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Molecular Nature of Spontaneous Modifications in *gacS* Which Cause Colony Phase Variation in *Pseudomonas* sp. Strain PCL1171

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***Pseudomonas* sp. strain PCL1171 displays colony phase variation between opaque phase I and translucent phase II colonies, thereby regulating the production of secondary metabolites and exoenzymes. Complementation and sequence analysis of 26 phase II mutants and of 13 wild-type phase II sectors growing out of phase I colonies showed that in all these cases the phase II phenotype is caused by spontaneous mutations in *gacA* or/and *gacS*. Mutation of *gac* reduced both the length of the lag phase and the generation time. Isolation and sequencing of the *gacS* genes from the phase II bacteria revealed one insertion as well as several random point mutations, deletions, and DNA rearrangements. Most phase II colonies reverted with a high frequency, resulting in wild-type *gacA* and *gacS* genes and a phase I phenotype. Some phase II bacteria retained the phase II phenotype but changed genotypically as a result of (re)introduction of mutations in either *gacA* or *gacS*. The reversion of *gacA* or *gacS* to the wild type was not affected by mutation of *recA* and *recB*. We conclude that in *Pseudomonas* sp. strain PCL1171, mutations in *gacA* and *gacS* are the basis for phase variation from phase I to phase II colonies and that, since these mutations are efficiently removed, mutations in *gac* result in dynamic switches between the "wild-type" population and the subpopulations harboring spontaneous mutations in *gacA* and or *gacS*, thereby enabling both populations to be maintained.**

Phase variation is a process of reversible, high-frequency phenotypic switching that is mediated by mutation, reorganization, or modification of DNA. This process is used by several bacterial species to generate population diversity that increases bacterial fitness and is important in niche adaptation (33). Phase variation can sometimes be observed by the appearance of morphologically distinct colonies or sectors within a colony (8, 12). In contrast to spontaneous mutations, which occur at a frequency of approximately 10^{-7} mutations per cell per generation, phase variation occurs at frequencies higher than 10^{-5} switches per cell per generation (12). Four mechanisms of phase variation are known (12): (i) slipped-strand mispairing, dependent on variations in the length of a repeat tract, switching a gene on or off as a result of frameshifts, or regulating the level of expression by altering promoter spacing; (ii) genomic rearrangements, based on invertible elements or recombination events resulting in gene conversions; (iii) differential methylation, based on the presence of methylation sites within a promoter, which can regulate the binding of regulatory proteins; and (iv) random unprogrammed variation, which can switch traits on and off via random reversible mutations (3).

Phase variation has been reported to regulate the production of pili (22), outer membrane proteins (22), flagella (13), fimbriae (1), surface lipoproteins and other surface-exposed structures (8, 12, 29), secondary metabolites (5, 38), and secreted enzymes such as proteases, lipases, and chitinases (5, 38). In a previous paper (38), we reported that out of 46 *Pseudomonas* strains antagonistic against the wheat-pathogenic fungus *Geaumannomyces graminis* pv. *tritici* R3-11A (27), 43 displayed colony phase variation. One of these strains, PCL1171, was selected for study of the molecular basis of phase variation. In this strain, antagonistic activity, morphology, and expression of secondary metabolites (such as a lipopeptide with antifungal activity), biosurfactant activity, and the exoenzymes chitinase, lipase, and protease are regulated via phase variation and expressed only in the phase I phenotype. Estimation of the phase variation frequencies showed approximately 5.0×10^{-5} and 9.0×10^{-2} switches per cell per generation for phase I to II and for phase II to I, respectively (38).

A preliminary genetic analysis of the phase variation mechanism in PCL1171 showed that transposon mutation of the *gacS* gene resulted in the phase II phenotype, whereas mutation of *mutS* strongly increased the frequency of switching between phase I and phase II (38). The *gacA*/*gacS* two-component regulatory system consists of a sensor kinase, GacS, and a reponse regulator, GacA, belonging to the FixJ family of transcriptional regulators (20); this system regulates secondary metabolism and production of exoenzymes (2, 15, 20, 28, 38). Phase variation via a homolog of *gacS*, *pheN*, which regulates pathogenicity and colony morphology in *Pseudomonas tolaasii*, is dependent on a spontaneous duplication in this gene (9). Furthermore, it has been reported that the *gacA*/*gacS* system is subject to the accumulation of mutations in several *Pseudomonas* spp. (4, 7, 20, 28, 34). However, neither reversibility of these *gac* mutations nor the molecular nature of mutations in the *gacS* gene has been reported. In this paper we analyze the role of *gacA*/*gacS* in phase switching and give a detailed description of the molecular nature of mutations accumulating in the *gacS* gene responsible for reversibility of colony phase variation.

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TABLE 1. Microbial strains and plasmids

Strain or plasmid	Characteristics	Reference or source
Bacterial strains		
PCL1171	Antagonistic *Pseudomonas* strain isolated from the rhizosphere of maize from a Mexican agricultural field; colony morphology varies between two distinct phases, defined as phases I (opaque) and II (translucent)	38
PCL1572	Derivative of PCL1171 in which a Tn*5luxAB* transposon is inserted into a *gacS* homolog; Km^r	38
PCL1586	PCL1171 derivative harboring a mutation in a *recA* homolog; Km^r	This study
PCL1589	PCL1171 derivative harboring a mutation in a *recB* homolog; Km^r	This study
S17-1	*E. coli* MM294, RP4-2 Tc::Mu-Km::T7 chromosomally integrated	36
DH5α	*E. coli endA1 gyrSA96 hrdR17*(r_K^- m_K^-) *supE44 recA1*; general-purpose *E. coli* host strain	10
Plasmids		
pRL1063a	Plasmid harboring a promoterless Tn*5luxAB* transposon and a p15A origin of replication; Km^r	40
pMP5285	pME3049 derivative, lacking the Hg^r gene, used for single homologous recombination; Km^r	19
pME6010	*E. coli*/*Pseudomonas* shuttle vector, stably maintained in *Pseudomonas* species, with an estimated copy number of 4 to 8; Tc^r	11
pMP6562	pME6010 harboring a 3.2-kb PCR product from strain PCL1171 which contains the *gacS* homolog from PCL1171; Tc^r	38
pMP5565	pME6010 harboring a 1.2-kb PCR product from *Pseudomonas* sp. strain PCL1446 which contains a *gacA* homolog	Kuiper et al., unpublished data
pMP6603	pME6010 harboring a 1.2-kb PCR product from *Pseudomonas* sp. strain PCL1446 containing a *gacA* homolog and a 3.2-kb PCR product from *Pseudomonas* sp. strain PCL1171 containing a *gacS* homolog	This study
pMP6604	pMP5285 harboring a 1.2-kb EcoRI fragment of a *recA* homolog of *Pseudomonas* sp. strain PCL1171	This study
pMP6605	pMP5285 harboring a 1.2-kb PCR fragment of a *recB* homolog of *Pseudomonas* sp. strain PCL1171	This study

