# Phase variation in Pseudomonas

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# Phase variation in *Pseudomonas*

Daniël van den Broek

Cover: an on-plate anti-fungal activity assay showing a phase I colony of *Pseudomonas* strain PCL1171 in which a phase II appears as a sector, inhibiting the growth of *Gaeumannomyces graminis* pv. *tritici* R3-11A.

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Phase variation in *Pseudomonas*

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Daniël van den Broek

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Phase variation in *Pseudomonas*, by D. van den Broek

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*Heaven and Hell are just one thing*
*Heaven is what you make of it,*
*Hell is what you go through.*

*Wu-tang*

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# CHAPTER 1

# General introduction

## 1.1 Introduction

Crop loss due to phytopathogenic fungi is an important economical problem in modern agriculture. Crop protection against various diseases is mainly dependent on the use of chemical pesticides (50). Due to growing concerns about negative environmental and health effects of these products, the use of chemicals is being restricted. For example, in 2003 the European Union has banned 60% of the chemical pesticides which were allowed in 1996. This measure increased the demand for alternatives. A number of alternatives for the use of chemical pesticides is available, such as the use of pest-resistant plants. The use of these genetically engineered plants is limited, and although Europe has recently opened its markets for some products from genetically engineered plants, such products still have to cope with a strong negative public perception. Another alternative is the use of naturally occurring micro-organisms to control plant pathogens. Using microbes naturally antagonistic to plant pathogens will limit the need for pesticides. Some of these microbes produce anti-fungal factors. These are produced locally, in minimal amounts and are biodegradable (45, 51, 149). A number of micro-organisms are already in use as commercial products against a range of pathogens (45). One of the drawbacks of the use of micro-organisms to control plant diseases is the fact that these biocontrol agents do not always give consistent disease suppression (30, 67, 117, 120, 133).

## 1.2 Mechanisms of biocontrol

The discovery of naturally disease-suppressive soils led to the identification of bacteria suppressing plant diseases (82, 234, 235, 269). The activity of these suppressive soils is considered to be the result of the activity of these micro-organisms suppressing the pathogen in conjunction with the host plant (213). Four mechanisms have been described to be active in the suppression of pathogens (45, 241): (i) competition for niches and nutrients, (ii) predation and parasitism, (iii) antibiosis, and (iv) induction of systemic resistance in the host plant.

Competition for niches and nutrients is based on a failure of the pathogen to effectively compete with a biocontrol agent for sites on the root and/or for the available nutrients sources. This will limit the opportunities of the pathogens to propagate. The rhizosphere contains a relative high amount of nutrients as a result of exudate compounds leaking from the root (150, 153). Especially the intracellular junctions between root epidermal cells are thought to be rich in

nutrients (44). Therefore, efficient colonisation of these sites by beneficial organisms is considered to be a mechanism of biocontrol.
Predation and parasitism is based on the production of exo-enzymes which, for example, can degrade cell wall constituents of fungi, thereby enabling the biocontrol microbes to utilise the degradation products. Predation may act synergistically with anti-fungal metabolites (58, 65, 147), and includes, for example, the production of proteases (66, 70, 237), chitinases (68, 69, 221, 260), β-glucanases (118, 205), and cellulases (40).

Antibiosis via production of antifungal metabolites by micro-organisms is based on the direct inhibition of the growth of pathogens. These secondary metabolites are produced by a wide range of micro-organisms and are often directly responsible for the observed biocontrol activity of these strains. These metabolites include 2,4-diacetylphloroglucinol (9, 76, 126, 127, 259), phenazines (9, 44), pyrrolnitrin (103, 109), pyoluteorin (108, 164), and HCN (261).

Systemic resistence in plants can be induced by non-pathogenic rhizobacteria. This induced systemic resistence (ISR) is phenotypically similar to the induction of plant responses to the presence of pathogenic micro-organisms (systemically acquired resistance, SAR). Activation of ISR results in a state of physiological immunity towards fungal, viral, and bacterial attacks (256).

## 1.3 Biocontrol and root colonisation of *Pseudomonas* bacteria

Root colonisation is one of the prerequisites for efficient biocontrol activity (43). Root colonisation is defined as "the proliferation of micro-organisms in, on, or around the growing root" (54). In biocontrol this means that efficient colonisation of a plant root will result in bacteria being present at the right time at the right place to occupy potential niches on the root and to inhibit the propagation of pathogens. *Pseudomonas fluorescens* WCS356 (82) was isolated as an efficient root coloniser and was extensively studied for traits involved in root colonisation (151, 152). These studies show that, among other traits (46, 152), mutation of the *sss* gene, encoding a site-specific recombinase, reduced tomato root tip colonisation both in quartz sand and in potting soil and the occurrence of morphological variants (56). The observation that this enzyme also plays a role in phenotypic variation during rhizosphere colonisation was later confirmed for *Pseudomonas fluorescens* F113 (55). In addition, it was

shown that introduction of *sss* into *Pseudomonas fluorescens* WCS307 and *P. fluorescens* F113 improved root colonisation (55) and that mutation of a *sss* homologue in the phenazine-1-carboxamide producing bacterium *P. chlororaphis* PCL1391 abolished control of tomato foot and root rot by this strain (43). For *P. fluorescens* WCS365, it was hypothesised, that the mutation of *sss* locked the bacteria in a phenotypic state not well suited for competition in colonisation of the rhizosphere (56). In *P. fluorescens* F113 the *sss* gene plays a role in phenotypic variation. Analysis of three different variants, isolated from an *sss* mutant, showed that these variants have different root colonisation patterns (209).

The *xerC*/*sss* homologue is a member of the λ integrase family of site-specific recombinases (48, 102, 206). Site-specific recombinases have been described to promote homologous recombination between two small repeated DNA sequences. Depending on the orientation of the sequences, recombination results in inversion or excision of the DNA fragment between the small repeats (206). Such recombination processes can play a role in phase variation. For example, site specific recombinases can regulate the expression of type 1 fimbriae in *Escherichia coli* (1), flagella in *Salmonella typhimurium* (181), surface antigenic variation in *Mycoplasma penetrans* (105), and variation of the major outer membrane protein Omp1 of *Dichelobacter nodosus* (177) (Table 2). The observation that DNA rearrangements can influence efficient root colonisation, and therefore biocontrol (43, 55), prompted us to study phase variation as a possible reason for the inconsistency of field results.

## 1.4 Phase variation

Phase variation has been defined by Saunders et al. (211) as a process of reversible, high-frequency phenotypic switching that is mediated by DNA mutations, reorganisation or modification. Phase variation is used by several bacterial species to generate population diversity that increases bacterial fitness and is important in niche adaptation including immune evasion. Phase variation occurs at a high frequency of $>10^{-5}$ switches per cell per generation (98) and can result in reversible ON or OFF switching of traits or in the variation of surface phenotypes. The main advantage of phase variation, by altering the expression of traits or by varying the character of these traits, in the survival of micro-organisms, is the ability to adapt to an environment or to cope with host defense systems. Phase variation is one of the mechanisms enabling pathogens to

survive in the host by escaping the immune respons (52). This is illustrated by the fact that phase variation poses a problem in vaccine production due to the high frequency of variation in epitopes exposed by the pathogen (163, 188). Although phase variation, or antigenic variation, is primarily being associated with host-pathogen interactions, a number of reports describe phase variation in a broader context. These reports show that phase variation is also involved in the production of exo-enzymes and secondary metabolites (38, 250) indicating that phase variation can have a much broader impact on the ecology of bacteria, affecting a high number of traits and processes, and therefore phase variation is not only relevant in host-pathogen interactions but also in more ecological and industrial processes. The aim of this thesis is to study the mechanism of phase variation in antagonistic *Pseudomonas* bacteria in relation to efficient biocontrol of phytopathogens.

## 1.5 Mechanisms of phase variation

Phase variation is a phenomenon encompassing a variety of mechanisms. These can be divided into programmed and un-programmed variation (27). Programmed variation is characterised by two properties, (i) a family of genes encoding proteins with the same or similar function, which is combined with (ii) the ability to express only one of the gene family members at a time and alter the expression of these members from time to time (27). Programmed variation entails regulated DNA conversions as the result of slipped-strand mispairing (slipped-strand mispairing is, despite the fact that the variation is based on errors during DNA replication, considered to be programmed due to the requirement of a specific repeat tract) or genomic rearrangements (including inversions, deletions, recombinational events and gene conversions) but can also be epi-genetic when based on differential methylation. Un-programmed phase variation is based on DNA alterations through the accumulation of errors during DNA replication, imperfect DNA repair, the recombination between non-identical genes, or reassortment of gene segments if the genome is not in one piece (27).

A number of mechanisms of phase variation have been studied in detail in a range of micro-organisms. Four mechanisms of phase variation will be discussed, namely slipped-strand mispairing (§1.6.1), genomic rearrangements (§1.6.2), differential methylation (§1.6.3), and un-programmed phase variation (§1.6.4). Paradigms will be given for each of these mechanisms: the regulation

of opacity genes in *Neisseria gonorrhoeae* via slipped-strand mispairing (§1.6.1.1), variation of type 1 fimbriae in *E. coli* (§1.6.2.1) and type IV pili in *Neisseria* spp. via genomic rearrangements (§1.6.2.2), *pap* phase variation via differential methylation in *E. coli* (§1.6.3.1) and capsule variation in *Streptococcus pneumonia* via un-programmed, spontaneous duplications (§1.6.4.1).

## 1.6 Programmed variation

### 1.6.1 Slipped-strand mispairing

Slipped-strand mispairing uses short sequence repeats to regulate gene expression at the translational or transcriptional level. These repetitive DNA sequences are increasingly being identified in prokaryotes (6, 212, 243, 248) and can consist of homopolymeric repeat tracts or multimeric, heterogeneous repeats (98, 143). The stability of these repeat tracts is influenced by a number of factors. (i) The repeat number. With an increase in the number of repeats the mutation rate will increase. (ii) Repeat unit length. When the repeat unit is less than 5 bp the mutation rate will be suppressed by mismatch repair (MMR). (iii) The repeat sequence composition and the purity of the tract. (iv) DNA replication and processes associated with replication such as proofreading. (v) DNA transcription. (vi) MMR, which has a strong impact on the stability of the repeat tract (15-17, 77, 143, 148). Repeats associated with a single locus, present in the promoter region or within the coding region, can alter gene expression by changing the number of repeats (Fig. 1A). The number of repeats is varied via a RecA independent mechanism through the formation of heteroduplex DNA (H-DNA) which is induced by superhelical coiling (18, 72, 148). This H-DNA consists of a triple-stranded region, based on the formation of triple residue bonds within the repeat region, with as a result a single-stranded region which will stimulate slipped-strand mispairing (18, 98). Altering the number of repeats will result in an incomplete gene product due to a shift in reading frame (Fig. 1A).

In addition to regulation by an ON – OFF switch, slipped-strand mispairing mechanism can also regulate at the level of transcription. This regulation is mediated by the presence of repeats upstream of the encoding gene which, upon variation of the number of repeats, results in an increase or decrease in expression by varying the promoter spacing (Fig. 1B).

**Table 1.** Examples of phase variable traits regulated via slipped-strand mispairing

| Mechanism | Locus | Species | Property affected | Reference |
|---|---|---|---|---|
| Slipped-strand mispairing ON↔OFF | | | | |
| | *fucT2* (AF076779) | *H. pylori* | LPS antigenicity, Lewis Y antigen | (265) |
| | *igtG* (AF076919) | *N. gonorrhoeae* | LPS antigenicity, glycosyltransferase G | (10) |
| | *lic1A,2A,3A* (M37912-14) | *H. influenza* | LPS antigenicity | (104) |
| | *lgtC* (U32772) | *H. influenza* | LPS[a] antigenicity, | (104) |
| | *pilC* (Z49120) | *N. gonorhoeae/ N. meningitidis* | Fimbrial expression | (121) |
| | *siaD*(M95053) | *N. meningitides* | Capsular polysaccharides | (91) |
| | *cps* (AY250187) | *S. pneumonia* | Capsular polysaccharides | (257) |
| | *flhB* (AF031418) | *P. putida* | Flagellum export | (219) |
| | *opa* (P11296) | *N. gonorhoeae/ N. meningitidis* | Adhesion/invasion/ neutrophil interaction | (230) |
| | *bvgS* (M25401) | *Bordetella spp.* | Two component sensing | (231) |
| | *lob1* (U94833) | *H. somnus* | Antigenicity of LOS[b] | (113) |
| | *p78* (AF100324) | *M. fermentans* | ABC[c] transporter | (239) |
| | *tcpH* (X74730) | *V. cholerae* | ToxR regulon | (36) |
| | *hpuA* (AF031495) | *N. gonorrhoeae* | Haemoglobin binding outer membrane proteins | (41) |
| | *hmbR* (AF105339), *hpuAB* (U73112) | *N. meningitidis* | Haemoglobin receptors | (144) |
| | *hgpB* (AF022910) | *H. influenza* | Haemoglobin and haptoglobin binding | (119) |
| | *vlp*(U35016) | *M. hyorhinis* | Virulence? | (47) |
| | | *E. coli* | Unknown | (244) |

Abbreviations: [a] Lipo-poly saccharide (LPS), [b] Lipo-oligo saccharide (LOS), [c] ATP Binding Cassette (ABC)

**Table 1.** *Continued*

| Mechanism | Locus | Species | Property affected | Reference |
|---|---|---|---|---|
| Volume control OFF→ON$^{+}$/ON$^{++}$→OFF | | | | |
| | *opc* (A44611) | *N. meningitides* | Adhesion/invasion | (210) |
| | *porA* (P13415) | *N. meningitides* | Solute transport | (251) |
| | *hifAB* (U19730) | *H. influenza* | Fimbrial expression | (255) |
| | | *H. influenza* | Pilus expression | (172) |
| | *Hmw1a/2a* (U08876) | *H. influenza* | Adhesion | (12) |

Slipped-strand mispairing as a regulatory mechanism is present in a wide range of bacteria regulating various traits. Examples of traits regulated via slipped-strand mispairing are presented in Table 1.

#### 1.6.1.1 Opacity genes in *Neisseria gonorrhoeae*, regulated via slipped-strand mispairing

*Neisseria gonorrhoeae* is the causative agent of gonorrhoea in humans. The opacity proteins (Opa) play an important role in the host pathogen interactions (180). The expression of specific *opa* genes leads to the adherence to, and invasion of, epithelial cells (157), while other Opa proteins do not promote adherence but confer Opa-specific binding to human leukocytes (129, 199). Furthermore, the susceptibility to killing by normal human serum is influenced by the Opa proteins produced (42, 115). The variation of *opa* genes is confined to a RecA independent ON and OFF switching (179, 230), and a result of slipped-strand mispairing (230). Multiple copies of the *opa* genes exist within the genome. For example, *gonococci* posses the highest number of *opa* genes, approximately eleven to twelve (49), *meningococci* only posses three to four genes, and *Neisseria lactamica* only two (90). A high variety of proteins can be produced since each *opa* gene can be switched ON or OFF, and different combinations can be expressed (171). Independent *N. gonorrhoeae* isolates rarely posses *opa* genes of the same sequence (90), suggesting that the repertoire of variant *opa* genes within a population will be substantially larger than within a single strain (171). Major differences have been identified among these loci, which are confined to two short regions, designated hypervariable

regions 1 and 2 (4, 49, 89). The regulation of expression, switching loci ON and OFF, is based on changes in the number of pentameric repeat elements (5'-CTTCT-3') with which the expression state of the *opa* gene(s) changes. For example 6, 9 or 12 repeats are equivalent to an $Opa^+$ phenotype in which the gene is in frame (Fig. 1A). All other numbers (for example 7, 8 or 13) shift the gene out of frame, resulting in incomplete gene products and an Opa- phenotype (230) (Fig. 1A).

Apart from the high frequency switch between $Opa^-$ and $Opa^+$ phenotypes, the Opc outer membrane protein of *Neisseria meningitidis* undergoes a second form of regulation. A polyC tract is present adjacent to the promoter region. The number of bases in this repeat influences the promoter strength.

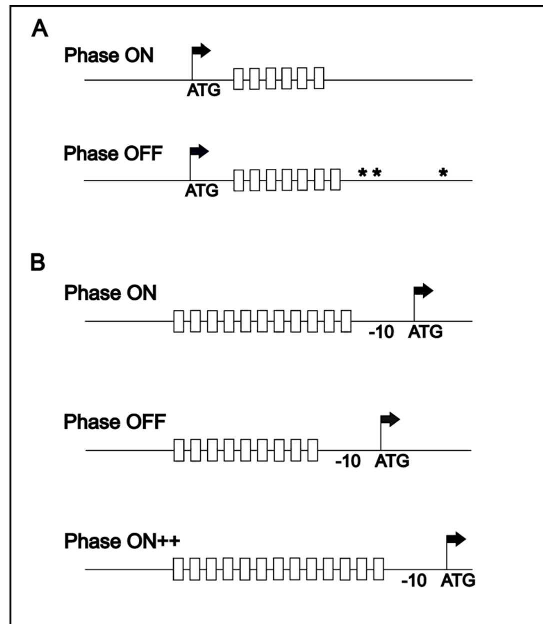


**Figure 1.** Model for phase variation via slipped-strand mispairing. A. Model for ON and OFF switching of traits via slipped-strand mispairing. Variations in the number of repeats (ڨ) within the coding region of the gene results in a shift of reading frame in or out of frame. A shift out of frame will introduce premature stop codons (*). B. Model for volume control via slipped-strand mispairing. Variations in the number of repeats within the promoter region of the gene will vary promoter -10 and -35 spacing, thereby increasing ($ON^{++}$) or decreasing (ON or OFF) promoter efficiency. For more details see text 1.6.1 and 1.6.1.1.

The expression of a promoter containing a polyC tract of 12 or 13 bases is 10 fold increased ($Opc^{++}$ phenotype) when compared to a promoter with a polyC tract of 11 or 14 bases ($Opc^+$ phenotype) (Fig. 1B). This is due to changes in promoter spacing. When the number of repeats exceeds 15 or becomes less than

10 no expression of opc is detected anymore (210) (Fig. 1B). This means slipped-strand mispairing can not only switch a gene ON and OFF but in addition can attenuate the promoter strength, controlling the amount of product formed.

### 1.7 Genomic rearrangements

Genomic rearrangements combine a wide range of processes involved in phase variation, these include inversions, deletions, gene duplication, and gene transfer using silent copies (recombinational deletion) (27). Control of expression of, for example, type 1 fimbriae in *E. coli* is based on the presence of inverted repeats and the action of site-specific recombinases (Fig. 2). The presence of inverted repeats within the promoter region facilitates the inversion of the promoter switching expression ON or OFF (1). On the other hand when the promoter itself is flanked by inverted repeats, as described for H1 and H2 flagellin genes of *Salmonella typhimurium*, different sets of genes can be expressed. One orientation of the promoter will result in the expression of *h2* and the repressor Rh1 of the *h1* promoter. Upon inversion both *h2* and *rh1* are no longer expressed, lifting the repression of *h1* by *rh1* (281).

A second form of variation based on genomic rearrangements, regulating for example variation of type IV pili in *N. gonorrhoeae* (122, 220) and the expression of surface proteins in *Borrelia spp.* (11), uses deletions, gene duplications and gene transfer to create a large potential of proteins to express. Although in many systems *recA* mutants are not yet available, those mutants analysed show that these rearrangements are dependent on the *recA* gene, and based on the deletion of one allele present in an active locus, which is subsequently replaced by transcriptionally inactive alleles present elsewhere on the genome (72, 98, 171). This is often combined with the presence of highly variable regions within these alleles, thereby increasing the variation potential of the gene product (171). Examples of traits regulated via genomic rearrangements are presented in Table 2. Two well studied examples of gene regulation via genomic rearrangements are variation of type 1 fimbriae in *E. coli* and variation of type IV pili by recombinational deletion in *Neisseria* species.

### 1.7.1 Type 1 fimbriae variation in *E. coli* by genomic rearrangements

Phase variation of type 1 fimbriae in *E. coli* plays an important role in the infection of the urinary tract, mediating the ability of *E. coli* to adhere to the uroepithelium (197). In general, fimbrial phase variation, in different organisms, can occur by at least four different mechanisms: (i) site-specific recombination (1), (ii) slipped-strand mispairing (274), (iii) general recombination or gene conversion (122, 236) and (iv) DNA methylation patterns (See §1.6.3.1). Phase variation of type 1 fimbriae in *E. coli* is based on a site-specific recombination event inverting a 314 bp segment of chromosomal DNA which includes the promoter for transcription of the gene encoding FimA, the structural subunit of the fimbriae (1) (Fig. 2). The inversion of this DNA fragment is dependent on the products of *fimB* and *fimE*, encoding site-specific recombinases sharing homology with the lambda integrase family of site-specific recombinases (165, 166). These recombinases recognise a 9 bp inverted repeat flanking the promoter region (1) (Fig. 2). FimB and FimE act independent of each other in the inversion. FimE will preferentially invert the promoter from the ON to the OFF configuration (229), while FimB can inverse the promoter both from ON to OFF as well as from OFF to ON (166). The expression of FimB and FimE is regulated by a histone like protein (H-NS), an Integration Host Factor (IHF) and the Leucine responsive protein (Lrp) (23, 60) (Fig. 2). According to the model (Fig. 2) binding of Lrp to three Lrp-binding sites present within the invertable region (22) changes the DNA conformation and facilitates inversion (203). Binding of the IHF will bend the DNA to align the recombinational sites to enable strand exchange (23) and, through action of either FimB or FimE, the promoter region is inverted. At this moment the exact mechanism is not clear, which is illustrated by the observation that locking the invertable region in an ON configuration by removal of one of the inverted repeats, did not abolish phase variable fimbrial expression (165).

### 1.7.2 *Neisseria* species and expression of variant type IV pili

*Neisseria* species are Gram negative diplococci, including a variety of commensal species beside the two pathogenic species *N. gonorrhoeae* and *N. meningitides* (171). Phase variation in these pathogenic *Neisseria* species is governed by a widespread regulation mechanism, affecting a high number of genes (227).

**Table 2.** Examples of phase variable traits regulated via genomic rearrangements

| Mechanism | Locus | Species | Property affected | reference |
|---|---|---|---|---|
| Site-specific inversion ON ↔ OFF | | | | |
| | *mrp* (Z32686) | *P. mirabilis* | Fimbrial expression | (280) |
| | *fimA* (Z37500) | *E. coli* | Fimbrial expression | (1) |
| | *omp1*(U02462) | *D. nodusus* | Major outer membrane protein | (177) |
| | *p35* family (L38250) | *M. penetrans* | Surface lipoprotein antigens | (105) |
| | *vsa* (U23947) | *M. pulmonis* | Surface antigens | (20) |
| | *vspA* (L81118) | *M. bovis* | Surface lipoprotein antigens | (154) |
| | *sapA* (AF071883) | *C. fetus* | S-layer expression | (71) |
| | *hsd1* (AF003541) | *M. pulmonis* | DNA restriction and modification properties | (74) |
| | hsd (AF076990) | *M. pulmonis* | DNA restriction | (73) |
| $ON_a/OFF_b \leftrightarrow ON_b/OFF_a$ | | | | |
| | *hin* (V01370) | *Salmonella spp.* | Flagellar expression | (281) |
| | *piv* (M34367) | *M. lacunata* | Type IV fimbriae | (96, 161) |
| Recombinational deletion ON ↔ OFF | | | | |
| | *pilE* (AF043652) | *N. gonorrhoeae/ N. meningitides* | Fimbrial expression | (89) |
| | *cap* (S62752) | *H. influenza* | Capsular polysaccharide production | |
| | *vsg* genes | *Trypanosome spp.* | Variable surface glycoproteins | (3, 13) |
| | *vsp* (AF396970 and AH008162) | *M. bovis* | Surface lipoproteins | (155) |
| | *vpma* (AF248865) | *M. agalactiae* | Surface proteins | (86) |
| | *vsp/vlp* (AF049852) | *Borrelia spp.* | Surface proteins | (11) |

Type IV pili are believed to be responsible for the initial attachement to the mucosal epithelium of the human host (180). Variation of type IV pili via homologous recombination is based on the presence of multiple, silent, variant

*pilS* genes, and one active *pilE* gene (89, 220, 236). The silent pilus genes contain a semivariable and a hypervariable region flanked by conserved regions involved in gene conversion between the *pilS* genes (89). This enables the bacteria to produce approximately $10^7$ variant PilE proteins (90). The rearrangements are RecA, RecO and RecQ dependent (135) and are promoted by RecJ (100).

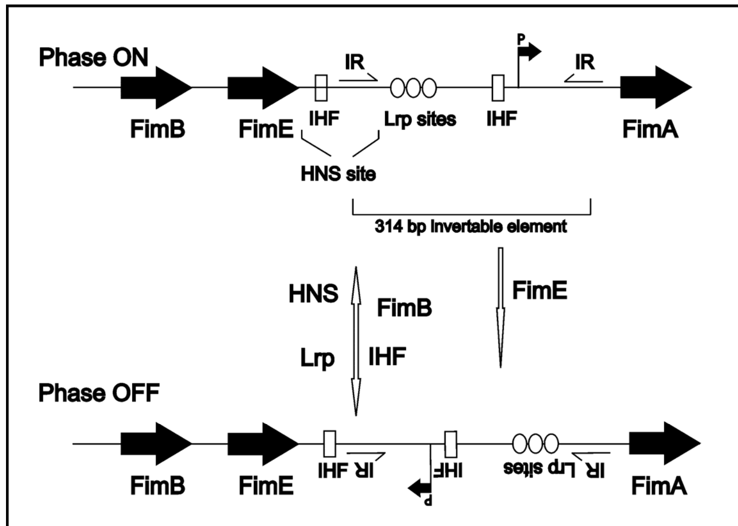


**Figure 2.** Model for phase variation via a 314 bp invertable element. Inversion of a 314 bp promoter fragment will switch expression of *fimA* ON or OFF. The inversion is facilitated by two site-specific recombinases FimE and FimB, recognising the two 9 bp inverted repeats (IR, the orientation is indicated with an arrow). FimE promotes the switch from ON to OFF, while FimB can invert the fragment in both directions. An Integration Host Factor (IHF) is required for efficient expression. Since mutation of one of the subunits of the IHF locks the expression of *fimA* either in an ON or the OFF configuration, the IHF is also involved in the inversion of the *fimA* promoter (60). Histone like protein (H-NS) is directly involved in suppression of the *fimB* gene, suppressing the inversion from OFF to ON (59, 184). The Leucine Responsive protein (Lrp) stimulates expression of *fimB* and slightly decreases expression of *fimE*, stimulating inversion in both directions as shown by a decrease in the frequencies of inversion, upon mutation of *lrp* (22). For more details see text 1.6.2.1.

Conserved regions encoding cysteine residues within the *pilS* genes are used in the recombination (110, 220), and all silent copies can donate their sequences to the expressed locus (220). The precise mechanism allowing this high frequency,

non-reciprocal chromosomal recombination between the different loci is still in discussion (110, 220).

