environmental microbiology

Environmental Microbiology (2010) **12**(11), 3007–3021 doi:10.1111/j.1462-2920.2010.02278.x

A subset of naturally isolated *Bacillus* strains show extreme virulence to the free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*

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Summary

The main food source of free-living nematodes in the soil environment is bacteria, which can affect nematode development, fecundity and survival. In order to occupy a reliable source of bacterial food, some nematodes have formed specific relationships with an array of invertebrate hosts (where bacteria proliferate once the hosts dies), thus forming a tritrophic system of nematode, bacteria and insect or other invertebrates. We isolated 768 *Bacillus* strains from soil (from Germany and the UK), horse dung and dung beetles and fed them to the genetically tractable free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus* to isolate nematocidal strains. While *C. elegans* is a bacteriovorous soil nematode, *P. pacificus* is an omnivorous worm that is often found in association with scarab beetles. We found 20 *Bacillus* strains (consisting of *B. cereus*, *B. weihenstephanensis*, *B. mycoides* and *Bacillus* sp.) that were pathogenic to *C. elegans* and *P. pacificus* causing 70% to 100% mortality over 5 days and significantly affect development and brood size. The most pathogenic strains are three *B. cereus*-like strains isolated from dung beetles, which exhibit extreme virulence to *C. elegans* in less than 24 h, but *P. pacificus* remains resistant. *C. elegans* Bre mutants were also highly susceptible to the *B. cereus*-like strains indicating that their toxins use a different virulence mechanism than *B. thuringiensis* Cry 5B toxin. Also, mutations in the *daf-2*/*daf-16* insulin signaling pathway do not rescue survival. We profiled the toxin genes (*bcet*, *nhe* complex, *hbl* complex, *pcpl*, *sph*, *cytK*, *piplc*, *hly2*, *hly3*, *entFM* and *entS*) of these three *B. cereus*-like strains and showed presence of most toxin genes but absence of the *hbl* complex. Taken together, this study shows that the majority of naturally isolated *Bacillus* from soil, horse dung and *Geotrupes* beetles are benign to both *C. elegans* and *P. pacificus*. Among 20 pathogenic strains with distinct virulence patterns against the two nematodes, we selected three *B. cereus*-like strains to investigate resistance and susceptibility immune responses in nematodes.

Introduction

Bacteria from the genus *Bacillus* are found in great abundance in the soil matrix, e.g. 10^4–10^6 per gram (Martin and Travers, 1989) as heat resistant spores (Nicholson, 2002). A subset of *Bacillus* are of medical and economic importance. For example, *Bacillus anthracis* is the causative agent of anthrax (Lew, 1995), *Paenibacillus larvae* is causative agent of American foulbrood disease in honeybees (de Maagd *et al*., 2001), which is responsible for large economic losses in the honey industry, and *B. cereus* is responsible for severe food poisoning (Granum and Lund, 1997). *Bacillus thuringiensis* (BT) is used as a commercial pesticide used to control insects in the orders Lepidoptera, Diptera and Coleoptera (Beegle and Yamamoto, 1992).

As well as insects, it has been speculated that nematodes may contribute to the evolution and/or spreading of *Bacillus* (in particular BT) (Wei *et al*., 2003), because nematodes are ubiquitous in the soil environment and many *Bacillus* species kill nematodes when fed spores or vegetative cells. For example, *B. firmus*, *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides* and *B. pumilus* are toxic to plant parasitic nematodes (*Heterodera* sp. and *Meloidogyne* sp.) (Siddiqui and Mahmood, 1999; Terefe *et al*., 2009) and BT crystal proteins (Cry 6A, 5B, 14A and 21A) can kill free-living and animal parasitic nematodes (Borgonie *et al*., 1996; Wei *et al*., 2003) although some have no effect.

The free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus* (Fig. 1A and B) have been developed as genetic model systems with a wealth of forward and reverse genetic tools and full genome sequence available (C. elegans Sequencing Consortium, 1998;

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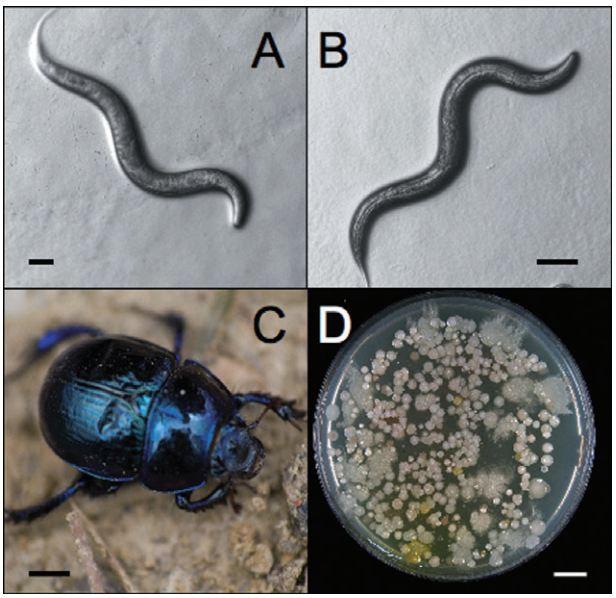

Fig. 1. Organisms used in this study. The nematodes *P. pacificus* (A) and *C. elegans* (B), *Geotrupes* sp. beetle (C) and *Bacillus* isolated from soil (D). Scale bars in A and B represent 100 μm, C represents 0.5 cm and D is 1 cm.

Dieterich *et al*., 2008). In nature, both these nematodes can be isolated from invertebrate hosts and soil samples from around the world, but they have a completely different ecology. *Caenorhabditis elegans* is a soil nematode that has occasionally been isolated from a range of invertebrates including isopods, slugs and snails (Mengert, 1953; Barriere and Felix, 2005; Caswell-Chen *et al*., 2005). In contrast, many *Pristionchus* species can be readily isolated from scarab beetles in an almost exclusive manner. For example, *P. pacificus* has been isolated from the oriental beetle in Japan and the USA (*Exomala orientalis*) (Herrmann *et al*., 2007), *P. maupasi* from cockchafers (*Melolontha* spp.), *P. entomophagus* from dung beetles (*Geotrupes* sp.) (Fig. 1C) in Europe (Herrmann *et al*., 2006a) and *P. uniformis* from the Colorado potato beetle (Herrmann *et al*., 2006b). These nematodes have a necromenic relationship with beetles whereby dauer stage nematodes infect a beetle host (Weller *et al*., 2010), wait for the beetle to die then resume growth and feed on microbes proliferating on the carcass. Once the food supply is depleted then the nematodes arrest in the dauer stage and move into the soil system to infect new adult beetles or soil dwelling grubs.

The usual food source under laboratory conditions for both *C. elegans* and *P. pacificus* is *Escherichia coli* strain OP50 (Brenner, 1974; Sommer *et al*., 1996). It remains unknown what bacteria *C. elegans* associates with in nature and only few studies have been carried out to date. Grewal (1991) isolated 10 bacteria species from the gut and cuticle of *C. elegans* including *Acinetobacter* sp., *Bacillus* sp., *Pseudomonas* sp. and *Enterobacter* sp. and showed varying levels of growth and reproduction when *C. elegans* was fed with each. In contrast, however, under natural conditions, *Pristionchus* species feed on a wealth of different pathogenic and non-pathogenic species isolated from both beetles and soil that can affect developmental rate, brood size, survival and behaviour (Rae *et al*., 2008). It has been shown that these two nematodes differ in their susceptibility to BT Cry 5B toxin (Wei *et al*., 2003) and the human pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Rae *et al*., 2008). This could be due to the abundance of detoxification genes in *P. pacificus* compared with *C. elegans* (Dieterich *et al*., 2008) or differences in morphology. *Caenorhabditis elegans* has a grinder (used for crushing bacterial cells) at the base of the pharynx but *P. pacificus* does not (Rae *et al*., 2008). The lack of grinder means *Pristionchus* species can ingest whole bacterial cells and disseminate bacteria to new areas (Chantanao and Jensen, 1969).

