Identification of new *Dickeya dadantii* virulence factors secreted by the type 2 secretion system.

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ABSTRACT

Dickeya are plant pathogenic bacteria able to provoke disease on a wide range of plants. A type 2 secretion system (T2SS) named Out is necessary for bacterial virulence. Its study in *D. dadantii* showed that it secretes a wide range of plant cell wall degrading enzymes, including pectinases and a cellulase. However, the full repertoire of exoproteins it can secrete has probably not yet been identified. Secreted proteins are first addressed to the periplasm before their secretion by Out. No secretion signal present on the protein allows the identification of substrates of a T2SS. To identify new Out substrates, we analyzed *D. dadantii* transcriptome data obtained in plant infection condition and searched for genes strongly induced encoding a protein with a signal sequence. We identified four new Out-secreted proteins: the expansin YoaJ, the putative virulence factor VirK and two proteins of the DUF 4879 family, SvfA and SvfB. We showed that SvfA and SvfB are required for full virulence of *D. dadantii* and showed that *svf* genes are present in a variable number of copies in other *Pectobacteriaceae*, up to three in *D. fanghzongdai*. This work opens the way to the study of the role of non-pectinolytic proteins secreted by the Out pathway in *Pectobacteriaceae*.

IMPORTANCE

The plant pathogen *Dickeya* rely on a type 2 secretion system named Out for their pathogenicity. Importance of plant cell wall degrading enzymes secreted by this system has been well studied. However, existence and role of other Out-secreted proteins has barely been investigated. By mining *D. dadantii* transcriptome data, we identified four new Out-secreted proteins. We showed that two of them, SvfA and SvfB, are necessary for the full virulence of the bacteria. These findings show that identification of all the proteins secreted by the *Dickeya* Out system is necessary for a better knowledge of the virulence of these bacteria.

INTRODUCTION

Soft rot *Pectobacteriaceae* (SRP), *Dickeya* and *Pectobacterium*, are plant pathogenic bacteria that can provoke disease on more than 35% of angiosperm plant orders, including both monocot and dicot plants (1). Among those, there is a wide range of plants of agronomic interest such as potato, rice, chicory, cabbage or ornementals on which they can cause severe losses. Symptoms are usually soft rot but these bacteria can provoke blackleg or wilting on aerial parts of potato. Recently, diseases on woody plants caused by *Dickeya* have been reported (2). There is no efficient way to fight these bacterial diseases. There are actually twelve species of *Dickeya* described, isolated either from infected plants (type strain of *D. chrysanthemi* isolated from *Chrysanthemum morifolium*, *D. dadantii* subsp. *dadantii* from *Pelargonium capitum*, *D. dadantii* subsp. *diffenbachiae* from *Dieffenbachia* sp., *D. dianthicola* from *Dianthus caryophillus*, *D. zeae* from *Zea mays*, *D. oryzae* from *Oryza sativa*, *D. paradisiaca* from *Musa paradisiaca*, *D. solani* from *Solanum tuberosum*, *D. fangzhongdai* from *Pyrus pyrifolia*, *D. poaceiphila* from *Saccharum officinarum*) (3)(4)(5)(6)(7) or from river of lake waters (*D. aquatica*, *D. lacustris* and *D. undicola*) (8)(9)(10). The role of protein secretion systems on the onset of the disease provoked by these bacteria has been recognized long ago (11). In contrast to many plant pathogenic bacteria, the type three Hrp secretion system is not the main determinant for SRP virulence (12). The main virulence factor for these bacteria is a type 2 secretion system (T2SS) named Out. It allows the secretion of enzymes that degrade the components of the plant cell wall, leading to the soft rot symptom distinctive of the disease. The first Out-secreted proteins to be identified were a set of pectinases and a cellulase which are easily detectable by simple enzymatic tests (13) (11). The pectinolytic secretome of the model strain *D. dadantii* 3937 has been studied in detail by cloning the genes of these easily

67 detectable enzymes. *D. dadantii* secretes by the Out machinery nine pectate lyases, one pectin
68 methylesterase, one pectin acetylesterase and one rhamnogalacturonate lyase (14). A proteomic
69 analysis of the secreted proteins by 2D gel electrophoresis allowed the identification of two
70 other secreted proteins, the feruloyl esterase FaeD and a protein with homology with a
71 *Xanthomonas campestris* avirulence protein AvrL (15). A search in *D. dadantii* of homologues
72 of proteins secreted by the Out T2SS of *Pectobacterium atrosepticum* (16) recently led to the
73 characterization of the metal binding protein IbpS (17). There is no strict host specificity for
74 Dickeya species, however some of them show a preference for some plant species. Since all the
75 pectinolytic enzymes studied in *D. dadantii* are present in most of other *Dickeya* species these
76 enzymes are probably not responsible for the host preference observed for these bacteria (18).
77 We hypothesized that additional T2SS-secreted proteins specific for some species might exist
78 and play a role in the host preference. To identify such proteins, we analyzed previously
79 published *D. dadantii* transcriptome data, looking for genes induced in plant infection
80 conditions and encoding proteins with a signal sequence. We identified several proteins
81 secreted by the Out machinery and showed that two proteins of the DUF4879 family, SvfA and
82 SvfB are *D. dadantii* virulence factors.
83
84 **MATERIAL AND METHODS**
85
86 **Bacterial strains and growth conditions**.

87 Bacterial strains, phages, plasmids and oligonucleotides used in this study are described in Table 2. *D.*
88 *dadantii* and *E. coli* cells were grown at 30 and 37°C respectively in LB medium or M63 minimal medium
89 supplemented with a carbon source (0.2%, w/v unless otherwise indicated). When required antibiotics
90 were added at the following concentrations: ampicillin, 100 mg l^{-1}, kanamycin and chloramphenicol, 25

91 mg l^{-1}. Media were solidified with 1.5% (w/v) agar. Transduction with phage ΦEC2 was performed
92 according to Résibois *et al.* (40)

93 **Mutant construction**.

