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Ralstonia solanacearum Virulence Increased Following Large Interstrain Gene Transfers by Natural Transformation

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Horizontal gene transfer (HGT) is a major driving force of evolution and is also likely to play an important role in the threatening emergence of novel pathogens, especially if it involves distantly related strains with substantially different pathogenicity. In this study, the impact of natural transformation on pathogenicity in six strains belonging to the four phylotypes of the plant-pathogenic bacterium *Ralstonia solanacearum* was investigated. The study focused on genomic regions that vary between donor and recipient strains and that carry genes involved in pathogenicity such as type III effectors. First, strains from *R. solanacearum* species complex were naturally transformed with heterologous genomic DNA. Transferred DNA regions were then determined by comparative genomic hybridization and polymerase chain reaction sequencing. We identified three transformant strains that acquired large DNA regions of up to 80 kb. In one case, strain Psi07 (phylotype IV tomato isolate) acquired 39.4 kb from GMI1000 (phylotype I tomato isolate). Investigations revealed that i) 24.4 kb of the acquired region contained 20 new genes, ii) an allelic exchange of 12 genes occurred, and iii) 27 genes (33.4 kb) formerly present in Psi07 were lost. Virulence tests with the three transformants revealed a significantincrease in the aggressiveness of BCG20 over its Psi07 parent on tomato. These findings demonstrate the potential importance of HGT in the pathogenic evolution of *R. solanacearum* strains and open new avenues for studying pathogen emergence.

Horizontal gene transfer (HGT) mechanisms (conjugation, transduction, and transformation) are well documented in prokaryotes and assumed to be one of the main forces behind the evolution of bacterial genomes. As a clue, most genomes display a mosaic structure, revealing that HGT frequently occurred over the course of evolution (Nakamura et al. 2004). However, although gene transfer could theoretically modify the fundamental physiology of bacteria by acquisition of genes conferring a wide variety of metabolic capabilities, symbiotic properties, and virulence, the actual impact of HGT on bacterial phenotypes is far less well established. Recently, selective pressure from plants following horizontal transfer of a symbiotic plasmid was found to modify the behavior of a phytopathogenic bacterium (Marchetti et al. 2010). The presence of this plasmid and spontaneous inactivation of a single regulatory gene caused the pathogen to exhibit some symbiotic traits. The emergence of new pathotypes or enhanced virulence in new hosts could be facilitated by recent mutations, including acquisitions of new genes. Virulence genes often occur in clusters called pathogenicity islands. Therefore, they could be transferred simultaneously to a new host, where they may confer new properties. For *Escherichia coli*, a normal resident of mammalian intestinal flora, the acquisition of a single pathogenicity island was sufficient to confer pathogenic properties on benign strains (Hacker and Kaper 2000). Lovell and associates (2009) documented pathogenicity-related genomic island transfer during the plant infection process by *Pseudomonas syringae* pv. *phaseolicola*. However, the plant defense hypersensitive response was triggered by the recipient bacteria but not by its wild parent; therefore, in this case, the transferred genes seemed to be disadvantageous and did not confer increased pathogenicity. Nevertheless, in more favorable circumstances, the transfer of genes essential for virulence, such as type III effectors, may have a marked impact on the pathogenicity of the recipient bacterium. Indeed, the acquisition of new effectors could help the bacterium to manipulate host cells to optimize inter- or intracellular environments so as to improve pathogen growth and dissemination, while subverting host defense responses (Grant et al. 2006).

DNA transfer efficiency between distantly related bacteria is known to be reduced by numerous factors, including ecological or behavioral isolation, obstacles to DNA entry, restriction enzymes, resistance to integration of divergent DNA sequences, reversal of recombination by mismatch repair, and functional incompatibility of recombined segments (Cohan 2002). However, it is likely that, in highly diverse taxa, transfers between phylogenetically remote bacteria provide a large potential source of genes that bacteria may use to dramatically modify their pathogenicity. Thus, in the framework of preventive studies carried out to evaluate the risk of new pathogen emergence caused by interspecies transfers, it is important to determine how easy, broad, and pathogenicity relevant interspecies gene transfers may be.

We performed such a survey with the plant-pathogenic bacterium *Ralstonia solanacearum*, which causes bacterial wilt disease. *R. solanacearum* is a species complex divided into

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*The *e*-Xtra logo stands for "electronic extra" and indicates that two supplementary tables are published online.

four phylotypes (Fegan and Prior 2005), which can infect more than 250 plant species, including economically and agriculturally important crops such as tomato, potato, or banana (Hayward 1991). Phylotypes display different pathogenic behaviors, and Wicker and associates (2007, 2009) recently isolated new strains with unconventional pathogenicity properties, which they suggested may have resulted from interphylotype HGT. *R. solanacearum* is prone to HGT because it is able to naturally develop a physiological state of competence required to exchange DNA by transformation under in vitro and in planta conditions (Bertolla et al. 1997, 1999). We found that this characteristic was shared by all phylotypes (Coupat et al. 2008), thus highlighting its potential role in rapid *R. solanacearum* pathogenicity evolution.

This study evaluated the effects of interphylotype gene transfer on the pathogenic evolution of *R. solanacearum*. We focused on the transfer of genomic regions carrying genes potentially involved in virulence. Frequencies and extents of gene transfers between phylotypes were determined. Comparative genomic hybridization (CGH) and sequencing allowed us to quantify the proportion of sequences subject to three types of genetic rearrangements: substitutive recombination, acquisition of new genomic regions, and deletion of genomic regions in the recipient genome. Finally, the impact of HGT on virulence evolution was evaluated in transformant strains.

RESULTS AND DISCUSSION

Construction and properties of donor and recipient strains.

