Two site-specific recombinases are implicated in phenotypic variation and competitive rhizosphere colonization in *Pseudomonas fluorescens*

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The biocontrol agent *Pseudomonas fluorescens* F113 undergoes phenotypic variation during rhizosphere colonization, and this variation has been related to the activity of a site-specific recombinase encoded by the *sss* gene. Here, it is shown that a second recombinase encoded by the *xerD* gene is also implicated in phenotypic variation. A putative *xerD* gene from this strain was cloned, and sequence analysis confirmed that it encoded a site-specific recombinase of the λ integrase family. Mutants affected in the *sss* or *xerD* genes produced a very low quantity of phenotypic variants compared to the wild-type strain, both under prolonged cultivation in the laboratory and after rhizosphere colonization, and they were severely impaired in competitive root colonization. Overexpression of the genes encoding either recombinase resulted in a substantial increment in the production of phenotypic variants under both culture and rhizosphere colonization conditions, implying that both site-specific recombinases are involved in phenotypic variation. Overexpression of the *sss* gene suppressed the phenotype of a *xerD* mutant, but overexpression of the *xerD* gene had no effect on the phenotype of an *sss* mutant. Genetic analysis of the phenotypic variants obtained after overexpression of the genes encoding both the recombinases showed that they carried mutations in the *gacA/S* genes, which are necessary to produce a variety of secondary metabolites. These results indicate that the Gac system is affected by the activity of the site-specific recombinases. Transcriptional fusions of the *sss* and *xerD* genes with a promoterless *lacZ* gene showed that both genes have a similar expression pattern, with maximal expression during stationary phase. Although the expression of both genes was independent of diffusible compounds present in root exudates, it was induced by the plant, since bacteria attached to the root showed enhanced expression.

INTRODUCTION

Site-specific recombinases catalyse genetic rearrangements related to multiple processes such as plasmid (Colloms *et al.*, 1990) and chromosome segregation (Blakely *et al.*, 1991), phage integration (reviewed by Groth & Calos, 2004) and phase variation (Dove & Dorman, 1994; Tominaga *et al.*, 1991). One of the best characterized is the *Escherichia coli* recombinase XerC, which forms an heterotetramer with the recombinase XerD (Ferreira *et al.*, 2003), and participates in chromosome segregation after replication (Blakely *et al.*, 1991, 1993), and in the segregation of different plasmids such as ColE1 (Colloms *et al.*, 1990; Blakely *et al.*, 1993) and pSC101 (Cornet *et al.*, 1994), resolving plasmid dimers.

The pseudomonads possess orthologues of genes encoding these site-specific recombinases. A gene from *Pseudomonas aeruginosa* affecting pyoverdin production, and named *sss*, was shown to be homologous to the *E. coli xerC* gene (Hofte *et al.*, 1994). The *sss* gene has also been cloned from different strains of *Pseudomonas fluorescens* (Dekkers *et al.*, 1998; Sánchez-Contreras *et al.*, 2002). An *sss* mutant of *P. fluorescens* WCS365 was affected in competitive rhizosphere colonization, and was displaced by the wild-type from the root tip of a variety of plants (Dekkers *et al.*, 1998). It has also been shown that introduction of extra copies of the *sss* gene can improve rhizosphere colonization (Dekkers *et al.*, 2000) and biocontrol abilities of different pseudomonads (Chin-a-Woeng *et al.*, 2000). A second recombinase that might function in conjunction with Sss is also present in pseudomonads, since the sequences of the *P. aeruginosa* PAO1 and *P. fluorescens* Pf0-1 genomes have shown the presence of genes homologous to the *E. coli xerD* genes. To date, these *xerD* homologues have not been cloned or analysed.

Phenotypic (phase) variation in pseudomonads occurs during rhizosphere colonization, and phenotypic variants showing a translucent and diffuse colony morphology have been isolated in laboratory cultures (Van Den Broek et al., 2003), and after rhizosphere passage (Sánchez-Contreras et al., 2002; Achouak et al., 2004). In P. fluorescens F113, the phenotypic variants isolated from the alfalfa rhizosphere showed enhanced motility, and their numbers were significantly reduced by a mutation in the sss gene (Sánchez-Contreras et al., 2002). Most of the few variants observed among the sss mutants also carried a secondary mutation affecting the Gac system (Sánchez-Contreras et al., 2002). The Gac system, forming a two-component regulatory system encoded by the gacA and the gacS (lemA) genes, regulates the production of secondary metabolites such as exoprotease and cyanide (Blumer et al., 1999). This system has also been implicated in phase variation in Pseudomonas sp. PCL1171 (Van Den Broek et al., 2003), since mutants in the gacS gene were locked in phase II, which is morphologically equivalent to the phenotypic variants produced by P. fluorescens F113 after rhizosphere colonization.

Due to the importance of phenotypic variation for rhizosphere colonization, and to improve biotechnological applications of these bacteria in biocontrol and rhizoremediation, the aim of this work was to characterize the xerD gene of P. fluorescens F113, and to determine its possible role in phenotypic variation and rhizosphere colonization.

METHODS

Bacterial strains, plasmids and growth conditions. Strains and plasmids used in this study are shown in Table 1. All the P. fluorescens strains are derivatives of the biocontrol strain F113, which was isolated from the sugarbeet rhizosphere (Shanahan et al., 1992). The F113 genebank used was constructed with partially EcoRI-digested genomic DNA cloned into the pLAFR3 cosmid. The sss and xerD mutants were generated by gene disruption using the suicide vector pK18mobsac (Schafer et al., 1994). Mutants were checked by Southern blotting. Overexpression of genes was achieved by cloning them under the control of the strong nptII promoter present in the pFAJ1709 plasmid (Dombrecht et al., 2001). All plasmids were mobilized to P. fluorescens by triparental matings, using pRK2013 as the helper plasmid (Figurski & Helinski, 1979).