94 To construct strain A6418 that contains a *svfA-uidA* fusion a 1.3 kb DNA fragment containing
95 *svfA* was amplified with primers 17176H+ and 17176A. The resulting fragment was inserted into
96 the pGEM-T plasmid (Promega). A XbaI site was created by site directed mutagenesis with the
97 primers 17176XbaF and 17176XbaR into the *svfA* coding sequence and a *uidA*-kanR cassette was
98 inserted into this XbaI site. To construct strain A6467 that contains a *svfB-uidA*-kanR fusion and
99 a CmR cassette a 2000bp DNA fragment containing *svfB* was amplified with the primers
100 15544L2+ and 15544L2-. The resulting fragment was inserted into the pGEM-T plasmid. A XmaI
101 site was created by site directed mutagenesis into *svfB* coding sequence with the primers
102 15544XmaF and 15544XmaR and a *uidA*-kanR cassette was inserted into this created unique XmaI
103 site. All the constructs were recombined into the *D. dadantii* chromosome according to Roeder
104 and Collmer (41). Recombinations were checked by PCR. His-tagged versions of the proteins
105 SvfA, SvfB, YoaJ and VirK were constructed by amplifying the corresponding genes with the
106 primers 17176H+ and 17176H-, 15544H+ and 15544H-, 14642H+ and 14642H-, VirKH+ and
107 VirKH-, respectively. The resulting DNA fragments were cloned into plasmid pGEMT.
108
109 **Secretion assays and Western blots.**
110 *D. dadantii* strains containing the plasmid to test were grown overnight in LB medium in the
111 presence of the appropriate antibiotic. Culture supernatant containing secreted proteins was
112 separated from cells by centrifugation at 10,000 g for 3 min, and both fractions were loaded onto
113 12% polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were next transferred onto
114 Immobilon P membrane (Merck) and probed with Ni-NTA-HRP.
115

Pathogenicity tests.

Bacteria were grown overnight in LB medium, centrifuged and resuspended at OD_{600} 1 in M63 medium. Potatoes were surface sterilized with 70% ethanol and dried. A hole was made with a pipette tip and 10 μl of bacteria were deposited in the hole which was covered with mineral oil. Potatoes were placed over a wet paper in a tray contained in a plastic bag to maintain moisture. After 48 h at 30°C, the weight of rotten tissue was measured.

Enzymatic assays.

β-glucuronidase assays were performed on toluenized extracts of cells grown to exponential phase using the method of Bardonnet *et al* (42) with *p*-nitrophenyl-β-D-glucuronate as the substrate.

RESULTS

Identification of new Out-secreted proteins

To have a more complete knowledge of the proteins secreted by the *D. dadantii* Out T2SS that could be involved in the pathogenicity process, we searched for candidate genes in recently published transcriptome data (19)(20). We selected genes strongly induced during plant infection and coding for a protein possessing a signal sequence. We retained the genes *Dda3937_01687*, *Dda3937_00585* (thereafter named SvfA and SvfB, respectively) and *Dda3937_00081* (also named *yoaJ*). We also retained VirK, a protein of unknown function with a signal sequence identified among the genes controlled by the transcriptional regulator PecS of many virulence factors (21). Each protein was tagged with a His-tag and its secretion was analyzed in the *D. dadantii* wild type strain and an *outD* mutant in which the Out machinery is not functional. The proteins SvfA, SvfB, YoaJ and VirK were detected in the supernatant of

142 the wild type strain but not of the mutant, demonstrating their secretion by the Out machinery
143 (Fig. 1). Production of these proteins from a gene cloned on a multicopy plasmid may explain
144 why secretion was not total in the wild type strain.
145
146 YoaJ is a PecS-regulated gene (21) and it was found among the most induced genes during
147 *Arabidopsis* infection or culture in the presence of plant extracts (19). It encodes a protein with
148 homology to expansins. These proteins are able to non-enzymatically loosen cell wall cellulose.
149 They are found in all plants where they have a role in cell wall extension and also in many plant
150 pathogenic microorganisms (22). Their role in virulence has been shown in *Ralstonia*
151 *solanacearum*, in *P. atrosepticum*, *P. Brasiliense* and in the plant pathogen *Erwinia*
152 *tracheiphila* (23) (24). The role of *D. dadantii* expansin is probably the same. VirK is a protein
153 of unknown function that has homologues in several plant pathogenic bacteria such as *R.*
154 *solanacearum*, *Agrobacterium tumefaciens, Lonsdalea* and *Xanthomonas*. VirK is controlled
155 by PecS and induced during Arabidopsis infection or culture in the presence of plant extracts
156 (21) (20). No symptom for the *D. dadantii virK* mutant was observed whatever the plant tested
157 (21). The two proteins SvfA and SvfB are studied in the next paragraphs.
158
159 **SvfA and SvfB are virulence factors**
160 *svfA* and *svfB* are among the most induced *D. dadantii* genes during Arabidopsis infection or
161 during culture of the bacteria in the presence of plant extracts (19). They are also strongly
162 expressed during maceration of potato tubers by *D. dianthicola* and *D. solani* (25). The two *D.*
163 *dadantii* proteins share 43% identity and 58% similarity in amino acid composition (Fig. 2).
164 SvfA is 187 amino acid long (165 for the mature form, 17.5 kDa) and SvfB is 198 amino acid
165 long (177 for the mature form, 18.9 kDa). These proteins belong to the DUF 4879 family of
166 proteins. Proteins of this family have no known function. YolA,a protein of the DUF 4879

family showing low homology with SvfB, is among the most highly secreted protein of *Bacillus subtilis* (26). YolA is also present in *B. cereus* and in the insect pathogen *B. thuringiensis*. *B. subtilis* YolA is shorter than SvfA and SvfB, missing the N-terminal more variable part (Fig. 2).

svfA and *svfB* mutants have been constructed and their pathogenicity has been tested on potato. The *svfA* mutant was significantly less aggressive than the wild type strain while the *svfB* mutant was not significantly affected (Fig. 3A). Virulence of the *svfA* mutant could be restored by introduction of a plasmid bearing the wild type *svfA* gene (Fig. 3B). Virulence of the double *svfA svfB* mutant was further reduced showing that the role of SvfB is additive to that of SvfA (Fig. 3A). Thus, genes *Dda3937_01687*and *Dda3937_00585* were named *svfA* and *svfB* for secreted virulence factor A and B.

All our attempts to overproduce the proteins SvfA and SvfB in order to purify them and to study more precisely their function were unsuccessful because their production was toxic to the bacterial cells engineered to overproduce them.