## 1.8 Differential methylation

Phase variation of *pap* fimbriae and expression of antigen 43 in *E. coli* is dependent on a differential DNA-methylation pattern and represent therefore an epigenetic mechanism of phase variation (98, 252). Methylation of GATC sites in the genome is dependent on deoxyadenosine methylase (*dam*) which binds to the GATC site and methylates adenosine at the $N^6$ position (187). Normally, methylation provides the organism with a regulatory mechanism for DNA repair, protection from restriction endonucleases, and timing and targeting of cellular events (160). Methylation of GATC sites within regions involved in gene regulation can inhibit or facilitate the binding of regulatory proteins at specific sites, and thus alter gene expression (183). Examples of gene expression, controlled via differential methylation, are presented in Table 3 Phase variation via differential methylation of a pyelonephritis-associated pilus has been studied extensively in *E. coli* and is the best described mechanism for regulation of this kind.

**Table 3.** Examples of phase variable traits regulated via differential methylation

| Mechanism | Locus | Species | Property affected | Reference |
|---|---|---|---|---|
| ON ↔ OFF | | | | |
| | *agn43* (U24429) | *E. coli* | Autoaggregation | (186) |
| | *pap* (X03391) | *E. coli* | Pilus expression | (25) |
| | *sfa* (M35273) | *E. coli* | S-pili | (254) |
| | *clp* (L48184) | *E. coli* | CS31A adhesive factor | (53) |
| | *pef* (AB041905) | *S. thymurium* | Fimbrial expression | (181) |

### 1.8.1 Pap phase variation in *E. coli* via differential methylation

In uropathogenic *E. coli* strains expression of the pyelonephritis-associated pilus (Pap) allows the bacteria to attach to uroepithelial cells, facilitating colonisation of the upper urinary tract (182, 185). The expression of *pap* is regulated via differential methylation. The *pap* operon consists of nine gene products involved in the assembly and expression of pilus complexes at the bacterial cell

surface (252). The regulation is dependent on the transcriptional regulators PapI and PapB and the global regulator Leucine responsive regulatory protein (Lrp), and also on the catabolite activator protein CAP, the histone like protein H-NS, and on deoxyadenosine methylase (Dam) activity (252) (Fig. 3). Phase variation of the *pap* operon is dependent on a reversible switch between an ON and OFF state which is controlled by differential methylation of two GATC sites present within the regulatory region of the *pap* locus (25) (Fig. 3). Methylation of $GATC_{1130}$, and an unmethylated $GATC_{1028}$ site, enables the expression of the *pap* locus. The reverse situation, in which $GATC_{1028}$ is methylated and $GATC_{1130}$ unmethylated, renders the operon inactive (25) (Fig. 3). Dam methylase activity is essential for this switch and mutation of the *dam* gene abolishes all *papA* transcription (25). Lrp is a 19 kDa DNA binding protein, which can bind within the *pap*-regulatory region to six Lrp binding sites (Fig. 3). Since the methylation sites are overlapping with the Lrp binding sites, binding of Lrp will prevent methylation (28, 29). Binding of Lrp to sites 1, 2, and 3 will protect $GATC_{1130}$ from methylation, rendering the system in an OFF configuration (25, 28) (Fig. 3). Alternatively, binding to sites 4 and 5 will render the system in an ON configuration by protection of $GATC_{1028}$ from methylation. Translocation of Lrp will switch the system to an ON or OFF configuration. This is facilitated by the PapI protein which, when bound to Lrp, reduces the affinity of Lrp for binding sites 1, 2, and 3 to 50% while increasing the affinity for binding sites 4 and 5 (183). Upon a subsequent translocation of the Lrp, the free GATC sites are methylated by *dam* activity.

This leads to the following model of *pap* phase variation (Fig. 3). Starting from a *pap* DNA fragment without any bound protein, a basal level of *papBA* expression can be detected (78, 253). Lrp will bind with the highest affinity to sites 1 to 3, protecting $GATC_{1130}$ from methylation. Subsequent methylation of $GATC_{1028}$ results in an OFF configuration. The switch to an ON configuration involves binding of PapI to Lrp, changing the affinity of the Lrp binding, which is only achieved by physical presence of $GATC_{1028}$ (183). Since the Lrp-PapI complex will bind to hemi-methylated DNA present after replication (183), it is hypothesised that DNA replication is involved to generate an unmethylated $GATC_{1028}$, to allow the Lrp to translocate (29, 183). Dam activity will methylate $GATC_{1130}$, and render the system in an ON configuration, thereby increasing transcription about eight fold (253). CAP plays a role in this process by interacting with RNA polymerase (252, 270). The physiological role of H-NS in *pap* expression is not yet clear, but is reported to be an important environmental

regulator for fimbrial transcription (273). Switching from an ON configuration to an OFF configuration occurs at a 100-fold higher frequency, and is likely to be the result of replication, after which sites 1 to 3 are hemi-methylated, allowing binding of Lrp, thereby protecting the new strand from methylation (252).

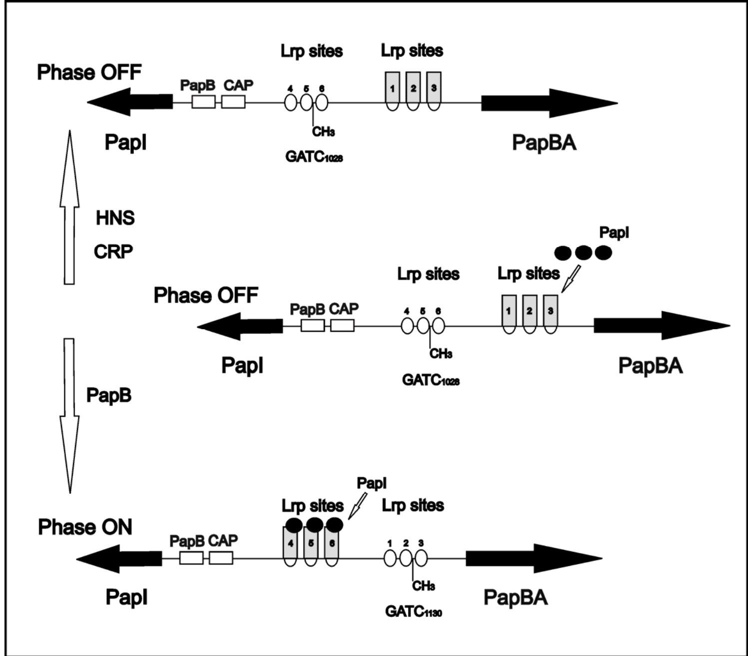


**Figure 3.** Model for phase variation via differential methylation. Differential methylation of two GATC sites, $GATC_{1028}$ and $GATC_{1130}$, regulates expression of *papBA*. Methylation of $GATC_{1130}$ will inhibit *papBA* expression. The regulation is based on competition for binding sites since Lrp and methylation sites overlap. Binding of PapI to Lrp will reduce the affinity for binding to sites overlapping with $GATC_{1130}$. The Lrp-PapI complex will preferentially bind to the sites overlapping $GATC_{1028}$, probably after replication, and thus facilitate methylation of $GATC_{1130}$ to enable *papBA* expression. Binding of CAP and PapB will lift H-NS suppression (78) and enable transcription of *papI*. In addition, binding of PapB upstream of the *papI* promoter will stimulate *papI* transcription (252). For more details see text 1.6.3.1.

## 1.9 Un-programmed variation

Random un-programmed variation is dependent on the introduction of mutations due to imperfect replication. For example viruses use this high plasticity to overcome host defenses and do not suppress spontaneous mutation. One of the draw-backs of a mechanism stimulating diversification based on imperfect replication is a high mutational load. Higher organisms had to evolve a mechanism of mutation while controlling the mutation rate using mechanisms like mismatch repair pathways (27, 217), or, although still under discussion, specific error prone DNA polymerases transcribing specific genomic regions (131, 167, 178, 238). Most organisms use this strategy to create diversity in for example antibody genes (81) but, this mechanism has also been suggested to play a role in adaptive evolution in microorganisms (178). The mutations accumulating in these regions can consist of small deletions (50 to 500bp), mismatches, and duplications (92, 175, 249, 263). Examples of spontaneous mutations in phase variation, switching genes ON and OFF are presented in Table 4. An example of phase variation via spontaneous, reversible duplications has been described in *Streptococcus pneumonia* to regulate capsule production.

### 1.9.1 Capsule phase variation in *Streptococcus pneumonia*

*Streptococcus pneumonia* is a Gram positive human pathogen causing otitis media, pneumonia, scepsis and meningitis. The presence of this pathogen is wide-spread. It is carried as a biofilm in the throat or nasopharynx. On average, carriage rates range from 40 to 50% in children and 20 to 30% in adults (8, 26, 84). The actual invasion of host cells by *pneumococci* is a multistage process initiated by adherence (26). The capsule plays a role in virulence, anti-phagocytosis and protective immunity (116, 196, 202, 226). Regulation of the capsule production is suggested to play a role in the transition from carriage to invasive disease (125, 202, 226). At a high frequency (between $10^{-6}$ and $10^{-3}$) small, transparent, acapsular colonies can be isolated from tissue-based biofilms, of which the majority can revert to the capsular, opaque phase upon subculturing to fresh plates (263). These opaque variants are unable to colonise the nasopharynx, while the translucent variants are stable and efficient colonisers (267). Cloning and sequencing of the first gene in the biosynthetic operon (*cap3A*, type 3 capsular polysaccharide) (7) revealed that random duplications of 11 to 239 bp, are present in different regions of the *cap3A* open reading frame in acapsular isolates, shifting the gene out of frame (263).

**Table 4.** Examples of phase variable traits regulated via un-programmed variation and traits for which the exact mechanism is unknown

| Mechanism | Locus | Species | Property affected | Reference |
|---|---|---|---|---|
| Spontaneous duplications ON ↔ OFF | | | | |
| | *cap3*, *cap8*, *tts* (Z12159, AJ239004, AJ131985) | *S. pneumoniae* | Capsule production | (263, 262) |
| | *pheN* (U95300) | *P. tolaasii* | Secondary metabolism /morphology | (92) |
| | D1 (X13547) | *Synechosystis spp.* | D1 protein photosystem II | (132) |
| Spontaneous mutations ON ↔ OFF | | | | |
| | *vir* locus | *B. bronchiseptica* | Virulence factors | (175) |
| | *gacA/S* (AY236957) | *Pseudomonas* sp. PCL1171 | Secondary metabolism and exo-enzymes | (249) |
| Mechanism unknown | | | | |
| | | *P. aeruginosa* | Motility/flagella / biofilm formation | (57) |
| | | *P. brassicacearum* | Root colonisation / flagella / exo-enzymes | (38) |
| | | *P. fluorescens* WCS365 | Root colonisation | (56) |
| | | *P. fluorescens* F113 | Root colonisation / motility / biofilm formation | (209) |

The same was shown for serotypes 8 and 37, in which spontaneous sequence duplications are responsible for phase variation of the capsular phenotype (262). The switch back to a capsular phase coincided with a precise excision of the duplications from the open reading frame (262, 263). Still unclear, in this example of phase variation, is the exact mechanism by which the capsule locus is switched ON again, which factors determine the switch OFF, and the relevance of this mechanism in disease.

### 1.10 Phase variation and *Pseudomonas*

Phase variation in *Pseudomonas* bacteria is a relatively unexplored phenomenon, but sofar some interesting examples of traits affected by phase

variation in pseudomonads have been described. In the pathogenic *P. aeruginosa* species, phase variation regulates the variation of the phosphocholine epitope of a 43 kDa protein in a temperature dependent manner (266), and the expression of type IV pili. Phase variation of these pili affects swimming, swarming and twitching motility and, as a result biofilm formation (57) (Table 4). *P. tolaasii* was shown to switch colony morphology and pathogenicity by a spontaneous duplication in *pheN* (92) (Table 4). With respect to non-pathogenic *Pseudomonas* species, phase variation has been described in *P. putida* DOT-T1E to control expression of the *flhB* gene via slipped-strand mispairing in response to environmental changes. FlhB is a protein involved in flagellin export. This type of regulation is probably not very conserved since for example in *P. putida* KT2440 this regulation was not observed (219). In addition, reversible phenotypic variants have been described in *P. fluorescens*, which are correlated with adaptation to heterogeneous growth conditions, which show that identical populations diversify morphologically under non restrictive conditions, resulting in niche specialists (193).

In several *Pseudomonas* sp. phase variation regulates the expression of exo-enzymes and plays a role in root colonisation (Table 4). In *Pseudomonas brassicacearum*, two morphologically distinct colonies (small mucoid phase I and large, flat nonmucoid phase II cells) have been isolated. An extra-cellular alkaline protease, a serine protease homolog and a lipase are only expressed in phase I cells. The genes coding for the protease and lipase are organised in a single operon, but a mechanism responsible for the ON and OFF switching of this operon is not yet described (38). Phase variation of *P. brassicacearum* affects root colonisation of *Arabidopsis thaliana*, the phase II bacteria show, due to an over production of flagellin, a higher ability to swim and swarm when compared to phase I bacteria. In root colonisation these bacteria are found at the root tip and on secondary roots, while the phase I bacteria are localized at the basal parts of the root. Based on these results phenotypic variation is suggested to be a strategy, increasing the colonisation ability of *P. brassicacearum* (2). The effect of phase variation on root colonisation was also suggested by the observation of a reduced competitive tomato root tip colonisation upon mutation of *sss* (a site specific recombinase) in *P. fluorescens* WCS365 (56). The link between phase variation, root colonisation and *sss* was also analysed in *P. fluorescens* F113. During root colonisation of alfalfa by *P. fluorescens* F113, phenotypic variants were isolated. The *sss* gene is responsible for the majority of the phenotypic variation, which is combined with a phenotypic selection for

gac mutations. Three morphologically different variants were isolated which showed a difference in colonisation pattern, and in the production of cyanide, exo-protease, and siderophores (209).

These examples show that phase variation in *Pseudomonas* sp. is regulating a wide range of traits, which affect biofilm formation, root colonisation, and production of secondary metabolites, suggesting that phase variation is a mechanism, relevant in the ecology and behaviour of these species.

## 1.11 Regulation of phase variation

To allow expression under relevant conditions, phase variation itself is often regulated by environmental factors. In pathogenic micro-organisms the expression can be linked to conditions in the host, at the site of infection. For example, phase variation of the type 1 fimbriae (§ 1.6.2.1) is influenced by temperature and medium composition such that expression of type 1 fimbriae is enhanced upon infection of mouse urinary tract by *E. coli* (233). FimB-promoted inversion is increased between 37 to 41 degrees, and FimE promoted ON to OFF inversion at lower temperatures (80, 218). Also *pap* phase variation (§ 1.6.3.1) is influenced by environmental factors. Temperature regulates expression of *pap* via RimJ, a *N*-acetyltransferase which modulates both *papBA* and *papI* expression. This results in the expression of pili at 37 degrees, and suppression of expression at 23 degrees (272). In addition, H-NS is thought to be an important environmental regulator for fimbrial expression allowing environmental factors such as low temperature, high osmolarity, glucose as a carbon source, and rich medium to influence phase variation and expression of *pap*. Mutation of H-NS lifted the negative effect on transcription by these factors (273). Since phase variation is influenced by specific conditions in the host it can regulate the expression of traits during infection and enable a pathogen to express specific traits at the right place. But, the regulation of phase variation is not only linked to infection processes. For example, phase variation is strongly influenced by stress conditions. This is illustrated by the observation that RpoS, a general stress response sigma factor facilitating adaptation to stress and controlling the expression of many genes during exponential and stationary growth (99), enhances the frequency of inversion in phase variation of type 1 fimbriae in *E. coli* and was shown to suppress expression of the *fimA* gene (61). In phase variation of a plasmid encoded fimbriae in *S. typhimurium* (*pef*), which is dependent on differential methylation, expression of *pef* was suppressed by

RpoS (181) and, in *Vibrio cholerae*, RpoS and RpoN affect the switch between the rugose and smooth colony variants (279). Under stress, or growth limiting conditions the frequency of stationary phase mutations will increase (131, 146). This is caused by down regulation of MMR (201, 247), spontaneous mutations of MMR components (106, 222, 223), and, possibly, the activity of error-prone polymerases like DinB (167, 232). As a result mechanisms like slipped-strand mispairing (201), introduction of spontaneous mutations (146) and genomic rearrangements (61) will be influenced and result in divers phenotypes enabling a population to cope with the growth limitations (31). It was suggested by Moxon et al. (178), that the combination of specific contingent regions and stable regions in the genome, facilitates efficient exploration of phenotypic solutions to unpredictable aspects of the (host) environment while minimizing deleterious effects on fitness. Thus, phase variation as a mechanism is first of all regulated by environmental factors, defining, for example, the site of infection and to cope with the host immune system. In addition, under stress or growth limiting conditions, it can lead to a diversification of a population, creating heterogeneous populations in order to survive an ever-changing and competitive environment.

## 1.12 Outline thesis

The research, presented in this thesis is aimed at the identification of the mechanism of phase variation in antagonistic *Pseudomonas* species in relation to biocontrol. It was hypothesised that the occurrence of frequent variations in for example the expression of antifungal metabolites or the switching between a rhizosphere competent and a less competent phase could influence biocontrol in the field. For this purpose a collection of antagonistic *Pseudomonas* bacteria was isolated from the rhizosphere of maize plants grown on agricultural fields in Totontepec Mixe, Oaxaca, Mexico. These strains were characterised for biocontrol traits and since the majority displayed phase variation between a phase I and phase II colony morphology, affecting the expression of major biocontrol traits, the effect of phase variation on biocontrol was studied (**Chapter 2**). The molecular basis of phase variation in *Pseudomonas* sp. PCL1171 was studied and led to the identification of a mechanism, in which the accumulation of spontaneous mutations in the *gacA* and *gacS* genes, and subsequent removal of these mutations, forms the basis for phase variation (**Chapter 3**). To identify factors involved in the accumulation of these

mutations in *gacA/S*, the role of GacS, RpoS and MutS in phase variation was studied (**Chapter 4**). Phase I and phase II are two morphological distinct phenotypes. In the identification of factors determining these differences *prtI* and *prtR* homologs were isolated. Their role in colony morphology and phase variation was analysed (**Chapter 5**). In chapter 6 the results are summarised and discussed.

# CHAPTER 2

# Biocontrol traits of *Pseudomonas* spp. are regulated by phase variation

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## ABSTRACT

Of 214 *Pseudomonas* strains isolated from maize rhizosphere, 46 turned out to be antagonistic, of which 43 displayed clear colony phase variation. The latter strains formed both opaque and translucent colonies, designated as phase I and phase II, respectively. It appeared that important biocontrol traits, such as motility and the production of antifungal metabolites (AFM), proteases, lipases, chitinases, and biosurfactants are correlated with phase I morphology and are absent in bacteria with phase II morphology. From a Tn*5luxAB* transposon library of *Pseudomonas* sp. strain PCL1171 phase I cells, two mutants exhibiting stable expression of phase II had insertions in *gacS*. A third mutant, which showed an increased colony phase variation frequency was mutated in *mutS*. Inoculation of wheat seeds with PCL1171 bacteria of phase I morphology resulted in efficient suppression of take-all disease, whereas disease suppression was absent with phase II bacteria. Neither the *gacS* nor the *mutS* mutant was able to suppress take-all, but biocontrol activity was restored after genetic complementation of these mutants. Furthermore, in a number of cases, complementation by *gacS* of wild-type phase II sectors to phase I phenotype could be shown. A PCL1171 phase I mutant defective in antagonistic activity appeared to have a mutation in a gene encoding a lipopeptide synthetase homologue and had lost its biocontrol activity, suggesting that biocontrol by strain PCL1171 is dependent on the production of a lipopeptide. Our results show that colony phase variation plays a regulatory role in biocontrol by *Pseudomonas* bacteria by influencing the expression of major biocontrol traits and that the *gacS* and *mutS* genes play a role in the colony phase variation process. Therefore phase variation not only plays a role in escaping animal defense but it also appears to play a much broader and vital role in the ecology of bacteria producing exoenzymes, antibiotics and other secondary metabolites.

## INTRODUCTION

In commercial agriculture, crop protection against phytopathogens relies heavily on chemical pesticides. There is a growing concern for negative health and environmental effects of such pesticides. For example, the European Union has decided that 60% of the chemical pesticides that were allowed in 1996 will be banned in 2003. Therefore, alternatives for the use of chemicals are needed. The use of genetically engineered disease-resistant plants is perceived poorly by the public, especially in Europe. Therefore, the use of microorganisms to control plant pathogens is the most attractive alternative. Sofar however, success in the field is limited due to variable results.

The control of phytopathogenic fungi by biocontrol microbes depends on a wide variety of traits, such as the production of antifungal metabolites (AFMs) (32, 44, 127, 164, 192, 240), production of exo-enzymes such as proteases, lipases, chitinases, and glucanases (32, 67, 245) production of hydrogen cyanide (HCN) (261), production of siderophores (142) of biosurfactants (228), and competitive root colonization (43, 152). Previous results have indicated that mutation of a *xerC*/*sss* homologue from the efficiently root colonizing *P. fluorescens* strain WCS365 resulted in a decrease in the frequency of colony phase variation and a severe decrease of its competitive root tip colonizing abilities (55, 56). The *xerC*/*sss* product has been reported to be involved in DNA rearrangements (48). Phase variation is a regulatory process by which bacteria undergo frequent and (often) reversible phenotypic changes resulting from genetic alterations in specific loci of their genome. Phase variation is based on structural changes at the DNA level and results in sub-populations of bacteria, as is often demonstrated by the presence of distinct morphological phases between colonies or within a colony (72, 98). In general, phase variation, thought of as a random event, occurs at frequencies of $>10^{-5}$ per generation (98). Phase variation, as a regulatory system, can influence the production of diverse traits such as the production of proteases and lipases (38), of pili (170), outer membrane proteins (170), fimbriae (1), surface lipoproteins (204), flagella (123), and other surface-exposed antigenic structures (72, 98).

The finding in our group that phase variation can negatively influence competitive root tip colonization (55, 56) and, therefore, biocontrol (43) has prompted us to study the influence of colony phase variation on other biocontrol traits.

## MATERIAL AND METHODS

### Microbial strains and plasmids

Bacterial strains and plasmids are listed in Table 1. *Pseudomonas* strains were grown on King's medium B (KB) (128) at 28°C. Solid growth media contained 1.8% (wt/v) agar (Difco Laboratories, Detroit, MI). Kanamycin, gentamicin, tetracycline, and cyclohexamide (Sigma, St Louis, MO) were added for antibiotic selection in final concentration of 50, 10, 40, and 100 µg/ml, respectively, when appropriate. Fungi were grown on KB or Potato Dextrose Agar (PDA) (Difco Laboratories, Detroit, MI). BM (Minimal Basic medium) (152) with 0.2% glycerol as carbon source was used for the screening for mutants without antagonistic activity.
For the isolation of *Pseudomonas* strains from the rhizosphere, roots from maize plants were shaken twice for 30 minutes in phosphate buffered saline (PBS) (208). The resulting suspensions were plated and grown overnight in *Pseudomonas* isolation medium (Difco Laboratories, Detroit, MI) at 28°C. Colony morphology and ARDRA (258) were used to identify the strains and select *Pseudomonas* spp.
For strain identification of PCL1171 phase I and phase II, colony PCR (275) was used for amplification of the 16S rDNA from colonies with a phase I or phase II morphology. The PCR products were sequenced by BaseClear (Leiden, The Netherlands) or ServiceXS (Leiden, the Netherlands) and analyzed for homologies using the BLAST (5).

### Measurement of phase variation frequencies

Bacteria with a phase I or phase II morphology were inoculated in a volume of 5 ml KB to an optical density at 620 nm (OD620) of 0.05 and grown shaking overnight at 28°C. By measuring the optical density and subsequent dilution and plating on KB medium, an average of 500 colonies per plate was obtained. For estimation of frequencies at least 1,500 colonies were counted. To obtain the frequency of switching, the number of switches was divided by the number of generations passed.

### Construction, selection, and complementation of mutants

A mutant library of strain PCL1171 phase I was constructed using the plasmid pRL1063a, which harbors a Tn*5* transposon with promotorless *luxAB* genes and

a kanamycin resistance marker (277). Electro-competent phase I cells were obtained by scraping the cells from the plates, washing them three times with sterile water, followed by two washings with 10% glycerol. pRL1063a plasmid DNA (1 to 2 μg) was used for electroporation of electro-competent cells using a pulser device (settings: 25 μF, 100Ω, and 2.5 kV) (Biorad Lab, Richmond, CA, U.S.A). The transformation mixture was grown in SOB medium (208) for 2 h and, subsequently, plated on selective medium and grown at 28°C. The obtained transposants were judged after at least two days of growth on KB plates for altered colony morphology. Mutants lacking colony phase variation or showing an increased frequency of colony phase variation were selected. Furthermore, mutants expressing a phase I morphology but that had lost their antagonistic activity were selected, using BM agar plates on which eight mutants were grown surrounding an inoculum of the fungus *G. graminis* pv. *tritici* (83). Mutants unable to inhibit the fungus were selected after 7 days of growth. DNA regions flanking the transposon were isolated by excision of the transposon from the chromosomal DNA of the transposants using *Eco*RI or *Cla*I, followed by ligation and transformation with *E. coli* strain DH5α. Since the Tn*5* transposon harbors an origin of replication (p15A), the plasmid can replicate and maintain itself in *E. coli*. The plasmids were reisolated. The flanking chromosomal regions were sequenced using unique primers oMP458 (5'-TACTAGATTCAATGCTATCAATTGAG-3') and oMP459 (5'-AGGAGGTCACATGGAATATCAGAT-3') directed outwards of the transposon ends. Sequencing was carried out by BaseClear (Leiden, The Netherlands) or ServiceXS (Leiden, the Netherlands). General DNA modification techniques were performed according to Sambrook et al. (208).

**Complementation of the *gacS* mutant strains**

Primers oMP658 (5'-GGAATTCAGGATGTCCATCAACACCA-3') and oMP618 (5'-GGAATTCATCGTTGATGAAGGCACACA-3') were used to amplify the complete *gacS* gene from PCL1171 by PCR. The obtained PCR fragment was cloned into pGEMTeasy (Promega Corp. Madison, WI, U.S.A) and was subsequently cloned in pME6010 using *Eco*RI. This construct, pMP6562, was used to transform PCL1563 and PCL1572 by electroporation. In addition, phase II bacteria of wild-type strains PCL1157, PCL1182, and PCL1184 phase II bacteria were complemented using pMP6562, and pMP5565 by mating.

**Analysis of cell envelope proteins and lipopolysaccharides**
To analyze LPS and membrane protein patterns, cells with a specific phase I or phase II morphology were harvested separately from plate after two days of growth at 28°C and re-suspended in 50 mM Tris-HCl, 2 mM EDTA pH 8.5. To isolate cell envelopes, cells were sonicated and centrifuged for 20 minutes at 2,700 rpm and for one hour at 10,000 rpm, to isolate preparations for the analysis of LPS and total membrane proteins, respectively. The obtained pellets were resuspended and stored in CE-buffer (2 mM Tris-HCl, pH 7.8). To visualize LPS patterns, the cell envelope preparation was incubated for 15 minutes at 100°C in 125 mM Tris/HCl pH 6.8, 4.0% SDS, 20% glycerol, and 0.02% bromophenol blue, followed by proteinase K treatment. The LPS fractions were separated in a denaturing 11% SDS-PAGE gel using a Mini-ProteanTM 3 Cell system (BioRad Lab). The LPS pattern was visualized by silver staining (246). Cell envelope proteins were denatured by adding β-mercaptoethanol to the cell envelope mixture to a final concentration of 0.1%, followed by incubation for 10 minutes at 100°C. Proteins were separated on a 11% SDS-PAGE denaturing gel using a Mini-ProteanTM 3 Cell system (BioRad Lab) and visualized with Coomassie-Blue staining (208).

