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The *Caenorhabditis elegans* assay: a tool to evaluate the pathogenic potential of bacterial biocontrol agents

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Abstract Bacterial biocontrol agents (BCAs) open up the possibility of controlling plant pathogens in an environmentally friendly way. Although they are naturally occurring microbes, some of them can cause diseases in humans. For successful registration it is necessary to test potentially adverse effects on the human health of at-risk candidates. Existing pathogenicity assays are cost-intensive, time-consuming and furthermore they are often inappropriate for facultative pathogens. We developed a new, fast and inexpensive bioassay on the basis of the nematode *Caenorhabditis elegans,* which is a well-accepted model organism to study bacterial pathogenicity. A selection of eight strains from clinical and environmental origin as well as potential and commercial BCAs from the genera *Bacillus, Pseudomonas, Serratia* and *Stenotrophomonas* were screened for their potential to kill the nematode in an *in vitro* agar plate assay. Furthermore, the motility and reproductive behaviour of nematodes exposed to strains were tested in comparison with those fed by the human pathogen *Pseudomonas aeruginosa* QC14-3-8 (positive control) and the negative control *Escherichia coli* OP50. Commercial as well as potential biocontrol strains did not display any adverse effects in all tests. In contrast, the *C. elegans* assay showed slight effects for clinical and environmental *Stenotrophomonas* strains. Results showed that the nematode *C. elegans* provides a model system to indicate the pathogenic potential of BCAs in a very early stage of product development.

Keywords Nematodes · *Caenorhabditis elegans* · BCAs · Antagonists · Pathogenicity · Screening

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Introduction

Biological control using antagonistic microorganisms towards plant pathogens offers sustainable and environmental friendly solutions for agriculture and horticulture (Weller 1988; Weller et al. 2002). Many studies showed the efficacy of biocontrol agents (BCAs) against plant diseases under field conditions (Timms-Wilson et al. 2000; Kurze et al. 2001; Grosch et al. 2005). Plant-associated antagonistic bacteria inhibit plant pathogens by various mechanisms, which include (i) inhibition by antibiotics, toxins and biosurfactants (antibiosis), (ii) competition for colonisation sites and nutrients, (iii) competition for minerals, e.g. iron through production of siderophores or efficient siderophore-uptake systems, (iv) degradation

of pathogenicity factors of the pathogen such as toxins, and (v) parasitism that may involve production of extracellular cell wall-degrading enzymes such as chitinases and β-1,3-glucanase (Whipps 2001; Compant et al. 2005; Haas and Défago 2005). Some antagonistic species of the genera *Burkholderia, Enterobacter, Herbaspirillum, Ochrobactrum, Pseudomonas, Serratia, Staphylococcus* and *Stenotrophomonas* are root-associated bacteria that can enter into mutualistic interactions with plant and human hosts (Parke and Gurian-Sherman 2001; Ribbeck-Busch et al. 2005). Mechanisms involved in the interaction between antagonistic plant-associated bacteria and their host plants are similar to those responsible for the pathogenicity of bacteria to humans (Rahme et al. 1995; Berg et al. 2005). The mechanisms of interaction with potential eukaryotic hosts are species- and strain-specific (Berg et al. 1996; 2002). To avoid the use of potential human pathogens in biological plant protection, it is necessary to evaluate the risk of each potential BCA.

Until now, the registration procedure for a BCA is based on rules originally developed for synthetic pesticides (EU Council Directive 91/414/EEC, see also www.rebeca-net.de). These methods, which were adopted from standardised tests for chemical-based agents are not only time-consuming and expensive, their results are difficult to assess for biological products. Traditional animal assays are also costly and time-consuming and furthermore subject to tight regulations and ethical considerations. Finally, pathogenicity and the mode of action of facultative pathogens such as *Burkholderia* and *Stenotrophomonas* cannot be analysed in traditional animal assays due to their opportunistic character. Therefore, alternative assays using non-mammalian hosts, such as the slime mould *Dictyostelium discoideum* (Alonso et al. 2004), the fruit fly *Drosophila melanogaster* (Vodovar et al. 2004), the zebrafish *Danio rerio* (Van der Sar et al. 2003) or the nematode *Caenorhabditis elegans* (Tan et al. 1999a) have been studied. This was possible because many human pathogens have a low species-specificity and can infect hosts ranging from insects and nematodes to fish, as well as other mammals (Kurz and Ewbank 2007). A rapidly growing number of human and animal pathogens including antagonistic bacteria have been shown to injure and kill nematodes (Aballay and Ausubel 2002; Ruiz-Díez et al. 2003; Cardona et al. 2005). In many cases, microbial genes important for full virulence in mammalian models are similarly required for maximum pathogenicity in nematodes (Sifri et al. 2005).