Here, we assessed the pathogencity of 768 naturally isolated *Bacillus* strains (at vegetative cell stage) sampled from soil (from Germany and the UK), dung beetles (*Geotrupes* sp.) and horse dung to investigate whether these nematode species differ in their susceptibility and to discover what proportion of *Bacillus* are virulent to nematodes. As well as effects on nematode survival we recorded the effects on development and fecundity. We tested whether available *C. elegans* Bre mutants (BT toxin resistant) and Daf mutants (abnormal dauer formation) were resistant to the most toxic *Bacillus* strains observed in our survey, which consisted of a group of three *B. cereus*-like strains. Mutations in the gene *daf-2* in *C. elegans* extends lifespan as well as causing resistance to the bacterial pathogen *Enterococcus faecalis* (Garsin *et al*., 2003). Mutations in Bre genes in *C. elegans* cause defects in the production of carbohydrate structures on arthroseries glycolipids which bind Cry 5B and hence make these mutants resistance to BT Cry 5B toxin (Griffitts *et al*., 2005). We also sequenced the 16S rRNA gene from all 768 strains to determine species identity and profiled the toxin genes from the most pathogenic *Bacillus* strains.

From our survey of 768 *Bacillus* strains, only 20 showed severe pathogenicity to nematodes with the most toxic causing mortality to *C. elegans* in less than 24 h. This represents, to our knowledge, the most virulent nematocidal *Bacillus* bacteria described in the literature. These *Bacillus* strains are toxic only to *C. elegans*, whereas *P. pacificus* is resistant. Most interestingly, mutations in Bre and Daf genes do not alter resistance to the *B. cereus*-like strains, indicating the presence of novel virulence mechanisms.

Results

Survey and analysis of Bacillus *strains*

To study the pathogenicity of naturally isolated *Bacillus* strains on nematodes, we isolated 768 strains from soil (from Germany and UK), horse dung and *Geotrupes* beetles (Fig. 1C and D). *Bacillus* strains were designated with a code beginning with D, DB, GS or US for horse dung, dung beetles, soil from Germany or soil from UK, respectively. Each strain was fed to *C. elegans* and *P. pacificus* and the 16S rRNA gene of all 768 *Bacillus* isolates was sequenced and analyzed to determine species identity (for full list see Supporting Tables S1–S4). In summary, the most abundant three strains found in soil from Germany were *Bacillus* sp. CM-B72 (60 isolates), *Bacillus* sp. RA51 (43 isolates) and *Bacillus weihenstephanensis* (25 isolates). From soil collected from the UK, we found *Bacillus* sp. CM-B72 (62 isolates), *B. cereus* (49 isolates) and *Bacillus* sp. RA51 (39 isolates) the most prevalent. From *Bacillus* isolated from *Geotrupes* beetles *Bacillus* sp. HSCC (45 isolates), *B. longisporus* (43 isolates) and *Bacillus* sp. CM-B72 (22 isolates) were the most common. The most common *Bacillus* from horse dung were *Bacillus* sp. (44 isolates), *Bacillus* sp. CM-B72 (29 isolates) and *B. cereus* (28 isolates). We also isolated common soil *Bacillus* sp. such as *B. mycoides*, *B. pumilus*, *B. licheniformis*, *B. subtilis*, *B. simplex* and BT although in small amount.

Caenorhabditis elegans *and* P. pacificus *show distinct pathogenicity patterns to* Bacillus *strains*

We categorized a *Bacillus* strain as being pathogenic as after 5 day feeding 25% of nematodes or less were still alive. From soil collected from Germany, six *Bacillus* strains were pathogenic to *C. elegans* and *P. pacificus* which significantly affected survival after 5 day exposure compared with the *Escherichia coli* OP50 control ($P > 0.001$) (Fig. 2A and B, see Supporting Table S5 for complete list of nematocidal *Bacillus* strains). Generally, these strains were both toxic to *C. elegans* and *P. pacificus* although strain numbers GS108 only affected *C. elegans* and not *P. pacificus* and only strain number GS158 killed *P. pacificus* and not *C. elegans*. We did not identify any *Bacillus* strains isolated from soil from the UK that caused any mortality to either nematode species.

When fed 192 *Bacillus* strains isolated from horse dung three *Bacillus* strains severely affected survival of *C. elegans* (Fig. 2C) ($P > 0.001$) and four strains caused mortality to *P. pacificus* (Fig. 2D) ($P > 0.001$). Both strain numbers D5 and D60 killed both nematodes but only D112 affected *C. elegans* and only D106 and D149 affected *P. pacificus*.

Seven strains of *Bacillus* isolated from dung beetles killed *C. elegans* after 5 day exposure (Fig. 2E) ($P > 0.001$). In contrast to *C. elegans*, *P. pacificus* was largely unaffected by these seven strains and survival was only significantly affected by *Bacillus* strain DB35 (Fig. 2F) ($P > 0.001$), a strain that did not affect *C. elegans*. The most pathogenic of the *Bacillus* isolated from dung beetles (strain numbers DB7, DB27 and DB73) were significantly more toxic than all other strains and killed 100% *C. elegans* within 24 h ($P > 0.001$). From all 768 *Bacillus* strains tested these three were most virulent. Sequence analysis revealed that DB7, DB27 and DB73 are most similar to *B. cereus* and we therefore designate them as *B. cereus*-like strains throughout the analysis. These strains are currently investigated in greater detail in collaboration with Dr A. Hoffmaster (CDC, Athens, Georgia). We decided to concentrate on these three strains in further experiments (see below).

In summary, the majority of soil, horse dung and *Geotrupes* beetles are largely non-nematocidal. However, those *Bacillus* strains that do kill nematodes vary in pathogenicity (100% mortality caused in under 24 h to 5 days) and in what nematode species they can kill i.e. *P. pacificus* is largely unaffected by DB7, DB27 and DB73.

Effects of 20 pathogenic Bacillus strains on fecundity on C. elegans *and* P. pacificus

Pathogenic *Bacillus* strains can be used as food source, so we exposed single virgin hermaphrodite *C. elegans* and *P. pacificus* to each nematode pathogenic *Bacillus* and analyzed development and fecundity. After 3 day exposure, the mean number of *C. elegans* and *P. pacificus* juveniles produced on our 20 pathogenic *Bacillus* strains was significantly lower than the *E. coli* OP50 control ($P < 0.001$) (Fig. 3A and B). Surprisingly, young adult *C. elegans* sporadically managed to produce offspring on the *B. cereus*-like strains DB7, DB27 and DB73 albeit at very low levels. *Pristionchus pacificus*, however, managed to produce significantly more juveniles compared with *C. elegans* on strains DB7, DB27 and DB73 ($P < 0.05$). Taken together, these 20 *Bacillus* strains severely affect survival and fecundity of *C. elegans* and *P. pacificus.*

Bacillus cereus *like strains are toxic to* C. elegans *Bre and Daf mutants*

Given the strong virulence of the *B. cereus*-like strains DB7, DB27 and DB73 on *C. elegans*, we started to investigate the virulence mechanisms. Mutations in glycolipid receptors make *C. elegans* resistant to BT Cry 5B toxins (i.e. Bre mutants) (Griffitts *et al.*, 2005). We wanted to know if DB7, DB27 and DB73 utilized a similar mechanism as BT Cry 5B and would hence be resistant to these *Bacillus*