HGT in the *R. solanacearum* species complex via the natural transformation mechanism required two concomitant conditions: i) development of the competence state in the recipient strain to allow fixing, uptake, and integration of transforming DNA; and ii) the presence of free, available, and functional DNA near competent bacteria. Our first step was to choose the best recipient strains of each phylotype that met several physiological and pathogenic criteria. Five strains belonging to phylotypes II, III, and IV—CMR34, JT516, CFBP3059, NCPPB1029, and Psi07 (Table 1)—were selected on the basis of their host plant range, aggressiveness on tomato plants ('Marmande'), and high competence rate. The second step was to optimize the detection of transferred genomic regions between phylotypes. For this purpose, comparative genomic hybridizations on DNA microarrays allowed us to precisely determine genomic regions that were absent or highly divergent in the genome of recipient strains in comparison with that of the donor, which was phylotype I strain GMI1000. Genomic comparison revealed that, overall, 13 to 26% of the GMI1000 genes were absent or highly divergent in the recipient strains (Table 1). Results obtained for strains CFBP3059 and Psi07 were similar to those obtained previously by Guidot and associates (2007). Moreover, CGH results were consistent with the previously reported phylotype classification, as analyzed by Guidot and associates (2007) (data not shown).

The complete annotated genome sequence of the GMI1000 donor strain (Salanoubat et al. 2002) was used to select, among absent or divergent DNA regions, those containing genes potentially involved in pathogenesis, such as type III effectors. We identified 55 regions corresponding to these criteria: 34 on the chromosome and 24 on the megaplasmid (Supplementary Table S1). Four regions were ultimately retained according to i) the size and gene content of the lacking region, ii) the degree of synteny of the neighboring DNA regions between GMI1000 and recipient strains, and iii) their distributions throughout the chromosome (Table 2). To detect and monitor HGT, the most common experimental approach involved the use of selective markers such as genes coding for resistance to antibiotics. The GMI1000 donor strain was tagged with a gentamicin resistance gene. Two strategies were implemented to tag each DNA region. The first one was to directly insert the gentamicin cassette into a GMI1000 region that was absent in recipient strains, while the second involved marker integration in regions fairly close to the divergent region but conserved in recipient strains (Table 2). Eight tagged clones from GMI1000 were constructed by natural transformation using plasmids carrying amplified genes disrupted by insertion of the gentamicin cassette near their centers. The correct cassette insertion in GMI1000 was checked by polymerase chain reaction (PCR) (data not shown).

Natural interphylotype transformation.

In order to evaluate DNA transfer between phylotypes, the five recipient strains (CMR34, JT516, CFBP3059, NCPPB1029, and Psi07) were naturally transformed with genomic DNA from the eight GMI1000 donor strains. These transformations allowed us to optimize the strategy localizing gene markers in

Table 1. Strains used in this study

Strain	Phylotype	Biovar	Genes conserved (%)[a]	Geographic origin	Host plant	Origin and localization of genomic modifications
Wild type						
GMI1000, RUN 54	I	3	100	French Guyana	Tomato	…
CMR34, RUN 147	II	2	83.4	Cameroon	Tomato	…
JT516, RUN 160	II	2T	80.4	Reunion island	Potato	…
CFBP3059, RUN 39	III	1	86	Burkina Faso	Eggplant	…
NCPPB1029, RUN 77	III	1	86	Reunion island	*Pelargonium capitatum*	…
Psi07, RUN 83	IV	2T	82	Indonesia	Tomato	…
Transformants						
BCG1	…	…	…	…	…	JT516 with RSc2123–RSc2155 region from GMI1000
BCG2 to BCG5	…	…	…	…	…	JT516 with RSc2124::Gm from GMI1000
BCG6	…	…	…	…	…	CMR34 with RSc2123–RSc2155 region from GMI1000
BCG7 to BCG10	…	…	…	…	…	CMR34 with RSc2124::Gm from GMI1000
BCG11 to BCG14	…	…	…	…	…	CFBP3059 with RSc2124::Gm from GMI1000
BCG15 to BCG19	…	…	…	…	…	NCPPB1029 with RSc2124::Gm from GMI1000
BCG20	…	…	…	…	…	Psi07 with RSc2123 - RSc2155 region from GMI1000

[a] Percentage of GMI1000 genes that exhibit an average $\log_2$ ratio above –2 for the signal on a microarray following hybridization of genomic DNAs of GMI1000 and the indicated strain.

the donor genome and to identify the most efficient donor DNA and the best donor DNA–recipient strain combination. Tagging the strain in lacking or divergent regions seemed to be inefficient for transformant selection. Genetic construction in RSc0227, RSc2130, RSc2581, and RSc3241 did not yield any transformants, and we concluded that homologous regions required for recombinations were probably too distant. The second strategy, which consisted of marker gene integration in the conserved genes RSc0230, RSc2124, RSc2570, and RSc3255, allowed recovery of transformant cells with phylotypes II, III, and IV (Table 3). In each transformant, the actual integration of chimeric constructions was verified by PCR (data not shown). The most efficient donor DNA was the GMI1000 gene RSc2124::Gm, which yielded transformants at a frequency reaching 3.7×10^{-7} transformants per recipient cell with the recipient strain NCPPB1029, and which gave a single clone with Psi07 (Table 3). The conserved RSc2124 gene encodes a transmembrane lipoprotein of unknown function. It is located at 4.9 kb of a specific 24-kb region of GMI1000 that has seven genes encoding proteins of the type III effector family or whose transcription is regulated by the HrpB/HrpG system (Occhialini et al. 2005; Poueymiro and Genin 2009; Valls et al. 2006).