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. *Pseudomonas* strains were grown in King's medium B (KB) (14) at 28°C. *Escherichia coli* strains were grown in Luria-Bertani medium (31) at 37°C. Solid growth media contained 1.8% (wt/vol) agar (Difco Laboratories, Detroit, Mich.). Kanamycin, tetracycline, and carbenicillin (Sigma, St. Louis, Mo.) were added for antibiotic selection in final concentrations of 50, 40, and 50 μg/ml, respectively. Growth in liquid medium was analyzed in triplicate by measurement of the optical density at 620 nm over time. The numbers of viable cells in the starting culture were counted. Growth experiments were carried out three times in triplicate.

Measuring frequencies of phase variation. Bacteria from a colony with phase I or phase II morphology were inoculated from a plate in 20 ml of KB and were grown overnight at 28°C. The morphology and initial number of bacteria were determined after dilution plating of an average of 500 cells per plate. At least 1,500 colonies were counted for the estimation of frequencies expressed as the number of switches per cell per generation.

Isolation and characterization of phase II Tn*5luxAB* transposon mutants. A mutant library of strain PCL1171 was constructed by using plasmid pRL1063a (38), which harbors a Tn*5* transposon with promoterless *luxAB* genes and a kanamycin resistance marker (40). Mutants with a phase II morphology were selected, replated several times, and grown in liquid medium to select those mutants locked in the phase II phenotype. General DNA recombinant techniques were performed according to the work of Sambrook and Russell (31).

Complementation of Tn*5luxAB* transposon mutants and wild-type phase II sectors. Phase II bacteria were complemented with pMP6562 (*gacS*) and pMP5565 (*gacA*) by parental mating. Primers oMP716 (5′-GGAATTCAGGATGTCCATCAACACCA-3′) and oMP717 (5′-GGAATTCATCGTTGATGAAGGCACACA-3′), each containing a HindIII site (GGAATTCA), were used to produce a 3.2-kb PCR fragment harboring a *gacS* homolog from *Pseudomonas* sp. strain PCL1171. The PCR product was cloned into pMP5565 by using HindIII. The resulting construct, pMP6603, harboring both the *gacA* and the *gacS* gene under the control of their own promoters, was electroporated into *E. coli* S17-1 (36) and used to transform phase II bacteria by parental mating. The morphology of the transformants was judged after 2 days of growth at 28°C on King's medium B (14) supplemented with tetracycline.

Analysis of the *gacS* gene of phase II bacteria. Primers oMP716 and oMP717 were used to amplify the complete *gacS* gene from phase II sectors and phase II mutants from PCL1171 by PCR. The PCR product was cloned into pMP5565 by using HindIII and transformed to PCL1572 (*gacS*::Tn*5luxAB*) by electroporation for complementation analysis. The morphology was judged after 2 days of growth at 28°C. Those constructs which did not restore the phase I phenotype in PCL1572 were selected. The *gacS* PCR product was sequenced by using oMP716 and oMP717; internal primers oMP698 (5′-ACCCAATCCCTGGAACAAC-3′), oMP699 (5′-GAGTTCCTGGCCAACATGAG-3′), oMP700 (5′-GAGCAGATGGGTGGTGAGAT-3′), and oMP701 (5′-CAAACCCTGCTGGAAGACAT-3′), annealing at nucleotide positions 220, 859, 1456, and 2047, respectively; and reverse primers oMP723 (5′-GGTCCACTTCAACACCACCTG-3′), oMP722 (5′-GTAATGCCGTTGGTCAGGTTC-3′), oMP721 (5′-GTTGATGATGCCCAACAGGT-3′), and oMP720 (5′-CCGATAGGTGGCTGATTGAG-3′), annealing at nucleotide positions 2353, 1692, 995, and 309 of the *gacS* sequence of PCL1171 (GenBank accession number AY236957), respectively. All mutations found were sequenced at least twice.

Isolation and construction of mutants. To isolate genes from PCL1171, a plasmid library was constructed. Chromosomal fragments of PCL1171 phase I bacteria, 3 to 5 kb long, were obtained after partial digestion using EcoRI. After ligation of the fragments into pBluescript, the clones were pooled and isolated. The partial *recA* gene was isolated from this library via PCR using two primers specific for pBluescript, T7 (5′-TAATACGACTCACTATAGGG-3′) and oMP61 (5′-GTTTTCCCAGTCACGAC-3′), and two internal primers for *recA*, oMP529 (5′-GGGGTACCAGCGCACCAGCAT-3′) and oMP530 (5′-GGAATTCCAGATCGAACGCCAATTCGG-3′). A second PCR was performed using the internal primers to select the correct PCR fragment. A *recA* mutant was constructed by using the PCR product of oMP61 and oMP530 to obtain a 1.2-kb EcoRI fragment of the *recA* gene which was cloned into the suicide plasmid pMP5285, resulting in pMP6604. This construct was electroporated into S17-1 (36) and transferred to PCL1171 by parental mating. After selection on kanamycin, the mutation of *recA* was confirmed via Southern hybridization (31) by using the AlkPhos direct labeling kit for detection (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom) according to the manufacturer's recommendations. The same approach was used to isolate the *recB* gene from PCL1171 by using internal primers oMP760 (5′-TTCGACAGCGGCAGCCTG

TTCACCCAGA-3′) and oMP761 (5′-AAGGCGTA(CT)AT(GC)GC(AG)TGC TTGGGGTCGCCGAT-3′). To construct a *recB* mutation, a 1.2-kb PCR product obtained by using oMP840 (5′-CAGGAATTCGAAGGTTGGTG-3′) and oMP761 was cloned into the pGEM-TEasy vector (Promega Crop., Madison, Wis.) and subsequently transferred to the suicide plasmid pMP5285 by using EcoRI, resulting in pMP6605. pMP6605 was electroporated into S17-1 (36) and transferred to PCL1171 via parental mating. After selection on kanamycin, the mutation of *recB* was confirmed by Southern hybridization (31). To determine the effects of *recA* and *recB* mutations on phase variation, phase II colonies were replated on King's medium B and the colony morphology was determined after at least 2 days of growth at 28°C.

