BRIEF COMMUNICATION

Title

Transcriptomic profiling reveals host-specific evolutionary pathways promoting enhanced fitness in the broad host range pathogen *Ralstonia pseudosolanacearum*

Authors

Rekha Gopalan-Nair[1], Françoise Jardinaud[1], Stéphane Genin*[,1] and Alice Guidot*[,1]

Addresses

[1]LIPME, Université de Toulouse, INRAE, CNRS, Castanet-Tolosan, France

***Corresponding authors:**

E-mails:

stephane.genin@inrae.fr

alice.guidot@inrae.fr

Abstract

The impact of host diversity on the genotypic and phenotypic evolution of broad-spectrum pathogens is a remaining issue. Here, we used populations of the plant pathogen *Ralstonia pseudosolanacearum* that were experimentally evolved on five types of host plants, either belonging to different botanical families or differing in their susceptibility or resistance to the pathogen. We investigated whether changes in transcriptomic profiles dissociated from genetic changes could occur during the process of host adaptation, and whether transcriptomic reprogramming was dependent on host type. Genomic and transcriptomic variations were established for 31 evolved clones that showed a better fitness in their experimental host than the ancestral clone. Few genomic polymorphisms were detected in these clones, but significant transcriptomic variations were observed, with a high number of differentially expressed genes (DEGs). In a very clear way, a group of genes belonging to the network of regulation of the bacterial virulence such as *efpR*, *efpH* or *hrpB*, among others, were deregulated in several independent evolutionary lineages and appeared to play a key role in the transcriptomic rewiring observed in evolved clones. A double hierarchical clustering based on the 400 top DEGs for each clone revealed two major patterns of gene deregulation that depend on host genotype, but not on host susceptibility or resistance to the pathogen. This work therefore highlights the existence of two major evolutionary paths that result in a significant reorganization of gene expression during adaptive evolution and underscore clusters of co-regulated genes associated to bacterial adaptation on different host lines.

Main body of text

Several studies have demonstrated the undeniable role of adaptive mutation fixation across generations to explain adaptation to changing environments or, in the case of pathogens, to new hosts [1, 2]. In a previous work, we conducted an experimental evolution of the plant pathogen *Ralstonia pseudosolanacearum* strain GMI1000 through serial passage experiment (SPE) into the stem of various plants during 300 bacterial generations [3]. Five biological SPE replicates were generated in parallel for each plant species thus generating five lineages of evolved clones per plant species. For each evolved clone, the adaptive advantage was compared to the ancestral clone through the measure of their Competitive Index during *in planta* in competition assays [3, 4]. The vast majority of the clones we obtained had significantly higher fitness gains than the ancestral clone. We then performed transcriptomic analyses of 10 clones evolved in the resistant tomato Hawaii 7996, which revealed a convergence towards a global rewiring of the virulence regulatory network [4]. As the evolution experiment was performed on several plant species [3], we sought to determine to what extent the transcriptional profiles mobilized for adaptation to tomato Hawaii were conserved for adaptation to other plants. We thus investigated genomic and transcriptomic variations in 21 additional clones obtained by experimental evolution of strain GMI1000 in either susceptible (tomato var. Marmande, eggplant var. Zebrina) and tolerant (bean var. blanc précoce, cabbage var. Bartolo) hosts.

We first investigated the genomic variations in the 21 clones compared to their ancestor by whole genome sequencing (Table 1). Both Illumina and Pacbio sequencing technologies were used for the detection of SNPs (Single Nucleotide Polymorphisms) or small InDels (Insertion-Deletion) and for the detection of large genomic rearrangements, respectively. These analyses revealed between zero and three genomic polymorphisms per

65 clone compared to the ancestor and included either SNPs, Indels, duplication, IS (Insertion
66 sequence) insertion or large deletion (Table 1). All these genomic polymorphisms were
67 confirmed by PCR amplification followed by Sanger sequencing or gel electrophoresis.

