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The Phytopathogen *Dickeya dadantii* (*Erwinia chrysanthemi* 3937) Is a Pathogen of the Pea Aphid†

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***Dickeya dadantii* (*Erwinia chrysanthemi*) is a phytopathogenic bacterium causing soft rot diseases on many crops. The sequencing of its genome identified four genes encoding homologues of the Cyt family of insecticidal toxins from *Bacillus thuringiensis*, which are not present in the close relative *Pectobacterium carotovorum* subsp. *atrosepticum*. The pathogenicity of *D. dadantii* was tested on the pea aphid *Acyrthosiphon pisum*, and the bacterium was shown to be highly virulent for this insect, either by septic injury or by oral infection. The lethal inoculum dose was calculated to be as low as 10 ingested bacterial cells. A *D. dadantii* mutant with the four cytotoxin genes deleted showed a reduced per os virulence for *A. pisum*, highlighting the potential role of at least one of these genes in pathogenicity. Since only one bacterial pathogen of aphids has been previously described (*Erwinia aphidicola*), other species from the same bacterial group were tested. The pathogenic trait for aphids was shown to be widespread, albeit variable, within the phytopathogens, with no link to phylogenetic positioning in the *Enterobacteriaceae*. Previously characterized gut symbionts from thrips (*Erwinia*/*Pantoea* group) were also highly pathogenic to the aphid, whereas the potent entomopathogen *Photorhabdus luminescens* was not. *D. dadantii* is not a generalist insect pathogen, since it has low pathogenicity for three other insect species (*Drosophila melanogaster*, *Sitophilus oryzae*, and *Spodoptera littoralis*). *D. dadantii* was one of the most virulent aphid pathogens in our screening, and it was active on most aphid instars, except for the first one, probably due to anatomical filtering. The observed difference in virulence toward apterous and winged aphids may have an ecological impact, and this deserves specific attention in future research.**

Dickeya dadantii (syn. *Erwinia chrysanthemi*, *Pectobacterium chrysanthemi*) (53), as well as many other erwinia species, is one of the phytopathogenic *Enterobacteriaceae* that can cause soft rot diseases in a wide range of economically important crops. These bacteria can survive in soils, from where they are transmitted to plants by water, miscellaneous insects, or cultural techniques. There is no recorded specific vector organism for such pathogens, although *Drosophila melanogaster* was claimed to be a potential vector for some *Erwinia* species (3). The common symptoms for *D. dadantii* infection, as well as other so-called pectinolytic soft rot *Erwinia*, are characterized by the rapid disorganization of parenchymatous tissues, mainly driven by pectic enzymes (38). Nevertheless, plant colonization by soft rot *Erwinia* is a multifactorial process requiring numerous additional factors, such as cellulases, iron assimilation, an Hrp type III secretion system, exopolysaccharides, motility, and proteins involved in resistance against plant defense mechanisms (58). The recent deciphering of the complete genome sequence of *D. dadantii*, strain 3937 (28), after that of *Pectobacterium carotovorum* subsp. *atrosepticum* (syn. *Erwinia atroseptica*), strain SCRI 1043 (5), allows for research on the complete genome and a comparative overview of potential virulence factors present in the soft rot *Erwinia* group (58).

In the genomic sequence of *D. dadantii*, four genes encoding homologues of insecticidal toxins, which were not present in *P. carotovorum* subsp. *atrosepticum*, were found, and these genes could play a role in the pathogenicity of the bacterium. They are clustered with a group of genes whose best hits are from different bacterial genera, including the entomopathogen *Photorhabdus luminescens* (28). However, no report exists on the pathogenic effect of any *D. dadantii* strain against an insect, including the fruitfly (3). To our knowledge, no organism has been recorded as a host outside the plant kingdom. Homologues of the identified toxin genes were only found in the gram-positive entomopathogen *Bacillus thuringiensis*, widely used for the biocontrol of insects and nematodes. The homologues of the *D. dadantii* genes fall into a minor family of *B. thuringiensis* spore/crystal genes, designated the *cyt* family (11), with recorded activity on a limited set of insect species, including some mosquitoes. After ingestion by the insect, and after the action of digestive endoproteases, the products of these genes have a cytolytic pore-forming activity on the gut membrane, leading to the bacterial invasion of the whole insect and its subsequent death (30, 49).

The presence of the Cyt-like toxin genes in the *D. dadantii* genome is intriguing and raises questions concerning their origin, their potential functions in *D. dadantii* physiology, and their eventual roles in the ecology of the bacterium. Since the only known activities of such toxins were on insect targets, we decided to test

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the possibility that *D. dadantii* displays entomopathogenic properties and, if so, whether the identified toxin(s) might participate, at least partly, in this new phenotype.

Since previous information on insect-*Dickeya* interactions is very scarce, we first decided to test four insect species from major orders of pest insects: Diptera (flies), Hemiptera (plant bugs), Coleoptera (beetles), and Lepidoptera (moths). *Drosophila melanogaster* (Diptera) was chosen due to its model status in insect-pathogen interactions and also because the only available information on insects and *D. dadantii* was a lack of immune response of a marker fruitfly strain to this bacterial challenge (3), without explicit study of its pathogenicity. Aphids (Hemiptera) are major crop pests and display a wide range of interactions with microbial organisms, going from their major vector status for plant viruses to their interactions with a guild of symbiotic microorganisms (34). However, there is no previous record of a natural bacterial pathogen of aphids, whereas a single report of experimental infection by a gut-resident bacterium, identified as *Erwinia aphidicola*, was published in 1997 (31). Nonetheless, and although aphids are known for harboring very limited gut microflora, aphids have been shown to naturally exhibit occasional gut populations of *Enterobacteriaceae*, including *Erwinia* sp. (29). The model pea aphid, *Acyrthosiphon pisum*, was therefore chosen for our screening purposes. Additional species were chosen from the Coleoptera (*Sitophilus oryzae*, the rice weevil) and Lepidoptera (*Spodoptera littoralis*, the cotton leaf-worm) orders. Our goal here was not a comprehensive study of the potential insect host-range of *D. dadantii* but rather the search for any experimentally tractable insect system that could be used to answer these questions. The aphid model proved to be satisfactory for these objectives and was consequently studied in more detail, including a small-scale screening of enterobacterial taxa covering both the target *Erwinia* phytopathogenic group, and some other related species, within the *Enterobacteriaceae*, showing documented interactions with insects.

MATERIALS AND METHODS

Bacterial strains, growth media, and 16S rRNA phylogenetic analysis. All of the bacterial strains used in the present study are listed in Table 1, including specific references to their known pathogenic phenotypes. They were mainly provided by the CFBP (the Collection Française de Bactéries Phytopathogènes, INRA Angers, France [http://www.brg.prd.fr/]). All strains were grown in LB medium at 30°C, except *Arsenophonus nasoniae*, which was grown in brain heart infusion medium (CM0225B; OXOID-France, Dardilly, France). EMBL accession numbers of the 16S rRNA gene from the species used are reported in Table 1. These sequences were used for the phylogenetic analysis performed on our bacterial panel. Neighbor-joining and Kimura distance methods were used on nearly complete 16S rRNA gene sequences (30 species, 1,231 informative sites, 1,000 bootstrap replicates). Analyses were performed with the seaview and phylo_win softwares (25).