All P. fluorescens strains were grown in SA medium (Scher & Baker, 1982) overnight at 28 °C. E. coli strains were grown overnight in Luria–Bertani (LB) medium (Bertani, 1951) at 37 °C. For the prolonged laboratory growth, bacteria were grown for 1 week in SA liquid medium with shaking, and the cultures were plated on solidified SA medium. When required, the following antibiotics were used at the indicated concentrations: rifampicin 100 µg ml^{-1}, tetracycline 10 µg ml^{-1} (for E. coli) or 70 µg ml^{-1} (for P. fluorescens), and kanamycin 25 µg ml^{-1} (for E. coli) or 50 µg ml^{-1} (for P. fluorescens). Exoprotease production was observed on skim-milk plates, as described by Sacherer et al. (1994). The same plates were used to assess pyoverdin production by inspection under UV light.

DNA manipulations and sequence analysis. Standard methods were used for DNA extraction and gene cloning (Sambrook et al., 1989). Southern blotting and colony hybridization were performed

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source or reference
Strains		
DH5α	General purpose E. coli strain	Boyer & Roulland-Dussoix (1969)
F113rif	P. fluorescens wild-type strain, Rif^r	Shanahan et al. (1992)
F113-sss2	F113rif sss mutant, Rif^r, Kan^r	Sánchez-Contreras et al. (2002)
F113-xerD	F113rif xerD mutant, Rif^r, Kan^r	This work
F113 sss::lac	F113rif sss::lacZ, Rif^r, Kan^r	This work
F113 xerD::lac	F113rif xerD::lacZ, Rif^r, Kan^r	This work
Plasmids		
pK18mobsacB	pUC18 derivative lacZ mob site sacB, Kan^r	Schafer et al. (1994)
pBG1261	pK18mobsac with an internal fragment of the xerD gene, used to generate F113-xerD	This work
pFAJ1709	Expression vector, Tet^r	Dombrecht et al. (2001)
pBG1457	pFAJ1709 derivative carrying sss under the control of the nptII promoter, used to overexpress sss	This work
pBG1442	pFAJ1709 derivative carrying xerD under the control of the nptII promoter, used to overexpress xerD	This work
pVIK112	lacZ reporter suicide vector, Kan^r	Kalogeraki & Winans (1997)
pBG1319	pVIK112 sss::lacZ transcriptional fusion	This work
pBG1306	pVIK112 xerD::lacZ transcriptional fusion	This work
pRK2013	Helper plasmid used in triparental matings, Kan^r	Figurski & Helinski (1979)
pME3066	A 1·65 kb BamHI–BglII fragment containing the gacA gene from P. fluorescens CHA0 in pVK100, Tet^r	Laville et al. (1992)
pEMH97	A 9·7 kb HindIII fragment containing the gacS gene from P. syringae pv. syringae B728a in pLAFR3, Tet^r	Hrabak & Willis (1992)

with a non-radioactive detection kit, and a chemiluminescence method was used to detect hybridization signals according to the instructions of the manufacturer (Boehringer Mannheim). PCR reactions were performed using the *Tth* enzyme (Biotools) under standard conditions. Primer sequences are available on request. DNA was custom sequenced by Centro Nacional de Investigaciones Oncológicas (CNIO; Madrid, Spain) and Sistemas Genómicos (Valencia, Spain). Sequence analysis was performed with software from the Genetics Computer Group (Madison, WI, USA) and the BLAST programs.

Protein sequences were aligned by using the multiple-sequence-alignment tool CLUSTAL W (Thompson *et al.*, 1994). The aligned sequences were studied by using genetic distance and bootstrap NJ tree for phylogenetic inference (1000 iterations). Ambiguous characteristics (where a deletion, insertion or unidentified state was recorded for any strain) were removed from the alignment data.

Rhizosphere colonization experiments. Alfalfa seeds were sterilized in 70 % ethanol for 2 min, diluted bleach (1:5) for 15 min, and then rinsed thoroughly with sterile distilled water. Seed vernalization was performed at 4 °C overnight, and germination was for 1 day at 28 °C. Germinated alfalfa seeds were sown in Leonard jar gnotobiotic systems (Villacieros *et al.*, 2003) using perlite as the solid substrate, and 8 mM KNO$_3$-supplemented Fahraeus (FP) medium (Fahraeus, 1957) as the mineral solution. After 2 days, alfalfa seeds were inoculated with $\sim$10^8 cells of the appropriate strains. In competition experiments, strains were inoculated at a 1:1 ratio. Plants were maintained in controlled conditions (16 h light at 25 °C, and 8 h dark at 18 °C) for 3 weeks. Bacteria were recovered from the rhizosphere by vortexing the root tips (last centimetre of the main root) for 2 min in a tube containing 5 ml 0·9 % NaCl, and plating the appropriate dilutions on SA agar containing antibiotics. Each experiment was performed in triplicate, and each replicate contained at least 20 plants. Statistical analysis was done with Sigma Plot 4 software (Windows).

Gene expression analysis. Transcriptional *lacZ* fusions of the *sss* and *xerD* genes were constructed by directionally cloning an internal fragment of the gene into the pVIK112 suicide vector (Kalogeraki & Winans, 1997). The construct was integrated into the F113 genome by triparental mating, and single homologous recombinants were checked by PCR and Southern blotting.