Expression of *svfA* and *svfB*

To try to identify the function of SvfA and SvfB, we analyzed the conditions in which their genes are expressed. We tested the effect of galacturonate and polygalacturonate, two compounds that are inducers of the expression of the main virulence factors, the pectate lyases, and of glucose, which represses it. We also analyzed the effect of mutations in genes controlling several aspects of *D. dadantii* virulence. KdgR represses the pectinase, pectin degradation and *out* genes (27). Its inducer is 2-keto-3-deoxygluconate, a polygalacturonate and galacturonate

192 catabolic derivative. PecS controls genes encoding the pectinases, diverse secreted protein, the
193 Out machinery and proteins involved in resistance to oxidative stress (28). PecT is a regulator
194 of the pectate lyase, motility and exopolysaccharide synthesis genes (29). Pir regulates
195 hyperinduction of pectate lyses in response to plant extracts (30). GacA, the regulator of the
196 two-component regulatory system GacA-GacS, is a global regulator required for disease
197 expression in response to the metabolic status of the bacteria (31). Expression of *svfA* was
198 slightly induced by polygalacturonate but not by galacturonate (Fig. 4A). However, expression
199 of this gene was not modified in a *kdgR* background indicating that induction by
200 polygalacturonate is not mediated by KdgR. Growth in the presence of chicory chunks strongly
201 induced s*vfA* expression as expected from transcriptomic data showing induction in the
202 presence of plant extract. A high concentration of glucose led to a strong induction of *svfA*
203 expression (Fig. 4A). This regulation is mediated by the catabolite repressor protein CRP since
204 a mutation in the *crp* gene derepressed *svfA* expression. Thus, Crp is a repressor of *svfA*.
205 Although it had not been previously identified as a PecS-regulated gene (21), *svf*A expression
206 is increased in a *pecS* background. A *pir* mutation provoked a weak derepression of *svfA*
207 expression. Neither PecT nor GacA significantly regulate *svfA* expression (Fig. 4A).
208
209 Regulation of *svfB* shows some similarity to that of *svfA*: it was not induced by galacturonate,
210 polygalacturonate or regulated by KdgR, it was induced by glucose and repressed by Crp, and
211 it was repressed by PecS (Fig. 4B). However, a few differences can be noted: in contrast to
212 what is observed with *svfA*, no induction by plant pieces was observed for *svfB* and PecT was
213 a repressor of *svfB* expression while Pir did not seem to control it (Fig. 4B).
214
215
216 **Occurrence of the new secreted proteins in other Dickeya species**

Presence of *svfA*, *svfB*, *virK* and *yoaJ* was searched in the genome of all the *Dickeya* type strains, and in a few *Pectobacterium* strains (Table 1). Presence and number of proteins of the DUF 4879 family is variable among *Dickeya* species. The gene *svfA* is present in all strains except *D. zeae*, *D. chrysanthemi*, *D. poaceiphila* and *D. paradisiaca*. The gene *svfB* is present in most species but is absent in *D. chrysanthemi*, *D. poaceiphila* and *D. paradisiaca*, *D. undicola* and *D. aquatica*. A third gene located next to *svfA* and probably resulting from a duplication is found in *D. fangzhongdai* and *D. undicola*. It was named *svfC*. It has 53% homology with *D. dadantii* SvfA and 34% with *D. dadantii* SvfB. SvfC is shorter that SvfA and SvfB (126 amino acid for the mature protein, 13.2 kDa) and has the same size as *B. cereus* YolA (Fig. 2). It possesses a signal sequence, indicating that it could also be secreted by the Out system. Thus, the number of genes of the DUF 4879 family in *Dickeya* strains varies from 0 to 3. Homologues of the *svf* genes can also be found in some *Pectobacterium* strains (Table 1). For example, two copies are present in *P. carotovorum* subsp *carotovorum*. However, even in a given species, the gene may be present or not (presence of a homologue of *svfB* in 10 out of the 23 *P. brasiliense* strains present in the ASAP data bank (https://asap.ahabs.wisc.edu/asap/home.php). Outside *Pectobacteriaceae*, homologues of *svfB* can be found in a few Gammaproteobacteriaceae, i.d. in some *Photorhabdus*, *Luteibacter* and *Pseudoalteromonas* strains. *yoaJ* is present in all *Dickeya* and *Pectobacterium* strains except *D. poaceiphila* and *D. paradisiaca*. *virK* is present in all Dickeya strains except in *D. aquatica* and absent in all *Pectobacterium* strains tested.

We also examined the presence or absence of genes of other non-pectinolytic proteins known to be secreted by a T2SS in *Dickeya* or *Pectobacterium*: *ibpS*, *nipE*, *xynA*, *avrL*/*avrM* (Table 1). IbpS is a metal binding protein that prevents ROS-induced killing of bacteria (17). NipE is a toxin that provoke plant cell death (32). XynA is a xylanase that was identified in a corn strain of *Dickeya zeae* previously named *Erwinia chrysanthemi* (33). AvrL is homologous to the

242 *Xanthomonas campestris* avirulence protein AvrL (15). Two very similar proteins, AvrL and
243 AvrM, are encoded by *D. dadantii* 3937. AvrL was named Svx in *P. atrosepticum* where a role
244 in virulence has been proven (34). However, its function in *Dickeya* has not been studied. IbpS
245 is present in almost all the species, except *D. paradisiaca*. NipE is absent in *D. paradisiaca* and
246 *D. poaceiphila*. Presence of XynA is variable in *Dickeya* strains and it is absent in
247 *Pectobacterium*. A variation in the presence and number of AvrL can be observed in Dickeya
248 strains (Table 1). Thus, the repertoire of T2SS-secreted protein known to be important for
249 virulence is very variable from species to species.
250
251
252 **DISCUSSION**
253 The T2SS of *Dickeya* and *Pectobacterium* is a major virulence factor of these bacteria. The
254 knowledge of the repertoire of secreted proteins is necessary to better understand the precise
255 mechanisms of virulence of these bacteria. These analyses have been undertaken with the model
256 strain *D. dadantii* 3937 and partially with *Pectobacterium atrosepticum* (35)(16). *Dickeya* and
257 *Pectobacterium* are characterized by their ability to degrade pectin and they are identified by
258 this characteristic on the semi selective Crystal Violet Pectate medium. They all secrete
259 enzymes capable of degrading pectin (pectate lyases, polygalacturonases, pectin
260 methylesterases). However, recent works show that other proteins are secreted by the Out T2SS
261 (15)(16). In the present work we used published transcriptome data to identify new potential
262 substrates of the *D. dadantii* T2SS. The most highly induced genes in a transcriptome
263 experiment of *D. dadantii* infecting *A. thaliana* are known virulence genes (*pelI*, *prtA*, *rhiE*,
264 *paeY*, *rhaD*, *ibpS*, etc...) (19). However, in this top list some genes have no known function.
265 The presence of a signal sequence in their product suggested that these proteins could be
266 substrates of the T2SS necessary for the infection process. We showed here that the proteins
267 SvfA, SvfB and YoaJ produced by genes present in the top list of those induced in Arabidopsis

