

# AstR–AstS, a new two-component signal transduction system, mediates swarming, adaptation to stationary phase and phenotypic variation in *Photorhabdus luminescens*

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*Photorhabdus luminescens* is an insect-pathogenic bacterium that forms a symbiosis with specific entomopathogenic nematodes. In this bacterium, a symbiosis-‘deficient’ phenotypic variant (known as the secondary variant or form II) arises at a low frequency during prolonged incubation. A knock-out mutant was generated of the regulator of a newly identified two-component regulatory system, designated AstR–AstS. Interestingly, this mutation altered the timing of phenotypic switching. Variant cells arose in the mutant strain several days before they did in the wild-type population, suggesting that AstRS is directly or indirectly involved in the genetic mechanism underlying variant cell formation. This mutation also affected motility and antibiotic synthesis. To identify AstRS-regulated genes, a comparative analysis using two-dimensional gel electrophoresis was performed. Seventeen proteins with modified synthesis in stationary phase were identified by mass spectrometry and shown to be involved in electron-transport systems, energy metabolism, iron acquisition and stress responses. The results imply that AstRS is involved in the adaptation of cells to the stationary phase, whilst negatively affecting the competitive advantage of form I cells. The link between AstRS-dependent stationary-phase adaptation and phenotypic variation is discussed.

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## INTRODUCTION

*Photorhabdus luminescens*, a Gram-negative luminescent gamma proteobacterium, forms an entomopathogenic symbiosis with soil nematodes belonging to the genus *Heterorhabditis*. This bacterium has a complex life cycle, with a symbiotic stage, in which bacteria colonize the intestinal tract of the nematodes, and a pathogenic stage, in which susceptible larval-stage insect prey are killed by the combined action of the nematode and the bacteria. After entering the insect host, the nematodes regurgitate

their bacterial symbionts into the insect haemocoel. Once released, the bacteria proliferate and produce exo- and endotoxins that kill the insect within 48 h. In addition, *P. luminescens* produces antibiotics that inhibit the growth of competing micro-organisms in the insect cadaver and enhance nematode reproduction by providing nutrients and other growth factors. After several rounds of reproduction, a new generation of infective nematodes, known as the infective juveniles (IJs), reacquire the bacteria and leave the insect carcass in search of new hosts (Boemare *et al.*, 1997).

*Photorhabdus* spp. have the somewhat unusual ability to exist in two phenotypically distinct forms, known as primary variant (form I) and secondary variant (form II) (Akhurst, 1980; Boemare & Akhurst, 1988; Boemare *et al.*, 1997). The two variants are equally pathogenic for the insects, but differ in a wide range of characteristics, including their biochemical properties and their colonial and cellular morphologies. Variant I is characterized by the production of antimicrobial agents, lipases, phospholipases,

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Abbreviations: 2D, two-dimensional; DH, dehydrogenase; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.

The GenBank/EMBL accession number for the *astRS* sequence reported in this paper is AJ510203.

proteases, pigmentation and bioluminescence. It has distinct colony morphology, adsorbs certain dyes and develops large intracellular protein crystal inclusions (Forst *et al.*, 1997). Most of these characteristics specific to the primary form are essential for the symbiotic interaction between the nematode and the bacterium (Joyce & Clarke, 2003). As expected, variant I is the bacterium normally found in association with the symbiotic infective-phase nematodes. The secondary variant either lacks or has reduced levels of the previously listed properties, and secondary variants from some strains are unable to support nematode growth and reproduction in the insect cadaver (Ehlers *et al.*, 1990). Upon entering the stationary phase, the secondary form also differs in its assimilation of nutrients and its vitamin requirements. Variant II cells maintain considerably higher levels of the major respiratory enzymes than do their variant I counterparts and have considerably higher levels of transmembrane proton-motive force (Boemare *et al.*, 1997; Smigielski *et al.*, 1994). As they display higher levels of cellular metabolism and respiration, variant II cells grow faster than variant I cells. Furthermore, following periods of starvation, variants II resume growth within 2–4 h of the addition of nutrients, compared to 14 h for variant I cells (Bleakley & Neelson, 1988; Boemare *et al.*, 1997). Variant II appears spontaneously at high frequency during the stationary period of *in vitro* culture or during nematode rearing on an artificial diet. Although the secondary form has also been observed *in vivo* during the reproduction in insects (Hurlbert, 1994), it is counter-selected by the infective juveniles which do not retain variant II bacteria in their intestinal tract. Stress conditions such as prolonged culture time, low oxygen levels, or low osmolarity in the liquid medium induce the formation of secondary cells (Boemare *et al.*, 1997; Krasomil-Osterfeld, 1995). Based on differences in levels of respiratory enzymes and lag times, it has been hypothesized that secondary cells might be better adapted for survival as free-living organisms in the soil (Boemare *et al.*, 1997) and that the secondary phenotype is a response by the bacterium to environmental conditions not favouring nematode association (French-Constant *et al.*, 2003).