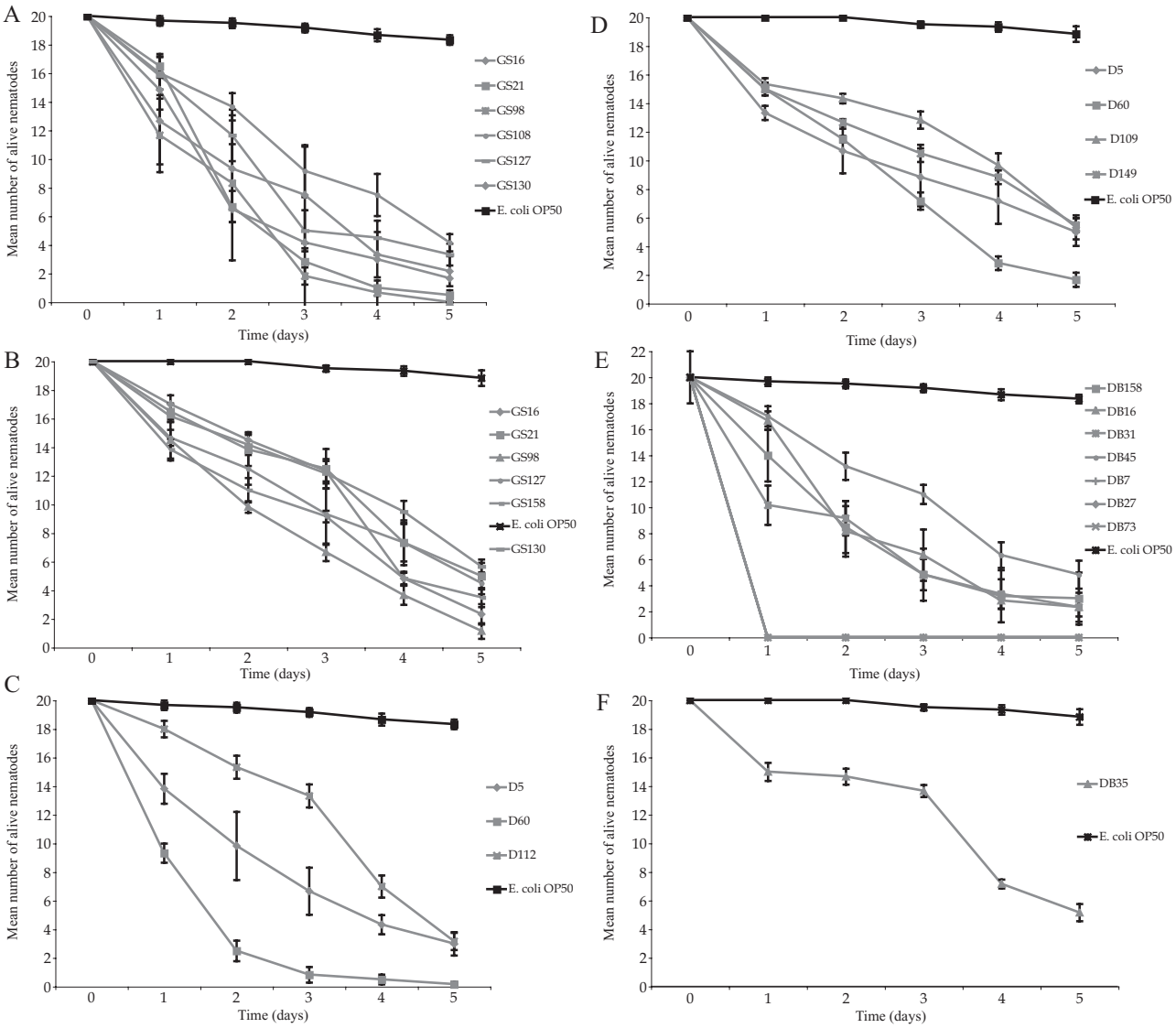

Fig. 2. Identification of nematode pathogenic *Bacillus* species. Mean number of alive *C. elegans* (A, C, E) and *P. pacificus* (B, D, F) exposed to *Bacillus* isolated from soil from Germany (A and B), horse dung (C and D) and *Geotrupes* sp. beetles (E and F) for 5 days. Bars represent ± one standard error.

strains. *Caenorhabditis elegans bre-1(ye4)*, *bre-2(ye31)* and *bre-3(ye26)* mutants exposed to DB7, DB27 and DB73 displayed the same survival dynamics as wild type *C. elegans* and 100% were dead after 24 h ($P < 0.001$, compared with *E. coli* OP50 control) (Fig. 4A and B, data not shown). Therefore, these *Bacillus* strains do not use the same pathogenic mechanism as BT Cry 5B toxin.

There are a number of *C. elegans* Daf mutants, e.g. *daf-2* that are resistant to bacterial pathogens (Garsin *et al*., 2003). We tested mutations in *daf-16(m27)*, *daf-2(e1368)*, *daf-12(m20)* and *age-1(hx546)* in the insulin signaling pathway that are known to affect longevity (Kenyon *et al*., 1993), dauer formation and stress response. *Caenorhabditis elegans daf-12*, although not known to have an effect on pathogenesis, was included because it represents the only Daf gene, for which there is a mutation available in *P. pacificus* (Ogawa *et al*., 2009). Interestingly, *daf-16(m27)* (Fig. 5B), *daf-2(e1368)* (Fig. 5C), *daf-12(m20)* (Fig. 5E) and *age-1(hx546)* (Fig. 5G) mutant worms are killed within 24 h of being fed DB7, DB27 and DB73 similar to *C. elegans* wild type (Fig. 5A) ($P > 0.05$, comparison of survival kinetics of mutants versus wild type). These results show that single gene mutations in the insulin-signaling pathway and *daf-12* have little effect on the survival on *C. elegans* when fed the three *B. cereus*-like strains. In contrast, *C. elegans daf-16(mg54), daf-2(e1370)* (Fig. 5D), *daf-12(m20), daf-2(m41)* (Fig. 5F), and *age-1(m333), daf-*

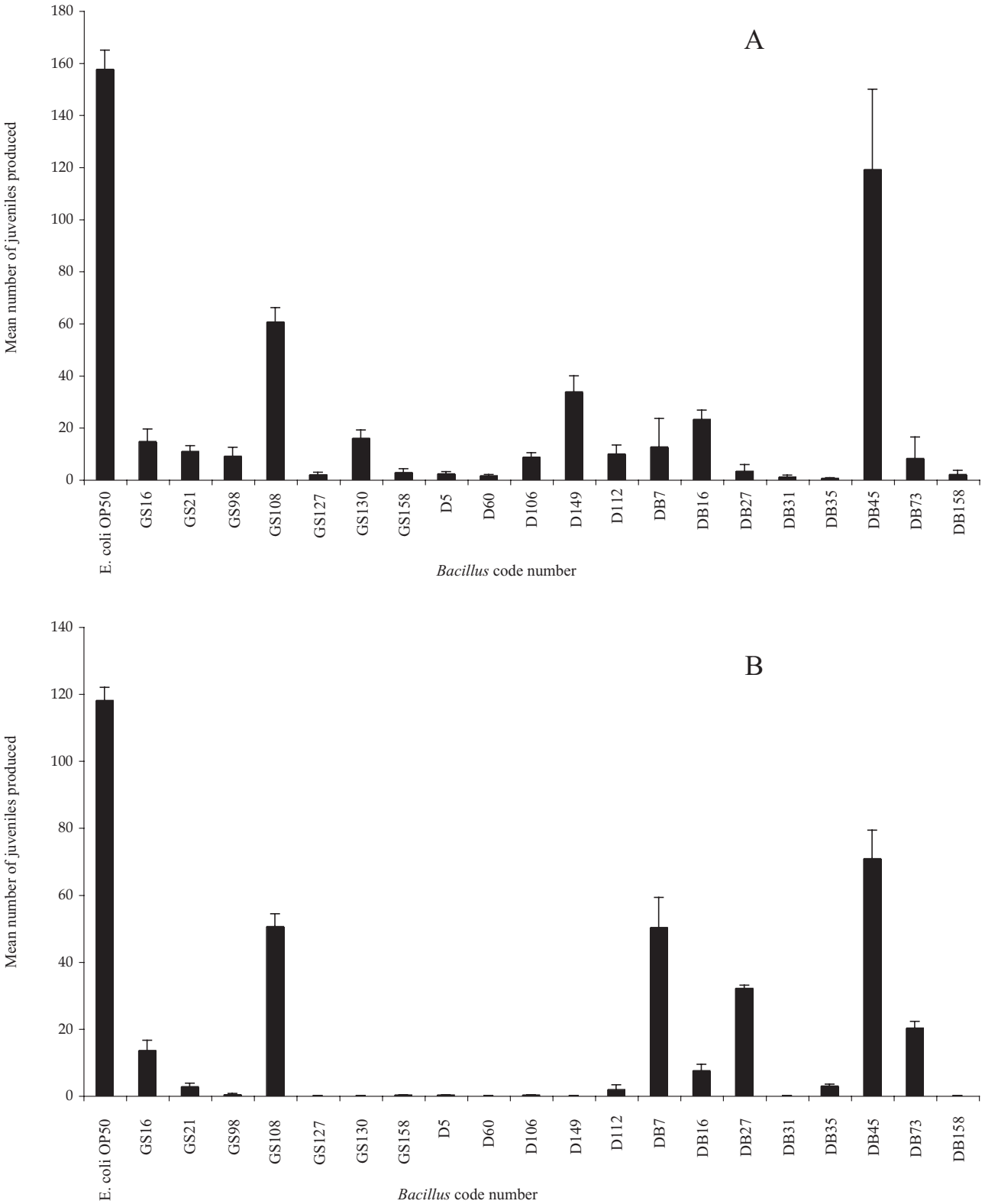

Fig. 3. Mean number of juveniles produced from single virgin hermaphrodite *C. elegans* (A) and *P. pacificus* (B) fed on 20 pathogenic *Bacillus* sp. and *E. coli* OP50 control for 3 days. Bars represent ± one standard error.