68 We then investigated the transcriptomic variations in the 21 evolved clones compared
69 to their ancestor by RNA-sequencing approach using Illumina technology. RNA were extracted
70 from bacterial cultures in MP medium supplemented with 10 mM glutamine and collected at
71 the beginning of stationary phase, in similar conditions as for Hawaii clones [4]. Three
72 biological replicates were conducted for each of the 21 clones and the GMI1000 ancestor
73 strain. Analysis of RNA-seq data revealed that all samples rendered between 0.7 and 1.3
74 million of GMI1000-mapped reads. Differentially expressed genes (DEGs) between the
75 evolved clones and the ancestral clone were considered as those presenting an FDR-adjusted
76 *p*-value (padj,FDR) < 0.05 (Table 1 and Supplementary Table S1). In the 21 new investigated
77 clones, when considering a log-fold change of expression I logFC I > 1, the number of DEGs
78 varied between 25 and 2368 and was not correlated to the number of mutations (Table 1). A
79 high number of DEGs was detected in the three clones evolved in eggplant Zebrina despite no
80 detected genetic alteration, confirming a previous observation that some transcriptomic
81 variations may be dependent on epigenetic modifications [4].

82 By looking more precisely at the DEGs, it appeared that there were groups of genes
83 that were deregulated frequently, even recurrently, in several independent evolutionary
84 lineages and regardless of the host considered (Table 1). In agreement with what was
85 observed for the Hawaii clones, we detected a significant down-regulation for the virulence
86 gene regulators *efpR, hrpB* and *prhP* [4] in 5, 2 and 4 of the newly 21 investigated clones,
87 respectively. In addition, we observed a significant up-regulation for the *efpH* gene, a virulence
88 regulatory gene homologous to *efpR* [5], but exclusively in clones evolved in tomato

89 Marmande and in cabbage. We also observed significant variations in the expression pattern
90 of genes involved in quorum-sensing-dependent virulence signaling pathways such as
91 *solI/solR* [6] in 14 clones; *rasI*/*rasR* [7] in 24 clones; as well as the lectin encoding genes *lecM*
92 and *rsl2* [8] in 21 clones. Genes involved in the denitrification pathway such as *narJ/narK1* and
93 *nosZ* [9] were exclusively up-regulated in the tomato Marmande and cabbage clones and *nasF*,
94 a nitrate transporter protein, was found up-regulated in 23 clones.

95 To go further in the analysis and increase its significance, we focused on the 400 top
96 DEGs for each clone (200 most up-regulated and 200 most down-regulated). The comparison
97 of these DEGs using a double hierarchical clustering is presented in Figure 1. This clustering
98 showed that the evolved clones can be separated into two groups according to the host on
99 which they have evolved, with two main branches clearly distinguishing the clones evolved on
100 tomato Marmande and cabbage on the one hand and the clones evolved on tomato Hawaii,
101 eggplant and bean on the other. This observation suggests that there are at least two major
102 patterns of gene deregulation associated with strain GMI1000 adaptation to its hosts, and that
103 these patterns is associated to the host genotype but not to the host susceptibility to bacterial
104 wilt disease. In terms of discriminating genes, it appears that the Tomato Marmande /
105 cabbage branch is associated with an upregulated expression of cluster (1) (Fig.1 and
106 Supplementary Table S1), which includes 180 genes, among which *efpH*, *narJ* and *nosZ*. The
107 analysis also confirmed down-regulation of part of the *hrpB* regulon (cluster 4) in several
108 clones evolved on Tomato Hawaii, eggplant and Bean, showing significant differences in
109 expression of several genes encoding type 3 effectors in Hawaii evolved clones [4].

