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1 **Full Title:**

2 **Genome wide identification of bacterial genes required for plant**
3 **infection by Tn-seq**

4

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9

10 **Short title:** *Dickeya dadantii* virulence genes in chicory

11

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15

16 **ABSTRACT**

17 Soft rot enterobacteria (*Dickeya* and *Pectobacterium*) are major pathogens that cause diseases

18 on plants of agricultural importance such as potato and ornamentals. Long term studies to

19 identify virulence factors of these bacteria focused mostly on plant cell wall degrading

20 enzymes secreted by the type II secretion system and the regulation of their expression. To

21 identify new virulence factors we performed a Tn-seq genome-wide screen of a transposon

22 mutant library during chicory infection followed by high-throughput sequencing. This

23 allowed the detection of mutants with reduced but also increased fitness in the plant.

24 Virulence factors identified differed from those previously known since diffusible ones

25 (secreted enzymes, siderophores or metabolites) were not detected by this screen. In addition
26 to genes encoding proteins of unknown function that could be new virulence factors, others
27 could be assigned to known biological functions. The central role of the FlhDC regulatory
28 cascade in the control of virulence was highlighted with the identification of new members of
29 this pathway. Scarcity of the plant in certain amino acids and nucleic acids required presence
30 of the corresponding biosynthetic genes in the bacteria. Their products could be targets for
31 the development of antibacterial compounds. Among the genes required for full development
32 in chicory we also identified six genes involved in the glycosylation of the flagellin FliC,
33 glycosylation, which in other plant pathogenic bacteria contributes to virulence.

34

35 **Author summary**

36 Identification of virulence factors of plant pathogenic bacteria has relied on the test of
37 individual mutants on plants, a time-consuming method. New methods like transcriptomic or
38 proteomic can now be used but they only allow the identification of genes induced during the
39 infection process and non-induced genes may be missed. Tn-seq is a very powerful method to
40 identify genes required for bacterial growth in their host. We used for the first time this
41 method in a plant pathogenic bacteria to identify genes required for the multiplication of
42 *Dickeya dadantii* in chicory. We identified about 100 genes with decreased or increased
43 fitness in the plant. Most of them had no previously described role in bacterial virulence. We
44 unveiled important metabolic genes and regulators of motility and virulence. We showed that
45 *D. dadantii* flagellin is glycosylated and that this modification confers fitness to the bacteria
46 during plant infection. Our work opens the way to the use of Tn-seq with bacterial
47 phytopathogens. Assay by this method of large collections of environmental pathogenic
48 strains now available will allow an easy and rapid identification of new virulence factors.

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52 **Introduction**

53 *Dickeya* are broad-host range phytopathogenic bacteria belonging to the
54 Pectobacteriaceae family [1] that provoke the soft rot disease on many plant species. They
55 are the cause of important losses on economically important crops such as potato, chicory and
56 ornamentals. Identification and studies on the virulence factors of these bacteria have been
57 performed mostly on the model strain *D. dadantii* 3937 and focused mainly on three
58 domains/aspects, known to be important for disease development: plant cell wall degrading
59 enzymes, the type III secretion system and iron metabolism [2]. Secretion of plant cell wall
60 degrading enzymes has long ago been identified as the bacteria main virulence factor. Many
61 studies focused on the identification and characterization of these secreted enzymes, mostly
62 pectinases [3], of the regulators controlling their production (*kdgR*, *pecS*, *pecT*, *hns*, *gacA*),
63 [4-8] of the genes whose expression is coregulated with that of the secreted enzyme genes [9,
64 10], and of the mechanism of their secretion by the type II secretion system [11]. Although of
65 a lesser importance for *Dickeya* virulence, the same type of approach has been used to
66 identify type III secretion system regulators and effectors [12] [13] [14]. Moreover,
67 struggling for iron within the plant is strong. *D. dadantii* acquires this metal through
68 production of two siderophores, chrysobactin and achromobactin [15] [16] [17]. Omics
69 approaches have also been used to identify genes induced during plant infection [18] [19]
70 [20]. These studies now provide a clearer picture on a complex network of factors required
71 for *D. dadantii* virulence [2, 21]. However, these approaches may have missed some
72 important factors not targeted by these analyses. More global screens need to be performed to
73 identify these factors. Libraries of transposon-induced mutants were tested on plants to find
74 mutants showing reduced virulence with *Pectobacterium carotovorum* and *atrosepticum*, two

75 other soft rot enterobacteria [22-24]. These studies identified auxotrophs, mutants defective
76 in production or secretion of exoenzymes and in motility. Other mutants with a more
77 complex phenotype were not characterized at this time. Moreover, the number of tested
78 mutants was limited by the necessity to test individually each mutant on plant. This type of
79 work has never been performed on *Dickeya* strains. To have a more complete view of the
80 genes required for the virulence of *Dickeya*, we used a high-throughput sequencing of a
81 saturated transposon library (Tn-seq) to screen tens of thousands random insertion mutants of
82 *D. dadantii* in laboratory medium and during infection of chicory. Tn-Seq involves creating
83 large transposon libraries, growing the mutants in a control and a selective condition,
84 sequencing the transposon insertion sites with next-generation sequencing, mapping sequence
85 reads to a reference genome and comparing the number of read in each gene in the two
86 conditions. Tn-seq has been extensively used to uncover essential genes required for mouse
87 colonization by human pathogens *Vibrio cholerae* [25], *Pseudomonas aeruginosa* [26] and
88 *Streptococcus pneumoniae* [27] or plant root colonization by *Pseudomonas simiae* [28] or
89 multiplication of *Pantoea stewartii* in corn xylem [29]. This latter bacteria relies on the
90 massive production of exopolysaccharides to block water transport and cause wilting. Thus,
91 Tn-seq is a very powerful method to identify genes required for bacterial growth in their host.
92 By applying this technique to screen a *D. dadantii* mutant library in chicory, we identified
93 metabolic pathways and bacterial genes required by a necrotrophic bacteria for growth *in*
94 *planta*. Among them, we found a cluster of genes required for flagellin glycosylation, a
95 modification known to be important for several plant pathogenic bacteria virulence.

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97

98 **Results and discussion**

99

100 **Characterization of *D. dadantii* 3937 *Himar1* transposon library**

101 Many tools are available to perform Tn-seq [30]. In order to perform a Tn-seq experiment
102 with *D. dadantii* 3937, we used a *Himar9* mariner transposon derivative carrying MmeI
103 restriction sites in the inverted repeats (IR) and a kanamycin resistance cassette between the
104 IRs [31]. We carried out a biparental mating between *E. coli* and *D. dadantii* on M63 agar
105 medium without carbon source and amino acids. We obtained approximately 300 000
106 colonies that were pooled. Subsequent DNA sequencing (see below) showed the presence of
107 transposon insertions in amino acid, vitamin, purine or pyrimidine biosynthesis pathways,
108 demonstrating that mating on M63 minimal medium does not prevent the obtention of
109 auxotroph mutants. To identify essential genes, mutants were grown in LB medium for
110 several generations. Two DNA libraries were prepared from two cultures and subjected to
111 high-throughput sequencing. The mariner transposon inserts into TA dinucleotides. The TPP
112 software [32] was used to determine the number of reads at each TA site for each biological
113 replicate. *D. dadantii* genome has 171,791 TA sites that can be targeted by the *Himar9*
114 transposase. Pairs of biological replicates were compared. 37,794 and 48,101 unique
115 insertions in TAs were detected in each sample, which corresponds to 22 and 28% density of
116 insertion respectively (Table 1). The average number of reads per TA is 88 and 75,
117 respectively. The results were reproducible with a Pearson correlation coefficient of 72%
118 (Fig. S1) The location of the unique insertions showed an even distribution around the
119 chromosome (Fig. 1A). For each gene, we calculated a log₂ fold change (FC) corresponding
120 to a ratio between the measured number of reads and the expected number of reads. The
121 density plot (Fig. 1B) indicates that essential and non-essential genes are easily
122 distinguishable, confirming the good quality of our Tn-seq libraries.

123 Then, gene essentiality of the Tn-seq input libraries was determined by using the TRANSIT
124 software [32]. We decided to use the Hidden Markov Model (HMM) which predicts

125 essentiality and non-essentiality for individual insertion sites since it has been shown to give
126 good prediction in datasets with density as low as 20% [32]. The HMM analysis led to the
127 identification of 665 genes essential for growth in LB (ES), representing 14% of the genes of
128 *D. dadantii* 3937, a number in the range of those found for this type of analysis with bacteria.
129 The transposon we used does not allow us to discriminate between the direct effect of the
130 insertion or a polar effect on downstream genes. Goodall et al [33] have shown that this
131 overestimates the number of essential genes. Thus 665 must be considered has an upper limit
132 of the number of essential genes.

133 552 genes were categorized as Growth Defect genes (GD, i.e. mutations in these genes lead
134 to loss of fitness), 125 as growth advantage genes (GA, i.e mutations in these genes lead to
135 gain of fitness) and 3320 as non-essential genes (NE) (Table S5 and Fig. 1B).

136

137 **TABLE 1** Tn-Seq analysis of *Dickeya dadantii* 3937

Mutant pool	Total no. of reads	No. of reads containing Tn end	No. of reads normalized^a	No. of mapped reads to unique TA sites	No. of mapped reads to unique TA sites after LOESS correction	Density (%)^b	Mean read count per TA^c
LB #1	23,152,186	22,647,343	18,748,028	13,166,770 (70 %)	12,904,900 (69 %)	28 %	75
LB #2	30,105,412	27,963,154	18,748,028	15,535,291 (83 %)	15,195,582 (81 %)	22 %	88
Chicory #1	18,925,029	18,748,028	18,748,028	17,535,146 (94 %)	14,906,888 (79 %)	24 %	87
Chicory #2	27,607,717	26,555,297	18,748,028	17,477,706 (93 %)	16,955,724 (90 %)	23 %	99

138

139 ^a The number of reads containing the sequence of a Tn end were normalized for each sample according to the Chicory #1140 ^b *Dickeya dadantii* 3937 genome has 171,791 TA sites. The density is the % of TAs for which mapped reads has been assigned by the TPP software.141 ^c The mean value of mapped reads per TA

142

143 **Genes necessary for chicory leaf maceration.** We used chicory leaf infection as a model to
144 identify *D. dadantii* genes required for growth in plant tissues. Biological duplicates were
145 performed to insure the reproducibility of the results. Each chicory was inoculated with 10^7
146 bacteria from the mutant pool and after 2 days more than 10^{10} bacteria were collected from
147 the rotten tissue. Sequencing transposon insertion sites in these bacteria followed by the TPP
148 analysis indicated a density of unique insertion in TAs comparable to that of the input
149 datasets (23-24%). Surprisingly, the results were more highly reproducible than in LB with a
150 very high Pearson correlation coefficient of 98% (Fig. S1).

