTp algorithm in the NCBI non-redundant database of protein sequences (2021-10). Matching sequences with *e* values less than 6×10^{-66} were retrieved and clustered using a 90% identity threshold through cd-hit (Li and Godzik 2006). A total of 47 sequences remained and were aligned using MAFFT (Katoh and Standley 2013). Phylogenetic trees were built using IQ-TREE (Nguyen et al. 2015). A total of 542 substitution models were tested (Kalyaanamoorthy et al. 2017) with 1,000 ultrafast bootstraps (Hoang et al. 2018). LG + F + R5 was identified as the best-fit model for the given data set based on Bayesian Information Criterion.

Using the above pipeline, two additional phylogenetic trees were generated using the domain sequence of a cellulase from the root-knot nematode *Meloidogyne incognita* and the pine wood nematode *Bursaphelenchus xylophilus*. Detailed information can be found in supplementary files, [Supplementary Material](#) online.

Evolutionary History of Cellulase Genes in the *Pristionchus* Lineage

Cellulase orthologs were identified using a phylogenomic data set that contains eight *Pristionchus* species and *M. japonica* (Prabh et al. 2018). We used OrthoFinder (Emms and Kelly 2015, 2019) for the identification of orthologous genes. Eight cellulase genes of *P. pacificus* were assigned into one ortholog set (orthogroup). Genes belonging to this orthogroup were examined using Pfam (Mistry et al. 2021) for putative domains, and the genes without a cellulase domain were removed manually. We produced multiple alignments of cellulase protein sequences using MUSCLE (Edgar 2004). The phylogenetic tree was inferred using FastME 2.0 (Lefort et al. 2015) with the LG model and 100 bootstraps. Cellulase genes were assigned into groups based on topology. Branches with low support value were assigned into the unknown group and were removed to improve the alignment and phylogeny.

Transcriptional Reporter Lines

Transcriptional reporter containing a 2-kb DNA fragment upstream of the *Ppa-cel-2* start codon, TurboRFP, and the *Ppa-rpl-23* 3’ UTR was assembled from PCR products using Gibson assembly kit following the manufacturer’s protocol. The assembled product was PCR-amplified, purified from the gel, and diluted in TE buffer to a final concentration of 5 ng/μl. This mix was directly used for injections. Progeny of injected hermaphrodites was screened for fluorescence. All fluorescent F1 (more than ten animals in total) showed RFP expression in

the pharyngeal gland cells. For consequent experiments, we used Ex[*cel-2p::TurboRFP*](*tuEX235*) line (RS2891) that had more than 95% transgene transmission rate.

CRISPR Knockouts

CRISPR knockouts were generated using previously published protocols in *P. pacificus* (Witte et al. 2015; Han et al. 2020). For CRISPR knockouts, CRISPR RNAs (crRNAs) were designed to target the second or third exon of the gene. In cases of ambiguous gene models, crRNAs were then designed in the exon that was 5' to the coding sequence of the cellulase domain. crRNAs and tracrRNA (Cat. No. 1072534) were synthesized by Integrated DNA Technologies (IDT), and the Cas9 endonuclease (Cat. No. 1081058) was purchased from IDT. The CRISPR/Cas9 complex was prepared by mixing 0.5 mg/ml Cas9 nuclease, 0.1 mg/ml tracrRNA, and 0.056 mg/ml crRNA in the TE buffer followed by a 10-min incubation at 37 °C. The *egl-20p::TurboRFP* plasmid (50 ng/μl) was added after the incubation as a coinjection marker (Han et al. 2020). Microinjections were performed following the standard practice using an Eppendorf microinjection system. Specific crRNA and primers for knockouts can be found in [supplementary table S5, Supplementary Material](#) online.

Imaging the Expression Pattern of *Ppa-cel-2p::TurboRFP*

Several individuals with representative expression patterns were mounted on 5% Noble Agar pads containing 0.3% NaN₃ and imaged using a Leica TCS SP8 confocal microscope. Images were processed using ImageJ.

To elucidate the change of *Ppa-cel-2p::TurboRFP* expression pattern due to the culture age, 20 RFP-positive gravid hermaphrodites were placed on a bacteria-seeded plate and allowed to lay eggs overnight. Then, 30–60 eggs were transferred to a plate without bacteria using a pick and bleached in 8 μl of the following solution: 150 μl HPLC-grade water, 50 μl household bleach (5% active chlorine), and 50 μl 5 M NaOH. After the bleaching solution had soaked into the agar, the eggs were transferred using a pick onto a fresh plate seeded with 100 μl *E. coli* OP50. The plates were incubated at 24.5 °C. On Day 4 postbleaching, the plates contained young adults of the first generation, and the food was plentiful. Over the course of the next few days, the food was gradually depleted, and the next generations of worms developed. By Day 11, the plates had no visible bacteria, and they mostly contained starved adults of the second and third generations. The pattern of fluorescence was examined using the Zeiss Axio Zoom.V16 microscope in 30–50 randomly selected adult hermaphrodites in each plate on Days 4, 7, 9, and 11 postbleaching. The experiment was biologically duplicated.