Aphid strain, breeding, and determination of LT_{50} after inoculation. Aphids used in these experiments were apterous parthenogenetic females of *Acyrthosiphon pisum* (clone LL01), a green clone collected from alfalfa fields near Lusignan (France) in 1988 and maintained since then in our laboratory on broad bean *Vicia faba* cv. Aquadulce in a phytotron (20°C, 16 h light/8 h dark). These insects, being exclusively phloem-feeders, may be reared on sterile liquid diets of fully known composition (AP3), as described for oral toxicology assays (50). The liquid diet, with or without a given bacterium species, was presented to the aphids between two Parafilm membranes, resulting in a food sachet (diameter, 3 cm; see Fig. S1A in the supplemental material). Prior to and after the 24-h oral infection period, the aphids were maintained on isolated broad beans at 20 to 22°C (Fig. S1C in the supplemental material). For technical constraints, the mutant complementation experiment was run at 18°C versus the standard 21°C assay, thereby affecting the absolute survival data but not the relative strain response.

LT_{50} (i.e., the median life duration after challenge, $t = 0$ taken at time of presentation to the infected food, i.e., the time to reach 50% mortality of the initial aphid population) is a valid measure of virulence as long as survivors are not included in its calculation (57). Thus, we used a classical actuarial survival analysis (Statview Package; SAS Institute), with daily life tables and censoring of survivors at the end of the experiment.

Inoculation of aphids by needle puncture infection. Bacterial infection was initiated by introducing the tip of a contaminated steel needle in the abdominal lateral part of young adult aphids (Fig. S1B in the supplemental material). For this purpose, 500 μl of an overnight LB bacterial culture were centrifuged (4,500 × *g*, 4°C, 5 min), the pellet was rinsed with water and centrifuged again. The tip of a sterile "00" entomological needle (0.25 mm in diameter) was plunged in the compact bacterial pellet and used to infect aphids. Thirty adult aphids were inoculated with each bacterial sample, and the needle was changed for each batch of aphids. Control aphids were inoculated in the same manner with a sterile needle. After inoculation, the aphids were maintained on broad beans, and survivors were counted every day (for up to 7 days or complete mortality).

Inoculation of aphids by ingestion. Third-instar aphid nymphs (L3) fed on broad beans were maintained for 24 h on the AP3 diet (50), containing bacteria at different concentrations, before being placed again on beans. For dose-response pathogenicity assays, an aliquot of an overnight LB culture corresponding to 10^8 bacteria was prepared, and dilutions in the AP3 diet were used to obtain infecting doses ranging from 10^8 to 10^3 bacteria/ml. For the general screening, only the 10^7-bacteria/ml dose was used. From each dilution, two replicates of 500 μl of AP3 diet containing bacteria were introduced in the standard Parafilm sachets. Twenty L4 aphid nymphs were placed on each sachet and maintained for 24 h at 20°C. Then, 30 infected nymphs were collected for further scoring on bean plants as described above.

Other insect bioassays with *D. dadantii* inoculation. In *D. melanogaster* assays, adult flies were pricked in the thorax (ventral face) with a needle dipped in a bacterial pellet or in a suspension in water (optical density [OD] = 0.8 and OD = 0.4). Thirty individuals were inoculated in each assay, plus an aseptic control. For oral infection, third-instar nymphs were inoculated according to the method of Basset et al. (3). Larvae were maintained for 3 to 24 h on a mixture of bacteria (OD = 200) and crushed banana before being transferred onto a standard corn-meal fly medium. Adult mortality or emergence were scored daily.

In *S. oryzae* bioassays, fourth-instar larvae were used after extraction from their wheat kernels. The injection took place in the posterior part of the larval body to avoid puncturing the enlarged midgut, and thereafter larvae, covered with flour to complete their development, were individually placed in 24-well boxes. A control aseptic puncture sample was also carried out. Oral infection was tested by feeding larvae with flour mixed with a bacterial suspension (10^8 bacteria/ml), including initial force-feeding by depositing the drop on the insect's head. The mortality was observed daily, as well as pupation and adult emergence.

S. littoralis was tested after a routine in vivo assay protocol previously used to screen the pathogenicity of different bacteria on this insect (27). Fifth-instar larvae were injected with 20 μl of a bacterial culture previously washed and diluted in phosphate-buffered saline. About 5×10^4 cells were first injected per larva, and another test batch with the aphid protocol was also carried out. Mortality was scored every 10 h after injection for 4 days. Per os toxicity was assayed on second-instar larvae, over 7 days, by incorporating the high-density bacterial suspension (10^8 bacteria/ml) in the agar-based diet supply.

Construction of the toxin deletion mutant. The four toxin genes are contiguous and were labeled ID16665, ID16664, ID16663, and ID16662 in the annotated *D. dadantii* genome (28). They were renamed *cytA*, *cytB*, *cytC*, and *cytD*, respectively. A 0.7-kb DNA fragment containing 100 bases of the coding sequence of *cytA* and the upstream region was amplified by PCR with the primers Am+ (CCTGAGGAATGATTAACAAAAACATGC) and Am− (AATCGCCTGGC CAACATATTTTGTCGG). The product was cloned into the pGEMT plasmid. A NotI-BglII fragment was extracted and inserted into the NotI and BglII sites of a derivative of plasmid pKO3 (42) containing a modified polylinker region, to give the plasmid pKOAm. The primers Av+ (GGCAGATCTGACCCAAGTC CTGCC) and Av− (TGATGGTGTCCAACTCGGAACGGG) were used to amplify a 0.6-kb DNA fragment downstream of *cytD*. This fragment was cloned into the pGEMT plasmid. A SalI-BglII fragment was extracted and cloned into plasmid pKOAm, digested by the same enzymes, to yield plasmid pKOAm-Av. A Kan^{res} cassette was inserted into the BglII site of plasmid pKOAm-Av to yield plasmid pKOAAK. This plasmid was introduced by electroporation into *D. dadantii*, and recombination of the construction into the chromosome was selected by growth in LB medium containing kanamycin and 5% (wt/vol) sucrose

TABLE 1. Bacterial strains used in the present study, together with the relevant taxonomic information, previously published plant or insect interaction phenotypes, and their pathogenicity indexes (per os and through septic injury assays) on the pea aphid *A. pisum*

