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Report

Bacterial Evolution by Genomic Island Transfer Occurs via DNA Transformation In Planta

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Summary

Our understanding of the evolution of microbial pathogens has been advanced by the discovery of "islands" of DNA that differ from core genomes and contain determinants of virulence [1, 2]. The acquisition of genomic islands (GIs) by horizontal gene transfer (HGT) is thought to have played a major role in microbial evolution. There are, however, few practical demonstrations of the acquisition of genes that control virulence, and, significantly, all have been achieved outside the animal or plant host. Loss of a GI from the bean pathogen *Pseudomonas syringae* pv. *phaseolicola* (*Pph*) is driven by exposure to the stress imposed by the plant's resistance response [3]. Here, we show that the complete episomal island, which carries pathogenicity genes including the effector *avrPphB*, transfers between strains of *Pph* by transformation in planta and inserts at a specific *att* site in the genome of the recipient. Our results show that the evolution of bacterial pathogens by HGT may be achieved via transformation, the simplest mechanism of DNA exchange. This process is activated by exposure to plant defenses, when the pathogen is in greatest need of acquiring new genetic traits to alleviate the antimicrobial stress imposed by plant innate immunity [4].

Results and Discussion

Transfer of PPHGI-1 between Strains of *Pseudomonas syringae* pv. *phaseolicola* In Planta

Strains of *Pseudomonas syringae* (*P.s.*) form a group of bacteria that are divided into a number of pathovars depending on the plant that they infect [5]. The pathogens manipulate their hosts by delivering effector proteins into the plant cell cytoplasm through the conserved type III secretion system [6]. Plants have evolved resistance proteins that recognize a subset of *P. s.* effectors, and these avirulence (Avr) proteins trigger a defensive hypersensitive reaction (HR), generating an antimicrobial environment and leading to the restriction of bacterial colonisation [7].

Several *avr* genes have been identified as controlling interactions between the bean pathogen *P.s.* pv. *phaseolicola* (*Pph*) and its host *Phaseolus vulgaris* [4, 5]. One, *avrPphB* (also named *hopAR1*), leads to the activation of the HR in bean cultivars such as Tendergreen (TG), which carry the *R3* resistance gene. In *Pph*, *avrPphB* is located on a 106 kb genomic island (GI), designated PPHGI-1 [3], that has a number of classic features described in other bacterial GIs; for example, it is present in the genomes of some strains but not others, is flanked by tRNA loci, and carries genes coding for DNA recombination and, potentially, conjugation [1, 3]. GIs have been found in a wide range of organisms, including examples in medically important pathogens [8], plant pathogens [9], and environmental bacteria [10]. PPHGI-1 is lost from the genome of *Pph* during infection of bean cultivars undergoing the HR, which drives the evolutionary selection of virulent *Pph* strains that no longer trigger *R3*-based resistance [3]. Excision of PPHGI-1 from *Pph* and generation of a circular episome occurs though specific recombination between the *att* locus in $tRNA^{lys}$ at either end of the GI and is mediated by the PPHGI-1-encoded integrase, XerC [3]. Here, we have aimed to investigate whether PPHGI-1 has the potential to be transferred between bacteria.

Transfer of PPHGI-1 from *Pph* 1302A (race 4) to *Pph* 1448A (race 6) was initially investigated via coinoculation of the donor and recipient strains into the resistant bean cultivar TG. The donor strain was *Pph* 1302A::NCR, which has a Kanamycin (kan)-resistance gene inserted into a noncoding region of PPHGI-1 [3], and the recipient strain was a spontaneous Rifampicin (rif)-resistant mutant of *Pph* 1448A (1448ARif). Overnight broth cultures of the donor and recipient were mixed in equal amounts and coinoculated into TG bean leaves (see Supplemental Experimental Procedures, available online). The plants were incubated in a growth cabinet at 23°C for 48 hr before the inoculated area was harvested, homogenized for extraction of the bacteria, and plated onto selective media containing antibiotics. After 3 days, a number of kan- and rif-resistant colonies were obtained, suggesting transfer of PPHGI-1 from 1302A::NCR to 1448ARif. Transfer frequency (the number of transconjugants divided by the number of recipients) was calculated to be $3.8 \pm 0.48 \times 10^{-8}$ (Table 1). We used plasmid profiles and the polymorphism in *avrPphE* (*hopX*) to show that transconjugants were *Pph* 1448ARif and not spontaneous *Pph* 1302ARif-resistant mutants (Figures 1A and 1B). The presence of PPHGI-1 in *Pph* 1448ARif was determined by polymerase chain reaction (PCR) of 15 genes spread along the island, confirming that PPHGI-1 remained intact after transfer to *Pph* 1448ARif (Table S1). PCR with previously used primers [3] also confirmed that PPHGI-1 is able to excise and form a circular intermediate from the *Pph* 1448ARif genome (Table S1).