are substrates of the Out T2SS. YoaJ belongs to the family of expansins, proteins that loosen cellulose fibers. Their role as a virulence factor has been shown in *P. brasiliense* and *P. atrosepticum* (23) and it probably has the same function in *D. dadantii* and other *Dickeya* species. No function could be predicted for SvfA and SvfB which belong to the DUF 4879 family of proteins. However, a reduction of virulence of a *svfA* mutant and a *svfA svfB* double mutant on potato could be observed, proving a role of the proteins in the bacterial pathogenicity. Although an additive effect of the mutations was observed, they could not have exactly the same function. The mutants should be tested on various hosts to detect potential differences. It can be supposed that each protein would be more active on one type or one family of plant. Presence of three DUF 4879 proteins in *D. fanghzongdai* could explain its wide host range, from orchid to pear trees. Presence of homologues of SvfA and SvfB in *Photorhabdus* and in *B. thuringiensis* strains, two insect pathogens, indicates that the role of these proteins is not restricted to plant virulence but may participate to a common process of bacterial pathogeny. We also showed here that the PecS-regulated protein VirK is secreted by Out. No role on virulence had been observed for this protein with the chicory leaf model of infection (21). Other models should be tested to find the role of this protein.

Regulation of expression of the *svfA* and *svfB* genes is atypical for a *D. dadantii* gene involved in pathogeny. While expression of most of the virulence factors is induced in the presence of pectin or its derivatives through the repressor KdgR and repressed by glucose, that of *svfA* and *svfB* is opposite: it is activated by glucose and not controlled by KdgR. Expression of *svfA* is induced in the presence of plant tissue. This pattern of regulation has been described for *ibpS*, which is also strongly induced in *A. thaliana* (17). This could correspond to conditions encountered during the early phases of infection: pectin has not yet been degraded and glucose and saccharose are plentiful in plant tissues. *svfA* and *ibpS* could be among the earliest gene to

293 be induced at the onset of infection, before the genes involved in pectin degradation. However,
294 regulation of these genes by PecS and PecT shows that *svfA* and *svfB* are fully integrated in the
295 network of regulators that controls *D. dadantii* virulence.
296
297 This work has extended our knowledge of the Out-dependent secretome of *D. dadantii*, showing
298 that besides pectinases several other proteins are secreted. If the number of pectinolytic
299 enzymes secreted is almost identical in the various *Dickeya* species, the number of additional
300 secreted proteins varies markedly. Among the proteins analyzed (Table 1), *D. paradisiaca* has
301 only one (VirK) while *D. fanghzongdai* has ten. All the intermediate combinations can be found
302 in the various species. There seems to be less variations in the *Pectobacterium* strains surveyed.
303 It is tempting to speculate that the presence/absence of these proteins could influence the host
304 preference of some *Dickeya* species, providing additional virulence factors favorable to infect
305 certain hosts. Works that compare *Dickeya* strains to understand what makes difference in their
306 host range or aggressivity often focus only on the presence of the six known types of secretion
307 systems without analyzing what proteins could be secreted (18)(36)(37). An exhaustive analysis
308 of the secreted proteins would be more informative.
309
310 Is there other T2SS-secreted proteins to be identified in *Dickeya* strains? No specific signal is
311 present on T2SS-secreted proteins that would allow their identification. 2D gels which were
312 used in previous studies performed on *D. dadantii* and *P. atrosepticum* to identify their
313 secretome have a limited sensitivity (15)(34). More sensitive methods such as liquid
314 chromatography-tandem mass spectrometry (LC-MS/MS) can now be used (38). However,
315 they give many false positive results since periplasmic and cytoplasmic proteins are often found
316 in the culture supernatant. The approach we used here allowed the identification of four new
317 secreted proteins. However, all these methods have a drawback. They can only detect proteins

in conditions where they are produced. For instance, the rhamnogalacturonate lyase RhiE could only be detected when the bacteria were cultivated in the presence of rhamnose (39). The genes encoding YoaJ and VirK were not induced in *D. dianthicola* grown on potato (25). Another problem is that a protein may not exist in the strain tested. An analysis of the secretome of several *Dickeya* strains grown in several conditions will be necessary to have a global view of all the additional virulence factors that can be secreted by *Dickeya* species and evaluate their potential role in pathogenicity.

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REFERENCES

1. Ma B, Hibbing ME, Kim H-S, Reedy RM, Yedidia I, Breuer J, Breuer J, Glasner JD, Perna NT, Kelman A, Charkowski AO. Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. Phytopathology. 2007; 97:1150–63.

2. Fujikawa T, Hatomi H, Ota N. Draft genome sequences of seven strains of *Dickeya dadantii*, a quick decline-causing pathogen in fruit trees, isolated in Japan. Microbiology Resource Announcements. 2020; 9:e00609-20.

3. Samson R, Legendre JB, Christen R, Saux MF-L, Achouak W, Gardan L. Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner *et al*. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. International Journal of Systematic and Evolutionary Microbiology. 2005; 55:1415–27.

4. Brady CL, Cleenwerck I, Denman S, Venter SN, Rodríguez-Palenzuela P, Coutinho TA, De Vos P. Proposal to reclassify *Brenneria quercina* (Hildebrand and Schroth 1967) Hauben *et al*. 1999 into a new genus, *Lonsdalea* gen. nov., as *Lonsdalea quercina* comb. nov., descriptions of *Lonsdalea quercina* subsp. *quercina* comb. nov., *Lonsdalea quercina* subsp. *iberica* subsp. nov. and *Lonsdalea quercina* subsp. *britannica* subsp. nov., emendation of the description of the genus *Brenneria*, reclassification of *Dickeya dieffenbachiae* as *Dickeya dadantii* subsp. *dieffenbachiae* comb. nov., and emendation of the description of *Dickeya dadantii*. International Journal of Systematic and Evolutionary Microbiology. 2012; 62:1592–1602.

5. van der Wolf JM, Nijhuis EH, Kowalewska MJ, Saddler GS, Parkinson N, Elphinstone JG, Pritchard L, Toth IK, Lojkowska E, Potrykus M, Waleron M, de Vos P, Cleenwerck I, Pirhonen M, Garlant L, Hélias V, Pothier JF, Pflüger V, Duffy B, Tsror L, Manulis S. *Dickeya solani* sp. nov., a pectinolytic plant-pathogenic bacterium isolated from potato (*Solanum tuberosum*). International Journal of Systematic and Evolutionary Microbiology. 2014; 64:768–74.