Classification[a]	Bacterial species[b]	AB[c]	Synonym	Strain[d]	EMBL accession no. (16S gene)	Pathogenicity[e]			LT_{50}[f] (day ± SE)		Reference(s) of pathotypes[g]
						Phyto +/−	Entomo +/−		Ingestion*	Injection	
							Patho	Immu			
Erwinia group											
Clust III	***Dickeya dadantii***	Dda1	*Erwinia chrysanthemi*	CFBP 3855 = 3937	AF520707	+	− (fruitfly)	−	2.56 ± 0.19	0.68 ± 0.12	53
Clust III	*Dickeya dadantii*	Dda2	*Erwinia chrysanthemi*	ident. (repeated)	AF520707	+	−	−	2.36 ± 0.24	0.60 ± 0.11	53
Clust III	*Dickeya paradisiaca*	Dpa	*Erwinia chrysanthemi* pv. *paradisiaca*	CFBP 3477	AF520710	+		+++	2.20 ± 0.36	1.39 ± 0.13	17
Clust III	*Brenneria quercina*	Bqu	*Erwinia quercina*	CFBP 3617	AJ223469	+		−	3.40 ± 0.73	1.46 ± 0.10	6
Clust I?	*Erwinia aphidicola*	Eap		CFBP 6829			+ (aphid)		2.00 ± 0.55	0.54 ± 0.10	31
Clust II	*Pectobacterium carotovorum* subsp. *carotovorum*	Pca1	*Erwinia carotovora*	CFBP 2046	AJ233411	+	− (fruitfly)	−	1.94 ± 0.17	0.75 ± 0.14	3
Clust II	*Pectobacterium carotovorum* subsp. *carotovorum*	Pca2	*Erwinia carotovora*	CFBP 2141	AJ233411	+	± (fruitfly)	+++	1.88 ± 0.16	1.06 ± 0.23	3
Clust II	*Pectobacterium carotovorum* subsp. *atrosepticum*	Pat1	*Erwinia atroseptica*	133A/8 UMG collection	AF373176	+		−	4.25 ± 1.09	1.44 ± 0.13	5
Clust II	***Pectobacterium carotovorum* subsp. *atrosepticum***	Pat2	*Erwinia atroseptica*	SCRI 1043	BX950851	+		−	5.33 ± 0.39	0.50 ± 0.09	5
Clust I	*Erwinia rhapontici*	Erh		SCRI 49-421	Z96087	+		−	>6	1.46 ± 0.13	37
Clust I	*Pantoea stewartii* subsp. *indologenes*	Pst	*Erwinia stewartii*	CFBP 3614	Y13251	+		−	2.17 ± 0.63	0.56 ± 0.10	1
Clust I	*Pantoea agglomerans*	Pag1	*Erwinia herbicola*	CFBP 4740	U80202	+			2.10 ± 0.38	1.25 ± 0.17	
Clust I	*Pantoea agglomerans*	Pag2	*Erwinia* sp. strain TAC2	TAC.XII.93.2	AF024613		Symbiont (thrips)		2.32 ± 0.17	0.50 ± 0.09	14, 15
Clust I	*Pantoea agglomerans*	Pag3	*Erwinia* sp. strain TAC8	TAC.XII.93.8	AF024612		Symbiont (thrips)		1.54 ± 0.10	0.50 ± 0.09	14, 15
Clust I	*Pantoea agglomerans*	Pag4	*Erwinia* sp. strain TAC Fo5	TAC 95.V.5	AY616177		Symbiont (thrips)		2.50 ± 0.10	0.50 ± 0.09	14, 15
Other											
Enterobacteriaceae											
	Escherichia coli	Eco1		XL1-Blue (Stratagene)	J01859	−	−	±	>8	ND	12
	Escherichia coli	Eco2		NM522 (Stratagene)	AY776275	−	−	±	4.60 ± 0.71	1.56 ± 0.10	36
	Escherichia coli	Eco3		Top 10 (Invitrogen)		−	−	±	4.17 ± 0.30	1.60 ± 0.33	
	Escherichia coli	Eco4		BL21		−	−	±	5.00 ± 0.43	3.38 ± 0.07	
	Proteus mirabilis	Pmi		UMG collection	AF008582				5.25 ± 0.94	0.60 ± 0.11	10
	Serratia marcescens	Sma1		UMG collection	AJ233431		+	±	2.62 ± 0.24	0.50 ± 0.09	39, 40
	Serratia marcescens	Sma2		JESM 267	M59160		+		1.75 ± 0.14	0.50 ± 0.09	39
	Serratia marcescens	Sma3		Db10	M59160		+ (fruitfly)		1.88 ± 0.16	0.50 ± 0.09	24, 39
	Serratia marcescens	Sma4		Db11	M59160		+	−	2.00 ± 0.28	0.50 ± 0.09	24, 39
	Photorhabdus luminescens	Plu		TT01	AJ007404	−	+ (bollworm)		>8	1.42 ± 0.13	20
	Xenorhabdus nematophilus	Xne		X1	X82251	−	+ (bollworm)		>8	1.35 ± 0.14	55, 56
	Arsenophonus nasoniae	Ana		LMG 12584 ATCC 49151	M90801	−	Son-killer (wasps)		>8	>8	26
Pseudomonadaceae											
	Pseudomonas syringae* pv. *tomato	Psy		CFBP 2212 type strain	AF326379	+ (tomato)			6.31 ± 0.44	1.41 ± 0.13	7
	Pseudomonas aeruginosa	Pae		CFBP 2466 type strain	Z76672	(+)	+ (multipathogen)	−	2.33 ± 0.29	0.68 ± 0.12	8, 22, 51

[a] Taxonomy information was taken from strain providers, mainly CFBP, and curated for the recent changes in the "*Erwinia*" group taxonomy (53); "*Erwinia*" clusters were as reported by Hauben et al. (33) and Euzéby et al. (21), which were in accordance with our phylogenetic analysis. Clust, cluster.

[b] Sequenced genomes are in boldface.

[c] AB, abbreviations used in our text and figures.

[d] Strain information is given from our main microbiological providers (CFBP, UMG, and LMG, see Materials and Methods), with additional strain nomenclature for fully sequenced species/strains and literature-referenced or commercially available strains (*Escherichia coli*).

[e] Pathogen phenotype previously known and published (see footnote *g*) for the strains used in this study. +, Known pathogens (for the given host); −, tested negative pathogens; blank or ND, unreported phenotypes for the given host categories. The immunity column (Immu) gives the *Drosophila* immune response to oral infection in the screening of Basset et al. (3).

[f] Aphid pathotype as quantified from the present *A. pisum* screening (LT_{50}, 50% lethal time [median survival time]). Due to the high doses used (*, dose = 10^7 bacteria/ml) and to the dose-response curves obtained on the limited set of taxa analyzed in more detail, bacteria with a pathogenicity index higher than 3 days are arbitrarily considered nonpathogens or weak pathogens.

[g] Select direct or review reference(s) for the main pathological phenotypes of the strains used in our study (see the appropriate section of Results for our rationale of strain selection and phylogenetic analyses).

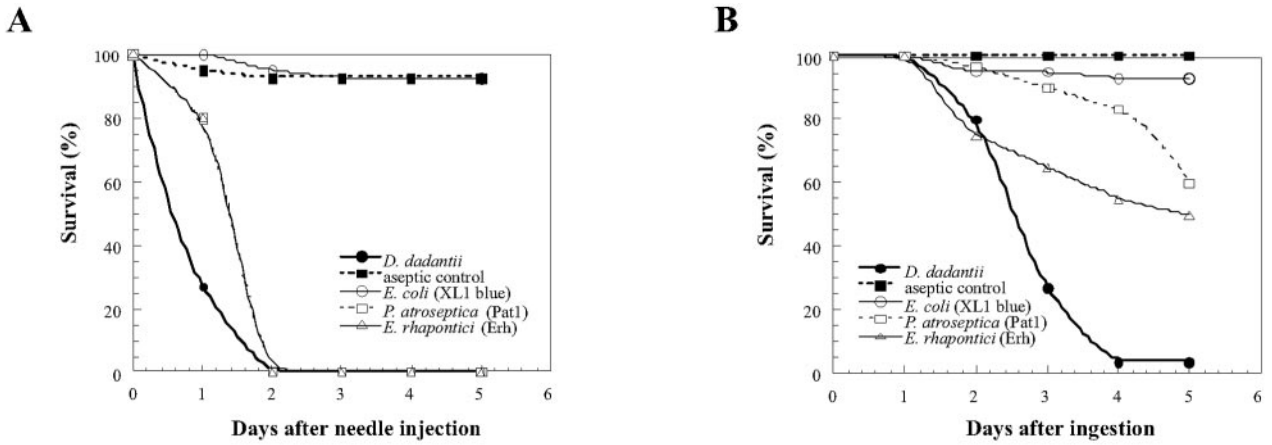


FIG. 1. Aphid survival after infection by *Dickeya dadantii* (*Erwinia chrysanthemi* 3937) and other enteric bacteria. Infection via septic challenge (needle infection) (A) or oral inoculation (ingestion, dose 10^7 bacteria/ml) (B) was evaluated. Bacterial strain abbreviations are as presented in Table 1.