110 Altogether, these data highlight a significant reorganization of gene expression
111 associated to adaptation of *R. pseudosolanacearum* to multihost species, which converged
112 toward two major patterns of gene deregulation according to the host genotype. In all cases,

113 a relatively small number of genes (including several transcriptional regulators such as *efpR*,
114 *efpH*, *hrpB*, *rasI*/*R*, *solI*/*R*, *rsl2*) seem to play a key role in these transcriptomic rewiring. The
115 distribution of these regulatory genes in different clusters of co-regulated genes opens
116 avenues for further characterization of these regulons and possible cross-regulations
117 associated to adaptive process to host plants.
118

119 ## Conflict of Interest

120 The authors declare no conflict of interest.
121

122 ## Acknowledgements

123 This work was supported by the French National Research Agency (grant number ANR-17-
124 CE20-0005-01). R.G.N was funded by a PhD fellowship from the French Laboratory of
125 Excellence project "TULIP" (grant number ANR-10-LABX-41; ANR-11-IDEX-0002-02).
126

127 ## References

128 1. Donegan MA, Coletta-Filho HD, Almeida RPP. Parallel host shifts in a bacterial plant
129 pathogen suggest independent genetic solutions. *Mol Plant Pathol* 2023.
130 2. Longdon B, Brockhurst MA, Russell CA, Welch JJ, Jiggins FM. The evolution and genetics
131 of virus host shifts. *PLoS Pathog* 2014; **10**: e1004395.
132 3. Guidot A, Jiang W, Ferdy J-B, Thébaud C, Barberis P, Gouzy J, et al. Multihost
133 Experimental Evolution of the Pathogen Ralstonia solanacearum Unveils Genes Involved
134 in Adaptation to Plants. *Mol Biol Evol* 2014; **31**: 2913–2928.

135 4. Gopalan-Nair R, Jardinaud M-F, Legrand L, Landry D, Barlet X, Lopez-Roques C, et al.
136 Convergent Rewiring of the Virulence Regulatory Network Promotes Adaptation of
137 Ralstonia solanacearum on Resistant Tomato. *Molecular Biology and Evolution* 2020.
138 5. Perrier A, Barlet X, Rengel D, Prior P, Poussier S, Genin S, et al. Spontaneous mutations in
139 a regulatory gene induce phenotypic heterogeneity and adaptation of Ralstonia
140 solanacearum to changing environments. *Environmental Microbiology* 2019; **21**: 3140–
141 3152.
142 6. Flavier AB, Ganova-Raeva LM, Schell MA, Denny TP. Hierarchical autoinduction in
143 Ralstonia solanacearum: control of acyl-homoserine lactone production by a novel
144 autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. *J Bacteriol*
145 1997; **179**: 7089–7097.
146 7. Yan J, Li P, Wang X, Zhu M, Shi H, Yu G, et al. RasI/R Quorum Sensing System Controls the
147 Virulence of Ralstonia solanacearum Strain EP1. *Appl Environ Microbiol* 2022; **88**:
148 e0032522.
149 8. Hayashi K, Kai K, Mori Y, Ishikawa S, Ujita Y, Ohnishi K, et al. Contribution of a lectin,
150 LecM, to the quorum sensing signalling pathway of Ralstonia solanacearum strain OE1-1.
151 *Mol Plant Pathol* 2019; **20**: 334–345.
152 9. Truchon AN, Dalsing BL, Khokhani D, MacIntyre A, McDonald BR, Ailloud F, et al. Plant-
153 Pathogenic Ralstonia Phylotypes Evolved Divergent Respiratory Strategies and Behaviors
154 To Thrive in Xylem. *mBio* 2023; **14**: e0318822.
155
156
157

158 ## Figure legend

159 **Figure 1** Double hierarchical clustering analysis of the 400 top Differentially Expressed Genes
160 (DEGs) in 31 evolved clones of *R. pseudosolanacearum*.
161 The 400 top DEGs (FDR<0.05) (200 most up-regulated and 200 most down-regulated) were
162 selected for each clone. Down- and up-regulated genes were binary coded, respectively -1
163 (blue) and 1(orange). The hclust function from the stats R package (R Development Core
164 Team, 2012) computed Euclidean distances between clone profiles. Clustering was computed
165 with Ward's D2 method and a heat map was generated with the gplots R package
166

