Role of RpoS and MutS in phase variation of *Pseudomonas* sp. PCL1171

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*Pseudomonas* sp. strain PCL1171 undergoes reversible colony phase variation between opaque phase I and translucent phase II colonies, which is dependent on spontaneous mutations in the regulatory genes gacA and gacS. Mutation of the mutS gene and constitutive expression of *rpoS* increases the frequency at which *gac* mutants appear 1000- and 10-fold, respectively. Experiments were designed to study the relationship between gacS, *rpoS* and mutS. These studies showed that (i) a functional gac system is required for the expression of *rpoS*, (ii) RpoS suppresses the expression of *mutS* and therefore increases the frequency of *gac* mutants, and (iii) upon mutation of *rpoS* and gacS, the expression of *mutS* is increased. Mutation of gacS abolishes suppression of *mutS* expression in stationary growth, suggesting that additional gac-dependent factors are involved in this suppression. In conclusion, inefficient mutation repair via MutS, of which the expression is influenced by gacAI/S itself and by *rpoS* in combination with other factors, contributes to the high frequency of mutations accumulating in gacAI/S. The role of RpoS in the growth advantage of a *gac* mutant was analysed, and mutation of *rpoS* only reduced the length of the lag phase, but did not affect the growth rate, suggesting a role for both RpoS and a reduction of metabolic load in the growth advantage of a *gac* mutant.

INTRODUCTION

Phase variation is a process of reversible high-frequency phenotypic switching that is mediated by mutations, reorganizations or other modifications of DNA. This process is used by several bacterial species to generate population diversity, which in turn increases bacterial fitness and plays a role in niche adaptation (Saunders et al., 2003). Phase variation in *Pseudomonas* sp. PCL1171 is dependent on the accumulation and subsequent removal of mutations in the *gacA* and *gacS* genes (van den Broek et al., 2005). These genes affect the production of secondary metabolites, such as a lipopeptide with antifungal activity, a biosurfactant, and the exo-enzymes chitinase, lipase and protease (van den Broek et al., 2003). The gacAI/gacS two-component regulatory system consists of a membrane-bound sensor kinase, GacS, and a response regulator, GacA. The latter protein belongs to the FixJ family of transcriptional regulators (Laville et al., 1992; Rich et al., 1994). The gac system regulates secondary metabolism and the production of exo-enzymes (Hrabak & Willis, 1993; Laville et al., 1992; Schmidli-Sacherer et al., 1997; Reimmann et al., 1997).

In several *Pseudomonas* species, the *gacA* and *gacS* genes are subject to spontaneous mutation, and *gac* mutants appear for example in nutrient-rich liquid medium (Bull et al., 2001; Duffy & Defago, 1995; van den Broek et al., 2003) and on plant roots (Sanchez-Contreras et al., 2002; Chancy et al., 2002; Schmidt-Eisenlohr et al., 2003; Achouak et al., 2004). In PCL1171, spontaneous mutation of the gacAI/S system is the basis for phase variation. Information on factors influencing the introduction of mutations in the *gac* genes of *Pseudomonas* as part of a phase-variation mechanism or by phenotypic selection are scarce.