Cells harbouring these constructs were grown in LB medium, and samples were taken at intervals. β-Galactosidase activity was determined according to Miller (1972).

Aqueous and methanolic root exudates were prepared by extracting roots from 100 5-day-old seedlings, growing on agar plates, in 10 ml solvent (distilled water or methanol) by shaking for 30 min. Exudates were used at a concentration of 10 µl ml^{-1}.

The reporter strains were also lawn plated on SA agar supplemented with X-Gal (40 µg ml^{-1}). Alfalfa seedlings, and wooden toothpicks impregnated with exudates, were laid on top of the bacterial lawn, and after 2 days incubation at 28 °C, plates were inspected microscopically.

RESULTS

Cloning of the *P. fluorescens* F113 *xerD* gene

Two primers derived from the sequence of the *xerD* gene from *P. aeruginosa* PAO1 were used to amplify genomic DNA from *P. fluorescens* F113. A PCR fragment of the expected size (308 bp) was obtained and sequenced. The
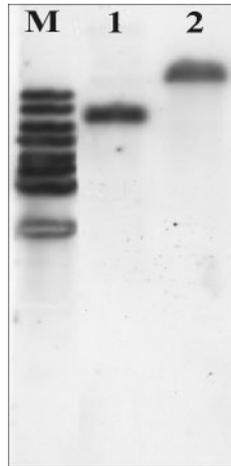
sequence showed that the amplified fragment corresponded to an internal fragment of the putative *xerD* gene, and it was used to screen an F113 gene library by hybridization. A hybridizing cosmid (pBG1386) was isolated, and primers designed from the sequence of the internal fragment of *xerD* were used to extend the sequence. The full sequence of the F113 *xerD* gene was obtained, and it has been deposited in GenBank (accession no. AY642384). Analysis of the sequence showed the presence of an ORF encoding a product of 298 aa that showed 79 % identity with the *xerD* gene from *P. aeruginosa* PAO1. The ORF also showed a high level of identity (95 %) with the *xerD* gene from the sequenced genome of *P. fluorescens* Pf0-1. The putative *P. fluorescens* F113 XerD sequence shows features typical of site-specific recombinases (Fig. 1A), possessing substantial homology with Sss, XerC and XerD proteins, and a conserved domain that includes the catalytic tyrosine residue close to the carboxyl terminus, typical of the λ integrase family of recombinases. Identification of this ORF as the F113 *xerD* gene was confirmed by cluster analysis, as the protein grouped with other recognized XerD proteins from several bacteria, including *E. coli* (Fig. 1B). Southern-blot analysis, using internal fragments of F113 *sss* and *xerD*, did not show the presence of additional copies of these genes in the F113 genome (Fig. 2).

Sss and XerD recombinases are implicated in phenotypic variation

F113rif derivatives, carrying mutations in the *sss* and *xerD* genes, were constructed by gene disruption. Despite several efforts, we were unable to construct a stable strain carrying a double mutation affecting both genes, suggesting that the presence of one or other of the recombinases is necessary for cellular viability. The appearance of phenotypic variants with a translucent colony morphology after prolonged laboratory cultivation, and after rhizosphere colonization, was assessed and compared to that of the wild-type strain (Table 2). Under prolonged laboratory cultivation conditions, the percentage of phenotypic variants produced by the three strains was very low, but higher in the wild-type strain. However, the appearance of phenotypic variants after rhizosphere colonization was very different (Table 2). While 19 % of the colonies recovered from the root tip of plants inoculated with the wild-type were phenotypic variants, less than 5 % of the colonies from root tips of plants inoculated with either of the mutants showed a variant morphology. These results show that both recombinases play a role in phenotypic variation during rhizosphere colonization. The ability of both mutants to form phenotypic variants was recovered after complementation by a wild-type copy of the genes cloned in pFAJ1709.

Derivatives overexpressing the site-specific recombinases were constructed by mobilizing plasmids into F113rif. These plasmids contained either of the genes encoding the recombinases, under the control of the strong *npt*II promoter. The strain overexpressing the *sss* gene (F113rif pBG1457) produced almost 90 % variants after prolonged

A

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XerD F113    KSLSPHTLRHAFATHLLNHGADLRVVQMLLGHSDLSTTQIYTHVARARLQDLHAKHHPRG 298
XerD E.coli  EKLSPHVLRHAFATHLLNHGADLRVVQMLLGHSDLSTTQIYTHVATERLRQLHQQHHPRA 298
Sss F113     QNLHPHMLRHSFASHLLESSQDLRAVQELLGHSDIKTTQIYTHLDFQHLATVYDSAHPRA 291
XerC E.coli  NHVHPHKLRHSFATHMLESSGDLRGVQELLGHANLSTTQIYTHLDFQHLASVYDAAHPRA 294
             : :  ** ***:**:*:*:  .  *** ** ****:::.*******:   :*  ::   ***.
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B

Fig. 1. Analysis of the *P. fluorescens* F113 *xerD* gene product. (A) Alignment of the C-terminal domain of the putative F113 XerD protein with the F113 Sss and the *E. coli* XerC and XerD proteins. The grey box indicates the conserved domain in the carboxyl terminus of the protein that includes the catalytic tyrosine residue (black box) present in an equivalent position in all these proteins. (B) Cluster analysis (bootstrap NJ tree) of the XerC (Sss) and XerD proteins from several Gram-negative bacteria. F113 proteins are shown in bold.

laboratory cultivation, and 97 % of the colonies recovered from the root-tip of plants inoculated with this strain were variants (Table 2). The strain overexpressing the *xerD* gene (F113rif pBG1442) also produced more variants than the wild-type after prolonged cultivation, although the percentage of variants was much lower than in the case of *sss* overexpression. Similarly, a significant percentage of the colonies recovered from the root-tip of plants inoculated with F113rif pBG1442 (*xerD* overexpression) showed variant morphology (Table 2).