**Analysis of biocontrol traits**
Antagonistic activity against the fungi *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Rhizoctonia solani*, *Rosellinia necatrix*, and *G. graminis* pv. *tritici* was analyzed, using an agar plate on which the fungus was inoculated in the center of a petri dish, whereas four bacterial strains were spot-inoculated at a distance of 2 to 3 cm. After 7 days of growth at 28°C the plates were examined for growth inhibition zones of the fungus surrounding the bacterial spot (83). For the detection of secreted bacterial protease, β-glucanase, lipase, and cellulase, 1.8% agar plates containing 5% skim milk, 0.1% lichenan (Sigma) (264), 2% Tween 80 (107), or 0.5% carboxymethylcellulose (94) were used, respectively. The plates were inspected for degradation zones as judged by clearing or precipitation zone in case of lipase activity, after 5 days of growth at 28°C.
For the detection of secreted chitinase activity chitinpentaose (Seikagaku, Tokyo, Japan) was O-acelytated with $^{14}C$-Acetyl CoA (Amersham Life Sciences, Cleveland, OH, U.S.A.) using the O-acetyl transferase NodL as described by Bloemberg et al. (21). Samples consisting of cell-free supernatant fluid of overnight cultures were loaded on a $NH_2F_{245s}$ Thin Layer

Chromatography plate (Merck, Darmstadt, Germany) and chromatographed using a 65% acetonitril/35% water (vol/vol) mixture. The distribution, e.g. breakdown, of chitinpentaose of radioactivity was measured after 4 to 7 days of exposure, using a Phosphor Imager (Biorad Lab).
Hydrogen cyanide was detected by growing the bacterial strains on agar plates in the presence of 3MM paper (2 x 2 cm) drenched in a solution of copper(II) ethyl-acetoacetate (5 mg/ml) and 4,4'-methylene-bis-(N,N-dimethylaniline) (5 mg/ml) (37). Hydrogen cyanide turns the indicator paper blueish purple.
Production of biosurfactant was determined using a drop-collapsing assay, in which a small amount of bacteria was taken from a bacterial colony with a toothpick and resuspended in 15 to 30 µl drops of water placed on parafilm. The presence of biosurfactant decreases the surface tension and therefore results in the collapse of the drop (114).
Bacteria were tested for motility after spot inoculating of cells in the middle of a plate containing 1/20 KB solidified with 0.3% agar. The plates were examined for the presence of migration zones after overnight incubation at 28°C (56).

**Table 1**. Microbial strains and plasmids

| Strains and plasmids | Characteristics | Reference or source |
|---|---|---|
| **Bacterial strains** | | |
| PCL1171 | Antagonistic *Pseudomonas* strain isolated from the rhizosphere of maize from Mexican agricultural fields. Colony morphology varies between two distinct phases, defined as phases I (opaque) and II (translucent). Model strain chosen for genetic studies | This study |
| PCL1152, 55, 57, 59,61, 63, 66, 69, 173, 75, 77, 80, 82, 84 | Other antagonistic *Pseudomonas* strains isolated from the rhizosphere of maize from Mexican agricultural fields. Colony morphologies vary with different frequencies between two distinct phases defined as phase I and phase II | This study |
| PCL1572 | Derivative of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a *gacS* homologue | This study |
| PCL1563 | Derivative of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a *gacS* homologue | This study |
| PCL1564 | PCL1572 complemented with pMP6562 (pME6010-*gacS*) | This study |

**Table 1.** *Continued*

| Strains and plasmids | Characteristics | Reference or source |
| --- | --- | --- |
| PCL1555 | Derivative of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a *mutS* homologue | This study |
| PCL1556 | PCL1555 complemented with pMCS5-*mutS* | This study |
| PCL1391 | *Pseudomonas chlororaphis*. Efficient biocontrol strain and good competitive colonizer of tomato roots, which produces phenazine-1-carboxamide. | (44) |
| PCL1666 | Derivate of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a lipopeptide synthetase homologue | This study |
| PCL1656 | Derivate of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into the thiolation domain of a lipopeptide synthetase homologue | This study |
| PCL1663 | Derivate of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a condensation domain of a lipopeptide synthetase homologue | This study |
| PCL1660 | Derivate of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a region preceding a adenylation domain of a lipopeptide synthetase homologue | This study |
| DH5α | *E. coli endA*1 *gyrSA*96 *hrdR*17(rK-mK-) *supE*44 *recA*1; general purpose *E. coli* host strain | (93) |
| **Fungal strains** | | |
| ZUM2407 | *Fusarium oxysporum* f.sp. *radicis-lycopersici*; causes tomato foot and root rot | IPO-DLO |
| 3R4FNA | *Rhizoctonia solani*; causes damping-off and fruit rot | IPO-DLO |
| *400* | *Rosellinia necatrix*; causes white root rot or Rosellinia root rot in a wide range of host plants | (189) |
| R3-11A | *Gaeumannomyces graminis* pv. *tritici*; causes take-all disease of wheat and of other cereals | (192) |
| **Plasmids** | | |
| pRL1063a | Plasmid harboring a promoterless Tn*5luxAB* transposon $Km^r$, and a p15A origin of replication | (277) |

**Table 1.** *Continued*

| Strains and plasmids | Characteristics | Reference or source |
|---|---|---|
| pGEM-T Easy | Vector system for cloning PCR products, $Cb^r$ | Promega, Madison USA |
| pME6010 | *E. coli* / *Pseudomonas* shuttle vector, stably maintained in *Pseudomonas* species, with an estimated copy number of 4-8, $Tc^r$ | (95) |
| pMP6562 | pME6010 harboring a 3.2kb PCR product from strain PCL1171 which contains the *gacS* homologue from PCL1171,$Tc^r$ | This study |
| pMP5565 | pME6010 harboring a 1.2kb PCR product from *Pseudomonas* sp. strain PCL1446 which contains a *gacA* homologue | Kuiper et al., unpublished data |
| pMCS5-*mutS* | pBBR1 MCS-5 containing the *mutS* gene from *P. aeruginosa*, $Gm^r$ | (190) |

**Attachment assays**

For root attachment experiments, tomato seeds were sterilized by incubating the seeds for 3 min in 5% sodium hypochlorite, followed by five rinses for 25 min in 20 ml of sterile water. Subsequently, the seeds were incubated for 3 min in 70% ethanol, followed by five rinses with sterile water. After a second incubation for 1 h in 5% sodium hypochlorite, the fluid was removed, and the seeds were left for 1 h in sterile water. The latter procedure was repeated once. Sterilized wheat and tomato seeds were stored on PNS (101) agar plates at 4°C and were allowed to germinate on PNS agar at 28°C. Seedlings were grown in a PNS solution in magenta vessels (Sigma, Bornhem, Belgium) holding a perforated stainless steel tray, for seven days at 20°C. Bacteria scraped from agar plates were resuspended in PBS to an $OD_{620}$ = 1.0. The roots were incubated for 45 minutes in the bacterial suspension, were removed and were washed in PBS to remove all nonattached bacteria. The roots were shaken vigorously for 15 minutes in a suspension of PBS in the presence of sand in an Eppendorff shaker (Eppendorff, Hamburg, Germany) to remove attached bacteria from the root. Appropriate dilutions of the suspensions were plated on KB agar. The number of colonies was determined after two days of growth at 28°C.

**Biocontrol of take-all of wheat caused by *Gaeumannomyces graminis* pv. *tritici***

The *G. graminis* pv. *tritici*-wheat system as described by Weller et al. (269) was used to test biocontrol activity. Briefly, an inoculum was prepared by growing *G. graminis* pv. *tritici* on sterilised oat for 3 to 4 weeks. The inoculum was dried overnight in a flow cabinet and stored at 4°C. The inoculum for the biocontrol assay was prepared following the method of Weller et al. (1985). A bacterial suspension ($2\times10^9$ CFU/ml) and a 2.0% (w/v) methylcellulose solution were mixed (1:1 vol/vol) and used to coat wheat seeds (*Triticium aestivum*, cultivar Residence). Wheat seeds were sown (nine seeds per pot) on a mixture of potting soil and chemically pure sand, in a 1:1 ratio, containing a predetermined percentage of inoculum, which results in 60 to 80% diseased plants, and covered with approximately 1 cm of inoculum-free potting soil. After 2 to 3 weeks of growth at 15°C, the number of diseased plants was determined based on the characteristic disease symptoms of take-all (192). Plants were scored as diseased or healthy.

## RESULTS

### Selection of antagonistic *Pseudomonas* spp. strains which undergo phase variation

A collection of 214 *Pseudomonas* strains was isolated from the rhizosphere of maize plants from an agricultural field in Totontepec Mixe, Oaxaca, Mexico. They were preliminary characterized as pseudomonads based on their growth on *Pseudomonas* isolation medium, colony morphology, and Amplified Ribosomal DNA Restriction Analysis (ARDRA). Using an antifungal activity plate assay (83), it was shown that 46 (21%) of the strains inhibited the growth of *Gaeumannomyces graminis* pv. *tritici* R3-11A, *Fusarium oxysporum* f.sp. *radicis-lycopersici*, *Rhizoctonia solani*, and *Rosellinia necatrix*. Another 33 strains (15%) showed slight antagonistic activity, i.e., the colonies were not overgrown by the fungus. The remaining 135 strains (63%) did not exhibit activity towards the fungal species tested.

Forty-three (93%) of the 46 selected strongly antagonistic strains showed colony phase variation, as judged after 4 days of growth on King's medium B (KB) agar at 28ºC. Two morphologically different colony types were found for all strains. Colonies, referred to as phase I, are thick and opaque (majority of colonies in Fig. 1A and B), whereas those of phase II are flat and translucent (Fig. 1C). After separation of the two different phases by re-streaking on KB agar and subsequent growth for two days at 28ºC, roughly three classes with distinct but somewhat fluctuating frequencies of phase variation could be distinguished. Fluctuating frequencies of phase variation could be distinguished in liquid culture with average frequencies of $>9.0 \times 10^{-2}$, around $10^{-3}$, and $<1.5 \times 10^{-4}$ switches per generation. For the latter class, consisting of strains PCL1152, PCL1157, PCL1159, PCL1166, PCL1169, PCL1177, PCL1182, and PCL1184, both colony types can be maintained separately. A low frequency of switching ($<5.0 \times 10^{-4}$) was observed from phase I to phase II, whereas a slightly higher switching frequency (around $10^{-3}$) was observed from phase II back to phase I. PCL1171 and PCL1173 exhibit a low frequency of switching ($<5.0 \times 10^{-4}$) from phase I to phase II. However, a high frequency ($>9.0 \times 10^{-2}$) for the reverse switch was observed, since restreaking of phase II colonies with a single phase appearance immediately resulted in phase I colonies out of which phase II sectors are again formed after two days of growth. For the most instable strains, PCL1155, PCL1161, PCL1163, PCL1175, and PCL1180, both phases are instable, and re-streaking of one of the phases always resulted in a mixture of

phase I and phase II colonies. Based on the differences in colony morphology and the distinct frequencies of phase variation, fifteen strains were selected (Table 1) for characterization of surface characteristics and the expression of biocontrol traits.

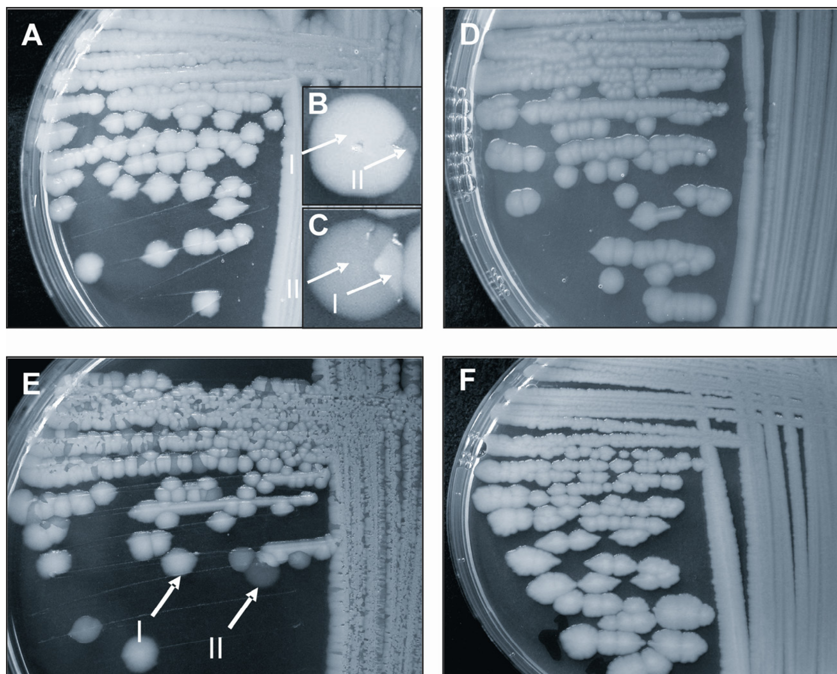



**Figure 1.** Colony phase variation of PCL1171 and its mutants. A, wild-type PCL1171, in which colonies with a phase I morphology are dominant; B, enlargement of a single colony of this strain in which phase II appears as a sector; C, enlargement of a single colony of this strain in which a phase I sector appears; D, stable phase II colony morphology of PCL1572 (Tn*5luxAB*::*gacS*); E, colony morphology of PCL1555 (Tn*5luxAB*::*mutS*) in which the frequency of colony phase variation is increased; F, colony morphology of PCL1556 (Tn*5luxAB*::*mutS*) complemented by pMCS5-*mutS* which decreases the frequency of colony phase variation of the mutant to wild-type levels. The arrows indicate phase I (I) and phase II (II) colonies, respectively.

## Biocontrol traits expressed in different phases

Each of the 15 selected strains showed a different lipopolysacharide (LPS) ladder pattern on SDS-PAGE (sodium dodecylsulfate polyacrylamide gelelectrophoresis), but no difference in LPS patterns were found between the

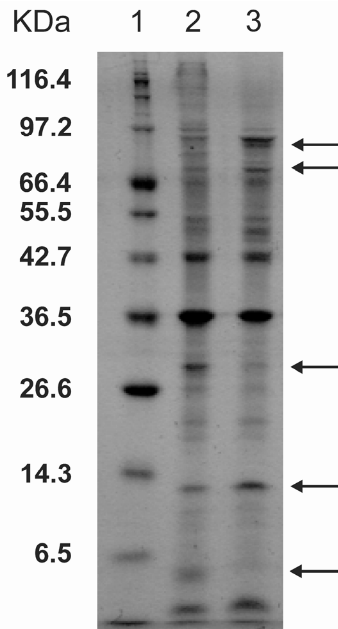



**Figure 2.** SDS-PAGE analysis of cell envelope proteins of phase I and phase II bacteria of strain PCL1171. SDS-PAGE gel of the cell envelope proteins isolated from colonies of PCL1171. Lane 1, protein markers (sizes indicated in kDa on the left); lane 2, cell envelope proteins expressed by phase I cells; lane 3, cell envelope proteins expressed by phase II cells (arrows indicate differences in expression of proteins between phase I and phase II cells). Clear differences in protein expression were observed for proteins with apparent molecular weights of 5, 12, 30, 72, and 84 kDa.

two colony phases of a single strain (data not shown). One of the strains, *Pseudomonas* sp. PCL1171, was examined for differences in cell envelope proteins between its two phases. SDS-PAGE analysis showed that proteins with apparent molecular masses of 5 and 30 kDa were enhanced in phase I, whereas proteins with apparent molecular masses of 12, 72, and 84 kDa were enhanced in phase II cells (Fig. 2). The ability of PCL1171 cells of the separate phases to attach to roots of wheat or tomato was analyzed in a time course, but no differences were observed. Both phase I and phase II bacteria were tested on motility plates. Overnight incubation of the bacteria resulted in a clear motility circle for phase II bacteria and in an irregular movement of the bacteria over the plate for phase I bacteria (Fig. 3).

Phase I and phase II bacteria of the 15 selected strains were tested in a plate assay for inhibition of the growth of the phytopathogenic fungi *G. graminis* pv. *tritici*, *Fusarium oxysporum* f.sp. *radicis-lycopersici*, *Rhizoctonia solani*, and *Rosellinia necatrix*. Only phase I bacteria inhibited growth of the fungal species tested. Furthermore, the production of chitinase and biosurfactant was also found to be correlated with phase I morphology for all 15 strains. Protease and lipase were primarily produced by bacteria with a phase I morphology, although, for seven strains, phase II bacteria still produce protease

or lipase activities, or both. None of the selected strains produced hydrogen cyanide, cellulase, or β-glucanase (Table 2).

**Preliminary genetic characterization of colony phase variation by strain PCL1171**

One of the 15 selected *Pseudomonas* strains, strain PCL1171, was chosen for preliminary genetic characterization of the colony phase variation phenomenon. This choice was based on the strain's relatively stable expression of phase I morphology on KB agar plates. Phase II sectors were only found after

**Table 2.** Phase variation characteristics and biocontrol traits of fifteen antagonistic *Pseudomonas* strains isolated form the rhizosphere of maize plants from an agricultural field, Totontepec Mixe, Mexico.

| Group [1,2] | Colony phase [3] | AFA [4] | Bio-surfactant | Chitinase | Protease | Lipase |
|---|---|---|---|---|---|---|
| A | I | + | + | + | + | + |
| | II | - | - | - | - | - |
| B | I | + | + | + | + | + |
| | II | - | - | - | + | - |
| C | I | + | + | + | + | + |
| | II | - | - | - | - | + |
| D | I | + | + | + | + | + |
| | II | - | - | - | + | + |

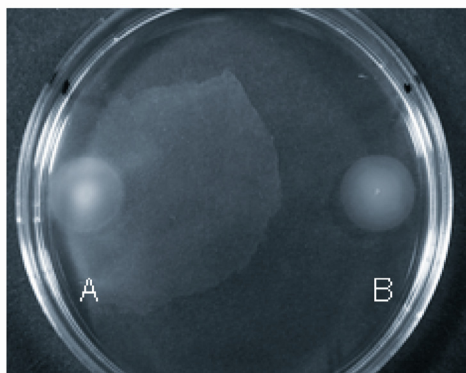
[1] Group A consists of strains PCL1155, PCL1157, PCL1169, PCL1171, PCL1177, PCL1180, PCL1182, and PCL1184, Group B consist of strains PCL1152, and PCL1163, Group C consists of strains PCL1159, and PCL1166, and group D consists of strains PCL1161, PCL1173, and PCL1175.

[2] None of the strains produced β-glucanase, cellulase, or hydrogen cyanide (HCN)

[3] Colony morphology, phase I (I) or phase II (II)

[4] Antifungal activity (AFA) towards *G. graminis* pv. *tritici* R3-11A, *F. oxysporum oxysporum* r.sp. *radicis-lycopersici*, *Rhizoctonia solani*, and *Rosellinia necatrix*.

**Figure 3.** Motility of PCL1171 phase I and phase II cells. Cells of PCL1171 phase I (A) and phase II (B) were inoculated on 1/20 King's medium B and grown overnight at 28°C.




approximately two days at the border of PCL1171 phase I colonies (Fig. 1A and B). Later, we observed that re-streaking of these phase II sectors coincided with a high frequency of switching back to a phase I phenotype, resulting in mainly phase I morphology on agar medium. The strain was further identified using polymerase chain reaction (PCR) amplification and subsequent sequencing of the 16S rDNA of phase I and phase II colonies, which yielded identical sequences. This sequence data has been submitted to Genbank under accession number AY236959. Comparison of these sequences with those in the Genbank database revealed similarity with sequences of *Pseudomonas* sp. RNA group I, which includes *P. aeruginosa*, *P. chlororaphis*, *P. fluorescens* biovars, and *P. putida*. Based on 16S rDNA sequence, similarity (up to 99 % identity) was found to a large group of *P. tolaasii* strains (with 100 % identity). However, this 16S rDNA sequence clearly branches off from these *Pseudomonas* species (data not shown) and is therefore considered to be closely related to *P. tolaasii* species.

A Tn*5luxAB* transposon library of phase I of strain PCL1171 was constructed. Mutants that exhibited a phase-locked colony morphology or an altered phase variation frequency were selected. Three mutants were selected out of 900 transposants. Two of these mutants, strains PCL1563 and PCL1572, appeared to be locked in a phase II colony morphology (Fig. 1D). Consistent with what we found for phase II cells of wild-type strain PCL1171, the mutants PCL1563 and PCL1572 did not produce protease, lipase, or biosurfactant and were not antagonistic (data not shown).

Sequencing of the regions flanking the Tn*5luxAB* transposon of mutants PCL1563 and PCL1572 revealed that their transposons had inserted at different

positions in the same gene (Fig. 4A). The mutated gene, predicted to encode a protein of 918 amino acids (aa), showed highest homology (82% identity and 89% similarity at the amino acid level) to the *gacS* gene product of *P. chlororaphis* (Genbank accession no.AAF06332) (Fig. 4A). Downstream of *gacS*, an open reading frame (ORF) transcribed in the same direction as *gacS*, was revealed, the predicted protein product of which shows 65% identity and 74% similarity at the amino acid level to D-lactate dehydrogenase of *P. aeruginosa* (PA0927) (Fig. 4A). Upstream of the *gacS* gene an ORF, transcribed in the opposite direction, was predicted to encode a protein with 50% identity and 70% similarity at amino acid level to a response regulator of a two component regulatory system (PA0929) (Fig. 4A). For complementation, a PCR product was constructed, containing the complete *gacS* homologue, including 390 bp upstream of the ATG to include the promoter region as well as 230 bp downstream of the stop codon, which includes a fragment of 169 bp of D-lactate dehydrogenase. This PCR fragment, cloned into pME6010 (estimated copy number 4 to 8) resulting in pMP6562, restored phase variation in strains PCL1563 and PCL1572 to the wild-type level. In addition, PCL1157, PCL1182, and PCL1184 were used to test whether a spontaneous phase II phenotype can be based on a *gacS* mutation. Phase II bacteria from strains PCL1157, PCL1182, and PCL1184, could be (partially) complemented using pMP6562. A mixture of phase I and phase II colonies was obtained on plate. Complementation using pMP5565 harbouring a *gacA* homologue from *Pseudomonas* sp. strain PCL1446 resulted in a mixture of phase I and phase II colonies (data not shown). Only phase II colonies were obtained after transformation of the empty parental vector.

The third mutant, strain PCL1555, displayed an increased switching frequency between phase I and II in comparison to the wild type (Fig. 1E), in such a way that neither of the phases could be obtained as colonies with a single phase appearance. Sequencing of the flanking regions of the Tn*5* insertion in PCL1555, showed that the transposon had inserted in a gene encoding a protein of 865 aa with 85% identity and 91% similarity at the amino acid level to the *mutS* gene product of *P. aeruginosa* (Genbank accession no. AE004782), which was therefore designated *mutS* (Fig. 4B). Sequencing downstream of the *mutS* gene revealed an ORF, transcribed in the same direction, the predicted protein product of which showed 92% identity and 94% similarity at the amino acid level to a hypothetical protein in *P. fluorescens* (Genbank accession no. ZP_00085195) (Fig. 4B). Upstream of the *mutS* gene, an ORF transcribed in the

opposite direction was predicted to encode a protein with 88% identity and 93% similarity at the amino acid level to a hypothetical protein of *P. fluorescens* (Genbank accession no. ZP_00085197) (Fig. 4B). After transformation of PCL1555 with pMCS5-*mutS*, which contains the complete *mutS* gene and a downstream 203-bp fragment of ferrodoxin A from *P. aeruginosa*, the phase variation frequency of PCL1555 was restored to wild-type levels (Fig. 1E and F). The sequence data of *gacS* and *mutS* has been submitted to the Genbank databases under accession no. AY236957 and AY236958, respectively.

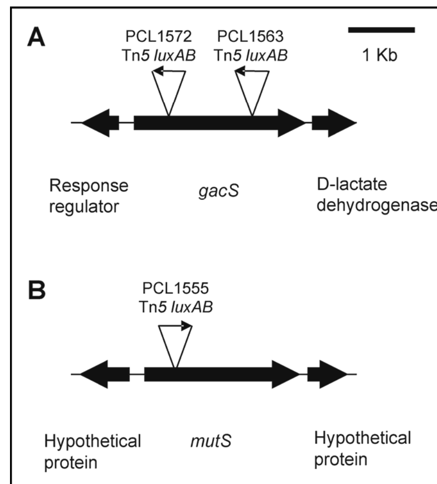



**Figure 4.** Schematic representation of the chromosomal regions of PCL1171 surrounding the transposon insertions of mutants A, PCL1572 (Tn*5luxAB*::*gacS*) and PCL1563 (Tn*5luxAB*::*gacS*) and of B, PCL1555 (Tn*5luxAB*::*mutS*). The arrows of the indicated genes and transposons indicate the direction of transcription.

**Effect of colony phase variation on biocontrol ability strain PCL1171**

Cells of the different colony phases of strain PCL1171 were tested for their biocontrol activity of wheat take all caused by *G. graminis* pv. *tritici*. Inoculation of the wheat seeds with phase I bacteria resulted in a significant reduction of the disease (Fig. 5A). PCL1171 phase I or phase II cells, tested in the absence of a pathogen, did not cause disease of wheat plants (data not shown). Inoculation of wheat seeds with phase II bacteria did not result in a statistically significant biocontrol, when compared to the untreated control seeds (Fig. 5A). Inoculation of the seeds using the well-described biocontrol strain, *P. chlororaphis* PCL1391 resulted in a significant suppression of the disease (Fig. 5A).

**Role of antagonism in biocontrol ability of strain PCL1171**

The Tn*5luxAB* transposon library of PCL1171 phase I was used to screen for genes involved in the antagonistic activity of PCL1171 towards *G. graminis* pv. *tritici*. A total of 2,000 mutants were screened for the loss of antagonistic activity in an antifungal plate assay (83). This screening only included mutants expressing phase I morphology with a phase variation frequency comparable to PCL1171. Four mutants, selected for the loss of antagonistic activity, were genetically characterized by sequencing the regions flanking the Tn*5luxAB* transposon. These sequences revealed that the transposon of all mutants had inserted in a lipopeptide synthetase homologue, but in different domains of this gene (88). The partially sequenced gene product showed highest homology to a syringomycin synthetase (Genbank accession no. AF47828). Domains in this gene include adenylation, thiolation and condensation domains, which are all needed to incorporate a single amino acid in the lipopeptide (158). Sequencing of the flanking regions of the transposon insertion in PCL1656 (2574 bp surrounding the Tn*5* with 75% identity and 64% similarity at the amino acid level), PCL1660 (942 bp surrounding the Tn*5* with 75% identity and 71% similarity at the amino acid level), PCL1663 (783 bp surrounding the Tn*5* with 62% identity and 53% similarity at the amino acid level), PCL1666 (1947 bp surrounding the Tn*5* with 50% identity and 60% similarity at the amino acid level) showed highest homology to thiolation, adenylation, and condensation domains, and a region preceding an adenylation domain, respectively, of lipodepsipeptide synthetase of *P. syringae* pv. *syringae*. In contrast to wild-type strain phase I cells, mutant PCL1666 phase I cells tested in the *G. graminis* pv. *tritici* - wheat system did not result in biocontrol (Fig. 5A).