Interphylotype gene transfer occurred at frequencies ranging from 5.1×10^{-9} to 2.8×10^{-7} (Table 3), consistent with previous interspecific genetic exchange results (Guidot et al. 2009; Mercier et al. 2007). Other studies demonstrated that transformation rates were dependent on physiological and molecular factors, such as the intrinsic transformability of bacteria and the level of sequence divergence between donor and recipient DNAs (Coupat et al. 2008; Mercier et al. 2007). Indeed, the transformation efficiency with the Psi07 strain, which displayed the highest gene divergence with GMI1000 (26%), was lower that with other recipient strains. Nevertheless, in spite of the fact that CFBP3059 and NCPBB1029 strains from phylotype III are closer to GMI1000 (phylotype I), with 13 and 14% of gene divergence, respectively, natural transformation was hard to obtain with these strains because they were transformed by only three of eight donor DNAs. The lower transformation rate of strains CFBP3059, NCPBB1029, and Psi07 could also be explained by experimental biases, including those related to the transformation protocol used, which was optimized for GMI1000. The transformability of the recipient strains appears to be a critical factor in the efficiency of HGT in the *R. solanacearum* species complex.

Length of DNA acquisitions determined by comparative genomic hybridization and sequencing.

Evaluation of the extent and content of gene flow that can be transferred between bacteria is a major factor in assessing the role of HGT in bacterial evolution. The nature and size of transferred DNA was evaluated in transformant cells obtained with the most efficient donor DNA RSc2124::Gm. The gene content of 20 transformants containing chimeric constructions, as previously verified by PCR, was analyzed by DNA microarray analysis (Table 4). This allowed us to detect HGT of new genes or alleles having sufficient nucleotide divergence between donor and recipient strains, to assess the number of genes transferred within these DNA blocks, and, finally, to characterize their functions. We considered that synteny in all strains (CMR34, JT516, CFBP3059, NCPPB1029, and Psi07) is conserved upstream and downstream of targeted regions, based on sequenced genomes IPO1609 (*not published*) and Psi07 (Remenant et al. 2010). Among the 20 transformants, only three (i.e., BCG1, BCG6, and BCG20) had acquired large DNA blocks (e.g., at least 29.4 kb) in conjunction with transfer of the resistance marker gene. Interestingly, the entire alternative codon usage region (ACUR) close to the RSc2124 gene was also transferred to the three transformants, thus confirm-

Table 2. Description of *Ralstonia solanacearum* target genes used to construct genomic donor DNA

Gene accession no.[a]	Gene annotation	Cloning site	Size of absent region (kb)	Distance to absent region (kb)	Putative function
RSc0227*	*avrrpm1*	*Eco*RI	9		Putative pseudogene (type III effector protein, Avrpm1 homologue)
RSc0230	…	*Eco*RI	…	1.4 to RSc0229	Putative membrane protein; putative LtrA domain (low temperature requirement A protein)
RSc2124	…	*Sma*I	…	4.9 to RSc2127	Conserved exported protein of unknown function
RSc2130*	…	*Psy*I	24		Type III effector protein (Skwp6)
RSc2570	*ada*	*Bam*HI	…	1.8 to RSc2573	Transcriptional regulator of DNA repair (AraC/Xyl family) (N-terminal); O6-Methylguanine-DNA methyltransferase
RSc2581*	*trbE*	*Nhe*I	47		Putative conjugal transfer protein Trbe
RSc3240*	…	*Bsu*BI	65		Conserved protein of unknown function
RSc3255	…	*Bgl*II	…	3.5 to RSc3252	Putative cytochrome of the thiosulfate-oxidizing multi-enzyme system, *sox*X homolog

[a] Genes indicated by an asterisk (*) were localized in the absent or divergent DNA regions, which were defined by comparative genomic hybridization on microarrays. Other genes were present in the genome of CMR34, JT516, CFBP3059, NCPBB1029, and PSI07 strains but were localized more and less close to the lacking or divergent DNA region.

Table 3. Transformation frequencies with genomic donor DNA

Donor DNA[a]	Recipient strain[b]					
GMI1000	**GMI1000**	**CMR34**	**JT516**	**CFBP3059**	**NCPBB1029**	**PSI07**
RSc0227::Gm*	ND	ND	3.0×10^{-8}	ND	ND	ND
RSc0230::Gm	3.8×10^{-8}	4.6×10^{-9}	7.2×10^{-8}	1.4×10^{-8}	2.8×10^{-7}	ND
RSc2124::Gm	2.8×10^{-8}	1.0×10^{-7}	3.4×10^{-8}	2.7×10^{-7}	3.7×10^{-7}	5.1×10^{-9}
RSc2130::Gm*	1.5×10^{-7}	8.7×10^{-9}	3.8×10^{-8}	ND	ND	ND
RSc2570::Gm	5.7×10^{-7}	1.4×10^{-8}	3.2×10^{-8}	ND	ND	ND
RSc2581::Gm*	3.3×10^{-8}	1.3×10^{-8}	3.4×10^{-8}	ND	ND	ND
RSc3240::Gm*	2.9×10^{-7}	5.2×10^{-8}	5.5×10^{-8}	ND	ND	ND
RSc3255::Gm	1.9×10^{-6}	2.8×10^{-8}	3.6×10^{-8}	1.4×10^{-8}	1.4×10^{-7}	ND

[a] Genes indicated by an asterisk (*) were localized in the absent or divergent DNA regions, which were defined by comparative genomic hybridization on microarrays.
[b] ND = transformation not detected (frequency $<10^{-10}$).

ing the hypothesis that ACUR can be transient genes with a high turnover rate in the genome and that they can often be transferred between *R. solanacearum* phylotypes (Guidot et al. 2009). In the present study, comparative genomic hybridization did not allow us to detect additional gene transfers outside of the region labeled by marker genes, contrary to our findings in previous studies (Coupat et al. 2008; Guidot et al. 2009).