Phenotypic variation in *Photorhabdus* is not a classical phase variation. The switch between the two forms appears to be unidirectional: only the transition from primary to secondary variant cells has been documented. Recent data indicate that several genetic loci affect phenotypic variation in different ways. Inactivation of either *cipA* or *cipB* genes, encoding the crystal proteins of *Photorhabdus* strain NC1, creates a variant cell type resembling the secondary variant in many respects (Bintrim & Ensign, 1998). Expression from a multicopy plasmid of *ner*, a gene which encodes a putative DNA-binding protein, switches the phenotype of the primary variant to that of the secondary (O'Neill *et al.*, 2002). Lastly, inactivation in secondary cells of a homologue of *hexA* restores the production of the primary-specific phenotypes, suggesting that secondary cells produce a repressor protein down-regulating the expression

of primary-specific phenotypes (Joyce & Clarke, 2003). These observations suggest the existence of a complex regulatory cascade of interacting genes controlling phenotypic variation in *P. luminescens*.

Entomopathogenic bacteria colonize diverse environments, including the nematode gut and the insect haemocoel, which have different physical and chemical properties. To establish culture in these locations, *P. luminescens*, like other bacterial pathogens (Hentschel *et al.*, 2000), has evolved two-component signal transduction systems to induce the expression of the sets of genes needed. These systems comprise a membrane-associated sensor kinase and a cytoplasmic transcriptional regulator. In response to an external stimulus, the sensor component is auto-phosphorylated at a conserved histidine residue in an ATP-dependent reaction. The phosphoryl group is then transferred to the regulator component, promoting its binding to DNA (Stock *et al.*, 2000). This study reports the identification of a new two-component signal transduction system, AstRS, which is involved in adaptation of *P. luminescens* cells to the stationary phase and which affects the phenotypic variation process. Mutation of the regulator component AstR reduces the competitive advantage of variant I cells during stationary-phase conditions and systematically induces an earlier transition to the secondary phenotype. We hypothesize that this system may be part of the regulatory cascade tightly controlling the decision to shift to the secondary form of *P. luminescens*.

## METHODS

**Bacterial strains, plasmids and growth conditions.** Permanent stocks of all strains were maintained at  $-80^{\circ}\text{C}$  in Luria–Bertani (LB) broth supplement with glycerol. The *P. luminescens* strains used were TT01 (Fischer-Le Saux *et al.*, 1999) and its *astR* disruptant PL2106 (this study). The *Escherichia coli* strains used were TG1 (Sambrook *et al.*, 1989) for plasmid maintenance and S17-1 (Simon, 1984) for conjugation. *E. coli* strains were routinely grown in LB at  $37^{\circ}\text{C}$ , whereas *P. luminescens* strains were grown at  $30^{\circ}\text{C}$  in Schneider medium (Bio-Whittek). The final concentrations (in  $\text{mg l}^{-1}$ ) of the antibiotics used for selection for both *E. coli* and *P. luminescens*, were as follows: gentamicin (Gm), 30; kanamycin (Kan), 20; chloramphenicol (Cm), 20. All experiments were performed in accordance with the European regulations concerning the contained use of Genetically Modified Organisms of Group I (agreement no. 2736 CAII).