6. Tian Y, Zhao Y, Yuan X, Yi J, Fan J, Xu Z, Hu B, De Boer SH, Li X. *Dickeya fangzhongdai* sp. nov., a plant-pathogenic bacterium isolated from pear trees (*Pyrus*

pyrifolia). International Journal of Systematic and Evolutionary Microbiology. 2016; 66:2831–35.

7. Wang X, He S-W, Guo H-B, Han J-G, Thin KK, Gao J, Wang Y, Zhang X-X. *Dickeya oryzae* sp. nov., isolated from the roots of rice. International Journal of Systematic and Evolutionary Microbiology. 2020; 70:4171–78.

8. Parkinson N, DeVos P, Pirhonen M, Elphinstone J. *Dickeya aquatica* sp. nov., isolated from waterways. International Journal of Systematic and Evolutionary Microbiology. 2014; 64:2264–6.

9. Hugouvieux-Cotte-Pattat N, Jacot-des-Combes C, Briolay J. *Dickeya lacustris* sp. nov., a water-living pectinolytic bacterium isolated from lakes in France. International Journal of Systematic and Evolutionary Microbiology. 2019; 69:721–26.

10. Oulghazi S, Pédron J, Cigna J, Lau YY, Moumni M, Van Gijsegem F, Chan K-G, Faure D. *Dickeya undicola* sp. nov., a novel species for pectinolytic isolates from surface waters in Europe and Asia. International Journal of Systematic and Evolutionary Microbiology. 2019; 69:2440–4.

11. Ji J, Hugouvieux-Cotte-Pattat N, Robert-Baudouy J. Molecular cloning of the *outJ* gene involved in pectate lyase secretion by *Erwinia chrysanthemi*. Molecular Microbiology. 1989; 3:285–93.

12. Yang C-H, Gavilanes-Ruiz M, Okinaka Y, Vedel R, Berthuy I, Boccara M, Chen JW-T, Perna NT, Keen NT. *hrp* genes of *Erwinia chrysanthemi* 3937 are important virulence factors. Molecular Plant-Microbe Interactions. 2002; 15:472–480.

386 13. Andro T, Chambost JP, Kotoujansky A, Cattaneo J, Bertheau Y, Barras F, Van Gijsegem
387 F, Coleno A. Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and
388 cellulase. Journal of Bacteriology. 1984; 160:1199–203.

389 14. Hugouvieux-Cotte-Pattat N, Condemine G, Shevchik VE. Bacterial pectate lyases,
390 structural and functional diversity. Environmental Microbiology Reports. 2014; 6:427–
391 40.

392 15. Kazemi-Pour N, Condemine G, Hugouvieux-Cotte-Pattat N. The secretome of the plant
393 pathogenic bacterium *Erwinia chrysanthemi*. Proteomics. 2004; 4:3177–3186.

394 16. Coulthurst SJ, Lilley KS, Hedley PE, Liu H, Toth IK, Salmond GPC. DsbA plays a critical
395 and multifaceted role in the production of secreted virulence factors by the phytopathogen
396 *Erwinia carotovora* subsp. *atroseptica*. Journal of Biological Chemistry. 2008;
397 283:23739–53.

398 17. Liu L, Gueguen-Chaignon V, Gonçalves IR, Rascle C, Rigault M, Dellagi A, Loisel E,
399 Poussereau N, Rodrigue A, Terradot L, Condemine G. A secreted metal-binding protein
400 protects necrotrophic phytopathogens from reactive oxygen species. Nature
401 Communications. 2019; 10:4853.

402 18. Potrykus M, Golanowska M, Hugouvieux-Cotte-Pattat N, Lojkowska E. Regulators
403 involved in *Dickeya solani* virulence, genetic conservation, and functional variability.
404 Molecular Plant-Microbe Interactions 2014; 27:700–11.

405 19. Pédron J, Chapelle E, Alunni B, Van Gijsegem F. Transcriptome analysis of the *Dickeya*
406 *dadantii PecS* regulon during the early stages of interaction with *Arabidopsis thaliana*.
407 Molecular Plant Pathology. 2018; 19:647–63.

20. Jiang X, Zghidi-Abouzid O, Oger-Desfeux C, Hommais F, Greliche N, Muskhelishvili G, Nasser W, Reverchon S. Global transcriptional response of *Dickeya dadantii* to environmental stimuli relevant to the plant infection. Environmental Microbiology. 2016; 18:3651–72.

21. Hommais F, Oger-Desfeux C, Van Gijsegem F, Castang S, Ligori S, Expert D, Nasser W, Reverchon S. PecS Is a global regulator of the symptomatic phase in the phytopathogenic bacterium *Erwinia chrysanthemi* 3937. *Journal of Bacteriology*. 2008; 190:7508–22.

22. Tancos MA, Lowe-Power TM, Peritore-Galve FC, Tran TM, Allen C, Smart CD. Plant-like bacterial expansins play contrasting roles in two tomato vascular pathogens. Molecular Plant Pathology. 2018; 19:1210–21.

23. Narváez-Barragán DA, Tovar-Herrera OE, Torres M, Rodríguez M, Humphris S, Toth IK, Segovia L, Serrano M, Martínez-Anaya C. Expansin-like Exl1 from *Pectobacterium* is a virulence factor required for host infection, and induces a defence plant response involving ROS, and jasmonate, ethylene and salicylic acid signalling pathways in *Arabidopsis thaliana*. Scientific Reports. 2020; 10:7747.

24. Rocha J, Shapiro LR, Kolter R. A horizontally acquired expansin gene increases virulence of the emerging plant pathogen *Erwinia tracheiphila*. Scientific Reports. 2020; 10:21743.

25. Raoul des Essarts Y, Pédron J, Blin P, Van Dijk E, Faure D, Van Gijsegem F. Common and distinctive adaptive traits expressed in *Dickeya dianthicola* and *Dickeya solani* pathogens when exploiting potato plant host. Environmental Microbiology. 2019. 21:1004–18.

26. Tjalsma H, Antelmann H, Jongbloed JDH, Braun PG, Darmon E, Dorenbos R, Dubois J-YF, Westers H, Zanen G, Quax WJ, Kuipers OP, Bron S, Hecker M, van Dijl JM.