**Role of phase variation genes in biocontrol**

The PCL1171 mutant derivatives PCL1572 (*gacS*::Tn*5luxAB*) and PCL1555 (*mutS*::Tn*5luxAB*) were tested in biocontrol. Neither of the mutants showed significant biocontrol activity (Fig. 5B and C). Complementation of PCL1572 and PCL1555 using pMP6562 and pMCS5-*mutS*, respectively, resulted in restoration of the wild-type colony phase I phenotype (Fig. 1F) as well as in corresponding levels of disease suppression (Fig. 5B and C).

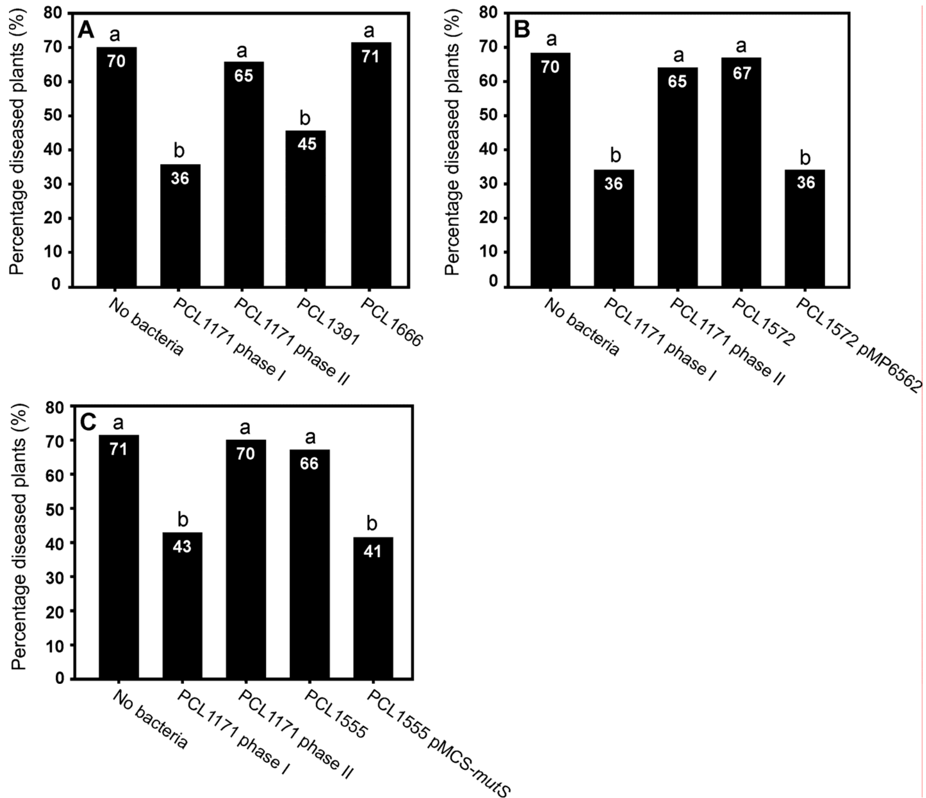



**Figure 5.** Biocontrol of wheat take all caused by *Gaeumannomyces graminis* pv. *tritici*. Wheat seeds were coated with bacteria and grown in a 1:1 mixture of potting soil and quartz sand amended with *G. graminis* pv. *tritici*. Seeds were coated with cells of the indicated wild type or mutant or with cells of the indicated phase. Experiments using PCL1171 phase I and phase II cells to coat the seeds, but without *G. graminis* pv. *tritici*, did not result in diseased plants. The negative control consisted of seeds coated without bacteria. After 12 days of growth the plants were scored for disease symptoms. For each strain, 108 plants were tested. Data were analyzed for significance using after arcsine square root transformations with analysis of variance followed by a Fischer's least significant difference test (p=0.05, n=12). Values with different letter indications denote a statistically significant difference. Similar results were obtained in a second experiment.

## DISCUSSION

### Isolation and preliminary characterisation of antagonistic *Pseudomonas* spp. strains which undergo phase variation

Performance of biocontrol microorganisms in the field is variable. Elucidation of the mechanism behind this phenomenon will contribute to defining the traits required for robust biocontrol strains and, therefore, enhanced performance. Our group has found that phase variation mediated by the *xerC*/*sss* gene has a profound effect on a *Pseudomonas* strain's ability for competitive root colonization (55, 56) and biocontrol (43). We therefore initiated a study on phase variation among *Pseudomonas* rhizosphere strains with the aim to identify other genes and traits involved in phase variation.

Maize in Totontepec Mixe, Mexico has been grown successfully for over 700 years without the application of chemicals. Since the climate in this region is warm and humid, conditions are ideal for the proliferation of fungi. A possible explanation for the excellent yields of maize could be a high incidence of biocontrol microbes protecting the plants against diseases caused by pathogenic fungi. Therefore, microbes derived from the maize rhizosphere of plants from this region were investigated. From 214 isolated putative *Pseudomonas* strains 46 isolates (21%) were found to inhibit the growth of a number of important crop pathogens including *G. graminis* pv. *tritici*, which causes wheat take-all, and *F. oxysporum* f.sp. *radicis*-*lycopersici*, which causes tomato foot and root rot. Indeed, this frequency of biocontrol strains is extremely high. For comparison, we previously found that the frequency of biocontrol pseudomonads in the rhizosphere of tomatoes from a commercial agricultural field in Andalusia (Spain) is approximately one percent (44).

A striking phenomenon was that 43 out of the 46 antagonistic isolates showed reversible colony phase variation (Fig. 1B and C). The reversible character of the colony phase variation is illustrated by the occurrence of phase II sectors originating from phase I colonies (Fig. 1B) and phase I sectors originating from phase II colonies (Fig. 1C). We selected 15 clearly different strains and tested these on the influence of phase variation on other biocontrol traits and found that the production of such diverse metabolites as antifungal metabolite, chitinase, biosurfactant, protease, and lipase are subject to phase variation. The majority of these molecules are synthesized by the opaque phase I colony form but not by the translucent form (Table 2). Other differences between these colony forms were found in motility (Fig. 3) and cell surface

proteins (Fig. 2). It should be noted that the difference in motility may be caused by the effect of phase variation on biosurfactant production since biosurfactant can influence motility by enabling bacteria to break the colony boundary more easily resulting in irregular swimming (169).

Since the majority of the factors mentioned in Table 2 are synthesized in opaque phase I cells under control of the *gac* system, it is likely that the factor determining opacity of phase I colonies is under the same control. Consistent with this notion is the finding that opacity proteins (*opa* genes) in *Neisseria gonorrhoeae* are also regulated by phase variation (230). Therefore, it is conceivable that one or more of the cell surface proteins which is/are relatively strongly expressed in phase I colonies (Fig. 2) are determining the colony opacity. It should also be realized that, if a strain does not produce this opacity factor, phase variation may occur but may not be visible as a change in colony morphology.

**Genetic characterization of colony phase variation of PCL1171**

One of the 15 strains, *Pseudomonas* sp. PCL1171, was chosen for a preliminary genetic characterization of colony phase variation. Since phase I colony morphology is dominant in PCL1171 (Fig. 1A), mutants locked in phase II (Fig. 1D) can relatively easily be detected. Both analyzed phase II-locked mutants appeared to have the transposon inserted in a *gacS* homologue (Fig. 4A). GacS is the sensor kinase of a two component regulatory system that, in combination with the response regulator GacA, controls the production of a wide range of secondary metabolites (271), including the production of AFM, chitinase, protease, HCN, and virulence factors (139, 156). The *gacS*/*gacA* regulatory system belongs to the FixJ family of transcriptional regulators (139). Our results indicate that the *gacA*/*gacS* system is one of the key players in phase variation, since mutation of *gacS* locks the bacteria in phase II. This is supported by the complementation by *gacS* and *gacA* of spontaneous phase II bacteria from wild-type strains PCL1157, PCL1182, and PCL1184.

Recent reports on *gacA*/*gacS* have shown that both genes are targets of point mutations, small deletions and insertions. For example, *P. fluorescens* grown in nutrient rich liquid media to stationary phase accumulates spontaneous stable *gacA* and *gacS* mutants (33). Furthermore a homologue of *gacS*, the *pheN* gene of *P. tolaasii*, was shown to be regulated by phase variation through an internal 661-bp duplication (92). Bull et al. (33) reported a selective advantage under laboratory conditions for the loss of *gacA* function which might represent

another example of phase variation via *gacA*/*gacS*. In addition, Chancey et al. (39) reported that *gacA*/*gacS* mutants can survive in the rhizosphere and, when present in wild-type populations, will increase the survival of these mixed populations. Also, in this case, phase variation could be the cause of these mixed populations. It is therefore conceivable that, in the heterogeneous and changing microenvironment of the rhizosphere, the ability to adapt by changing the expression of specific traits to reduce metabolic load via *gacA*/*gacS*, combined with the beneficial effect of these mutants on population survival in the rhizosphere, could be advantageous for the bacterium.

Mutant PCL1555 shows a strongly increased frequency of switching between phases I and II (Fig. 1E). Genetic analysis showed that its transposon is inserted into a homologue of the *mutS* gene (Fig. 4B). MutS is involved in methyl-directed recognition of DNA mismatches related to replication. MutS recognizes base mismatches and small insertions or deletion mispairs originating from replication. Upon recognition of these mismatches by MutS, a repair pathway involving MutLH is activated, resulting in excision of the mismatch by exonucleases. Strand specificity in excision and resynthesis of the excised strand is determined by the hemimethylated state of the DNA (174). Mutation of *mutS* is reported to result in the persistence of mutations due to the lack of repair (35). For example, mutation of the *mutS* gene resulted in a 100- to 1,000-fold increase in the frequency of mutations found in *E. coli* (106). Whereas our results strongly suggest that the *gacA*/*gacS* system is a key regulator in colony phase variation, we hypothesize that the high frequency of phase variation in *mutS* mutant PCL1555 is the result of the lack of repair of mutations in the *gacA* and *gacS* genes. Considering the data obtained for our *gacS* and *mutS* mutants we hypothesize that introduction of mutations in *gacA*/*gacS* is the basis for the phase I to phase II switch. It is likely that the reverse switch from phase II to phase I, is based on repair of these mutations in *gacA*/*gacS*, and is likely to involve *mutS*.

**Effect of colony phase variation on biocontrol ability strain PCL1171**

It appeared that PCL1171 phase I cells, but not phase II cells, are active as a biocontrol agent of wheat take-all (Fig. 5). The activity of phase I cells of PCL1171 is even slightly better than that of the well-known tomato foot and root rot biocontrol strain *P. chlororaphis* PCL1391 (Fig. 5A) (44). This is the first report which shows that strain PCL1391 also controls a disease of a monocot plant. The *gacS* mutant PCL1572, which only exhibits phase II

morphology (Fig. 1D), is as inactive in biocontrol as phase II wild-type cells (Fig. 5B). Both phase I colony appearance and biocontrol activity by the mutant (Fig. 5B) are restored by genetic complementation. In conclusion, there exists a strong correlation between phase I and biocontrol ability. These results show that PCL1171 requires a functional Gac system for efficient biocontrol of wheat take all. Previously, it was shown that a *gacA* mutant of *P. fluorescens* CHAO exhibited biocontrol in a Ggt system, presumably due to the (upregulated) production of siderophores, which is not dependent on GacA (215). Also, *mutS* mutant PCL1555 is impaired in biocontrol and, in this case, this phenomenon can be restored by genetic complementation (Fig. 5C). Possible advantageous effects of a high mutation rate on the fitness of cells are short-term effects. In contrast, in the long term, especially in heterogeneous environments, a high mutation rate will reduce the overall fitness due to the high mutation load (85). It is therefore conceivable that the high mutation frequency in mutant PCL1555 leads to a higher percentage of disabled cells, which are poorly rhizosphere competent and therefore, will hardly contribute to biocontrol.

**Role of antagonism in biocontrol ability of strain PCL1171**

Mutant PCL1666 lacks antagonistic activity (Fig. 5A) but is still expressing a wild-type phase I morphology. Genetic analysis of this mutant showed that its transposon is inserted in a homologue of a lipopeptide synthetase gene. This gene has been described as being responsible for the production of a lipodepsipeptide (19, 88). Some lipopeptides are known as host-specific toxins that play an important role in the virulence of, for example, *P. syringae* (19). In this context, it should be stressed that PCL1171 phase I cells, tested in a biocontrol experiment in the absence of *G. graminis* pv. *tritici*, did not cause disease symptoms on wheat (data not shown). The lack of antagonistic activity of mutant PCL1666 (Fig. 5A) is consistent with published data which show that a number of lipopeptides, including syringomycin, can have fungicidal activity (19, 242). Based on our observations, we conclude that the production of the antifungal metabolite of PCL1171 is a prerequisite for the biocontrol activity of this strain. The genetic analysis of PCL1666 strongly suggests that the mutation resides in the structural gene for a lipopeptide, which is switched off in phase II and that this is one of the reasons for the lack of biocontrol activity of this phase variant. This hypothesis is supported by the observation that both the phase switch in strain PCL1171 (Fig. 5A) as well as the production of a variety of

toxins by *P. syringae* (14) and lipopeptide production in *Pseudomonas* sp. strain DSS73 (134) is dependent on *gacS* activity.

We have shown that *Pseudomonas* sp. PCL1171 can control wheat take-all and that one of the important biocontrol traits is the production of an AFM (Fig. 5A), likely to be a lipopeptide in nature. Since phase variation may be a frequently occurring phenomenon that hampers the optimal production of the AFM of PCL1171 as well as optimal colonization and biocontrol of *P. chlororaphis* PCL1391 (43, 56), phase variation may be a major factor in the inconsistent biocontrol observed in several field trails (214, 268). Thus, phase variation not only plays a role in escaping animal and human defence by enabling pathogens to adapt to heterogeneous or hostile environments (140, 204) but it also appears to play a vital and much broader role in the ecology of bacteria producing exoenzymes, antibiotics, and other secondary metabolites. We plan to investigate the genetic basis of phase variation in more detail in order to determine which traits are involved in regulation of phase variation and which target genes are regulated by it. Hopefully this research will result in more insight in traits required for robustness of biocontrol strains. The result may also be of importance for the efficient production of industrial enzymes such as protease and lipase, and of antibiotics and biosurfactants. Similarly, since vaccines frequently contain cell surface proteins of which the synthesis can be under control of phase variation, an enhanced control of phase variation may be used to more efficiently produce purer and less expensive vaccines.

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# CHAPTER 3

# Molecular nature of spontaneous modifications in *gacS* which cause colony phase variation in *Pseudomonas* sp. PCL1171

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## ABSTRACT

*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 exo-enzymes. Complementation and sequence analysis of twenty-six phase II mutants and of thirteen 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*. The 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 remained in the phase II phenotype but changed genotypically as the result of (re-)introduction of mutations in either *gacA* or *gacS*. The reversion of *gacA/S* to the wild type was not affected by mutation of *recA* and *recB*. We conclude that in *Pseudomonas* sp. 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 sub-populations harbouring spontaneous mutations in *gacA* and or *gacS*, thereby enabling both populations to be maintained.

## INTRODUCTION

Phase variation is a process of reversible, high-frequency phenotypic switching that is mediated by mutation, reorganization or modification of DNA. Phase variation is used by several bacterial species to generate population diversity that increases bacterial fitness and is important in niche adaptation (211). Phase variation can sometimes be observed by the appearance of morphologically distinct colonies or sectors within a colony (72, 98). 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 (98). Four mechanisms of phase variation are known (98). (i) Slipped-strand mispairing, dependent on variations in the length of a repeat tract, switching a gene on or off as a result of frame-shifts, or regulating the level of expression by altering promoter spacing. (ii) Genomic rearrangements, based on invertable 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 un-programmed variation, which can switch traits on and off via random reversible mutations (27).
Phase variation has been described to regulate the production of pili (170), outer membrane proteins (170), flagella (123), fimbriae (1), surface lipoproteins and other surface exposed structures (72, 97, 204), secondary metabolites (38, 250), and of secreted enzymes such as proteases, lipases and chitinases (38, 250). In a previous paper (250), we described that out of forty six *Pseudomonas* strains antagonistic against the wheat pathogenic fungus *Gaeumannomyces graminis* pv. *tritici* R3-11A (192), forty three displayed colony phase variation. One of these strains, PCL1171, was selected to study 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 exo-enzymes chitinase, lipase, and protease are regulated via phase variation, and only expressed in the phase I phenotype. Estimation of the phase variation frequencies showed approximately $5.0 \times 10^{-5}$ and $9.0 \times 10^{-2}$ switches per cell per generation for phase I to II and for phase II to I, respectively (250).
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 (250). 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 (138), and regulates secondary metabolism and production of exo-enzymes (24, 130, 138, 200, 250). Phase variation via a homolog of *gacS*, *pheN*, which regulates pathogenicity and colony morphology in *P. tolaasii*, is dependent on a spontaneous duplication in this gene (92). Furthermore, it has been described that the *gacA/S* system is subject to the accumulation of mutations in several *Pseudomonas* sp. (33, 64, 138, 200, 216). However, neither reversibility of these *gac* mutations nor the molecular nature of mutations in the *gacS* gene, have been reported. In this paper we analyse the role of *gacA/S* in a 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.

## 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) (128) at 28°C. *E. coli* strains were grown in Luria-Bertani (LB) medium (208) at 37°C. Solid growth media contained 1.8% (wt/vol) agar (Difco Laboratories, Detroit). 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 analysed in triplicate by measurement of the optical density (OD620) in time. The numbers of viable cells in the starting culture were counted. The 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 plate in 20 ml of KB and grown overnight at 28°C. The morphology and the initial number of bacteria was determined after dilution plating of an average of 500 cells per plate. At least 1,500 colonies were counted for the estimation of frequencies as number of switches per cell per generation.

**Table 1**. Microbial strains and plasmids

| Strains and plasmids | 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) | (250) |
| PCL1572 | Derivate of PCL1171 in which a Tn*5luxAB* transposon is inserted into a *gacS* homologue, $Km^r$ | (250) |
| PCL1586 | PCL1171 derivate harboring a mutation in a *recA* homolog, $Km^r$ | This study |
| PCL1589 | PCL1171 derivate harboring a mutation in a *recB* homolog, $Km^r$ | This study |
| S17-1 | *E. coli* MM294, RP4-2 Tc::Mu-Km::T7 chromosomally integrated | (224) |
| DH5α | *E. coli endA*1 *gyrSA*96 *hrdR*17(rK-mK-) *supE*44 *recA*1; general purpose *E. coli* host strain | (93) |
| **Plasmids** | | |
| pRL1063a | Plasmid harboring a promoterless Tn*5luxAB* transposon $Km^r$, and a p15A origin of replication | (277) |
| pMP5285 | pME3049 derivate, lacking the $Hg^r$ gene,used for single homologous recombination, $Km^r$ | (137) |
| pME6010 | *E. coli* / *Pseudomonas* shuttle vector, stably maintained in *Pseudomonas* species, with an estimated copy number of 4-8, $Tc^r$ | (95) |
| pMP6562 | pME6010 harboring a 3.2kb PCR product from strain PCL1171 which contains the *gacS* homologue from PCL1171,$Tc^r$ | (250) |
| pMP5565 | pME6010 harboring a 1.2kb PCR product from *Pseudomonas* sp. strain PCL1446 which contains a *gacA* homologue | Kuiper et al. unpublished data |
| pMP6603 | pME6010 harboring a 1.2kb PCR product from *Pseudomonas* sp. PCL1446 containing a *gacA* homologue and a 3.2kb PCR product from *Pseudomonas* sp. PCL1171 containing a *gacS* homolog | This study |
| pMP6604 | pMP5285 harbouring a 1.2kb *Eco*RI fragment of a *recA* homologue of *Pseudomonas* sp. PCL1171 | This study |
| pMP6605 | pMP5285 harbouring a 1.2kb PCR fragment of a *recB* homologue of *Pseudomonas* sp. PCL1171 | This study |

**Isolation and characterisation of phase II Tn*5luxAB* transposon mutants**

A mutant library of strain PCL1171 was constructed using plasmid pRL1063a (250), which harbours a Tn*5* transposon with promoterless *luxAB* genes and a

kanamycin resistence marker (277). 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 Sambrook et al. (208).

**Complementation of Tn*5luxAB* transposon mutants and wild-type phase II sectors**

Phase II bacteria were complemented using pMP6562 (*gacS*) and pMP5565 (*gacA*) by parental mating. Primers oMP716 (5'-GGAATTCAGGATGTCCATCAACACCA-3') and oMP717 (5'-GGAATTCATCGTTGATGAAGGCACACA-3') both containing a *Hind*III site (GGAATTCA) were used to obtain a 3.2 kb PCR fragment harbouring a *gacS* homologue from *Pseudomonas* sp. PCL1171. The PCR product was cloned into pMP5565 using *Hind*III. The resulting construct pMP6603, harbouring both the *gacA* and the *gacS* gene under their own promoters, was electroporated into *E. coli* S17-1 (224) and used to transform phase II bacteria by parental mating. Morphology of the transformants was judged after two days of growth at 28°C on King's medium B (128) 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 using *Hind*III and transformed to PCL1572 (*gacS*::Tn*5luxAB*) by electroporation for complementation analysis. Morphology was judged after two 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 using oMP716 and oMP717, and internal primers oMP698 (5'-ACCCAATCCCTGGAACAAC-3'), oMP699 (5'-GAGTTCCTGGCCAACATGAG-3'), oMP700 (5'-GAGCAGATGGG TGGTGAGAT-3'), oMP701 (5'-CAAACCCTGCTGGAAGACAT-3') annealing at nucleotide position 220, 859, 1456, 2047, respectively, and reverse primers oMP723 (5'-GGTCCACTTCAACACCACCTG-3'), oMP722 (5'-GTAATGCCGTTGGTCAGGTTC-3'), oMP721 (5'-GTTGATGA TGCCCAACAGGT-3'), 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 found mutations were sequenced at least two times.

**Isolation and construction of mutants**

To isolate genes from PCL1171 a plasmid library was constructed. Chromosomal fragments of PCL1171 phase I bacteria, 3-5 kb in length, were obtained after partial digestion using *Eco*RI. 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'-GTTTTCCCA GTCACGAC-3'), and two internal primers for *recA* oMP529 (5'-GGGGTACC AGCGCACCAGCAT-3') and oMP530 (5' GGAATTCCAGATCGAACGCC AATTCGG-3'). A second PCR was performed using the internal primers to select the correct PCR fragment. A *recA* mutant was constructed using the PCR product of oMP61 and oMP530 to obtain a 1.2 kb *Eco*RI fragment of the *recA* gene which was cloned into the suicide plasmid pMP5285 resulting in pMP6604. This construct was electroporated into S17-1 (224) and transferred to PCL1171 by parental mating. After selection on kanamycin, the mutation of *recA* was confirmed via Southern hybridisation (208) using the AlkPhos direct labelling kit for detection (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's recommendations. The same approach was used to isolate the *recB* gene from PCL1171 using internal primers oMP760 (5'-TTCGACAGCGGCAGCCTGTTCACCCAGA-3') and oMP761 (5'-AAGGC GTA(CT)AT(GC)GC(AG)TGCTTGGGGTCGCCGAT-3'). To construct a *recB* mutation a 1.2 kb PCR product obtained using oMP840 (5'-CAGGAAT TCGAAGGTTGGTG-3') and oMP761 was cloned into the pGEM-TEasy Vector (Promega Crop., Madison, WI, USA) and subsequently transferred to the suicide plasmid pMP5285 using *Eco*RI, resulting in pMP6605. pMP6605 was electroporated into S17-1 (224) and transferred to PCL1171 via parental mating. After selection on kanamycin, the mutation of *recB* was confirmed via Southern hybridisation (208). To determine the effect of *recA* and *recB* mutation on phase variation, phase II colonies were re-plated 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 $6.0x10^{-5}$ and $8.2x10^{-2}$ 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, using pRL1063a (277). 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 can be isolated which are more stable. In contrast to the $8.2x10^{-2}$ switches per cell per generation as observed for the wild type, no switch back to the phase I phenotype was observed in these phase II mutants. These mutants will further be referred to 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 (250), 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 the plasmids pMP6562 (harboring a *gacS* homolog of PCL1171), pMP5565 (harboring a *gacA* homologue of *Pseudomonas* sp. PCL1446), and pMP6603 (harboring both a *gacA* and *gacS* homologue). It appeared that out of 26 mutants, 13 could be complemented to the phase I phenotype using pMP6562 (*gacS*) and the other 13 mutants could be complemented to the phase I phenotype using pMP5565 (*gacA*). Complementation using pMP6603, harboring both the *gacA* and the *gacS* genes, restored the phase I phenotype in all 26 mutants. Introduction of the empty parental vector pMP6010 did not alter the phase II phenotype. Subsequent re-streaking 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 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 when compared to the wild type (50 ± 4.2 min vs. 60 ± 4.2 min, respectively) (Fig. 1). Measuring numbers of CFU's 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 higher number of dead cells in the culture of the wild type.

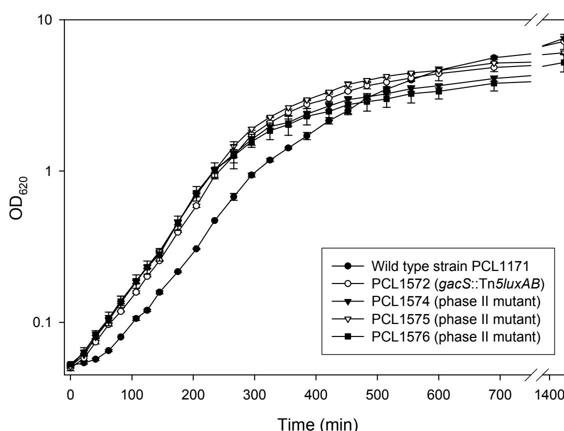


**Figure 1.** Growth curve of wild-type *Pseudomonas* sp. PCL1171, its Tn*5luxAB* transposon derivate PCL1572, and three stable phase II mutants (PCL1574, PCL1575, and PCL1576) in King's medium B. The number of CFU's was determined at the start of growth. The results indicated that the difference in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

**Molecular basis of phase II phenotype of phase II mutants**

Since, only mutations accumulating in the *gacA* gene have been analyzed (33), we decided to focus on the role of *gacS* in phase II variation of *Pseudomonas* sp. PCL1171. We isolated the *gacS* gene from ten 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 indeed the mutations interfered with the 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 to 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 (a$A_{329}$c→a$G_{329}$c) (Table 2 and Figs. 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 using pMP5562 (*gacS*) (Table 2), complementation of PCL1572 (*gacS*::Tn*5luxAB*) 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 re-isolated from colonies in which the *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 using pMP5565 (*gacA*). This changed PCL1575 derivate was designated as PCL1575a (Table 2). In addition, PCL1575 phase II colonies were found which could neither be complemented using pMP5565 nor by using pMP6562. One such derivate was designated as PCL1575b (Table 2). Sequencing of the *gacS* gene of PCL1575a after PCR amplification showed that the point mutation identified in PCL1575 has been removed ($T_{154}$aa→$C_{154}$aa) in PCL1575a while maintaining a phase II phenotype. This point mutation was still present in PCL1575b ($T_{154}$aa) (Table 2).