Caenorhabditis elegans is a free-living terrestrial nematode that feeds on bacteria in its environment (Beale et al. 2006). Under simple growth conditions, self-reproducing hermaphrodites grow up to 1 mm in length. They are transparent, and therefore easy to observe under standard light microscopy. Approximately 40% of genes associated with human disease have homologues in the *C. elegans* genome (Culletto and Sattelle 2000). The duration of the developmental cycle of the nematode is two to three weeks under optimal living conditions. The post-embryonic development comprises four different stages of juveniles (J1 – J4) which differ in size in comparison to the adult worm. Extensive information about *C. elegans* research is available in databases (www.wormbook.org, www.wormbase.org).

The objective of this study was to evaluate *C. elegans* as a model to assess human pathogenicity of potential BCAs. The slow-killing method was used to analyse opportunistic bacterial pathogens (Köthe et al. 2003; Ruiz-Díez et al. 2003). Using this assay, we analysed a representative number of bacterial antagonists against fungal plant pathogens from different phylogenetical groups and origins including commercial BCAs.

Materials and methods

Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. *Escherichia coli* OP50 was kindly provided by Leo Eberl (Zürich, Switzerland). In the laboratory *E. coli* OP50 is used to maintain worm strains (Tan et al. 1999b; Köthe et al. 2003; Beale et al. 2006). The non-pathogenic *E. coli* strain OP50 was also used as negative control in pathogenicity assays as well as the human pathogenic *Pseudomonas aeruginosa* QC14-3-8 as a positive control.

Nematode assays

The nematode *C. elegans* N2 was kindly provided by Leo Eberl (Zürich, Switzerland). The nematode mortality assay (slow killing of *C. elegans*) was

Table 1 Strains used in the assay and their antagonistic and plant growth promoting mechanisms

Species	Strain number	Origin	Antagonistic properties against[1]	Bioactive metabolites	Growth at 37°C	References
Pseudomonas aeruginosa (positive control)	QC14-3-8	Geocaulosphere of potato, Quedlinburg, Germany	*E. carotovora, V. dahliae, R. solani*	Indol-3-acetic acid	+	Lottmann et al. (1999)
Escherichia coli (negative control)	OP50		-	n.d.; uracil requiring mutant of *E. coli*	+	Brenner (1974)
Bacillus subtilis	B2g	Rhizosphere of oilseed rape; Biestow, Germany	*P. ultimum, R. solani*	Siderophores, Glucanase	+	Marten et al. (2000); Berg et al. (2000)
Pseudomonas fluorescens	L13-6-12	Rhizosphere of potato, Groß Lüsewitz, Germany	*P. betae, R. solani, S. rolfsii, V. dahliae*	Indol-3-acetic acid	+	Lottmann et al. (2000); Scherwinski et al. (2007)
Pseudomonas trivialis (1)	3Re2-7	Endorhiza of potato; Bonn, Germany	*R. solani, V. dahliae*	Indol-3-acetic acid, Proteases, Siderophores, DAPG	+	Berg et al. (2005); Scherwinski et al. (2007, 2008)
Pseudomonas trivialis (2)	RE*1-1-14	Rhizosphere of sugar beet; Hilprechtshausen, Germany	*P. betae, R. solani, S. rolfsii*	Chitinase, Glucanase, Proteases	−	Zachow et al. (2008)
Serratia plymuthica (1)	3Re4-18	Endorhiza of potato; Rostock, Germany	*P. betae, R. solani, S. rolfsii, V. dahliae*	Cellulase, Chitinase, Glucanase, Pectinase, Proteases, Siderophores	+	Berg et al. (2005); Scherwinski et al. (2006)
Serratia plymuthica (2)	HRO-C48	Rhizosphere of oilseed rape, Rövershagen, Germany	*R. solani, V. dahliae*	Chitinases, Proteases, Siderophores, Indol-3-acetic acid	+	Kalbe et al. (1996); Kurze et al. (2001)
Stenotrophomonas maltophilia	C5	Human; Copenhagen, Denmark	*C. albicans*	Siderophores, Proteases, Chitinases, Antibiotics, Hemolysine, zot Toxin	+	Berg et al. (1999); Minkwitz & Berg (2001); Ribbeck-Busch et al. (2005); Hagemann et al. (2006)
Stenotrophomonas rhizophila	P69	Rhizosphere of oilseed rape; Rostock, Germany	*V. dahliae*	Siderophores, Glucanase, Proteases, Hemolysine	+	Berg et al. (1996, 1999); Minkwitz & Berg (2001); Wolf et al. 2002; Ribbeck-Busch et al. (2005)