RESULTS

Isolation and preliminary characterization of stable phase II Tn*5luxAB* transposon mutants. The frequency of switching between phase I and phase II was determined for PCL1171 at 6.0×10^{-5} and 8.2×10^{-2} switches per cell per generation for phase I to phase II and for phase II to phase I, respectively. To identify genes involved in phase variation, we constructed a Tn*5luxAB* transposon library from PCL1171, which has a strong preference for the phase I phenotype, by using pRL1063a (40). From this library, 26 Tn*5luxAB* transposon mutants expressing a stable phase II phenotype were selected out of approximately 1,200 transformants. No mutants expressing a stable phase I phenotype were isolated. Observations from both the mutant library and growth of the wild type on solid medium indicated that the majority of the phase II bacteria switch back with a high frequency to the phase I phenotype, while occasionally phase II bacteria which are more stable can be isolated. In contrast to the 8.2×10^{-2} switches per cell per generation observed for the wild type, no switch back to the phase I phenotype was observed in these phase II mutants. These mutants are referred to below as stable phase II mutants. Sequencing of the flanking regions of several transposon insertions of these stable mutants showed homology to genes with widely different functions (data not shown). Since we had previously observed that a Tn*5* insertion in *gacS* could lock PCL1171 in the phase II phenotype (38), we tested the possibility that, in addition to the Tn*5luxAB* insertion, at least some of the mutants had accumulated mutations in *gacS* or *gacA*. For this test we used plasmids pMP6562 (harboring a *gacS* homolog of PCL1171), pMP5565 (harboring a *gacA* homolog of *Pseudomonas* sp. strain PCL1446), and pMP6603 (harboring both a *gacA* and a *gacS* homolog). It appeared that out of 26 mutants, 13 could be complemented to the phase I phenotype by using pMP6562 (*gacS*) and the other 13 could be complemented to the phase I phenotype by using pMP5565 (*gacA*). Complementation using pMP6603, harboring both the *gacA* and the *gacS* gene, restored the phase I phenotype in all 26 mutants. Introduction of the empty parental vector pMP6010 did not alter the phase II phenotype. Subsequent restreaking of the complemented mutants resulted in phase I colonies from which phase II sectors appeared as in the wild type.

To study the effect of a *gacS* mutation on growth, we compared the growth curve of the previously identified *gacS* Tn*5* mutant PCL1572 and those of three other phase II mutants, PCL1574, PCL1575, and PCL1576, with that of wild-type phase I bacteria (Fig. 1). It appeared that mutation of the *gacS* gene reduced the length of the lag phase and reduced the generation time compared to that for the wild type (50 ± 4.2 versus 60 ± 4.2 min, respectively) (Fig. 1). Counting the numbers of CFU present at the start of growth showed no differences between the *gacS* mutants and the wild type, indicating that the reduced lag phase was not due to a higher number of dead cells in the wild-type culture.

Molecular basis of phase II phenotype of phase II mutants. Since only mutations accumulating in the *gacA* gene have been analyzed (4), we decided to focus on the role of *gacS* in phase II variation of *Pseudomonas* sp. strain PCL1171. We isolated the *gacS* genes from 10 phase II mutants. Nine of the isolated *gacS* genes, cloned into pMP5565 (*gacA*), could not restore the phase I phenotype in PCL1572, showing, as a control, that the mutations indeed interfered with GacS function. The *gacS* gene isolated from PCL1574 did restore the phase I phenotype in a small number of transformants. The *gacA* gene was included in the screening to eliminate the effect of possible mutations in the *gacA* gene of PCL1572. Complementation using a wild-type *gacS* gene in pMP5562 always restored PCL1572 to the phase I phenotype. Three of the mutated *gacS* genes were sequenced and aligned with the wild-type *gacS* sequence. The *gacS* genes from strains PCL1574 and PCL1575 were found to harbor a point mutation (Table 2). The *gacS* gene from PCL1576 harbored a 115-bp inversion, which was flanked by an inverted repeat (5′-C_{327}AGCAG-3′ and 5′-C_{442}TGCTG-3′). This repeat was created due to a point mutation at nucleotide position 329 ($aA_{329}c \rightarrow aG_{329}c$) (Table 2 and Fig. 2C). The inversion of the 115-bp fragment introduced a stop codon at amino acid position 134 of the predicted GacS protein.

Instability of mutations in *gacA* and *gacS*. The stable phase II mutant PCL1574 could be complemented to the phase I phenotype by using pMP5562 (*gacS*) (Table 2); complementation of PCL1572 (*gacS*::Tn*5luxAB*) by using the *gacS* gene isolated from PCL1574 resulted in a mixture of phase I and phase II colonies. Since this *gacS* gene did not restore the phase I phenotype in all PCL1572 transformants, the construct harboring the *gacS* gene from PCL1574 was reisolated from colonies in which *gacS* did complement PCL1572. Sequencing of the *gacS* gene from these phase I colonies showed that the point mutation was no longer present.

Similar observations were made for the stable phase II mutant PCL1575. This strain harbors a point mutation in its *gacS* gene resulting in a premature stop codon (Table 2). Whereas initially introduction of the wild-type *gacS* gene completely restored the phase I phenotype in this mutant, the introduction of pMP6562 (*gacS*) in subsequent complementations also resulted in a number of phase II colonies. These could be complemented by using pMP5565 (*gacA*). This changed PCL1575 derivative was designated PCL1575a (Table 2). In addition, PCL1575 phase II colonies were found which could be complemented neither by using pMP5565 nor by using pMP6562. One such derivative was designated PCL1575b (Table 2). Sequencing of the *gacS* gene of PCL1575a after PCR amplification showed that the point mutation identified in PCL1575 had been removed (T_{154}aa→C_{154}aa) in PCL1575a while a phase II phenotype was maintained. This point mutation was still present in PCL1575b (T_{154}aa) (Table 2).