**Molecular basis of phase II phenotype in wild-type strain PCL1171**

To test whether mutations in the *gac* system also form the molecular basis for the highly unstable phase II phenotype in wild-type strain PCL1171, the *gacS* gene from phase II sectors was amplified using 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*) and the resulting construct was used to complement PCL1572 (*gacS*::Tn*5luxAB*). Out of thirteen *gacS* genes isolated from these phase II sectors, five were unable to complement PCL1572 (Table 2). One, named 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 the 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 non-complementing *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* gene from sector 4 and sector 18 was found to harbor one and two point mutations, respectively (Table 2), while the *gacS* from sector 9 harbored a +1 frame shift due to a one 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, re-isolated 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 re-streaking coincides with a high frequency ($8.2 \times 10^{-2}$ switches per cell per generation) switch back to the phase I phenotype. This high frequency enabled us to analyze the *gacS* gene from phase II sectors after switching back to the phase I phenotype. The three mutations previously identified in phase II sector 1

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

| **Origin** | **Mutation in *gacS* gene**[a] | | **Functional gene** | | **Remarks** |
|---|---|---|---|---|---|
| | 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 |
| PCL1575 | $C_{154}aa – T_{154}aa$ | $Q_{52}$ – stop | + | - | *gacS* mutation |
| PCL1575a | - | - | - | + | *gacA* mutation |
| PCL1575b | $C_{154}aa – T_{154}aa$ | $Q_{52}$ – stop | - | - | *gacA* and *gacS* mutation |
| PCL1576 | $aA_{329}c – aG_{329}c$<br>115 bp inversion | $N_{110} – S_{110}$<br>$N_{110} – R_{147}$<br>(stop134) | + | - | Inverted repeat<br>Inversion[d] |
| Wild-type phase II sectors[c] | | | | | |
| Sector 1 | 12 bp deletion<br>$cT_{263}t – cC_{263}t$<br>$gA_{1813}g – gG_{1813}g$ | $A_{54}PL_{56}$<br>$L_{88} – P_{88}$<br>Silent | nd | - | Deletiond and point mutations |
| Sector 3 | nd | nd | nd | - | *gacS* mutation |
| Sector 4 | $C_{1981}cg – T_{1981}cg$ | $P_{661} – S_{661}$ | nd | - | Point mutation |
| Sector 9 | ct_caat -ct$T_{309}$caat | $L_{103}$ - frameshift | nd | - | 1 bp insertion[d] |
| Sector 16 | $tT_{2231}a – tC_{2231}a$<br>307 bp deletion<br>$ggC_{663} – ggA_{663}$ | $L_{744} – S_{744}$<br><br>Silent | nd | - | Tandem repeat<br>Deletion[d] |
| Sector 18 | $cT_{182}g – cC_{182}g$<br>$gC_{1124}c – gT_{1124}c$ | $L_{61} – P_{61}$<br>$A_{375} – V_{375}$ | nd | - | Point mutations |
| 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] Functional *gacA/S* component was determined by the introduction of pMP6562 (*gacS*) and pMP5565 (*gacA*). '+' indicates which component is functional, '-' indicates which component harbours mutations and is non-functional. In addition, strains PCL1565, PCL1568, and another 8 phase II mutants had a functional *gacA* gene (*gacS* mutants), while PCL1564, PCL1573, PCL1566, PCL1567, and another 9 phase II mutants had a functional *gacS* gene (*gacA* mutants).

[c] Functional *gacA/S* component was determined by complementation of PCL1572 (*gacS*::Tn*5luxAB*) by the *gacS* gene isolated from a phase II sector. '+' indicates that a

phase I morphology was restored in PCL1572, '-' indicates that a phase II morphology maintained after introduction of the *gacS* gene. In addition, the *gacS* gene isolated from another 5 sectors did complement PCL1572 (*gacS*::Tn*5luxAB*), suggesting that mutation of the *gacA* gene was responsible for the phase II phenotype in these sectors. nd, not determined.
[d] See Figure 1

(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 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, using the suicide construct pMP6604, which resulted in strain PCL1586. Strain PCL1586 (*recA*::km$^r$) showed a phase I phenotype from which phase II sectors originated with a high frequency. After re-plating 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. Re-plating 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 exo-enzymes (250).

Since we observed that mutation of the *gacS* gene locked the bacteria in a phase II phenotype (250), we studied the role of *gacA/S* in phase variation. Complementation analysis of a total of thirty-nine phase II derivates 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. 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. (33) (point mutations, a 3 bp deletion and a 1bp insertion), the *gacS* gene of PCL1171 also harbored a 12 and 307 bp deletion and a 115 bp inversion (Table 2 and Fig. 2), showing that a large diversity of mutations, both in nature and location, can accumulate in the *gacA* and *gacS* genes (Table 2 and Fig. 2). We observed that not all mutations in the *gacS* gene, for example single mutations in the periplasmic loop (e.g. sector 2), affected GacS function, which is consistent with the observation that deletion of this domain did not affect GacS function in *P. fluorescens* CHAO (282).

Accumulation of mutations in *gacA* and *gacS* was reported previously for *P. syringae* pv. *syringae* strain B728a (200), *P. fluorescens* CHAO (33, 64, 138), *P. aureofaciens* 30-84 (39), and *P. chlororaphis* isolate SPR044 (216). However, for none of these a reversion to the wild-type phenotype was 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, 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 that the identified deletion 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 plate, can switch back to the phase I phenotype.

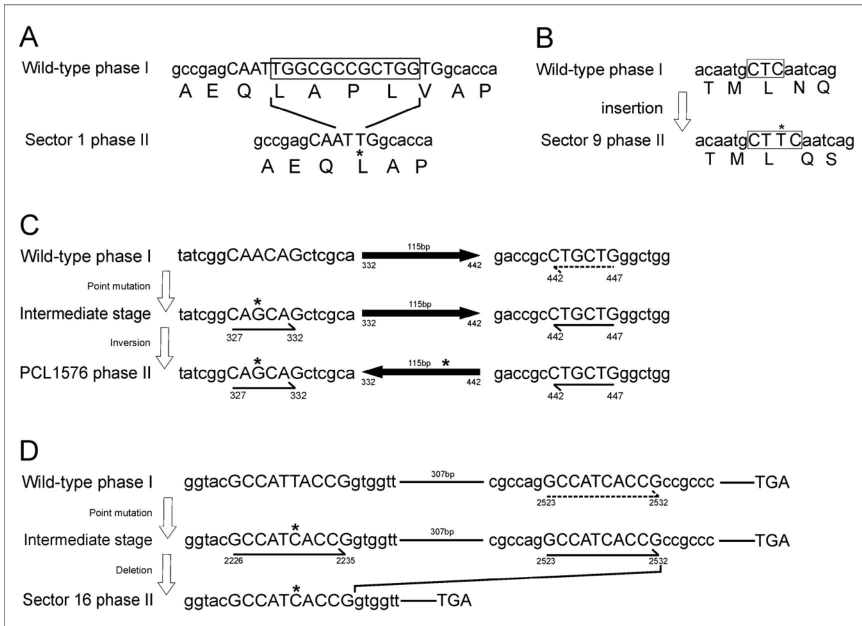


**Figure 2.** Complex mutations identified in *gacS* of *Pseudomonas* sp. PCL1171. Mutations identified in sector 1 (A), sector 9 (B), PCL1576 (C), and sector 16 (D), harbouring a 12 bp deletion, a 1 bp insertion, a 115 bp inversion, and a 307 bp deletion, respectively are shown. Panels A and B show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

Occasionally more stable phase II bacteria can be isolated. The isolation of stable, phase II derivates of which the genetic basis switched between mutations in *gacA*, *gacS* and in both *gacA* and *gacS* (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 occurs either completely, resulting in a switch to the phase I phenotype, or, in the case of a

stable phase II phenotype, mutations cannot be removed, or the removal coincides 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 an increased growth competitiveness, especially under conditions where stationary and exponential growth often switch as may be expected colonies and in the rhizosphere. This explanation is consistent with the suggestion by Schmidt-Eisenlohr et al. (216) 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* sub-population does not replace the wild-type population in culture, as found in PCL1171 shows the effect of the reversibility of the *gac* phenotype and indicates that, at least under lab conditions, there is no direct environmental selection in favor of these mutants.

**What is the mechanism of phase variation in PCL1171?**

According to literature phase variation can regulate gene expression via four mechanisms: (i) slipped-strand mispairing, (ii) differential methylation, (iii) genomic rearrangements, and (iv) random un-programmed variation (27, 98). To our knowledge, only two molecular mechanism of phase variation in *Pseudomonas* sp. have been described. In *P. putida* DOT-T1E the expression of flagella is controlled via slipped-strand mispairing (219), and in *P. tolaasii*, a 661 bp spontaneous, reversible duplication in *pheN*, controls colony morphology and pathogenicity (92). In addition, although no mechanism is described, the *sss* gene, encoding a site specific recombinase, was indicated to play a major role in the phenotypic variation of *P. fluorescens* F113 during root colonisation. The latter variation is combined with a selection for *gac* mutants (209).

Considering the mechanism of phase variation via *gac*, none of the sequenced *gacA/S* genes harbors repeat tracts. Since phase variation in PCL1171 is dependent on mutation of *gac*, it is not epigenetic. Therefore, slipped-strand mispairing and differential methylation can be excluded as the major basis of phase variation via *gacA/S* 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 (206) in mutation of these *gac* genes. But, since these rearrangements only could take place after the occurrence of point mutations, introducing repeats (Fig. 2) which can be recognized by site specific recombinases, we conclude that genomic rearrangements only play a minor, secondary role in phase variation of PCL1171. This 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 (209).

In PCL1171, the accumulation of mutations in *gacA/S* was previously suggested to be suppressed by MutS-dependent mismatch repair since mutation of the *mutS* gene resulted in a dramatic increase of the phase variation frequency (250). Furthermore, based on the mechanism of repair (174), 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/S*. In addition, the spontaneous mutation rate in PCL1171, measured by the frequency of spontaneous rifampicin resistant mutants, increases upon mutation of *mutS* from $5x10^{-7}$ to $3x10^{-5}$ mutants per cell per generation (S. de Weert, personal communication). This frequency is comparable to the frequency of phase variation in the wild-type ($6x10^{-5}$ 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/S*. This observation supports the hypothesis that the *gacA* and *gacS* genes are not hot-spots for mutations. But, under growth limiting conditions micro-organisms can increase their mutation frequency (131, 178). 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 un-programmed variation based on the introduction and restoration of random point mutations, insertions, deletions, and rearrangements in *gacA/S*. Un-programmed variation is not dependent on specific DNA features, and spontaneous duplications and deletions have been described to control for example capsule genes in *Streptococcus pneumonia* (262), the pathogenicity and colony morphology in *P. tolaasii* and *Ralstonia solanacearum* (92, 191), and virulence of *Bordetella bronchiseptica* (175).

Un-programmed 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

mentioned strains has been elucidated. A preliminary study on the reversion of the phase II phenotype in PCL1171 focused at 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 (136) and in some cases in phase variation (98, 135, 168). Mutation of neither *recA* nor *recB* affected the ability of phase II bacteria to switch back to the phase I phenotype. Although this result cannot exclude 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* (225) where RecA was not needed for the removal of the duplication in *pheN* (225).

**Acknowledgements**

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# CHAPTER 4

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

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## ABSTRACT

*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, 1,000- 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 *gacA/S* itself and by *rpoS* in combination with other factors, contributes to the high frequency of mutations accumulating in *gacA/S*. The role of RpoS in the growth advantage of a *gac* mutant was analyzed and mutation of *rpoS* only reduced the length of the lag-phase but did not affect the growth rate, suggesting a role for 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 that increases bacterial fitness and plays a role in niche adaptation (211). Phase variation in *Pseudomonas* sp. PCL1171 is dependent on the accumulation and subsequent removal of mutations in the *gacA* and *gacS* genes (249). These genes affect production of secondary metabolites such as a lipopeptide with antifungal activity, a biosurfactant, and the exo-enzymes chitinase, lipase, and protease (250). The *gacA*/*gacS* two- component regulatory system consists of a membrane-bound sensor kinase GacS and a reponse regulator GacA. The latter protein belongs to the FixJ family of transcriptional regulators (138, 200). The *gac* system regulates secondary metabolism and production of exo-enzymes (111, 138, 198, 215).

In several *Pseudomonas* sp. the *gacA* and *gacS* genes are subject to spontaneous mutation and *gac* mutants appear for example in nutrient-rich liquid medium (33, 63, 250) and on plant roots (39, 216). In PCL1171, spontaneous mutation of the *gacA/S* system is the basis for phase variation. Information on factors influencing the introduction of mutations in the *gac* genes of *Pseudomonas* sp., as part of a phase variation mechanism or phenotypic selection, are scarce.

Previously we have reported that MutS-dependent mismatch repair plays a role in phase variation (249, 250). MutS is involved in methyl-directed recognition of DNA mismatches related to replication, which include base mismatches and small insertion and deletion mispairs (174). Expression of MMR systems can be negatively influenced by stress or growth limiting conditions, resulting in increased genetic and population diversity (131). For example, in *E. coli mutS* is negatively regulated by the general stress response sigma factor RpoS (247). In *Pseudomonas* the regulatory link between RpoS and MutS, and the role of these proteins in bacterial phase variation has 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 phase variation of *Pseudomonas* sp. PCL1171.

## MATERIAL 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) (128) at 28°C. *E. coli* strains were grown in LB (208) at 37°C. Solid growth media contained 1.8% (wt/vol) agar (Difco Laboratories, Detroit). Kanamycin, tetracycline, gentamycin, and carbenicilin (Sigma, St. Louis) were added for antibiotic selection in final concentrations of 50, 40, 10, and 50 µg/ml, respectively. Growth in liquid medium was analyzed in triplicate by measuring the optical density ($OD_{620}$) in time. To determine the numbers of bacteria at the start of growth, samples of the culture were plated and the number of viable cells was established as colony forming units.

**Construction of mutants**
PCL1587 (*rpoS*::$km^r$) was constructed using pMP7418, the suicide plasmid pMP5285 harboring a 0.5 kb PCR product from the *rpoS* gene of *P. chlororaphis* strain PCL1391. pMP7418 was transferred to PCL1171 by parental mating. Mutation of *rpoS* was confirmed by Southern hybridisation (208) using the AlkPhos direct labelling kit for detection (Amersham Biosciences, Buckinghamshire, England) according to the manufacturer's recommendations. Colony morphology was judged after at least two days of growth at 28°C. Parental mating with *E. coli* S17-1 (224), harboring pMP7420 (pBBR1MCS5 harboring $P_{tac}$*rpoS*; G. Girard, unpublished), was used to introduce pMP7420 into PCL1171 and PCL1555, resulting in PCL1585 and PCL1590, respectively.

**Expression studies using a bioluminescent Tn*5luxAB* reporter**
The expression of the *mutS* gene was measured using the promoterless *luxAB* genes (277) inserted behind the *mutS* promoter. Bacteria were inoculated in 20 ml of KB to an optical density ($OD_{620}$) between 0.05 and 0.1, and grown under aeration at 28°C. Growth was monitored by measuring the optical density. To determine gene expression, 100 µl samples were taken in triplicate. A volume of 100 µl of a solution containing 0.2% *n*-decyl-aldehyde (Sigma, St. Louis, MO) and 2.0% bovine serum albumin (Sigma, St. Louis, MO) solution was added. After mixing, bioluminescence was determined in time using a MicroBeta 1450 TriLux luminescence counter (Wallac, Turku, Finland) and normalized to luminescence counts per $OD_{620}$ unit.

**Table 1.** Microbial strains and plasmids

| Strains and plasmids | Characteristics | Reference |
|---|---|---|
| **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). | (250) |
| PCL1555 | Derivative of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a *mutS* homolog; $Km^r$ | (250) |
| PCL1572 | Derivative of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a *gacS* homolog; $Km^r$ | (250) |
| PCL1587 | Derivative of PCL1171 in which a *rpoS* mutation is introduced using pMP7418; $Km^r$ | This study |
| PCL1585 | Derivative of PCL1171 harboring pMP7420; $Gm^r$ | This study |
| PCL1590 | Derivative of PCL1555 harboring pMP7420; $Km^r$, $Gm^r$ | This study |
| S17-1 | *E. coli* MM294, RP4-2 Tc::Mu-Km::T7 chromosomally integrated | (224) |
| **Plasmids** | | |
| pMP5285 | pME3049 derivate, lacking the $Hg^r$ gene, used for single homologous recombination; $Km^r$ | Kuiper et al., un-published data |
| pME6010 | *E. coli* / *Pseudomonas* shuttle vector, stably maintained in *Pseudomonas* species, with an estimated copy number of 4-8; $Tc^r$ | (95) |
| pMP6603 | pME6010 harboring a 1.2 kb PCR product from *Pseudomonas* sp. PCL1446 containing a *gacA* homologue and a 3.2 kb PCR product from *Pseudomonas* sp. PCL1171 containing a *gacS* homologue | (249) |
| pMP7418 | pMP5285 harboring a 0.5 kb PCR fragment of the *rpoS* gene from *P. chlororaphis* PCL1391 | G. Girard un-published data |
| pMP7420 | pBBR1MCS5 harboring a *rpoS* homologue of *P. chlororaphis* PCL1391 expressed under a constitutive *tac* promoter | G. Girard un-published data |

**Quantification of the frequencies of phase variation**

A volume of 20 ml 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 analyzed and the initial number of bacteria was determined. At least 1,500 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 of KB medium were inoculated from colonies on plate and grown under aeration at 28°C. A 5 ml sample was harvested at an $OD_{620}$ of 0.35, and the second sample was taken after 24h. To stop RNA degradation, 0.625 ml of ice-cold EtOH/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 RNeasy mini kit including an on column DNaseI digestion (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's recommendations. Total RNA was stored at -20°C. The amount of isolated RNA was measured at $OD_{480}$ using an Ultrospec 2100pro photospectrometer (Amersham Biosciences, Buckinghamshire, England). Real-time PCR was performed using a real time PCR machine (Lightcycler, Roche GmbH, Penzberg, Germany) on 500 ng of total RNA using the Lightcycler RNA master SYBR Green I (Roche GmbH, Penzberg, Germany) 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 harbors a mutation in the *rpoS* gene, and the *rpoS* over-expressing strain PCL1585 was obtained by the introduction of pMP7420 (pBBR1MCS5-derivative harboring a *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 a *rpoS* mutant of PCL1171 appeared to be of two classes with respect to their rate of reversal to phase I. In the first class only phase I colonies were found after re-plating 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 10-fold, but had no significant effect on the frequency of the reverse switch (Table 2). Complementation of phase II colonies from PCL1585 (PCL1171 $P_{tac}$*rpoS*) with pMP6603 (pME6010 harboring both the *gacA* and *gacS* gene under their own promoters) (249) 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*

Real-time polymerase chain reaction (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 4-fold in stationary phase cells when compared to exponentially growing bacteria (Table 3). In mutant PCL1572 (*gacS*::Tn*5luxAB*), locked in phase II, the expression of rpoS in the exponential phase was approximately

5-fold lower than in phase I wild-type cells whereas only a small induction of *rpoS* expression was observed in the stationary phase as compared to the exponential phase (Table 3).

**Table 2.** Influence of *rpoS* and *mutS* on the frequency of colony phase variation[a]

| Strain | Frequency of phase variation (switch per cell per generation) | |
|---|---|---|
| | Phase I → Phase II | Phase II → Phase I |
| PCL1171 (wildtype) | $6.4x10^{-5}$ | $8.2x10^{-2}$ |
| PCL1587 (*rpoS*::km$^r$) | $3.8x10^{-6}$ | -[b] |
| PCL1585 (PCL1171 $P_{tac}$*rpoS*) | $6.2x10^{-4}$ | $6.9x10^{-2}$ |
| PCL1555 (*mutS*::Tn*5luxAB*) | $8.3x10^{-2}$ | $6.2x10^{-2}$ |
| PCL1590 (PCL1555 $P_{tac}$*rpoS*) | $7.8x10^{-2}$ | $2.0x10^{-2}$ |

[a] The frequencies are shown as the number of switches per cell per generation
[b] Phase II colonies of PCL1587 (*rpoS*::km$^r$) appeared to be of two classes, with respect to their reversal rate to phase I phenotype. One class was locked in the phase II phenotype, while in the other class only phase I colonies were found after re-plating from culture.

**RpoS regulates *mutS***

The effect of a *mutS* mutation on the frequency of switching was analysed using PCL1555 (*mutS*::Tn*5luxAB*). Mutation of *mutS* increased the switching frequency from phase I to phase II approximately 1,000-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 introduction of pMP7420 ($P_{tac}$*rpoS*) in PCL1555 (*mutS*::Tn*5luxAB*). Constitutive expression of *rpoS* did not alter the frequency of switching from phase I to phase II (Table 2).

The expression of the *mutS* gene during growth could be studied in PCL1555 (*mutS*::Tn*5luxAB*) (250) which harbors a Tn*5* transposon with promoterless *luxAB* genes (277). Expression of *mutS* in different growth stages of strain PCL1555 was compared with its expression in its derivate strain PCL1590 harboring pMP4720 (pBBR1MCS5 $P_{tac}$*rpoS*). In PCL1555, the *mutS* gene is highly expressed during the exponential growth phase, but expression was decreased approximately 2-fold upon transition to stationary growth (Fig. 1).

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

| Strain | Relative expression levels in exponential and stationary growth phases[a] | | | | | |
|---|---|---|---|---|---|---|
| | *gacS* | | *rpoS* | | *mutS* | |
| | Exp. | Stat. | Exp. | Stat. | Exp. | Stat. |
| PCL1171 (wildtype) | 1 | 0.62 ±0.2 | 1 | 4.0 ±0.7 | 1 | 0.44 ±0.2 |
| PCL1572 (*gacS*::Tn*5luxAB*) | 0[b] | 0 | 0.20 ±0.1 | 0.30 ±0.2 | 2.7 ±1.4 | 2.5 ±0.5 |
| PCL1587 (*rpoS*::km$^r$) | nd[c] | nd | 0 | 0 | 3.4 ±1.2 | 1.4 ±0.2 |
| PCL1555 (*mutS*::Tn*5luxAB*) | nd | nd | nd | nd | 0 | 0 |

a Expression levels, determined using RT-PCR, are related to the expression level of the indicated gene during exponential growth in the wild-type situation
b No detectable expression
c nd, not determined

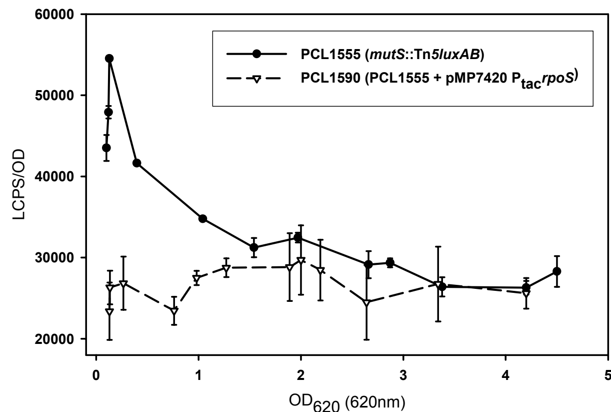


**Figure 1.** Expression of *mutS*, and the influence of constitutive *rpoS* expression, as a function of growth phase. Expression of the *mutS* gene in PCL1555 (*mutS*::Tn*5luxAB*) and in PCL1590 (PCL1555 + P$_{tac}$*rpoS*) was measured using the bioluminescent Tn*5luxAB* reporter in the *mutS* gene. The Y-axis shows the number of luminsescence counts per second (LCPS) divided by the optical density (OD).

In strain PCL1590, in which *rpoS* is 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 2- to 3- fold in the stationary phase, whereas in PCL1555 (*mutS*::Tn*5luxAB*), used as a control, expression was abolished (Table 3). In the *rpoS* mutant PCL1587 (*rpoS*::km$^r$) the expression of *mutS* was 2-to 4-fold increased compared to that of the wild type in the exponential phase (Table 3). In the stationary phase *mutS* expression in PCL1587 (*rpoS*::km$^r$) was 2- to 3- fold reduced compared to expression in the exponential phase (Table 3).

### *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 24h of growth as shown by RT-PCR (Table 3). Expression of *gacS* in PCL1572 (*gacS*::Tn*5luxAB*) was abolished (Table 3). In the *gacS* mutant PCL1572, the expression of *mutS* was 2- to 3-fold increased in both exponential and stationary phases, when compared to the wild type (Table 3).

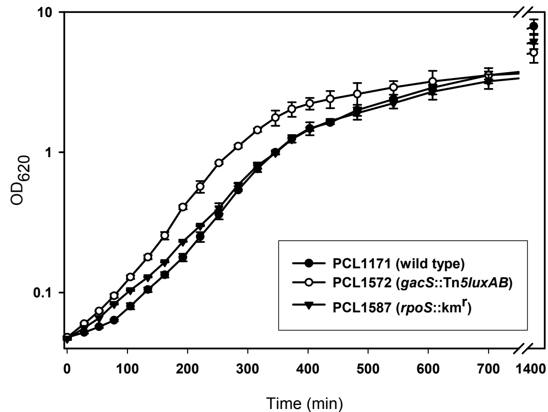


**Figure 2.** Growth of *Pseudomonas* sp. PCL1171 and its derivates PCL1572 (*gacS*::Tn*5luxAB*) and PCL1587 (*rpoS*::km$^r$) in liquid KB-medium.

### Influence of *gacS* and *rpoS* on growth

When the growth of the wild-type strain PCL1171 was compared to that of PCL1572 (*gacS*::Tn*5luxAB*) a reduction in the length of the lag-phase and a decrease in generation time (from 60 ± 4 to 50 ± 4 min. respectively) was

observed for PCL1572, and this mutant reached a lower cell density after 24h 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

Results of a previous paper (250) indicated that MutS-dependent mismatch repair plays a central role in colony phase variation in *Pseudomonas* sp. PCL1171, which occurs via the accumulation of spontaneous reversible mutations in *gacA* and *gacS* (249). The mutations accumulated in the *gacA/S* genes result in a growth advantage under certain conditions (249). To identify genetic factors involved in the accumulation of mutations in the *gacA/S* genes, we analyzed the roles of *gacA/S*, *rpoS* and *mutS* in phase variation of strain PCL1171, by analyzing their effects on the phase variation frequency and expression of each other.