[1] *C. albicans* = *Candida albicans*; *E. carotovora* = *Erwinia carotovora*; *P. betae* = *Phoma betae*; *P. ultimum* = *Pythium ultimum*; *R. solani* = *Rhizoctonia solani*; *S. rolfsii* = *Sclerotium rolfsii*; *V. dahliae* = *Verticillium dahliae*

performed as described by Köthe et al. (2003) with modifications. Briefly, 100 µl of an overnight culture of the bacterial test strains were plated on 6.0 cm-diam nematode growth medium (NGM) agar plates. After spreading the bacterial culture on the agar surface, plates were incubated at 30°C for 24 h to form bacterial lawns. Plates were then inoculated with approximately 25 hypochlorite-synchronised J2 juveniles of *C. elegans*. The nematodes were age-synchronised with the bleaching procedure. Adults, juveniles and eggs grown for 2.5 days on *E. coli* OP50 at 20°C were harvested by washing the NGM agar plate with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 6 g NaCl, 5 g $MgSO_4$ in 1 l H_2O, pH 6.0). The nematode suspensions were mixed with a bleaching solution (600 µl Aqua distilled, 500 µl 12% NaOCl, 400 µl 6 N NaOH) and gently shaken for 8 min. After 1 min at 3500 rpm the supernatant was discarded and the pelleted eggs washed twice with 1 ml M9 buffer as mentioned above. After a final centrifugation, the pellet containing nematode stages and dead nematodes was resuspended in 150 µl M9

buffer and the suspension placed on NGM agar plates with the food strain OP50 and incubated at 20°C. Second-stage juveniles (J2) freshly hatched from the eggs were collected after 48 h and used for the experiment.

The actual number of the initially introduced J2 juveniles was determined with a WILD M3B microscope (Heerbrugg, Switzerland) at 16-fold magnification. After counting, plates were incubated at 20°C and scored for live nematodes every 24 h at the same time points. Nematodes were considered dead when they failed to respond to touch. In order to determine the killing rate of *C. elegans* the number of dead worms, as well as the emergence of eggs and juveniles were recorded. Susceptible nematodes became inactive and did not show any response to contact. The nematodes became elongated and disintegrated. In all cases, experiments were stopped after 5 days, when the initial nematodes were indistinguishable from their progeny. All experiments were carried out at least three times with three replicates.

The evaluation of pathogenicity of bacteria to *C. elegans* was assessed according to Cardona et al. (2005). The authors established that a given strain was pathogenic for the nematode if one of the following criteria was met: (i) a diseased appearance at day 2, which included reduced locomotive capacity and the presence of an distended intestine; (ii) percentage of live nematodes at day 2 $\leq$ 50%; and (iii) total number of nematodes at day 5 $\leq$ 50. The presence of any one, two or three of these criteria was scored to differentiate mild from severe infections. A pathogenic score (PS 1, 2 or 3) was given based on the number of criteria met.