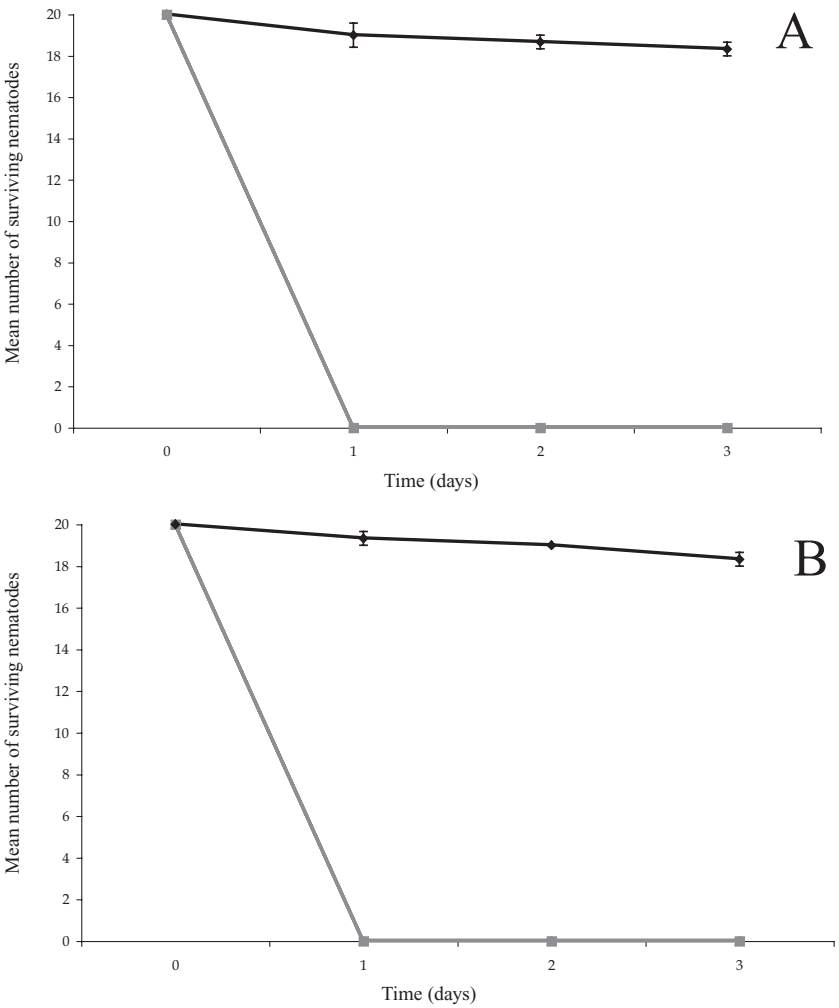

Fig. 4. Mean survival of *C. elegans* wild type (A) and *bre-1* (B) exposed to the most pathogenic *Bacillus* strains DB7 (dark grey squares) isolated from *Geotrupes* dung beetles and *E. coli* OP50 (black diamonds) for 3 days. Bars represent ± one standard error.

16(m26) (Fig. 5H) double mutants die, but have slightly lengthened survival dynamics compared with wild type. There are significant differences between wild type survival and *daf-16(mg54); daf-2(e1370)* on day 1 exposed to DB7 ($P < 0.05$), 27 ($P < 0.05$) but not DB73 ($P > 0.05$). This is also true for *age-1(m333); daf-16(m26)* on day 1 exposed to strain DB73 ($P < 0.05$) compared with wild type. Also, there are significantly more survivors of *daf-12(m20); daf-2(m41)* exposed to DB7 and DB73 ($P < 0.05$), compared with wild type on day 1. Although there are significant differences on day 1, these mutants all die and there are no survivors on day 3, so generally the effect is very minimal and is not comparable to published effects found on other bacteria, e.g. *E. faecalis* (Garsin *et al*., 2003).

Effect of 20 nematode pathogenic Bacillus strains *on* Beauveria bassiana

Pristionchus nematodes are known to feed on whole bacterial cells and to expel them up to 27 h after ingestion, potentially meaning that these nematodes can transport bacteria to new hosts and new areas (Chantanao and Jensen, 1969; Rae *et al*., 2008). We therefore wanted to investigate whether there were other potential antagonistic effects of the pathogenic bacteria against other organisms. One such candidate in soil ecosystems is the entomopathogenic fungus *Beauveria bassiana*. *Beauveria bassiana* has been found infecting *Melolontha* grubs with *Pristionchus* species present (Herrman, Rae and Sommer, unpubl. data), although the effect this fungus has on *Pristionchus* viability when growing on these infected beetles is unknown. We investigated the ability of each of the 20 nematocidal *Bacillus* strains to suppress growth of *B. bassiana* in an *in vitro* dual culture assay. As a control we also tested the effect of 20 randomly picked non-nematocidal *Bacillus* sp. on fungal suppression.

All nematocidal *Bacillus* strains inhibited growth of *B. bassiana* significantly compared with the control, including the strongest nematocidal *B. cereus*-like strains DB7, DB27 and DB73, but there was no significant difference between these three strains ($P > 0.05$) (Fig. 6A). The