151 In order to test the statistical significance of the identified genes conferring to *D. dadantii* a
152 loss or a gain of fitness *in planta*, we performed the RESAMPLING (permutation test)
153 analysis of the TRANSIT software. The RESAMPLING method is a variation of the classical
154 permutation test in statistics that sums the reads at all TA sites for each gene in each
155 condition. It then calculates the difference of the sum of read-counts between the input (LB)
156 and output (chicory) datasets. The advantage of this statistical method is to attribute for each
157 gene an adjusted p-value (q-value). Genes with a significant difference between total read-
158 counts in LB and chicory achieve a q-value ≤ 0.05 . The method also calculates a \log_2 fold-
159 change (\log_2 FC) for each gene based on the ratio of the sum of read counts in the output
160 datasets (chicory) versus the sum of read counts in the input (LB) datasets [32]. Applied to
161 our Tn-seq datasets and selecting only genes achieving a FDR adjusted p-value (q-value) \leq
162 0.05, we identified 122 genes out of 4666 required for fitness *in planta*, as shown with the
163 volcano plot of RESAMPLING results comparing replicates grown in LB versus *in planta*
164 (Fig. S2). For these 122 genes, we applied an additional cutoff by removing 20 genes with a
165 mean read count in LB < 5 (less than 5 reads in average / TA). These genes were categorized
166 as ES or GD in LB. We also removed from the analysis 6 genes with a \log_2 FC comprised
167 between -2 and 2. By applying all these criteria, we retained only 96 genes for a further

168 analysis (Table 2). 92 of them were identified as GD genes in the chicory ($\log_2FC \leq 2$), the 4
169 left as GA genes in the chicory ($\log_2FC \geq 2$). A possible polar effect for genes being part of an
170 operon is analysed in Table 2: if a GD gene is upstream of another GD gene in the same
171 operon, a polar effect of insertions in the first gene on the second one cannot be excluded.
172 Some of these genes, in bold in Table 2, were already known to play a role in *D. dadantii*
173 virulence, confirming the validity of the Tn-seq approach. Using the Kyoto Encyclopedia of
174 Genes and Genomes (KEGG) [34], we discovered that certain metabolic pathways and
175 biological functions are very important for growth in chicory (Table S4). We highlight some
176 of them in the next sections of the article.

177

Locus ^a	Gene ^a	Function	HMM State in LB ^b	RESAMPLING					In operon ^e	genes in operon (state) ^b	
				No. of TAs ^c	Mean reads ^d		log ₂ FC ^c	q-value ^f			
				LB	Chicory	ΔSum					
Dda3937_00335	<i>glpD</i>	glycerol-3-phosphate dehydrogenase	GD	33	650	0	-11,706	-12.56	0.00	N	
Dda3937_03379	<i>purL</i>	phosphoribosylformyl-glycineamide synthetase	NE	73	378	0	-21,944	-11.91	0.00	N	
Dda3937_03564	<i>opgG</i>	Glucans biosynthesis protein G precursor	GA	40	1976	1	-90,843	-11.41	0.00	Y	<i>opgG (-11.41) opgH (-9.79)</i>
Dda3937_00244	<i>purH</i>	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	NE	37	145	0	-2,896	-11.25	0.00	Y	<i>purD (-1.66) purH (-11.25)</i>
Dda3937_00432	<i>hflK</i>	FtsH protease regulator	GD	28	339	0	-4,060	-11.12	0.03	Y	<i>hflK (-11.12) hflC (+0.06) yjeT (-1.38)</i>
Dda3937_02515	<i>purM</i>	phosphoribosylaminoimidazole synthetase	NE	21	344	0	-6,188	-10.57	0.00	Y	<i>purM (-10.57) purN (0)</i>
Dda3937_02627		4-hydroxythreonine-4-phosphate dehydrogenase	NE	26	129	0	-2,065	-10.06	0.00	Y	<i>Dda3937_02627 (-10.06) Dda3937_02626 (-3.77)</i>
Dda3937_00004	<i>guaB</i>	IMP dehydrogenase	NE	33	151	0	-3,915	-9.97	0.00	N	
Dda3937_03563	<i>opgH</i>	Glucans biosynthesis glucosyltransferase H	GA	62	1409	2	-90,073	-9.79	0.00	Y	<i>opgG (-11.41) opgH (-9.79)</i>
Dda3937_01284	<i>pyrB</i>	aspartate carbamoyltransferase	NE	17	159	0	-1,910	-9.68	0.00	Y	<i>pyrB (-9.68) pyrI (+1.33)</i>
Dda3937_03924	<i>rffG</i>	dTDP-glucose 4,6-dehydratase	NE	23	317	1	-3,167	-9.38	0.02	Y	<i>rffG (-9.38) rffH (-3.49) rfbC (-0.53) rfbD (-0.91)</i>
Dda3937_01389	<i>carB</i>	carbamoyl-phosphate synthase large subunit	NE	48	249	0	-7,967	-9.23	0.00	N	
Dda3937_03299	<i>acrA</i>	MexE family multidrug efflux RND transporter periplasmic adaptor subunit	NE	34	196	0	-5,860	-9.03	0.00	Y	<i>acrA (-9.03) acrB (-8.9)</i>
Dda3937_03300	<i>acrB</i>	multidrug efflux system protein	NE	89	422	1	-31,986	-8.90	0.00	Y	<i>acrA (-9.03) acrB (-8.9)</i>
Dda3937_03258	<i>pyrE</i>	orotate phosphoribosyltransferase	NE	14	175	0	-2,788	-8.81	0.00	N	
Dda3937_02336	<i>nlpI</i>	lipoprotein	GD	33	27	0	-601,000	-8.69	0.00	N	
Dda3937_02506	<i>nlpB (bamC)</i>	outer membrane protein assembly factor BamC	NE	20	47	0	-841,000	-8.69	0.00	Y	<i>dapA (+2.02) bamC (-8.69)</i>
Dda3937_04018	<i>pta</i>	phosphate acetyltransferase	GD	36	579	2	-10,400	-8.59	0.02	N	
Dda3937_03554	<i>pyrC</i>	dihydro-orotase	NE	25	343	1	-7,534	-8.44	0.00	N	
Dda3937_04573	<i>lpxM</i>	acyl (myristate) transferase	NE	33	63	0	-1,764	-8.31	0.00	N	
Dda3937_01116	<i>glnG</i>	Nitrogen regulation protein NR(I), Two-component system	NE	26	39	0	-629,000	-8.22	0.00	Y	<i>glnL (-0.2) glnG (-8.22)</i>
Dda3937_02099	<i>purF</i>	amidophosphoribosyltransferase	NE	32	107	0	-2,779	-8.19	0.00	Y	<i>purF (-8.19) cvpA (-1.92)</i>
Dda3937_04019	<i>ackA</i>	acetate kinase A and propionate kinase 2	NE	29	45	0	-1,063	-8.16	0.00	Y	<i>Dda3937_04020 (-2.48) ackA (-8.16)</i>
Dda3937_02189	<i>yejM</i>	Membrane-anchored periplasmic protein, alkaline phosphatase superfamily	GA	34	4160	15	-99,478	-8.08	0.00	Y	<i>yejL (0) yejM (-8.08)</i>
Dda3937_01390	<i>carA</i>	carbamoyl-phosphate synthase small subunit	NE	21	69	0	-956,000	-8.05	0.00	N	
Dda3937_01426	<i>ptsI</i>	Phosphoenolpyruvate-protein phosphotransferase of PTS system	NE	33	45	0	-1,176	-7.85	0.00	Y	<i>crr (-2.66) ptsI (-7.85) ptsH (0)</i>
Dda3937_00161	<i>cysQ</i>	3'(2'),5'-bisphosphate nucleotidase	NE	16	44	0	-434,000	-7.81	0.02	N	
Dda3937_00210	<i>cysI</i>	sulfite reductase beta subunit	NE	40	252	1	-7,515	-7.65	0.00	Y	<i>cysH (-8.93) cysI (-7.65) cysJ (-6.25)</i>
Dda3937_04075	<i>lysR</i>	LysR family transcriptional regulator	NE	13	2385	13	-18,976	-7.51	0.00	N	
Dda3937_02526	<i>yidR</i>	conserved protein	NE	18	50	0	-591,000	-7.50	0.00	N	
Dda3937_03888	<i>metB</i>	Cystathionine gamma-synthase	NE	21	118	1	-1,881	-7.34	0.01	Y	<i>metB (-7.34) metL (-3.23)</i>
Dda3937_00195	<i>relA</i>	(pppGpp synthetase I/GTP pyrophosphokinase	NE	55	256	2	-11,683	-7.12	0.00	Y	<i>relA (-7.12) rumA (-1.33)</i>
Dda3937_02532	<i>lfeR</i>	Fructose repressor FruR, Lacl family	NE	15	399	3	-4,756	-7.04	0.00	N	
Dda3937_02226	<i>fliF</i>	Flagellar M-ring protein fliF	NE	46	476	4	-18,898	-7.02	0.00	Y	<i>fliF (-7.02) fliG (-4.26) fliH (-3.92) fliI (-6.56) fliJ (-5.44) fliK (-4.71)</i>
Dda3937_02206	<i>flgE</i>	Flagellar hook protein flgE	NE	50	597	5	-29,608	-7.00	0.00	Y	<i>flgE (-7) flgF (-4.76) flgG (-5.91)</i>
Dda3937_04507	<i>gnd</i>	phosphogluconate dehydrogenase (NADP(+)-dependent, decarboxylating)	GD	36	7	0	-190,000	-6.91	0.00	N	
Dda3937_00697	<i>degQ</i>	Protease	NE	28	80	1	-956,000	-6.87	0.01	N	
Dda3937_03631	<i>trxB</i>	thioredoxin-disulfide reductase	GD	25	16	0	-257,000	-6.85	0.03	N	
Dda3937_00361	<i>yrjF (igaA)</i>	intracellular growth attenuator protein	GD	38	22	0	-430,000	-6.78	0.03	N	
Dda3937_00588	<i>cysB</i>	Transcriptional dual regulator, O-acetyl-L-serine-binding protein	NE	29	90	1	-2,504	-6.75	0.00	N	
Dda3937_03783	<i>prc</i>	carboxy-terminal protease for penicillin-binding protein 3	NE	46	243	2	-11,557	-6.71	0.00	Y	<i>prc (-6.71) proQ (-1.82)</i>
Dda3937_00433	<i>hflX</i>	predicted GTPase	GD	27	16	0	-187,000	-6.69	0.04	N	
Dda3937_03427	<i>fliC</i>	flagellar filament structural protein (flagellin)	NE	33	96	1	-1,520	-6.61	0.03	N	
Dda3937_02223	<i>fliI</i>	Flagellum-specific ATP synthase fliI	NE	42	236	3	-7,009	-6.56	0.00	Y	<i>fliF (-7.02) fliG (-4.26) fliH (-3.92) fliI (-6.56) fliJ (-5.44) fliK (-4.71)</i>
Dda3937_04419	<i>hdjR</i>	DNA-binding transcriptional regulator	NE	29	117	1	-3,241	-6.34	0.00	N	
Dda3937_00209	<i>cysJ</i>	sulfite reductase alpha subunit	NE	41	180	2	-6,746	-6.25	0.00	Y	<i>cysH (-8.93) cysI (-7.65) cysJ (-6.25)</i>
Dda3937_02209	<i>flgH</i>	Flagellar L-ring protein flgH	NE	23	586	8	-13,875	-6.22	0.01	Y	<i>flgH (-6.22) flgI (-5.49) flgJ (-7.16)</i>
Dda3937_02246	<i>fabF</i>	beta-ketoacyl-[acyl-carrier-protein] synthase II	GD	41	10	0	-273,000	-6.15	0.00	N	