Previously, we have reported that MutS-dependent mismatch repair affects phase variation (van den Broek et al., 2003, 2005). MutS is involved in the methyl-directed recognition of DNA mismatches related to replication, which include base mismatches and small insertion and deletion mispairs (Modrich, 1991). The expression of mismatch repair systems can be negatively influenced by stress or growth-limiting conditions, resulting in increased genetic and population diversity (Kivisaar, 2003). For example, in *Escherichia coli*, mutS is negatively regulated by the general stress-response sigma factor RpoS (Tsui et al., 1997). In *Pseudomonas*, the regulatory link between RpoS and MutS and the role of these proteins in bacterial phase variation have not been studied. To identify and understand genetic factors which influence the high
frequency of spontaneous mutations accumulating in gacA/S, and the switch from phase I to phase II, we studied the regulatory relationship between gacA/S, rpoS, and mutS in the phase variation of Pseudomonas sp. PCL1171.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. Pseudomonas strains were grown in King’s medium B (KB) (King et al., 1954) at 28°C. E. coli strains were grown in LB (Sambrook & Russell, 2001) at 37°C. Solid growth media contained 1-8% (w/v) agar (Difco). Kanamycin, tetracycline, gentamicin and carbenicillin (Sigma) were added for antibiotic selection at final concentrations of 50, 40, 10 and 50 μg ml⁻¹, respectively. Growth in liquid medium was analysed in triplicate by measuring OD₆₂₀ (cuvette path length, 1 cm, using a Pharmacia Biotech Novaspec II) over time. To determine the number of bacteria, samples of the culture were plated, and the number of viable cells was established as colony-forming units.

Construction of mutants. PCL1587 (rpoS::km') was constructed using a 0-7 kb PCR product of the rpoS gene of Pseudomonas chlororaphis strain PCL1391. The PCR product was obtained using oMP768, AA(AC)GAAG(GCT)GCGGGAGTTTGAC, and oMP769, GT(A/G)TG(GC)AGCAAGGTCTTGTCCGA, and cloned into pMP5285 to form pMP7418 (G. Girard, unpublished results). pMP7418 was transferred to PCL1171 by parental mating. Mutation of rpoS was confirmed by Southern hybridization (Sambrook & Russell, 2001) using the AlkPhos direct labelling kit for detection (Amersham Biosciences) according to the manufacturer’s recommendations. Colony morphology was judged after at least 2 days of growth at 28°C. Parental mating with E. coli S17-1 (Simon et al., 1986) harbouring pMP7420 (pBBR1MCS-5 harbouring the rpoS gene of P. chlororaphis PCL1391 under a constitutive tac promoter; G. Girard, unpublished results), was used to introduce pMP7420 into PCL1171 and PCL1555, resulting in PCL1585 and PCL1590, respectively.

Expression studies using a bioluminescent Tn5luxAB reporter. The expression of the mutS gene was measured using the promoterless luxAB genes (Wolk et al., 1991) inserted behind the mutS promoter. Bacteria were inoculated in 20 ml KB to an OD₆₂₀ between 0.05 and 0.1, and grown under aeration at 28°C. Growth was monitored by measuring OD₆₂₀. To determine gene expression, 100 μl samples were taken in triplicate. A 100 μl volume of a solution containing 0-2% n-decyl-aldehyde (Sigma) and 2-0% BSA (Sigma) was added. After mixing, bioluminescence was determined over time using a MicroBeta 1450 TriLux luminescence counter (Wallac) and normalized to luminescence counts per OD₆₂₀ unit.

Quantification of the frequencies of phase variation. A 20 ml volume of KB medium was inoculated with bacteria from a colony with phase I or phase II morphology and the bacteria were grown overnight at 28°C. After dilution plating of an average of 500 cells per plate, the morphology was analysed and the initial number of bacteria was determined after overnight growth. At least 1500 colonies were examined and counted for the estimation of frequencies as number of switches per cell per generation.

Isolation of total RNA and real-time PCR (RT-PCR). Erlenmeyer flasks containing 20 ml KB medium were inoculated from colonies on
agiar plates and grown under aeration at 28 °C. A 5 ml sample was harvested at an OD<sub>560</sub> of 0.35, and a second sample was taken after 24 h. To stop RNA degradation, 0.625 ml of ice-cold ethanol/phenol was added. Cells were spun down, and the pellet was frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated using the Qiagen RNaseasy mini kit, including an on-column DNaseI digestion (Qiagen), according to the manufacturer’s recommendations. Total RNA was checked on-gel and stored at −20 °C. The amount of isolated RNA was measured at OD<sub>480</sub> using an Ultrospec 2100pro photodensitometer (Amersham Biosciences). Reverse transcription, followed by RT-PCR, was performed using an RT-PCR machine (Lightcycler, Roche) on 500 ng of total RNA using the Lightcycler RNA master SYBR Green I (Roche) according to the manufacturer’s recommendations. All experiments and each measurement were carried out at least three times.