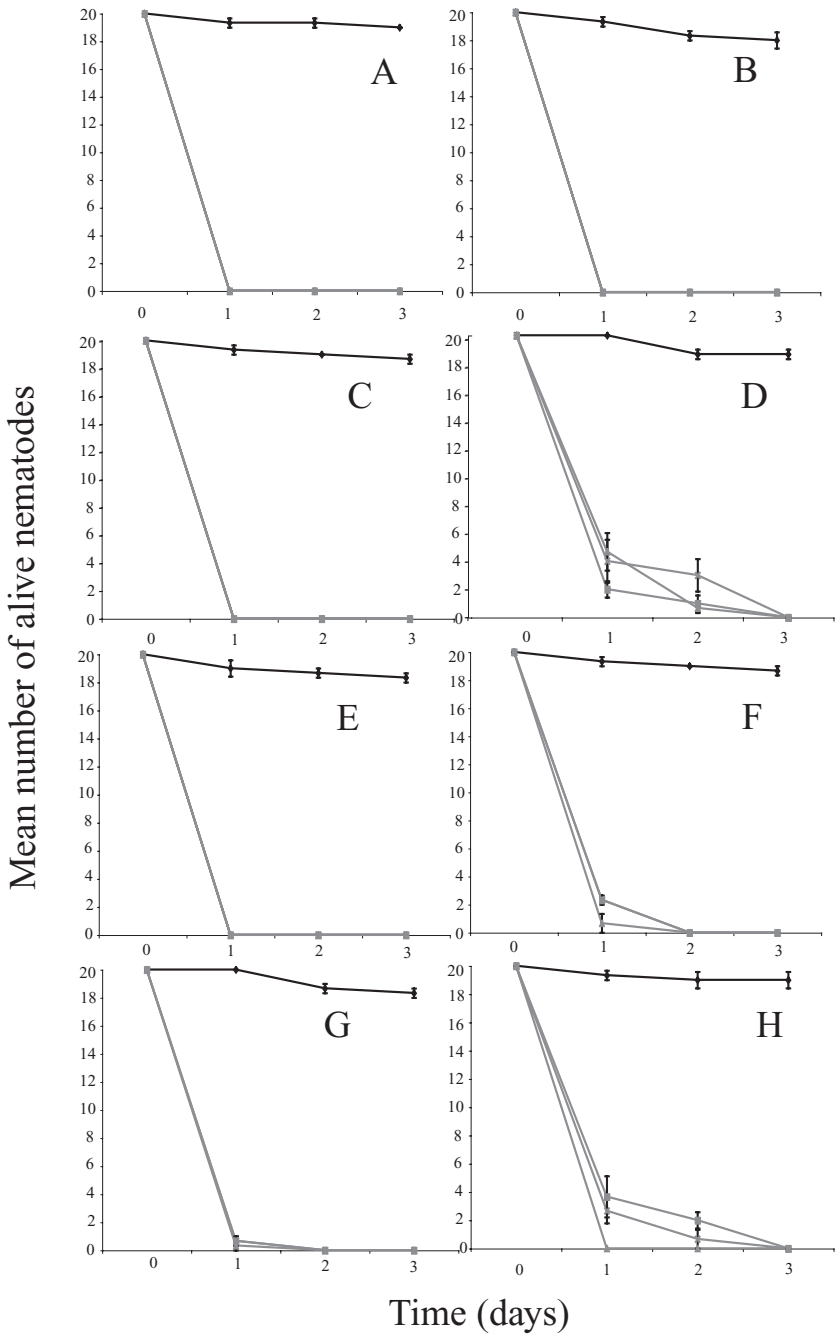

Fig. 5. Mean survival of *C. elegans* wild type (A), *daf-16* (B), *daf-2* (C), *daf-16*, *daf-2* (D), *daf-12* (E), *daf-12*, *daf-2* (F), *age-1* (G) and *age-1*, *daf-16* (H) exposed to the most pathogenic *Bacillus* strains DB7 (dark grey squares), DB27 (dark grey triangles) and DB73 (dark grey crosses) isolated from *Geotrupes* dung beetles and *E. coli* OP50 control (black diamonds) for 3 days. Bars represent ± one standard error.

strongest inhibitory effect (percentage inhibition > 60%) of *Bacillus* isolated from *Geotrupes* beetles was caused by DB31 (a strain of *B. cereus*).

The strength of fungal antagonism caused by nematocidal *Bacillus* strains differed significantly ($P < 0.05$) and so we classified this effect based on the percentage value of inhibition (see Supporting Table S6). The majority of strains belong to strong and very strong antagonists, while no weak antagonists were present. The highest percentage of strong and very strong antagonists was present in *Bacillus* isolated from *Geotrupes* beetles (87.5%) and *Bacillus* isolated from soil from Germany (85.7%), followed by horse dung *Bacillus* isolates (60%).

In contrast to the nematocidal strains, 20 randomly chosen non-nematocidal strains showed weaker or nearly no fungal antagonism (Fig. 6B). Specifically, non-nematocidal strains had a minimum and maximum inhibition of 5.92% ± 3.62% and 25.70% ± 6.51%, respectively, whereas nematocidal strains had a minimum and maximum inhibition of 19.84% ± 1.15% and 59.57% ± 0.43%, respectively. Also when the numbers of nematocidal and non-nematocidal *Bacillus* sp. that

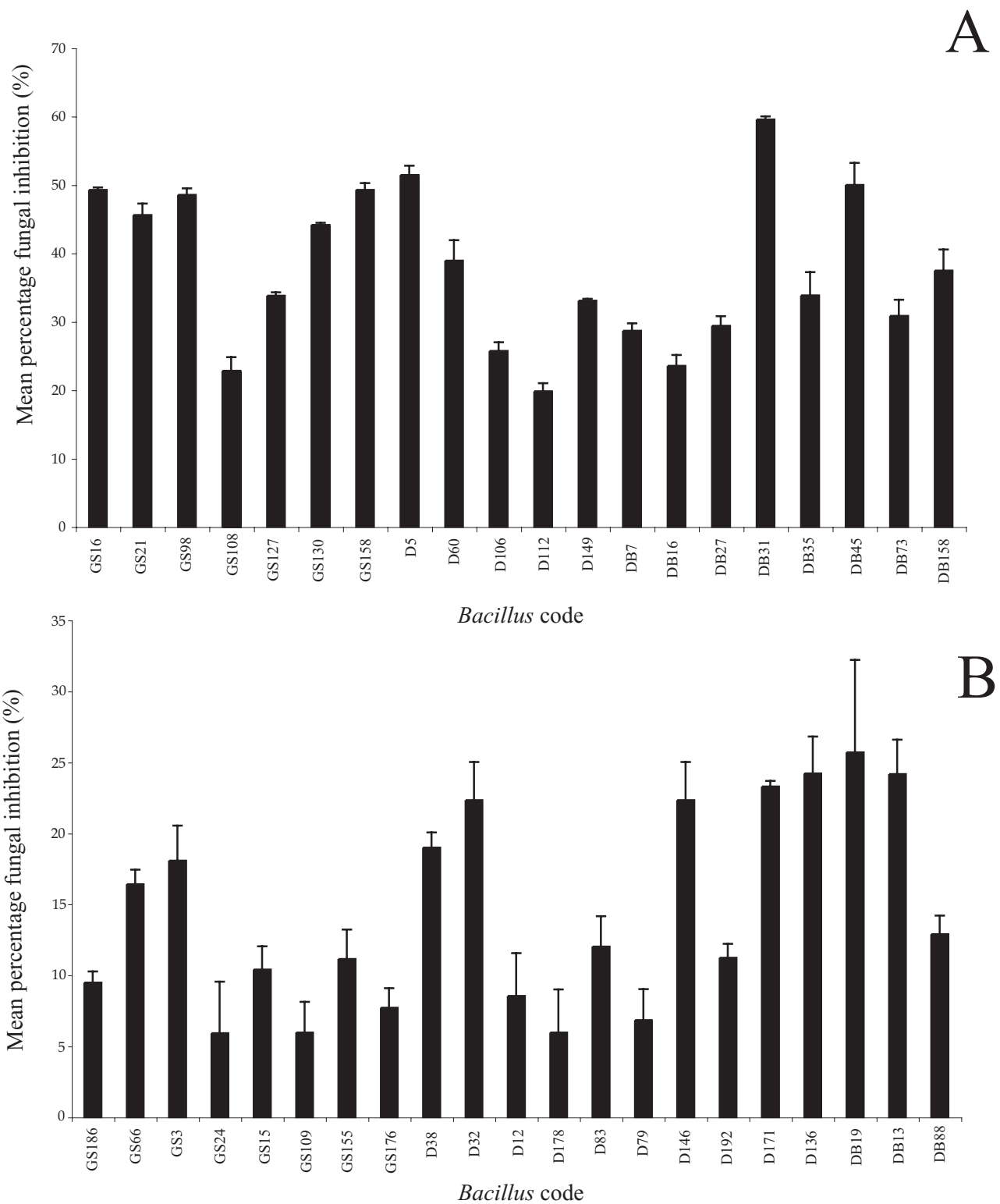

Fig. 6. Mean percentage inhibition of *B. bassiana* exposed to 20 nematocidal (A) and non-nematocidal (B) *Bacillus* sp. isolated from initial survey. Bars represent ± one standard error.

caused strong and very strong inhibitory effects on *B. bassiana* were compared, there were significantly more found in the nematocidal isolates ($P > 0.05$, see Supporting Table S6). Taken together, there is a strong overlap in nematode pathogenicity and in anti-fungal suppression.

Characterization of B. cereus *toxin genes and toxins*

We profiled the known toxin genes (see Fig. 7A) present in our nematocidal *Bacillus* isolates (Fig. 7B). The three *B. cereus*-like strains DB7, DB27 and DB73 are toxin gene rich and almost identical in profile even though they were isolated from separate *Geotrupes* beetles. All three strains tested positive for *nheA*, *nheB*, *nheC*, *pcpl* (*cerA*), *sph* (*cerB*), *piplc*, *cytK*, *hly3*, *entFM*, *entS* and four of five primer combinations for the *bceT* gene. We could only detect one component of the *hbl* complex (*hblC*) in DB7 and DB27 and none in DB73. Also DB7 and DB27 tested positive for *hly2* but DB73 did not. Although only six of 20 nematocidal strains are *B. cereus* (see Supporting Table S5) some of the other *Bacillus* strains have tested positive for specific *B. cereus* genes and therefore share same components of similar virulence mechanisms.

