RESEARCH LETTER

Genomic and phenotypic characterization of a nonpigmented variant of *Pantoea vagans* biocontrol strain C9-1 lacking the 530-kb megaplasmid pPag3

Theo H.M. Smits[1], Fabio Rezzonico[1], Cosima Pelludat[1], Alexander Goesmann[2], Jürg E. Frey[1] & Brion Duffy[1]

[1]Division of Plant Protection, Agroscope Changins-Wädenswil ACW, Wädenswil, Switzerland; and [2]CeBiTec, University of Bielefeld, Bielefeld, Germany

Correspondence: Theo H.M. Smits, Division of Plant Protection, Agroscope Changins-Wädenswil ACW, Schloss, Postfach, CH-8820 Wädenswil, Switzerland. Tel.: +41 44 783 6111; fax: +41 44 783 6305; e-mail: theo.smits@acw.admin.ch

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Abstract

A 530-kb megaplasmid pPag3 contributing 10.8% of the total genome of *Pantoea vagans* biocontrol strain C9-1 was sequenced. A rare nonpigmented variant C9-1W was obtained and shown to have lost pPag3, but retained all other plasmids (pPag1, pPag2). Phenotypic characterization of the variant confirmed the function of several annotated genes that may influence ecological fitness and efficacy. Metabolic profiling revealed important plasmid-based carbon utilization phenotypes. Plasmid loss resulted in thiamine auxotrophy, absence of carotenoid pigmentation, desferrioxamine diffusible siderophore biosynthesis, inherent ampicillin resistance and expression of AI-1 quorum-sensing signaling. This confirmed the functional expression of the corresponding genes located on pPag3 in *P. vagans*.

Introduction

Pantoea is a diverse genus, with most species considered to be ubiquitous plant epiphytes and often isolated from a wide range of other environmental habitats (e.g., soil, water, insect/animal gut and clinical samples). Some plant isolates have demonstrated strong beneficial activity as biological control agents for pre- and postharvest fungal and bacterial diseases (Braun-Kiewnick *et al.*, 2000; Bonaterra *et al.*, 2005; Francés *et al.*, 2006). The type species, *Pantoea agglomerans*, has undergone extensive taxonomic rearrangement emerging from the *Enterobacter agglomerans–Erwinia herbicola* complex (Ewing & Fife, 1972; Rezzonico *et al.*, 2009). Recently, several closely related species, such as *P. vagans*, have been newly described based on molecular analysis (e.g., multilocus sequence analysis, DNA–DNA hybridization) (Brady *et al.*, 2008, 2009; Rezzonico *et al.*, 2009).

Strain C9-1 is an important biocontrol agent (Ishimaru *et al.*, 1988; Johnson & Stockwell, 1998) that is registered in the United States and Canada as Blight Ban C9-1 (NuFarms America). It is one of the most effective commercial agents against fire blight, a major threat to global pome fruit production caused by the related enterobacterium *Erwinia amylovora* (Johnson & Stockwell, 1998). Recently, C9-1 has been reassigned from *P. agglomerans* to the novel species *P. vagans* based on its *gyrB* sequence (Brady *et al.*, 2009; Rezzonico *et al.*, 2009).

Pantoea agglomerans and *P. vagans* isolates are generally considered nonpathogenic. Most *P. agglomerans* isolates lack virulence determinants such as type III secretion systems (T3SS), while some contain a T3SS described as a nonpathogenic type (Rezzonico *et al.*, 2009). The phytopathogenicity of subspecies *P. agglomerans* pv. *gypsophilae* and pv. *betae* can be attributed to recently acquired large plasmids that carry a pathogenic type of T3SS and other virulence determinants (Ezra *et al.*, 2000; Mor *et al.*, 2001; Guo *et al.*, 2002; Manulis & Barash, 2003; Nissan *et al.*, 2006). Virulence and ecological fitness genes in phytopathogenic *P. agglomerans* pathovars, *Pantoea stewartii* and *Pantoea ananatis* are regulated by an autoinducer-1 quorum-sensing system involving *N*-acyl-homoserine lactone (AHL) signals (von

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Bodman *et al.*, 2003; Morohoshi *et al.*, 2007; Chalupowicz *et al.*, 2008, 2009).