A strain was considered non-pathogenic when no symptoms of disease were observed during the course of the infection experiment (pathogenicity score PS 0). Additionally, the influence of the bacteria on motility and propagation of the nematode was monitored for 120 h as follows. An evaluation score (ES 1 to 8) was given based on the number of criteria met: ES: 1 = no progeny (no eggs or hatching worms), 2 = eggs, 3 = second generation, 4 = second generation no longer distinguishable from the first generation, 5 = slow movement, 6 = accumulation in batches, 7 / 8 = after 120 h no / complete digestion of the bacterial layer by the nematodes. If one of the conditions 1 or 5 to 7 were observed the strain was excluded from further screenings in case it was human pathogenic.

Statistics

For each nematode assay, the survival rate was calculated and the killing kinetics of *C. elegans*, which describes the number of living worms over the whole experiment, was statistically analysed by ANOVA (analysis of variance) using SPSS version 10.0 for Windows. If ANOVA showed statistical significance, individual means were then compared using Tukey's pairwise comparison.

Results

Effects on mortality of *C. elegans* by BCAs, potential BCAs and antagonistic strains

First, the aim was to analyse the pathogenic potential of the strains which are already commercialised BCAs, *Bacillus subtilis* B2g, *Serratia plymuthica* HRO-C48, and *Pseudomonas trivialis* 3Re2-7 (Table 1, Fig. 1a). *Bacillus subtilis* B2g and *P. trivialis* 3Re2-7 showed no negative effect on *C. elegans.* The third biocontrol strain *S. plymuthica* HRO-C48 caused a mortality rate of approximately 23% after 72 h but showed no adverse effect on progenies and movement. All commercial strains were scored with a pathogenicity score of PS 0.

The potential BCAs, which are still in product development, were represented by *P. fluorescens* L13-6-12, *P. trivialis* RE*1-1-14, and *S. plymuthica* 3Re4-18. There were no significant differences in mortality of *C. elegans* between these three strains and in comparison to treatments with the negative control *Escherichia coli* OP50 (pathogenicity score 0; Fig. 1b). Also, reproductive behaviour of the nematode was similar to the negative control with *E. coli* OP50 and not affected significantly by the BCAs. In contrast, mortality of *C. elegans* was significantly higher in applications of *P. aeruginosa* QC14-3-8 after 24 h (pathogenicity score 3). The nematodes were not able to produce eggs and showed retardation in movement.

Furthermore, a clinical (*S. maltophilia* C5) and non-clinical isolate (*S. rhizophila* P69) from the genus *Stenotrophomonas* were analysed in the *C. elegans*