Pathogenicity tests on bean pods demonstrated that transfer of PPHGI-1 caused a change in the virulence of *Pph* 1448ARif from virulent to avirulent on bean cultivar TG, as expected from the acquisition of *avrPphB* (Table S1). Repeated passage of *Pph* 1448ARif::PPHGI-1 through TG led to loss of PPHGI-1 from the 1448ARif genome at the same rate as from its progenitor *Pph* 1302A, providing evidence that PPHGI-1 retains mobility in planta after transfer to *Pph* 1448ARif (Table S1).

Integration of PPHGI-1 into *att* Site 2 in the *Pph* 1448ARif Genome

The *Pph* 1448A genome (accession number NC005773) contains two 52 bp *att* sites with the same sequence as the

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Table 1. Transformation Occurs In Planta but Not In Vitro at 23°C: Transfer Frequency of PPHGI-1 to *Pph* Strain 1448ARif under Various Conditions, All after Incubation for 48 Hours

Condition	PPHGI-1 Donor	Response to Infection	Mean Transfer Frequency ± SEM ($\times 10^{-8}$)
Bean leaves			
TG	1302A::NCR cells	HR	3.80 ± 0.48a
TG	1302A::NCR DNA		3.95 ± 0.27a
TG	1302A::NCR DNA 1 in 3 dilution		2.42 ± 0.25b
TG	1302A::NCR nonviable cells		3.60 ± 0.45a
CW	1302A::NCR cells	Disease	2.25 ± 0.79bc
CW	1302A::NCR DNA		3.57 ± 0.39a
CW	1302A::NCR DNA 1 in 3 dilution		2.30 ± 0.23bc
Other plants			
Arabidopsis	1302A::NCR cells	Basal resistance	1.20 ± 0.22c
Arabidopsis	1302A::NCR DNA		1.60 ± 0.19c
Tobacco	1302A::NCR cells	HR	2.70 ± 0.45ab
Tobacco	1302::NCR DNA		2.60 ± 0.47ab
In culture			
LB broth 23°C	1302A::NCR cells		0
LB broth 4°C	1302A::NCR cells		0.38 ± 0.13d
MM broth 23°C[a]	1302A::NCR cells		0
MM broth 4°C	1302A::NCR cells		0

Bean cv Tendergreen (TG) leaves develop a hypersensitive reaction (HR) after inoculation with 1302A; whereas cv Canadian Wonder (CW) is susceptible to 1302A and develops disease symptoms. An HR develops in tobacco but not in *Arabidopsis* after inoculation with *Pph*. Additional abbreviations are as follows: MM, minimal medium; LB, Luria Bertani. Means are from four replicates. Different letters indicate significant differences at $p < 0.05$, assessed with Student's t test.

[a] The same result was observed with M9 minimal medium and *Hrp*-inducing medium (HIM) with fructose, sucrose or glucose as the carbon source.