167 ## Supplementary material

168 **Supplementary Table S1** RNAseq raw data and analysis for all wild-type (wt) GMI1000 strain
169 and all 31 experimentally evolved clones transcripts detected in minimal medium
170 supplemented with 10mM glutamine. The 400 top DEGs identified for each clone and used for
171 the double hierarchical clustering analysis are indicated by a cross in the 'Top DEGs' column.
172 For each clone, the top DEGs are encoded -1.00 for the down-regulated genes, 1.00 for the
173 up-regulated genes and 0.00 for the genes not differentially regulated compared to the wt
174 strain. The gene cluster to which the 400 top DEGs in each 31 evolved clone belong is given in
175 the 'cluster (k=9)' column. RNAseq data analysis for the Zeb26c2 and Zeb26c4 clones are given
176 in grey at the end of the table. The number of DEGs for these two clones were not sufficient
177 to be included in the double hierarchical clustering analysis.

178

Table 1 List of evolved clones with their genomic polymorphisms and transcriptomic variations of known virulence determinants

Experimental host	Lineage	Evolved clone	Mean CI	*Mutations*	no. of DEGs (I logFC I > 1; p-value FDR <0.05)	Varying expression of known virulence determinants														Reference
						efpR	*efpH*	*hrpB*	*prhP*	*solI*	*solR*	*rasI*	*rasR*	*rsl2*	*lecM*	*narJ*	*narK1*	*nasF*	*nosZ*	
Tomato var. Marmande (suceptible host)	A	Mar26a1[a]	5.6	*RSc2508* $^{IS,-12}$ *tktA* ^{R326G} *RSp0128-0154* $^{Del\ 33kb}$	**1390**		Up (4.43)			Down (-2.75)	Down (-2.92)	Up (2.07)		Down (-8.50)		Up (3.03)	Up (2.99)	Up (3.19)	Up (3.24)	Guidot et al. 2014; this study
	A	Mar26a2	5.4	*RSc2508* $^{IS,-120}$	**1424**		Up (4.38)			Down (-3.22)	Down (-3.21)	Up (2.20)		Down (-9.28)		Up (2.98)	Up (2.73)	Up (3.20)	Up (3.08)	Guidot et al. 2014; this study
	B	Mar26b2[a]	3.9	*phcS* ^{T26M}	**2368**		Up (4.10)			Down (-2.40)		Up (2.05)		Down (-2.79)	Down (-4.45)	Up (1.92)	Up (2.81)	Up (3.18)	Up (4.96)	Guidot et al. 2014; this study
	D	Mar26d2	5.7	*RSc2508* $^{IS,-120}$	**1387**	Down (-2.24)	Up (4.00)							Down (-9.57)		Up (2.69)	Up (2.43)	Up (2.87)	Up (2.59)	Guidot et al. 2014; this study
	E	Mar26e1[a]	3.4	*RSc2508* $^{IS,-120}$	**2174**	Down (-2.35)	Up (3.66)		Down (-2.25)	Down (-2.56)	Down (-4.32)		Up (1.97)	Down (-10.6)	Down (-2.13)	Up (2.58)		Up (2.43)	Up (1.87)	Guidot et al. 2014; this study
	E	Mar26e3	6.3	*RSc2508* $^{IS,-12}$ *RSp1466* $^{In\ 8\ nt,-256}$	**1371**		Up (4.10)			Down (-2.54)	Down (-3.75)	Up (2.20)		Down (-9.16)		Up (2.92)	Up (2.55)	Up (2.98)	Up (2.85)	Guidot et al. 2014; this study
Eggplant var. Zebrina (suceptible host)	B	Zeb26b1	2.7	*RSp0083* ^{IS,1}	**332**							Up (1.88)								Guidot et al. 2014; this study
	B	Zeb26b5	3.7		**239**	Down (-1.30)								Down (-1.56)				Up (1.41)		Guidot et al. 2014; this study
	C	Zeb26c2	2.1	*RSp0127* ^{F91L}	**92**															Guidot et al. 2014; this study
	C	Zeb26c3	1.6	*RSp0127* ^{F91L}	**335**			Down (-1.06)						Down (-1.00)				Up (1.27)		Guidot et al. 2014; this study
	C	Zeb26c4	2.1	*dld* ^{R135S}	**25**															Guidot et al. 2014; this study
	D	Zeb26d1	0.9[ns]		**353**			Down (-1.00)												Guidot et al. 2014; this study
	E	Zeb26e1	3.6		**515**				Down (-1.28)	Down (-2.97)	Down (-3.65)	Up (1.46)		Down (-11.3)						Guidot et al. 2014; this study
Bean var. Blanc Précoce (tolerant host)	A	Bean26a4	6.1	*RSc2508* ^{A394i} *rpoB* ^{D428Y}	**1952**					Down (-3.53)	Down (-4.43)		Up (2.38)	Down (-10.5)	Down (-2.59)					Guidot et al. 2014; this study
	A	Bean26a5	6.5	*RSc2508* $^{A394(-)*}$	**1940**					Down (-3.53)	Down (-5.06)		Up (2.44)	Down (-10.8)	Down (-2.59)					Guidot et al. 2014; this study
	C	Bean26c1[a]	6.6	*efpR* ^{P93Q} *purF* $^{G-88A}$	**897**	Down (-5.95)						Up (2.20)		Down (-3.67)	Down (-3,34)					Guidot et al. 2014; this study