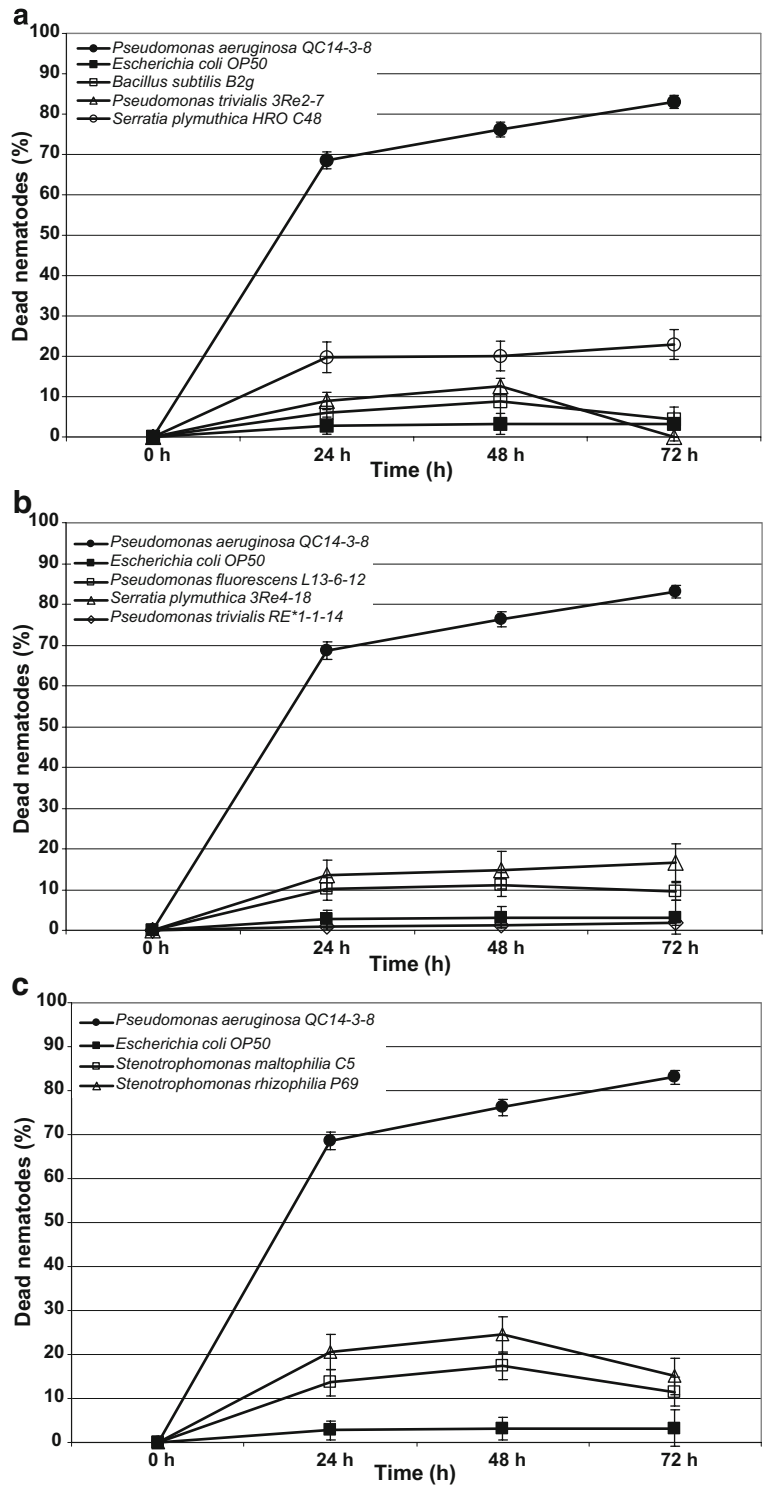

Fig. 1 Activity of *Caenorhabditis elegans* fed with different BCAs under slow killing conditions. Nematodes grew on NGM and fed on *Pseudomonas aeruginosa* QC14-3-8 (positive control, black circle) and *Escherichia coli* OP50 (negative control, black square), respectively. **a** Commercial BCAs: *B. subtilis* B2g (white square), *S. plymuthica* HRO-C48 (white diamond), and *P. trivialis* 3Re2-7 (white triangle). **b** BCAs which are still in biological control product development: *Pseudomonas fluorescens* L13-6-12 (white square), *P. trivialis* RE*1-1-14 (white diamond), and *Serratia plymuthica* 3Re4-18 (white triangle). **c** *Stenotrophomonas* isolates: environmental strain *S. rhizophila* P69 (white triangle), the clinical isolate *S. maltophilia* C5 (white square). Data points represent means ± standard errors of at least three independent experiments with three repetitions

assay. The rate of killing by the environmental strain was slightly higher but not significantly different to the clinical strain. Both strains exhibited different degrees of pathogenicity in comparison to the BCAs. They were scored with PS 1, because of their retardation of velocity of movement (Fig. 1c).

Effects on movement and propagation of *C. elegans* by BCAs, potential BCAs and antagonistic strains

Because of nematode desiccation and to ensure results, the effects of the BCAs on movement and propagation of *C. elegans* were monitored and summarised with scores ES 1 to 8 in Table 2. The positive control *P. aeruginosa*, completely inhibited propagation of *C. elegans* (ES 1) whereas the negative control *E. coli* OP50 had no effect on the number and development of eggs and juveniles (Table 2). *Pseudomonas aeruginosa* reduced movement of *C. elegans* (ES 5). Escape behaviour was observed in treatments with *P. aeruginosa*; the nematodes moved away from the bacterial lawn to the margins of the Petri dishes (data not shown). The negative control *E. coli* OP50 did not affect motility, movement and reproduction of *C. elegans* (ES 2, 3, 4). None of the biocontol strains (*B. subtilis* B2g, *P. fluorescens* L13-6-12, *P. trivialis* 3Re2-7, *P. trivialis* RE*1-1-14, *S. plymuthica* 3Re4-18, *S. plymuthica* HRO-C48) influenced *C. elegans* negatively; the BCAs were used as feed by *C. elegans,* as shown for *B. subtilis* B2g. After 120 h, the nematodes started to digest the *B. subtilis* B2g from fissures and the margin of the bacterial lawn where the nematodes accumulated. If there were no more detectable bacterial cells on the Petri dish the nematode was able to digest the bacteria. Both *Stenotrophomonas* strains significantly reduced motility of *C. elegans* (ES 5), but did not have any negative effect on the number of eggs and the development of progeny (Table 2).