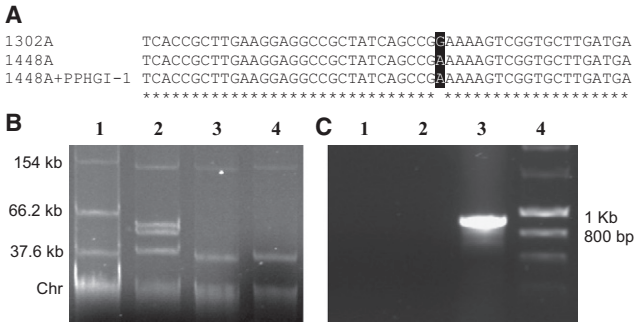

Figure 1. Transfer of PPHGI-1 from *Pph* Strain 1302A::NCR to *Pph* Strain 1448ARif In Planta

(A) Sequencing *avrPphE* confirms recipient identity. ClustalW multiple sequence alignment of a section of sequence of *avrPphE* from *Pph* 1448ARif, *Pph* 1448ARif::PPHGI-1, *Pph* 1302A::NCR. The single-nucleotide polymorphism between *Pph* 1302A and *Pph* 1448A is highlighted in black.

(B) Plasmid profiles are retained by the recipient strain. Lanes are as follows: 1, *E.coli* 39R861 marker strain containing plasmids of known size; 2, *Pph* 1302A::NCR; 3, *Pph* 1448ARif; 4, *Pph* 1448ARif::PPHGI-1; Chr, chromosomal DNA.

(C) Location of PPHGI-1 insertion site in *Pph* 1448ARif recipient. PCR amplification of the junction at which PPHGI-1 integrates into the *Pph* 1448ARif chromosome with the use of primer pair attJF2 and xerCJF. Primer attJF2 included part of *att* site 2 in *Pph* 1448A, and primer xerCJF included part of PPHGI-1. Lanes are as follows: 1, *Pph* 1302A::NCR; 2, *Pph* 1448ARif; 3, *Pph* 1448ARif::PPHGI-1; 4, DNA molecular size marker.

att borders of PPHGI-1 in *Pph* 1302A (tRNAlys genes designated PSPPH_0745 and PSPPH_3762, site 1 and site 2, respectively). For determining which *att* site might integrate PPHGI-1, primer pairs (Table S2) were designed to amplify across putative integration junctions that included the tRNA locus (*att* site) of *Pph* 1448A and either the left-hand or the right-hand border of PPHGI-1. PCR of 15 different 1448ARif::PPHGI-1 isolates showed that PPHGI-1 integrated preferentially into *att* site 2 (14 strains) in comparison to *att* site 1 (one strain). Figure 1C shows the amplification of PPHGI-1 from *att* site 2 in one transformant (see also Figure S1).

Transfer of PPHGI-1 Occurs via Transformation

The three mechanisms by which DNA can be transferred between bacteria are conjugation, transformation, and transduction. There are a number of reports of horizontal gene transfer (HGT) between bacteria exploiting these methods. For example, plasmids transfer via conjugation in the rhizosphere [11] and on the leaf surface [12]. Some bacteria are also naturally competent for transformation; for example, *Acinetobacter* sp. [13]. and *Ralstonia solanacearum* [14]. Horizontal transfer of GIs has been demonstrated experimentally in vitro but *not* within a host. For example, the high-pathogenicity island of *Yersina* [15, 16] and the *Salmonella* GI1 [17, 18], by conjugation, as well as the *P. aeruginosa* pathogenicity island PAPI-1, by an undetermined mechanism [19]. Transduction has been demonstrated for the *Vibrio cholerae* pathogenicity island (VPI), which can be transferred between O1 serogroup strains via a generalized transducing phage CP-T1 [20]. None of these studies has demonstrated GI transfer during infection of a host.

For investigating whether transfer of PPHGI-1 occurred via conjugation or transformation, donor *Pph* 1302A::NCR cells were heat killed (80°C, 15 min) and then coinoculated with *Pph* 1448ARif into TG leaves. Transfer of PPHGI-1 occurred at the same frequency as that of viable cells (Table 1), demonstrating transfer by transformation rather than by conjugation. For confirmation of this route, circularized DNA (expected to include PPHGI-1) was extracted from *Pph* 1302A::NCR and mixed with *Pph* 1448ARif cells before inoculation. Transfer of PPHGI-1 occurred at frequencies similar to those of viable cells (Table 1), supporting the conclusion that transformation was indeed the mechanism of transfer. The differential transformation rates recorded in resistant TG and susceptible Canadian Wonder (CW) leaves with the use of live bacteria were not observed with the plasmid DNA donor (Table 1). Significantly, no transfer was observed when plasmid DNA was mixed with *Pph* 1448ARif cells and incubated in vitro at 23°C. These results suggested that *Pph* 1448ARif becomes naturally competent in planta. In order to test whether the competent cells take up other DNA and not specifically PPHGI-1, we mixed cells of *Pph* 1448ARif with pBBR1MCS-2 [21] DNA and inoculated them either into a 10 ml Luria Bertani (LB) broth or into a TG leaf and incubated them for 24 hr before plating them onto selective media. No transformants were observed from the in vitro culture, whereas a transformation frequency of $2.25 \pm 0.99 \times 10^{-7}$ was recorded in planta. These results show that the plant environment greatly increases the transformation competency of 1448A, as well as enhancing excision of the GI during the HR.