Dda3937_00301	<i>uvrD</i>	ATP-dependent DNA helicase UvrD/PcrA	NE	42	29	0	-678,000	-6.11	0.00	N	
Dda3937_02212	<i>flgK</i>	Flagellar hook-associated protein flgK	NE	63	116	2	-4,808	-6.07	0.00	Y	<i>flgK</i> (-6.07) <i>flgL</i> (-5.58)
Dda3937_04046	<i>purU</i>	Formyltetrahydrofolate deformylase	NE	28	51	1	-1,105	-5.84	0.00	N	
Dda3937_03965	<i>flhA</i>	predicted flagellar export pore protein	NE	49	106	2	-3,532	-5.80	0.00	Y	<i>flhE</i> (-0.89) <i>flhA</i> (-5.8) <i>flhB</i> (-5.31) <i>Dda3937_04633</i> (-1) <i>cheZ</i> (-3.29) <i>cheY</i> (-4.52) <i>cheB</i> (-5.14) <i>cheR</i> (-4.67)
Dda3937_02205	<i>flgD</i>	Flagellar basal-body rod modification protein flgD	NE	22	227	4	-4,905	-5.73	0.01	Y	<i>flgB</i> (-3.45) <i>flgC</i> (-6.38) <i>flgD</i> (-5.73)
Dda3937_01352	<i>leuC</i>	3-isopropylmalate dehydratase large subunit	NE	21	139	3	-2,457	-5.73	0.01	Y	<i>leuA</i> (-4.69) <i>leuB</i> (-4.63) <i>leuC</i> (-5.73) <i>leuD</i> (-6.26)
Dda3937_02784	<i>flhC</i>	Flagellar transcriptional activator flhC	NE	20	477	9	-11,222	-5.66	0.01	Y	<i>flhC</i> (-5.66) <i>flhD</i> (-4.1)
Dda3937_02782	<i>motB</i>	Flagellar motor rotation protein motB	NE	40	109	2	-4,067	-5.55	0.01	Y	<i>motA</i> (-5.06) <i>motB</i> (-5.55) <i>cheA</i> (-4.89) <i>cheW</i> (-5.39)
Dda3937_02210	<i>flgI</i>	Flagellar P-ring protein flgI	NE	26	163	4	-3,191	-5.49	0.00	Y	<i>flgH</i> (-6.22) <i>flgI</i> (-5.49) <i>flgJ</i> (-7.16)
Dda3937_02222	<i>fliJ</i>	Flagellar protein fliJ	NE	14	182	4	-2,486	-5.44	0.03	Y	<i>fliF</i> (-7.02) <i>fliG</i> (-4.26) <i>fliH</i> (-3.92) <i>fliI</i> (-6.56) <i>fliJ</i> (-5.44) <i>fliK</i> (-4.71)
Dda3937_02219	<i>fliM</i>	Flagellar motor switch protein fliM	NE	27	143	3	-3,339	-5.40	0.00	Y	<i>fliL</i> (-4.17) <i>fliM</i> (-5.4) <i>fliN</i> (-4.78) <i>fliO</i> (-6.89) <i>fliP</i> (-4.78) <i>fliQ</i> (-3.12) <i>fliR</i> (-4.56)
Dda3937_02774	<i>flhB</i>	Flagellar biosynthesis protein flhB	NE	32	186	5	-4,712	-5.31	0.00	Y	<i>flhE</i> (-0.89) <i>flhA</i> (-5.8) <i>flhB</i> (-5.31) <i>Dda3937_04633</i> (-1) <i>cheZ</i> (-3.29) <i>cheY</i> (-4.52) <i>cheB</i> (-5.14) <i>cheR</i> (-4.67)
Dda3937_02777	<i>cheB</i>	Chemotaxis response regulator protein-glutamate methyltransferase CheB	NE	31	282	8	-7,682	-5.14	0.00	Y	<i>flhE</i> (-0.89) <i>flhA</i> (-5.8) <i>flhB</i> (-5.31) <i>Dda3937_04633</i> (-1) <i>cheZ</i> (-3.29) <i>cheY</i> (-4.52) <i>cheB</i> (-5.14) <i>cheR</i> (-4.67)
Dda3937_02783	<i>motA</i>	Flagellar motor rotation protein motA	NE	24	39	1	-834,000	-5.06	0.00	Y	<i>motA</i> (-5.06) <i>motB</i> (-5.55) <i>cheA</i> (-4.89) <i>cheW</i> (-5.39)
Dda3937_00565	<i>tonB</i>	TonB protein	NE	14	106	3	-2,062	-5.00	0.05	N	
Dda3937_00427	<i>fbp</i>	fructose-bisphosphatase	GA	33	805	27	-28,026	-4.92	0.01	N	
Dda3937_02781	<i>cheA</i>	Chemotaxis protein CheA	NE	50	151	5	-5,838	-4.89	0.00	Y	<i>motA</i> (-5.06) <i>motB</i> (-5.55) <i>cheA</i> (-4.89) <i>cheW</i> (-5.39)
Dda3937_03422		Carbamoyl-phosphate synthase small subunit	NE	43	379	13	-11,713	-4.85	0.02	Y	<i>Dda3937_03422</i> (-4.85) <i>Dda3937_03421</i> (-0.71)
Dda3937_02577	<i>lysA</i>	diaminopimelate decarboxylase	NE	23	332	0	-3,989	-4.79	0.00	N	
Dda3937_02207	<i>flgF</i>	Flagellar basal-body rod protein flgF	NE	21	35	1	-671,000	-4.76	0.00	Y	<i>flgE</i> (-7) <i>flgF</i> (-4.76) <i>flgG</i> (-5.91)
Dda3937_02230	<i>fliD</i>	Flagellar hook-associated protein fliD	NE	47	93	3	-2,506	-4.75	0.00	N	
Dda3937_04301	<i>leuA</i>	2-isopropylmalate synthase	NE	36	35	1	-944,000	-4.69	0.02	Y	<i>leuA</i> (-4.69) <i>leuB</i> (-4.63) <i>leuC</i> (-5.73) <i>leuD</i> (-6.26)
Dda3937_02778	<i>cheR</i>	Chemotaxis protein methyltransferase CheR	NE	30	462	18	-8,882	-4.67	0.05	Y	<i>flhE</i> (-0.89) <i>flhA</i> (-5.8) <i>flhB</i> (-5.31) <i>Dda3937_04633</i> (-1) <i>cheZ</i> (-3.29) <i>cheY</i> (-4.52) <i>cheB</i> (-5.14) <i>cheR</i> (-4.67)
Dda3937_02228	<i>fliT</i>	Flagellar biosynthesis protein fliT	GD	16	8	0	-95,000	-4.63	0.05	Y	<i>fliS</i> (-6.36) <i>fliT</i> (-4.63)
Dda3937_04404	<i>leuB</i>	3-isopropylmalate dehydrogenase	NE	16	285	12	-3,835	-4.63	0.05	Y	<i>leuA</i> (-4.69) <i>leuB</i> (-4.63) <i>leuC</i> (-5.73) <i>leuD</i> (-6.26)
Dda3937_02214	<i>fliR</i>	Flagellar biosynthesis protein fliR	NE	33	268	11	-5,653	-4.56	0.00	Y	<i>fliL</i> (-4.17) <i>fliM</i> (-5.4) <i>fliN</i> (-4.78) <i>fliO</i> (-6.89) <i>fliP</i> (-4.78) <i>fliQ</i> (-3.12) <i>fliR</i> (-4.56)
Dda3937_03727	<i>kduI</i>	4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase	NE	26	70	3	-2,015	-4.54	0.03	N	
Dda3937_03267		O-antigen, teichoic acid lipoteichoic acids export membrane protein	ES	107	89	4	-1,181	-4.33	0.05	Y	<i>Dda3937_03267</i> (-4.33) <i>Dda3937_03268</i> (-1.07)
Dda3937_00415	<i>epd</i>	D-erythrose 4-phosphate dehydrogenase	NE	26	316	16	-4,793	-4.27	0.02	N	
Dda3937_02337	<i>pmp</i>	polynucleotide phosphorylase/polyadenylase	GD	50	5	0	-105,000	-3.97	0.00	N	
Dda3937_01683	<i>purK</i>	N5-carboxyaminoimidazole ribonucleotide synthase	NE	16	90	0	-722,000	-3.49	0.01	Y	<i>purE</i> (-5.75) <i>purK</i> (-3.49)
Dda3937_00689	<i>yrbF</i> (<i>mIaF</i>)	predicted toluene transporter subunit	GA	9	1254	114	-15,962	-3.47	0.01	Y	<i>yrbF</i> (-3.47) <i>yrbE</i> (-1.48) <i>yrbD</i> (-3.09) <i>yrbC</i> (-2.81) <i>yrbB</i> (-0.24)
Dda3937_02829	<i>helD</i>	DNA helicase IV	NE	26	99	9	-1,803	-3.46	0.01	N	
Dda3937_02252	<i>ptsG</i>	PTS system glucose-specific IICB component	NE	37	81	8	-2,928	-3.38	0.03	N	
Dda3937_00726	<i>tolC</i>	transport channel	NE	34	184	0	-3,304	-3.35	0.00	N	
Dda3937_02363	<i>clpA</i>	ATP-dependent Clp protease ATP-binding subunit	NE	44	64	8	-1,793	-3.02	0.03	Y	<i>clpS</i> (-2.07) <i>clpA</i> (-3.02)
Dda3937_02470	<i>corC</i>	magnesium and cobalt ions transport	NE	13	159	21	-1,377	-2.90	0.02	Y	<i>lnt</i> (+3.02) <i>corC</i> (-2.09)
Dda3937_00692	<i>yrbC</i> (<i>mIaC</i>)	predicted ABC-type organic solvent transporter	GA	23	740	106	-16,493	-2.81	0.01	Y	<i>yrbF</i> (-3.47) <i>yrbE</i> (-1.48) <i>yrbD</i> (-3.09) <i>yrbC</i> (-2.81) <i>yrbB</i> (-0.24)
Dda3937_02045	<i>emvC</i>	murein hydrolase activator	NE	17	71	12	-825,000	-2.59	0.00	N	
Dda3937_01807	<i>nuoM</i>	NADH-quinone oxidoreductase subunit M	NE	29	57	10	-1,130	-2.47	0.03	Y	<i>nuoN</i> (-2.01) <i>nuoM</i> (-2.47)
Dda3937_03668	<i>sufB</i>	Fe-S cluster assembly protein	NE	32	116	21	-3,581	-2.44	0.00	Y	<i>sufB</i> (-2.44) <i>sufA</i> (-1.47)
Dda3937_02080	<i>trkH</i>	Potassium uptake protein	NE	36	65	13	-1,047	-2.33	0.05	Y	<i>pepQ</i> (-0.21) ; <i>yigZ</i> (+0.1) <i>trkH</i> (-2.33) <i>hemG</i> (+1.15)
Dda3937_03042	<i>fet</i>	ferrichrysoactin outer membrane receptor	NE	80	244	51	-14,622	-2.25	0.01	N	
Dda3937_01287	<i>argI</i>	Ornithine carbamoyltransferase	NE	24	279	59	-4,383	-2.23	0.03	N	
Dda3937_02456	<i>rsmC</i>	global regulatory protein RsmC	NE	10	116	221,705	2,659,067	10.90	0.028	N	
Dda3937_03858	<i>gcpA</i>	hypothetical protein	GA	55	3728	140,136	9,002,975	5.23	0.00	N	
Dda3937_03971	<i>mltD</i>	outer membrane-bound lytic murein transglycosylase D	NE	46	276	10,885	445,590	5.30	0.00	N	
Dda3937_00363	<i>mrcA</i>	penicillin-binding protein 1A (PBP1A)	NE	53	85	468	16,879	2.47	0.021	N	