(42). Deletion of the four toxin genes was confirmed by PCR. The Δ*cyt* mutant obtained was named A4664. DNA manipulations were performed according to standard protocols (52). The four genes were also amplified by using the primers Am+ and Av−. The product was cloned into the pGEMT plasmid to give plasmid pGEAA2, which was used to complement the A4664 mutant.

RESULTS

***D. dadantii* (*E. chrysanthemi*) is an experimental pathogen for the pea aphid.** When *A. pisum* was infected by *Erwinia* sp., either by septic challenge or by oral infection, its survival was greatly reduced (Fig. 1). By injecting *Erwinia* into the adult hemocoel, aphids survived no more than 2 days (Fig. 1A). *D. dadantii* (Dda) was more virulent than either *P. carotovorum* subsp. *atrosepticum* or *Erwinia rhapontici* (twin curves), with more than 73% mortality observed on the first day for Dda versus only 20% with the other strains. Conversely, *Escherichia coli* injection (XL1-Blue strain) or aseptic punctures did not affect aphid survival.

When L3 aphid nymphs were fed first on artificial media containing different bacterial doses for 24 h and thereafter on broad beans, the pathogenicity of *Erwinia* strains was also observed; preliminary studies led us to choose a dose of 10^7 bacteria/ml for further screening (Fig. 1B). After 4 days, 100% mortality of aphids fed on *D. dadantii* was observed. In the same conditions, aphids fed on other taxa were less affected (only 40% mortality after 5 days), and aphids ingesting *E. coli* survived almost as well as controls fed on aseptic media.

The characteristics of pathogenicity were variable with the different strains, both in color and in symptoms. Typically, with *D. dadantii*, aphids died very quickly and homogeneously (low variability in LT_{50}); they turned black and eventually were hanging by their stylets under the leaves, highlighting the intensity of final pathogenic stress (Fig. S1D to F in the supplemental material). With other bacteria, aphids fell on the soil before death, and their color changed from green to gray and black within in a few hours. Not all tested strains displayed this quick succession nor this final color (Fig. S1E in the supplemental material).

The pathogenic trait is both widespread and variable within the *Enterobacteriaceae*. Since the responses of *A. pisum* to septic challenges with the first tested soft rot *Erwinia* were different, we decided to test other species from the same bacterial clade (*Enterobacteriaceae*) for their pathogenicity in *A. pisum*, as well as two outgroup *Pseudomonadaceae*. We focused on typical phytopathogens (*Erwinia* sp., *Pectobacterium* sp., *Pseudomonas syringae*, etc.) and on culturable bacteria with potential interactions with insects, such as known pathogens and symbionts (*Arsenophonus nasoniae*, *Photorhabdus* sp, *Xenorhabdus* sp., *Pantoea* sp. thrips symbionts, etc.). Some multipathogens (*Pseudomonas aeruginosa* and *Serratia marcescens*), as well as laboratory *E. coli* K-12 derivative strains (XL1-Blue, Top 10, and NM522), were also included as landmarks in our *A. pisum* screening (Table 1). For all strains, we tried to summarize in Table 1 the pathogen phenotypes and links to selected references relevant to our field of investigation.

Aphid survival parameters (i.e., the $LT_{50} \pm$ the standard error [SE]) were calculated for each bacterial strain for both needle infection and ingestion at the screening dose of 10^7 bacteria/ml (Table 1). Some species were highly pathogenic in the two assays: *D. dadantii*, *D. paradisiaca*, *E. aphidicola*, *P. aeruginosa*, *S. marcescens*, *P. agglomerans*, *P. stewartii*, and *P. carotovorum* subsp. *carotovorum*. Others were less pathogenic by ingestion than by infection: *P. carotovorum* subsp. *atrosepticum*, *E. rhapontici*, *Proteus mirabilis*, *P. syringae* pv. tomato, and *E. coli* (NM522 and Top 10) strains. In these strains, the gut barrier could be a significant line of defense against the bacteria. It was interesting to note that *Photorhabdus luminescens* and *Xenorhabdus nematophilus*, which are known insect pathogens, were not pathogenic in *A. pisum* by ingestion ($LT_{50} > 8$ days) and were not among the most virulent pathogens by injection into the aphid hemocoel.

Only *A. nasoniae* (a sex-manipulating symbiont of the wasp *Nasonia vitripennis*) and *E. coli* (XL1-Blue) were not pathogenic in either assay, and most aphids survived 8 days after the challenge. This corroborated the use of XL1 blue as a control toxin carrier in other insect systems (12) and the conclusions of gut elimination in the pea aphid with this *E. coli* strain (31).

Examining the taxonomic position (Fig. 2) of the most virulent species and strains used in our screening did not reveal