Proteomics of protein secretion by *Bacillus subtilis*: Separating the "secrets" of the secretome. Microbiology and Molecular Biology Reviews. 2004; 68:207–33.

27. Condemine G, Robert-Baudouy J. Tn*5* insertion in *kdgR*, a regulatory gene of the polygalacturonate pathway in *Erwinia chrysanthemi*. FEMS Microbiology Letters. 1987; 42:39–46.

28. Reverchon S, Nasser W, Robert-Baudouy J. *pecS*: a locus controlling pectinase, cellulase and blue pigment production in *Erwinia chrysanthemi*. Molecular Microbiology. 1994; 11:1127–39.

29. Surgey N, Robert-Baudouy J, Condemine G. The *Erwinia chrysanthemi pecT* gene regulates pectinase gene expression. Journal of Bacteriology. 1996; 178:1593–99.

30. Nomura K, Nasser W, Kawagishi H, Tsuyumu S. The *pir* gene of *Erwinia chrysanthemi* EC16 regulates hyperinduction of pectate lyase virulence genes in response to plant signals. Proceedings of the National Academy of Sciences. 1998; 95:14034–39.

31. Lebeau A, Reverchon S, Gaubert S, Kraepiel Y, Simond-Côte E, Nasser W, Van Gijsegem F. The GacA global regulator is required for the appropriate expression of *Erwinia chrysanthemi* 3937 pathogenicity genes during plant infection. Environmental Microbiology. 2008; 10:545–59.

32. Laasik E, Põllumaa L, Pasanen M, Mattinen L, Pirhonen M, Mäe A. Expression of *nipP.w* of *Pectobacterium wasabiae* is dependent on functional *flgKL* flagellar genes. Microbiology. 2014; 160:179–86.

33. Keen NT, Boyd C, Henrissat B. Cloning and characterization of a xylanase gene from corn strains of *Erwinia chrysanthemi*. Molecular Plant-Microbe Interactions. 1996; 9:651-7.

453 34. Corbett M, Virtue S, Bell K, Birch P, Burr T, Hyman L, Lilley K, Poock S, Toth I,
454 Salmond G. Identification of a new quorum-sensing-controlled virulence factor in *Erwinia*
455 *carotovora* subsp. *atroseptica* secreted via the type II targeting pathway. Molecular Plant-
456 Microbe Interactions. 2005; 18:334–42.

457 35. Mattinen L, Nissinen R, Riipi T, Kalkkinen N, Pirhonen M. Host-extract induced changes
458 in the secretome of the plant pathogenic bacterium *Pectobacterium atrosepticum*.
459 Proteomics. 2007; 7:3527–37.

460 36. Ališ Š, Pédron J, Dreo T, Van Gijsegem F. Genomic characterisation of the new *Dickeya*
461 *fangzhongdai* species regrouping plant pathogens and environmental isolates. BMC
462 Genomics. 2019; 20:34.

463 37. Xu P, Wang H, Qin C, Li Z, Lin C, Liu W, Miao W. Analysis of the taxonomy and
464 pathogenic factors of *Pectobacterium aroidearum* L6 using whole-genome sequencing
465 and comparative genomics. Frontiers in Microbiology. 2021; *a*12:679102.

466 38. Burtnick MN, Brett PJ, DeShazer D. Proteomic Analysis of the *Burkholderia*
467 *pseudomallei* type II secretome reveals hydrolytic enzymes, novel proteins, and the
468 deubiquitinase TssM. Infection and Immunity. 2014; 82:3214–26.

469 39. Laatu M, Condemine G. Rhamnogalacturonate lyase RhiE is secreted by the Out system
470 in *Erwinia chrysanthemi*. Journal of Bacteriology. 2003; 185:1642–9.

471 40. Resibois A, Colet M, Faelen M, Schoonejans E, Toussaint A. ϕEC2, a new generalized
472 transducing phage of *Erwinia chrysanthemi*. Virology. 1984; 137:102–12.

473 41. Roeder DL, Collmer A. Marker-exchange mutagenesis of a pectate lyase isozyme gene in
474 *Erwinia chrysanthemi*. Journal of Bacteriology. 1985; 164:51–6.

475 42. Bardonnet N, Blanco C. 'uidA-antibiotic-resistance cassettes for insertion mutagenesis,
476 gene fusions and genetic constructions. FEMS Microbiology Letters. 1992; 93:243–7.

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Fig 1: Identification of new secreted proteins by *D. dadantii*. Wild-type and *outD* mutant strains containing plasmid bearing the gene of the selected protein were grown overnight in LB medium. The supernatant (S) and cellular (C) fractions were separated by SDS-PAGE. After blotting, the proteins were detected with Ni-NTA-HRP.

Fig. 2: Alignment of Svf proteins. The sequences of *D. dadantii* SvfA (Dda3937_01687) and SvfB (Dda3937_00585), *D. fanghzongdai* SvfC (CVE23_15565), *B. cereus* WP-193674364.1 and *Photorhabdus asymbiotica* CAQ86327.1, without their signal sequence, were aligned with Clustal omega. Identical residues are indicated by a star and chemically equivalent residues by a double dot.

Fig. 3. Virulence of *svfA* and *svfB* mutants. A. Potatoes were infected with the wild type strain, and the *svfA*, the *svfB* and the *svfA svfB* mutants. Rotten tissue was weighed after 48 h. B. Complementation of the *svfA* mutation. Potatoes were infected with the wild type strain, the *svfA* mutant containing the empty plasmid pBBRGm and the *svfA* mutant containing the pBBRGm plasmid bearing *svfA*. Rotten tissue was weighed after 48h. Statistical tests were performed using the Wilcoxon- Mann-Whitney test. The *p*-value were compared with an alpha risk of 4%. $p < 0.001$=***, $p < 0.005$=**, $p < 0.01$=*.

Fig 4 : Expression of *svfA* and *svfB* in various growth conditions. A. The *D. dadantii* strain A6418 containing the *svfA-uidA* fusion and its derivative strains containing an additional regulatory mutation were grown in M63 medium in the presence of the indicated compounds (Y = glycerol, G = glucose, A = galacturonate, PGA = polygalacturonate, E = chicory chunks).