Discussion

In this study, the mortality of *C. elegans* (slow killing assay) was used to assess the potential human pathogenicity of biocontrol strains. All investigated strains showed significantly less pathogenicity to *C. elegans* compared to the well-known facultative human pathogen *P. aeruginosa*. However, subtle differences in the level of pathogenicity were found. These differences as well as advantages and disadvantages of the *C. elegans* assay for our approach will be discussed in detail.

Bacteria are able to interact with their hosts by various mechanisms. One of the first steps is the recognition and the colonisation of the rhizosphere or

Table 2 Pathogenicity scores and further remarks of J4 larval-stage *Caenorhabditis elegans* during the 120 h experiment

Species of biocontrol strain	Strain number	Behaviour score S[2] during the development of the nematodes						
		PS[1]	0 h	24 h	48 h	72 h	96 h	Remarks 0 – 120 h
Pseudomonas aeruginosa (positive control)	QC 14-3-8	3	1	1	1	1	1	5, 7
Escherichia coli (negative control)	OP50	0	1	2	2, 3	2, 3	2, 4	6, 8
Bacillus subtilis	B2g	0	1	2	2, 3	2, 3	2, 3	8
Pseudomonas fluorescens	L13-6-12	0	1	2	2, 3	2, 3	2, 4	6,8
Pseudomonas trivialis (1)	3Re2-7	0	1	2	2, 3	2, 4	2, 4	5, 7
Pseudomonas trivialis (2)	RE*1-1-14	1	1	2	2, 3	2, 3	2, 4	6, 8
Serratia plymuthica (1)	3Re4-18	0	1	2	2, 3	2, 3	2, 4	8
Serratia plymuthica (2)	HRO-C48	0	1	2	2, 3	2, 3	2, 4	8
Stenotrophomonas maltophilia	C5	1	1	1	2	2, 3	2, 4	5, 6, 7
Stenotrophomonas rhizophila	P69	1	1	2	2, 3	2, 3	2, 4	5, 6, 7

[1] Pathogenicity score PS 0, no signs of disease; 1, 2 and 3, one two or three symptoms of disease, respectively

[2] Evaluation score ES: 1 = no progeny (no eggs or hatching worms), 2 = eggs, 3 = second generation, 4 = second generation no longer distinguishable from the first generation, 5 = slow movement, 6 = accumulation in batches, 7 / 8 = after 120 h no / complete digestion of the bacterial layer by the nematodes

the attachment to human tissue (Compant et al. 2005). The biocontrol strains used in the study colonised rhizospheres (Lottmann et al. 2000; Kurze et al. 2001; Scherwinski et al. 2008; Zachow unpublished results). During the screening, the bacteria were not able to colonise *C. elegans;* in contrast the nematode was able to digest the bacteria. Vaitkevicius et al. (2006) suggested that killing of *C. elegans* by *Vibrio cholerae* requires direct contact with bacterial cells. In this way, exoenzymes, toxins and antibiotics interact in direct contact with the nematode. Some of our strains are known for the production of antifungal antibiotics and toxins, e.g. *Serratia* for pyrrolnitrin and *Stenotrophomonas* for maltophilin and *zot* toxin (Liu et al. 2007; Hagemann et al. 2006). However, in the assay, they showed no or only minor effects on *C. elegans*.