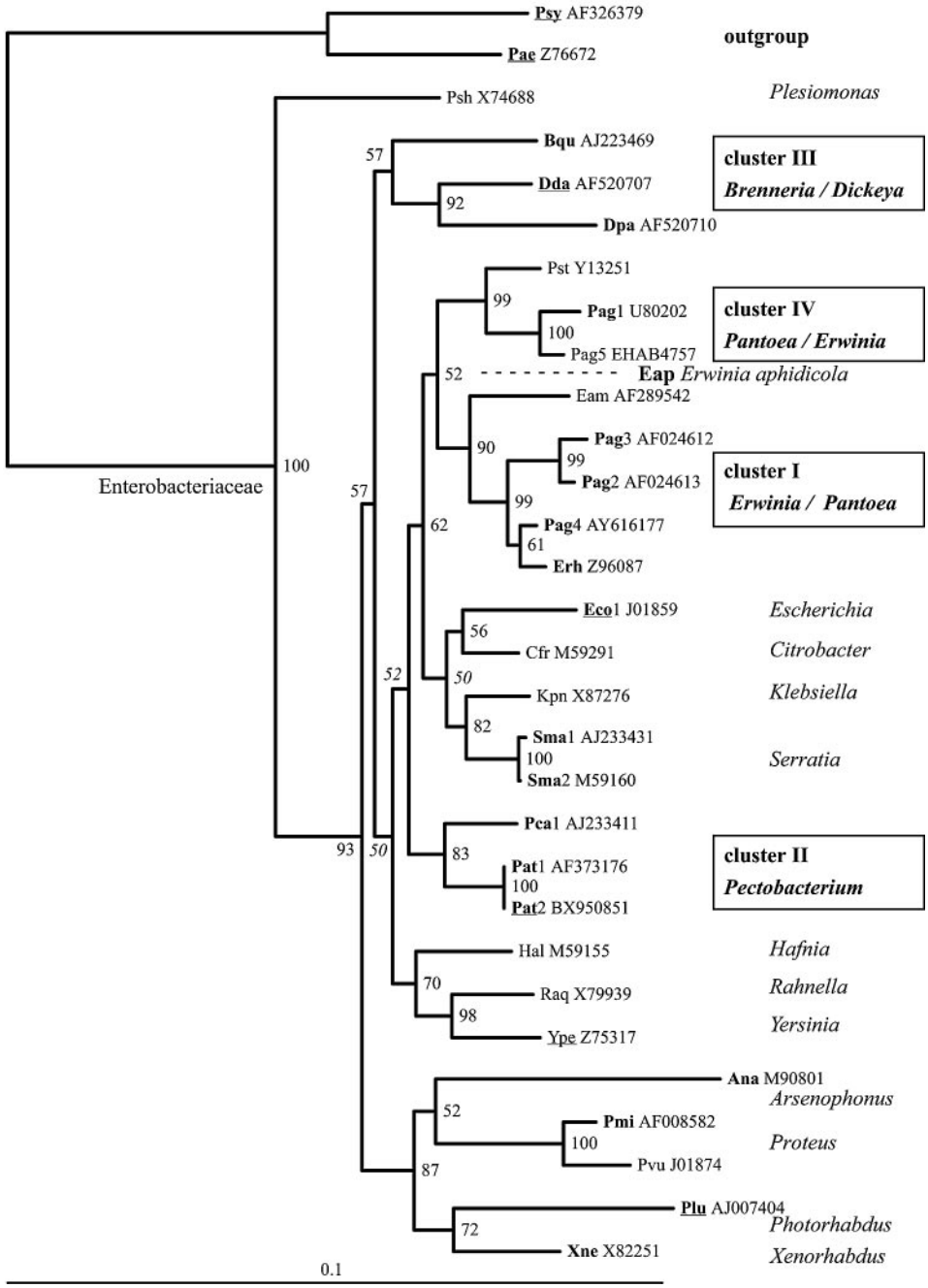


FIG. 2. 16S rRNA phylogenetic tree of tested bacterial strains and additional representative taxa from the *Enterobacteriaceae* (*Pseudomonas* as an outgroup). Taxa with fully sequenced representatives are underlined, and taxa tested in aphid bioassays are in boldface. See the text for the full phylogenetic methodology (1,231 sites, gap removal, neighbor-joining method, Kimura distance, and 1,000 boostrap replicates). Abbreviations of the additional *Enterobacteriaceae* strains not included in Table 1: Cfr, *Citrobacter freundii*; Eam, *Erwinia amylovora*; Hal, *Hafnia alvei*; Kpn, *Klebsiella pneumoniae*; Pag5, *Pantoea agglomerans*; Psh, *Plesiomonas shigelloides*; Pvu, *Proteus vulgaris*; Raq, *Rahnella aquatilis*; Ype, *Yersinia* pestis. *Erwinia aphidicola* was positioned manually by a phylogeny on groEL. Clusters I, II, III, and IV refer to the classification of Hauben et al. (33), with a refinement of the positioning of the *Dickeya* clade.

any link of the aphid pathogenicity trait with phylogenetic positioning. At most, some groups may appear to share similar high-virulence or low-virulence traits (e.g., respectively, the *Dickeya* clade or the *Proteus/Arsenophonus/Photorhabdus/Xenorhabdus* clade).

***Dickeya dadantii* is a virulent aphid pathogen that is active at a low inoculum dose.** The dose-response mortality of *A. pisum* to oral infection by a subset of our bacterial sample was analyzed (Fig. S2A and B in the supplemental material). Among the phytopathogens, *D. dadantii* proved to be one of

TABLE 2. Pathogenicity assays for *D. dadantii* (*E. chrysanthemi* 3937) in insects from four different orders

Insect species	Insect order	*D. dadantii* administration			
		Needle injection		Ingestion	
		% Mortality (*n*)	Mean LT_{50} (days) ± SE	% Mortality (*n*)	Mean LT_{50} (days) ± SE
A. pisum	Hemiptera	100 (30)	0.6 ± 0.11	100 (30)	2.36 ± 0.24
D. melanogaster	Diptera	0 (90)		0 (120)	
S. littoralis	Lepidoptera	0 (40)		0 (40)	
S. oryzae	Coleoptera	60 (24)	3.0 ± 0.86	0 (24)	

the most pathogenic strains for *A. pisum*, since its virulence was conserved at the weakest concentrations tested (10^5 and 10^4 bacteria/ml, respectively). At low doses, only the aphid pathogen *E. aphidicola* and, surprisingly, some *P. agglomerans* TAC strains (thrips symbionts) proved to be slightly more virulent than *D. dadantii* (Fig. S2B in the supplemental material).

Almost all aphid instars are equally susceptible to *D. dadantii*. Since larval aphid guts were shown to be rarely infected by bacteria (29), we decided to test the age dependency of the pea aphid sensitivity to *D. dadantii* oral infection. Parthenogenetic apterous aphids of diverse ages and stages were subjected to the standard assay (10^7 bacteria/ml) before they resumed their development on broad beans. All aphid instars were equally sensitive to *D. dadantii* by ingestion (LT_{50} in days = 2.62 ± 0.11, 2.60 ± 0.11, 2.87 ± 0.17, 2.64 ± 0.13, and 2.63 ± 0. 15 for the L2, L3, and L4 larval stages and 1- and 6-day-old adults, respectively), except for the first stage that escaped contamination ($LT_{50} > 8$ days). This "resistance" of the first-instar nymphs could be interpreted by a drastic mechanical filtering of bacterial inoculum by the alimentary canal, as was previously suggested to explain natural infections (29). Since internal stylet sections of the pea aphid are only 0.6 μm in the first instar, and *Erwinia* sp. are straight rods 0.5 to 1 μm wide by 1 to 3 μm long, a strong bacterium-filtering process might be responsible for the observed "immunity" of neonate aphids, disappearing at the second instar.

***D. dadantii* is not a generalist insect pathogen.** Other insects were tested for their susceptibility to *D. dadantii*. Protocols were adapted to allow comparisons with our assay, as well as other standard procedures (*Drosophila, Spodoptera*). In particular, oral inoculation was only performed on fully growing stages with continuous feeding on high-inoculum diets (doses were always the maximum tolerated by the insect biology and practical considerations, including humidity control, ensuring *Dickeya* viability). *D. dadantii* was not a pathogen for *D. melanogaster* or *S. littoralis*. It was only deleterious for *S. oryzae* by injection and not by oral ingestion (Table 2). It should be noted that weevil larvae were quite sensitive to needle puncture injury, since observed control mortality was ca. 15 to 25% with *S. oryzae*, in contrast to the bollworm, drosophila, and aphid assays, where mortality was negligible in aseptic conditions (see Fig. S1H in the supplemental material for aphids).

Therefore, *D. dadantii* does not seem to be a generalist pathogen for insects, although care should be taken regarding the limited range of our insect assays. It displayed instead a unique high pathogenicity for aphids.

***D. dadantii* is less virulent toward young winged aphids.** Since aphids may be potential vectors for *D. dadantii*, we decided to test whether wing polymorphism could influence the fate of infection. In the pea aphid, wing production is induced by overcrowding and reduced plant quality experienced by the parthenogenetic mother (46). Progeny subsequent to this treatment are triggered to follow alate adult development, with an altered behavioral and physiological program: the last nymphal instars are produced with distinct anatomical features (N4, versus apterous-producing L4 nymphs), and they feed and give rise to nonfeeding alate adults programmed for an obligate flying phase, the completion of which turns the winged individuals to another feeding phase on new host plants. We tested aphids before and after the potential flying phase, in both developmental lineages (apterous and alate), therefore assaying old feeding nymphs (L4 or N4), as well as 6-day-old adults having completed (alate) or not (apterous) the flying or fasting stage. Our standard assay results (Fig. 3) clearly show that alate aphids were less sensitive to *D. dadantii* infection, with almost 50% of the population surviving after 6 days and with reduced pathogenicity indices: the LT_{50}s (days) were 2.4 ± 0.16 (L4), 2.5 ± 0.11 (N4), 2.6 ± 0.15 (apterous adults), and 3.7 ± 1.2 (alate adults), the latter group differing significantly after survival analysis ($P < 0.001$, log-rank statistics). It should be noted that this experiment was repeated at a lower inoculum dose, with similar results (10^5 bacteria/ml [data not shown]). Also, since feeding differences exist between the two development stages tested, the protocol was optimized and checked to ensure that all aphids were feeding equally within the 24-h feeding period, thus confirming the absence of the inoculation bias.