Molecular basis of phase II phenotype in the wild-type strain PCL1171. To test whether mutations in the *gac* system also form the molecular basis for the highly unstable phase II

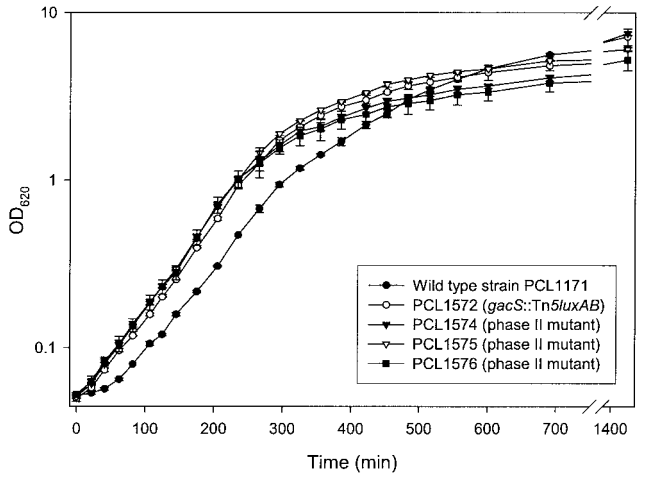

FIG. 1. Growth curves of wild-type *Pseudomonas* sp. strain PCL1171, its Tn*5luxAB* transposon derivative PCL1572, and three stable phase II mutants (PCL1574, PCL1575, and PCL1576) in King's medium B. The number of CFU was determined at the start of growth. The results indicated that the difference in the length of the lag phase is not due to differences in numbers of viable cells.

phenotype in the wild-type strain PCL1171, the *gacS* gene from phase II sectors was amplified by PCR. Since analysis of the stable phase II mutants showed that a phase II phenotype could be the result of mutations in either *gacA* or *gacS* (Table 2), and since wild-type phase II sectors cannot be maintained due to the instability of the phase II phenotype, we needed a method to distinguish between *gacA* and *gacS* mutations. For this purpose the amplified *gacS* gene from phase II sectors was cloned into pMP5565 (containing a wild-type *gacA* gene) and the resulting construct was used to complement PCL1572 (*gacS*::Tn*5luxAB*). Of 13 *gacS* genes isolated from these phase II sectors, 5 were unable to complement PCL1572 (Table 2). One, termed sector 16, resulted in a mixture of phase I and phase II colonies, whereas the other seven *gacS* genes complemented PCL1572 to the phase I phenotype, showing that in the latter seven sectors GacS function was not affected and that the phase II phenotype is most likely caused by a mutation in the *gacA* gene.

As controls, the *gacS* genes from sectors 2 and 5, which could restore the phase I phenotype in PCL1572, were sequenced. Sector 2 harbored one point mutation, while no changes were identified in the *gacS* gene isolated from sector 5 (Table 2).

Analysis of five out of the six noncomplementing *gacS* genes revealed various mutations (Table 2). The *gacS* gene from sector 1 harbored a total of three mutations, a 12-bp deletion in combination with two point mutations, including one silent mutation (Table 2 and Fig. 2A). The *gacS* genes from sectors 4 and 18 were found to harbor one and two point mutations, respectively (Table 2), while the *gacS* gene from sector 9 harbored a +1 frameshift due to a 1-nucleotide insertion (Table 2 and Fig. 2B), resulting in the introduction of stop codons downstream of the insertion. The *gacS* gene isolated from sector 16, reisolated from PCL1572 colonies not complemented by this gene, harbored a 307-bp deletion from nucleotide positions 2236 to 2542. A 10-bp tandem repeat (5′-G_{2226} CCATCACCG-3′ and 5′-G_{2523}CCATCACCG-3′) was identified at the borders of the deletion as the result of a single point mutation ($tT_{2231}a \rightarrow tC_{2231}a$) (Table 2 and Fig. 2D). The *gacS* gene from sector 16, isolated from PCL1572 colonies complemented to the phase I phenotype, did not harbor any mutations. In addition, a silent point mutation was identified (Table 2).

Phase II sectors from PCL1171 are highly unstable, and restreaking coincides with a high frequency (8.2×10^{-2} switches per cell per generation) of switching back to the phase I phenotype. This high frequency enabled us to analyze the *gacS* gene from phase II sectors after a switch back to the phase I phenotype. The three mutations previously identified in phase II sector 1 (a 12-bp deletion and two point mutations) were absent in phase I bacteria growing from sector 1 phase II cells. Also, all *gacS* mutations present in cells of sector 4 (single point mutation), sector 9 (1-bp insertion), sector 16 (307-bp deletion), and sector 18 (two point mutations) had been restored to the wild-type *gacS* sequence after switching to the phase I phenotype.

Role of *recA* and *recB* in phase variation. Based on the observation that a wide variety of mutations in the *gacS* gene, in both stable and unstable phase II bacteria, could be restored efficiently to the wild-type *gacS* sequence, we hypothesized that large DNA rearrangements could play a role in the switch from phase II to phase I. To test this hypothesis, a *recA* mutation was introduced into PCL1171, by using the suicide construct pMP6604, resulting in strain PCL1586. Strain PCL1586 (*recA*::Km^r) showed a phase I phenotype from which phase II sectors originated with a high frequency. After replating of these phase II sectors, phase I colonies and phase II colonies with phase I sectors were found.

The suicide construct pMP6605 harboring a *recB* fragment was used to introduce a *recB* mutation in PCL1171. Mutation of *recB* reduced the viability of the cells, which, however, still showed phase variation resulting in single phase I and phase II colonies. Replating of the phase II colonies resulted in phase I colonies and phase II colonies.

DISCUSSION

Molecular nature of mutations in *gacS*. *Pseudomonas* sp. strain PCL1171 displays a high frequency of reversible phase variation between a thick, opaque phase I and a thin, translucent phase II colony, thereby regulating secondary metabolism and production of exoenzymes (38). Since we observed that mutation of the *gacS* gene locked the bacteria in a phase II phenotype (38), we studied the role of *gacA*/*gacS* in phase variation. Complementation analysis of a total of 39 phase II derivatives showed that mutations in *gacA* (20 mutants) and *gacS* (19 mutants) are responsible for the phase II phenotype (Table 2). Introduction of pMP6603 (*gacA gacS*) restored all mutants to a phase I phenotype, showing that spontaneous mutation of the *gacA*/*gacS* two-component regulatory system forms the basis of the switch from phase I to phase II in phase variation of *Pseudomonas* sp. strain PCL1171.