The plasmids overexpressing the genes encoding the site-specific recombinases were also mobilized into the *sss* and *xerD* mutants to generate strains mutated in one of the recombinases, but overexpressing the other. As shown in Table 2, overexpression of the *sss* gene in a XerD⁻ background resulted in a high proportion of phenotypic variants after prolonged cultivation. Therefore, overexpression of *sss* suppressed the phenotype of a *xerD* mutant, indicating that the XerD recombinase is necessary for the wild-type level of phenotypic variation when Sss is produced at a physiological (wild-type) level, but that it plays a marginal role when *sss* is expressed at higher levels. Conversely, the overexpressed *xerD* gene could not suppress the phenotype of the *sss* mutant, as the number of phenotypic variants after prolonged cultivation and rhizosphere passage remained very low. These results show that the Sss recombinase plays a more important role in phenotypic variation than the

Fig. 2. Southern blot analysis of *Eco*RI-digested total genomic DNA from *P. fluorescens* F113. Lane 1, *sss* probe; lane 2, *xerD* probe; M, molecular mass markers (1 kb ladder).

XerD recombinase, although the latter is necessary for wild-type level of phenotypic variation when *sss* is expressed at a physiological level.

## The Gac system is affected by site-specific recombinase activity

The colony morphology of phenotypic variants resembles the morphology of mutants in the GacA/GacS two-component system (Sánchez-Contreras *et al.*, 2002). To test the Gac phenotype of the variants produced by

Table 2. Percentage of phenotypic variants appearing under laboratory cultivation and rhizosphere colonization conditions

Values are means of three experiments $\pm$ SD.

Strain	Phenotypic variants (%)	
	Lab. cultivation	Root tip
F113rif	$0\cdot25\pm0\cdot05$	$19\cdot00\pm4\cdot36$
F113-sss2	$<0\cdot1$	$4\cdot37\pm1\cdot21$
F113-xerD	$<0\cdot1$	$4\cdot11\pm2\cdot72$
F113rif pBG1457 (*sss* overexpression)	$89\cdot50\pm0\cdot71$	$97\cdot00\pm1\cdot83$
F113rif pBG1442 (*xerD* overexpression)	$11\cdot76\pm6\cdot95$	$39\cdot97\pm3\cdot32$
F113-xerD pBG1457	$91\cdot68\pm4\cdot31$	$69\cdot47\pm8\cdot56$
F113-sss2 pBG1442	$<0\cdot1$	$2\cdot23\pm0\cdot21$

overexpression of both recombinases, 17 variants from the *sss* overexpression experiment, 15 variants from the *xerD* overexpression experiment, and 10 colonies with wild-type morphology obtained from the overexpression experiments were randomly selected and tested for exoprotease and pyoverdin production. *gac* mutants in *P. fluorescens* do not produce exoprotease, and they produce higher levels of the siderophore pyoverdin than the wild-type (Sánchez-Contreras *et al.*, 2002; Duffy & Defago, 2000; Blumer *et al.*, 1999; Sacherer *et al.*, 1994). None of the variants in this study produced a halo in plates containing skim milk, indicating the lack of exoprotease activity (Fig. 3B, D), while all the wild-type colonies produced a halo. Similarly, all the variants produced greater fluorescence when plates
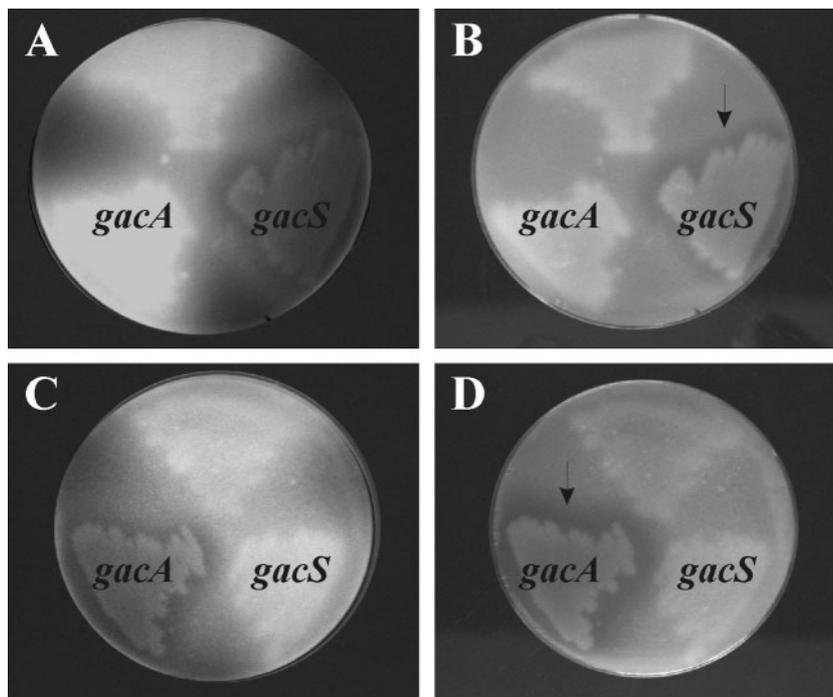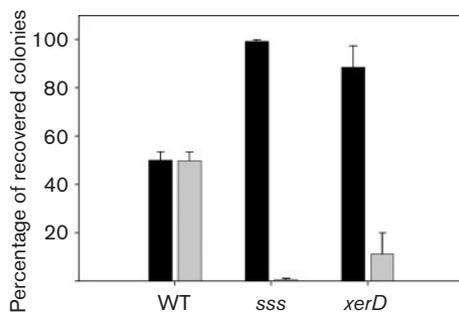