A

Gene	Gene code	Primer sequences (5'-3')	Reference
bceT	BCET 1	CGTATCGGTCGTTCACTCGG	Agata *et al* . (1995)
	BCET 2	AGCTTGGAGCGGAGCAGACT	
	BCET 3	GTTGATTTTCCGTAGCCTGGG	
	BCET 4	TTTCTTTCCCGCTTGCCTTT	
	BCET 5	TTACATTACCAGGACGTGCTT	
	BCET 6	TGTTTGTGATTGTAATTCAGG	
hblA (B)	HBLA1	GTGCAGATGTTGATGCCGAT	Hansen and Hendriksen (2001)
	HBLA2	ATGCCACTGCGTGGACATAT	
hblC (L2)	L2A	AATGGTCATCGGAACTCTAT	Hansen and Hendriksen (2001)
	L2B	CTCGCTGTTCTGCTGTTAAT	
hblD (L1)	L1A	AATCAAGAGCTGTCACGAAT	Hansen and Hendriksen (2001)
	L1B	CACCAATTGACCATGCTAAT	
nheA	nheA 344 S	TACGCTAAGGAGGGGCA	Hansen and Hendriksen (2001)
	nheA 843 A	GTTTTTATTGCTTCATCGGCT	
nheB	nheB 1500 S	CTATCAGCACTTATGGCAG	Hansen and Hendriksen (2001)
	nheB 2269 A	ACTCCTAGCGGTGTTCC	
nheC	nheC 2820 S	CGGTAGTGATTGCTGGG	Hansen and Hendriksen (2001)
	nheC 3401 A	CAGCATTCGTACTTGCCAA	
pcpl (cerA)	CERA 1	ACTGAGTTAGAGAACGGTAT	Hendriken *et al* . (2006)
	CERA 2	CGCTTACCTGTCATTGGTGT	
sph (cerB)	CERB 1	TCGTAGTAGTGGAAGCGAAT	Hendriken *et al* . (2006)
	CERB 2	AGTCGCTGTATGTCCAGTAT	
cytK	CK-F-1859	ACAGATATCGGKCAAAATGC	Guinebretiere *et al* . (2002)
	CK-R-2668	TCCAACCCAGTTWSCAGTTTC	
piplc	phosC 1	CGCTATCAAATGGACCATGG	Hansen *et al* . (1998)
	phosC 2	GGACTATTCCATGCTGTACC	
hly II	BcHlyII-S	AGAAGGAGTGGCTGTCTGTA	Hendriken *et al* . (2006)
	BcHlyII-A	TTCTTTCCAAGCAAAGCTAC	
hly III	BCHEM 1	AATGACACGAATGACACAAT	Hendriken *et al* . (2006)
	BCHEM 3	ACGATTATGAGCCATCCCAT	
entFM	ENTA F	ATGAAAAAAGTAATTTGCAGG	Minnaard *et al.* (2007)
	ENTA R	TTAGTATGCTTTTGTGTAACC	
entS	TY123 F	GGTTTAGCAGCAGCTTCTGTAGCTGGCG	Minnaard *et al.* (2007)
	TY125 R	GTTTCGTTAGATACAGCAGAACCACC	
hblB	hblB F	AAGCAATGGAATACAATGGG	Minnaard *et al.* (2007)
	hblB R	AATATGTCCCAGTACACCCG	

B

Toxin	Gene	Primer code	DB7	DB16	DB27	DB31	DB35	DB45	DB73	DB158	GS16	GS21	GS98	GS108	GS127	GS130	GS158	D5	D60	D109	D112	D149
B. cereus enterotoxin	*BCET*	1+3	+	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	BCET	1+4	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	BCET	2+3	+	+	+	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	-	+
	BCET	2+4	(+)	-	(+)	-	-	-	(+)	-	+	-	-	-	-	+	-	-	(+)	-	-	-
	BCET	5+6	+	+	+	-	-	+	+	-	-	+	+	-	-	+	-	-	-	+	-	-
Hemolysin BL	*hblA*	7+8	-	-	-	-	+	-	-	+	-	-	+	-	+	-	-	-	-	+	+	-
	hblB	35+36	-	-	-	+	+	-	-	-	-	-	+	-	+	-	-	-	-	+	+	-
	hblD	9+10	-	+	-	+	+	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+
	hblC	11+12	+	-	+	+	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+
Non-hemolytic enterotoxin	*nheA*	13+14	+	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+
	nheB	15+16	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	nheC	17+18	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+
Phospholipases	*pcpl(cerA)*	19+20	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-
	sph(cerB)	21+22	+	+	+	(+)	+	-	+	-	-	+	+	-	+	+	+	+	+	-	+	+
	piplc	25+26	+	+	+	-	+	+	+	-	-	+	+	-	+	-	+	-	+	+	+	-
Cytotoxin K	*cytK*	23+24	+	-	+	-	-	(+)	+	+	-	-	-	-	-	-	-	-	-	-	(+)	-
Hemolysin 2	*hly2*	27+28	+	+	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	+	+	+
Hemolysin 3	*hly3*	29+30	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+
Enterotoxin FM	*ent FM*	31+32	+	+	+	-	+	-	+	+	-	+	-	-	+	+	-	-	+	+	+	-
Enterotoxin S	*ent S*	33+34	+	-	+	-	+	-	+	-	-	+	+	-	+	+	-	-	-	-	+	+

Fig. 7. *Bacillus cereus* primers used in this study (A). Presence (positive sign) or absence (negative sign) of each *B. cereus* toxin gene (B). Positive sign in brackets is where faint band was detected.

Discussion

There have been few studies on the effects naturally isolated *Bacillus* sp. have on free-living nematodes as most of the research has concentrated on animal parasitic nematodes (Kotze *et al*., 2005; Cappello *et al*., 2006) and plant parasitic nematodes (Li *et al*., 2007, 2008; Terefe *et al*., 2009). This is primarily to discover novel alternate biocontrol methods used to combat nematodes and rival current chemical nematocides. In our survey, the majority of *Bacillus* are not pathogenic to *C. elegans* and *P. pacificus* as we only identified 20 strains (out of 768 collected) that show pathogenicity towards these nematodes. Previous studies have concentrated on a number of *Bacillus* species that are virulent to nematodes, e.g. *B. firmus* (Terefe *et al*., 2009), *Brevibacillus laterosporus* (Huang *et al*., 2005), *B. nematocida* (Niu *et al*., 2006) and BT (Schulenburg and Muller, 2004). Huang and colleagues (2005) and Niu and colleagues (2006) showed that *B. nematocida* and *Br. laterosporus* protease production is the major factor in causing nematode death. In our case, however, the system is based on screening through isolates that cause death after food uptake through the intestine. We did not test the *Bacillus* spore stage, the life stage that is the most pathogenic to insects and is applied in pest control of insects. One reason for concentrating on vegetative cells rather than spores is that our previous work indicated that *Pristionchus* nematodes could successfully suppress spore germination in the intestine (Rae *et al*., 2008). Similarly, these nematodes can even use *Bacillus* spores as food source under laboratory conditions (R. Rae, unpubl. Obs.).

The three *B. cereus*-like strains DB7, DB27 and DB73 show remarkable pathogenicity towards *C. elegans* and begin to die after 8 h and are all dead after 16 h (I. Iatsenko, unpubl. obs.). From our knowledge this is one of the most toxic bacteria isolated so far when examined for pathogenicity to *C. elegans*, when grown under standard conditions using NGM medium. Other bacteria can exhibit the same fast killing dynamics but specifically have to been grown on other media to enhance this effect, e.g. *P. aeruginosa* grown on fast-killing medium kills *C. elegans* in under 24 h (see Tan *et al*., 1999). When fed *S. aureus*, *P. aeruginosa*, *Photorhabdus luminescens* or *Xenorhabdus nematophila C. elegans* begins to die rapidly after 24 h exposure (Tan *et al*., 1999; Begun *et al*., 2005; Rae *et al*., 2008) but with our *B. cereus*-like strains the pathogenicity process is much quicker.