pDIA606 was constructed via a two-step PCR method. Briefly, the kanamycin-resistance gene of pUC4K (Amersham Pharmacia Biotech) was amplified by PCR with oligonucleotides kan5 (5'-AATTTCTGCCATTCATCCGCCACGTTGTGTCTCAAAATCTC-3') and kan3 (5'-TTGATCGGCACGTAAGAGGTATCCAGCCAGAAAGTGAGG-GAGC-3'), resulting in a 1250 bp DNA fragment. Two 1075 bp DNA fragments containing either the 5' upstream region of *astR* or the end of the coding region of *astR* and the downstream region were also amplified using genomic DNA from *P. luminescens* TT01 and either oligonucleotides AstA3 (5'-AAACTGCAGGATGCTGGTAG-CAACAGCGC-3') and AstA4 (5'-ACCTCTTACGTGCCGATCAAG-GCAATGTTGCCAGTATCGTA-3'), or AstA5 (5'-GCGGATGAAT-GGCAGAAATTGGACTAAGCAGACGATGAGCG-3') and AstA6 (5'-CGGGATCCGCGATTCACTGCCACATTGA-3'). The first 20

bases of primers AstA4 and AstA5 are complementary to primers kan5 and kan3, respectively. After purification and quantification, 100 ng samples of each of the three previously amplified fragments were mixed and used as template to generate a new 3360 bp DNA fragment using oligonucleotides AstA3 and AstA6. The latter amplicon, which corresponds to an *astR::kan* fragment, was purified, cut with *Pst*I and *Xba*I and ligated to the pJQ200KS vector (Quandt & Hynes, 1993) to yield pDIA606. To construct pDIA608, a DNA fragment containing the *astR* gene was generated by PCR with primers AstS3 (5'-AAACTGCAGCTGGCAAGGTATCGCACTGC-3') and AstA6, and genomic DNA from *P. luminescens* TT01. The resulting 1700 bp fragment was purified with the QIAquick PCR purification kit (Qiagen), cut with *Pst*I (present in primer AstS3) and *Eco*RI (located inside the amplified sequence), and cloned into the pSU18 vector (Bartolomé *et al.*, 1991) in the same orientation as the *lacZ* promoter.

**DNA manipulations.** Chromosomal DNA preparations, ligations, electrophoresis and Southern blotting were carried out according to standard procedures (Sambrook *et al.*, 1989). Plasmid DNA was isolated with the GenElute Plasmid Miniprep kit (Sigma). Restriction enzymes were obtained from Roche and enzymic reactions were purified with the MinElute Reaction Cleanup kit (Qiagen). pDIA606 was introduced into *P. luminescens* by conjugal transfer, whereas pDIA608 was electroporated. *E. coli* and *P. luminescens* were transformed by electroporation using a Bio-Rad gene pulser according to standard procedures (Sambrook *et al.*, 1989).

**Mutant strain construction.** Strain PL2106 was created via allelic exchange with pDIA606 (which contains a kanamycin-resistance cassette in the *astR* coding region). pDIA606 was transformed into *E. coli* S17-1 and introduced into *P. luminescens* by mating. Cm<sup>R</sup>Gm<sup>S</sup>Sac<sup>R</sup> exconjugants were selected on proteose peptone agar [1% (w/v) proteose peptone, 0.5% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) agar] containing 2% (w/v) sucrose. These exconjugants had undergone allelic exchange and lost the wild-type copy of *astR* and the plasmid vehicle. Insertions were confirmed by Southern blot hybridization using a PCR-amplified digoxigenin (DIG)-labelled *astR* gene probe obtained by PCR with oligonucleotides AstA5 and AstA6 and the PCR DIG probe Synthesis kit (Roche).

**Handling of RNA.** Total RNA was prepared from 10 ml cultures of *E. coli* and *P. luminescens* as previously described (Derzelle *et al.*, 2002). Primer extension reactions were performed by standard procedures (Sambrook *et al.*, 1989) with some modifications as previously described (Derzelle *et al.*, 2002). Ten nanograms of end-labelled primer was annealed with 50 µg total RNA and reverse transcription was performed with AMV reverse transcriptase (Roche) at 42 °C for 90 min. As a reference, sequencing reactions were performed with the ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham) with the same primer as used to map the 5' termini of *astR* mRNA, AstA4. Quantification of band intensities was performed using the PDQUEST software package (PDI, Humington Station) as follows. A large and identical area including the band to be quantified was delimited for each primer extension line. The background noise of the gel was subtracted and band intensity values were normalized: the raw quantity of each band was divided by the total intensity value of all the pixels measured in the area.