Fig. 3. Complementation analysis of *P. fluorescens* F113 phenotypic variants. (A, B) A variant complemented by a cloned *gacS* gene. (C, D) A variant complemented by the *gacA* gene. (A, C) Viewed under UV light to test for the amount of pyoverdin produced. (B, D) Viewed under white light to observe the presence of a halo due to exoprotease activity on skim-milk plates. Arrows point to haloes.

were observed under UV light, indicating that they produce more pyoverdin (Fig. 3A, C). All the wild-type colonies showed a normal level of pyoverdin production. These results indicate that the Gac system is affected by site-specific recombinase activity, and that the variant phenotype is, at least partially, due to mutations in the genes encoding this system. A complementation analysis (Fig. 3) using the cloned *gacS* gene from *Pseudomonas syringae* pv. *syringae* B728a (Hrabak & Willis, 1992), and the cloned *gacA* gene from *P. fluorescens* CHA0 (Laville *et al.*, 1992), showed that 13 of the variants from the *sss* overexpression experiment, and 11 of the variants from the *xerD* over-expression experiment, were complemented for exoprotease and pyoverdin production by the cloned *gacS* gene (Fig. 3A, B). Three variants from each experiment were complemented by the *gacA* gene (Fig. 3C, D), and one variant from each experiment was not complemented by either gene (*gacA* or *gacS*). These results suggest that both *gacA* and *gacS* are affected by site-specific recombinase activity.

Sss and XerD are required for competitive rhizosphere colonization

An *sss* mutant of *P. fluorescens* WCS365 has been shown to be defective in tomato root colonization, indicating that the *sss* gene plays an important role in rhizosphere colonization (Dekkers *et al.*, 1998). In order to investigate the putative role of the *xerD* gene in rhizosphere colonization, the *sss* and *xerD* mutants were used to perform competitive rhizosphere colonization experiments with the wild-type strain. Alfalfa seedlings were inoculated with a 1:1 mixture

of the wild-type strain and one of the mutants. As shown in Fig. 4, both mutants were displaced from the alfalfa root tip by the wild-type strain, indicating that both recombinases are required for competitive colonization.

The *sss* and *xerD* genes are rhizosphere-induced

Disruption of the *sss* and *xerD* genes with an internal fragment of the gene cloned in the plasmid pVIK112
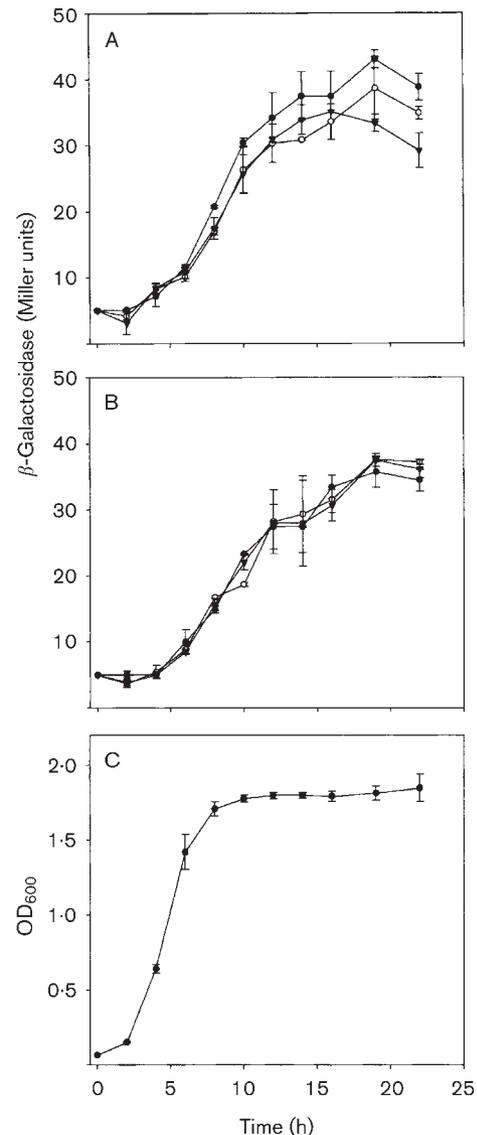

Fig. 4. Competitive root colonization by *P. fluorescens* F113 and derivatives harbouring mutations in the *sss* and *xerD* genes. A wild-type strain tagged with the same vector used to generate the mutants was used as a competitor for the wild-type strain. The wild-type strain was used as the competitor for the mutants. Plants were inoculated 1:1 with the test strain and the competitor, and, after 3 weeks, root tips were collected and the bacteria were cultured. Grey bars represent the percentage of colonies recovered from the test strains, and black bars represent the percentage of colonies recovered from the competitor (wild-type) strain. Results are shown as means ±SD. The mean number of recovered bacteria per gram of root tip was $7{\cdot}37 \times 10^6$, the range being from $1{\cdot}85 \times 10^6$ to $1{\cdot}77 \times 10^7$.

Fig. 5. Time-course expression (Miller units) of the genes encoding the two site-specific recombinases in the absence and presence of root exudates. (A) F113 *sss*::*lac*. (B) F113 *xerD*::*lac*. ● No exudate, ○ aqueous exudate, ▼ methanolic exudates. (C) Growth curve of *P. fluorescens* F113 in LB medium. No differences in growth were observed for the derivatives used in expression experiments in any treatment. Results are shown as means ±SD.