180 ^a Genes for which a role in *D. dadantii* virulence has been described before are in bold. Underlined genes have been deleted to study the mutants in further analysis.

181 ^b State of each gene in LB defined by the TRANSIT software using an Hidden Markov Model: NE, Non-Essential ; GD, Growth-Defect ; E, Essential ; GA, Growth-Advantage.

182 ^c Mean reads per TA site for a gene in each growth condition

183 ^d Difference of reads between chicory and LB growth condition

184 ^e Ratio of reads between chicory and LB condition expressed in log₂

185 ^f P-values adjusted for multiple comparisons using the Benjamini-Hochberg procedure (See Transit manual)

186 ^g Presence of the gene in an operon (Yes or No)

187 ^h Operon structure determined by analysis of *D. dadantii* 3937 RNA-seq datasets from Jiang X *et al*, Environ Microbiol. 2016 Nov;18(11):3651-3672. Log₂FC for each gene in operon are indicated in brackets, genes considered to be essential in chicory are indicated in bold (q-value <0.05).

188

189 **Analysis of the genes of *D. dadantii* required for plant colonization.**

190 **(i) Metabolism.** Chicory appears as an environment in which amino acids, nucleic acids and
191 some vitamins (pyridoxal) are scarce. Of the 92 genes identified as GD genes *in planta*, 8 are
192 involved in purine and 7 in pyrimidine metabolisms (Table S4). In the purine metabolism
193 pathway, the inosine monophosphate (IMP) biosynthesis pathway that produces IMP from L-
194 glutamine and 5-phosphoribosyl diphosphate is particularly important for *D. dadantii in*
195 *planta* since 5 out of the 10 genes of this pathway are significantly GD genes *in planta* (Fig.
196 2). IMP is the precursor of adenine and guanine. Next, IMP can be converted in xanthosine
197 5'-phosphate (XMP) by the IMP dehydrogenase GuaB. *guaB* gene is also a GD gene *in*
198 *planta*, with a strong log₂FC of -10.06 (Fig. 2). In the pyrimidine synthesis, the uridine
199 monophosphate (UMP) biosynthesis pathway that converts L-glutamine to UMP, a precursor
200 of uracyl, is very important *in planta* since *carAB*, *pyrB*, *pyrC* and *pyrE*, involved in this
201 enzymatic pathway, are all required for growth *in planta* (Fig. 2). This pyrimidine
202 biosynthesis pathway is specific to bacteria. It is noteworthy that in the human pathogen *S.*
203 *pneumoniae*, mutants of this pathway have a fitness defect in the nasopharynx of infected
204 mice [27]. Hence, it looks that the pyrimidine biosynthesis pathway is particularly important
205 for multiplication of some bacterial species in the host.

206 Mutants in genes involved in the synthesis of sulfur-containing amino acids (*cysIJQ*, *metB*),
207 lysine (*lysA*) and leucine (*leuABC*) are disadvantaged in chicory (Table 2 and Fig. 3A). These
208 amino acids are known to be present in low concentration in plant tissues. Other amino acids
209 seem to be present in quantity sufficient for growth of *D. dadantii* auxotrophs. Low level of
210 certain amino acids probably induces the stringent response in the bacteria. Reduced growth
211 in the plant of the *relA* mutant, unable to synthesize the alarmone ppGpp, supports this
212 hypothesis. Glucose is the main sugar present in plant tissue, present as a circulating sugar or
213 a cellulose degradation product. Mutants in the PTS glucose transport system genes *ptsI* and

214 *ptsG* have a reduced growth in bacteria (Table 2) showing its importance as a carbon source
215 *in planta*.

216 Degradation of cell wall pectin by a battery of extracellular enzymes is the main determinant
217 of *Dickeya* pathogenicity. Mutants unable to produce or to secrete these enzymes by the type
218 II secretion system were not disfavored in chicory since these mutants could use for their
219 growth the pectin degradation compounds produced by enzymes secreted by other bacteria.
220 The redundancy of oligogalacturonate specific porins (KdgM and KdgN) and inner
221 membrane transporters (TogT and TogMNABC) allow entry of these compounds into the
222 bacteria even in a mutant in one of these transport systems. However, *kduI* mutants, blocked
223 in the intracellular part of the pectin degradation pathway, have a limited growth *in planta*,
224 confirming the importance of the pectin degradation pathway in the disease progression.

225 **(ii) Stress resistance.** Plant is an hostile environment for the bacteria that has to cope with
226 antimicrobial peptides, ROS, toxic compounds and acidic pH [35]. We observed that the
227 pump AcrABTolC, that can efflux a wide range of compounds [36], is important for survival
228 in chicory (Fig. S3). Stress can lead to the accumulation of phospholipids in the outer
229 membrane. This accumulation makes the bacteria more sensitive to small toxic molecules
230 [37]. Such a phospholipid accumulation probably occurs when the bacteria infect chicory
231 since *miaC* and *miaF* mutants, which are unable to prevent phospholipid accumulation in the
232 outer membrane, have a reduced growth in plant. Production of exopolysaccharides (EPS)
233 was shown to protect the bacteria during the first steps of infection [9]. We observed that
234 *rffG* and *wzx* mutants unable to synthesize EPS have a growth defect in chicory. A set of
235 genes required to repair or degrade altered proteins (*clpA*, *degQ*, *trxB*) are also important for
236 survival *in planta*. No gene directly involved in detoxification of ROS was detected in our
237 analysis. However, ROS can create DNA damage. The two helicases involved in DNA repair,
238 UvrD and HelD, give growth advantage in plant. Osmoregulated periplasmic glycans (OPG)

239 are polymers of glucose found in the periplasm of α , β and γ -proteobacteria. Their exact role
240 is unknown but their absence leads to avirulence in certain bacteria such as *D. dadantii* [38].
241 This absence induces a membrane stress that is sensed and transduced by the Rcs envelope
242 stress response system. This system controls the expression of many genes, including those
243 involved in motility, and those encoding plant cell wall degrading enzymes through the
244 RsmA-RsmB system [39-41]. Thus, mutants defective in OPG synthesis are expected to have
245 a reduced virulence. Indeed, in our experiment, mutants in the two genes involved in OPG
246 synthesis, *opgG* and *opgH* were non competitive in chicory (Table 2).