**Swarming capacity.** Tryptone swarm plates containing 1% (w/v) Bacto-Tryptone, 0.5% (w/v) NaCl and 0.3% (w/v) Bacto-Agar were used to test bacterial motility as previously described (Bertin *et al.*, 1999). Cell concentration of each culture was measured and adjusted to OD<sub>600</sub> 2 before 5 µl of the culture was applied to the swim plate.

**Antibiotic plate assay.** LB plates were spot inoculated with 24 h-old-broth cultures of each strain to be tested and incubated for 3 days. Ten millilitres of sterile soft agar was allowed to cool to 45 °C before being inoculated with 100 µl indicator strain culture (OD<sub>600</sub> 0.2). After mixing, it was poured onto the plates, which had just been exposed to chloroform for 2 h to kill the spotted colonies. An inhibition zone around a spot indicated the production of antibiotics.

**Bacterial survival assays.** Schneider medium (10 ml in a 250 ml glass Erlenmeyer flask) was inoculated with 0.3 ml of an overnight culture of *P. luminescens* strain TT01 grown in LB medium at 30 °C and 140 r.p.m. and incubated under the same conditions. Samples were removed periodically, diluted in Schneider medium and plated on nutrient agar plates, which were then kept in the dark at 30 °C. Starvation survival was measured by taking samples every 48 h and determining c.f.u. ml<sup>-1</sup>.

**In vivo pathogenicity assays.** The pathogenicity assays were performed on the common cutworm *Spodoptera littoralis* as previously described (Givaudan & Lanois, 2000). Briefly, a 20 µl sample of exponentially growing bacteria was diluted (10<sup>-4</sup>) in PBS and injected into the haemolymph of 20 fifth-instar larvae of *S. littoralis* reared on an artificial diet. The larvae were then individually incubated at 23 °C for up to 96 h, and the number of c.f.u. determined by plating dilutions on LB agar. About 1000–5000 bacterial cells were injected into insects. Insect death was monitored every 5 h.

**Analytical two-dimensional (2D) gel electrophoresis.** Exponential- and stationary-phase cells (50 ml) were harvested by centrifugation. The cell pellets were washed with ED minimal medium (120 mM potassium phosphate buffer, 3 mM trisodium citrate) and resuspended in 1 ml distilled water. After DNase and RNase treatment, cells were disrupted with an FP120 FastPrep Cell disruptor (Bio 101) (twice for 30 s at maximum speed with 1 min intervals on ice). Cell debris was removed by ultracentrifugation for 60 min at 90 000 g.

Isoelectric focusing (IEF) was done with the horizontal Multiphor II system (Pharmacia) at 20 °C (Gorg *et al.*, 1987, 1988). For analytical gels, 60 µg protein was solubilized in 400 µl rehydration solution [0.5% (v/v) Pharmalyte 3–10, 8 M urea, 65 mM DTT, 2% (v/v) Nonidet P40], and loaded onto an 18 cm pH 4–7 L immobilized pH gradient strip (IPG) using the in-gel rehydration technique (Rabilloud *et al.*, 1994). For preparative gels, 120 µg protein was solubilized as mentioned above. For both analytical and preparative gels, focusing was performed for 3 h at 300 V, 1 h at 750 V, 30 min at 1500 V, 16 h at 2500 V and 2 h at 3500 V (total = 50 kVh). The IPGs were equilibrated as previously described (Gorg *et al.*, 1987). The second dimension was performed with 11.5% (w/v) SDS-polyacrylamide gels using the Protean II xi 2D Multicell system (Bio-Rad). Proteins were stained with silver nitrate and gels were digitized using a JX-330 scanner (Sharp). Digitized 2D gel patterns were edited and matched using the PDQUEST software package.

To account for non-specific variations, a minimum of six gels were run for both strains and conditions (exponential or stationary phase) using two independent protein preparations extracted from two independent cultures. Protein levels were expressed as percentage volume, which corresponds to the percentage ratio between the volume of a single spot and the total volume of all spots present in a gel. The mean intensity values of each spot were calculated on at least three gels. Spots showing large variation between replicates were not considered.