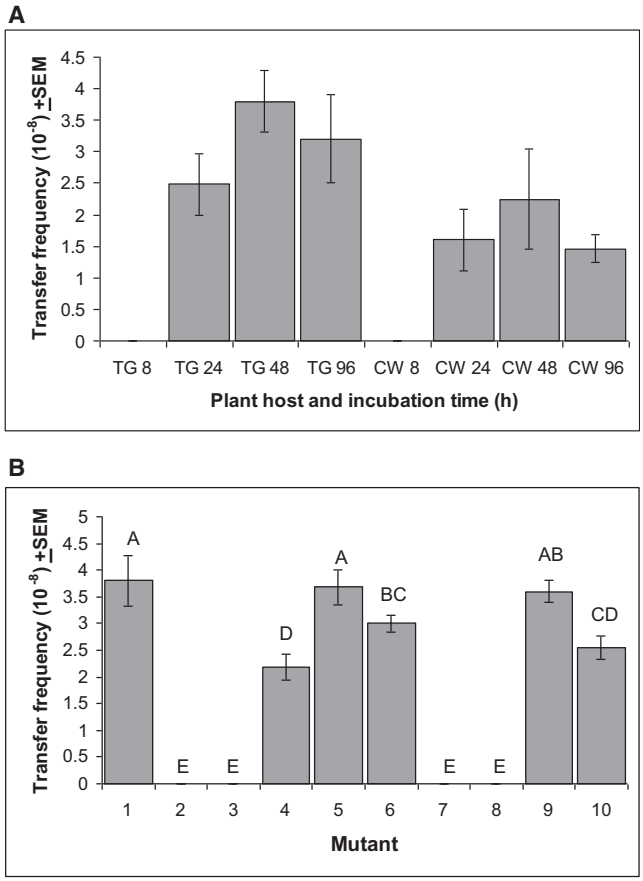

Figure 2. Effect of Incubation Time and Selected Mutations in PPHGI-1 on Transfer of the Island from *Pph* 1302A::NCR to *Pph* 1448ARif

(A) Transfer of PPHGI-1 was analyzed after 8, 24, 48, and 96 hr in both resistant Tendergreen (TG) and susceptible Canadian Wonder (CW) bean cultivars. No transfer was observed after 8 hr. Statistical analysis of data combined for each cultivar revealed significant differences between the two plant hosts. Student's t test $p < 0.05$.

(B) Insertional mutants were created in nine potentially important genes from PPHGI-1, and the effects of mutation on transfer frequency of PPHGI-1 were analyzed. Equal amounts of mutagenized *Pph* 1302A::NCR and *Pph* 1448ARif were mixed at a concentration of 8×10^8 cells per ml, coinoculated into TG, and incubated for 48 hr. Data are presented as follows: 1, wild-type 1302A::NCR and insertional mutants created in genes; 2, *xerC* (ORF.100); 3, ORF.99; 4, bacteriophytochrome (ORF.29); 5, *virB4* (ORF.84); 6, *pilN* (ORF.18); 7, *rulB* (ORF.96); 8, *xerD* (ORF.54); 9, topoisomerase (ORF.56); 10, *avrPphB* (ORF.16). All genes are designated as in Pitman et al. [3]. Statistical analysis by ANOVA and Student's t test showed significant differences in transfer frequencies between 1302A::NCR (column 1) and certain mutants ($p < 0.05$), as indicated by different letters above bars. Data are means ± standard error of the mean (SEM) from four replicates.