247 **(iii) Iron uptake.** *D. dadantii* produces two types of siderophores, achromobactin and
248 chrysobactin, that are required for the development of maceration symptoms in the iron
249 limited environment of plant hosts [42]. Once iron loaded, the siderophores are imported into
250 the bacteria. Import through the outer membrane requires a specific outer membrane channel
251 and the energy transducing complex formed by TonB ExbB and ExbD. While the absence of
252 synthesis of one of the siderophores can be compensated by the presence of siderophore
253 secreted by other bacteria in the growth medium, mutants of the TonB complex are totally
254 unable to acquire iron and thus are unable to grow in the plant. In accordance, *tonB* was
255 essential in chicory while the genes coding for siderophore synthesis or secretion were not.
256 Similarly a mutant devoid of the iron-loaded chrysobactin transport gene (*fct*) is non-
257 competitive.

258 **(iv) Regulation.** Mutants in several genes controlling virulence factor production have a
259 growth defect in the plant. The master regulator FlhDC acts as a regulator of both flagella and
260 virulence factor synthesis in many bacteria such as *Yersinia ruckeri*, *Edwardsiella tarda* and
261 *Ralstonia solanacearum* [43-45]. In *D. dadantii* FlhDC has recently been shown to control, in
262 addition to flagellar motility, type III secretion system and virulence factor synthesis through
263 several pathways [46]. We observed that *flhC* gives a growth advantage in chicory. In

264 addition, we uncovered that some genes regulating *flhDC* in other bacteria regulate *D.*
265 *dadantii* virulence, probably by controlling *flhDC* expression. *rsmC* is a poorly characterized
266 gene in *D. dadantii* but that has been studied in *Pectobacterium carotovorum*. It negatively
267 controls motility and extracellular enzyme production through modulating transcriptional
268 activity of FlhCD [47]. HdfR is a poorly characterized LysR family regulator that controls the
269 *std* fimbrial operon in *S. enterica* and FlhDC expression in *E. coli* [48]. *rsmC* mutants were
270 overrepresented in the chicory (Fig. 3B), indicating a gain of virulence for these mutants.
271 *hdfR* conferred fitness benefits during growth in chicory and could also act in *D. dadantii* as
272 activator of *flhDC* expression.

273 The GGDEF proteins are c-di-GMP synthase. Their genes are often located next to their
274 cognate EAL diguanylate phosphodiesterase gene. *ecpC* (*yhjH*) encodes an EAL protein that
275 was shown to activate virulence factor production in *D. dadantii* [49]. *gcpA*, which is located
276 next to *ecpC* encodes a GGDEF protein. Role of *gcpA* in *D. dadantii* virulence has recently
277 been described [50]. We observed that *gcpA* mutants (Dda_03858) were overrepresented in
278 chicory (Table 2). This increased virulence, a phenotype opposite to that described for the
279 *ecpC* mutants, indicates that overproduction of c-di-GMP could reduce *D. dadantii* virulence.

280 Of the eighteen regulators of the LacI family present in *D. dadantii*, four of them were found
281 to be involved in plant infection [51]. One of those, LfcR, which has been found important
282 for infection of chicory, Saintpaulia and *Arabidopsis*, was identified as important for chicory
283 infection in our experiment. LfcR is a repressor of adjacent genes [51]. Surprisingly none of
284 these genes appeared to play a role for chicory infection suggesting that other targets of LfcR
285 probably remain to be discovered.

286 Finally, it is noteworthy to mention that the *ackA* and *pta* genes are GD *in planta*. These
287 genes constitute the reversible Pta-AckA pathway. The steady-state concentration of acetyl-
288 phosphate (acetyl-P), a signaling molecule in bacteria, depends upon the rate of its formation

289 catalyzed by Pta and of its degradation catalyzed by AckA [52]. The GD phenotype of *D.*
290 *dadantii* *ackA* and *pta* mutants during infection suggests that acetyl-P might play a crucial
291 signaling role in the adaptation of *D. dadantii* to the plant tissue.

292 **(v) Motility.** Motility is an essential virulence factor of *D. dadantii* required to move on the
293 surface of the leaf, enter the wounds and propagate into the plant tissue [53-55]. Accordingly,
294 all the genes required for flagella synthesis, the flagella motor and genes regulating their
295 synthesis (*flhC*, *flhD*, *fliA*) (see above) are necessary for fitness during chicory infection (Fig.
296 3C and 5A). All the genes responsible for the transduction of the chemotaxis signal (*cheA*, *B*,
297 *R*, *W*, *X*, *Y* and *Z*) also confer a benefit *in planta* (Table 2). No methyl-accepting
298 chemoreceptor gene mutant was found. Like other environmental bacteria, *D. dadantii*
299 encodes many such proteins (47). They probably present some redundancy in the recognized
300 signal which prevented their detection by our screen.

301

302 ***D. dadantii* flagellin is modified by glycosylation**

303 A group of six genes located between *fliA* and *fliC* retained our interest since insertions in
304 these genes led to a growth defect in chicory (Fig. 4A). This effect does not result from
305 insertions in the first gene of the group since they are not expressed in operon [56].
306 Dda3937_03424 encodes an O-linked N-acetylglucosamine transferase and Dda3937_03419
307 encodes a protein with a nucleotide diphospho sugar transferase predicted activity. The other
308 ones could be involved in the modification of sugars (predicted function of Dda3937_03423:
309 nucleotide sugar transaminase, Dda3937_03422: carbamoyl phosphate synthase,
310 Dda3937_03421: oxidoreductase; Dda3937_03420: methyltransferase). Their location let
311 suppose that this group of genes could be involved in flagellin glycosylation. Analysis by
312 SDS-PAGE of FliC produced by the wild type, and the Dda3937_03424 and Dda3937_03419
313 mutants, showed that in the two latter strains the molecular weight of the protein diminished

314 (Fig. 4B). The molecular weight determined by mass spectroscopy was 28,890 Da for
315 FliC_{A4277}, 31,034 Da for FliC_{A3422} and 32170 Da for the WT FliC. Thus, in the wild type
316 strain FliC is modified by the products of the genes Dda_03424 to Dda_03419, probably by
317 multiple glycosylation with a disaccharide. Absence of modification did not modify *D.*
318 *dadantii* motility (data not shown). The flagellin of the plant pathogens *Pseudomonas*
319 *syringae* pv *tabaci* and *Burkholderia cenocepacia* are also glycosylated and absence of this
320 modification lowered the ability of these bacteria to cause disease on tobacco and
321 *Arabidopsis*, respectively [57, 58]. Accordingly, in *D. dadantii*, FliC modification appears
322 important for multiplication of the bacteria in plant.

323

324 **Additional genes could be involved in virulence**

325 Several genes have a log₂FC >4 or <-4 but do not satisfy the statistical permutation test
326 adjusted by the false discovery rate method (q-value) (table S6). However, most of them
327 belong to the categories described above and could be required for growth *in planta*. Among
328 those with a log₂FC < -4 can be found genes involved in amino acid and nucleic acid synthesis
329 (*cysH*, *ilvC*, *pyrF*, *pyrD*, *purC*, *thrC*, *metA*, *cysK*, *lysC*), flagella and motility (*flgJ*, *fliO*, *flgC*,
330 *fliS*, *flgG*, *flgA*, *flgL*, *cheW*, *fliN*, *fliP*, *fliK*, *fliG*, *fliL*), pectin and glucose metabolism (*kduD*,
331 *pgi*), EPS synthesis (*gmd*), flagella glycosylation (*vioA*) and regulation (*zur*, *ecpC* and the
332 general RNA chaperone *hfq*).

333 Among the genes with a log₂FC > 4, three regulators can be noticed: *pecS*, *pecT* and
334 *Dda3937_00840*. *pecS* and *pecT* are known regulators of *D. dadantii* controlling the
335 expression of many factors involved in virulence [5, 6]. Thus, their mutation could confer an
336 increased fitness of the bacteria in chicory. *D. dadantii* possesses a functional *expI-expR*
337 quorum sensing system which does not seem to control plant virulence factor production
338 [59]. However, several LuxR family regulator genes which are not associated with a *luxI*

339 gene are present in the genome of the bacteria. Mutants of one of them (*Dda3937_00840*) are
340 overrepresented in the chicory. Its product is probably a repressor of genes conferring an
341 increased fitness *in planta*.

342

343 **Validation of the Tn-seq results.**

344 To validate the Tn-seq results, we performed coinoculation experiments in chicory leaves
345 with the wild type strain and various mutants in GA genes (*gcpA* and *rsmC*) or GD genes
346 (*hdfR*, *clpSA*, *metB*, *flhDC*, *purF*, *cysJ*, *degQ*, *pyrE*, *carA*, *leuA*, *guaB*, *purL*, *lysA*) in a 1/1
347 ratio. We calculated a competitive index (CI) by counting the number of each type of bacteria
348 in the rotten tissue after 24 h. We confirmed the ability of the $\Delta rsmC$ and $\Delta gcpA$ to overgrow
349 the wild type strain. At the opposite, the wild type strain overgrew the in frame deletion
350 mutants that were tested. The lowest competitive index were observed with mutants in
351 biosynthetic pathways such as $\Delta leuA$, $\Delta guaB$, $\Delta purL$, $\Delta lysA$.

352 Amino acid auxotroph mutants (Cys^- , Leu^- , Met^- and Lys^-) tested by coinoculation
353 experiments could be phenotypically complemented *in planta*. Addition of both the non-
354 synthesized amino acid and the auxotroph mutant within the wound totally or almost
355 completely suppressed the growth defect of the auxotroph mutant *in planta* (Fig. S4). This
356 confirmed the low availability of certain amino acids in chicory. This result confirmed that
357 Tn-seq is a reliable technique to identify genes involved in plant colonization and virulence.