179

Table 1 (continued) List of evolved clones with their genomic polymorphisms and transcriptomic variations of known virulence determinants

Experimental host	Lineage	Evolved clone	Mean CI	*Mutations*	no. of DEGs (I logFC I > 1; p-value FDR <0.05)	Varying expression of known virulence determinants														Reference
						efpR	*efpH*	*hrpB*	*prhP*	*solI*	*solR*	*rasI*	*rasR*	*rsl2*	*lecM*	*narJ*	*narK1*	*nasF*	*nosZ*	
Cabbage var. Bartolo (tolerant host)	B	Cab36b1	4.1	*RSp0955* $^{IS, -1}$ *flhB* $^{Dup\ 21\ nt,\ 1129}$	**1494**	Down (-2.47)	Up (4.44)		Down (-2.36)		Down (-2.06)	Up (2.23)		Down (-6.37)		Up (2.13)	Up (2.24)	Up (3.22)	Up (3.58)	Guidot et al. 2014; this study
	B	Cab36b2	4.9	*RSc2508* $^{IS, 76}$ *RSp0955* ^{IS,} *flhB* $^{Dup\ 21\ nt,\ 1129}$	**2038**		Up (4.26)		Down (-2.12)	Down (-2.87)	Down (-3.75)	Up (2.67)		Down (-8.91)		Up (2.79)	Up (2.14)	Up (2.84)	Up (2.45)	Guidot et al. 2014; this study
	C	Cab36c2	8.8	*spoT* ^{A219P} *RSc2428* $^{C-2}$ *RSc2573-2622* $^{Del\ 44.4kb}$	**1740**		Up (4.34)					Up (2.32)				Up (2.84)	Up (2.57)	Up (2.92)	Up (3.30)	Guidot et al. 2014; this study
	D	Cab36d1	3.5	*phcS* ^{Y106C} *flgB* $^{Del\ 12\ nt,}$ *RSc2573-2622* $^{Del\ 44.4kb}$	**2309**		Up (4.94)			Down (-2.38)	Down (-2,64)			Down (-8.35)	Down (-4.43)	Up (2.33)	Up (2.82)	Up (3.64)	Up (4.41)	Guidot et al. 2014; this study
	E	Cab36e3	9.4	*RSc2573-2622* $^{Del\ 44.4kb}$	**1515**		Up (4.31)			Down (-2.34)	Down (-3.59)	Up (2.69)		Down (-9.43)		Up (2.59)	Up (2.73)	Up (2.83)	Up (3.20)	Guidot et al. 2014; this study
Tomato var. Hawaii (resistant host)	A	Haw35a1	8.6	*soxA1* ^{C639R}	**1227**	Down (-3,65)			Down (-1.28)	Down (-2.49)		Up (1.87)		Down (-4.46)	Down (-3.42)			Up (1.52)		Gopalan-Nair et al. 2020
	A	Haw35a4	7.2		**187**							Up (1.38)						Up (1.08)		Gopalan-Nair et al. 2020
	B	Haw35b1	6.5	*RSp1574* ^{V95L}	**478**							Up (2.17)		Down (-2.44)	Down (-1.58)		Up (1.56)	Up (1.60)		Gopalan-Nair et al. 2020
	B	Haw35b4	12.9	*RSp1574* ^{V95L} *prhP* $^{IS, -6}$	**503**			Down (-1.38)	Down (-6.36)			Up (2.26)		Down (-1.80)				Up (1.59)		Gopalan-Nair et al. 2020
	C	Haw35c1	4.2		**902**	Down (-1,91)		Down (-1.68)		Down (-1.62)	Down (-1.12)	Up (2.21)		Down (-4.86)	Down (-1.01)		Up (1.52)	Up (1.16)		Gopalan-Nair et al. 2020
	C	Haw35c2	4.0		**272**			Down (-1.56)				Up (2.13)						Up (1.41)		Gopalan-Nair et al. 2020
	D	Haw35d3	5.4		**125**							Up (1.57)						Up (1.30)		Gopalan-Nair et al. 2020
	D	Haw35d5	4.1		**269**							Up (1.40)		Down (-0.98)				Up (1.38)		Gopalan-Nair et al. 2020
	E	Haw35e1	3.8	*RSp1136* $^{C-218A}$	**245**							Up (1.28)						Up (1.00)		Gopalan-Nair et al. 2020
	E	Haw35e3	5.4	*RSp1136* $^{C-218}$ *RSc3094* ^{R162R}	**212**							Up (1.53)						Up (1.17)		Gopalan-Nair et al. 2020