Recombination sites were localized by PCR and sequence walking in regions flanking the putative transferred region. This analysis confirmed the acquisition of new genes in the recipient strains, while also demonstrating the replacement of genes in recipient strains by their donor allele counterpart. This led us to reassess the size of the transferred DNA blocks, which ranged from 39.4 to 78.9 kb. In this case of substitutive recombination, DNA replacements affect regions with similar size and gene content in donor and recipient genomes. For this kind of genetic exchange, we propose the reciprocal gene transfer (RGT) concept, which only generates acquisition of single-nucleotide polymorphisms (SNP). In BCG20, RGT produced 601 SNP distributed in 12 new alleles. In all, 11 of 12 alleles also displayed amino acid dissimilarities with their recipient counterparts, representing potential sources of phenotypic modification (Table 5).

In addition, based on crossover evidence, we assumed that recipient genes located within the exchanged regions had been lost during transfer events. This loss of genes specific to the recipient strain was not detectable on the microarray because the one used was designed to detect donor genes only. Therefore, potential losses of recipient-specific genes were investigated by PCR. No amplification was obtained with BCG20 when looking for recipient-specific genes RPSI07_1288 and RPSI07_1300, whereas amplifications were obtained as expected with the Psi07 strain (data not shown). Because RPSI07_1288 and RPSI07_1300 flank a recipient-specific region of 33.4 kb, it is likely that the entire region was absent in BCG20. This suggests that, in BCG20, a DNA loss of approximately 33.4 kb occurred simultaneously to the integration of 24.4 kb of foreign DNA, corresponding to a loss and gain of 27 and 20 genes, respectively, in the same genomic region (Fig. 1). In conclusion, there were three kinds of genetic rearrangement in BCG20: acquisition of novel genomic regions, substitutive recombinations (i.e., allele exchanges), but also deletion of genomic regions in transformant genomes.

For BCG20, the correlation between homologous recombination sites and χ sequences (Fall et al. 2007) was evaluated but no statistically significant link could be established. The GC content variations were also analyzed in the 100-kb region around the acquired region. Majewski and Cohan (1999) hypothesized that there might be a critical stability of the bond between the invasive end and the recipient DNA that would be necessary to initiate recombination. This stability was characterized by a relatively high GC content. For the four recombination sites between BCG20 and GMI1000, no relationship between high GC content and recombination sites was evident. However, as expected, SNP density analysis demonstrated that recombination sites were localized in regions with strong homology (<0.05 SNP per 100 bp). This result is in accordance with studies on the mismatch repair system in *R. solanacearum* which recognizes and eliminates mispairs, which are the main barrier to recombination between divergent sequence, limiting interspecific gene exchange (Mercier et al. 2007).

Transformant aggressiveness.

To determine whether gene acquisition, allelic replacement, and gene loss could increase transformant aggressiveness, tomato and eggplant plantlets were inoculated with BCG1, BCG6, and BCG20, their respective wild-type strains Psi07, CMR34, and JT516, and the donor strain GMI1000 RSc2124::Gm. As expected, wild-type strain GMI1000 completely wilted tomato and eggplant plantlets 9 and 6 days postinoculation, respectively. Strains CMR34 and JT516 from phylotype II and III, as well as their respective transformants, were as aggressive as GMI1000 on both tomato and eggplant. Psi07 from phylotype IV was not very aggressive on either tomato or eggplant compared with GMI1000, with respective disease indexes of 2.6 and 2.0 for Psi07 and 4.0 for GMI1000 at 9 days postinfection ($P < 0.001$) (Fig. 2). Interestingly, the transformant strain BCG20 had a significantly higher aggres-

Table 4. Detection by comparative genomic hybridization on microarrays of acquired regions in recombinant clones[a]

Recipient strains and recombinant clones	Localization of genomic modifications	No. of ORFs (size of DNA modified [bp])	Verification
JT516			
BCG1	Rsc2117-2189	72 (78,441)	PCR sequencing
BCG2	**RSc2124**	1	PCR
BCG3	**RSc2124**	1	PCR
BCG4	**RSc2124**	1	PCR
BCG5	**RSc2124**	1	PCR
CMR34			
BCG6	RSc2123-2155	23, (40,307)	PCR sequencing
BCG7	**RSc2124**	1	PCR
BCG8	**RSc2124**	1	PCR
BCG9	**RSc2124**	1	PCR
BCG10	**RSc2124**	1	PCR
CFBP3059			
BCG11	**RSc2124**	1	PCR
BCG12	**RSc2124**	1	PCR
BCG13	**RSc2124**	1	PCR
BCG14	**RSc2124**	1	PCR
NCPPB1029			
BCG15	**RSc2124**	1	PCR
BCG16	**RSc2124**	1	PCR
BCG17	**RSc2124**	1	PCR
BCG18	**RSc2124**	1	PCR
BCG19	**RSc2124**	1	PCR
Psi07			
BCG20	RSc2123-2155	23 (39,441)	PCR sequencing

[a] Marker genes used for selection of recombinant clones appear in bold. RSc = gene identity from the *Ralstonia solanacearum* GMI1000 chromosome; ORF = open reading frame; PCR = polymerase chain reaction.

siveness than the wild-type recipient strain Psi07 on tomato ($P < 0.001$, 5 days postinoculation), with an average disease index of 3.7 at 9 days postinoculation (Fig. 2). However, BCG20 was not significantly more aggressive than the recipient strain Psi07 on eggplant (2.5 and 2, respectively), suggesting that the increased aggressiveness of BCG20 depends on the tested host plant.