Several *Pantoea* species are yellow pigmented (Grimont & Grimont, 2005) due to production of carotenoids (Sandmann *et al.*, 1990; Hundle *et al.*, 1994). Nonpigmented variants have been reported, arising spontaneously at a low frequency (10^{-2}–10^{-3}) after extended cultivation on nutrient-rich laboratory media (Chatterjee & Gibbins, 1971; Gantotti & Beer, 1982; Lindh *et al.*, 1991). These reports described physiological changes, such as thiamine deficiency and negative reactions with citrate or maltose, and lack of reversion to a wild-type phenotype, suggesting that such phenotypic changes are due to plasmid loss, although this has never been confirmed experimentally.

We have found that *P. vagans* C9-1 carries three plasmids: two plasmids of 168 kb (pPag1) and 166 kb (pPag2), and the 530-kb megaplasmid named pPag3 (Smits *et al.*, 2009). The phenotypic effects of these plasmids in *P. vagans* C9-1 have not been described previously. Sequence analysis of the megaplasmid revealed that carotenoid biosynthesis is encoded on plasmid pPag3. We obtained a nonpigmented variant of *P. vagans* C9-1 that lost the ability to synthesize thiamine and metabolize maltose, features that were also encoded by plasmid pPag3 genes. The aim of this study was to use the nonpigmented variant that lacks pPag3, representing over 10% of the total genome, in order to confirm functional phenotypes for annotated plasmid genes.

Materials and methods

Strains, growth media and conditions

Bacteria were routinely grown at 28 °C on Luria–Bertani (LB) (Sambrook *et al.*, 1989). Carbon source (glucose, sorbitol, maltose or sucrose) and thiamine (5 μg mL^{-1}) utilization assays were conducted in amended M9 minimal medium (Sambrook *et al.*, 1989). Resistance to ampicillin (2.5–200 μg mL^{-1}) or tellurite (50 μg mL^{-1}) was determined on amended LB agar. Siderophore biosynthesis was assessed on chrome azurol S (CAS) agar (Schwyn & Neilands, 1987) with 5 μL of an LB preculture spotted onto the medium. The AHL biosensor strain *Agrobacterium tumefaciens* NTL4[pZLR4] was grown in LB medium containing 30 μg mL^{-1} gentamicin (Luo *et al.*, 2003).

Plasmid curing

The method of Gantotti & Beer (1982) was used to generate a nonpigmented variant of *P. vagans* C9-1. An LB culture of C9-1 wild type was incubated at 38 °C for 24 h, and 10^{-5}–10^{-6} dilutions were plated onto LB agar and incubated at 37 °C for 5 days. The nonpigmented variant C9-1W that was obtained was tested for the presence of the three C9-1 plasmids using PCR. Oligonucleotides (Supporting Information, Table S2) were synthesized by Sigma-Genosys (Steinheim, Germany). The HotStarTaq Master Mix kit (Qiagen, Hilden, Germany) was used as described by the supplier. Chromosomal DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). PCR was performed as described elsewhere (Innis *et al.*, 1990). PCR products were visualized on 1.5% agarose gels (Sambrook *et al.*, 1989).

Metabolic profiling

The metabolic profiles of *P. vagans* C9-1 and C9-1W were obtained using Biolog GN2 and AN plates (Hayward, CA). Precultures were grown in M9 medium with 5 mM glucose and allowed to grow to the late stationary phase to ensure complete substrate utilization. Cultures were centrifuged at 4000 ***g*** and the cell pellets were washed once before resuspending in a fresh M9 medium. The attenuance at 600 nm ($A_{600\,nm}$) was set to 0.15 and each microtiter plate well was inoculated with 100 μL of this bacterial suspension. The plates were scored after 1, 2 and 5 days of incubation at 28 °C.