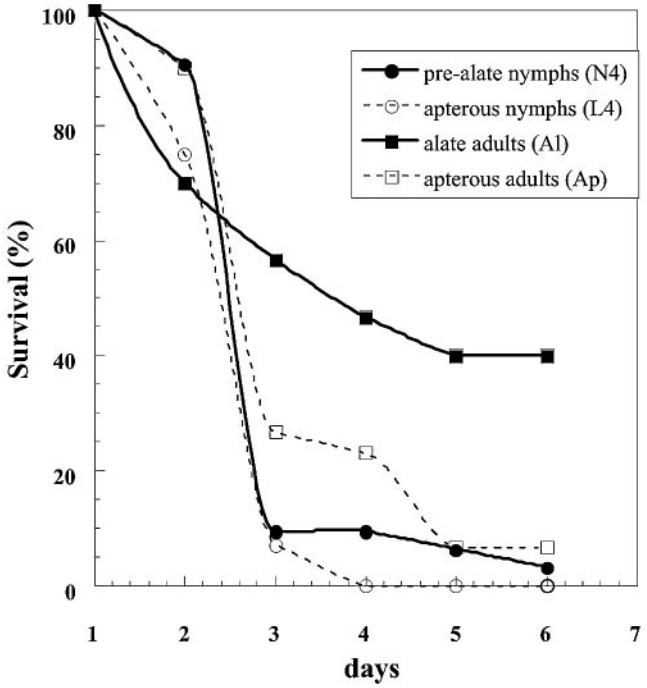


FIG. 3. Sensitivity of winged versus apterous developmental lineages of the pea aphid to *D. dadantii* oral infection at L4 and N4 nymphal stages and reproducing adult stages, 6 days after imaginal molt.

TABLE 3. *A. pisum* virulence assays for *D. dadantii* (*E. chrysanthemi* 3937) wild-type strain and A4664 mutant (Δ*cytABCD*) after septic inoculation or oral challenge at different doses

Assay and dose (bacteria/ml)	LT_{50} ± SE (days)		Log-rank test result (*P*)[a]
	D. dadantii wild type	*D. dadantii* Δ*cyt* mutant	
Injection	1.4 ± 0.13	1.4 ± 0.14	0.75
Ingestion			
10^7	2.1 ± 0.25	3.0 ± 0.97	0.001
10^6	2.5 ± 0.16	5.2 ± 0.75	<0.0001
10^5	2.5 ± 0.13	3.9 ± 0.40	<0.0001
10^4	2.8 ± 0.88	>8	0.019

[a] Wild-type versus mutant survival comparison.

The *D. dadantii* deletion mutant showed a reduced virulence for *A. pisum*. When inoculations were performed in parallel with *D. dadantii* strain 3937 and its mutant derivative A4664 (with *cytABCD* deleted), we observed a significant reduction in the virulence of the Δ*cyt* A4664 strain (Table 3), in particular for medium inoculation doses that should be the most relevant to potential natural contaminations. This experiment was repeated three times with similar results, especially regarding the significance of differences at intermediate doses, while the lowest and highest doses showed less marked differences (data not shown). No significant difference between strains was detected for septic injury assays (Table 3). When the mutant was complemented by the pGEAA2 plasmid containing the four *cyt* genes, the LT_{50}s of the rescued mutant were similar to those of the wild-type 3937 strain, reducing by almost 1 day, at any dose, the median survival of aphids infected with the 4664 mutant. This is exemplified by the data obtained at the standard screening dose of 10^7 bacteria/ml (performed in a phytotron at 18°C for technical constraints; Fig. 4).

DISCUSSION

We demonstrated here, for the first time, that the common phytopathogenic bacterium *Dickeya dadantii* (*Erwinia chrysanthemi*) is also a potent experimental pathogen of an aphid (*A. pisum*). The only previous data related to the insect pathogenicity of *Erwinia* strains also concerned the pea aphid but with *Erwinia aphidicola* (not reported as a phytopathogen) (31), as well as another species pair: *D. melanogaster* and *Pectobacterium carotovorum* (4). Since these three strains do not belong to the same phylogenetic cluster (Fig. 2), we might expect that the insect pathogenicity traits are widespread within this bacterial group. By screening for aphid pathogenicity on a large panel of bacteria belonging to this group, we were able to show that some of them are highly virulent against the pea aphid, such as *D. dadantii*, *P. carotovorum pv. carotovorum*, *E. aphidicola*, and *Pantoea agglomerans*. However, this prevalence does not raise the whole bacterial clade to the status of generalist insect pathogens. *Drosophila* was shown to be more resistant than the pea aphid to many species or strains being mainly susceptible or, more exactly, immune responsive to *P. carotovorum* and *Dickeya paradisiaca* species (4).

Two ecological traits might be related to the higher susceptibility of aphids, namely, the presence of associated symbionts (the obligate *Buchnera*, or different widespread facultative bacteria) and the restriction of aphids to a specific diet, the phloem sap, which is largely protected from the external plant microflora. These traits are old determinants of aphid ecology and might have shaped these insects' immune response. Conversely, many *Enterobacteriaceae* were not serious pathogens for the pea aphid (such as *E. coli* strains) or displayed only reduced virulence, at least via the oral infection route (*P. carotovorum* subsp. *atrosepticum*, *Brenneria quercina*, and *Erwinia rhapontici*). Also, several bacteria from the *Proteus* clade did not display pathogenicity for the aphid by oral contamination, including *Arsenophonus nasoniae* (inducing the son-killer trait in wasps) and the emblematic insect pathogens, which are also nematode symbionts, *Photorhabdus* and *Xenorhabdus*. This surprising result might be related to the peculiar trophic niche of aphids, which imposes specific constraints, such as high osmolarity due to elevated sucrose levels (up to 1 M in the phloem), contributing to their protection from otherwise potent insect pathogens. *Photorhabdus luminescens* is, however, known to be halo- and osmotolerant (54), emphasizing that pathogenicity is a complex trait governed by multiple unrelated pathways. Moreover, this *Proteus* clade has also been characterized as a bacterial group related to many obligate intracellular animal symbionts of different evolutionary ages (9), including two aphid secondary symbionts newly defined as bacterial taxa (13, 45). This may indicate a mitigation of pathogenic and symbiotic traits within this large group, both evolving through horizontal gene transfers, such as those that occurred between *Yersinia* and *Photorhabdus* (20), or through phage-included toxins in aphid secondary symbionts (44).