358

359 **Conclusion**

360 This Tn-seq experiment highlights new factors required for *D. dadantii* successful rotting of
361 chicory. Many genes known to be important for pathogenesis were not found in this screen
362 because their products are secreted and can be shared with other strains in the community.
363 This includes all the proteins secreted by the type II secretion system and small molecules

364 such as siderophores and butanediol. Other categories of genes were not found: for example,
365 no genes involved in response to acidic or oxidative stresses were identified. Chicory has
366 been described as an inadequate model to study the response of *D. dadantii* to oxidative stress
367 [60]. Similarly, the type III *hrp* genes were not identified in our study. The Hrp system is not
368 necessary for *D. dadantii* virulence and in our experimental conditions (high inoculum on
369 isolated chicory leaves) the necrotrophic capacities of *D. dadantii* (production of plant cell
370 wall degrading enzymes) is probably sufficient to provoke the disease. Our results also
371 uncover some unknown aspects of the infection process. Struggle for iron availability
372 between plant and bacterial pathogens has been well described. However, a competition for
373 amino acids and nucleic acid seems also to take place in the plant. The amount of nucleic
374 acids and of the cysteine, leucine, methionine, threonine and isoleucine amino acids is too
375 low in chicory to allow an efficient multiplication of bacteria defective in their biosynthesis.
376 Some enzymatic steps involved in their synthesis are specific to bacteria and fungi. Thus,
377 they could be good targets for the development of specific inhibitors [61] to fight *D. dadantii*.
378 Regulation of *D. dadantii* virulence has been extensively studied [2, 21]. However, new
379 regulatory genes controlling virulence were also detected in this study. An orphan LuxR
380 family regulator seems to play an important role in virulence. New members of the FlhDC
381 regulation pathway were also detected. A few genes of unknown function remain to be
382 studied.

383 *D. dadantii* can infect dozens of plants. Besides chicory, *D. dadantii* virulence tests are
384 usually performed on potato plant, tuber or slices, *Arabidopsis thaliana*, saintpaulia or celery.
385 Metabolic status or reaction defenses of these model plants are all different and bacterial
386 genes required for a successful infection will probably differ in each model. Testing several
387 of them will allow to determine the full virulence repertoire of the bacteria.

388 While Tn-seq has been used to study genes required for the infection of animals, there has
389 been no genome-wide study of factors necessary for a necrotrophic plant pathogen to develop
390 and provoke disease on a plant. Besides the genes of known function described in the Result
391 section, this study allowed the identification of several genes of unknown function required
392 for chicory rotting. Repetition of this experiment with other strains or on other plants will tell
393 if these genes encode strain or host specific virulence factors.

394

395 **Methods**

396 **Bacterial strains and growth conditions.** Bacterial strains, phages, plasmids and
397 oligonucleotides used in this study are described in Table S1 to Table S3. *D. dadantii* and *E.*
398 *coli* cells were grown at 30 and 37°C respectively in LB medium or M63 minimal medium
399 supplemented with a carbon source (2 g/L). When required antibiotics were added at the
400 following concentration: ampicillin, 100 µg/L, kanamycin and chloramphenicol, 25 µg/L.
401 Media were solidified with 1.5 g/L agar. Transduction with phage PhiEC2 was performed
402 according to [62]. The motility of each mutant was compared with that of the wild-type strain
403 on semisolid (0.4%) LB agar plates as previously described [63].

404 **Construction of the transposon library**

405 Five mL of an overnight culture of *D. dadantii* strain A350 and of *E. coli* MFDpir/pSamEC
406 were mixed and centrifuged 2 min at 6000 g. The bacteria were resuspended in 1 mL of M63
407 medium and spread onto a 0.45 µm cellulose acetate filter placed on a M63 medium agar
408 plate. After 8h, bacteria were resuspended in 1 mL M63 medium. An aliquot was diluted and
409 spread onto LB agar + kanamycin plates to estimate the efficiency of mutagenesis. The other
410 part was inoculated in 100 mL of LB medium + kanamycin and grown for 24 h at 30°C. To
411 confirm that the bacteria that grew were *D. dadantii* strains with a transposon but without

412 plasmid pSamEC, we checked that all the grown bacteria were kan^R, amp^S and
413 diaminopimelate (DAP) prototrophs (MFDpir is DAP⁺). The bacteria were frozen in 40%
414 glycerol at -80°C and represent a library of about 300 000 mutants.

415 **DNA preparation for high-throughput sequencing**

416 An aliquot of the mutant library was grown overnight in LB medium + kanamycin. To
417 identify essential genes in LB, the culture was diluted 100-fold in LB and grown for 6 h. To
418 infect chicory, the overnight culture was centrifuged and resuspended at OD₆₀₀ = 1 in M63
419 medium. Chicories cut in half were inoculated with 10 µL of this bacterial suspension and
420 incubated at 30°C with maximum moist. After 60 h, the rotten tissue was collected and
421 filtered through a cheesecloth. The bacteria were collected by centrifugation and washed
422 twice in M63 medium. DNA was extracted from 1.5 mL aliquots of bacterial suspension
423 adjusted to OD₆₀₀1.5 with the Promega Wizard Genomic DNA purification kit. Next steps of
424 the DNA preparation methods were adapted from [26]. All DNA gel-extraction were
425 performed onto a blue-light transilluminator of DNA stained with gel-green (Biotium) to
426 avoid DNA mutation and double-stranded breaks. 50 µg of DNA samples were digested with
427 50 U MmeI in a total volume of 1.2 mL for one hour at 37°C according to manufacturer's
428 instructions, then heat-inactivated for 20 minutes at 80°C, purified (QIAquick, PCR
429 purification kit Qiagen) and concentrated using a vacuum concentrator to a final volume of
430 25 µL. Digested DNA samples were run on a 1% agarose gel, the 1.0–1.5 kb band containing
431 the transposon and adjacent DNA was cut out and DNA was extracted from the gel according
432 to manufacturer's instructions (Qiaquick Gel Extraction Kit, Qiagen). This allowed recovery
433 of all the fragments containing genomic DNA adjacent to transposons (1201 bp of
434 transposable element with 32-34 bp of genomic DNA). A pair of single-stranded
435 complementary oligonucleotides containing an unique 5-nt barcode sequence (LIB_AdaptT
436 and LIB_AdaptB) was mixed and heated to 100°C, then slowly cooled down in a water bath

437 to obtain double-stranded adaptors with two-nucleotide overhangs. 1 µg DNA of each sample
438 was ligated to the barcoded adaptors (0.44 mM) with 2000 U T4 DNA ligase in a final
439 volume of 50 µL at 16°C overnight. Five identical PCR reactions from the ligation product
440 were performed to amplify the transposon adjacent DNA. One reaction contained 100 ng of
441 DNA, 1 unit of Q5 DNA polymerase (Biolabs), 1X Q5 Buffer, 0.2 mM dNTPs, 0.4 µM of the
442 forward primer (LIB_PCR_5, which anneals to the P7 Illumina sequence of the transposon)
443 and the reverse primer (LIB_PCR_3, which anneals to the P5 adaptor). Only 18 cycles were
444 performed to keep a proportional amplification of the DNA. Samples were concentrated
445 using a vacuum concentrator to a final volume of 25 µL. Amplified DNA was run on a 1.8%
446 agarose gel and the 125 bp band was cut-out and gel extracted (QIAquick, PCR purification
447 kit Qiagen). DNA was finally dialysed (MF-Millipore™ Membrane Filters) for 4 hours.
448 Quality control of the Tn-seq DNA libraries (size of the fragments and concentration) and
449 High-throughput sequencing on HiSeq 2500 (Illumina) was performed by MGX (CNRS
450 sequencing service, Montpellier). 6 DNA libraries were multiplexed on one flow-cell. After
451 demultiplexing, the total number of reads was comprised between 18 and 31 millions (Table
452 1).

453

454 **Bioinformatics analysis:**

455 Raw reads from the fastQ files were first filtered using cutadapt v1.11 [64] and only reads
456 containing the *mariner* inverted left repeat (ACAGGTTGGATGATAAGTCCCCGGTCTT)
457 were trimmed and considered *bona fide* transposon-disrupted genes. Trimmed reads were
458 then analyzed using a modified version of the TPP script available from the TRANSIT
459 software v2.0.2 [32]. The mapping step was modified to select only reads mapping uniquely
460 and without mismatch in the *D. dadantii* 3937 genome (Genbank CP002038.1). Then, the
461 counting step was modified to accurately count the reads mapping to each TA site in the

462 reference genome according to the Tn-seq protocol used in this study. Read counts per
463 insertion were normalized using the LOESS method as described in [65]. We next used the
464 TRANSIT software (version 2.0) to compare the Tn-seq datasets.

465

466 **Strain construction.** To construct the A4277 strain, gene Dda3937_03424 was amplified
467 with the oligonucleotides 19732+ and 19732-. The resulting fragment was inserted into the
468 pGEM-T plasmid (Promega). A *uidA*-kan^R cassette [66] was inserted into the unique AgeI
469 site of the fragment. The construct was recombined into the *D. dadantii* chromosome
470 according to [67]. Recombination was checked by PCR. To construct the in-frame deletion
471 mutants, the counter-selection method using the *sacB* gene was used [68]. The suicide
472 pRE112 plasmid containing 500 bp of upstream and downstream DNA of the gene to delete
473 was transferred by conjugation from the *E. coli MFDpir* strain into *D. dadantii* 3937.
474 Selection of the first event of recombination was performed on LB agar supplemented with
475 chloramphenicol at 30 µg/L. Transconjugants were then spread on LB agar without NaCl
476 supplemented with 5 % sucrose to allow the second event of recombination. In-frame
477 deletions were then checked by auxotrophy analysis and/or by PCR (Dreamtaq polymerase,
478 Thermofisher). In order to discriminate mutants from the wild strain during coinoculation
479 experiments, a Gm^R derivative of the WT strain was constructed by insertion of the mini-
480 Tn7-Gm into the *attTn7* site (close to the *glmS* gene) [69]. A 3937 Gm^R strain was made by
481 coelectroporation of pTn7-M [69] and pTnS3 [70] plasmids into *D. dadantii* 3937 strain. The
482 mini-Tn7-Gm delivered by the pTn7-M vector (suicide plasmid in *D. dadantii*) is inserted
483 into the *attTn7* site (close to the *glmS* gene) of recipient strain thanks to pTnS3 plasmid
484 encoding the Tn7 site-specific transposition pathway. The Gm^R strain obtained was then
485 checked by PCR using *attTn7-Dickeya3937-verif* and 3-Tn7L primers (Table S3).