Autoinducer bioassay for the detection of AHL biosynthesis

For AHL bioassays, cell-free filtrates (150 μL) from stationary-phase cultures of *P. vagans* (16 h, 28 °C) were combined with 150 μL of a washed stationary-phase culture of *A. tumefaciens* NTL4[pZLR4] (Luo *et al.*, 2003) (16 h, 28 °C) in a fresh LB medium containing 0.1% 5-bromo-4-chloro-3-indolyl β-galactoside (X-Gal). The production of AHL was determined qualitatively by observing a change to blue in the color of the microculture over the course of 3 days.

Plasmid sequence analysis

The genome sequence of plasmid pPag3 from *P. vagans* C9-1 (Smits *et al.*, 2009) was annotated using GenDB (Meyer *et al.*, 2003) and was deposited at GenBank (Accession number CP001895). Sequence manipulations were performed using the Lasergene package (DNASTAR, Madison, WI). Additional BLAST searches (Altschul *et al.*, 1990) were performed at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and discussion

The sequence of pPag3

The genome sequence of *P. vagans* C9-1 (Smits *et al.*, 2009) revealed a 530-kb plasmid, designated pPag3, encoding the carotenoid biosynthesis cluster *crtEXYIBZ* (Pvag_pPag30170–Pvag_pPag30175) as the most prominent feature. The encoded proteins share 91–97% sequence identity to the respective proteins of *P. agglomerans* pv. *milletiae* Wist 801 (GenBank: AB076662) and 70–89% to those of

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P. ananatis 20D3^T (Misawa *et al.*, 1990). The plasmid also carries four thiamine biosynthesis genes (*thiOSGF*; Pvag_pPag30158–Pvag_pPag30161) and a complete maltose metabolism gene cluster (Pvag_pPag30206–Pvag_pPag30215). The genes have an identical order as in other Enterobacteria (i.e., *Escherichia coli*) (Blattner *et al.*, 1997; Dippel & Boos, 2005).

Based on the sequence annotation, the genetic information encoded on pPag3 corresponds with the previously described phenotypic characteristics of nonpigmented variants of *P. agglomerans* (i.e., thiamine deficiency, lack of maltose utilization, no pigmentation) (Chatterjee & Gibbins, 1971; Gantotti & Beer, 1982; Lindh *et al.*, 1991), indicating that the plasmids in both species likely have similar features. In addition, when multiplying the size of pPag3 (530 kb) with the average molecular weight of a base pair (660 Da), the molecular weight of pPag3 obtained is in agreement with the observed 350 MDa plasmid reported from *P. agglomerans* (ex *E. herbicola*) Eh112Y (Gantotti & Beer, 1982).

Curing of plasmid pPag3 and characterization of the variant

A nonpigmented variant of *P. vagans* C9-1, designated C9-1W, was obtained (Fig. 1). The identity of C9-1W as a derivative rather than as a contaminant was confirmed with 100% *gyrB* sequence identity compared with C9-1. The distinctive white colony color is attributable to loss of the carotenoid biosynthetic gene cluster located on pPag3. Genotyping with multiple primer pairs targeting parts of the three plasmids in *P. vagans* C9-1 confirmed the absence of pPag3 and the presence of pPag1 and pPag2.

The plasmid sequence data identified a thiamine biosynthetic cluster (*thiOSGF*). Whether these are required for thiamine biosynthesis was unclear because several other genes (*thiBCDEIJKLMPQ*) known from pathways in prokaryotes (Begley *et al.*, 1999; Settembre *et al.*, 2003) are found scattered on the *P. vagans* C9-1 chromosome (Smits *et al.*, 2009). When tested on glucose-amended minimal media, C9-1W only grew with a thiamine supplement. This confirms that plasmid-borne *thiOSGF* are essential for thiamine autotrophy in *P. vagans* C9-1, and may explain thiamine auxotrophy reported for the nonpigmented variant, plasmid-cured *P. agglomerans* (Chatterjee & Gibbins, 1971; Gantotti & Beer, 1982). Substitution of glucose with maltose in the minimal medium resulted in no growth regardless of the presence/absence of thiamine. This further demonstrates that maltose utilization (Dippel & Boos, 2005) is conferred by genes located on pPag3 (Pvag_pPag30206–Pvag_pPag30215). When *P. vagans* C9-1W was grown with sucrose or sorbitol as the sole carbon sources, thiamine was again found to be the critical parameter for growth. This confirms the thiamine auxotrophy of *P. vagans* C9-1W, and also the retention of pPag1 (containing sucrose metabolic genes) and pPag2 (containing sorbitol metabolic genes) in the variant.