Strains with additional mutations were grown with glycerol as a carbon source except the *crp* mutant that was grown with 0.2% glucose. ß-glucuronidase activity was measured with *p*-nitrophenyl-ß-D-glucuronate. B. Similar experiment for the *D. dadantii* strain A6467 containing the *svfB-uidA* fusion and its derivative strains containing an additional regulatory mutation. Activities are expressed in µmoles of *p*-nitrophenol produced per minute and per milligram of bacterial dry weight $\pm$ standard deviation. Data are expressed as the mean (n = 6) from six independent experiments. Statistical tests were performed using the Wilcoxon- Mann-Whitney test. The *p*-value were compared with an alpha risk of 4%. $p < 0.001$=***, $p < 0.005$=**, $p < 0.01$=*.

Table 1: Presence of Out-secreted proteins in various *Dickeya* and *Pectobacterium* strains.

Table 2: Oligonucleotides and strains used in this study.

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522 Table 1 : Presence of Out-secreted proteins in various *Dickeya* and *Pectobacterium* strains.
523

Strain	*SvfA*	*SvfB*	*SvfC*	*YoaJ*	*VirK*	*IbpS*	*NipE*	*XynA*	*AvrL*
D. dadantii	1	1	0	1	1	1	1	1	2
D. diffenbachiae	1	1	0	1	1	1	1	0	2
D. fangzhongdai	1	1	1	1	1	1	1	1	2
D. solani	1	1	0	1	1	1	1	1	2
D. zeae	0	1	0	1	1	1	1	1	1
D. oryzae	1	1	0	1	1	1	1	1	0
D. dianthicola	0	1	0	1	1	1	1	0	1
D. undicola	1	0	1	1	1	1	1	0	1
D. lacustris	1	0	0	0	0	1	1	0	2
D. aquatica	1	0	0	1	0	1	1	0	2
D. chrysanthemi	0	0	0	1	1	1	1	0	1
D. paradisiaca	0	0	0	0	1	0	0	0	0
D. poaceiphila	0	0	0	0	1	1	0	1	0
P. atrosepticum	0	0	0	1	0	1	1	0	1
P. carotovorum	1	1	0	1	0	1	1	0	1
P. parmentieri	0	0	0	1	0	1	1	0	1
P. polaris	0	0	0	1	0	1	1	0	1

524
525 The strains used in this study are *D. dadantii* 3937, *D. aquatica* 174/2, *D. chrysanthemi*
526 ATCC 11663, *D. dadantii* subsp *dieffenbachiae* NCPPB 2976, *D. dianthicola* NCPPB 453, *D.*
527 *fanghzongdai* DSM 101947, *D. lacustris* S29, *D. oryzae* ZYY5, *D. paradisiaca* ATCC 33242,
528 *D. poaceiphila* NCPPB 569, *D. solani* IPO 2222, *D. undicola* 2B12, *D. zeae* NCPPB 2538, *P.*
529 *atrosepticum* ATCC 33260, *P. carotovorum* subsp. *carotovorum* ATCC 15713, *P.*
530 *parmentieri* RNS08.42.1A and *P. polaris* NIBIO 1006. The presence and number of proteins
531 detected by search of the corresponding gene in the genome in each strain is indicated.
532

Table 2: Oligonucleotides and strains used in this study

Oligonucleotides

17176H+	CCTCCTGAGATTAGAGAGAG
17176A	CGCGCCGGTGTTTTTCTTGCG
17176H-	GGTTAGTGATGGTGATGGTGATGCTGAATATTGAGCGACGTGC
17176XbaF	GAACAGGATGTGTCCTCTTCTAGACACAAAGCGCTGCGTGCG
17176XbaR	CGCACGCAGCGCTTTGTGTCTAGAAGAGGACACATCCTGTTC
15544L2+	CTAAGAATCAGTCAGTTTGCG
15544L2-	AGGCAGATAACGCTACTCGCC
15544H+	ATTTATACTCGCCACCGATGC
15544XmaF	GCCTTGCGAGCCCCCGCTCCCGGGCTGTCTCGGGTTACCGTG
15544XmaR	CACGGTAACCCGAGACAGCCCGGGAGCGGGGGCTCGCAAGGC
15544H-	GGTTAGTGATGGTGATGGTGATGTTTAATATAGGTCTGTGTGAAC
14642H+	GGTGAGGAATAATTCTGGCC
14642H-	GGTTAGTGATGGTGATGGTGATGAGGCAGTTGTACTTTTCCAG
VirKH+	TGCCGTATGTGATAGTCACG
VirKH-	CCTTAGTGATGGTGATGGTGATGTTGCTTGAAGAAGCGGATATC

Dickeya dadantii strains

3937	Wild type	Laboratory collection
A5653	*outD* ::CmR	Laboratory collection
A6417	*svfB* ::CmR	This work
A6418	*svfA* ::*uidA*-kanR	This work
A3838	*kdgR* ::Mu-CmR	(19)
A3845	*pecS*::Mu-CmR	(28)
A3846	*pecT*::CmR	(29)
A3849	*pir*::CmR	Laboratory collection
A4237	*gacA*::CmR	(31)
A6434	*kdgR*::Mu-CmR *svfA*::*uidA*-kanR	This work
A6435	*pecS*::Mu-CmR *svfA* :*uidA*-kanR	This work
A6436	*pecT*::CmR *svfA*::*uidA*-kanR	This work
A6436	*pir*::CmR *svfA*::*uidA*-kanR	This work

A6437	*gac*A::CmR *svfA*::*uidA*-kanR	This work
A6467	*svfB*::*uidA*-kanR	This work
A6469	*kdgR*::Mu-CmR *svfB*::*uidA*-kanR	This work
A6470	*pecS*::Mu-CmR *svfB*::*uidA*-kanR	This work
A6471	*pecT*::CmR *svfB*::*uidA*-kanR	This work
A6472	*pir*::CmR *svfB*::*uidA*-kanR	This work
A6473	*gacA*::CmR *svfB*::*uidA*-kanR	This work