Determination of Optimum Conditions for Transfer of PPHGI-1

We determined the optimum conditions for transfer of PPHGI-1 (Figure 2A). Frequency was highest 48 hr after inoculation and was significantly higher in the resistant bean variety TG than in the susceptible CW over time. The nonhost plants *Arabidopsis* (Nd-0) and tobacco were also tested, and although transfer was observed, it was at a lower level than than in TG or CW (Table 1). An incubation temperature of 23°C was used for all in planta experiments, and at this temperature, no transformants were observed in vitro. However, incubation at 4°C in vitro produced a few transformants (transfer frequency of $0.38 \times 10^{-8} \pm 0.13$), 10-fold lower than that observed in planta. The best transfer conditions were determined to be in planta coinoculation with the use of the resistant bean cultivar TG and incubation for 48 hr at 23°C.

Which PPHGI-1 Genes Are Required for Transfer?

Nine genes within PPHGI-1, including those that are homologous to genes for DNA recombination and conjugation, were tested for their potential involvement in HGT (Figure 2B). Gene-specific knockout mutations of the PPHGI-1 genes were constructed, and coinoculations were carried out with the use of these mutants and *Pph* 1448ARif into leaves of the resistant bean cultivar TG under optimal transfer conditions. No transformants were found after mutation of the integrase encoding *xerC* and *xerD* genes; ORF 99, which is transcriptionally linked to *xerC*; and *rulB*, which is involved in DNA repair. Importantly, each of these mutations also prevented PPHGI-1 from forming a circular intermediate (data not shown), indicating that they are probably involved in island excision or stability as a circular molecule. None of the other mutants was impaired in its ability to form a circular intermediate. However, some mutations in PPHGI-1 reduced but did not prevent transfer to *Pph* 1448ARif; for example, this was observed in bacteriophytochrome and *pilN* mutants. Significantly, mutation of *avrPphB* reduced transfer frequency by approximately 30%, to the same frequency as that observed in susceptible host CW (Figure 2B and Table 1). This result was consistent with the *avrPphB* mutant failing to cause the HR in TG.

Transfer of PPHGI-1 In Vitro with the Use of Apoplastic Fluids

Plant factors released during the HR appeared to promote HGT. We therefore tested the effect of intercellular (apoplastic) fluids recovered from bean leaves on transformation frequency in culture. Transfer of PPHGI-1 was observed in vitro if minimal medium was supplemented with apoplastic fluid collected both before and after bacterial inoculation. Without the plant extracts, no transfer was detected at 23°C in vitro. Transfer was significantly higher when media were supplemented with fluids from leaves of the resistant cultivar TG that was undergoing the HR than from susceptible cultivar CW or uninoculated tissues (Table 2). In all cases, the addition of apoplastic fluid increased levels of transformation.

Conclusion

We have shown that the horizontal transfer of a GI (PPHGI-1) between strains of the plant pathogen *Pph* occurs in planta. Previously, GIs have been shown to transfer between bacteria in vitro but not in the host during the infection process [15–19]. Yacoubi et al. [22] have demonstrated the transfer of a plasmid containing a pathogenicity gene, *pthB*, in planta through conjugative transfer, but we show for the first time that a plant pathogen GI transfers in vivo via a natural transformation process. Other bacteria have also been shown to be naturally competent for transformation. For example, by the construction of strains that contained identical DNA sequences in the donor and recipient genomes, *Acinetobacter* sp. was shown to become competent to take up antibiotic genes released from *Ralstonia solanacearum* in planta [13]. Coupat et al. [14] also showed that *Ralstonia solanacearum* is transformable in vitro and in planta, having the ability to acquire small fragments of exogenous DNA. The transfer of the large GI,

Table 2. Promotion of Transformation by Plant Extracts: Transfer Frequency of PPHGI-1 from 1302A::NCR into *Pph* 1448ARif in Cultures Supplemented with Apoplastic Fluid

Source of Apoplastic Fluid	Dilution Factor of Apoplastic Fluid[a]	Mean Transfer Frequency ± SEM ($\times 10^{-8}$)
Uninoculated TG leaves	1 in 10	0.80 ± 0.08c
Uninoculated TG leaves	1 in 5	1.05 ± 0.12b
Uninoculated TG leaves	1 in 2	1.40 ± 0.11b
TG leaves undergoing the HR	1 in 2	2.50 ± 0.11a
Uninoculated CW leaves	1 in 2	1.20 ± 0.24b
Diseased CW leaves	1 in 2	1.40 ± 0.42b

Apoplastic fluids were recovered from leaves of uninoculated cvs Tendergreen (TG) or Canadian wonder (CW), from TG undergoing the HR to *Pph* 1302A, and from CW undergoing a disease response after inoculation with *Pph* 1448ARif. HR denotes hypersensitive reaction.