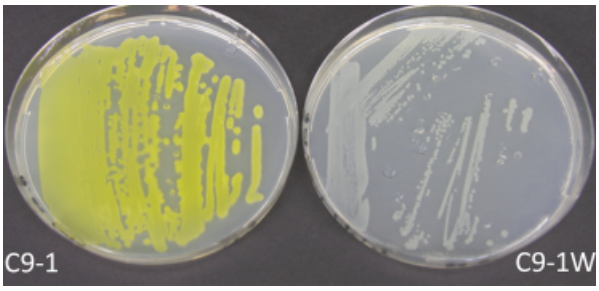

Fig. 1. *Pantoea vagans* C9-1 (left) and its nonpigmented variant C9-1W (right). The lack of yellow pigmentation could be attributed to the absence of the carotenoid biosynthesis gene cluster (*crtEXYIBZ*; Pvag_pPag30170–Pvag_pPag30175).

Ampicillin resistance

Plasmid pPag3 also contains two genes with high sequence identity to a β-lactamase *bla* and its cognate regulator *ampR* (Pvag_pPag30395–Pvag_pPag30396). Ampicillin resistance has been reported to occur commonly in *P. agglomerans* clinical isolates (Cruz *et al.*, 2007). Unlike the wild-type strain *P. vagans* C9-1, variant C9-1W was sensitive to ampicillin at 200 μg mL^{-1}, suggesting that a functional β-lactamase is encoded on the plasmid.

Autoinducer biosynthesis

Autoinduction mediated by AHL signals has been well described in the plant pathogen *P. stewartii* ssp. *stewartii* (von Bodman *et al.*, 2003) and has been reported recently in the pathogens *P. agglomerans* pv. *gypsophilae* and *P. ananatis* (Morohoshi *et al.*, 2007; Chalupowicz *et al.*, 2008). Based on the sequence homology to the *pagRI* genes of *P. agglomerans* pv. *gypsophilae* (Chalupowicz *et al.*, 2008; Rezzonico *et al.*, 2009) the transcriptional regulator *pagR* and the AHL-synthase *pagI* genes (Pvag_pPag30141–Pvag_pPag30142) have been identified on plasmid pPag3.

Using an *A. tumefaciens* biosensor (Shaw *et al.*, 1997), AHL production was tested. For this purpose, the plant pathogenic strain *P. ananatis* LMG 2665 was added to the assay as a positive control. *Pantoea vagans* C9-1 has a positive autoinducer functional activity, but yields a weaker signal in the biosensor assay than *P. ananatis* LMG 2665 (Fig. 2). The variant *P. vagans* C9-1W lost this activity (Fig. 2), confirming that this strain is not able to produce detectable AHLs. Although the chromosome also contains a putative AHL synthase, located next to the *sdiA* gene encoding a LuxR-type transcriptional regulator (Lindsay & Ahmer, 2005; Smits *et al.*, 2009), it can be concluded from

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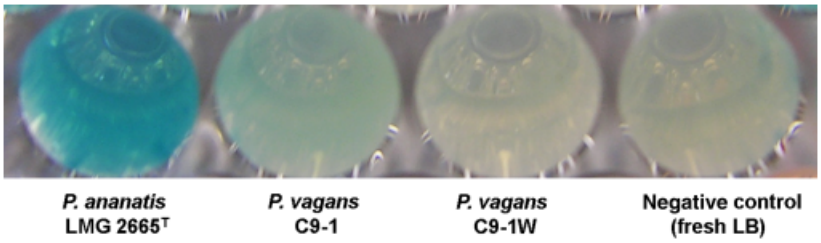