RESULTS

RpoS plays a role in phase variation of Pseudomonas sp. PCL1171

To study the possible role of the general stress-response sigma factor RpoS in the regulation of MutS-dependent mismatch repair in PCL1171, and its role in the accumulation of mutations in gacA/S, strains PCL1587 and PCL1585 were constructed. Strain PCL1587 harbours a mutation in the rpoS gene, and the rpoS-overexpressing strain PCL1585 was obtained by the introduction of pMP7420 (pBBR1MCS-5 derivative harbouring an rpoS homologue expressed under the constitutive tac promoter) into PCL1171. Wild-type strain PCL1171 displays a high frequency of switching from phase I to phase II, whereas the switch-back frequency to phase I is even higher (Table 2). On plates, rpoS mutant PCL1587 was found primarily as phase I colonies, out of which phase II sectors appeared. Mutation of rpoS decreased the frequency of the switch from phase I to phase II (Table 2). Phase II colonies of an rpoS mutant of PCL1171 appeared to be of two classes with respect to their rate of reversion to phase I. In the first class, only phase I colonies were found after replating the start mix from liquid culture, whereas in the other class, cells appeared to be locked in the phase II phenotype (Table 2).

Constitutive expression of rpoS in the wild-type, studied using pMP7420, increased the frequency of phase variation from phase I to phase II tenfold, but had no significant effect on the frequency of the reverse switch (Table 2). Complementation of phase II colonies from PCL1585 (PCL1171 P<sub>tac</sub>rpoS) with pMP6603 (pME6010 harbouring both the gacA and gacS genes under their own promoters) (van den Broek et al., 2005) restored the phase I phenotype in these bacteria. Introduction of the parent vector pME6010 did not affect the phase II phenotype in these phase II colonies.

Expression of rpoS is dependent on growth phase and gac

RT-PCR was used to study the effect of gacA/S on rpoS expression in the exponential and stationary growth phases. It appeared that in wild-type strain PCL1171, rpoS expression was increased approximately fourfold in stationary phase cells compared to exponentially growing bacteria (Table 3). In mutant PCL1572 (gacS::Tn5luxAB), locked in phase II, the expression of rpoS in the exponential phase was approximately fivefold lower than in phase I wild-type cells, and no induction of rpoS expression was observed in the stationary phase compared to the exponential phase (Table 3).

RpoS regulates mutS

The effect of a mutS mutation on the frequency of switching was analysed using PCL1555 (mutS::Tn5luxAB). Mutation of mutS increased the switching frequency from phase I to phase II approximately 1000-fold, whereas the switch from phase II to phase I was unaltered (Table 2). To study the effect of constitutive rpoS expression on the frequency of phase variation in a mutS background, strain PCL1590 was constructed by the introduction of pMP7420 (P<sub>tac</sub>rpoS) into PCL1555 (mutS::Tn5luxAB). Constitutive expression of rpoS did not alter the frequency of switching from phase I to phase II, whereas the frequency from phase II to phase I was slightly decreased (Table 2).