To further test whether the observed changes of *cel-2p::TurboRFP* expression patterns were caused by starvation or aging, we bleached well-fed adult *P. pacificus* nematodes to synchronize the development stage of offspring and placed the bleached eggs OP50 bacterial lawn. Four days later, the nematodes developed into adults and were washed off from the plates. The washed nematodes were placed into two conditions: regular NGM plates with OP50 (nonstarved); NGM

plates with no bacteria (starved). We examined the nematodes for expression patterns under the two conditions 11 days postbleaching. The experiment was biologically duplicated.

Fluorescence-Based Assays for Cellulase Activity Quantification

Cellulase activity was quantified using a simplified fluorescence-based assay (Enzchek, Invitrogen Cat. No. E33953). To compare relative cellulase activity from the secretion and within the nematode tissue, one 10-cm NGM plate of hermaphrodites was washed three times with M9 buffer to remove bacteria. These nematodes were resuspended in 2 ml of 0.5× M9 buffer and incubated at 22 °C, 220 rpm for 48 h. After spun down, the supernatant was extracted and concentrated to 0.2 ml using an Eppendorf concentrator plus. The remaining nematodes were then frozen and crushed. The supernatant and crushed tissues were separated and resuspended to 0.2 ml with M9 buffer and measured for the cellulase activity, respectively. Fifty microliters of fluid from each treatment was used for cellulase activity testing via Enzchek. H₂O, Enzchek digestion buffer, and/or denatured fluid (heated at 80 °C for 10 min) were used as negative control, whereas the *Aspergillus niger* cellulase (Sigma-Aldrich, Cat. No. C1184) was used as positive control. A 30-min reaction at 20 °C was conducted. The fluorescence signal was read using a Tecan plate reader. Readings of the denatured fluid from each treatment were used as the baseline, since we found that the cloudiness from the tissue debris in the solution can affect reading.

To measure the cellulolytic activity of *P. pacificus* cellulase single mutants, nematodes from one 10-cm NGM plate of the designated mutant were washed. These nematodes were filtered through double 120 μl nylon nets where adult hermaphrodites were kept. These hermaphrodites were resuspended in 2 ml of 0.5× M9 buffer and incubated at 22 °C 220 rpm for 48 h. One milliliter of the supernatant was then concentrated to 0.3 ml as mentioned above. A Bradford assay (ThermoFisher, Cat. No. 23236) was conducted to determine protein concentration in the supernatant. The fluorescence reading from a given sample was normalized to its protein concentration, and the relative value to the wild-type activity was presented. All assays using EnzChek were technically and biologically duplicated.

Biochemical Characterization of *P. pacificus* Cellulase Using LC-MS

To characterize biochemical features of *P. pacificus* cellulases using LC-MS, one 10-cm NGM plate of well-fed hermaphrodites was washed free of bacterial and resuspended in 2 ml of 0.5× M9. They were incubated at 22 °C and 220 rpm for 48 h. The supernatant was extracted and concentrated as above. Fifty microliters of the supernatant was used to 1) be directly examined for presence of glucose or cellobiose (fig. 3, rows 3–4); 2) react with 4% carboxymethylcellulose sodium salt (CMC, Sigma-Aldrich, Cat. No. C5678) at 30 °C for 8 h (fig. 3, rows 6–7); and 3) denature at 80 °C for 10 min before reacting with 4% CMC at 30 °C for 8 h (fig. 3, rows 8–9). The *A. niger* cellulase (3,000 mU/ml) was used as positive control under the condition as above (fig. 3, row 5). Three milliliters of the product from the above treatments was analyzed for

glucose and cellobiose in negative ion mode using a Dionex Ultimate 3000 UPLC instrument coupled to a Bruker Impact II ultrahigh-resolution qTOF mass spectrometer equipped with an electrospray ionization (UPLC ESI-qTOF-HRMS). A mixed solution of H₂O + 0.1% formic acid and acetonitrile (3% ACN + 0.1% formic acid) was used to elute the LC-MS system equipped with a chromatographic reverse phase C18 column (Agilent Eclipse XDB-C18, 250 × 4.6 mm, 5 μm) with a flow rate of 0.4 ml/min. A sodium formate solution (250 ml isopropanol, 1 ml formic acid, 5 ml 1 M NaOH in 500 ml H₂O) was applied as a calibration solution. The Compass DataAnalysis software was used to calibrate, process, and analyze MS data. The experiment was performed in duplicate.

