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 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), fimbriniae (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 Geaumanomycetes 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 response 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.
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 Tn5luxABC transposon is inserted into a gacS homolog; Km’ | 38 |
| PCL1586           | Derivative harboring a mutation in a recA homolog; Km’ | This study |
| PCL1589           | PCL1171 derivative harboring a mutation in a recB homolog; Km’ | This study |
| S17-1             | *E. coli* MM294, RP4-2 Ce::Mu-Km::T7 chromosomally integrated | 36 |
| **DH5α**         | *E. coli* endA1 gyrA96 hrdR17 (rK– mK–) supE44 recA1; general-purpose *E. coli* host strain | 10 |
| **Plasmids**     |                 |                     |
| pRL1063a         | Plasmid harboring a promoterless Tn5luxABC transposon and a p15A origin of replication; Km’ | 40 |
| pMP5285          | pME3049 derivative, lacking the Hg’ gene, used for single homologous recombination; Km’ | 19 |
| pME6010          | *E. coli*/Pseudomonas shuttle vector, stably maintained in *Pseudomonas* species, with an estimated copy number of 4 to 8; Tc’ | 11 |
| pMP6562          | pME6010 harboring a 3.2-kb PCR product from strain PCL1171 which contains the gacS homolog from PCL1171; Tc’ | 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% (w/v) agar (Difco Laboratories, Detroit, Mich.). Kanamycin, tetracycline, and carbencillin (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 *Tn5luxABC* transposon mutants.** A mutant library of strain PCL1171 was constructed by using plasmid pRL1063a (38), which harbors a Tn5 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 located in the phase II phenotype. General DNA recombinant techniques were performed according to the work of Sambrook and Russell (31).

**Complementation of *Tn5luxABC* 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’-GGATTCCAGTACAGATGTTCCAGAAG GCACACA-3’) and oMP717 (5’-SGAATTCGTTGCACTGATGAAAG GCACACA-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, pMP6031, 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::Tn5luxABC) 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 pMP716 and oMP717; internal primers oMP98 (5’-ACCCATCTCTGGAACAATC-3’), oMP99 (5’-GAGGTGGTCTTCGGCCCAACATGAG-3’), oMP100 (5’-TTCGACAGCGGCACCATG-3’), and oMP101 (5’-CCAGATGTCGAGACCATG-3’), annealing at nucleotide positions 220, 859, 1456, and 2047, respectively; and reverse primers oMP723 (5’-GTTCCACACTCAACACCATG-3’), oMP722 (5’-GTAATGCGGTGTCAGGTAGT-3’), oMP721 (5’-GGTGTAGTGCGCCC AACAGT-3’), and oMP720 (5’-CCGATACTGGTCTGAGTAC-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 the *Bluescript* T7 (5’-TACTACCATGCTTACAGAC-3’ and 5’-GTCGACTGGGATC-3’) and two internal primers for recA, oMP529 (5’-GGGGTACCCGGAAGTACGATG-3’) and oMP530 (5’-GGTATCAGACCCGGAACGATG-3’). The 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 pMP604. 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’-TTCGAGAAGGCGAAGACGCGTGGC...
TTACCCAGA-3') and oMP761 (5'-AAGCCGTA(CT)AT(GC)CG(A)GAGTGC TTGGGTTGCGCCAT-3'). To construct a recB mutation, a 1.2-kb PCR product was obtained by using oMP840 (5'-CAGGATTGCAAGGTTGGT-3') and oMP761 cloned into the pGEM-Teasy vector (Promega Corp., Madison, Wis.) and subsequently transferred to the suicide plasmid pMP5265 by using EcoRI, resulting in pMP6605. pMP6605 was electroporated into S17-1 (36) and transferred to PCL171 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 Tn5luxAB 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 Tn5luxAB transposon library from PCL1171, which has a strong preference for the phase I phenotype, by using pRL1063a (40). From this library, 26 Tn5luxAB 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 Tn5 insertion in gacS could lock PCL1171 in the phase II phenotype (38), we tested the possibility that, in addition to the Tn5luxAB insertion, at least some of the mutants had accumulated mutations in gacS or gacA. For this we used plasmids pMP5662 (harboring a gacS homolog of PCL1171), pMP5565 (harboring a gacA homolog of Pseudomonas sp. strain PCLI446), 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 pMP5662 (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 Tn5 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 PCLI171. 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 PCLI574 and PCLI575 were found to harbor a point mutation (Table 2). The gacS gene from PCLI576 harbored a 115-bp inversion, which was flanked by an inverted repeat (5’-C442TGCTG-3’). This repeat was created due to a point mutation at nucleotide position 329 (aA329c→aG329c) (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::Tn5luxAB) by using the gacS gene isolated from PCLI574 resulted in a mixture of phase I and phase II colonies. Since this gacS gene did not restore the phase I phenotype in all PCLI572 transformants, the construct harboring the gacS gene from PCLI574 was reisolated from colonies in which gacS did complement PCLI1572. 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 initial introduction of the wild-type gacS gene completely restored the phase I phenotype in this mutant, the introduction of pMP5562 (gacS) in subsequent complementations also resulted in a number of phase II colonies. These could be complemented by using pMP5565 (gacA). This changed PCLI575 derivative was designated PCLI575a (Table 2). In addition, PCLI575 phase II colonies were found which could be complemented neither by using pMP5565 nor by using pMP6562. One such derivative was designated PCLI575b (Table 2). Sequencing of the gacS gene of PCLI575a after PCR amplification showed that the point mutation identified in PCLI575 had been removed (T154aa→C154aa) in PCLI575a while a phase II phenotype was maintained. This point mutation was still present in PCLI575b (T154aa) (Table 2).