The expression of the mutS gene during growth could be studied in PCL1555 (mutS::Tn5luxAB) (van den Broek et al., 2003), which harbours a Tn5 transposon with promoterless luxAB genes (Wolk et al., 1991). Expression of mutS at different growth stages of strain PCL1555 was compared with expression in its derivative strain PCL1590 harbouring pMP4720 (pBBR1MCS-5 P<sub>tac</sub>rpoS). In PCL1555, the mutS gene is highly expressed during the exponential growth phase, but expression was decreased approximately twofold upon transition to stationary growth (Fig. 1). In strain PCL1590, in which rpoS is

Table 2. Influence of rpoS and mutS on the frequency of colony phase variation

Frequencies are shown as the number of switches per cell per generation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Frequency of phase variation for:</th>
<th>Phase I→</th>
<th>Phase II→</th>
<th>Phase II→</th>
<th>Phase I→</th>
</tr>
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<tbody>
<tr>
<td>PCL1171 (wild-type)</td>
<td></td>
<td>6.4 × 10&lt;sup&gt;−5&lt;/sup&gt;</td>
<td>8.2 × 10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL1587 (rpoS::km&lt;sup&gt;+&lt;/sup&gt;)</td>
<td></td>
<td>3.8 × 10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td></td>
<td>6.9 × 10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PCL1585 (PCL1171 P&lt;sub&gt;tac&lt;/sub&gt;rpoS)</td>
<td></td>
<td>6.2 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL1555 (mutS::Tn5luxAB)</td>
<td></td>
<td>8.3 × 10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PCL1590 (PCL1555 P&lt;sub&gt;tac&lt;/sub&gt;rpoS)</td>
<td></td>
<td>7.8 × 10&lt;sup&gt;−2&lt;/sup&gt;</td>
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</table>

*Phase II colonies of PCL1587 (rpoS::km<sup>+</sup>) appeared to be of two classes with respect to their reversion rate to the phase I phenotype. One class was locked in the phase II phenotype, while in the other class only phase I colonies were found after replating from culture.
constitutively expressed, the expression of mutS during exponential growth was as low as during stationary growth (Fig. 1).

To determine the effect of rpoS on the expression of mutS in more detail, RT-PCR of mutS was used. In wild-type strain PCL1171, mutS was expressed during exponential growth, and expression was reduced approximately two to threefold in the stationary phase, whereas in PCL1555 (mutS::Tn5luxAB), the expression of mutS was two to fourfold increased compared to that of the wild-type in the exponential phase (Table 3). In the phase variation of strain PCL1171 by analysing mutS::Tn5luxAB reporter in the mutS gene. The gacS and rpoS genes result in a growth advantage under certain conditions (van den Broek et al., 2005). To identify genetic factors involved in the accumulation of mutations in the gacA/S genes, we examined the roles of gacA/S, rpoS and mutS in the phase variation of strain PCL1171 by analysing constitutively expressed, the expression of mutS during exponential growth was as low as during stationary growth (Fig. 1).

Expression levels determined using RT-PCR are related to the expression level of the indicated gene during exponential growth in the wild-type. Exp., exponential phase; Stat., stationary phase; 0, no detectable expression; ND, not determined.

Table 3. Relative expression of gacS, rpoS and mutS in Pseudomonas sp. PCL1171 and derivatives during exponential and stationary growth

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative expression levels in exponential and stationary growth phases for:</th>
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<tbody>
<tr>
<td></td>
<td>gacS</td>
</tr>
<tr>
<td></td>
<td>rpoS</td>
</tr>
<tr>
<td></td>
<td>mutS</td>
</tr>
<tr>
<td>PCL1171 (wild-type)</td>
<td>Exp. 1 Stat. 0-62±0-2</td>
</tr>
<tr>
<td>PCL1572 (gacS::Tn5luxAB)</td>
<td>Exp. 1 Stat. 0-20±0-1 0-30±0-2</td>
</tr>
<tr>
<td>PCL1587 (rpoS::km³)</td>
<td>ND 0-4±0-7</td>
</tr>
<tr>
<td>PCL1555 (mutS::Tn5luxAB)</td>
<td>ND 3-4±1-2 1-4±0-2</td>
</tr>
</tbody>
</table>

In wild-type phase I cells, the gacS gene is primarily expressed during the transition from exponential to stationary growth (data not shown), after which the expression decreases to a 1-6-fold lower level after 24 h of growth, as shown by RT-PCR (Table 3). Expression of gacS in PCL1572 (gacS::Tn5luxAB) was abolished (Table 3). In the gacS mutant PCL1572, the expression of mutS was two to threefold increased in both exponential and stationary phases compared to the wild-type (Table 3).