486

487 **Protein techniques.** Flagella were prepared from overnight LB grown cells. Bacteria
488 were pelleted, resuspended in 1/10 volume of water and passed 20 fold through a needle
489 on a syringe. Cells and cells debris were removed by centrifugation 5 min at 20 000 x g
490 [63]. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

491

492 **Celery inoculation experiments.** Wild Type and A4277 (glycosylation) mutant were grown
493 overnight in M63 + glycerol medium. Bacteria were washed in M63 medium and the OD₆₀₀
494 was adjusted to 1.0. Bacteria were diluted 10-fold in the same medium. 10 µL of the bacterial
495 suspension were inoculated into leaves in a hole made with a pipet tip. The wound was
496 covered with mineral oil and the leaves were incubated at 30°C at high humidity for 2 days
497 (celery). Length or rotten tissue was measured.

498

499 **Coinoculation experiments.** To determine the competitive index of the mutants, the wild
500 type strain and the mutant to test were grown overnight in M63 + glycerol medium. Bacteria
501 were washed in M63 medium and the OD₆₀₀ was adjusted to 1.0. Bacteria were mixed to a
502 1:1 ratio and diluted 10-fold. For complementation experiments *in planta*, the dilution was
503 performed in M63 medium with 1mM of required amino acid. 10 µL of the mixture were
504 inoculated into chicory leaves. The wound was covered with mineral oil and the leaves were
505 incubated at 30 °C at high humidity. After 24 h the rotten tissue was collected, homogenized,
506 diluted in M63 and spread onto LB and LB + antibiotic plates. After 48 h at 30°C, colonies
507 were counted. The competitive index is the ratio (number of mutant bacteria/number of WT
508 bacteria) in the rotten tissue / (number of mutant bacteria/number of WT bacteria) in the
509 inoculum. For the genes whose absence confers a growth advantage in chicory according to
510 the Tn-seq experiment, in frame deletions were realized in a WT strain. The other mutants
511 were realized in the 3937 Gm^R strain.

512 **Nucleotide sequence accession numbers.** The transposon sequence reads we obtained have
513 been submitted to the ENA database under accession number PRJEB20574.

514

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520

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787 **Legend of figures**

788 **Fig. 1. Quality control of the Tn-seq *D. dadantii* 3937 libraries.** (A) Frequency and
789 distribution of transposon sequence reads across the entire *D. dadantii* 3937 genome. The
790 localization of transposon insertions shows no bias throughout the genome of *D. dadantii*
791 3937. B) Density plot of \log_2FC (measured reads/expected reads per gene).

792

793 **Fig 2. Scheme of the purine and pyrimidine biosynthesis pathways in *D. dadantii* that**
794 **produce XMP (purine metabolism) and UMP (pyrimidine metabolism) from L-**
795 **glutamine.** In red are indicated the growth defect genes in chicory that pass the permutation
796 test ($q\text{-value} \leq 0.05$). In bold are genes for which GD phenotype was tested and confirmed
797 with in frame deletion mutants. The \log_2FC of read numbers between chicory and LB for
798 each gene is indicated in bracket. Some genes do not pass the permutation test (in black) but
799 have a strong negative \log_2FC . PRPP: 5-phosphoribosyl-1-pyrophosphate ; GAR: 5'-
800 phosphoribosyl-1-glycinamide ; FGAM: 5'-phosphoribosyl-*N*-formylglycinamide ; AIR: 5'-
801 phosphoribosyl-5-aminoimidazole ; CAIR: 5'-phosphoribosyl-5-aminoimidazole carboxylic
802 acid ; SAICAR: 5'-phosphoribosyl-4-(*N*-succino-carboxamide)-5-aminoimidazole ; AICAR:
803 5-aminoimidazole-4-carboxamide ribonucleotide ; IMP: inosine monophosphate ; XMP:
804 xanthine monophosphate ; UMP: uridine monophosphate.

805

806 **Fig 3. Examples of essential and important genes revealed by Tn-seq.** Number of reads at
807 each transposon location in the sample grown in LB or in chicory. Data are averaged from
808 biological replicates and normalized as described in the method section. Three regions of the
809 genome representative of Tn-seq results are shown, with the predicted genes represented at
810 the bottom of each panel. Peaks represent read number at TA sites. Black arrows represent
811 genes that pass the permutation test ($q\text{-value} \leq 0.05$). Small arrows indicate the presence of
812 promoter (A) Essentiality of leucine biosynthetic genes in chicory. (B) Insertions in the 5'
813 region of *rsmC* generate growth advantage for the bacteria in chicory. (C) Importance of
814 genes involved in motility for growth in chicory.

815

816 **Fig 4. Modification of FliC revealed by Tn-seq analysis and SDS-PAGE.** (A) Importance
817 of 6 genes located between *fliA* and *fliC* for growth in chicory. Log_2FC are indicated in
818 bracket. Dda3937_03425 and Dda3937_03426 are duplicated transposase genes that have
819 been removed from the analysis. Black arrow: GD in chicory ($q\text{-value} \leq 0.05$); white arrow:
820 genes that do not pass the permutation test ($q\text{-value} > 0.05$). Small arrows indicate the
821 presence of promoter. (B) Analysis by SDS-PAGE of FliC produced by the wild type (lane
822 1), the A3422 (lane 2) and A4277 (lane 3) strains. (C) Maceration of celery leaves by the
823 Wild Type and A4277 (glycosylation) mutant. Length of rotten tissue was measured 48 h
824 post infection. Boxplot were generated by BoxPlotR from 9 data points. The calculated
825 median value is 109 for the WT strain, 40 for the A4277 strain. Center lines show the
826 medians; box limits indicate the 25th and 75th percentiles as determined by R software;
827 whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

828

829 **Fig 5. Competitive Index (CI) of several mutant strains.** CI values were determined in
830 chicory leaves as described in Methods. Each value is the mean of 5 experiments. Center
831 lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R
832 software; whiskers extend 1.5 times the interquartile range from the 25th and 75th
833 percentiles, outliers are represented by dots. $n = 5$ sample points. Numbers above the boxes
834 indicate the average competitive index in Log_{10} . * indicates a significant difference relative to
835 the WT ($p < 0.05$, Welch's t-test).

836

837 **Supporting information legends**

838 **Fig S1. Biological reproducibility of the Tn-seq results.** Pairs of Tn-seq assay results are
839 compared, with the total number of reads per gene plotted. Analysis of DNA samples
840 corresponding to two independent cultures of the mutant pool grown (A) in LB medium
841 (correlation coefficient $R = 0.72$) and (B) in chicory (correlation coefficient $R = 0.98$). Values
842 represent average numbers of reads per gene from the pairs of biological replicates.

843 **Fig S2. Volcano plot of resampling results comparing replicates grown in chicory**
844 **versus in LB.** Significant hits have $q < 0.05$ or $-\log_{10} q > 1.3$. Growth defect (GD) or growth
845 advantage (GA) genes are indicated by a red frame.

846 **Fig S3. *acrAB* are essential in chicory.** Number of Tn-seq reads at each insertion site in the
847 *acrA acrB* region in samples grown in LB or in chicory. Data are averaged from biological
848 replicates and normalized as described in Methods. *dnaX* which encodes both the tau and
849 gamma subunits of DNA polymerase is represented by a grey arrow. *dnaX* is essential gene in
850 LB. *acrAB* represented by grey arrows are GD in chicory ($q\text{-value} \leq 0.05$).

851 **Fig S4. Complementation of auxotroph mutants *in planta*.** Each leaf was inoculated with
852 10^6 bacteria. Length of rotten tissue was observed after 24h. Bacteria were injected into the

853 wounded leaf with or without amino acid. Center lines show the medians; box limits indicate
854 the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the
855 interquartile range from the 25th and 75th percentiles, outliers are represented by dots. n = 5
856 sample points. Numbers above the boxes indicate the average competitive index in Log_{10} . *
857 indicates a significant difference relative to the WT ($p < 0.05$, Welch's t-test). ** Indicates an
858 absence of significant difference relative to the WT ($p > 0.05$, Welch's t-test).

859

860 **Table S1: bacterial strains used in this study**

861 **Table S2: plasmids used in this study**

862 **Table S3: oligonucleotides used in this study**

863 **Table S4 : number of genes implicated in KEGG pathway**

864 **Table S5: raw data of the HMM and resampling analysis by transit**

865 **Table S6: List of genes with $\log_2\text{FC} < -2$ or > 2 but with q-value > 0.05**

866

Fig.1

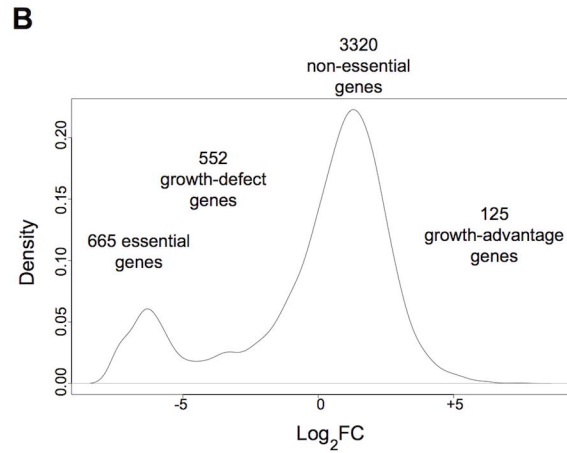
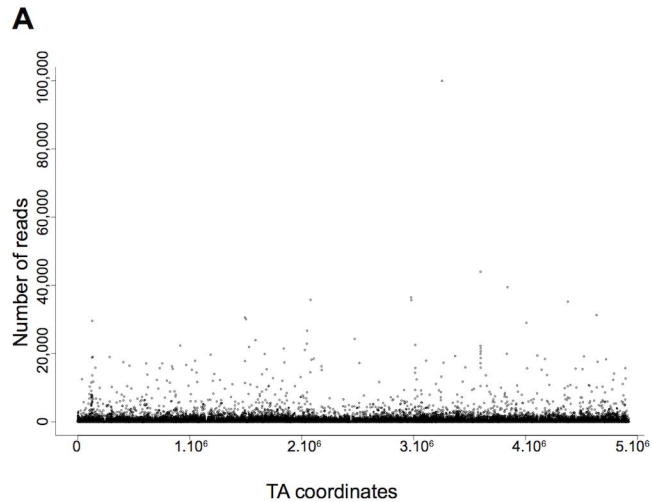