The *gacS* genes isolated from phase II bacteria harbored random point mutations, deletions, DNA rearrangements, and an insertion (Table 2 and Fig. 2). In addition to the mutations identified in the *gacA* gene of *Pseudomonas fluorescens* CHAO by Bull et al. (4) (point mutations, a 3-bp deletion, and a 1-bp

TABLE 2. Spontaneous modifications in the *gacS* gene isolated from phase II sectors and phase II mutants of *Pseudomonas* sp. strain PCL1171

Origin	Mutation in *gacS* gene[a]		Functional gene		Remarks	Affected GacS domain
	Nucleotide	Amino acid	*gacA*	*gacS*		
Phase II Tn*5luxAB* mutants[b]						
Wild-type phase I	–	–	+	+	No mutations	
PCL1574	$cT_{899}c$–$cG_{899}c$	L_{300}–R_{300}	+	–	Point mutation	Phosphotransfer
PCL1575	$C_{154}aa$–$T_{154}aa$	Q_{52}–stop	+	–	*gacS* mutation	Periplasmic loop
PCL1575a			–	+	*gacA* mutation	
PCL1575b	$C_{154}aa$–$T_{154}aa$	Q_{52}–stop	–	–	*gacA* and *gacS* mutations	Periplasmic loop
PCL1576	$aA_{329}c$–$aG_{329}c$	N_{110}–S_{110}	+	–	Inverted repeat	Periplasmic loop
	115-bp inversion	N_{110}–R_{147} ($stop_{134}$)			Inversion[d]	
Wild-type phase II sectors[c]						
Sector 1	12-bp deletion	$A_{54}PL_{56}$	ND[e]	–	Deletion[d] and point mutations	Periplasmic loop
	$cT_{263}t$–$cC_{263}t$	L_{88}–P_{88}				
	gA_{1813g}–$gG_{1813}g$	Silent				
Sector 3	ND	ND	ND	–	*gacS* mutation	
Sector 4	$C_{1981}cg$–$T_{1981}cg$	P_{661}–S_{661}	ND	–	Point mutation	Phosphotransfer
Sector 9	ct_caat–$ctT_{309}caat$	L_{103}–frameshift	ND	–	1-bp insertion[d]	Periplasmic loop
Sector 16	$tT_{2231}a$–$tC_{2231}a$	L_{744}–S_{744}	ND	–	Tandem repeat	Phosphotransfer
	307-bp deletion				Deletion[d]	
	ggC_{663}–ggA_{663}	Silent				
Sector 18	$cT_{182}g$–$cC_{182}g$	L_{61}–P_{61}	ND	–	Point mutations	Periplasmic loop
	$gC_{1124}c$–$gT_{1124}c$	A_{375}–V_{375}				Phosphotransfer
Sector 5			ND	+	*gacA* mutation	
Sector 2	$cT_{443}g$–$cC_{443}g$	L_{148}–P_{148}	ND	+	*gacA* mutation	

[a] Numbers indicate positions in the wild-type *gacS* sequence.

[b] The functional *gacA*/*gacS* component was determined by the introduction of pMP6562 (*gacS*) and pMP5565 (*gacA*). A plus sign indicates which component is functional; a minus sign indicates which component harbors mutations and is nonfunctional. In addition, strains PCL1565, PCL1568, and another eight phase II mutants had a functional *gacA* gene (*gacS* mutants), while PCL1564, PCL1573, PCL1566, PCL1568, and another eight phase II mutants had a functional *gacA* gene (*gacS* mutants), while PCL1564, PCL1573, PCL1566, PCL1567, and another nine phase II mutants had a functional *gacS* gene (*gacA* mutants).

[c] The functional *gacA*/*gacS* component was determined by complementation of PCL1572 (*gacS*::Tn*5luxAB*) by the *gacS* gene isolated from a phase II sector. A plus sign indicates that a phase I morphology was restored in PCL1572; a minus sign indicates that a phase II morphology was maintained after introduction of the *gacS* gene. In addition, the *gacS* genes isolated from another five sectors did complement PCL1572 (*gacS*::Tn*5luxAB*), suggesting that mutation of the *gacA* gene was responsible for the phase II phenotype in these sectors.

[d] See Fig. 1.

[e] ND, not determined.

insertion), the *gacS* gene of PCL1171 also harbored a 12- and a 307-bp deletion and a 115-bp inversion (Table 2 and Fig. 2), showing that a large diversity of mutations, both in nature and in location, can accumulate in the *gacA* and *gacS* genes (Table 2 and Fig. 2). We observed that not all mutations in the *gacS* gene affected GacS function (for example, single mutations in the periplasmic loop [e.g., sector 2] did not), a finding consistent with the observation that deletion of this domain did not affect GacS function in *P. fluorescens* CHAO (41).

Accumulation of mutations in *gacA* and *gacS* has been reported previously for *Pseudomonas syringae* pv. *syringae* strain B728a (28), *P. fluorescens* CHAO (4, 7, 20), *Pseudomonas aureofaciens* 30-84 (6), and *Pseudomonas chlororaphis* isolate SPR044 (34). However, for none of these was a reversion to the wild-type phenotype reported. In contrast, in our strain the introduction and removal of mutations in *gac* is the basis of the observed phase variation, and the majority of the phase II cells switch back to the phase I phenotype at a high frequency, which correlates with the complete removal of the mutations from the *gacS* gene. Interestingly, in sector 16 we found that the 307-bp deletion was restored upon the switch to the phase I phenotype. But, since complementation of PCL1572, by using the *gacS* gene from sector 16, resulted in a mixture of phase I and II colonies, it is likely that a mixture of *gacS* genes was already present before PCR amplification. Therefore, we cannot exclude the possibility that the deletion identified cannot be repaired. This deletion mutant could be part of a subpopulation of stable phase II bacteria, whereas the majority of the bacteria in this sector harbor other mutations (e.g., *gacA*) and, as observed on plates, can switch back to the phase I phenotype.