**MALDI-TOF mass spectrometry and database searching.** MS analyses were performed using a matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) Voyager-DE-STR mass

spectrometer (Applied Biosystems), operated in positive ion reflector mode. Protein spots of interest were cut out and digested with trypsin (Roche) as described previously (Shevchenko *et al.*, 1996). Peptide mixtures were desalted with ZipTip C18 (Millipore) and analysed using a saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (Sigma) in acetonitrile containing 1% trifluoroacetic acid (Sigma) (50/50, v/v). The trypsin autolysis peptides were used as internal calibrants. Peptides were selected in the mass range 800–3000 Da. A local copy of the MS-FIT program, developed by the University of California at San Francisco, was used to search the *P. luminescens* database (Duchaud *et al.*, 2003). Search parameters were as follows: monoisotopic masses, maximum allowed peptide mass error of 50 p.p.m., consideration of one incomplete cleavage per peptide, and oxidation of methionine. No restrictions on molecular mass or pI were made. A minimum of four matching peptides was required for protein identification in the database. To identify low-molecular-mass proteins, post-source decay (PSD) experiments were performed with the MALDI instrument.

The amino acid sequence similarity search was carried out using the BLASTP software (Altschul *et al.*, 1990; Altschul & Lipman, 1990).

## RESULTS

### Identification of the AstR–AstS two-component system

The genome sequencing of *P. luminescens* TT01 was recently completed. Nineteen two-component regulatory systems were found (Duchaud *et al.*, 2003; GenBank accession no. BX470251). One of these, here called AstRS ('Adaptation to Stationary-phase Regulator and Sensor'), showed some similarity with the known two-component system BvgA–BvgS of *Bordetella* spp., a member of a family of signal-transducing proteins that communicate via a four-step His–Asp–His–Asp phosphorelay (Uhl & Miller, 1996). Furthermore, the predicted sensor and regulator are similar in size to the *Bordetella parapertussis* proteins BvgS and BvgA (1203 vs 1238 residues and 208 vs 209 residues, respectively) and share 28.3% and 21.3% identity (and 47% and 48% conservative replacement) at the amino acid level (Fig. 1). BvgS is a complex membrane-bound sensor kinase containing a periplasmic region at its N-terminus and several distinct cytoplasmic domains: the linker, histidine kinase, response regulator and second transmitter domains (Uhl & Miller, 1996). *P. luminescens* AstS exhibited a similar domain organization (Fig. 1b, c). The first two domains that participate in the putative phosphorelay (first transmitter and receiver) are the most conserved (38.1% and 39.3% identity), especially the sequences flanking the phosphorylation site of each domain. Most of the differences were found in the putative periplasmic domain, delineated by two hydrophobic *in silico*-predicted transmembrane sequences (TM) predicted by TopPred II (Claros & von Heijne, 1994). BvgA and AstR are both typical response regulators, each containing an N-terminal receiver domain and a C-terminal helix–turn–helix motif (Uhl & Miller, 1996).

The only match found with BLASTP for the sensor was with BvgS of *Bordetella*; however, other regulators exhibited

higher identity with AstR, including a probable two-component response regulator from *Pseudomonas aeruginosa* (PA3714) (41.4% identity) and the RcsB of *Proteus mirabilis* or *Erwinia amylovora* (38.0%). A search for homologous proteins in *Escherichia coli* identified a group of typical bacterial response-regulator proteins belonging to two-component regulatory systems such as RcsB (37.3% identity), NarP (27.3%), EvgS (24.6%), UhpA (23.1%) and UvrY (21.4%). The match with BvgAS is of great interest as the BvgAS system coordinates the transcription of a large set of genes and operons that function during the infectious cycle in *Bordetella* spp. It mediates a morphogenic programme involving the coordinated regulation of virulence genes as well as alteration in cell shape, surface structures and colony morphology. The similarities shared by the *astRS* locus of *P. luminescens* and the *bvgAS* locus of *Bordetella* were therefore analysed further.