(Kalogeraki & Winans, 1997) generated transcriptional fusions of these genes with a promoterless *lacZ* gene. These strains were used to investigate the regulation of the genes encoding both recombinases.

As shown in Fig. 5(A), the expression level of the *sss* gene was low, and was dependent on the growth phase (Fig. 5C), being lower during exponential growth and increasing during the stationary phase. When bacteria harbouring this fusion were grown in the presence of aqueous or methanolic root extracts, no differences in expression were observed, indicating that expression of the *sss* gene does not depend on diffusible compounds exuded by the plant root. Fig. 5(B, C) shows that the expression level and pattern of the *xerD* gene were similar to those of *sss*, also being independent of compounds present in the root extracts.

However, both fusions were significantly induced by the presence of the plant root. As observed in Fig. 6, cells grown attached to the rhizoplane showed expression of the *lacZ* reporter. Expression was observed only in the bacteria growing in the close vicinity of the root, indicating again that the induction is not due to any diffusible compound. Furthermore, bacteria growing attached to toothpicks impregnated with aqueous or methanolic extracts did not show induction (data not shown), ruling out that attachment was the only cause of induction. It is therefore possible that a non-diffusible compound present in the root is an inducer of both *sss* and *xerD* expression.

DISCUSSION

The Sss recombinase from pseudomonads is an orthologue of XerC (Hofte *et al.*, 1994), and it has been implicated in phenotypic variation (Sánchez-Contreras *et al.*, 2002) and rhizosphere colonization (Dekkers *et al.*, 1998; Sánchez-Contreras *et al.*, 2002). We have sequenced and cloned the *xerD* gene from the rhizosphere colonizer *P. fluorescens* F113, and sequence analysis has shown that it encodes a site-specific recombinase homologous to its *E. coli* counterpart.

A mutant affected in the *xerD* gene showed the same phenotype as an *sss* mutant with regard to phenotypic variation, producing a very low number of phenotypic variants after rhizosphere colonization. This indicates that not only Sss, but also that XerD is necessary to produce the genetic rearrangements leading to the appearance of variants. Considering that in *E. coli* the XerC/XerD hetero-tetramer is necessary for recombination (Ferreira *et al.*, 2003), it seems reasonable to assume that in pseudomonads, Sss and XerD form a heterotetramer responsible for an increase in phenotypic variation. However, we have found differences in the phenotype of strains overproducing either recombinase. Overexpression of either of the genes encoding the two recombinases led to an increase in the proportion of phenotypic variants, although the impact of overproduction of Sss was much higher than overproduction of XerD. Furthermore, overproduction of Sss could overcome the phenotype of a *xerD* mutant, indicating that at high cellular concentration, Sss alone (forming either homodimers or homotetramers) can catalyse recombination events that give rise to variants. Conversely, over-expression of the *xerD* gene was not enough to suppress the *sss* mutation, suggesting that there is a strict requirement of the Sss recombinase for the increase in phenotypic variation. However this interpretation is unlikely because of the increase in the number of phenotypic variants obtained after overproduction of XerD at constant and possibly low levels (see below) of Sss. Furthermore, we have been unable to construct a stable double mutant affecting both recombinases, suggesting that one or other of them is necessary for chromosome segregation after replication. The finding that, in contrast to *E. coli* (Hendricks *et al.*, 2000), single mutants grew at the same rate as the wild-type (data not shown), and with a normal cell morphology, indicate that either of the recombinases can function independently.

Analysis of the genotype of phenotypic variants has shown that all of them carry mutations in the Gac system. Mutations in different variants affected either the *gacS* or the *gacA* genes, although in two variants, the affected gene could not be determined. It is likely that these two variants are affected in both genes, although it is also possible that they are affected in genes acting downstream in the *gac* regulatory cascade. These results could suggest that the
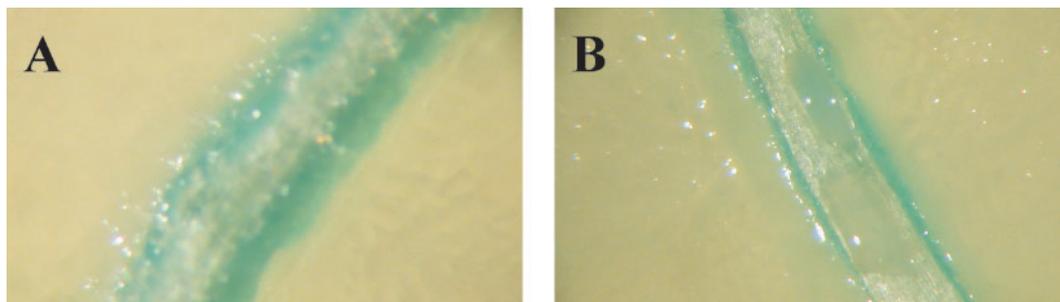
Fig. 6. Induction of the genes encoding both site-specific recombinases in the alfalfa rhizosphere. *β*-Galactosidase activity was visualized by the appearance of a blue precipitate in X-Gal containing plates. (A) F113 *sss*::*lac*. (B) F113 *xerD*::*lac*.

gacA and *gacS* genes are a target for the site-specific recombinases. However, considering that these genes are located in different parts of the genome, it is unlikely that the activity of the recombinases would result in the accumulation of diverse mutations in both genes. Therefore, we propose an indirect effect of the site-specific recombinases on the Gac system. The relationship between the Gac system and phenotypic variation has been shown by Van Den Broek *et al.* (2003). These authors demonstrated that *Pseudomonas* sp. PCL1171 transposon-generated mutants in the *gacS* gene were locked in the variant colony morphology. It has also been shown that a phenotypic variant of *P. fluorescens* F113, isolated from the alfalfa rhizosphere, carried a point mutation in the *gacA* gene (Sánchez-Contreras *et al.*, 2002). In the same report, the few phenotypic variants appearing in the rhizosphere after colonization by an *sss* mutant were defective in the Gac system.