In the BCG20 transformant, virulence assays demonstrated that HGT between microbial pathogens can lead to emergence of new pathotypes on tomato plants. In this case, 39.4 kb of foreign sequences containing 20 new open reading frames and 11 new alleles were integrated in the genome of Psi07, and 33.4 kb was lost. These genetic variations, independently or in combination, could mediate the appearance of new pathogenic

Table 5. Acquired and lost genes in BCG20 transformant strains

Open reading frames	Gene name	Length (bp)	Function
Acquired genes			
RSc2127	*dalR*	947	Transcriptional regulator
RSc2128	*xylB*	1,475	Xylulose kinase
RSc2129	*dalB*	1,397	D-arabinitol 4-dehydrogenase
RSc2130	…	2,312	Type III effector protein (Skwp6)
RSc2131	…	404	Protein of unknown function
RSc2132	…	956	Conserved protein of unknown function
RSc2133	…	272	Putative transmembrane protein
RSc2134	…	311	Conserved protein of unknown function
RSc2135	…	467	Conserved exported protein of unknown function
RSc2136	…	1,058	Putative porin signal peptide protein
RSc2137	…	464	Glucose-6-phosphate isomerase
RSc2138	…	377	Putative helix-turn-helix transcription regulator protein
RSc2139	…	3,191	Awr type III effector family protein
RSc2140	*ugpC*	1,109	*sn*-Glycerol-3-phosphate transport ATP-binding protein
RSc2141	…	698	Putative HAD-superfamily hydrolase subfamily IA transmembrane protein
RSc2142	…	860	Putative transmembrane ABC transporter protein
RSc2143	…	953	Putative transmembrane ABC transporter protein
RSc2144	…	1,325	Putative sugar-binding periplasmic signal peptide protein
RSc2145	*kbaZ*	1,355	D-tagatose-1,6-bisphosphate aldolase subunit KbaZ
RSc2146	*scrK*	956	Putative fructokinase protein
RSc2147	*polS*	770	Sorbitol dehydrogenase
Gene replacement			
RSc2123	*maeB*	2,321	NAD(P)-dependent malic enzyme; bifunctional: Malate dehydrogenase/phosphate acetyltransferase
RSc2124	…	641	Conserved exported protein of unknown function
RSc2125	*pepN*	2,717	Aminopeptidase N
RSc2126	*fbp*	1,016	Fructose-bisphosphatase
RSc2148	…	482	Conserved protein of unknown function, Peptidoglycan-binding LysM domain
RSc2149	…	1,031	Putative esterase
RSc2150	…	305	Conserved protein of unknown function
RSc2151	…	401	Conserved exported protein of unknown function
RSc2152	*greB*	563	Transcription elongation factor (Transcript cleavage factor)
RSc2153	…	2,207	Putative bifunctional: (p)ppGpp synthetase II
RSc2154	*rpoZ*	203	DNA-directed RNA polymerase subunit omega
RSc2155	*gmk*	665	Guanylate kinase (GMP kinase)
Gene lost			
RPSI07_1279	…	506	Conserved exported protein of unknown function
RPSI07_1280	…	716	Conserved hypothetical protein
RPSI07_1281	…	1,625	Putative type II secretion system protein E (GspE)
RPSI07_1282	…	1,193	Putative type II secretion system protein F (GspF)
RPSI07_1283	…	407	Putative type II secretion system protein G (GspG)
RPSI07_1284	…	476	Conserved exported protein of unknown function
RPSI07_1285	…	386	Putative general secretion pathway GspG-like
RPSI07_1286	…	476	Putative general secretion pathway GspG-related transmembrane protein
RPSI07_1287	…	488	Conserved exported protein of unknown function
RPSI07_1288	…	478	Conserved exported protein of unknown function
RPSI07_1289	…	2,279	Putative type II secretory pathway, GspD-related protein
RPSI07_1290	…	146	Protein of unknown function
RPSI07_1291	…	4,106	Putative rhs-related transmembrane protein
RPSI07_1292	…	2,321	Exported protein of unknown function, RHS repeat
RPSI07_1293	…	407	Protein of unknown function
RPSI07_1294	…	950	Putative transposase
RPSI07_1295	…	428	Protein of unknown function
RPSI07_1296	…	299	Protein of unknown function
RPSI07_1297	…	101	Protein of unknown function
RPSI07_1298	…	596	Protein of unknown function
RPSI07_1299	…	1,682	Putative transporter
RPSI07_1300	…	1,664	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP(+))
RPSI07_1301	*lldD*	1,151	L-lactate dehydrogenase, FMN-linked
RPSI07_1302	*dctD*	1,328	Transcriptional response regulator, C4-dicarboxylate transport response regulator
RPSI07_1303	…	1,985	Putative sensor histidine kinase, transcriptional regulator of C4-dicarboxylate transport (DctB)
RPSI07_1304	…	626	Ferredoxin
RPSI07_1305	*nadB*	1,631	LL-aspartate oxidase (LASPO; quinolinate synthetase B)
RPSI07_1306	*nuoF*	1,265	NADH-quinone oxidoreductase subunit F