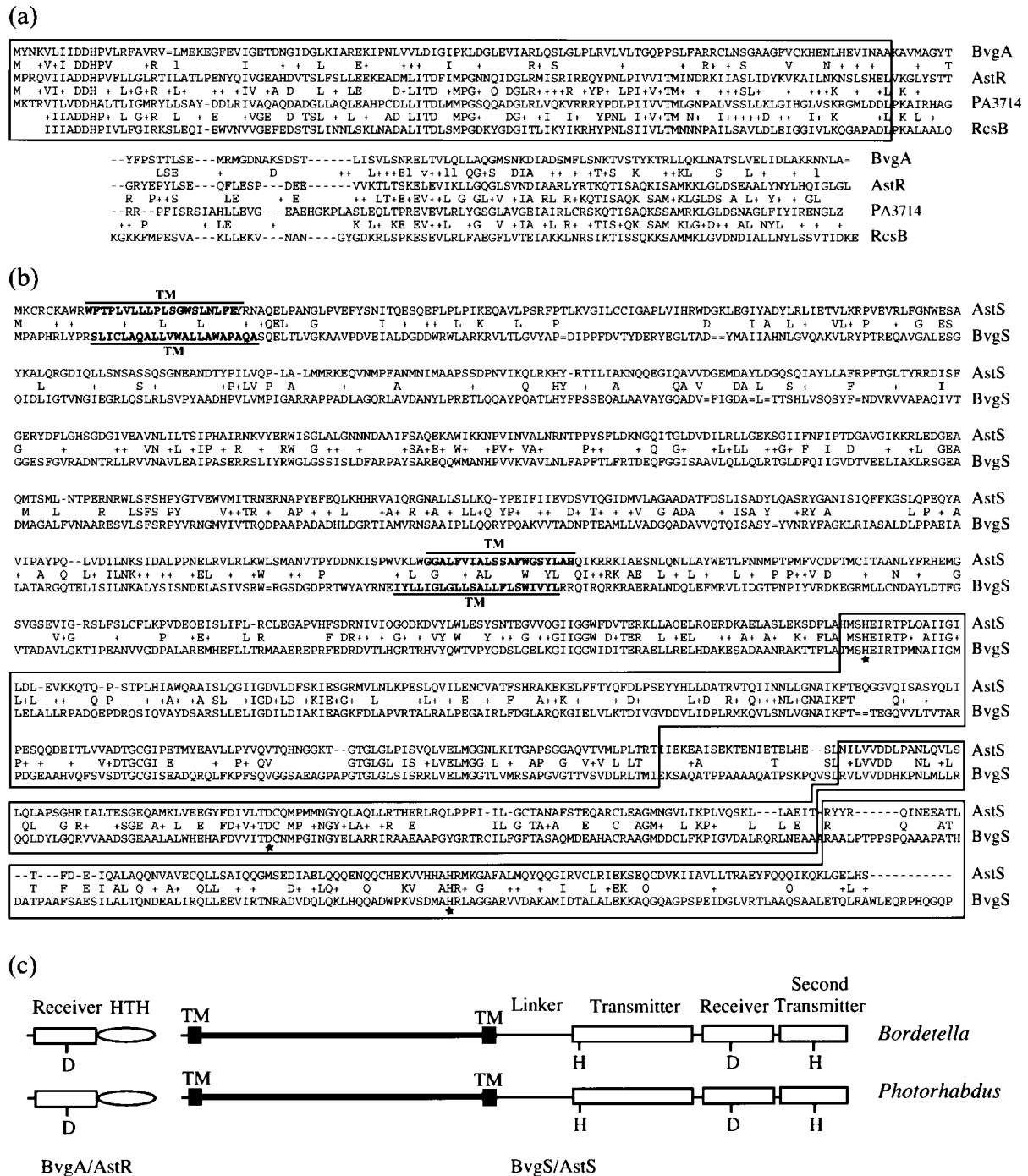
### Characterization and expression of the *astRS* operon from *P. luminescens*

*astS* starts three bases downstream of the coding sequence of *astR*, suggesting that these two genes form a single transcriptional unit. To map the transcription start site of this putative operon, primer extension analysis was performed with 40  $\mu$ g RNA. Total RNA was extracted from *P. luminescens* during the exponential growth phase and hybridized with primer BvgA4, which is specific to *astR*. The start point is located at a guanosine residue located 72 bp upstream of the translation start codon of *astR* (Fig. 2a) and is preceded by very poor –35 and –10 consensus sequences (TCGCTT-17 bp-GAAAAT).