Influence of gacS and rpoS on growth

Mutation of gac results in a growth advantage (van den Broek et al., 2005; Chancey et al., 2002). The latter authors hypothesize this advantage to be dependent on the reduced expression of rpoS. When the growth of the wild-type strain PCL1171 was compared to that of PCL1572 (gacS::Tn5luxAB), a reduction in the length of the lag phase and a decrease in generation time (from 60±4 to 50±4 min) was observed for PCL1572, and this mutant reached a lower cell density after 24 h of growth (Fig. 2). Mutation of the rpoS gene in PCL1587 slightly reduced the length of the lag phase and, compared to the wild-type, a reduction in the growth rate was observed (the generation time increased from 60±4 to 93±2 min) (Fig. 2).

DISCUSSION

The results of a previous paper (van den Broek et al., 2003) indicate that MutS-dependent mismatch repair plays a role in colony phase variation in Pseudomonas sp. PCL1171, which occurs via the accumulation of spontaneous reversible mutations in gacA and gacS (van den Broek et al., 2005). The mutations accumulated in the gacA/S genes result in a growth advantage under certain conditions (van den Broek et al., 2005). To identify genetic factors involved in the accumulation of mutations in the gacA/S genes, we examined the roles of gacA/S, rpoS and mutS in the phase variation of strain PCL1171 by analysing constitutively expressed, the expression of mutS during exponential growth was as low as during stationary growth (Fig. 1).

gacS and mutS expression in the wild-type and in a gacS mutant

In wild-type phase I cells, the gacS gene is primarily expressed during the transition from exponential to stationary growth (data not shown), after which the expression decreases to a 1-6-fold lower level after 24 h of growth, as shown by RT-PCR (Table 3). Expression of gacS in PCL1572 (gacS::Tn5luxAB) was abolished (Table 3). In the gacS mutant PCL1572, the expression of mutS was two to threefold increased in both exponential and stationary phases compared to the wild-type (Table 3).

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their effects both on phase-variation frequency and on each other's expression.

The introduction of pMP7420 (PtacrpoS) into PCL1171 resulted in a tenfold increase in the frequency of switching from phase I to phase II, while mutation of rpoS decreased the frequency of phase variation (Table 2). The conclusion that rpoS increases the accumulation of mutations in gacA/S was further supported by the introduction of pMP6603 (harbouring gacA and gacS) into phase II colonies of PCL1585 (PCL1171 PtacrpoS), which appeared to restore the phase I phenotype.

An even stronger effect on phase-variation frequency was observed by mutating mutS, which increased the frequency of switching from phase I to phase II 1000-fold (Table 2). Additional overexpression of rpoS, by introducing pMP7420 (PtacrpoS), did not further increase this frequency (Table 2), indicating that the effect of RpoS in phase variation is mainly dependent on its regulatory effect on mutS. Subsequent studies showed that constitutive expression of rpoS suppresses the transcription of the mutS gene (Fig. 1), whereas mutation of rpoS increases the expression of mutS (Table 3), thereby increasing and decreasing, respectively, the frequency of gac mutants (Table 2). We conclude that mutS expression is directly or indirectly repressed by RpoS in PCL1171, thereby increasing the phase-variation frequency. Considering the role of RpoS as a sigma factor (Loewen et al., 1998), this regulation is likely to be indirect. Our results show that changes in mutS expression directly correlate with the frequency at which gac mutants appear (Tables 2 and 3), indicating that the levels of MutS-dependent repair are a determinant for the frequency of phase variation.