Among the previously identified insect symbionts (Table 1), the case of the thrips gut symbionts (*P. agglomerans*: Pag2,

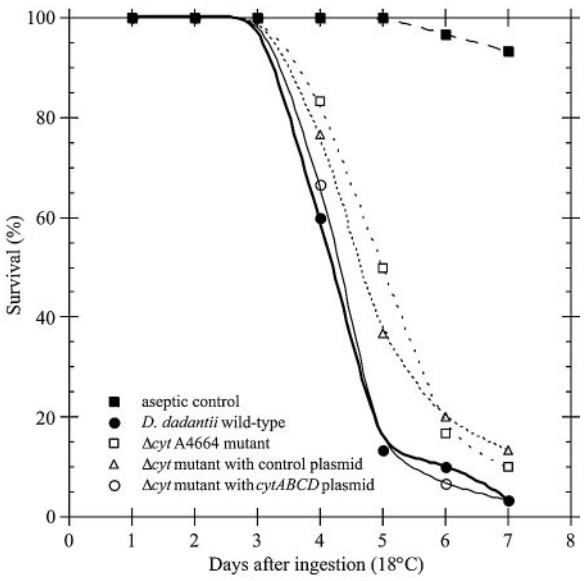


FIG. 4. Survival of pea aphids in response to wild-type, *cytABCD*-defective, and plasmid-complemented mutants. LT_{50}s of strains (in days, at 18°C, standard assay dose): wild type, 4.2 ± 0.25; *cyt*-defective A4664, 5.0 ± 0.39; A4664 *cyt* rescued, 4.3 ± 0.21 ($P = 0.011$; survival analysis). The A4664 mutant complemented with the control pGEMT plasmid did not differ from its untransformed A4664 control (LT_{50} = 4.7 ± 0.24 days).

Pag3, and Pag4 strains) deserves special interest. These bacteria were identified as ubiquitous hindgut microflora of different thrips species (Thysanoptera, a sister order to the plant-sucking Hemiptera), and they are transmitted through cyclic recolonization of the insect gut, without maternal inheritance (15). They have been shown to exert a beneficial influence in some insect host-plant combinations (16). Interestingly, these bacteria are highly pathogenic to the pea aphid, and this raises questions regarding the mechanistic and ecological bases of these associations. Bacterium-mediated insect plant interactions, including interspecific competition between aphids and thrips, are likely to be largely influenced by the diversity of pathogenic phenotypes harbored by such microorganisms.

The sequence homology analysis of the four toxin genes (*cyt* cluster) found in *D. dadantii* shows that they are all similar to the Cyt2Aa1 toxin genes of *Bacillus thuringiensis*, a member of the evolutionarily related Cyt toxins from this species (30). The involvement of the *cyt* gene cluster in the virulence of *D. dadantii* against *A. pisum* is clearly demonstrated and will be investigated further. Although blastp results were all significant ($10^{-7} <$ hit expects $< 10^{-41}$), protein identities over the hits on Cyt2Aa1 (Uniprot Z14147) ranged from 26% (CytB-Dda) to 43% (CytA-Dda). The Cyt2Aa1 toxin, also called CytB-Btk or 29-kDa toxin in previous reports, was shown to kill the larvae of some mosquito species by making pores in the epithelial cell membrane of the insect midgut (41). The G+C content of the four open reading frames has values of between 40 and 45%, falling between those of the *B. thuringiensis* toxin (27% G+C) and those of typical *D. dadantii* genes (55% G+C). The research of such *cyt* sequences by blasting GenBank revealed that, up to now, no other gram-negative bacterium has been shown to harbor these toxins. Therefore, wide-ranging horizontal transfer between gram positive and gram negative is probably at the origin of these genes in *D. dadantii*. Ecological interactions between phytopathogenic *Erwinia* and *B. thuringiensis* have been described through a quorum-sensing interaction termed signal interference (18), which may be an indication of functional exchanges and long-term cohabitation between the two taxa in the plant or soil environment.

It was clear, however, that the *D. dadantii* Δ*cyt* mutant still displayed significant virulence toward the pea aphid (Table 3) and therefore that other virulence genes or factors are involved in the pathogenicity of *D. dadantii*. None of the Cyt-like toxins were found in the genome of *P. carotovorum* subsp. *atrosepticum* (5), but this bacterium was also able to kill *A. pisum*, albeit less conspicuously than *D. dadantii*. Conversely, another insect virulence factor (Evf, also a pore-forming toxin) was identified in *P. carotovorum* subsp. *carotovorum* as being largely responsible for the pathogenicity of this bacterium against *Drosophila*, but it has not been found in the genome of *D. dadantii*. All of these phytopathogenic soft rot *Erwinia* spp. are therefore able to enhance their insect pathogenicity, with divergent specificities, through different sets of unrelated toxins and targets. We should also mention that the toxin-defective mutant strain was as infectious as the wild type in our septic challenge assay. Thus, the Cyt-like toxins may be involved in the early pathogenic steps related to the infection of the aphid digestive tract, a finding in agreement with the demonstrated cellular target of their *B. thuringiensis* homologues.

Our results demonstrate the experimental pathogenicity of *D. dadantii* to the pea aphid, but no inference can be made on the ecological importance of this trait, especially in the epidemiology of soft rot diseases. Aphids have been shown to harbor very limited gut microflora in laboratory conditions (19, 29), but there are very few reports based on natural populations, and there is no mention of pathogenicity in older publications describing aphid gut bacteria (47). The only report on a bacterial pathogen of aphids concerns *E. aphidicola* (31, 32), which was not investigated further in natural conditions. Although phylogenetically distinct (Fig. 2), these two species display similar pathogenic phenotypes on the aphid. When the minimum inoculum dose is calculated from published ingestion rates of the pea aphid on the same diet (23), *D. dadantii* seems to be able to infect an aphid through fewer than 10 ingested bacterial cells (nymphs at the inoculated L4 stage ingest about 1.1 μl per day, which is ca. 150% of their average weight). This low value is probably enough to be relevant in natural conditions, either from surface samplings or from ingestion of contaminated vascular tissues (58), such as phloem (on which aphids feed), or more likely from contaminated xylem (from which aphids are able to ingest sap for long durations under diverse conditions of stress). After acquisition, the bacterial population probably grows in the aphid gut as demonstrated for *E. aphidicola* (31), and crosses the gut barrier before killing the insect. More histological experiments need to be conducted to investigate this further.

From our present knowledge of the very fast infection process and from anatomical considerations of the aphid mouthparts, it is extremely unlikely that the bacteria may be inoculated back to the plant by the stylet canals, as might occur with other homopteran species vectors of bacterial diseases (leafhoppers) and as occurs widely with the persistent viruses transmitted by aphids after an internal cycle through the insect salivary glands. Size filtering through the stylet food canal is probably an important element of the interaction between aphids and bacteria, and although hypothesized early in the search for insect vectors of phytopathogenic erwiniae (48), the vector status of aphids in the spread of bacterial disease is still unclear. This is true even for widely investigated diseases such as the fire blights caused by *Erwinia amylovora* (35), which was not included in our survey because of its quarantine status. Two elements introduced by our study are likely to renew interest in this question: (i) the aphid pathogenicity demonstrated for many of the surveyed phytopathogens and (ii) the potential differential virulence toward aphid morphs with very different impacts on the dispersal potential. The fact that a vectored pathogen should kill its vector to pursue its infection cycle is uncommon, but parasite-induced vector death occurs in many parasitic systems (2). It is not documented in the field of insect vectoring of plant microbial pathogens, although the case of replicative plant viruses, inducing immune reactions in their insect vectors (43), appears as an intermediate situation.