Occasionally, more-stable phase II bacteria can be isolated. The isolation of stable phase II derivatives whose genetic basis switched between mutations in *gacA*, mutations in *gacS*, and mutations in both (Table 2) shows that even within such a "stable" phase II phenotype, mutations still can be removed and (re)introduced. Our results indicate that all mutations in the *gacS* and *gacA* genes of PCL1171 can be restored spontaneously. This may occur completely, resulting in a switch to the phase I phenotype, but, in the case of a stable phase II phenotype, mutations may not be removable, or the removal may coincide with the introduction of new mutations.

Growth experiments using *gac* mutants (Fig. 1) showed that mutation of *gac* decreases the length of the lag phase and increases the growth rate. Therefore, one explanation for the accumulation of *gac* mutants at a high frequency is increased growth competitiveness, especially under conditions where stationary and exponential growth often switch, as may be ex-

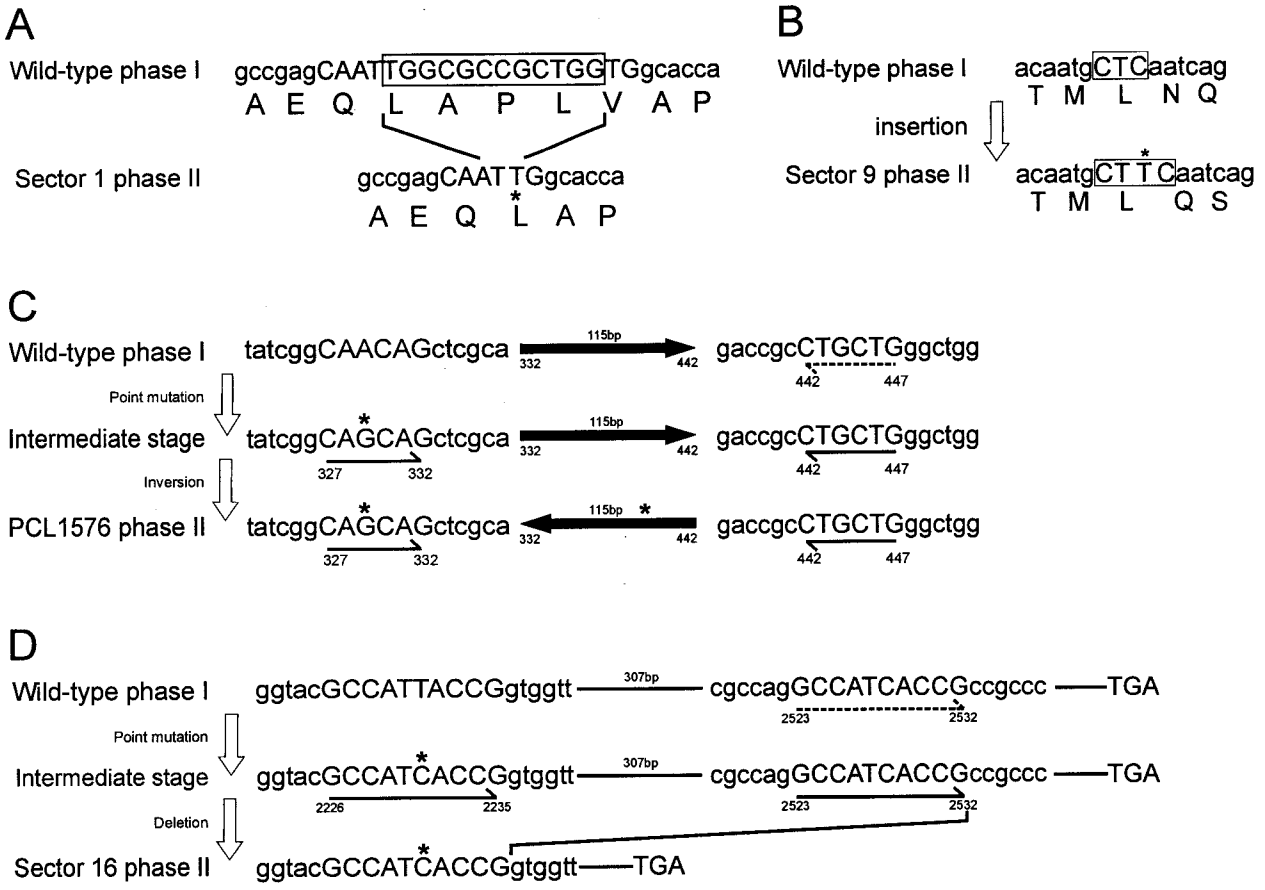

FIG. 2. Complex mutations identified in *gacS* of *Pseudomonas* sp. strain PCL1171. Mutations identified in sector 1 (A), sector 9 (B), PCL1576 (C), and sector 16 (D), harboring a 12-bp deletion, a 1-bp insertion, a 115-bp inversion, and a 307-bp deletion, respectively, are shown. (A and B) The phase I wild-type situation and, upon switching to a phase II phenotype, the corresponding situation in the *gacS* gene. The changes at the DNA and amino acid levels are indicated. (C and D) Wild-type situation of a phase I phenotype in which, due to point mutations (asterisked), an inverted repeat and a tandem repeat (indicated by half arrows), respectively, are created at an intermediate stage. A subsequent inversion and a deletion based on these repeats result in the situations in the *gacS* gene present in PCL1576 and in sector 16. All nucleotide positions correspond to those of the wild-type *gacS* gene.

pected in colonies and in the rhizosphere. This explanation is consistent with the suggestion by Schmidt-Eisenlohr et al. (34) for *P. chlororaphis* isolate SPR044 that having a mixed population could, based on the growth characteristics of the *gac* mutants, provide a competitive advantage, especially in changing and heterogeneous environments. The observation that the *gac* subpopulation does not replace the wild-type population in culture, as found for PCL1171, shows the effect of the reversibility of the *gac* phenotype and indicates that, at least under laboratory conditions, there is no direct environmental selection in favor of these mutants.