The effect of mutation of gac on rpoS expression was analysed using PCL1572 (gacS::Tn5luxAB). It appears that a functional gacA/S system is necessary for the expression of rpoS (Table 3). This suggests that in a phase II phenotype, RpoS will be present in limited amounts or even not at all. That this is likely has been shown for gac mutants of Pseudomonas fluorescens Pf-5 (Whistler et al., 1998), P. chlororaphis isolate SPR044 (Schmidt-Eisenlohr et al., 2003) and P. chlororaphis O6 (Kang et al., 2004).

The expression of mutS was increased in the gacS mutant PCL1572 in both exponential and stationary phase bacteria compared to the wild-type (Table 3), indicating that in a phase II phenotype (in which gacA and/or gacS is mutated), MutS-dependent repair is increased. Under these conditions, the mutation frequency will decrease, thereby limiting possible negative effects of prolonged high mutation rates (Giraud et al., 2001). In addition to our previous observations, which exclude the effect of the nature of the mutations accumulated in gac (van den Broek et al., 2005), this suggests that the switch from phase II to phase I is also independent of MutS or of mutations compensating or restoring the initial mutations. The increase in mutS expression is probably the result of the low RpoS levels in these mutants (Table 3). However, since the repression of mutS in stationary phase, as observed in an rpoS mutant, was no longer observed in a gacS mutant, it is likely that, in addition to RpoS, other gacA/S-dependent factors are involved in the stationary phase repression of mutS (Table 3). Our results suggest that the high frequency of mutations accumulating in gacA/S is the result of inefficient repair by MutS, as a consequence of rpoS expression and possibly additional unknown factor(s), which are in addition all influenced by the mutation of gacA/S itself.

Since we have previously observed a growth effect of the mutation of gac (van den Broek et al., 2005), and since this has been hypothesized to be the result of altered rpoS expression (Chancey et al., 2002), we tested the growth behaviour of an rpoS mutant. Both in our strain PCL1171 (van den Broek et al., 2005) and in P. chlororaphis strain SPR044 (Schmidt-Eisenlohr et al., 2003), mutation of the gacA/S gene abolishes rpoS expression (Table 3). In PCL1171, mutation of the rpoS gene slightly decreases the length of the lag phase, but not as drastically as in a gac mutant (Fig. 2). In addition, the generation time decreases compared to that of the wild-type. The same differences in growth were observed in minimal medium (data not shown). In contrast to the above hypothesis, in strain PCL1171, the lack of RpoS can only partially explain the growth behaviour of a gac mutant. We hypothesize that the selective advantage of spontaneous gac mutants is a combination of the lack of RpoS and of the lowered expression of other (RpoS-independent) genes regulated by gac, resulting in a reduction of metabolic load.

Our observation, that RpoS plays a role in phase variation in Pseudomonas sp. PCL1171, indicates that the accumulation of mutations in gacA/S will be influenced by stress and stationary growth conditions. Consistent with this is the observation that the avoidance of nutrient stress
liquid cultures increases the genetic stability of gacA/S in P. fluorescens (Duffy & Defago, 2000). Increasing the frequency of mutation of gacA/S under growth-limiting conditions will result in a subpopulation with a high growth rate which is able to reinitiate growth more easily (Fig. 2). The appearance of these mutants under laboratory conditions is likely to be the result of the increased growth competitiveness of Pseudomonas syringae (Duffy & Defago, 2000). Increasing the frequency of mutation of the gacA gene in Pseudomonas syringae will result in a subpopulation with a high growth rate which is able to reinitiate growth more easily (Fig. 2). The appearance of these mutants under laboratory conditions is likely to be the result of the increased growth competitiveness (van den Broek et al., 2005). In a heterogeneous and highly competitive environment, such as the rhizosphere, this might enable a population to be more successful in competition and to establish itself via these mutants in new less-limiting environments. Since this trait is combined with the possibility of reversion to the phase I phenotype, the gac mutants can, when conditions improve, switch back to the phase I phenotype to express secondary metabolites and exo-enzymes.

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