Finally, the recognition of the pea aphid and *D. dadantii* as a new experimental multipathosystem may have importance in comparative physiopathology, next to seminal models such as *Drosophila*, which has recently proved its importance in the investigation of virulence factors of many human or plant bacterial pathogens such as *Pseudomonas*, *Salmonella*, *Vibrio*, and *Erwinia*. It may also be noted that the potential finding of a

B. thuringiensis toxin with aphid specificity, which has been claimed in several instances (59) without convincing follow-ups, may also be an important outcome of the present work.

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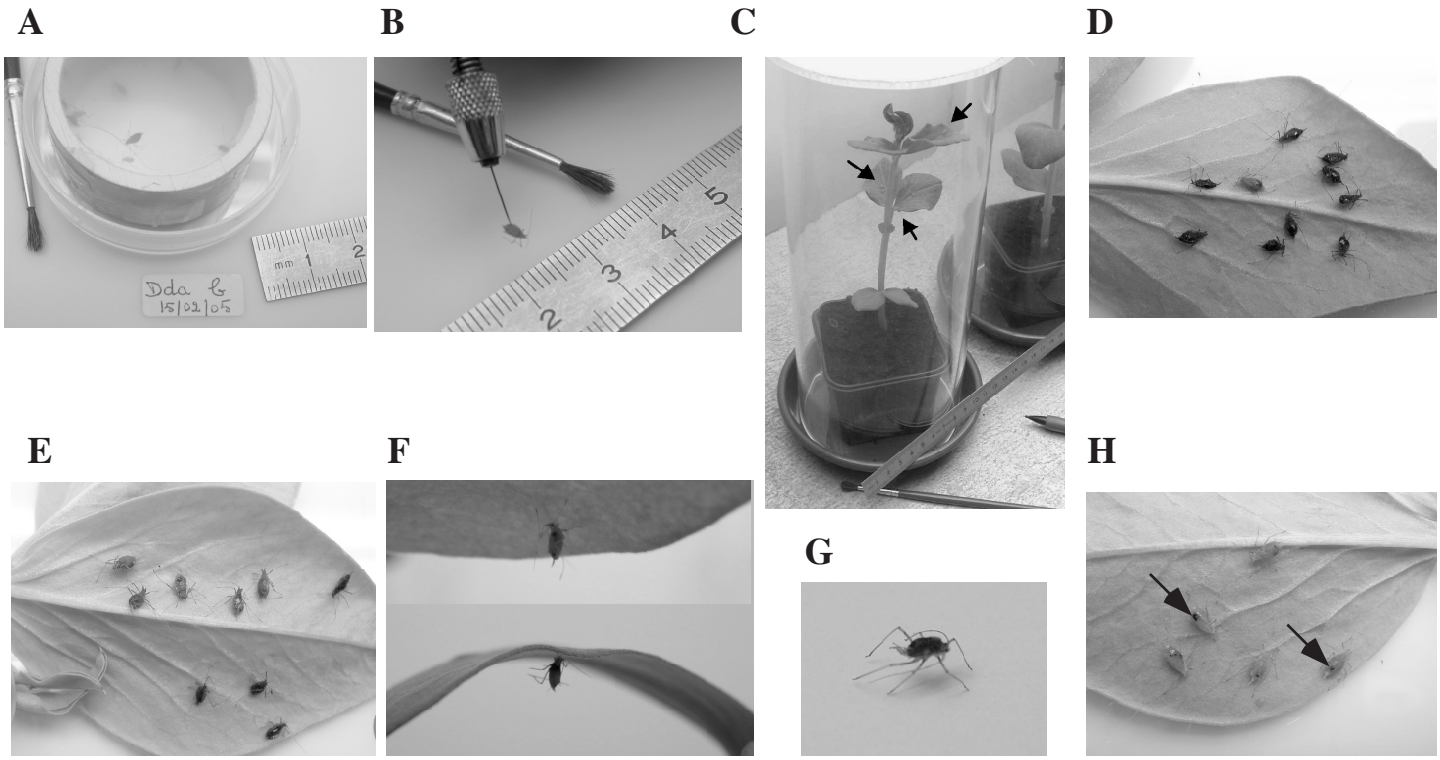
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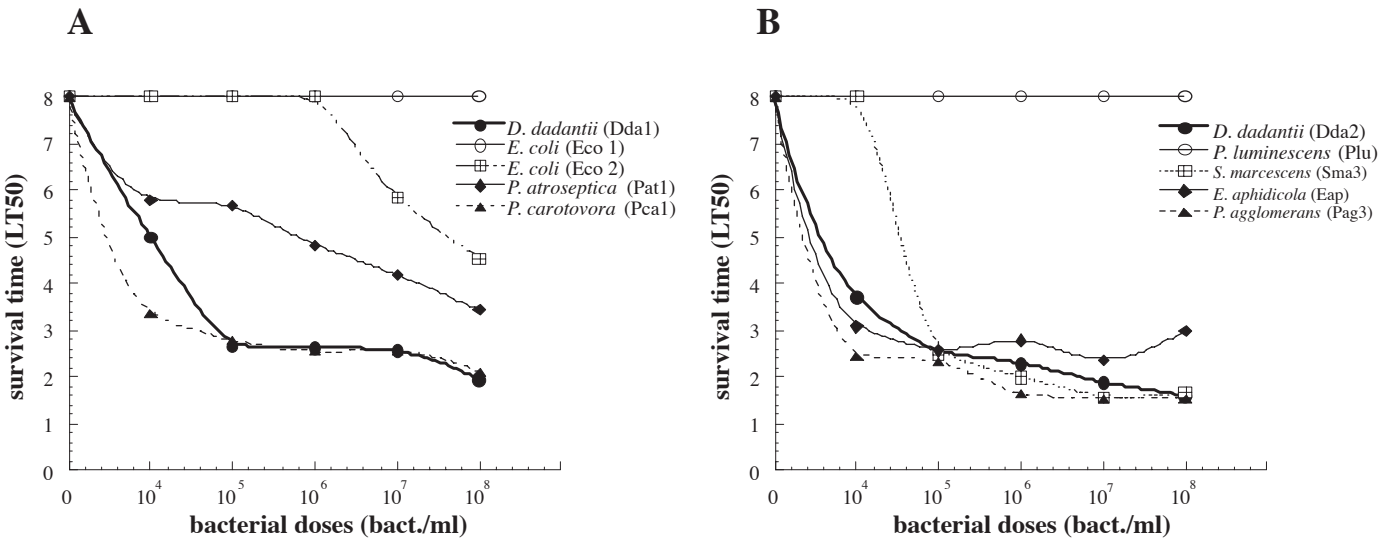
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Figure S1 of supplementary material



Infection protocols (A, B, C) and phenotypes of dead aphids after infection (D, E, F, G). A: Parafilm® sachet cages for oral inoculation; B: needle inoculation; C: plant cages allowing simple scoring (arrows) without leaf handling; D: aphids dead from oral inoculation by *Dickeya dadantii*; E: aphids dead from oral inoculation by *Pectobacterium carotovorum subsp. atrosepticum*; F: occasional aphids observed hanging dead from *D. dadantii* oral infection, tethered to the plant by their mouthparts; G; aphids dead from molting failure, infrequently observed from oral *D. dadantii* infections; H: aphids surviving *E.coli* puncture infections; healed wounds indicated (arrows).

Figure S2 of supplementary material



Survival time dose-response curves of the pea aphid from per os infection assays with a selected sample of the bacterial panel. A: phytopathogens, B: recorded pathogens or symbionts of insects (*D. dadantii* included in both graphs for comparison; data from the independent assays Dda1 and Dda2, see Table 1); bacterial strain abbreviations as detailed in Table 1.