We can also see from our study that virulence to nematodes varies between *B. cereus* strain and nematode species. In our study, we isolated 108 *B. cereus* strains, yet only six have a strong effect on *C. elegans* or *P. pacificus*. It is know that *B. cereus* induced food poisoning in humans varies from strain to strain (Granum, 1997) and this also seems to be the case with pathogenicity in nematodes as well.

The *B. cereus* group consists of six recognized species including *B. cereus*, BT, *B. anthracis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* (Stenfors Arnesen *et al*., 2008). *Bacillus cereus* causes human food poisoning consisting of diarrhea and abdominal distress or nausea and vomiting and can cause a variety of infections including endophthalmitis, bacteremia, septicemia, endocarditis, salpingitis, cutaenous infections, pneumonia and meningitis (Drobniewski, 1993; Logan and Turnbull, 1999; Rasko *et al*., 2005). *Bacillus cereus* toxins include pore-forming cytoxins haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe) and cytotoxin K (CytK) (Beecher and MacMillan, 1991; Lund and Granum, 1996; Lund *et al*., 2000), which are activated by the transcriptional regulator PlcR (Lereclus *et al*., 1996; Gohar *et al*., 2002). The three *B. cereus*-like strains DB7, DB27 and DB73 contain a wealth of toxin genes, but we were unable to amplify the enterotoxin hemolysin BL (HBL) gene by standard Polymerase chain reaction (PCR) primers that work well for other strains. The presence of this gene seems to be variable among strains. For example, Hansen and Hendriksen (2001) only found the HBL complex present in 11 of 22 *B. cereus* strains tested and Mantynen and Lindstrom (1998) found *hblA* in 52% of *B. cereus* strains. It is not surprising that these genes from the HBL complex are present in our other nematocidal *Bacillus* as it has been reported that they have been identified in BT, *B. mycoides*, *B. weihenstephanensis*, *B. pseudomycoides* and *B. anthracis* (Ryan *et al*., 1997; Pruss *et al*., 1999; Hansen and Hendriksen, 2001). As we recorded presence of the majority of genes tested, it remains to be discovered which factors might be responsible for causing rapid nematode mortality.

Pristionchus pacificus differs in susceptibility to *S. aureus*, *P. aeruginosa* (Rae *et al*., 2008) and BT toxin Cry 5B (Wei *et al*., 2003) and in our study we were able to show that this is also true for a number of *Bacillus* species identified in our screening procedure. The main reasons for this are currently unknown but hyper susceptible mutants are being isolated to discover what genes are integral to *P. pacificus* immunity (Rae, unpubl. Obs.).

Mutations in *daf-2* create resistance to gram-negative and gram-positive pathogens such as *E. faecalis*, *P. aeruginosa* and *S. aureus* (Garsin *et al*., 2003). However, we have found that *C. elegans daf-2* does not enhance resistance to our *B. cereus* strains, therefore, although resistance to pathogens can be conferred through suppression of *daf-2* it strongly depends on bacterial species. Surprisingly, we found that *daf-2; daf-16* and *age-1; daf-16* double mutants showed a slight resistance to *Bacillus* DB27. One potential reason for this finding might

be that DAF-16 has pleiotropic effects and is part of several signaling systems involved in stress response (Kenyon, 2010). We also found that *C. elegans* Bre mutants were also susceptible to the *B. cereus*-like strains. This suggests that mutations in glycolipids that stop Cry 5B from binding to the intestinal cells does not stop the virulence process of *B. cereus*.

We have shown that some soil and dung derived *Bacillus* not only cause death to *C. elegans* and *P. pacificus* but also suppress *B. bassiana* using *in vitro* assays. In nature bacteria, nematodes and fungi share the same ecological niche in soil or on beetles. To survive in these 'microbial jungles' bacteria must protect themselves against predators (nematodes) as well as to suppress other competitors (fungi and other bacteria). Some bacteria have been shown to live in symbiosis with beetles and play and provide an important role in their life. Mutualistic associations with microorganisms are widespread in insects, and the microbes serve an array of functions for their insect hosts, including protective services (Lundgren *et al*., 2007). For instance, Colorado potato beetle isolates belonging to the genera *Pantoea* sp*., Enterobacter* sp*., Pseudomonas* sp. and *Bacillus* sp. inhibited growth of the entomopathogenic fungus *B. bassiana in vitro*. They have also been shown to protect the beetle against the entomopathogenic nematode *Heterorhabditis marelatus* by suppressing its bacterial symbiont *Photorhabdus temperata*, which is responsible for the killing of the beetle (Blackburn *et al*., 2008). In addition, some *Pseudomonas* sp*., Serratia* sp. and *Bacillus* sp. strains have been isolated from oral secretions of spruce beetles and when tested inhibited the growth of potentially pathogenic fungi associated with beetles (Cardoza *et al*., 2006). These bacteria also affect nematodes and it was therefore assumed that the bacteria might serve as a potential defense against nematodes and fungi. In our assays, we showed that nematocidal *Bacillus* strains also inhibit *B. bassiana*, which may point to the potential protective role of these bacteria.

Recently, Weller and colleagues (2010) profiled the nematode community from *Geotrupes* dung beetles sampled from the Schönbuch forest, Tübingen (location where we also sampled). They found that these beetles are infected with several different nematode species from the genera including *Pelodera*, *Koerneria*, *Strongyloidea* and *Spirurida* as well as *Pristionchus* species. When *Geotrupes* beetles die microorganisms, such as bacteria and fungi, proliferate on the beetle cadaver. It is at this point that resident nematodes classified as 'necromenic', e.g. *Pristionchus* and not *C. elegans*, can exit from the dauer stage and feed upon this feast for development, growth and nutrition. In order to survive these toxic conditions then nematodes must be able to tolerate a wealth of toxic bacteria. *Pristionchus pacificus* is a member of the Diplogastrid family and *C. elegans* is part of the Rhabditidae family and are thought to have diverged over 280–430 MYA (Dieterich *et al*., 2008). *Pristionchus pacificus* has evolved the ability to tolerate and digest pathogenic bacteria such as *P. aeruginosa*, *S. aureus* (Rae *et al*., 2008), BT Cry 5B (Wei *et al*., 2003) and three strongly nematocidal strains of *B. cereus* (from this study). The dramatic expansion of the detoxification machinery in the *P. pacificus* genome relative to *C. elegans* points to nematode adaptation possible due to digestion of bacteria without grinder and/or presence in hostile beetle host environments. By using forward and reverse genetic tools, both of which are available in *P. pacificus*, the molecular mechanisms associated with this tolerance can be identified in future studies.

Experimental procedures

Nematode, bacteria and fungal maintenance

Nematodes (*C. elegans* N2 Bristol strain and *P. pacificus* RS2333 strain) were maintained on NGM (Nematode Growing Media) agar plates seeded with 200–300 µl *E. coli* OP50 and stored at 20°C. Individual *Bacillus* strains were grown overnight in 5 ml LB at 30°C. *Beauveria bassiana* was isolated from an infected cock chafer (*Melolontha* spp.) from Kaferwald near Karlsruhe, Germany and maintained on potato dextrose agar (PDA) at room temperature. *Caenorhabditis elegans bre-1*(*ye4*), *bre-2* (*ye31*), *bre-3*(*ye26*)), *daf-2*(*e1368*, *daf-12*(*m20*), *daf-16*(*m27*) and *age-1*(*hx546*), as well as *daf-16*(*mg54*); *daf-2*(*e1370*), *daf-2*(*m41*); *daf-12* (*m20*) and *daf-16* (*m26*); *age-1* (*m333*) double mutants were obtained from the *Caenorhabditis* Genetic Centre (CGC), Minnesota.