Fig. 2. Detection of AHL production *Pantoea vagans* C9-1 and its nonpigmented variant C9-1W using the *Agrobacterium tumefaciens* biosensor assay. As a positive control, *Pantoea ananatis* LMG 2665^T was used; fresh LB medium was used as a negative control.

the results of the biosensor assay that this chromosomal gene is not involved in the synthesis of the AHLs that can be detected with the *A. tumefaciens* biosensor.

The PagRI quorum-sensing system plays a central role in the virulence of *P. agglomerans* pv. *gypsophilae* by regulating the expression of the T3SS (Chalupowicz *et al.*, 2009). Its role in the ecological behavior of *P. vagans* and *P. agglomerans* strains that have functional *pagRI* genes is currently unknown (Rezzonico *et al.*, 2009). The fact that most nonpathogenic strains lack a T3SS, however, suggests that *pagRI* may have additional non-virulence-related functions in phytopathogenic pathovars.

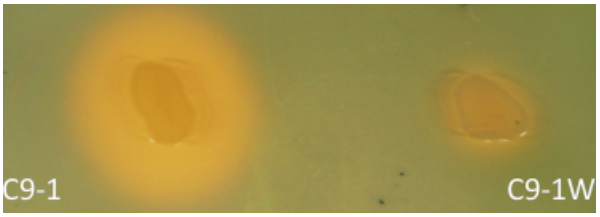

Fig. 3. CAS agar plate with *Pantoea vagans* C9-1 (left) and its nonpigmented variant C9-1W (right). The lack of a large halo around C9-1W can be explained by the absence of the biosynthetic genes for desferrioxamine (*dfoJACS*; Pvag_pPag30339–Pvag_pPag30342). The weak signal indicates the production of chromosomally encoded enterobactin.

Iron acquisition

Siderophores are small molecules that bind Fe^{3+} with a high affinity and are synthesized by bacteria under iron starvation. The genome of *P. vagans* C9-1 contains biosynthetic genes for the catecholate siderophore enterobactin (*ent-fep*) and the hydroxamate siderophore desferrioxamine E (*dfoJACS*), which were reported to be produced by the strain (Feistner & Ishimaru, 1996). Siderophore biosynthesis can be an important biocontrol trait, as the strain may be able to compete with phytopathogens for the already limited supply of iron *in planta*.

When spotted onto CAS siderophore indicator plates (Schwyn & Neilands, 1987), *P. vagans* C9-1 produces a large halo, indicative of siderophore synthesis, while variant C9-1W produces a small halo, just around the colony (Fig. 3). This difference can be attributed to the absence of the pPag3-encoded *dfoJACS* gene cluster (Pvag_pPag30339–Pvag_pPag30342), which confers the ability of desferrioxamine production to the strain.

Metabolic profiling using Biolog plates

Pantoea vagans C9-1W was compared with the wild-type strain *P. vagans* C9-1 for its substrate utilization pattern as determined by Biolog GN2 and AN plate assays (see for selected data Table S1). In general, growth with some of the compounds appeared to be slower than with the wild-type strain, and it cannot be excluded that this is influenced by the thiamine auxotrophy (thiamine was added for C9-1W to the minimal medium in the Biolog assays) or by the physiological differences in growth caused by the absence of pPag3.