What is the mechanism of phase variation in PCL1171? According to the literature, phase variation can regulate gene expression via four mechanisms: (i) slipped-strand mispairing, (ii) differential methylation, (iii) genomic rearrangements, and (iv) random unprogrammed variation (3, 12). To our knowledge, only two molecular mechanisms of phase variation in *Pseudomonas* spp. have been described. In *Pseudomonas putida* DOT-T1E, the expression of flagella is controlled via slipped-strand mispairing (35), and in *P. tolaasii*, a 661-bp spontaneous, reversible duplication in *pheN* controls colony morphology and pathogenicity (9). In addition, although no mechanism is described, the *sss* gene, encoding a site-specific recombinase, was suggested to play a major role in the phenotypic variation of *P. fluorescens* F113 during root colonization. The latter variation is combined with selection for *gac* mutants (32).

Considering the mechanism of phase variation via *gac*, none of the sequenced *gacA*/*gacS* genes harbors repeat tracts. Since phase variation in PCL1171 is dependent on mutation of *gac*, it is not epigenetic. Therefore, slipped-strand mispairing or differential methylation can be excluded as the major basis of phase variation via *gacA*/*gacS* in PCL1171. Genomic rearrangements were present in some *gacS* genes isolated from phase II bacteria and were based on the presence of inverted or tandem repeats (Table 2 and Fig. 2). This suggests a role for site-specific recombinases (30) in mutation of these *gac* genes. But, since these rearrangements could take place only after the occurrence of point mutations, introducing repeats (Fig. 2) which can be recognized by site-specific recombinases, we con-

clude that genomic rearrangements play only a minor, secondary role in the phase variation of PCL1171. This conclusion differs from what has been reported for *P. fluorescens* F113, in which a site-specific recombinase is responsible for the majority of the phenotypic variation (32).

In PCL1171, the accumulation of mutations in *gacA*/*gacS* was previously suggested to be suppressed by MutS-dependent mismatch repair, since mutation of the *mutS* gene resulted in a dramatic increase in the frequency of phase variation (38). Furthermore, based on the mechanism of repair (23), all point mutations identified in *gacS* (Table 2) could, upon introduction, have been recognized by MutS. This suggests that inefficient repair via MutS contributes to the mutations we found in *gacA*/*gacS*. In addition, the spontaneous mutation rate in PCL1171, measured by the frequency of spontaneous rifampin-resistant mutants, increases upon mutation of *mutS* from 5×10^{-7} to 3×10^{-5} mutants per cell per generation (S. de Weert, personal communication). This frequency is comparable to the frequency of phase variation in the wild type (6×10^{-5} switches per cell per generation), supporting the notion that inefficient repair of replication-related mismatches via MutS is responsible for the accumulation of random mutations in *gacA*/*gacS*. This observation supports the hypothesis that the *gacA* and *gacS* genes are not hot spots for mutations. Under growth-limiting conditions, microorganisms can increase their mutation frequency (16, 25). Under these conditions, *gac* mutants could be selected for, either on the basis of their growth characteristics or by preventing restoration of the mutations. Our observations suggest that the mechanism of phase variation in PCL1171 is random and unprogrammed variation based on the introduction and restoration of random point mutations, insertions, deletions, and rearrangements in *gacA*/*gacS*. Unprogrammed variation is not dependent on specific DNA features, and spontaneous duplications and deletions have been reported to control, for example, capsule genes in *Streptococcus pneumoniae* (39), pathogenicity and colony morphology in *P. tolaasii* and *Ralstonia solanacearum* (9, 26), and the virulence of *Bordetella bronchiseptica* (24).

Unprogrammed phase variation, as observed in PCL1171, is reversible in the sense that the original sequence of the wild-type gene is restored. At this moment no molecular mechanism for the restoration of the mutations in the strains mentioned has been elucidated. A preliminary study on the reversion of the phase II phenotype in PCL1171 focused on the possible role of homologous recombination. To test this idea, we constructed a RecA and a RecB mutant. Both genes are involved in homologous recombination (18) and, in some cases, in phase variation (12, 17, 21). Neither *recA* mutation nor *recB* mutation affected the ability of phase II bacteria to switch back to the phase I phenotype. Although this result cannot exclude the possibility that recombinational processes are involved in this phase variation, it shows that RecA and RecB are not essential for the repair of mutations in *gacS* and *gacA* of PCL1171. A similar observation was made for *P. tolaasii* (37), where RecA was not needed for the removal of the duplication in *pheN* (37).