[a] M9 minimal medium was diluted with apoplastic fluid. All cultures were incubated at 23°C, static for 96 hr. Means are from four replicates. Different letters (a, b, c) indicate significant differences at $p < 0.05$, obtained with Student's t test. No transfer was detected in minimal medium without apoplastic fluid.

PPHGI-1, highlights the potential for the movement of pathogenicity islands (PAIs) by transformation within infected hosts.

The work described here demonstrates that transfer of PPHGI-1 between *Pph* strains by transformation requires four distinct processes: excision of the island from the chromosome, release of the circular episome from the bacterium, relocation into competent bacterial cells, and integration into a specific *att* site. The discovery that transfer frequencies are reduced during coinoculation with a *pil* mutant donor (Figure 2B) indicates the possibility of conjugation being involved, but we show compelling evidence that transformation is sufficient for island transfer to recipient bacteria. In particular, transfer was achieved from purified DNA recovered via plasmid extraction protocols that recovered circularized PPHGI-1. The low level of excised episome that occurs in *Pph* grown in culture may have been sufficient for transfer [3], although stress (e.g., during the heat treatment used for killing bacteria) may have enhanced excision of PPHGI-1. The higher rates of transformation observed during the resistant reaction in TG leaves are probably associated with stress-induced excision of the GI and release of DNA after bacterial cell death within the antimicrobial environment generated by the HR. The lower rates of transformation observed during the disease reaction in CW reflect the lack of bacterial stress within the benign conditions of susceptible leaf tissue. Nonhost resistance in tobacco and *Arabidopsis* also promoted transfer of PPHGI-1. We have no evidence that the third component of the transfer process, transformation competence, is itself enhanced by plant resistance, because donor DNA was transformed at similar rates in both resistant and susceptible leaves.

It may, however, be simplistic to use general "stress" to explain enhanced excision and transfer of the GI. The enhancement of transformation frequencies in vitro by the addition of apoplastic fluids to minimal medium indicates that specific plant metabolites released from cells into intercellular space may condition bacteria to undertake the transfer process. Importantly, apoplastic fluids are known to support rapid bacterial multiplication, whether recovered from resistant or susceptible plant reactions. We suggest, therefore, that sublethal stress and exposure to specific but as yet unknown plant metabolites leads to island excision, as well as competence for DNA acquisition.

The role of HGT in microbial evolution has largely been inferred from bioinformatic analysis of bacterial genomes [4]. The dynamics and mechanisms of gene exchange, particularly the transfer of GIs within host tissues, has rarely been addressed. Use of the *Pph* and bean model system has proved beneficial in allowing mobilization of PPHGI-1 to be demonstrated within the plant, initially through following loss of the *avrPphB* avirulence gene [3] and now through the novel demonstration of HGT by in planta transformation. Under the conditions of our experiments, paradoxically, uptake of *avrPphB* within the GI disadvantages the recipient bacteria within resistant cultivar TG leaves because it leads to the triggering of the defensive HR. However, genes on PPHGI-1 may have advantages to 1448ARif at other stages of the disease cycle that have not been tested in this investigation. The principle demonstrated here would also apply to GIs and PAIs that carry beneficial traits for the bacteria leading to the evolution of virulence. Further work is needed to confirm that the transfer of other bacterial GIs occurs via transformation during plant or animal infections, to assess the contribution of the process to the evolution of microbial pathogenicity, and to exploit our understanding of HGT for durable disease control.

Supplemental Data

Supplemental Data include detailed Supplemental Experimental Procedures, one figure, and three tables and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01590-5.

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