The Sss recombinase has been shown to be essential for competitive root colonization of the potato, radish, wheat and tomato rhizospheres by *P. fluorescens* WCS365 (Dekkers *et al.*, 1998). Here, we show the need of this recombinase for competitive colonization of the alfalfa rhizosphere by another biocontrol strain, *P. fluorescens* F113. This strain is an efficient colonizer of all plants tested so far, including alfalfa (Villacieros *et al.*, 2003), sugarbeet (Delany *et al.*, 2001; Shanahan *et al.*, 1992), pea (Naseby & Lynch, 1999) and tomato (Simons *et al.*, 1996). It is likely that the activity of site-specific recombinases is a requirement for competitive rhizosphere colonization of a large variety of plants by *P. fluorescens*. We have also shown that an additional recombinase, XerD, is required for competitive rhizosphere colonization. The finding that the proportion of phenotypic variants depends directly on the cellular amount of recombinases, and the higher proportion of variants after rhizosphere passage, indicates that phenotypic variants are selected in the rhizosphere, and it makes a clear link between recombinase activity, phenotypic variation and competence for rhizosphere colonization. It is interesting to note that rhizosphere colonization has been shown to be necessary for biocontrol (Chin-a-Woeng *et al.*, 2000), and that biocontrol traits have been shown to be regulated by phase variation in pseudomonads (Van Den Broek *et al.*, 2003).

Expression of the genes encoding the two recombinases under laboratory growth conditions was low, as expected for genes whose products might alter the genome structure. The expression pattern showed that the lower level of expression occurred during the exponential phase, probably reflecting the low amounts of recombinases required for chromosome segregation, even in an actively growing population. The expression level was higher during the stationary phase, when cell division is restricted. It is possible that larger amounts of recombinases during this period are related to the stress-induced adaptive mutagenesis that appears during stationary phase in bacteria (Wright, 2004). It is important to note that it has been shown that after prolonged growth, *gacA* and *gacS* mutants

accumulate in *P. fluorescens* CHA0 batch cultures (Duffy & Defago, 2000). It is likely that this accumulation of mutants, with a colony morphology and genotype resembling recombinase-induced variants, is partially due to the overproduction of the recombinases during long-term stationary-phase conditions.

Genes encoding the two recombinases are induced in the alfalfa rhizosphere, although no diffusible inducer could be detected in root exudates. Since attachment to toothpicks impregnated with exudates did not induce these genes, it is likely that a non-diffusible inducer present in the plant root is responsible for the increased gene expression. Following a strategy to identify plant-induced genes of *P. syringae*, Marco *et al.* (2003) found that one of the five plant-induced genes detected corresponded to a *xerD* homologue, suggesting that site-specific recombinases might also play a role in pathogenic plant–microbe interactions. Rhizosphere induction also indicates that besides phenotypic selection of variants during rhizosphere colonization, there is also an increase in the production of variants, since we have shown that the proportion of variants is dependent on the expression level of the recombinases. Dekkers *et al.* (2000) have shown that overexpression of the *sss* gene can improve root colonization by different *P. fluorescens* strains. Our results show that the *xerD* gene offers new possibilities to improve rhizosphere colonization and, possibly, biotechnological applications of pseudomonads.

ACKNOWLEDGEMENTS

REFERENCES

Achouak, W., Conrod, S., Cohen, V. & Heulin, T. (2004). Phenotypic variation of *Pseudomonas brassicacearum* as a plant root-colonization strategy. *Mol Plant–Microbe Interact* **17**, 872–879.

Bertani, G. (1951). Studies on lysogenesis. 1. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* **62**, 293–300.

Blakely, G., Colloms, S., May, G., Burke, M. & Sherratt, D. (1991). *Escherichia coli* XerC recombinase is required for chromosomal segregation at cell division. *New Biol* **3**, 789–798.

Blakely, G., May, G., McCulloch, R., Arciszewska, L. K., Burke, M., Lovett, S. T. & Sherratt, D. J. (1993). Two related recombinases are required for site-specific recombination at *dif* and *cer* in *Escherichia coli* K12. *Cell* **75**, 351–361.

Blumer, C., Heeb, S., Pessi, G. & Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci U S A* **96**, 14073–14078.

Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**, 459–472.

Chin-a-Woeng, T. F. C., Bloemberg, G. V., Mulders, I. H. M., Dekkers, L. C. & Lugtenberg, B. J. J. (2000). Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chloraphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant–Microbe Interact* **13**, 1340–1345.

Colloms, S. D., Sykora, P., Szatmari, G. & Sherratt, D. J. (1990). Recombination at ColE1 *cer* requires the *Escherichia coli xerC* gene product, a member of the *λ* integrase family of site-specific recombinases. *J Bacteriol* **172**, 6973–6980.

Cornet, F., Mortier, I., Patte, J. & Louarn, J. M. (1994). Plasmid pSC101 harbors a recombination site, *psi*, which is able to resolve plasmid multimers and to substitute for the analogous chromosomal *Escherichia coli* site *dif*. *J Bacteriol* **176**, 3188–3195.