Molecular basis of phase II phenotype in the wild-type strain PCLI171. To test whether mutations in the gac system also form the molecular basis for the highly unstable phase II
phenotype in the wild-type strain PCL1171, the \textit{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 \textit{gacA} or \textit{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 \textit{gacA} and \textit{gacS} mutations. For this purpose the amplified \textit{gacS} gene from phase II sectors was cloned into pMP5565 (containing a wild-type \textit{gacA} gene) and the resulting construct was used to complement PCL1572 (\textit{gacS::Tn5luxAB}). Of 13 \textit{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 \textit{gacS} genes complemented PCL1572 to the phase I phenotype, showing that in the latter seven sectors \textit{GacS} function was not affected and that the phase II phenotype is most likely caused by a mutation in the \textit{gacA} gene.

As controls, the \textit{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 \textit{gacS} gene isolated from sector 5 (Table 2).

Analysis of five out of the six noncomplementing \textit{gacS} genes revealed various mutations (Table 2). The \textit{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 \textit{gacS} genes from sectors 4 and 18 were found to harbor one and two point mutations, respectively (Table 2), while the \textit{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 \textit{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\textsubscript{2226}CCATCACC\textsubscript{3}' and 5'-G\textsubscript{2523}CCATCACC\textsubscript{3}') was identified at the borders of the deletion as the result of a single point mutation (tT\textsubscript{2231}a\textrightarrow{}tC\textsubscript{2231}a) (Table 2 and Fig. 2D). The \textit{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 \times 10^{-2} switches per cell per generation) of switching back to the phase I phenotype. This high frequency enabled us to analyze the \textit{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 \textit{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 \textit{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 \textit{gacS} gene, in both stable and unstable phase II bacteria, could be restored efficiently to the wild-type \textit{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 \textit{recA} mutation was introduced into PCL1171, by using the suicide construct pMP6604, resulting in strain PCL1586. Strain PCL1586 (\textit{recA::Km'}) 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 \textit{recB} fragment was used to introduce a \textit{recB} mutation in PCL1171. Mutation of \textit{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 \textit{gacS}.** \textit{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 \textit{gacS} gene locked the bacteria in a phase II phenotype (38), we studied the role of \textit{gacA}/\textit{gacS} in phase variation. Complementation analysis of a total of 39 phase II derivatives showed that mutations in \textit{gacA} (20 mutants) and \textit{gacS} (19 mutants) are responsible for the phase II phenotype (Table 2). Introduction of pMP6603 (\textit{gacA} \textit{gacS}) restored all mutants to a phase I phenotype, showing that spontaneous mutation of the \textit{gacA}/\textit{gacS} two-component regulatory system forms the basis of the switch from phase I to phase II in phase variation of \textit{Pseudomonas} sp. strain PCL1171.

The \textit{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 \textit{gacA} gene of \textit{Pseudomonas fluorescens} CHAO by Bull et al. (4) (point mutations, a 3-bp deletion, and a 1-bp...
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. aeruginosa strain 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 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.

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-

### 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 | Functional gene | Remarks | Affected GacS domain |
|--------|-----------------------|-----------------|---------|----------------------|
|        | Nucleotide | Amino acid | gacA | gacS |                              |
| Phase II Tn5luxAB mutants⁶ | | | | | |
| Wild-type phase I | | | | | |
| Wild-type phase II sectors⁵ | | | | | |
| Sector 1 | 12-bp deletion cT₄₆₁t-cC₄₆₁³ | A₄₆PL₆₆ | + | + | No mutations |
| Sector 3 | ND | | | | Periplasmic loop |
| Sector 4 | C₉₉₁g-T₉₉₁⁵ | P₆₆₁-S₆₆₁ | ND | | gacS mutation |
| Sector 9 | ct-frameshift | L₇₄₄-S₇₄₄ | ND | | Deletion⁷ |
| Sector 16 | 307-bp deletion ggC₆₆₃–ggA₆₆₃ | Silent | | | |
| Sector 18 | 1-bp insertion | L₆₁-P₆₁ | ND | | Point mutations |
| Sector 2 | 443g–cC₄₄₃³ | L₁₄₈-P₁₄₈ | ND | | gacA mutation |

### Notes
- Numbers indicate positions in the wild-type gacS sequence.
- 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 gacS gene (gacS mutants), while PCL1564, PCL1573, PCL1566, PCL1568, and another eight phase II mutants had a functional gacA gene (gacA mutants).
- The functional gacA/gacS component was determined by complementation of PCL1572 (gacS: Tn5luxAB) 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:: Tn5luxAB), suggesting that mutation of the gacA gene was responsible for the phase II phenotype in these sectors.

### References
- Pseudomonas chlororaphis CHAO (4, 7, 20), Pseudomonas fluorescens P. fluorescens pv. syringae strain B728a (28), P. fluorescens CHAO (4, 7, 20), Pseudomonas aureofaciens 30-84 (6), and Pseudomonas chlororaphis isolate SPR044 (34).
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 phe notypic 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 $5 \times 10^{-7}$ to $3 \times 10^{-5}$ mutants per cell per generation (S. de Weert, personal communication). This frequency is comparable to the frequency of phase variation in the wild type ($6 \times 10^{-5}$ 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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