Growth with maltose and maltotriose is abolished in *P. vagans* C9-1W due to the lack of the complete *mal* operon (Pvag_pPag30206–Pvag_pPag30215). Cellobiose, arbutin and salicin tested negative when using in C9-1W, but positive with the wild-type strain C9-1. These substrates are transported over the cytoplasmic membrane and channelled into the central pathways via a phosphotransferase system and a phosphohydrolase, respectively (An *et al.*, 2004, 2005). These functions are putatively encoded by two gene clusters on pPag3, *bglBFG* (Pvag_pPag30318–Pvag_pPag30320) and *ascBFG* (Pvag_pPag30345–Pvag_pPag30437). The plasmid pPag3 contains the *gabTP* genes (Pvag_pPag30456–Pvag_pPag30457) (Niegemann *et al.*, 1993), described for their role in the uptake (GapP) and the initial transamination of γ-aminobutyrate (GABA) to succinate semialdehyde (GapT), which is subsequently channelled into the TCA cycle. Growth with GABA is retarded in C9-1W compared with the wild type, but not absent. Therefore, it is likely that there is an alternative pathway for growth with GABA in *P. vagans* C9-1W.

Growth with many organic acids is either retarded or absent in *P. vagans* C9-1W. This might partly be caused by the thiamine deficiency mentioned above. In addition, as plasmid pPag3 encodes several proteins involved in the uptake and conversion of organic acids, the lack of these functions may also contribute to these phenotypes in *P. vagans* C9-1W. The same may be true for the observed

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Table 1. Presence of amplicons for pPag3 in *Pantoea vagans* strains

Amplicon	C9-1	LMG 24199^T	LMG 24196	LMG 24195
A	+	–	–	–
B	+	–	–	–
C	+	–	–	–
pagRI	+	+	+	+
D	+	+	+	+

Primer combinations and amplicon positions are listed in Table S2.

delay or the absence of growth with some of the amino acids, for which putative transporter- and conversion-encoding genes are also encoded on pPag3. However, as a direct link between annotated genes and a certain phenotype cannot be made based only on bioinformatic analysis, these observations remain hypothetical until further data are collected.

Presence and composition of pPag3 in other *P. vagans* strains

A spontaneous nonpigmented variant of *P. vagans* strain LMG 24196 was obtained on a rich medium plate under normal laboratory conditions. This variant was tested with the primers for *pagRI* (Rezzonico *et al.*, 2009) with no amplification, in contrast to a positive amplification in the wild-type parent LMG 24196 and the other two *P. vagans* strains (LMG 24195 and LMG 24199^T) (Brady *et al.*, 2009). This indicates that these autoinducer genes are also plasmid-borne in this strain.

Four PCR primer sets targeting pPag3 in genes encoding hypothetical proteins (amplicons A–C) and within the putative TonB-dependent siderophore receptor gene *fepA* (amplicon D) (Table 1) were used to screen the *P. vagans* strains. *Pantoea vagans* C9-1 was positive for all four amplicons, whereas strains LMG 24199^T, LMG 24195 and LMG 24196, were positive for amplicon D, but negative for amplicons A–C (Table 1). Amplicons A–C are within a 20 kb region on pPag3 (Table S2), suggesting that this part of the plasmid was acquired recently by *P. vagans* C9-1.

Conclusions

We have described here some phenotypic features for which the predicted genes are spread over the 530-kb plasmid pPag3 of *P. vagans* C9-1. This study confirms that plasmid loss can occur in *P. vagans* C9-1, albeit at a low frequency, even under conditions designed to obtain variants (e.g., rich media), as has been observed in *P. agglomerans* strains (Chatterjee & Gibbins, 1971; Gantotti & Beer, 1982; Lindh *et al.*, 1991). Several phenotypes that are lost along with the loss of plasmid pPag3 may be important for the ecological fitness of *P. vagans* C9-1, disfavoring the selection of non-pigmented variants in natural environments. Chief among these are carotenoid pigmentation that can protect against environmental stresses (Dussault *et al.*, 2008; Johler *et al.*, 2010) and thiamine and siderophore biosynthesis that may improve competitiveness (Temple *et al.*, 2004; Dubuis *et al.*, 2006).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Comparison of substrate spectrum between *P. vagans* C9-1 and the nonpigmented variant C9-1W using BIOLOG GN2 and AN plates.
Table S2. PCR primers used for gene amplification.

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