Soil/horse dung and Geotrupes *sp. sampling regime*

Dung beetles (*Geotrupes* sp.) were collected from the Schönbuch forest (Tübingen, Germany). Fresh horse dung heaps were excavated thoroughly and any dung beetles found were placed in non-airtight plastic tubes and immediately transported back to the laboratory. Samples of horse dung were also taken at this location. Soil samples were collected from surrounding agricultural farmland, grassland, rhizosphere of clover (*Trifolium* sp.), moss (*Polytrichum commune*), mixed coniferous woodland (*Abies* and *Picea* sp.), and from leaf litter from deciduous forest floor (mainly Ash, *Fraxinus* sp.). Soil samples were treated similarly to *Geotrupes* sp. collection. One hundred soil samples were collected from the UK from farmland, grassland, coniferous and deciduous forest and coastal habitats.

Soil/horse dung and Geotrupes *sp.* Bacillus *isolation*

Soil and horse dung samples (approximately 10–30 g) were mixed vigorously with PBS (Phosphate Buffered Saline) for 2 min. One millilitre of soil/buffer mix was then heated to 80°C for 10 min to kill all resident bacteria apart from heat resistant

Bacillus spores. Samples (50–100 μl) were then spread on LB plates and incubated overnight at 25°C. One hundred collected *Geotrupes* sp. were washed in PBS for 5 min to remove any adhering horse dung and then immediately chopped into small pieces using sterile scissors and mixed with 1–2 ml PBS. The resultant solution was then heated and treated as described above. After an overnight period of growth single *Bacillus* colonies were streaked onto fresh LB plates and in the following days could be used in nematode feeding assays, long-term storage procedures and DNA extraction. In total, we picked 768 strains of *Bacillus* compromising of 192 from four sampling types (soil from UK and Germany, horse dung and *Geotrupes* beetles).

Assays for assessment of Bacillus effects on nematodes

To assess the pathogenicity of *Bacillus* strains to nematodes 80 μl of overnight *Bacillus* cultures were spread evenly over the surface of six NGM plates and incubated at 25°C overnight. The following morning 20 L4 stage *P. pacificus* and *C. elegans* were placed onto three separate plates and survival was recorded daily for 5 days. Every 2 days nematodes were transferred onto fresh *Bacillus* NGM plates to prevent confusion in differentiating between tested worms and their offspring. This procedure was repeated for *C. elegans* Bre and Daf mutants. The survival of nematodes fed *E. coli* OP50 was also tested using the same procedures as a control. To test the effect of *Bacillus* affecting brood size of each nematode species 30 μl of each *Bacillus* was added to the middle of six NGM plates and left to grow overnight at 25°C. Three single virgin *C. elegans* and *P. pacificus* hermaphrodites were placed in the centre of the bacterial spot and placed in an incubator at 25°C. The number of offspring produced by each hermaphrodite was then counted after 4 days. Experiments were repeated twice.

Bacillus DNA extraction, sequencing and toxin gene profiling

Each *Bacillus* strain was grown overnight at 30°C in 5 ml LB Broth. *Bacillus* DNA was extracted using the MasterPure gram-positive DNA purification kit (Epicentre, Madison, USA). The PCR amplification of bacterial 16S rRNA gene was carried out in 20 μl reactions using primer set 27f (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492r (5′ TACGGYTACCTTGTTACGACTT 3′) (Lane, 1991) and also internal primers (Forward 5′ CGTGCCAGCAGCCGCGGTAATA CGTA 3′ and Reverse 5′ ACTCCTACGGGAGGCAGCAGT 3′. Thermal cycling conditions were as follows: 3 mins at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 55°C and 1.5 min at 72°C there was then a final step of 8 min at 72°C. Reactions consisted of 2 μl 10 ×PCR Buffer, 2 μl 2 mM dNTPs, 1 μl 10 μM 27f, 1 μl 10 μM 1492r, one unit of *Taq* DNA polymerase, 12.8 μl H_2O and 1 μl of bacterial DNA. The PCR amplicons were visualized by standard agarose gel electrophoresis (Sambrook *et al*., 1989). Products of *Bacillus* 16S rRNA gene were diluted 10–20 fold and added to the Big Dye terminator sequencing mix (Applied Biosciences, USA), which contained the sequencing primers previously used for initial amplification. Sequencing reactions typically contained 0.4 units Big Dye, 2 μl 5 × Sequencing Buffer, 1 μl primer (10 μM), 1 μl DNA (previously diluted) and 5.6 μl H_2O. Thermal cycling condition were thus: 96°C for 30 s, followed by 50 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Resultant gene sequences of *Bacillus* strains were aligned using Seqman (DNA Star) and compared with GenBank database sequences using Blastn searches using sequence similarity matches at 90%.

Also the toxin genes of our 20 toxic *Bacillus* strains isolated in our study were profiled. Specifically, we looked for the genes *bceT, nhe*, *hbl*, *pcpl*, *sph* (*cerB*), *cytK*, *piplc*, *pcpl* (*cerA*), *hlyII*, *hlyIII*, *entFM* and *entS*, which have been implicated in outbreaks of food poisoning (Guinebretiere *et al*., 2002) (see Fig. 7A for primers used). A typical reaction for each toxin gene consisted of 2 μl 10 ×PCR Buffer, 2 μl 2 mM dNTPs, 1 μl of each primer (10 μM), 0.3 μl *Taq* and 12.8 μl H_2O. PCR conditions were used according to Hendriksen *et al*., (2006). The PCR amplicons were visualized to determine presence or absence of each toxin gene.

All gene sequences from 768 *Bacillus* sequenced were submitted to GenBank and can be accessed using accession numbers HM566450–HM567157.

Fungi Antagonism study by dual-culture plate method

Methods to study antagonistic properties of nematode pathogenic *Bacillus* strains exposed to *B. bassiana* were followed by Swain and Ray (2009). One 10-mm disk of pure culture of *B. bassiana* was placed at the centre of a Petri plate (10 cm) containing PDA. A circular line made with a 6 cm diameter Petri plate dipped in a suspension of *Bacillus* strains was placed surrounding the fungal inoculum. In total, 20 nematocidal strains were tested and also compared with 20 randomly picked non-nematocidal strains. Plates were cultured for 144 h at 25°C and growth diameter of the fungus was measured and compared with control growth where the bacterial suspension was replaced by sterile distilled water. Each experiment was run in triplicate and repeated at least two times. Results are expressed as means % inhibition ± S.D. of the growth of *B. bassiana* in the presence of any of the *Bacillus* isolates. Percentage of inhibition was calculated using the following formula: % inhibition = (1 – (fungal growth in the presence of *Bacillus*/control growth)) × 100.

Statistical analysis

Raw counts of survival of nematodes (*P. pacificus, C. elegans* wild type, Bre and Daf mutants) fed each *Bacillus* isolates was analyzed using Two way Analysis of Variance (ANOVA). Nematode fecundity and fungal inhibition were analyzed using a One-Way ANOVA and differences between means were analyzed using Least Significant Difference (LSD) after corrected for comparing multiple comparisons using the Bonferroni method. Unpaired and paired student *t*-tests were used to compare number of juveniles in fecundity assays of *C. elegans* and *P. pacificus*,comparing survival of Daf mutants and wild type *C. elegans* and also counts of *Bacillus* that show fungal suppression from nematocidal and non-nematocidal strains.

Acknowledgements

We are extremely grateful for sequence analysis advice and help from Amit Sinha and Dr Werner Mayer. Dr Dan Bumbarger took the photo of a *Geotrupes* beetle. We thank the *Caenorhabditis elegans* Genetic Centre for *C. elegans* mutants. This research was funded by the Max Planck Society.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Analysis of 16S rRNA sequences of 192 *Bacillus* isolates from soil from UK.
Table S2. Analysis of 16S rRNA sequences of 192 *Bacillus* isolates from soil from Schönbuch, Tübingen, Germany.
Table S3. Analysis of 16S rRNA sequences of 192 *Bacillus* isolates from horse dung from Schönbuch, Tübingen, Germany.
Table S4. Analysis of 16S rRNA sequences of 192 *Bacillus* isolates from *Geotrupes* dung beetles soil from Schönbuch, Tübingen, Germany.
Table S5. Identification of nematocidal *Bacillus* strains from German soil, horse dung and *Geotrupes* beetles based on analysis of 16S rRNA gene.
Table S6. Classification of nematocidal and non-nematocidal *Bacillus* that inhibit growth of the entomopathogenic fungus *B. bassiana*.

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