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REFERENCES

1. **Abraham, J. M., C. S. Freitag, J. R. Clements, and B. I. Eisenstein.** 1985. An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **82:**5724–5727.
2. **Blumer, C., S. Heeb, G. Pessi, and D. Haas.** 1999. Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc. Natl. Acad. Sci. USA **96:** 14073–14078.
3. **Borst, P.** 2003. Mechanisms of antigenic variation: an overview, p. 1–15. *In* A. Craig and A. Scherf (ed.), Antigenic variation. Elsevier Academic Press, London, United Kingdom.
4. **Bull, C. T., B. Duffy, C. Voisard, G. Defago, C. Keel, and D. Haas.** 2001. Characterization of spontaneous *gacS* and *gacA* regulatory mutants of *Pseudomonas fluorescens* biocontrol strain CHAO. Antonie Leeuwenhoek **79:**327–336.
5. **Chabeaud, P., A. de Groot, W. Bitter, J. Tommassen, T. Heulin, and W. Achouak.** 2001. Phase-variable expression of an operon encoding extracellular alkaline protease, a serine protease homolog, and lipase in *Pseudomonas brassicacearum*. J. Bacteriol. **183:**2117–2120.
6. **Chancey, S. T., D. W. Wood, E. A. Pierson, and L. S. Pierson III.** 2002. Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30–84 in the wheat rhizosphere. Appl. Environ. Microbiol. **68:**3308–3314.
7. **Duffy, B. K., and G. Defago.** 2000. Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. **66:**3142–3150.
8. **Dybvig, K.** 1993. DNA rearrangements and phenotypic switching in prokaryotes. Mol. Microbiol. **10:**465–471.
9. **Han, B., A. Pain, and K. Johnstone.** 1997. Spontaneous duplication of a 661 bp element within a two-component sensor regulator gene causes phenotypic switching in colonies of *Pseudomonas tolaasii*, cause of brown blotch disease of mushrooms. Mol. Microbiol. **25:**211–218.
10. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
11. **Heeb, S., Y. Itoh, T. Nishijyo, U. Schnider, C. Keel, J. Wade, U. Walsh, F. O'Gara, and D. Haas.** 2000. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. Mol. Plant-Microbe Interact. **13:**232–237.
12. **Henderson, I. R., P. Owen, and J. P. Nataro.** 1999. Molecular switches—the ON and OFF of bacterial phase variation. Mol. Microbiol. **33:**919–932.
13. **Josenhans, C., K. A. Eaton, T. Thevenot, and S. Suerbaum.** 2000. Switching of flagellar motility in *Helicobacter pylori* by reversible length variation of a short homopolymeric sequence repeat in *fliP*, a gene encoding a basal body protein. Infect. Immun. **68:**4598–4603.
14. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44:**301–307.
15. **Kitten, T., T. G. Kinscherf, J. L. McEvoy, and D. K. Willis.** 1998. A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. Mol. Microbiol. **28:**917–929.
16. **Kivisaar, M.** 2003. Stationary phase mutagenesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. Environ. Microbiol. **5:**814–827.
17. **Koomey, M., E. C. Gotschlich, K. Robbins, S. Bergstrom, and J. Swanson.** 1987. Effects of *recA* mutations on pilus antigenic variation and phase transitions in *Neisseria gonorrhoeae*. Genetics **117:**391–398.
18. **Kowalczykowski, S. C., D. A. Dixon, A. K. Eggleston, S. D. Lauder, and W. M. Rehrauer.** 1994. Biochemistry of homologous recombination in *Escherichia coli*. Microbiol. Rev. **58:**401–465.
19. **Kuiper, I., G. V. Bloemberg, S. Noreen, J. E. Thomas-Oates, and B. J. Lugtenberg.** 2001. Increased uptake of putrescine in the rhizosphere inhibits competitive root colonization by *Pseudomonas fluorescens* strain WCS365. Mol. Plant-Microbe Interact. **14:**1096–1104.
20. **Laville, J., C. Voisard, C. Keel, M. Maurhofer, G. Defago, and D. Haas.** 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc. Natl. Acad. Sci. USA **89:** 1562–1566.
21. **Mehr, I. J., and H. S. Seifert.** 1998. Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation and DNA repair. Mol. Microbiol. **30:**697–710.
22. **Meyer, T. F., C. P. Gibbs, and R. Haas.** 1990. Variation and control of protein expression in *Neisseria*. Annu. Rev. Microbiol. **44:**451–477.
23. **Modrich, P.** 1991. Mechanisms and biological effects of mismatch repair. Annu. Rev. Genet. **25:**229–253.
24. **Monack, D. M., B. Arico, R. Rappuoli, and S. Falkow.** 1989. Phase variants of *Bordetella bronchiseptica* arise by spontaneous deletions in the *vir* locus. Mol. Microbiol. **3:**1719–1728.
25. **Moxon, E. R., P. B. Rainey, M. A. Nowak, and R. E. Lenski.** 1994. Adaptive evolution of highly mutable loci in pathogenic bacteria. Curr. Biol. **4:**24–33.
26. **Poussier, S., P. Thoquet, D. Trigalet-Demery, S. Barthet, D. Meyer, M. Arlat,**

and A. Trigalet. 2003. Host plant-dependent phenotypic reversion of *Ralstonia solanacearum* from non-pathogenic to pathogenic forms via alterations in the *phcA* gene. Mol. Microbiol. **49:**991–1003.
27. **Raaijmakers, J. M., and D. M. Weller.** 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. Mol. Plant-Microbe Interact. **11:**144–152.
28. **Rich, J. J., T. G. Kinscherf, T. Kitten, and D. K. Willis.** 1994. Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. J. Bacteriol. **176:**7468–7475.
29. **Rosengarten, R., and K. S. Wise.** 1990. Phenotypic switching in mycoplasmas: phase variation of diverse surface lipoproteins. Science **247:**315–318.
30. **Sadowski, P.** 1986. Site-specific recombinases: changing partners and doing the twist. J. Bacteriol. **165:**341–347.
31. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Sanchez-Contreras, M., M. Martin, M. Villacieros, F. O'Gara, I. Bonilla, and R. Rivilla.** 2002. Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. J. Bacteriol. **184:** 1587–1596.
33. **Saunders, N. J., E. R. Moxon, and M. B. Gravenor.** 2003. Mutation rates: estimating phase variation rates when fitness differences are present and their impact on population structure. Microbiology **149:**485–495.
34. **Schmidt-Eisenlohr, H., A. Gast, and C. Baron.** 2003. Inactivation of *gacS* does not affect the competitiveness of *Pseudomonas chlororaphis* in the *Arabidopsis thaliana* rhizosphere. Appl. Environ. Microbiol. **69:**1817–1826.
35. **Segura, A., A. Hurtado, E. Duque, and J. L. Ramos.** 2004. Transcriptional phase variation at the *flhB* gene of *Pseudomonas putida* DOT-T1E is involved in response to environmental changes and suggests the participation of the flagellar export system in solvent tolerance. J. Bacteriol. **186:**1905–1909.
36. **Simon, R., M. O'Connell, M. Labes, and A. Puhler.** 1986. Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. Methods Enzymol. **118:**640–659.
37. **Sinha, H., A. Pain, and K. Johnstone.** 2000. Analysis of the role of *recA* in phenotypic switching of *Pseudomonas tolaasii*. J. Bacteriol. **182:**6532–6535.
38. **van den Broek, D., T. F. C. Chin-A-Woeng, K. Eijkemans, I. H. Mulders, G. V. Bloemberg, and B. J. J. Lugtenberg.** 2003. Biocontrol traits of *Pseudomonas* spp. are regulated by phase variation. Mol. Plant-Microbe Interact. **16:**1003–1012.
39. **Waite, R. D., J. K. Struthers, and C. G. Dowson.** 2001. Spontaneous sequence duplication within an open reading frame of the pneumococcal type 3 capsule locus causes high-frequency phase variation. Mol. Microbiol. **42:** 1223–1232.
40. **Wolk, C. P., Y. Cai, and J. M. Panoff.** 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc. Natl. Acad. Sci. USA **88:**5355–5359.
41. **Zuber, S., F. Carruthers, C. Keel, A. Mattart, C. Blumer, G. Pessi, C. Gigot-Bonnefoy, U. Schnider-Keel, S. Heeb, C. Reimmann, and D. Haas.** 2003. GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. Mol. Plant-Microbe Interact. **16:**634–644.