The introduction of pMP7420 ($P_{tac}$*rpoS*) into PCL1171 resulted in a 10-fold 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 (harboring *gacA* and *gacS*), into phase II colonies of PCL1585 (PCL1171 $P_{tac}$*rpoS*), 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 1,000-fold (Table 2). Additional over-expression of *rpoS*, by introducing pMP7420 ($P_{tac}$*rpoS*), 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 (Figs. 1 and 3), whereas mutation of *rpoS* increased the expression of *mutS* (Fig. 3 and 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 (Fig. 3). Considering the role of RpoS as a sigma factor (145) 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 (Fig. 3 and Tables 2 and 3), indicating that levels of MutS-dependent repair are a determinant for the frequency of phase variation.

The effect of mutation of *gac* on *rpoS* expression was analyzed using PCL1572 (*gacS*::Tn*5luxAB*). It appeared that a functional *gacA/S* system is necessary for the expression of *rpoS* (Fig. 3 and 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, was shown for *gac* mutants of *rpoS* expression in *gac* mutants were described for *P. fluorescens* Pf-5 (271), *P. chlororaphis* isolate SPR044 (216), and *P. chlororaphis* O6 (124).

The expression of *mutS* was increased in the *gacS* mutant PCL1572 in both exponential and stationary growing bacteria when 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 (Fig. 3) (85).

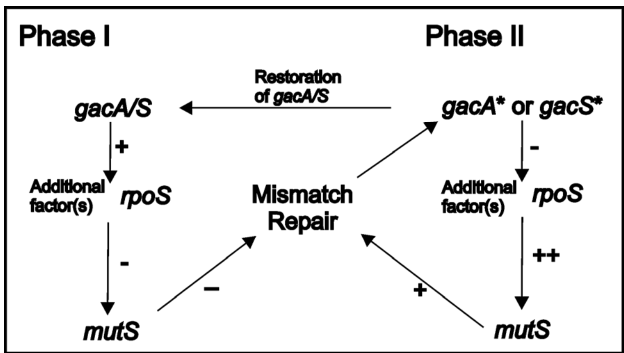


**Figure. 3.** Model for the genetic regulation of spontaneous mutations accumulating in *gac*. A schematic representation of the regulatory roles of GacA/S, RpoS and MutS in phase variation of PCL1171. Phase I cells harbor intact *gacA* and *gacS* genes that are required for expression of *rpoS*, which, in combination with additional factors, in stationary phase negatively regulates *mutS* expression. Inefficient repair of mutations due to down regulation of MutS, results in a decreased repair of spontaneous mutation which will result in the accumulation of mutations in *gacA/S* (indicated with an asterix). As a result the cell switches to the phase II phenotype. Mutation of the *gacA/S* genes decreases *rpoS* expression and a subsequent increase in the expression of *mutS*, thus limiting the mutation rate.

This increase 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 a *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 stationary phase repression of *mutS*. Our results show that the high frequency of mutations accumulating in *gacA/S* is the result of inefficient repair via MutS, as a result of expression of *rpoS*, and possibly, additional, unknown factor(s), which are, in addition, all influenced by the mutation of *gacA/S* itself (Fig. 3).

Since we previously observed a growth effect of the mutation of *gac* (249), and this was for *P. chlororaphis* SPR044 (39) hypothesized to be the result of altered *rpoS* expression, we tested the growth behavior of a *rpoS* mutant. Both in our strain PCL1171 (249) and in *P. chlororaphis* strain SPR044 (216) mutation of the *gacS* gene abolishes *rpoS* expression (Table 3). In PCL1171 mutation of the *rpoS* gene slightly decreased the length of the lag-phase, but not as drastic as observed in a *gac* mutant (Fig. 2). In addition, the generation time decreased when compared to the wild type. The same differences in growth were observed in minimal medium (data not shown). In contrast to the 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 a reduction 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 avoiding nutrient stress in liquid cultures increases the genetic stability of *gacA/S* in *P. fluorescens* (64). 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 re-initiate growth more easily (Fig. 2). The appearance of these mutants under laboratory conditions is likely to be the result of the increased growth competitiveness (249). 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 to revert 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

## Acknowledgements

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# CHAPTER 5

# The extra-cytoplasmic sigma factor PrtI affects phase variation-related colony morphology of *Pseudomonas* sp. PCL1171

Daan van den Broek, Marino Marinkovic, Thomas F.C. Chin-A-Woeng, Guido V. Bloemberg, Ben J.J. Lugtenberg

## ABSTRACT

*Pseudomonas* sp. PCL1171 displays a reversible phase variation between thick opaque phase I and a thin translucent phase II colonies. Spontaneous mutations in the two-component regulatory system formed by *gacA* and *gacS*, results exclusively in phase II colonies. In a screening to identify genes that determine phase I and/or phase II morphology we selected a mutant that shows only phase II morphology but that could not be complemented by *gacA*/*gacS*. Introduction of *gacA*/*gacS* resulted in an intermediate colony morphology, designated as phase I', which is less translucent than phase II, and not opaque like the phase I colonies. Genetic analysis showed that the transposon in this mutant was inserted in a *prtR* homolog, transcriptionally coupled to an upstream *prtI* homolog, encoding a sigma factor. Analysis of cell envelope proteins, lipopolysaccharide, flagella, and extra-cellular polysaccharides (EPS) showed that mutation of *prtR* interfered with EPS production. The phase I' phenotype appeared to be dependent on the absence of negative regulation of PrtI: constitutive expression of *prtI* in the wild-type resulted in a phase I' morphology. PrtI does not affect EPS in a *gacS* or in a *rpoS* background suggesting that these genes play a role in the regulation of this sigma factor. Our results indicate that EPS production in PCL1171 is involved in the determination of the colony morphology, but, by itself is not sufficient to determine the difference between a phase I and phase II morphology.

## INTRODUCTION

*Pseudomonas* sp. PCL1171 displays phase variation between a thin translucent phase II and a thick opaque phase I. In addition to the morphological difference, secondary metabolites such as an anti-fungal factor, biosurfactant and exo-enzymes such as protease, lipase and chitinase are only expressed in the phase I phenotype. Previously, we showed that phase variation of PCL1171 is dependent on spontaneous, reversible mutations in *gacA*/*gacS* (250). The *gacA*/*gacS* two-component regulatory system consists of a cognate sensor kinase GacS and a reponse regulator GacA, the latter protein belongs to the FixJ family of transcriptional regulators (138, 200) which regulate secondary metabolism and the production exo-enzymes (24, 111, 138, 215).
The biochemical basis of colony morphology in bacterial species can be diverse. For example, expression of an extra-cellular polysaccharide (EPS) matrix determines colony morphology in *Vibrio cholerae* (195), *V. parahaemolyticus* (75), and in *P. aeruginosa* (87). Furthermore, the composition of the bacterial capsule polysaccharides (79, 176) and outer membrane proteins (141) can influence the colony morphology.
Phase variation is often described to result in morphologically distinct colonies (72, 98). In several *Pseudomonas* species colony morphology is affected by mutation of *gac* (92, 209, 250). Phase variation via *gac* is, to our knowledge, only described in *Pseudomonas* sp. PCL1171 (249, 250) and for a homolog of *gacS* in *Pseudomonas tolaasii* (92). In these strains, no genes, except *gacA*/*gacS*, have been identified which affect colony morphology.
In the present study a phase II locked transposon mutant was identified that could not be complemented by *gacA*/*gacS*. Here we describe our attempts to identify genes and traits other than *gacA*/*gacS* involved in determining the colony morphology of PCL1171.

## MATERIAL 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) (128) at 28°C. Solid growth media contained 1.8% (wt/vol) agar (Difco Laboratories, Detroit). Kanamycin, tetracycline,

gentamycin and carbenicillin (Sigma, St. Louis) were added for antibiotic selection in final concentrations of 50, 40, 10 and 50 µg/ml, respectively.

**Table 1.** Microbial strains and plasmids

| Strains and plasmids | Characteristics | Reference |
|---|---|---|
| **Bacterial strains** | | |
| PCL1171 | Antagonistic *Pseudomonas* strain isolated from the rhizosphere of maize from Mexican agricultural fields. Colony morphology varies between two distinct phases, defined as phases I (opaque) and II (translucent). | (250) |
| PCL1578 | Derivate of PCL1171 harboring pMP6611 ($P_{tac}$*prtI*), $Gm^r$ | This study |
| PCL1580 | Derivate of PCL1588 (*prtR*::Tn*5luxAB*) harboring pMP6611 ($P_{tac}$*prtI*), $Km^r$ $Gm^r$ | This study |
| PCL1579 | Derivate of PCL1572 (*gacS*::Tn*5luxAB*) harboring pMP6611 ($P_{tac}$*prtI*), $Km^r$ $Gm^r$ | This study |
| PCL1581 | Derivate of PCL1587 (*rpoS*::$km^r$) harboring pMP6611 ($P_{tac}$*prtI*), $Km^r$ $Gm^r$ | This study |
| PCL1588 | Derivate of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a *prtR* homolog, $Km^r$ | This study |
| PCL1572 | Derivative of PCL1171 in which a promoterless Tn*5luxAB* transposon is inserted into a *gacS* homolog, $Km^r$ | (250) |
| PCL1587 | Derivative of PCL1171 in which a *rpoS* mutation is introduced using pMP7418, $Km^r$ | van den Broek et al., *In Press* |
| PCL1591 | Derivate of PCL1171 in which a *prtI* mutation is introduced using pMP6606, $Km^r$ | This study |
| PCL1597 | Derivate of PCL1588 (*prtR*::Tn*5luxAB*) harboring pMP6603 (*gacA*/*gacS*), $Km^r$, $Tc^r$ | This study |
| S17-1 | *E. coli* MM294, RP4-2 Tc::Mu-Km::T7 chromosomally integrated | (224) |

**Table 1.** *Continued*

| plasmids | Characteristics | Reference |
|---|---|---|
| pMP5285 | pME3049 derivate, lacking the Hg$^r$ gene, used for single homologous recombination, Km$^r$ | Kuiper et al., unpublished data |
| pME6010 | *E. coli* / *Pseudomonas* shuttle vector, stably maintained in *Pseudomonas* species, with an estimated copy number of 4-8, Tc$^r$ | (95) |
| pMP6603 | pME6010 harboring a 1.2 kb PCR product from *Pseudomonas* sp. PCL1446 containing a *gacA* homolog and a 3.2 kb PCR product from *Pseudomonas* sp. PCL1171 containing a *gacS* homolog. | (249) |
| pMP6609 | Suicide construct pMP5285 harboring a 200 bp *Eco*RI fragment from the *prtI* gene | This study |
| pMP6610 | Suicide construct pMP5285 harboring a 700 bp *Eco*RI fragment from the *alga* gene | |
| pMP6611 | pBBRMCS5 harboring the complete *prtI* gene under a constitutive *tac* promoter | This study |
| pMP7420 | pBBRMCS5 harboring a *rpoS* homologue of *P. chlororaphis* PCL1391 expressed under a constitutive *tac* promoter | G. Girard et al., submitted |

**Isolation and characterization of PCL1588 (*prtR*::Tn*5luxAB*)**

A mutant library of strain PCL1171 was constructed using plasmid pRL1063a (250), which harbors a Tn*5* transposon with promoterless *luxAB* genes and a kanamycin resistence marker (277). Mutants expressing the phase II phenotype were re-plated several times and grown in liquid medium to select those mutants locked in the phase II phenotype. DNA regions flanking the transposon were isolated by recovering the transposon with its flanking regions from the chromosomal DNA of the transposants using *Eco*RI or *Cla*I, followed by ligation and transformation to *E. coli* strain DH5α. Since the Tn*5* transposon harbors an origin of replication (p15A), the plasmid can replicate and maintain itself in *E. coli*. The flanking chromosomal regions were sequenced using primers oMP458 (5'-TACTAGATTCAATGCTATCAATTGAG-3') and oMP459 (5'-AGGAGGTCACATGGAATATCAGAT-3') directed outwards of

the transposon ends. Sequencing was carried out by ServiceXS or BaseClear (Leiden, The Netherlands). General DNA modification techniques were performed according to Sambrook et al. (208).

**Analysis of cell envelope proteins and lipopolysaccharides**

To analyse lipopolysacharide (LPS) and membrane protein patterns, cells of different phases were separately harvested from plates after two days of growth at 28°C and resuspended in 50 mM Tris/HCl - 2 mM EDTA, pH 8.5. Cell suspensions were sonicated and centrifuged for 20 minutes at 2,700 rpm. The resulting supernatant fluid was centrifuged for one hour at 10,000 rpm. The obtained pellets were resuspended and stored in CE (cell envelope)-buffer (2 mM Tris/HCl, pH 7.8). To visualize LPS patterns, the cell envelope preparation was incubated for 15 min at 100°C in 125 mM Tris/HCl, pH 6.8 - 4.0% SDS – 20% glycerol - 0.02% bromophenol blue, followed by a proteinase K (Sigma, St. Louis) treatment. The LPS fractions were separated in a denaturing 11% SDS-PAGE gel using a Mini-ProteanTM 3 Cell system (BioRad lab., Richmond, CA). The LPS pattern was visualized by silver staining (246). Cell envelope proteins were denatured by adding β-mercaptoethanol to the cell envelope mixture to a final concentration of 0.1%, followed by incubation for 10 minutes at 100°C. Proteins were separated on a 11% SDS-PAGE denaturing gel using a Mini-ProteanTM 3 Cell system (BioRad lab. Richmond CA) and visualized using Coomassie-Blue staining (208).

**Analysis of extra-cellular polysaccharides**

Extra-cellular polysaccharides were isolated following the protocol of Enos-Berlage et al. (75). Briefly, bacteria were grown on solid medium for 72 hours at 28°C and the distinct morphological colony phases were harvested and resuspended separately in 4.5 ml PBS. After vigorous shaking, the samples were incubated under shaking at 28°C for two times 1.5 hours. Samples were spun down, and the supernatant was treated by proteinase K (200 µg/ml) for 1.5 hour at 56°C. Four and a half ml of a 1:1 (v/v) phenol-chloroform mixture was added and the precipitate was spun down. This step was repeated, after which 2.5 volume of ethanol was added to the supernatant to precipitate the EPS. The pellet was washed using 70% ethanol, and resuspended in MilliQ. The phenol-sulfuric acid method of Dubois et al. (62) was used to quantify the amount of sugar. The EPS was visualized using a denaturing 11% SDS-PAGE gel, with a

5% stacking gel and visualized using a silver staining (246) (Biorad Lab., Richmond, CA).

**Construction of pMP6611 (pBBR1MCS5 $P_{tac}$*prtI*)**

The chromosomal regions flanking the Tn*5luxAB* transposon of PCL1588 after reisolation of the transposon (see above) were used as a template to amplify *prtI* by polymerase chain reaction (PCR). The primers oMP833 (5'-TTCGCTGGGGGCAGGCTGATCATT-3') and oMP822 (5'-TT*GACA*ATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGA TAACAATTTCACACAGGAAACAGCTAAATGCATGAACTCGACGAA CAGTTACGTGAACTCATCCCCAGGTTGCGGCGTTTTGCC-3') used for *prtI* amplification, harbored the *tac* promoter sequence directly in front of the ATG of *prtI*. The 600 bp PCR product was inserted into the pGEM-TEasy Vector (Promega Crop., Madison, WI), and subsequently cloned into pBBR-MCS5 using *Apa*I and *Sac*I resulting in pMP6611. The PCR fragment was sequenced to exclude mutations occurring during the PCR process. Subsequently, pMP6611 was transferred to S17-1 by electroporation and the resulting strain was used to transfer pMP6611 by parental mating to *Pseudomonas*.

**Construction of mutants**

PCL1591 (*prtI*::$km^r$) was constructed by homologous recombination using pMP6609, which consists of a 200 bp *Eco*RI *prtI* fragment from the PCR product resulting from primers oMP802 (5'-GTGAACTCATCCCCAGGTTG-3') and oMP803 (5'-ATGTCGGCGACTTCCTTGTA-3') cloned into the suicide vector pMP5285. PCL1594 (*algA*::$km^r$) was constructed using pMP6610, which consists of a 700 bp *Eco*RI, *algA* fragment from the PCR product of oMP830 (5'-ATGTTCCTGTTCCGCGCCAGCCGCT(AT)CCT-3') and oMP831 (5'-AGCGGCCGCCAATGTCCACCGAGTCGTA-3') cloned into pMP5285. The plasmids pMP6609 and pMP6610 were transferred to S17-1 and introduced into PCL1171 by parental mating. Correct incorporation into the chromosome was checked by Southern hybridization.

## RESULTS

### Isolation and characterization of a stable PCL1171 phase II Tn*5luxAB* mutant

PCL1588 was isolated from a Tn*5luxAB* transposon library of PCL1171 as a mutant expressing a phase II phenotype. In contrast to previously isolated mutants (249), transformation of PCL1588 with pMP5565 (*gacA*), pMP6562 (*gacS*) or pMP6603 (*gacA* and *gacS*) resulted in an intermediate morphology of phases I and II, further referred to as phase I'. In the non-transformed PCL1588, phase I' sectors were observed after growth for several days. The phase I' morphology is less translucent than that of phase II (Fig. 1B), but morphologically also different from wild-type phase I colonies (Fig. 1A) since the colonies are not opaque (Fig. 1C). Phenotypic characterization of PCL1588 showed that this mutant had neither lost its anti-fungal activity, nor its capacity to produce biosurfactant, protease, or lipase, all of which are characteristic for the phase I phenotype. So apparently only its colony morphology was affected.

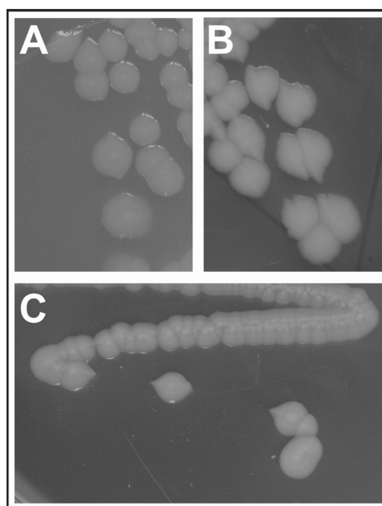


**Figure 1.** Colony morphologies of wild-type strain PCL1171 and its derivate phase I'. The colony morphologies of the wild-type phase I (Panel A), the wild-type phase II (Panel B) is shown, and the intermediate phase I' (Panel C) are shown.

Re-isolation of the Tn*5luxAB* transposon and subsequent sequencing of the chromosomal regions flanking the transposon showed that the transposon had inserted in an ORF which encodes a protein showing highest homology to the *prtR* gene product (73% identity and 78% similarity at the amino acid level) of *P. fluorescens* (Genbank accession number AAF81073). Using the Entrez conserved domains database (159) the predicted protein was shown to align

with domains of predicted anti-sigma factors (COG5662, COG03806, and COG3712). Therefore it seems reasonable to predict that in PCL1171 the identified open reading frame encodes a transmembrane anti-sigma factor (34). Sequence analysis of the region downstream of this open reading frame revealed another open reading frame, transcribed in the opposite direction and encoding a protein showing highest homology to a hypothetical protein (73% identity and 80% similarity at amino acid level) of *P. fluorescens* (Genbank accession number AAF81076). Sequence analysis of the region upstream of the *prtR* gene identified an open reading frame transcribed in the same direction, showing highest homology (83% identity and 86% similarity at amino acid level) to the *prtI* gene product of *P. fluorescens* (Genbank accession number AAF81076). Using the Entrez conserved domains database (159) the protein aligned with RpoE (COG1595), a well known ECF sigma factor in *E. coli* required for high temperature survival (112). In addition it aligned with a sigma 70-r2 and r4 domain (pfam04542 and pfam04545) involved in the binding of the -10 and -35 promoter recognition sites. Therefore it seems reasonable to predict that in PCL1171 the identified open reading frame encodes an extracytoplasmic-function (ECF) sigma factor (34). The region upstream of the *prtI* gene (approximately 1 kb) did not show any substantial ORF and/or amino acid similarity to data base entries. Sequence analysis showed, similar to other known ECF sigma factors and their regulators (112), that the stop codon of the *prtI* was overlapping with the start codon of the *prtR*, indicating that transcription of the *prtI* and *prtR* genes in PCL1171 is coupled (Fig. 2).

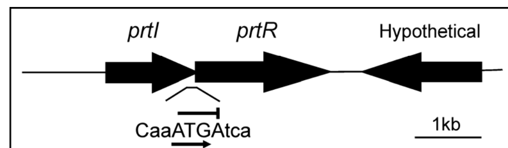


**Figure 2.** Genomic organization of the *prtI/R* operon in *Pseudomonas* strain PCL1171.

After prolonged growth of PCL1588 (*prtR*::Tn*5luxAB*) in the absence of kanamycin, phase I sectors appeared which had grown out of phase II and phase I' colonies. Re-plating of these phase I sectors on plates containing kanamycin showed that these bacteria had lost their kanamycin resistence. Re-plating on plates without antibiotic showed growth of bacteria with a phase I morphology indistinguishable to that of the wild-type strain. No kanamycin resistant phase I sectors could be isolated. We conclude that spontaneous removal of the

transposon coincided with a switch back to a phase I phenotype, showing that the phase II phenotype of PCL1588 (*prtR*::Tn*5luxAB*) is indeed dependent on mutation of *prtR*.

**Effect of *prtR* on cell surface traits**

To study the role of *prtR* in phase I and phase II colony morphology, cell-envelope proteins, outer membrane proteins, lipopolysaccharide (LPS) ladder patterns, flagella, and production of EPS were analyzed. Previously, analysis of cell envelope proteins expressed in phase I and phase II morphologies had shown similar patterns with small differences in the amounts of a number of proteins produced (250). Mutation of the *prtR* gene in PCL1588 (*prtR*::Tn*5luxAB*) did not appear to have a detectable effect on the cell envelope protein pattern (data not shown) compared to those of phase I and phase II wild-type cells. Furthermore, analysis of LPS ladder patterns and production of flagella did not show detectable differences between wild-type phase I, wild-type phase II, PCL1572 (*gacS*::Tn*5luxAB*) and PCL1588 (*prtR*::Tn*5luxAB*). Analysis of the EPS showed that the EPS of PCL1588 (*prtR*::Tn*5luxAB*) (Fig. 3, lane 3) differed from the EPS isolated from wild-type phase I (Fig. 3, lane 1), wild-type phase II cells (data not shown), and PCL1572 (*gacS*::Tn*5luxAB*) (Fig. 3, lane 2). The EPS of PCL1588 was only present as smaller fragments in the lower part of the SDS-PAGE gel (Fig. 3, lane 3). Introduction of pMP6603 (*gacA*/*gacS*) into PCL1588 (*prtR*::Tn*5luxAB*), resulted in strain PCL1597 which expressed a phase I' morphology. The EPS isolated from PCL1597 (Fig. 3, lane 5) was indistinguishable from that of the wild-type (Fig. 3, lane 1).

**Characterization of a *prtI* mutant**

Mutation of the *prtI* gene using pMP6609 resulted in strain PCL1591 (*prtI*::$km^r$). This strain expresses phase I morphology and produces antifungal activity, biosurfactant, proteases, and lipase. In addition, the EPS isolated from PCL1591 (*prtI*::$km^r$) (Fig. 3, lane 9) did not differ from the EPS isolated from wild-type phase I bacteria (Fig. 3, lane 1).

To study the role of PrtI in the phenotype of PCL1588, we constructed pMP6611, a pBBR1MCS5 vector harboring the *prtI* gene under a constitutive tac promoter. pMP6611 ($P_{tac}$*prtI*) was introduced into PCL1171 (wild-type phase I) and PCL1591 (*prtI*::$km^r$). These derivates appeared to express a phase I' morphology, whereas introduction of the empty parental vector pBBR1MCS5 did not alter the phase I phenotype of these strains. After prolonged growth of PCL1578 (PCL1171 $P_{tac}$*prtI*) it appeared that the phenotype was not stable since

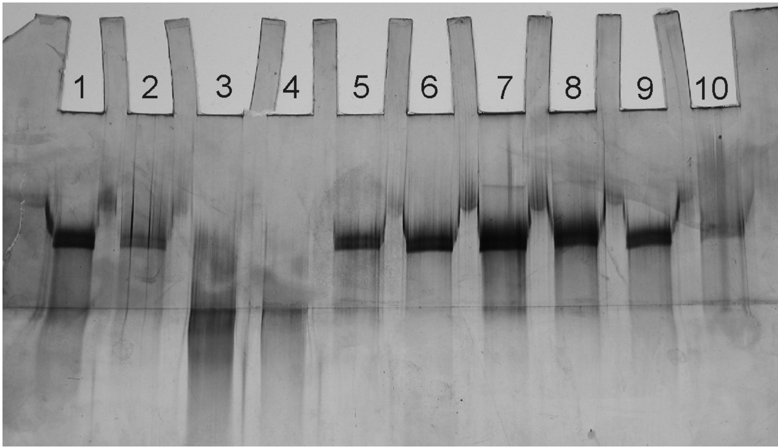


**Figure 3.** Analysis of EPS using SDS-PAGE. The EPS of (1) the wild-type strain PCL1171 with a phase I morphology was compared with these of (2) the stable pShase II mutant PCL1572 (*gacS*::Tn*5luxAB*) and (3) PCL1588 (*prtR*::Tn*5luxAB*). In addition, the EPS's from (4) PCL1580 (PCL1588 (*prtR*::Tn*5luxAB*) + $P_{tac}$*prtI*), (5) PCL1588 (*prtR*::Tn*5luxAB*) + pMP6603 (*gacA*/*gacS*), (6) PCL1588 (*prtR*::Tn*5luxAB*) + $P_{tac}$*prtI* + *gacA*/*gacS*, (7) PCL1578 (wild-type strain PCL1171 + $P_{tac}$*prtI*), (8) wild-type strain PCL1171 + $P_{tac}$*prtI* + pMP6603 (*gacA*/*gacS*), (9) PCL1591 (*prtI*::km$^r$), and (10) PCL1579 (PCL1572 (*gacS*::Tn*5luxAB*) + $P_{tac}$*prtI*), were analyzed.

phase I sectors were found growing out of the phase I' colonies. This was also observed upon the introduction of pMP6603 (*gacA*/*gacS*) and of the parental vector pME6010 into PCL1578 (PCL1171 $P_{tac}$*prtI*), in all cases the result was a mixture of phase I, phase II and phase I' colonies.

The introduction of pMP6611 ($P_{tac}$*prtI*) into PCL1588 (*prtR*::Tn*5luxAB*), resulting in strain PCL1580 (PCL1588 + $P_{tac}$*prtI*), did not restore the wild-type EPS pattern (Fig. 3, lane 3). However, the subsequent introduction of pMP6603 (*gacA*/*gacS*) resulted in a phase I' phenotype and the restoration of the wild-type EPS pattern (Fig. 3, lane 5 and 6).