traits. Specifically, transfer of type III GMI1000 effectors RSc2130, RSc2132, and RSc2139, could be one explanation for the increased aggressiveness on tomato in the BCG20 transformant. However, Mukaihara and Tamura (2009) recently showed that RSc2130 and RSc2139 had no PIP box or –10 sequence, typical features of *hrp* regulation. Thus, these effectors would not be regulated by HrpB and could not be translocated into the plant cell. Therefore, the contribution of other transferred or lost genes in pathogenic evolution cannot be excluded. For example, among genes acquired or replaced in the BCG20 transformant, 28% of the encoded proteins were involved in carbohydrate metabolism. These metabolic changes could have consequences on its ability to invade new ecological niches (Ricard et al. 2006), especially in modifying its host spectrum. Six of the genes lost during the natural transformation event are possibly involved in the type II secretion system (Table 5), which is implicated in secretion of cell-wall-degrading exoenzymes such as pectinmethylesterase or endoglucanase. We hypothesize that the redundancy of this system in the GMI1000 and Psi07 genomes, where three clusters were found, offset the gene loss and led to a complementation of their functions (Preston et al. 2005). In the plant defense setting, the deletion of genomic regions in the recipient genome during the recombination step could also lead to the loss of effectors potentially recognized by plant defenses in a gene-for-gene manner, and could avoid the initiation of defense mechanisms (Grant et al. 2006). In BCG20, no open reading frames identified as coding for a type III effector or listed in the effector repertoires from *R. solanacearum* GMI1000 and BS048 tested by Wroblewski and associates (2009) were detected among the lost genes. Finally, genes directly involved in the aggressiveness of *R. solanacearum* strains on tomato still have to be identified, which will be the focus of a follow-up project. Nevertheless, these 39.4 and 33.4 kb of acquired and lost sequences represent good candidates to explain the host-specific pathogenic determinants because their transfer or loss modify aggressiveness on tomato but not on eggplant.

The evolution of plant–pathogen relationships is complex because of the ability of a pathogen to induce an infectious disease but also because plant defense mechanisms are varied, thus facilitating modification of their resistance levels. The "zigzag" model proposed by Jones and Dangl (2006) partially reflects this relationship. These authors suggest that natural selection drives pathogens to avoid plant defense responses by either shedding or diversifying the effector gene recognized by resistance proteins or by acquiring additional effectors that suppress effector-triggered immunity. Plant–microbe interactions are then governed by an arms race between pattern recognition receptors and resistance genes in plants and effectors in microbial pathogens (Boller and He 2009). The genetic rearrangements described in this study are consistent with the arms race concept, thus explaining the emergence of new pathogens. Therefore, it appears that one of main forces behind the evolution of the *R. solanacearum* genome and pathogenesis could be the result of: i) a subtle balance between loss and acquisition of foreign DNA during interspecific gene transfers and ii) transfer of new alleles via acquisition of SNP during RGT.

In conclusion, we focused on the role of HGT on pathogenic evolution in *R. solanacearum.* We demonstrated that large DNA regions of up to 80 kb can be naturally transferred between two phylotypes and that weakly aggressive strains could gain significant virulence following HGT among distantly related strains. Our results indicate that HGT is a potentially important mechanism of bacterial genomic plasticity that can increase their host range and aggressiveness on plants. The characterization of these HGT in vitro represents the first step in the validation process of our strategies for detecting gene flow, which will then be transposed to in planta conditions.

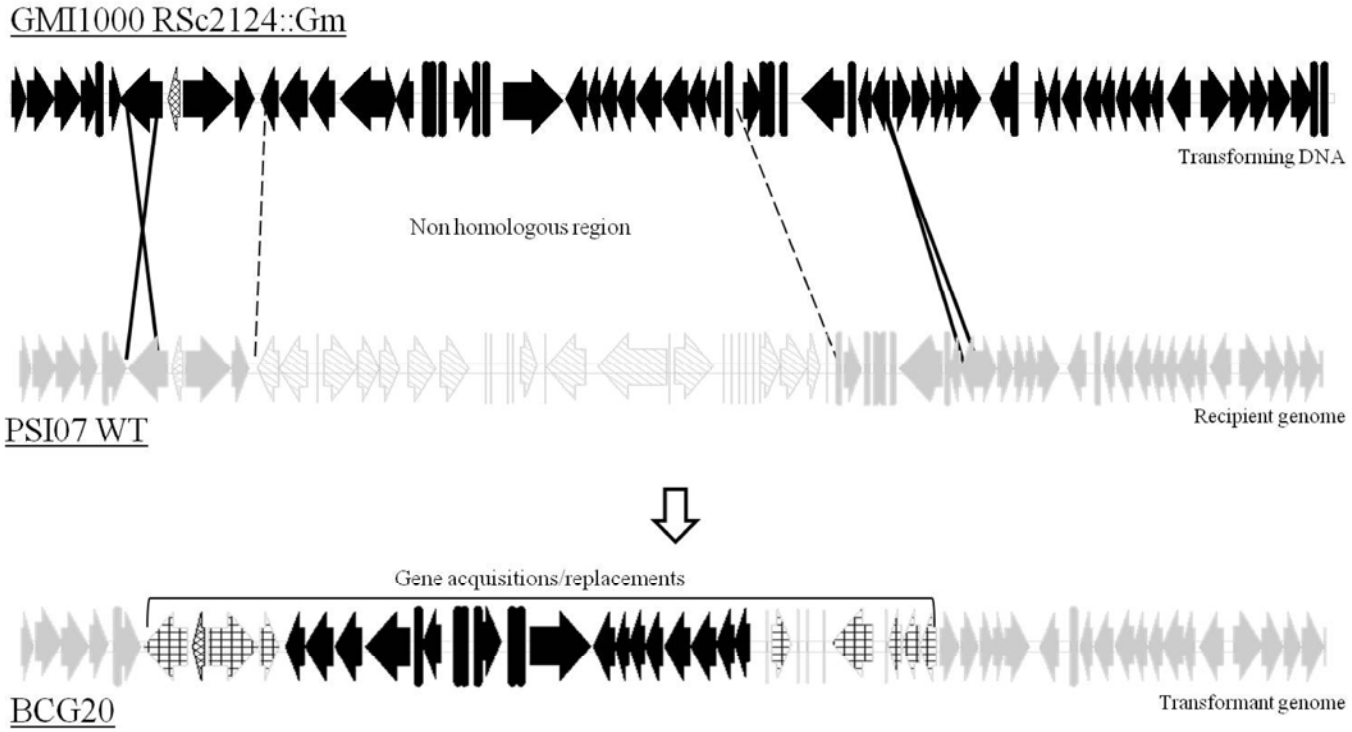

Fig. 1. Schematic representation of gene acquisitions and replacements in the transformant BCG20. A nonhomologous region in the Psi07 genome was replaced by natural transformation with a 40-kb region from the GMI1000 genome to generate the BCG20 transformant. More precisely, a DNA loss of approximately 33.4 kb, corresponding to 27 genes (in gray), occurred simultaneously with the integration of 39.4 kb of foreign (GMI1000) sequences containing 20 new open reading frames (in black) and 11 new alleles (dashed). Crosses represent the sites of crossover between the GMI1000 transforming DNA and the Psi07 genome.