Expressions of Recombinant Cellulase Proteins in *P. pacificus*

Ppa-CEL-2, Ppa-CEL-4, Ppa-CEL-5.1, and Ppa-CEL-6 were selected and expressed in *E. coli* following the protocol from Grüner et al. (2016). Briefly, synthetic *E. coli* K-12 codon optimized DNA strings (<https://eu.idtdna.com/CodonOpt>) corresponding to the cellulase cDNA sequences lacking the putative signal peptide were cloned into the pNEA-NpM vector (Diebold et al. 2011). The resulting recombinant cellulases are tagged N-terminally with the maltose-binding protein (MBP). All proteins were expressed in *E. coli* BL21 Star (DE3) cells (Invitrogen, Cat. No. C601003) grown in LB medium at 37 °C till OD600 approximately 0.3, and then induced with IPTG overnight at 20 °C and 180 rpm. The following purification steps were performed at 4 °C. Cells were pelleted and resuspended in the 50 mM HEPES (pH 7.3), 150 mM NaCl, 2 mM DTT, DNase I (5 μg/ml), lysozyme (1 mg/ml), and protease inhibitor cocktail (Roche). Cells were then lysed by sonication. The recombinant cellulases were purified from cleared bacterial lysates using amylose resin (New England Biolabs, Cat. No. E8021). The purified MBP-tagged cellulases were analyzed on 10% SDS-PAGE.

To test whether the expressed proteins were cellulolytic active, lysates were used in diffusion assays. Holes of 0.8 mm diameter were drilled on a 1% agar plate supplemented with 0.5% CMC plate and 80 μl of the lysate was inoculated into the hole. Eighty microliters of bacterial lysate expressing only MBP was used as negative control, and 40 μl of 300 mU/ml cellulase from *A. niger* was used as positive control. After overnight incubation at 37 °C, the plate was stained with 0.1% Congo red solution for 30 min and destained with 1 M NaCl for three times.

Cellulase Gene Expressions on Different Bacteria Using RT-qPCR

Wild-type *P. pacificus* were grown on *E. coli* OP50, and bacteria isolated from La Réunion island including *Pseudomonas* sp. strain LRB26, *Acinetobacter* sp. strain LRB80, *Comamonas* sp. strain LRB103, and *Wautersiella* sp. strain LRB104 (Akduman et al. 2018). The expression levels of *Ppa-cel-1*, *Ppa-cel-2*, and *Ppa-cel-3* were determined using RT-qPCR, and relative expression levels to the internal reference gene *Ppa-Y45F10D.4* and *Ppa-cdc-42* are presented (Schuster and Sommer 2012). A one-step RT-qPCR kit was used following the manufacturer's protocol (New England Biolabs, Cat. No.

E3005). Specific primers can be found in [supplementary table S6, Supplementary Material](#) online.

Reproduction Assays

Details of modified NGM plates used in this study can be found in [supplementary table S3, Supplementary Material](#) online.

All *E. coli* strains were inoculated into LB broth and incubated at 37 °C for 15 h. One hundred microliters of the designated bacterial culture was seeded onto the standard or modified NGM plate. Plates were kept at 20 °C for 3 days before use. The *P. pacificus* wild-type strain PS312 or cellulase octuple mutant RS3762 grown on standard NGM OP50 were bleached and placed on an unseeded NGM plate for hatching. Hatched J2 animals (as P0s) were then maintained as individual lines on their own designated plates and incubated at 20 °C. After approximately 72 h, the P0 was transferred onto a new plate. Offspring from both plates were counted. All experiments were biologically duplicated.

Developmental Speed Assays

Wild type and octuple mutant animals were bleached and maintained as individual lines as above. After approximately 72 h when each PS312 P0 had laid around 30 eggs, P0s from all treatments were removed from the plates. The stages of F1 stages were determined when the majority of PS312 F1s on *E. coli* OP50 had reached the J4 stage (~90 h after the removal of P0s). All experiments were performed in duplicate, and for each treatment between 14 and 28 P0s were examined for the developmental speed of their offspring.

Motility Assays

Bleached eggs were placed on standard NGM plates with 3-day-old designated bacteria. When the nematodes developed into young adults, they were moved to a standard NGM with a 2-day-old bacterial lawn. A 5-min video was taken after at least 1 h of recovery. A Zeiss Discovery V12 stereo microscope with a Zeiss AxioCam was used to record the video and the video was analyzed using the software tierpsy-tracker (Javer et al. 2018) to measure the distance of path conducted by each nematode.