**Effect of constitutive expression of *prtI* and *rpoS***

To study the regulation of *prtI/R*, pMP6611 ($P_{tac}$*prtI*) was introduced into the *gacS* mutant PCL1572 (stable phase II) and the rpoS mutant PCL1578 (expressing a phase I morphology). This resulted in strains PCL1579

(*gacS*::Tn*5luxAB* + $P_{tac}$*prtI*) and PCL1581 (*rpoS*::km$^{r}$ + $P_{tac}$*prtI*), respectively. The phase II morphology was not affected by the constitutive expression of the prtI gene in PCL1572 (*gacS*::Tn*5luxAB*). The subsequent introduction of pMP6603 (*gacA*/*gacS*) into strain PCL1579 (*gacS*::Tn*5luxAB* + $P_{tac}$*prtI*) resulted in a mixture of phase I and phase I' colonies. Introduction of the empty vector, pMP6010, did not alter the phase II morphology of PCL1579 (*gacS*::Tn*5luxAB* + $P_{tac}$*prtI*). The EPS isolated from PCL1579 (*gacS*::Tn*5luxAB* + $P_{tac}$*prtI*) (Fig. 3, lane 10) was not altered when compared to that of PCL1572 (*gacS*::Tn*5luxAB*) (Fig. 3, lane 2).
Similarly, the phase I morphology of PCL1587 (*rpoS*::km$^{r}$) was not affected by the constitutive expression of the prtI gene. In a second approach pMP7420 (pBBR1MCS5 $P_{tac}$*rpoS*) was introduced into PCL1588 (*prtR*::Tn*5luxAB*). This derivate appeared to have a phase I' phenotype.

## DISCUSSION

Phase variation in *Pseudomonas* sp. PCL1171, observed as a switch in colony morphology, is based on the spontaneous mutation of *gacA* and/or *gacS* and subsequent restoration of the wild-type sequence. It regulates secondary metabolism and exo-enzyme production (249, 250). A Tn*5luxAB* mutant, PCL1588 was isolated which (i) expresses phase II morphology, and (ii) expresses a phase I' morphology upon the introduction of *gacA*/*gacS* (in contrast to other phase II mutants in which *gacA*/*gacS* restores a phase I morphology), and (iii) produces secondary metabolites and exo-enzymes which is normally correlated with the phase I phenotype. Based on these observations we hoped to identify a gene determining colony morphology.

Sequence analysis of this mutant showed that the Tn*5* transposon was inserted in a *prtR* homolog. The transposon was lost spontaneously, which correlated with a switch back to phase I morphology. Sequencing showed that the *prtR* gene is part of an operon, with an upstream gene showing homology to the *prtI* gene (Fig. 2). The same genetic organisation is present in *P. fluorescens* LS107d2 (34) (Genbank acc. no. AF228766) and *P. putida* KT2440 (Genbank acc. no. NP_745033 and NP_745032).

Transmembrane anti-sigma factors, such as *prtR*, interact with extra-cytoplasmic function (ECF) sigma factors of the $\sigma^{70}$ family (34). A membrane-localized anti-sigma factor functions as a sensor and signaling molecule

allowing an adaptive response to specific environmental changes (173). These changes include temperature, heat shock, ion levels, oxidation, but the sigma factors are also activated upon mutations affecting multidrug efflux pumps or misfolding of outer membrane proteins (194). When PrtR is not induced, the ECF sigma factor is bound to the anti-sigma factor, located at the inner membrane. Upon induction the sigma factor is released, and will associate with RNA polymerase core enzymes and thereby regulate the transcription of genes to cope with the sensed stress (194). ECF sigma factors identified in *Pseudomonas* include PrtI (34), PvdS (276), AlgU (278), and FecI (162), which regulate the synthesis of protease, pyoverdine, and alginate, and iron acquisition (194), respectively. Sequence analysis and the presence of conserved domains indicate that in PCL1171 the *prtR* and *prtI* homologs encode an anti-sigma factor and an ECF sigma factor, respectively.

Analysis of the regulatory relation between PrtR and PrtI indicates that the phenotype of PCL1588 is dependent on the absence of negative regulation of PrtI. Based on (i) the characteristics of and homology to known anti-sigma factors, and the observations that (ii) the mutation of the *prtI* gene did not alter the phase I morphology, (iii) the introduction of pMP6611 ($P_{tac}$*prtI*) altered the colony morphology of the wild-type strain from a phase I to a phase I' morphology, and (iv) the introduction of pMP6611 into PCL1588 did not affect the phenotype, including the altered EPS, of this mutant, we hypothesize that PrtR is negatively regulating PrtI. Furthermore, the sigma factor PrtI plays a role in the phase I and phase II colony morphology of PCL1171, via a direct or indirect effect on the synthesis or secretion of EPS in PCL1171. Interestingly, no differences in the EPS pattern were detected when the pattern obtained from wild-type phase I colonies was compared with the EPS isolated wild-type phase II colonies or from the *gacS* mutant PCL1572. This indicates that EPS alone is not responsible for the difference in colony morphology between phase I and phase II. In addition, the introduction of pMP6603 (*gacA*/*gacS*) into PCL1588 resulted in a phase I' morphology and restored a wild-type EPS pattern. We hypothesize that regulation of PrtI, in addition to PrtR, could account for these results. Therefore we studied the regulatory relationship between GacS, RpoS, and PrtI. In contrast to changes caused by the introduction of pMP6611 into the wild-type, the phase II morphology of PCL1572 (*gacS*::Tn*5luxAB*), and the phase I morphology of PCL1587 (*rpoS*::km$^r$), was not altered by pMP6611.

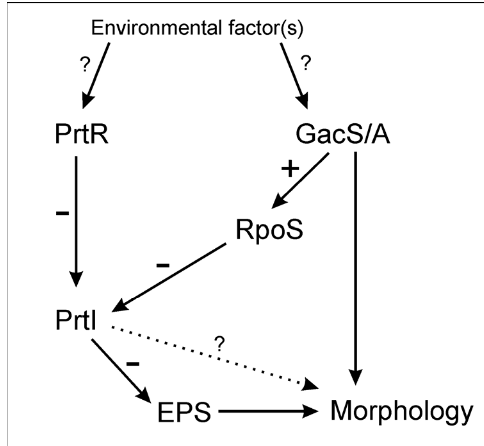


**Figure 4.** Work hypothesis for the regulation of colony morphology of PCL1171. Phase colony morphology of PCL1171 is based on spontaneous mutation of *gacA/S*, resulting in the phase II morphology. In addition EPS is regulated via a membrane bound anti-sigma factor PrtR and its ECF sigma factor PrtI. In the absence of regulation of PrtI via PrtR or RpoS the EPS is altered (Fig. 3). But since the EPS pattern is not always correlated with the expression of a phase I morphology but results in an intermediate phase I' morphology the colony morphology is likely to be dependent on other PrtI or GacA/S regulated factors in combination with EPS.

This shows that GacS and/or RpoS play a role in the regulation of PrtI. Based on these observations and since (i) the introduction of pMP7420 ($P_{tac}$*rpoS*) into PCL1588 restored a phase I' morphology, and (ii) the fact that in *Pseudomonas gacA/S* is needed for the expression of *rpoS* (249), we hypothesize that the regulation of PrtI is most likely influenced by RpoS. Our results indicate that the PrtI is not only regulated via PrtR, but that the presence of RpoS also prevents the activity of PrtI. This is supported by the observation that the EPS is only affected in the combination of a *prtR* mutation and additional mutations in *gac*, illustrated by the restoration of the EPS pattern in PCL1588 (Fig 3. lanes 5 and 6) after introduction of pMP6603.

Our results show that the restoration of the EPS pattern is not always correlated with the expression of a phase I morphology, but results in a phase I' morphology. Therefore we hypothesize that PrtR and PrtI are regulating additional factors involved in colony morphology of PCL1171. This hypothesis implies that the phase I colony morphology of PCL1171 is not dependent on a single factor such as EPS, but on multiple factors (Fig. 4).

## ACKNOWLEDGEMENTS

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# CHAPTER 6

# Summary and general discussion

## 1.1 Introduction

Plants secrete a variety of compounds into the soil, making the rhizosphere a relatively rich nutritional environment when compared to the surrounding soil (150, 153). As a result, the rhizosphere is inhabited by a wide range of organisms including protozoa, nematodes, mycorrhizae, bacteria, and fungi. These organisms present in the rhizosphere are facing a heterogeneous and competitive environment. As a result, micro-organisms have developed traits to improve their success in the rhizosphere. Examples of such competitive traits are increased growth rates (241), efficient root colonisation (43), and the production of secondary metabolites and/or lytic exo-enzymes aimed at limiting growth of other species (58, 147). Micro-organisms can thereby suppress the propagation of phytopathogens in the rhizosphere, and as a result control plant diseases (45, 241).

In modern agriculture plant diseases are mainly suppressed by the application of chemical pesticides. Many of these chemicals are difficult to degrade and therefore maintained in the environment for long periods. Some of these are harmful and toxic to humans and other non-target organisms. This has led to the political decision, on national and international (e.g. EU) level, to limit the input of these chemicals into the environment. This measure increased the demand for alternatives. The use of micro-organisms to control phytopathogens is a promising and environmental friendly alternative for the use of chemical pesticides. At this moment, worldwide hundreds of commercial products, based on micro-organisms, are available for the control of plant diseases. But, the commercial application of this alternative is still limited, due to variable results under practical conditions. To improve the performance of biocontrol inoculants it is essential to understand mechanisms or processes affecting the efficiency and reliability of these bacteria as biocontrol agents.

One of the factors which affects biocontrol is a site-specific recombinase (56, 209), encoded by the *sss* gene. Mutation of *sss* severely decreased the occurrence of morphological variants and the root colonisation ability of *P. fluorescens* WCS365 (56) while this mutation in *P. chlororaphis* PCL1391 abolished the biocontrol ability of this strain (43). In addition, the *sss* gene was indicated to play a major role in the phenotypic variation of *P. fluorescens* F113 during root colonization although a genetic mechanism was not described (209). The involvement of a site specific recombinase in these processes suggested that mechanisms including DNA alterations could influence root colonisation and therefore the performance of biocontrol agents. The characterization of a set

of antagonistic pseudomonads indicated that colony phase variation frequently occurs in rhizobacteria (Chapter 2). Phase variation is defined as a process of reversible, high-frequency phenotypic switching that is mediated by DNA mutation, reorganisation or modification. Most known examples of phase variation are described in the context of host-pathogen interactions (Chapter 1), but phase variation occurs in various environments and can affect a variety of processes. It is, for example, used by bacteria to generate population diversity in order to increase fitness and it is important in niche colonization and adaptation, including immune evasion (211). Since phase variation is a strategy of pathogenic micro-organisms to evade host defense systems it poses a problem in vaccine production (163). In addition, in industrial production processes of microbial molecules, yields can be negatively influenced by the formation of non-producing sub-populations. Understanding a process such as phase variation can be an essential step towards controlling or limiting the effect of such a mechanism on medical, industrial and ecological processes, in this case the control of phytopathogens. Therefore, the aim of this thesis was to study the mechanism of phase variation in antagonistic *Pseudomonas* bacteria in relation to efficient biocontrol of phytopathogens.

The results described in this thesis contribute to the understanding of a molecular mechanism and the regulation of phase variation in *Pseudomonas* bacteria. A novel example of un-programmed phase variation is described, based on spontaneous mutation of the global regulatory *gacA* and *gacS* genes, which affects the major biocontrol traits of *Pseudomonas* PCL1171. This mechanism of phase variation affected the biocontrol activity of PCL1171, but also seems to serve as a mechanism to improve fitness and success in a competitive environment such as the rhizosphere.

### 1.2 Influence of phase variation on biocontrol

Some agricultural fields in Tontotepec, Oaxaca Mixe, Mexico have a history of over 700 years of sustainable agriculture. Despite the humid climate, which should be beneficial for the growth of (phytopathogenic) fungi, excellent crop yields can be obtained from these fields without the application of chemicals. A possible explanation for the natural suppressivenes of these fields was provided by the isolation of a high percentage of *Pseudomonas* strains, from maize roots, which are antagonistic against important phytopathogenic fungi (21 % of the isolated strains). Almost all of these antagonists display colony phase variation.

Two distinct colony morphologies could be observed on plates, namely a thick opaque phase I and a thin translucent phase II (Chapter 2). The phenotypic characterization of a phase I and phase II phenotype showed that the production of secondary metabolites and exo-enzymes is correlated with a phase I phenotype (Chapter 2). The production of secondary metabolites and exo-enzymes by PCL1171 phase I cells resulted in a significant suppression take-all of wheat, caused by *Gaeumannomyces graminis* pv. *tritici*. Seed inoculation using PCL1171 phase II cells, or with cells of a PCL1171 mutant derivative with a higher frequency of phase variation, did not result in biocontrol activity (Chapter 2).

The antagonistic activity of PCL1171, both on plate and in a bioassay, was shown to be dependent on the expression of an antifungal metabolite. This metabolite is presumably a lipopeptide since (i) mutation of a homolog of a lipopeptide synthase abolished the antagonistic activity, while maintaining the phase I phenotype, and (ii) lipopeptides have been described to have anti-fungal activity in *Pseudomonas fluorescens* DR54 (242) (Chapter 2). We conclude that the expression of a lipopeptide is a prerequisite for biocontrol activity in PCL1171 and that the absence of this lipopeptide in the phase II phenotype is the main reason for a lack of biocontrol activity of this phenotype (Chapter 2).

The structural basis of the phase I and phase II colony morphology in *Pseudomonas* PCL1171 is described in Chapter 5. In a number of other *Pseudomonas* species colony morphology is regulated by phase variation. For example, phase variation of *P. tolaasii* results in smooth and rough colonies (92), while in *P. fluorescens* F113 (209), and *Pseudomonas brassicacearum* (38) translucent and opaque colonies can be isolated. The data presented suggests a role for EPS in the colony morphology of PCL1171. The EPS was affected by the expression of an extra-cellular sigma factor PrtI, of which the effect was influenced by Gac and RpoS, and its membrane bound anti-sigma factor PrtR. Based on the results the phase I colony morphology of PCL1171 is not determined by a single factor such as EPS, but probably by multiple factors (Chapter 5).

In conclusion, phase variation plays a role in efficient control of take-all of wheat and therefore could affect the reliability of a biocontrol strain. This was illustrated by the observation that the application of phase I cells suppressed the effect of *G. graminis* pv. *tritici* on wheat, in contrast to phase II cells or a derivate with a high frequency of variation. Phase variation, associated with the production of anti-fungal metabolites and secondary metabolism,

affecting the control of pathogenic fungi has not been described before. Our findings show that phase variation should be considered in a broader ecological context, affecting for example a process such as biocontrol and the production of secondary metabolites and exo-enzymes.

## 1.3 The molecular mechanism of phase variation in *Pseudomonas* sp. PCL1171

In *Pseudomonas* only two molecular mechanism of phase variation have been described previously. In *P. putida* DOT-T1E the expression of flagella is controlled via slipped-strand mispairing (219), and in *P. tolaasii*, a 661 bp spontaneous, reversible duplication in *pheN*, controls colony morphology and pathogenicity (92).

The data presented in Chapters 2 and 3 show that spontaneous mutation of the global regulatory genes *gacA* and *gacS* is responsible for the switch from phase I to phase II. The presence of intact *gacA* and *gacS* genes is directly correlated with a phase I phenotype and the production of secondary metabolism and exo-enzymes. Production of secondary metabolites and exo-enzymes is also affected by spontaneous mutation of the global regulatory genes *gacA* and *gacS* in other *Pseudomonas* species, but in these reports the mutations are not described as part of a reversible process such as phase variation (33, 63, 64, 138, 200, 209, 216).

In Chapter 3 the spontaneous modifications in the *gacS* gene of PCL1171 were analysed. The mutations accumulated in a set of *gacS* mutant derivatives included point mutations, an insertion, deletions, and an inversion. The mutations identified in *gacA* (33) and *gacS* of PCL1171 (Chapter 3 and Fig. 1) show that mutation of *gac* is random, both in the location and nature of the mutation. Based on these observations and the involvement of mismatch repair (MMR) (Chapters 2, 3 and 4), it is concluded that phase variation of PCL1171 is an example of un-programmed spontaneous mutations (Chapter 3). The mechanism of phase variation that is described for PCL1171 is novel in the sense of its occurrence in *Pseudomonas*, the nature of the mutations, and the targets of mutation. In PCL1171, *P. tolaasii* (92), and *Ralstonia solanacearum* (191) global regulatory genes are mutated, other examples of un-programmed variation involve the spontaneous mutation of biosynthetic genes such as the capsule locus in *Streptococcus pneumoniae* (263), and the *vir* locus of *Bordetella bronchiseptica* (175).

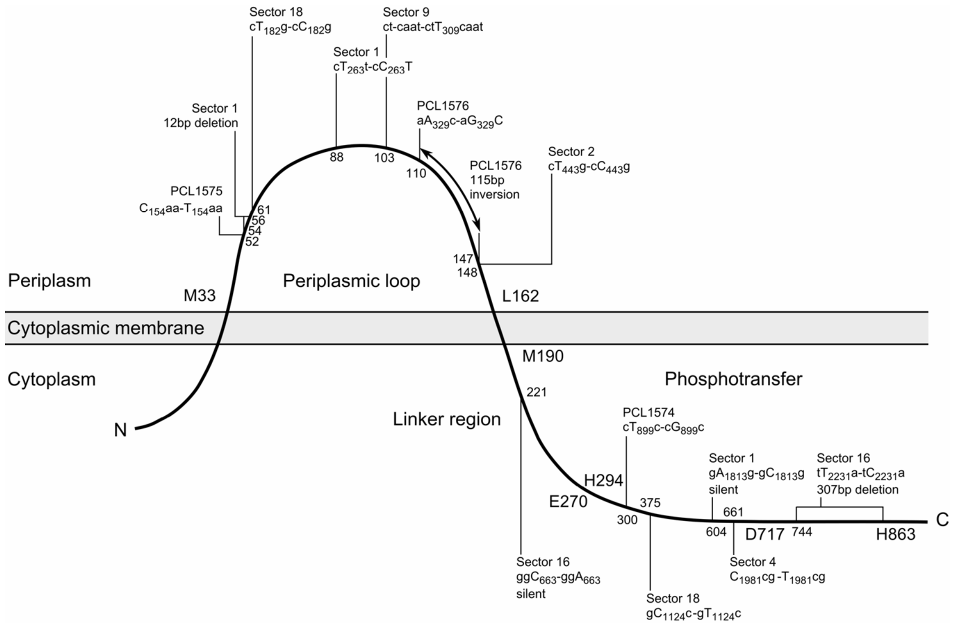


**Figure 1.** Spontaneous mutations identified in the *gacS* gene from phase II transposon mutants (PCL1574, PCL1575, and PCL1576) and wild-type phase II sectors (sector 1, 2, 4, 9, 16, and 18) from *Pseudomonas* sp. PCL1171. The different domains of the GacS protein, the periplasmic loop ($M_{33}$ – $L_{126}$), the linker region ($L_{162}$ – $E_{270}$), and the phosphotransfer domain at the C-terminal end (from E270) harbouring three phosphorylation sites ($H_{294}$, $D_{717}$, and $H_{863}$) (282) are indicated.

In *Pseudomonas* PCL1171 two classes of phase II bacteria were isolated, unstable sectors (as observed in single colonies, see Chapter 2, Fig. 1) and stable phase II mutants. Both classes are based on spontaneous mutation of *gac* genes, in which mutations were spontaneously restored to the wild-type sequence. The stability of the phase II phenotype, observed in one class of mutants, is the result of restoration and simultaneous introduction of new mutations (Chapter 3). The molecular mechanism responsible for this difference in stability of the phase II phenotype classes is not clear, but is not likely to be dependent on the nature or the location of the mutations, which is random for both classes (Chapter 3 and Fig. 1). Only one mutation was identified, a 307 bp deletion in the *gacS* gene, which is likely to lock bacteria in the phase II phenotype (Chapter 3). It can be hypothesized that spontaneous mutation or

regulation of components of a restoration mechanism responsible for the switch from phase II to phase I, prevents these bacteria to return efficiently to a phase I phenotype.

In most cases, as described in Chapters 2, 3 and 4, the phase II phenotype in PCL1171 is reversible. Wild-type phase II bacteria switch back with a remarkably high frequency to a phase I phenotype, restoring the wild-type *gacA* and *gacS* genes (Chapters 3 and 4). The molecular mechanism responsible for the genetic restoration of the mutated gac genes is not known. Preliminary analysis of this switch showed that RecA and RecB are not essential for the restoration of a phase I phenotype. This indicates that large DNA rearrangements are not essential for the restoration of wild-type *gac* sequences. To our knowledge, for all described examples of un-programmed variation in *P. tolaasii* (92), *S. pneumonia* (263), *R. solanacearum* (191), and *B. bronchiseptica* (175), the mechanism for the restoration to the wild-type is unknown. Interestingly, in *P. tolaasii*, RecA was needed for the introduction of the mutation (225), but this is not the case in PCL1171 (Chapter 3). Quantification of the frequency of reversion showed that the switch from phase II to phase I was only affected in a *rpoS* mutant (Chapter 4). This mutation either locks the bacteria in a phase II phenotype, or increases the instability of the phase II bacteria. This is an interesting observation since in a phase II phenotype the *rpoS* gene is not expressed (Chapter 4), possibly resulting in a situation in which the switch to a phase I phenotype is either stimulated or prevented.

### 1.4 Phase II cells of *Pseudomonas* sp. PCL1171 have a growth advantage

Growth experiments presented in Chapters 3 and 4 show that mutation of *gac* in PCL1171 reduced the length of the lag-phase and increased the growth rate. This is consistent with observations in *Pseudomonas chlororaphis* (216). The data presented in Chapter 4 suggests that this growth effect is dependent on a combination of (i) a lack of RpoS activity (as is the case in a *gac* mutant (Chapter 4)) and (ii) a *gac* dependent reduction in metabolic load. Based on the observation that mutation of *gac* increases the growth rate, we hypothesize that the isolation of high numbers of *gac* mutants under laboratorium conditions can be the result of an enrichment of these mutants during culturing (Chapter 3). Still, the percentage of *gac* mutants remains limited, most likely due to the reversibility of the phenotype and the absence of a strong and specific selection

for these mutants under laboratorium conditions. The effect of increased growth competitiveness will be much more apparent under conditions where stationary and exponential growth often switch, as may be expected in colonies and in the rhizosphere. This explanation is consistent with the suggestion by Schmidt-Eisenlohr et al. (216) 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. Phase variation via spontaneous mutation of *gac* could therefore provide a population with a competitive advantage.

Spontaneous *gac* mutants have been isolated in a range of antagonistic, rhizosphere competent *Pseudomonas* species (33, 63, 64, 139, 200, 209, 216). Up till now only stable *gac* mutants have been isolated from these strains. This could be due to the growth advantage of these mutants or the lack of a restoration mechanism in these species. In PCL1171 a small number of the isolated *gac* mutants was not observed to revert to the wild-type. Since (i) *gac* mutants have been isolated in a wide range of antagonistic and rhizosphere competent *Pseudomonas* species, (ii) mutation of *gac* results in a growth advantage and in most selections only stable mutants will be selected for, (iii) *gac* mutations are reversible in PCL1171, it should be considered that phase variation via spontaneous mutation of *gac* is a conserved mechanism in soil-borne *Pseudomonas* species aimed at improving their success in the rhizosphere.

### 1.5 Regulation of the phase variation frequency in *Pseudomonas* sp. PCL1171

In most examples of phase variation the underlying mechanism is regulated by environmental factors to allow a pathogen to express specific traits under the right conditions (see Chapter 1). Regulation of the frequency of phase variation (the frequency of spontaneous mutation of *gac*) in PCL1171 was studied (Chapters 2, 3 and 4). In Chapter 2 a *mutS* mutant is described. Mutation of the *mutS* gene results in a strong increase in the frequency of phase variation (appr. 1000-fold (Chapter 4)). Further analysis of the role of MutS-dependent mismatch repair (MMR) in phase variation of PCL1171 showed that inefficient MMR directly increases the frequency of *gac* mutants (Chapters 2, 3, and 4). In addition, since the frequency of spontaneous mutations in a *mutS* mutant was of the same order as the wild-type phase variation frequency (Chapter 3),

inefficient MMR can be considered (one of) the main mechanism for the switch from phase I to II.
Our results show that *mutS* expression is, directly or indirectly, negatively regulated by RpoS. This RpoS dependent regulation is combined with at least one other (sofar unknown) *gac* dependent factor (Chapter 4). In a phase II phenotype, *rpoS* is not expressed (due to mutation of *gac*) and as a result *mutS* expression is increased when compared to the wild-type (Chapter 4 and Fig. 2). An increase in *mutS* expression should again suppress the introduction of mutations and thereby limit the effect of a high mutational load due to long term absence of MMR. This hypothesis is supported by the observation of a limited number of mutations present in the *gacA* and *gacS* genes and that in almost all mutants either the *gacA* or the *gacS* gene is mutated (Chapters 2 and 3).

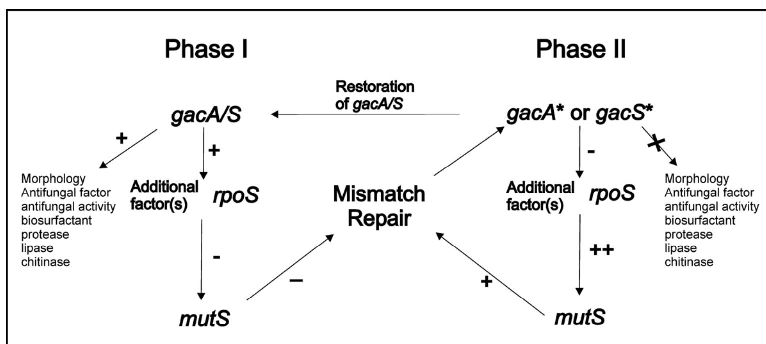


**Figure 2.** Work model for the genetic regulation of spontaneous mutations accumulating in *gac*. A schematic representation of the regulatory roles of GacA/S, RpoS and MutS in phase variation of PCl1171. Phase I cells harbor intact *gacA* and *gacS* genes that are required for expression of *rpoS*, which, in combination with additional factors, in stationary phase negatively regulates *mutS* expression. Inefficient repair of mutations due to down regulation of MutS results in a decreased repair of spontaneous mutation which in turn will result in the accumulation of mutations in *gacA/S* (indicated with an asterix). As a result the cell switches to the phase II phenotype. Mutation of the *gacA/S* genes decreases *rpoS* expression and the subsequent increase in the expression of *mutS*, thus limiting the mutation rate. In addition RpoS is hypothesized to affect both the introduction and the restoration of *gacA*/*gacS*.

The involvement of RpoS in phase variation of PCL1171 suggests that the spontaneous mutation of *gac* is influenced by stress conditions; under such conditions the expression of *rpoS* will increase (99). In addition, the frequency of the switch from phase II to phase I was affected in a *rpoS* mutant (Chapter 4), suggesting a role for RpoS in both mutation and restoration of *gac*. We hypothesize that under growth limiting conditions, for example nutrient stress, MMR will be suppressed resulting in an increase in the frequency of spontaneous mutation and the frequency of *gac* mutants (Fig. 2). This is supported by the observation that a lack of nutrient stress in liquid cultures increases the stability of the *gac* genes (64).