Dekkers, L. C., Phoelich, C. C., Van Der Fits, L. & Lugtenberg, B. J. J. (1998). A site-specific recombinase is required for competitive root colonization by *Pseudomonas fluorescens* WCS365. *Proc Natl Acad Sci U S A* **95**, 7051–7056.

Dekkers, L. C., Mulders, I. H. M., Phoelich, C. C., Chin-a-Woeng, T. F. C., Wijfjes, A. H. M. & Lugtenberg, B. J. J. (2000). The *sss* colonization gene of the tomato–*Fusarium oxysporum* f. sp. *radicis-lycopersici* biocontrol strain *Pseudomonas fluorescens* WCS365 can improve root colonization of other wild-type *Pseudomonas* spp. bacteria. *Mol Plant–Microbe Interact* **13**, 1177–1183.

Delany, I. R., Walsh, U. F., Ross, I., Fenton, A. M., Corkery, D. M. & O'Gara, F. (2001). Enhancing the biocontrol efficacy of *Pseudomonas fluorescens* F113 by altering the regulation and production of 2,4-diacetylphloroglucinol. Improved *Pseudomonas* biocontrol inoculants. *Plant Soil* **232**, 195–205.

Dombrecht, B., Vanderleyden, J. & Michiels, J. (2001). Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in Gram-negative bacteria. *Mol Plant–Microbe Interact* **14**, 426–430.

Dove, S. L. & Dorman, C. J. (1994). The site-specific recombination system regulating expression of the type-1 fimbrial subunit gene of *Escherichia coli* is sensitive to changes in DNA supercoiling. *Mol Microbiol* **14**, 975–988.

Duffy, B. K. & Defago, G. (2000). Controlling instability in *gacS–gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. *Appl Environ Microbiol* **66**, 3142–3150.

Fahraeus, G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J Gen Microbiol* **16**, 374–381.

Ferreira, H., Butler-Cole, B., Burgin, A., Baker, R., Sherratt, D. J. & Arciszewska, L. K. (2003). Functional analysis of the C-terminal domains of the site-specific recombinases XerC and XerD. *J Mol Biol* **330**, 15–27.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proc Natl Acad Sci U S A* **76**, 1648–1652.

Groth, A. C. & Calos, M. P. (2004). Phage integrases, biology and applications. *J Mol Biol* **335**, 667–678.

Hendricks, E. C., Szerlong, H., Hill, T. & Kuempel, P. (2000). Cell division, guillotining of dimer chromosomes and SOS induction in resolution mutants (*dif*, *xerC* and *xerD*) of *Escherichia coli*. *Mol Microbiol* **36**, 973–981.

Hofte, M., Dong, Q. G., Kourambas, S., Krishnapillai, V., Sherratt, D. & Mergeay, M. (1994). The *sss* gene product, which affects pyoverdin production in *Pseudomonas aeruginosa* 7NSK2, is a site-specific recombinase. *Mol Microbiol* **14**, 1011–1020.

Hrabak, E. M. & Willis, D. K. (1992). The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. syringae on bean is a member of a family of two-component regulators. *J Bacteriol* **174**, 3011–3020.

Kalogeraki, V. S. & Winans, S. C. (1997). Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. *Gene* **188**, 69–75.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G. & Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root-rot of tobacco. *Proc Natl Acad Sci U S A* **89**, 1562–1566.

Marco, M. L., Legac, J. & Lindow, S. E. (2003). Conditional survival as a selection strategy to identify plant-inducible genes of *Pseudomonas syringae*. *Appl Environ Microbiol* **69**, 5793–5801.

Miller, J. H. (1972). *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Naseby, D. C. & Lynch, J. M. (1999). Effects of *Pseudomonas fluorescens* F113 on ecological functions in the pea rhizosphere are dependent on pH. *Microb Ecol* **37**, 248–256.

Sacherer, P., Defago, G. & Haas, D. (1994). Extracellular protease and phospholipase-C are controlled by the global regulatory gene *gacA* in the biocontrol strain *Pseudomonas fluorescens* CHA0. *FEMS Microbiol Lett* **116**, 155–160.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sánchez-Contreras, M., Martin, M., Villacieros, M., O'Gara, F., Bonilla, I. & Rivilla, R. (2002). Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J Bacteriol* **184**, 1587–1596.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. & Puhler, A. (1994). Small mobilizable multipurpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69–73.

Scher, F. M. & Baker, R. (1982). Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to Fusarium wilt pathogens. *Phytopathology* **72**, 1567–1573.

Shanahan, P., O'Sullivan, D. J., Simpson, P., Glennon, J. D. & O'Gara, F. (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl Environ Microbiol* **58**, 353–358.

Simons, M., Vanderbij, A. J., Brand, I., Deweger, L. A., Wijffelman, C. A. & Lugtenberg, B. J. J. (1996). Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Mol Plant–Microbe Interact* **9**, 600–607.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Tominaga, A., Ikemizu, S. & Enomoto, M. (1991). Site-specific recombinase genes in three *Shigella* subgroups and nucleotide-sequences of a *pinB* gene and an invertible B segment from *Shigella boydii*. *J Bacteriol* **173**, 4079–4087.

Van Den Broek, D., Chin-a-Woeng, T. F. C., Eijkemans, K., Mulders, I. H. M., Bloemberg, G. V. & Lugtenberg, B. J. J. (2003). Biocontrol traits of *Pseudomonas* spp. are regulated by phase variation. *Mol Plant–Microbe Interact* **16**, 1003–1012.

Villacieros, M., Power, B., Sánchez-Contreras, M. & 8 other authors (2003). Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil* **251**, 47–54.

Wright, B. E. (2004). Stress-directed adaptive mutations and evolution. *Mol Microbiol* **52**, 643–650.