RNA-Seq Library Preparation

Both wild type and the octuple mutant, which were previously reared on *E. coli* OP50, were bleached. Bleached eggs from each strain were placed onto standard NGM plates with 3-day-old bacterial lawns of *E. coli* OP50 and *E. coli* K-12 AR3110, respectively. Nematodes were collected when they grew to the second generation where the majority reached the young adult stage but before the depletion of the bacterial food source. This process was performed in triplicate. RNA extraction was performed using the Direct-zol RNA Miniprep Kits (Zymoresearch, Cat. No. R2053). Library preparation was performed using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, Cat. No. E7765) with 500–1,000 ng RNA input following the manufacturer's protocol "for use with NEBNext Poly(A) mRNA Magnetic Isolation Module." Library quality was analyzed

on an Agilent Bioanalyzer and sequencing was performed on an Illumina HiSeq 3000 machine.

Statistics and Bioinformatics

All reproduction assays were analyzed using two-way ANOVA, in which *P. pacificus* strains and bacteria strains (or CMC supplementation) were treated as the two factors. One-tailed Student's *t*-test and Mann–Whitney rank test were used to test the effect from bacteria strains or CMC supplementation between the *P. pacificus* wild type and the cellulase octuple mutant. ANOVA tests were conducted using the python package “pingouin” (<https://pingouin-stats.org/>), whereas *t*-tests and Mann–Whitney rank tests were conducted using the python package “SciPy” (<https://scipy.org/>). Developmental stages were analyzed using permutational multivariate analysis of variance (PERMANOVA) followed by pairwise comparisons between selected treatments. Tests were conducted using R package PERMANOVA and pairwiseAdonis. The expression patterns of *Ppa-cel-2p::TurboRFP* were analyzed using Fisher–Freeman–Halton test, which was conducted by the python package FisherExact. To construct the phylogeny of nematodes, the 1:1 orthologous were identified using OrthoFinder (Emms and Kelly 2015, 2019), and the phylogeny was inferred using IQ-TREE (Nguyen et al. 2015). To evaluate the selection of cellulase genes, branch- and site-specific selection tests were conducted using PAML (v.4) from the CodeML module (Yang 2007). The log-likelihood values of the model comparisons and their statistical significance were summarized in [supplementary tables S1 and S2, Supplementary Material online](#).

Reads from RNA-seq experiments were mapped to the *P. pacificus* genome (pristionchus.org, version: El Paco) using Hisat2 (version 2.1.0; Kim et al. 2015). Transcripts were quantified using featureCounts (Liao et al. 2014) based on the El Paco annotation version 3. To determine biological processes that were differentially expressed between *P. pacificus* wild type and the octuple mutant on different *E. coli* strains, we performed a GSEA (Subramanian et al. 2005). The *P. pacificus* gene sets were adapted from established *C. elegans* gene sets (WormEnrichr; Chen et al. 2013; Kuleshov et al. 2016). *P. pacificus* orthologous genes to *C. elegans* were determined using OrthoFinder (Emms and Kelly 2015, 2019), and these orthologs were assigned to the corresponding gene groups (referred as GO terms). The python package GSEAPy (<https://gseapy.readthedocs.io/en/latest/>) was used for the analysis.

Supplementary Material

[Supplementary data](#) are available at *Molecular Biology and Evolution* online.

Acknowledgments

We thank Dr Regine Hengge and Alexandra Possling for providing *E. coli* K-12 strains. We thank Christian Feldhaus for microscopy and the genome center at the Max-Planck Institute for sequencing services. Drs Christian Roedelberger, Matthias Herrmann, James W. Lightfoot,

Nickolas Youngblut, Hassan Salem, Adrian Streit, and Mr. Wenfei Xian provided valuable suggestions to the manuscript. We also thank the assistance from Drs Michael W. Werner, Matthias Herrmann, Mr. Chris Weiler, and other members in the Sommer lab. We thank four anonymous reviewers whose comments have greatly improved this manuscript. This work was supported by the Max-Planck Society and a Humboldt Research Fellowship for postdoctoral researchers from the Alexander von Humboldt Foundation (to Z.H.).

Author Contributions

Z.H., B.S., and V.S. performed most of the experiments with technical help from H.W. W.-S.L. performed bioinformatics analyses. C.I., C.D., and A.B. performed biochemistry and analytical chemistry experiments. Z.H., B.S., V.S., and R.J.S. wrote the manuscript. R.J.S. supervised the project.

Data Availability

The data underlying this article are available within the National Center for Biotechnology Information (NCBI) GenBank database (<https://www.ncbi.nlm.nih.gov/genbank>), and all GenBank accession numbers are listed in [Supplementary Material](#). Genes from *Pristionchus* spp. can be found in <http://pristionchus.org/>, and gene IDs are listed in [Supplementary Material](#). Raw reads for transcriptomic experiments have been submitted to NCBI SRA database under accession PRJNA746144.

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