Therefore, the second step will be demonstrating whether these phenotypic changes also occur within *R. solanacearum*'s natural habitat. In planta, bacteria are driven by exposure to the stress imposed by the plant's resistance response. Further studies will be conducted to evaluate the selection pressure imposed by the plant on HGT.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The phylotype I GMI1000 strain was chosen as DNA donor strain because its genome is completely sequenced and it was considered a highly virulent strain. Strains belonging to phylotypes II, III, and IV were used as recipient (Table 1). Strains CMR34 and JT516 strains were selected for phylotype II, CFBP3059 and NCPPB1029 for phylotype III, and Psi07 for phylotype IV. *R. solanacearum* wild-type and transformant strains were grown on B solid or liquid medium at 28°C (Boucher et al. 1985). *E. coli* cells were grown at 37°C on Luria-Bertani liquid or solid medium.

DNA microarray technology.

Microarray description. The microarray used in this study was described by Occhialini and associates (2005). All genes of GMI1000 were spotted twice on the microarray. It represents 5,074 65- or 70-mer oligonucleotides, which correspond to the 3,442 genes and 1,678 genes, respectively, described for the chromosome and megaplasmid of this strain. A set of 10 oligonucleotides corresponding to five *Corynebacterium glutamicum* genes plus "blank" controls in which buffer without oligonucleotide were spotted on this microarray served as negative controls.

DNA labeling and hybridization. Hybridizations on DNA microarrays were carried out as described by Guidot and associates (2007). Genomic DNA (2 µg), extracted with the Nucleospin tissue kit (Macherey Nagel, Düren, Switzerland), was labeled with Cy5 or Cy3 fluorescent dye (GE Healthcare, Piscataway, NJ. U.S.A.). Labeled DNA was purified with the CyScribe GFX Purification kit (GE Healthcare) and hybridization experiments were done in a Lucidea automated slide processor (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). For each experiment, Cy5-labeled DNA from the donor GMI1000 wild-type strain was hybridized with Cy3-labeled DNA from one of the recipient strains (wild-type strains or transformant clones). Hybridization and washing conditions were as described by Guidot and associates (2007). Each hybridization experiment was performed in duplicate.

Array scanning and analysis. Hybridized microarrays were scanned using the GenePix 4000A dual-channel (635- and 532-nm) confocal laser scanner (Axon Instruments, Union City, CA, U.S.A.) with a resolution of 10 nm/pixel. Scanning parameters were the same a those used by Guidot and associates (2007). Signals from individual arrays were quantified using ImaGene 5.6.1 software and analyzed with Genesight 3.5.2 software (BioDiscovery Inc., El Segundo, CA, U.S.A.). Empty spots and spots with impurities, high local background fluorescence, or weak intensity compared with the signal observed for hybridization to the negative controls were excluded from the analysis. As described by Guidot and associates (2007), for each spot, the ratio of the hybridization signal of the recipient strain versus the donor GMI1000 strain was calculated and $\log_2$ transformed, and the values thus obtained were normalized by subtracting the mean $\log_2$ ratio value calculated on a set of 2,690 conserved genes in *R. solanacearum* strains. Finally, the average $\log_2$ ratio value of the four spots representing each gene (two slides with two spots per gene) was calculated and used for further analysis. The lists of GMI1000 genes present in the recipient strains (wild-type strains and recombinant clones) were established by selecting genes for which the calculated average $\log_2$ ratio was above –2. The lists of GMI1000 genes transferred into the recombinant clones were established by comparing lists of the GMI1000 genes that were present in the wild-type strains and recombinant clones. In this study, we assumed that genomic synteny was the same in all *R. solanacearum* strains tested when localizing recombination events along the genome. All primary data from the microarray experiments as well as experimental protocols used are available from the ArrayExpress depository (accession numbers E-MEXP-2660).

Plasmid and genomic constructions.

The RSc0227, RSc0230, RSc2124, RSc2130, RSc2570, RSc2581, RSc3240, and RSc3255 genes from *R. solanacearum* GMI1000 strain were amplified by PCR with specific primers (Supplementary Table S2) to generate fragments of approximately 2 kb. Amplifications were performed with 200 ng of genomic DNA in the presence of 10% (wt/vol) dimethyl sulfoxide and *Platinum Taq* DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.). Fragments were then cloned into pGEM-T easy plasmid (Promega Corp., Madison, WI, U.S.A.). Resulting plasmids were digested around the middle of the GMI1000 fragment and ligated with gentamicin resistance gene *aac*C3-IV extracted by appropriate digestion of pMGm