Fig.2

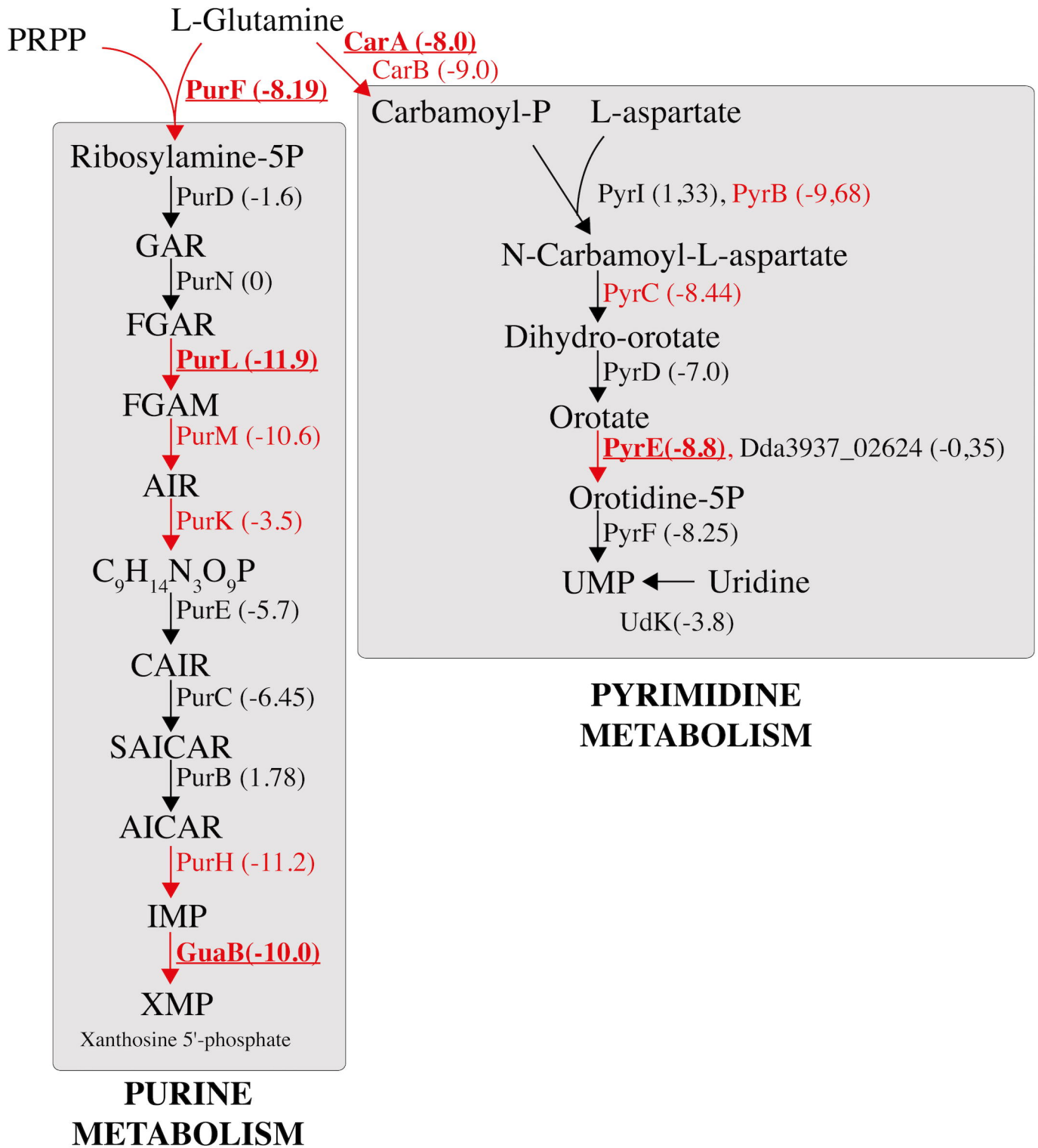
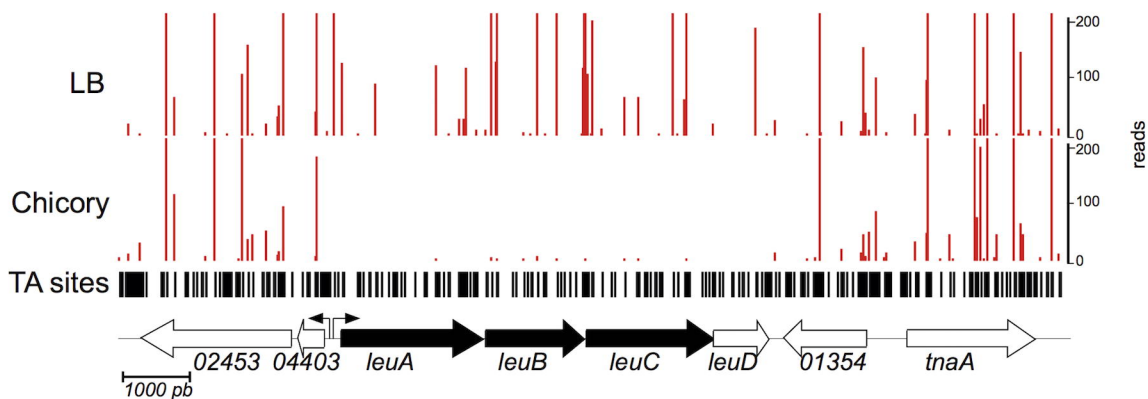
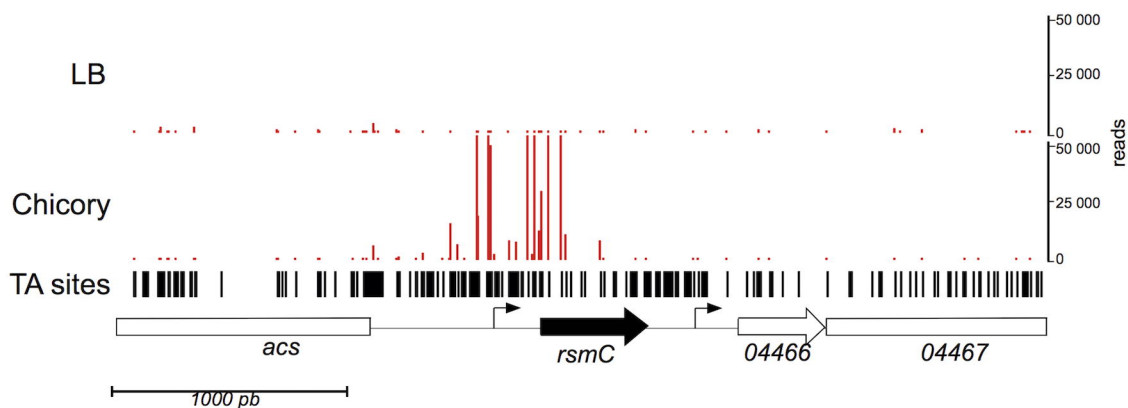


Fig.3

A



B



C

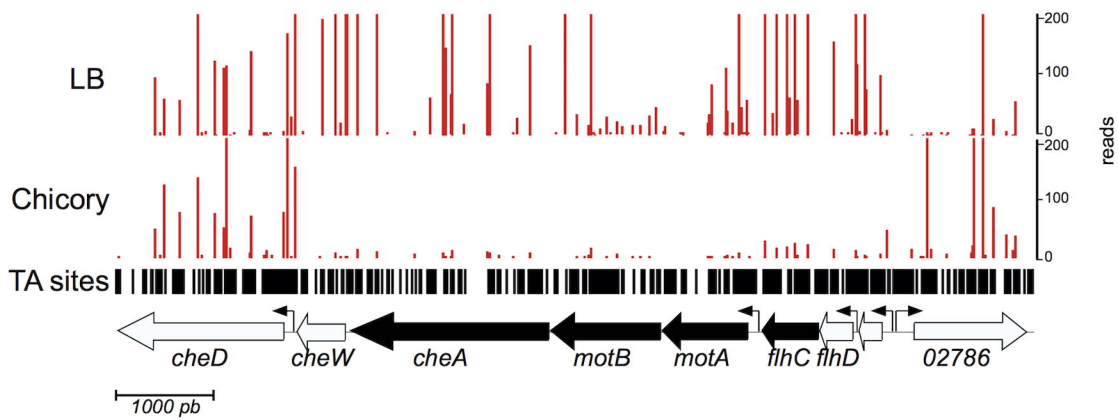
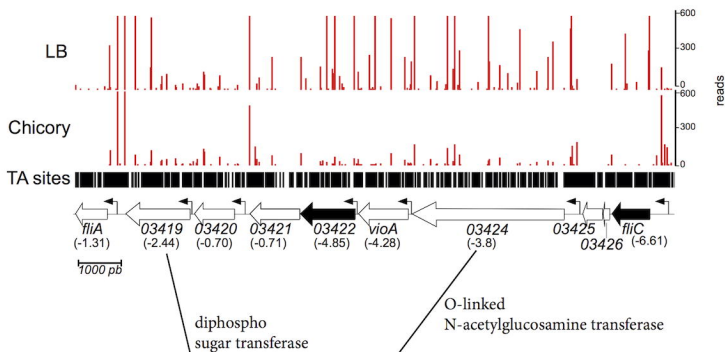
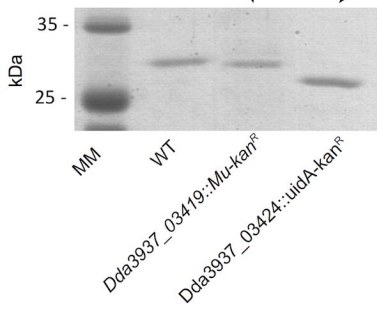


Fig. 4

A



B



C

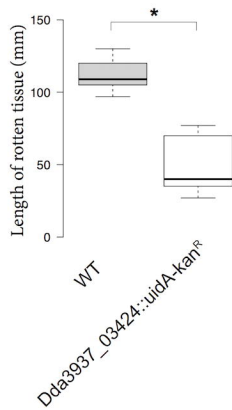


Fig. 5