Increasing the frequency of spontaneous mutation can be one of the strategies of micro-organisms to cope with stresses (131, 178). In the rhizosphere conditions can show strong fluctuations. For example the availability of nutrients, the humidity, temperature, and the presence of other organisms creates a stressful environment. Therefore, surviving in the rhizophere will depend on adequate responses to these stresses. A strategy can be to increase the frequency of spontaneous mutation, via the down-regulation of MutS-dependent repair pathways (for example through RpoS), resulting in sub-populations of *gac* mutants. Considering the growth advantages of a *gac* mutant under these conditions, a population including these mutants should be able to cope more easily with changes in the conditions, maintain their presence in the rhizosphere and enable them to colonize new niches more efficiently.

### 1.6 Role of phase variation in the rhizosphere

In a heterogeneous and changing environment such as the rhizosphere, organisms are in a constant struggle to maintain themselves on the plant root or colonize new niches. It can be hypothesized that populations of *Pseudomonas* bacteria present in the rhizosphere or on the plant root, harbor subpopulations of *gac* mutants (33, 39, 139, 216, 250, 250). These *gac* mutants have a significant growth advantage over wild-type bacteria ((216) and Chapters 3 and 4). The presence of these subpopulations could enable the population to react more efficiently to changes or stress conditions.

The presence of *gac* mutants in the rhizosphere has two consequences. First these mutants can provide a population with an advantage: (i) an increase in the growth rate can improve success in the rhizosphere, (ii) the possibility to re-initiate growth more efficiently will increase the chance to maintain a niche

when conditions fluctuate and improve success in competition for new niches, (iii) when conditions become limiting for a *Pseudomonas* population, mutation of *gac* could decrease the metabolic load, and (iv) recent work on phenotypic variation of *Pseudomonas brassicacearum* showed that an overproduction of flagella in a phase II phenotype, resulted in an improved root colonisation pattern of a phase II phenotype (2). Therefore, these mutants are likely to be more successful in the colonization of new niches and in maintaining themselves under fluctuating conditions in the rhizosphere.

Secondly, the mutants have a negative effect on the production of secondary metabolites and exo-enzymes as the basis for the suppression of other organisms and efficient biocontrol activity. But, we suggest that a combination of these two consequences for example in a mixture of phase I and phase II bacteria is not necessarily negative for biocontrol. This is illustrated by the observation that the presence of high numbers of phase variable strains on the maize roots isolated from the agricultural sites in Tontotepec, Oaxaca Mixe, Mexico still results in a natural protection of the plants against phytopathogens (Chapter 2). An optimal mixture is possible since the frequency at which mutations are introduced into *gac* is relatively low and these *gac* mutants are part of a reversible mechanism. Therefore a population can combine the production of secondary metabolites of a phase I phenotype with the growth advantage of a phase II phenotype. The absence of an optimal mixture of phase I and phase II cells, for example a high frequency of phase variation or a stable phase II phenotype, was shown to abolish biocontrol (Chapter 2).

Phase variation as described in this thesis, is a strategy to improve success in a heterogeneous, competitive and ever changing environment like the rhizosphere. As such, phase variation can improve the efficiency of biocontrol and enable a strain to establish itself along the root more easily, at more sites and in larger numbers. Especially since phase II bacteria are not locked in this phenotype, the production of secondary metabolites and exo-enzymes, and therefore biocontrol is not lost upon mutation of *gac*. Therefore, the presence of a subpopulation of phase II bacteria will not necessarily abolish biocontrol. But, considering the results presented in this thesis, phase variation has been identified as an important factor in efficient biocontrol and could influence the reliability of biocontrol agents. Biocontrol activity of PCL1171 is lost when (only) phase II bacteria or a derivate with an increased phase variation frequency were applied to wheat seeds (Chapter 2). Since these mutants cannot maintain the hypothesized optimal mixture of wild-type and *gac* mutants we

could show that phase variation can have a strong impact on the biocontrol and when these situations occur in the field, they are likely to affect the biocontrol.

### 1.7 Utilisation

The aim of this thesis was to study phase variation and design a strategy to control this mechanism in order to improve biocontrol activity in *Pseudomonas*. Our results suggest that locking bacteria in the phase I phenotype will not necessarily increase the efficiency of control since it will include a loss of competitive advantage. However, since a mutant strain locked in a phase I phenotype was not isolated and could not be constructed, the effect of such a mutation remains hypothetical. In Chapter 2 we describe the isolation of a *mutS* mutant with a 1000-fold increased phase variation frequency, and a *gacS* mutant locked in a phase II phenotype. In both situations biocontrol was abolished, indicating that a moderate frequency of phase variation, and therefore an optimal ratio of wild-type bacteria and *gac* mutants, is not necessarily a disadvantage in the control of phytopathogens. Based on the results described in Chapters 3 and 4 this frequency of phase variation optimal for biocontrol is likely to be dependent on the regulation of MMR activity. MMR will be optimal when stress is prevented and *rpoS* is not highly expressed. Duffy et al., (64) showed that the addition of certain nutrients (i.e., zinc, copper, cobalt, manganese, and ammonium molybdate) increased the stability of *gac*. Therefore, growth limitation and conditions such as temperature, minerals and humidity could affect phase variation. The optimal environmental conditions for biocontrol by limiting spontaneous mutation of *gac* have not yet been determined. Alternatively the stability of *gacA* and *gacS* can be increased genetically by over-expression of *mutS*, or mutation of *rpoS* (Chapter 4).

Under certain circumstances phase variation can form a serious problem, for example in vaccine and enzyme production. Since the variation of epitopes, which is the major problem in vaccine production (163), is in most cases not based on an ON or OFF switching as described in this thesis, I will not focus on utilisation of the presented results with respect to vaccine production. Phase variation in industrial production processes can affect the overall yield when subpopulations of non-producing bacteria are formed. As for example shown by Duffy et al. (64), an up-scaling of production size using *Pseudomonas fluorescens*, can exponentially increase the size of the *gac* mutant sub-population even up to 61% of the total viable cells. It is likely that nutrient

stress, growth limitation and therefore the expression of *rpoS*, and suppression of MMR, account for this observation. Although in most cases the production of microbial metabolites is transferred to microbial species optimal for production purposes, to delay, prevent or reduce phase variation (i) optimal growth conditions offering sufficient nutrients to prevent stationary growth should be applied, or (ii) genetically modified strains should be constructed, for example by mutation of *rpoS*, or a constitutive expression of *mutS*.

### 1.8 Concluding remarks

In this thesis we describe the mechanism of phase variation of a rhizosphere-competent *Pseudomonas* strain. Phase variation in these bacteria affects secondary metabolism, the production of exo-enzymes, and -as a result- biocontrol of take-all of wheat. But, although phase variation strongly influences the expression of biocontrol traits, within a competitive, heterogeneous, and changing environment like the rhizosphere phase variation can be beneficial for strains to establish and maintain themselves in sufficiently large numbers on the plant root. The advantage of a sub-population of *gac* mutants is based on their growth effect which could improve the succes in competition or survival under fluctuating and stressfull conditions (Chapters 3 and 4). This strategy is employed under stress conditions (Chapter 4) in which the growth advantage will be most effective. Phase variation is likely to affect biocontrol in the absence of an equilibrium between phase I (production of secondary metabolites and exo-enzymes) and phase II bacteria (growth advantage) on the root and could thereby affect the reliability of a biocontrol agent in the field. In conclusion, phase variation is a phenomenon, aimed at adaptation of a population, either in host-pathogen interactions by evading the host defense systems, or in interactions between organisms in a competitive and heterogeneous environment to improve success in competition.

# CHAPTER 7

# Nederlandse samenvatting

## Inleiding

De bescherming van gewassen tegen ziekteverwekkers speelt een belangrijke rol in de moderne landbouw. Bescherming is tot op heden vooral gebaseerd op het gebruik van chemische bestrijdingsmiddelen. Deze pesticiden zijn echter vaak slecht afbreekbaar en blijven daardoor lang actief in het milieu. Een bijkomend probleem is een veelal aspecifieke werking, waardoor het milieu en de gezondheid van de mens wordt bedreigt. Op nationaal en internationaal niveau is dan ook besloten het gebruik van chemische bestrijdingsmiddelen te beperken. Om gewassen tegen ziekteverwekkers te blijven beschermen zijn efficiënte alternatieven noodzakelijk. Een veelbelovend alternatief is het gebruik van micro-organismen. De beoogde micro-organismen zijn oorspronkelijk ontdekt in bodems die van nature de groei van schimmels onderdrukken. De werking van deze bodems is gebaseerd op de aanwezigheid van specifieke ziekteonderdrukkende micro-organismen. Door middel van niche-exclusie, de productie van antischimmelfactoren, predatie, of door de activering van de plantenverdedigingsrespons onderdrukken deze biocontrole bacteriën de effecten van ziekteverwekkers (45, 241). Wereldwijd zijn er op dit ogenblik meer dan honderd producten op basis van deze biocontrole stammen commercieel verkrijgbaar. Redenen waarom deze stammen nog niet uitgebreid worden toegepast zijn o.a. het moeizaam tot stand komen van regelgeving en de variabele betrouwbaarheid in het veld. Veel research richt zich op het identificeren van factoren of mechanismen die verantwoordelijk zijn voor de variatie in de betrouwbaarheid van deze vorm van gewasbescherming. Ons onderzoek richt zich op *Pseudomonas* bacteriën, een microbiële species die onder andere algemeen voorkomt op het worteloppervlak. Eén van deze stammen, *Pseudomonas fluorescens* WCS365, staat bekend om zijn uitstekende kolonisatie-eigenschappen (83). Eén van de bij kolonisatie betrokken eigenschappen in deze stam is gebaseerd op de werking van een recombinase (*sss*). Mutatie van het *sss*-gen onderdrukte het voorkomen van morfologische varianten, het competitief vermogen de wortel te koloniseren (56) en de biocontrole-activiteit (43). Recombinases zijn betrokken bij DNA-recombinatie en -herschikking, bijvoorbeeld door inversies en deleties, en worden vaak geassocieerd met de vorming van koloniesectoren (72, 98). Deze resultaten suggereren dat een mechanisme zoals koloniefasevariatie een belangrijke oorzaak zou kunnen zijn van de eerder genoemde variabiliteit van biocontrole.

Fasevariatie is gedefinieerd als een met een hoge frequentie voorkomend (> $10^{-5}$ wisselingen per cel per generatie) fenomeen dat gebaseerd is op

DNA -mutaties, -reorganisaties, of -modificaties met als resultaat reversibele fenotypische variatie (98, 211). Dat een dergelijk mechanisme voorkomt bij micro-organismen in de rhizosfeer bleek uit de isolatie van 214 *Pseudomonas* bacteriën van de wortels van mais. Zesenveertig van deze stammen vertoonden antagonistische activiteit tegen verschillende ziekteverwekkers. Drieënnegentig procent van deze antagonisten vertoonden koloniefasevariatie. Fasevariatie is voornamelijk beschreven als een proces in de context van een gastheer – pathogeen-interactie (bijv. het ontwijken van de immuunrespons) (52). Dit fenomeen kan een verscheidenheid aan eigenschappen reguleren en wordt ook gebruikt door micro-organismen om diversiteit binnen een populatie te verhogen en speelt een belangrijke rol in adaptatie en kolonisatie van niches (207, 211). Fasevariatie vormt een belangrijk probleem in de productie van vaccins (163) en kan de opbrengst van industriële productieprocessen negatief beïnvloeden. Het laatste is gebaseerd op de vorming van subpopulaties die de beoogde metabolieten niet tot expressie brengen. Het begrijpen van fasevariatie is een essentiële stap om de nadelige effecten op medische, industriële en ecologische processen te kunnen beperken.

Het doel van dit proefschrift is het bestuderen en begrijpen van het mechanisme van fasevariatie in antagonistische *Pseudomonas* bacteriën in relatie tot de efficiënte controle van plantpathogenen. De resultaten beschreven in dit proefschrift dragen bij aan het begrip van fasevariatie in *Pseudomonas*. We ontdekten dat een mechanisme gebaseerd op een reversibele spontane mutatie van een tweecomponenten regulatiesysteem daaraan ten grondslag ligt. Met de spontane mutatie van deze genen produceren de *Pseudomonas* bacteriën geen secundaire metabolieten en extracellulaire enzymen meer. Omdat deze eigenschappen de basis vormen voor de biocontrole-activiteit van deze stammen heeft dit een belangrijke invloed op de efficiëntie van de biocontrole. Deze spontane mutanten blijken echter ook een significant groeivoordeel te hebben in vergelijking met de wildtype-bacteriën. Omdat het proces omkeerbaar is kan de fasevariatie een competitief voordeel opleveren voor de totale populatie van deze *Pseudomonas* biocontrolebacteriën. De resultaten zoals deze zijn beschreven in dit proefschrift worden hier per hoofdstuk besproken.

**Hoofdstuk 2** beschrijft de isolatie en karakterisatie van een collectie *Pseudomonas* bacteriën geïsoleerd van de wortels van maïs. Zesenveertig van de 214 geïsoleerde stammen (21%) onderdrukte de groei van verscheidene plantpathogene schimmels, waaronder *Gaeumannomyces graminis* pv. *tritici* en

*Fusarium oxysporum* f. sp. *radicis lycopersici* die respectievelijk take-all van tarwe en voet- en wortelrot bij tomaat veroorzaken. Drieenveertig van deze antagonistische stammen vertonen koloniefasevariatie. Deze stammen vormen twee kolonietypen, nl. een ondoorschijnende fase I en een doorschijnende Fase II. Dat de ene fase uit de andere fase wordt gevormd blijkt uit de isolatie van fase-I-kolonies met fase-II-sectoren en fase-II-kolonies met fase-I-sectoren. Uit analyse van deze verschillende kolonievormen bleek dat belangrijke eigenschappen voor biocontrôle alleen tot expressie komen in een fase-I-fenotype. Deze eigenschappen omvatten de productie van (i) een lipopeptide met antischimmel activiteit, (ii) een biosurfactant, en (iii) de extracellulaire enzymen protease, lipase, en chitinase. Door tarwezaden te coaten met cellen van een bepaalde stam en op te groeien in aanwezigheid van een ziekteverwekker, kan de mate van ziektebescherming door de bewuste stam worden bepaald. Voor deze experimenten en voor de verdere moleculaire analyse van het mechanisme van fasevariatie werd één van de antagonistische stammen geselecteerd. Stam PCL1171 vertoont een sterke antagonistische activiteit en een duidelijke fasevariatie met een voorkeur voor een fase-I-fenotype. Alleen de aanwezigheid van bacteriën met een fase-I-fenotype resulteert in een efficiënte controle van take-all. Wanneer bacteriën met een fase-II-fenotype werden gebruikt wordt de ziekte niet onderdrukt. De biocontrole activiteit van PCL1171 bleek afhankelijk te zijn van de productie van een lipopeptide. De mutatie in een lipopeptide synthetase had geen effect op de fase-I-morfologie of fenotype behalve op de antagonistische activiteit van PCL1171. Afwezigheid van dit lipopeptide in een fase-II-fenotype is hoogstwaarschijnlijk de reden voor de afwezigheid van biocontrole activiteit in dit fenotype.

De genetische basis van fasevariatie werd bestudeerd na constructie van een transposon bank van *Pseudomonas* PCL1171 fase I bacteriën. Drie mutanten werden geselecteerd: twee op grond van een stabiel fase-II-fenotype en één op grond van een verhoogde frequentie van fasevariatie. Mutatie van *gacS* bleek verantwoordelijk te zijn voor een stabiel fase-II-fenotype. GacS vormt samen met GacA een tweecomponenten systeem dat het secundaire metabolisme en de productie van extracellulaire enzymen in *Pseudomonas* reguleert. Een centrale rol voor *gacA/S* in fasevariatie bleek uit het herstel van het fase-I-fenotype in fase II kolonies na de introductie van een wildtype-*gacA*-of-*gacS*-gen.

Mutatie van *mutS* in PCL1171 resulteerde in een sterke verhoging van de frequentie van fasevariatie. MutS is de eerste stap in het herstel van DNA-mismatches die ontstaan ten gevolge van fouten in de replicatie van chromosomaal DNA. Analyse van de biocontrole activiteit van deze twee mutanten laat zien dat zowel een stabiel fase-II-fenotype als een verhoogde frequentie van fasevariatie leiden tot een verlies van de biocontrole eigenschap. Uit de resultaten blijkt dat (i) fasevariatie een grote invloed op de biocontrôle-activiteit van *Pseudomonas* kan hebben, dat (ii) de *gacA/S*-genen een centrale rol spelen in het mechanisme van fasevariatie, en dat (iii) de frequentie van variatie wordt onderdrukt door MutS-afhankelijk mismatchherstel.

**Hoofdstuk 3** richt zich op de moleculaire basis van de rol die de genen *gacA/S* in fasevariatie in *Pseudomonas* PCL1171 spelen. Herstel van het fase-I-fenotype na de introductie van wildtype-*gacA*-en/of-*gacS*-genen in zesentwintig fase-II-mutanten en dertien fase-II-sectoren liet zien dat in al deze gevallen het fase II fenotype gebaseerd is op mutaties in het *gacA*- of *gacS*-gen. Reeds in een eerdere publicatie zijn verschillende puntmutaties in het *gacA*-gen geïdentificeerd (33). In het *gacS*-gen werden verschillende puntmutaties, deleties, een insertie en een inversie geïdentificeerd. In bijna alle gevallen werden deze mutaties spontaan en efficiënt verwijderd, waarmee een fase-I-fenotype werd hersteld. Daarnaast werden enkele (meer) stabiele fase-II-mutanten geïsoleerd. Verdere analyse van deze laatste mutanten liet zien dat mutaties wisselden tussen het *gacA*- en *gacS*-gen via een stadium waarin beide genen mutaties bevatten. In beide klassen kunnen de mutaties in *gac* worden verwijderd ook al valt dit dus niet noodzakelijk samen met een switch terug naar een fase-I-fenotype. Ten gevolge van het sterk reversibele karakter van de mutaties in de *gac* genen is het aannemelijk dat er een dynamische relatie tussen de wildtype-populatie en de *gac* subpopulatie bestaat.

Om het mechanisme achter het herstel van de wildtype *gacA*- en *gacS*-nucleotidevolgorde te achterhalen werd de switch van fase II naar fase I bekeken in zowel een *recA*- als in een *recB*-mutant. RecA en RecB spelen een rol in DNA-herschikkingen die ten grondslag zouden kunnen liggen aan het verwijderen van de mutaties uit *gac*. Echter nóch de mutatie van *recA*, nóch van *recB* heeft een effect op de switch terug naar fase I, waaruit blijkt dat DNA-herschikking niet essentieel is in deze switch.

De groeieigenschappen van alle geteste *gac*-mutanten vertoonden in vergelijking met het wildtype een verkorte lag-fase en een hogere groeisnelheid.

Spontane mutatie van *gac* leidt dus tot een groeivoordeel en daarmee mogelijk ook tot een competitief voordeel. De *gac*-mutanten zijn echter niet in staat om de wildtypepopulatie weg te concurreren. Dit is hoogst waarschijnlijk het gevolg van (i) de afwezigheid van specifieke, sterke selectie onder laboratoriumcondities en van (ii) de reversibiliteit van het fase-II-fenotype.

In **Hoofdstuk 4** richten we ons op de regulatie van fasevariatie in *Pseudomonas*. In hoofdstuk 2 is een *mutS*-mutant beschreven. De mutatie van *mutS* leidt tot een hoge frequentie van fasevariatie. In hoofdstuk 3 bleek ook dat de frequentie van spontane mutaties in een *mutS* mutant overeenkomt met de normale frequentie van fasevariatie in het wildtype. Het is dus aannemelijk dat de introductie van DNA mutaties (zoals mismatches, puntmutaties, kleine inserties) en daarmee de switch van fase I naar fase II afhankelijk is van de activiteit van MutS. Aangezien de activiteit van mismatchherstelmechanismen vaak wordt beïnvloed door stress is de relatie tussen de algemene sigmafactor RpoS, die een rol speelt in stressrespons, *gacA*/*gacS*, en *mutS* bekeken. Dat een dergelijke relatie bestaat werd duidelijk toen we vonden dat niet alleen de mutatie van *mutS* de frequentie van fasevariatie met een factor 1000 verhoogde, maar dat ook mutatie van *rpoS* de frequentie 10 maal verhoogde. Uit de experimenten bleek dat (i) een functioneel *gac*-systeem essentieel is voor de expressie van *rpoS*, (ii) RpoS de expressie van *mutS* onderdrukt waardoor de frequentie van *gac*-mutanten toenam, (iii) de expressie van *mutS* was verhoogd in *gacS*- en *rpoS*-mutanten. Daarnaast bleek dat in een *gacS*-mutant de *mutS* expressie in de stationaire groeifase, in tegenstelling tot die van het wildtype en van een *rpoS*-mutant, in het geheel niet meer werd onderdrukt. Dit suggereert dat de regulatie van *mutS* gebaseerd is op RpoS, *gacA*/*gacS*, en tenminste één andere, door *gac*-gereguleerde, factor. De regulatie van *mutS* heeft een direct effect op de frequentie van gac mutanten en daarmee op de frequentie van fase variatie. Analyse van de groeieigenschappen van een *rpoS* mutant en een *gacS* mutant liet zien dat het groeivoordeel van een *gac*-mutant is gebaseerd op een combinatie van afwezigheid van RpoS, en een *gac*-afhankelijke reductie van de metabole last.

**Hoofdstuk 5** richt zich op de moleculaire basis van de morfologie van een fase I en fase II fenotype. De stabiele fase-II-mutant PCL1588 bleek na introductie van *gacA*/*gacS* niet, zoals alle andere fase-II-mutanten en sectoren, een fase-I-fenotype tot expressie te brengen maar een intermediaire kolonie

morfologie. Deze fase-I'-koloniemorfologie onderscheidt zich door een morfologie die doorschijnender is dan een fase I maar ook minder doorschijnend dan een fase II. Het transposon in PCL1588 bevindt zich in het *prtR*-gen dat een operon vormt met het transcriptioneel daarmee gekoppelde *prtI*-gen. PrtI is een extracellulaire sigmafactor en PrtR is een membraan gebonden antisigma factor. Om het fenotype van PCL1588 te bepalen werden de celenvelop eiwitten, de lipopolysaccharides (LPS), flagellen en extracellulaire polysacchariden (EPS) bestudeerd. De mutatie van *prtR* had alleen invloed op het EPS. Het fase-I'-fenotype lijkt te berusten op de afwezigheid van negatieve regulatie door PrtR. Dit blijkt bijvoorbeeld uit de expressie van een fase-I'-morfologie bij een constitutieve expressie van *prtI* in het wildtype PCL1171. In een *gacS*- en een *rpoS*-achtergrond blijkt het effect op het EPS te zijn verdwenen. Alleen de afwezigheid van negatieve regulatie van PrtR en RpoS (door mutatie van *rpoS* of met hetzelfde effect *gacS*, zie hoofdstuk 4) had effect op het EPS. Onze conclusie is dat het EPS een rol speelt in de fase I en fase II koloniemorfologie van PCL1171 maar niet de enige factor is die de koloniemorfologie bepaald.

**Conclusie**. Dit proefschrift beschrijft een fasevariatiemechanisme gebaseerd op de spontane mutatie van een algemeen tweecomponenten *gacS*/*gacA*-regulatiesysteem. Deze mutanten zijn niet in staat secundaire metabolieten en extracellulaire enzymen te produceren. Uit de resultaten blijkt dat de reversibele switch tussen wildtype fase I en fase II bacteriën een belangrijke factor is in gewasbescherming door micro-organismen. Deze mutanten hebben ook bepaalde groeivoordelen, nl. een verkorte lag-fase en een hogere groeisnelheid in de exponentiële fase. In een heterogeen en competitieve omgeving zoals de rhizosfeer, kan dit een voordeel zijn en de populatie in staat stellen sneller op veranderingen in te spelen en de concurrentie met andere organismen aan te gaan. Doordat RpoS, door de regulatie van *mutS* expressie en daarmee in de vorming van *gac*-mutanten, een centrale rol speelt in fasevariatie, zullen stressomstandigheden leiden tot de formatie van *gac*-mutanten. Onder deze omstandigheden zal een grotere diversiteit en een subpopulatie van *gac*-mutanten een voordeel zijn om de stresscondities het hoofd te kunnen bieden. Echter, een te hoge frequentie van fasevariatie of een te grote populatie fase-II-bacteriën heeft negatieve gevolgen voor de biocontrôle. Hiermee is niet alleen aangegeven dat fase variatie een fenomeen is dat in staat is variatie in de veldresultaten te introduceren, maar ook dat een evenwicht tussen fase-I- en

fase-II-bacteriën niet noodzakelijk negatief maar ook mogelijk voordelig kan zijn voor gewasbescherming. Zo'n evenwicht is aannemelijk ten gevolge van de reversibiliteit van de fenotypes. Dat de fasevariatie niet noodzakelijk een negatieve factor is in gewasbescherming blijkt ook uit de jarenlange succesvolle opbrengsten van de velden waar deze pseudomonaden zijn geïsoleerd. Dat mutatie van gac een groeivoordeel met zich meebrengt en er *gac*-mutanten zijn geïsoleerd in antagonistische, rhizosfeer competente, *Pseudomonas* soorten, suggereert dat spontane mutatie van *gac* een geconserveerd mechanisme is. Het is echter niet uitgesloten dat ook in deze andere gevallen, zoals in PCL1171, de mutaties reversibel zijn en dat fase variatie via *gac* een geconserveerd mechanisme is, dat voorkomt in *Pseudomonas* soorten in de bodem, met als doel hun succes in de rhizosfeer te vergroten.

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# Curriculum vitae

Daniël van den Broek werd geboren op 5 oktober 1976 te Leiden. Het VWO-diploma werd behaald aan het Visser 't Hooft Lyceum te Leiden in 1995, waarna met de studie biologie aan de Universiteit Leiden werd gestart. In 1997 kwam hier de studie wijsbegeerte van een bepaald wetenschapsgebied bij. De studie biologie met als specialisatie moleculaire biologie, werd in 1999 cum laude afgerond met onderzoeksstages binnen het Instituut Moleculaire Plantkunde (later het Instituut Biologie Leiden) onder leiding van Dr. G. V. Bloemberg en Prof. Dr. B.J.J. Lugtenberg, en binnen de moleculaire genetica onder leiding van Prof. Dr. J. Brouwer. In 1999 werd dit promotie onderzoek gestart binnen de sectie Microbiologie van het Instituut voor Biologie aan de Universiteit Leiden, waarvan dit proefschrift in 2005 het product is. Ditzelfde jaar werd de studie Wijsbegeerte van een bepaald wetenschapsgebied afgerond. Vanaf 1 januari 2005 is hij werkzaam als klinisch chemicus in opleiding in het Meander Medisch Centrum in Amersfoort.

## Publications

**Thomas F.C. Chin-A-Woeng, D. van den Broek, G. de Voer, K. M. van der Drift, S. Tuinman, J. E. Thomas-Oates, B.J.J. Lugtenberg, and G. V. Bloemberg.** 2001. Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium. Mol.Plant Microbe Interact. **14**:969-979.

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