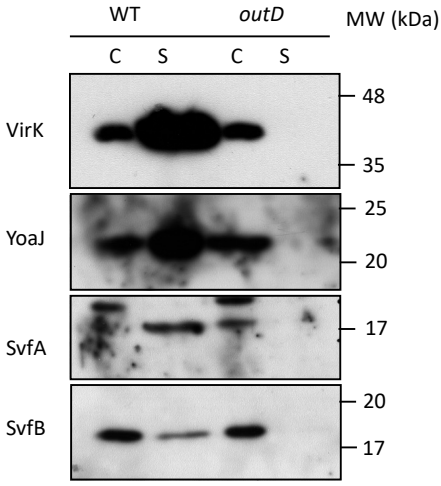


Fig. 1

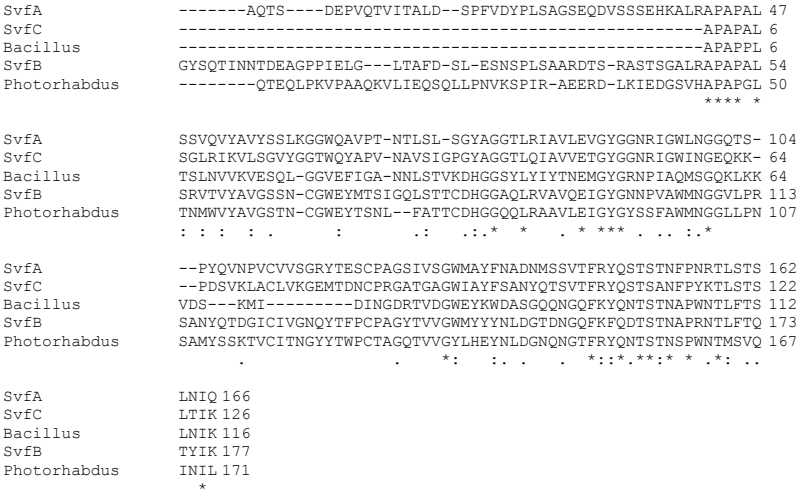
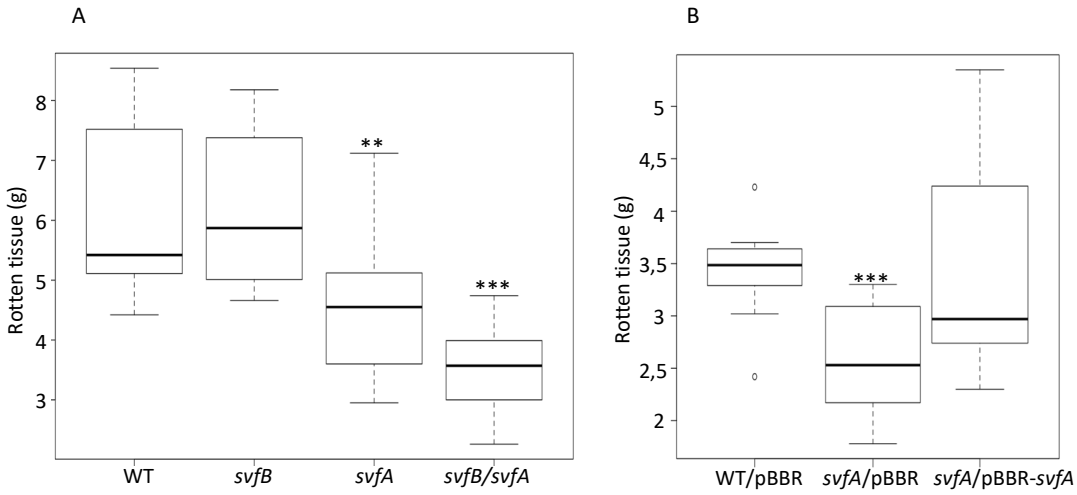


Fig . 2

595



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597 Fig. 3

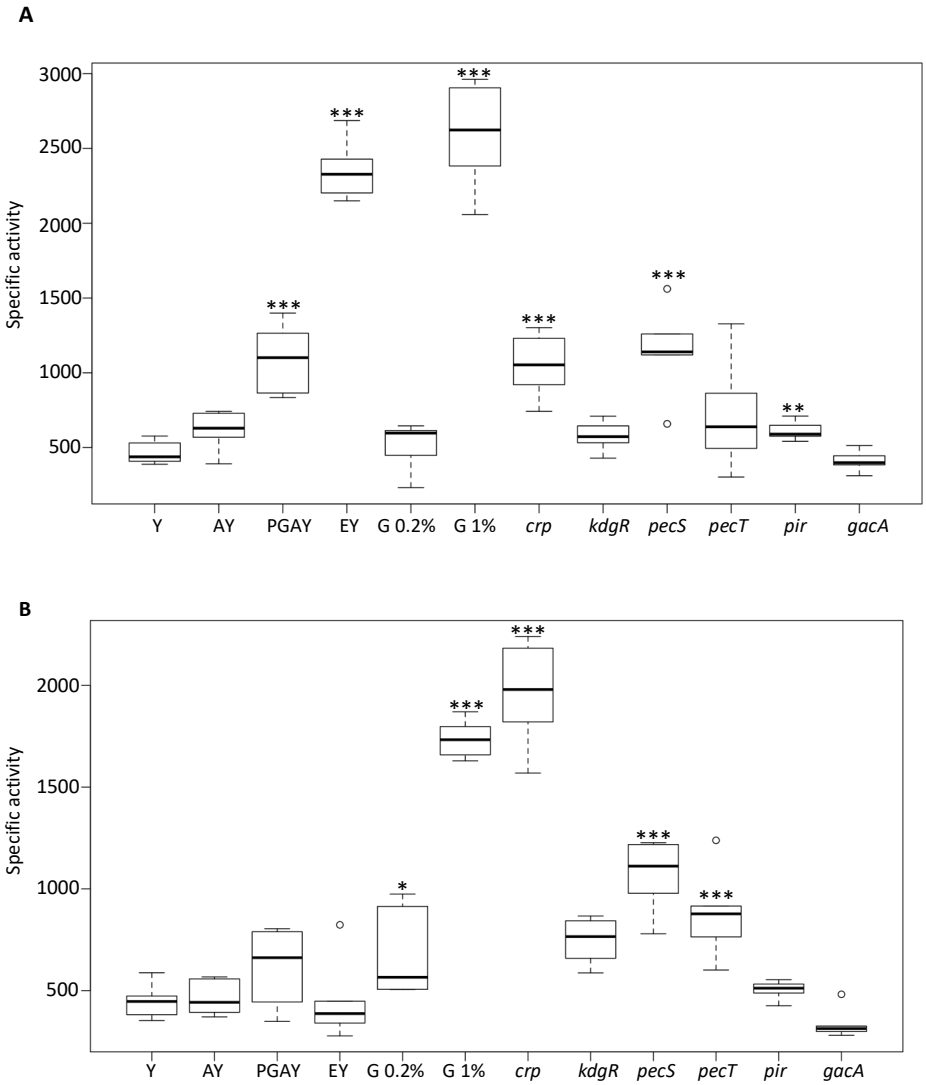

Fig. 4