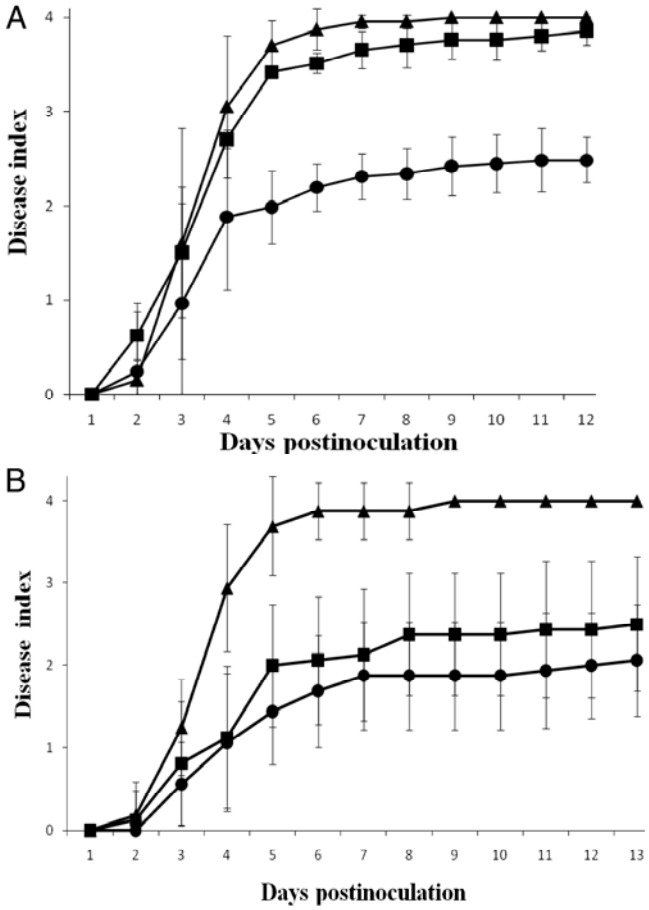

Fig. 2. Virulence of the BCG20 transformant on **A, '**Marmande' tomato and **B, '**Zebrina' eggplant. Inoculations were done by stem injection of GMI1000 (black triangle), PSI07 wild-type (black circle), and BCG20 (black square) strains. Plants were rated daily on a 0-to-4 disease index scale, where 1 = one leaf wilted, 2 = two or three leaves wilted, 3 = all the leaves are wilted, and 4 = the stem is bent. Values are averages of 42 plants ± standard deviation, and the experiments were performed in triplicate.

(Brau et al. 1984). The donor strain GMI1000 was then naturally transformed by the pRSc0227::Gm, pRSc0230::Gm, pRSc2124::Gm, pRSc2130::Gm, pRSc2570::Gm, pRSc2581::Gm, pRSc3240::Gm, and pRSc3255::Gm plasmids in order to construct mutant strains with allelic replacement into targeted genomic DNA regions. All DNA manipulations, including restriction enzyme digestions, ligations, and *E. coli* TOP10 cell transformations (Invitrogen), were carried out using standard procedures.

Natural transformation.

R. solanacearum strains were transformed according to the protocol described by Bertolla and associates (1997) with some modifications. Competent cells (50 µl) were transformed with 200 ng of plasmid DNA or 400 ng of genomic DNA. Five different recipient strains belonging to phylotypes II, III, and IV (CMR34, JT516, CFBP 3059, NCPPB 1029, and Psi07) were naturally transformed by the genomic DNA from GMI1000 strains gRSc0227::Gm, gRSc0230::Gm, gRSc2124::Gm, gRSc2130::Gm, gRSc2570::Gm, gRSc2581::Gm, gRSc3240::Gm, and gRSc3255::Gm. The transformation frequencies were defined by the number of colonies growing on the appropriate selective media and exhibiting the expected DNA fragments after PCR amplification, divided by the total number of viable bacteria. For each transformation experiment, the spontaneous mutation rate was determined by plating on selective medium containing gentamicin (12 µg/ml) and using transformation controls carried out without DNA.

Transformant verification.

Integration of chimeric constructions of the gentamicin-resistant plasmid series into the *R. solanacearum* GMI1000 genome was confirmed by PCR using primer sets for each target gene. Moreover, the sensitivity of the gentamicin-resistant clones to ampicillin confirmed the absence of replicative plasmids in the transformant.

Recombinants from CMR34, JT516, CFBP3059, NCPPB-1029, and Psi07 were also checked by PCR analysis using primers in the *acc3*-IV gene, the target gene, and different genes around the target gene. Acquisition of large DNA regions originating from GMI1000 were then detected by CGH on microarrays. For clones in which large GMI1000 DNA regions were acquired, recombination sites between transforming DNA and the genome of recipient strains were investigated and accurately localized by PCR amplification, cloning, and sequencing.

Plant inoculation.

Tomato (Marmande; Vilmorin, La Ménitré, France) or eggplant ('Zebrina'; INRA Avignon, France) plantlets at the four-leaf stage were inoculated by stem injections. Bacterial suspensions were performed in sterile water and approximately 100 µl of 10^7 CFU/ml of this suspension was inoculated in the stem. Plants were incubated in a growth cabinet (Sanyo, Leicestershire, U.K.) at 28°C, 80% humidity, and with 16 h of daylight and 8 h of moonlight, alternately. The disease index was evaluated for each plant as described by Roberts and associates (1988), where 0 = no symptoms, 1 = one leaf wilted, 2 = two or three leaves wilted, 3 = all leaves wilted, and 4 = stem folded. Experiments were done in triplicate on 14 plants per strain, yielding a total of 42 plants.

Statistical analysis.

Disease progress curve results were analyzed using repeated measures and analysis of variance over the time course of the experiment. Tukey's honestly significant difference multiple comparisons test was used to compare disease index means between strains on specific days during the assay. For strain Psi07, the transferred DNA region, including recombination sites and 20 kb of the neighboring sequence, were analyzed. The GC content, χ sequence, and SNP density distributions were evaluated in 100-bp (GC content and SNP density) or 500-bp (χ sequence density) windows along the 100-kb sequence. All analyses were performed using R software and, especially, the SeqIn software package was used (Charif and Lobry 2007).

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