First developed for Gram-negative bacteria, Garsin et al. (2001) applied the *C. elegans* assay to Gram-positive bacteria. A number of Gram-positive human pathogens including *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* killed *C. elegans* but other species like *Enterococcus faecium* or *Streptococcus pyogenes* did not exhibit a negative effect on the nematode. Under the same conditions, *B. subtilis* PY79 did not increase mortality (Garsin et al. 2001). In contrast, Qiuhong et al. (2006) reported killing of *C. elegans* by *Bacillus* spp. with a serine protease as the responsible factor. In our study, *B. subtilis* B2g did not display any negative effect on *C. elegans*. The example of *Bacillus* confirmed strain-specificity, which was found for antagonistic mechanisms of plant-associated bacteria (Berg et al. 2002), also for the effect in the *C. elegans* assay. Altogether, all Gram-negative and -positive BCAs showed no substantial effects on health and reproduction of *C. elegans* except for the positive control *P. aeruginosa* QC14-3-8 (Lottmann et al. 1999).

Although no pathogenic potential of BCAs was detected, the *C. elegans* assay showed an effect of *Stenotrophomonas* strains on the nematode. *Stenotrophomonas* strains were reported as promising BCAs as well as opportunistic pathogens (Berg et al. 1999; Minkwitz and Berg 2001; Ribbeck-Busch et al. 2005). Interestingly, the environmental *S. rhizophila* decreased the velocity of the nematode movement more than the clinical isolate *S. maltophila*. For the clinical *Stenotrophomonas* strain it is assumed that a phage insertion carrying genes for the *zot* toxin enhances its pathogenicity (Hagemann et al. 2006). The environmental strain produces no antibiotics, but shows proteolytic and haemolytic activity (Kalbe et al. 1996), which could be responsible for this reaction. Due to the fact that the nematode was slightly sensitive to both *Stenotrophomas* strains tested, we suggest further detailed studies considering strain specificity.

The application of the *C. elegans* assay faces some limitations. The degree of pathogenicity depends on the quantity of the bacterial inoculum which can potentially infect the nematode. This was shown for *P. aeruginosa*, *P. fluorescens*, *Serratia marcescens*, *Burkholderia cepacia*, *B. pseudomallei*, *B. thailandensis*, *Salmonella* spp., and *Bacillus megaterium* (Labrousse et al. 2000; Tan and Ausubel 2000). In this study, we applied an overnight culture with approximately 10^7 cells ml^{-1} in an appropriate thin cell layer in order to standardise the applied bacterial inoculum and the exposure of the nematode to the inoculum. This concentration allowed the detection of differences in survival among different strains of *C. elegans* after 24 h (Schulenburg and Ewbank 2004). Furthermore, mortality was also influenced by the developmental stage of the applied nematodes. Adult worms were more sensitive and died faster than J4 juveniles. Therefore, we applied J2 juveniles exactly 48 h after egg preparation in the mortality assay. BCAs with known and intended nematicidal activites such as *B. thuringiensis*, pose another problem. Lastly, it is difficult to correlate the pathogenic potential in *C. elegans* and humans without experimental data for both. Until now, such a study does not exist. But there are studies for *P. aeruginosa* (Lee et al. 2006) and for *Burkholderia* (Cardona et al. 2005) showing that human-associated strains of this genus caused effects in *C. elegans*. In interpreting the results it should be also considered that *C. elegans* uses a sophisticated chemosensory system to identify food and olfactory learning as a mechanism to avoid pathogens (Beale et al. 2006). Data show that after a short exposure to *P. aeruginosa*, *C. elegans* learns to avoid it when subsequently exposed (Zhang et al. 2005, this study). Escape behaviour was found for the worm cultivated with *P. aeruginosa* QC14-3-8 but not for *Stenotrophomonas*. However, the final evaluation of the health of the nematode and its ability to digest the bacteria as a safe nutrition source at the end of the experiment is important.

On the other hand, use of the *C. elegans* assay for development of a biocontrol product can have several advantages. In contrast to the existing laborious methods, the assay is fast, standardised and inexpensive. Results, which are carefully interpreted, can be useful for biocontrol approaches. If negative effects on eukaryotes are found, we have to delve in more detail and analyse the mechanisms involved, and also the effect in classical vertebrate assays. We therefore postulate that the *C. elegans* assay has potential during initial screening for BCAs where it serves as a new tool to identify effects against eukaryotes at a very early stage of product development.

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