Note - The name of the evolved clone indicates the plant species, the number of serial passages, the lineage and the number of the clone. The CI (Competitive Index) value is indicated for each evolved clone and was measured *in planta* in competition with the ancestral GMI1000 clone in our previous works (Guidot et al. 2014; Gopalan-Nair et al. 2020). In the Mutation column, the gene ID or gene name and the modification type is indicated. For SNPs inside the coding sequence, the protein modification is indicated with the original amino acid followed by the position of the SNP and by the new amino acid. For SNPs upstream the start codon of a gene, the original nucleotide is indicated followed by the position of the SNP from the start codon and by the new nucleotide. For small insertion (In), deletion (Del) and duplication (Dup), the size of the modification is indicated followed by the position of the modification. For IS insertion (IS), the position of the insertion is indicated upstream the start codon or in the coding sequence of the gene. For the varying expression of known virulence determinants, the log fold change (logFC) value is given in brackets. Genes targeted by both mutation and transcriptomic deregulation are highlighted in yellow. [a]Genomes of these clones were established with Illumina-seq technology in Guidot et al., 2014; DEGs, Differentially expressed genes; [*]Single nucleotide deletion; ns, not significantly different from the ancestral clone; nt, nuclotides.

400 top DEGs for each clone
Cluster (k=9)
1
2
3
4
5
6
7
8
9
Tomato var. Hawaii_a1
Tomato var. Hawaii_c1
Bean var. Blanc Precoce_c1
Tomato var. Hawaii_b1
Tomato var. Hawaii_b4
Eggplant var. Zebrina_d1
Tomato var. Hawaii_c2
Tomato var. Hawaii_a4
Eggplant var. Zebrina_b1
Bean var. Blanc Precoce_a4
Bean var. Blanc Precoce_a5
Eggplant var. Zebrina_e1
Tomato var. Hawaii_e1
Tomato var. Hawaii_e3
Tomato var. Hawaii_d5
Tomato var. Hawaii_d3
Eggplant var. Zebrina_b5
Eggplant var. Zebrina_c3
Tomato var. Marmande_d2
Tomato var. Marmande_e3
Cabbage var. Bartolo_b2
Cabbage var. Bartolo_e3
Tomato var. Marmande_a1
Tomato var. Marmande_a2
Cabbage var. Bartolo_b1
Tomato var. Marmande_e1
Tomato var. Marmande_b2
Cabbage var. Bartolo_c2
Cabbage var. Bartolo_d1
Evolved clones
Up regulated genes
Down regulated genes