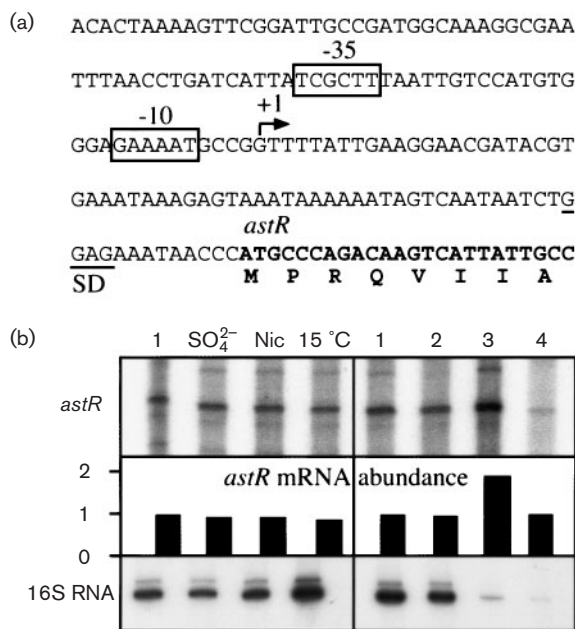
We then measured the levels of *astR* mRNA during a complete growth cycle at 30 °C (Fig. 2b). This revealed that the abundance of the *astR* mRNA is quite stable during the bacterial growth cycle, with an increase (about two-fold) upon entry into stationary phase. Unlike the *astR* transcript, 16S rRNA drastically decreased in abundance at the end of the exponential phase. Differences in environmental conditions, such as the presence of sulphate or nicotinic acid, or growth at low temperature, are known to modulate BvgS activity in *Bordetella* spp. To explore the sensing function of AstS in *P. luminescens*, we investigated the effect of various concentrations of nicotinic acid and MgSO<sub>4</sub>, and growth at low temperatures (15 and 20 °C), on *astRS* expression. We hypothesized that if AstRS is autoregulated as BvgAS, then a signal to which AstS responds should alter the level of *astR* transcript. No significant difference in transcript abundance was detected in such conditions (Fig. 2b), suggesting that AstRS may control a completely different regulon to BvgAS.

### Construction and phenotypic characterization of an *astR* mutant

To obtain information about the functional role of the AstRS two-component system in *P. luminescens*, the regulator component AstR was inactivated by allelic exchange.



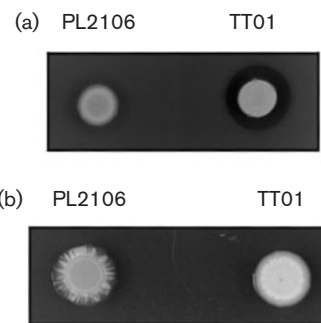
**Fig. 1.** (a) Sequence alignment of *P. luminescens* regulator AstR with other two-component response regulators: *Bordetella parapertussis* BvgA, *Pseudomonas aeruginosa* PA3714 probable regulator and *Proteus mirabilis* RcsB. Putative receiver domains that contain the catalytic site and the site of phosphorylation (D) are boxed. The site of phosphorylation is marked in bold. The C-terminal regions contain the putative helix–turn–helix motifs thought to mediate sequence-specific DNA binding. (b) Sequence alignment of the BvgS sensor from *B. parapertussis* and the AstS sensor from *P. luminescens*. Domains that participate in the phosphorelay (histidine kinase, response regulator and second transmitter domains) are boxed and the site of phosphorylation in each domain is marked by a star. Hydrophobic transmembrane sequences (TM) are underlined and written in bold. (c) Comparative schematic diagram of the BvgAS and AstRS systems. Domains that participate in the phosphorelay (transmitter, receiver, second transmitter) are boxed in this order and their site of phosphorylation labelled. Periplasmic domains are represented by thick lines and are delineated by two hydrophobic transmembrane sequences (TM), indicated by black boxes. The linker region joins the periplasmic domain to cytoplasmic domain. The C-terminal helix–turn–helix motifs (HTH) of BvgA and AstR are shown. All domains were delimited by similarity with those of BvgAS of *Bordetella* spp.



**Fig. 2.** (a) Nucleotide sequence of the 5'-region of *astR* from *P. luminescens*. Promoter sequences (-35 and -10 boxes) are boxed and the transcriptional start site is indicated by an arrow (+1). The Shine-Dalgarno sequence (SD) is underlined. The first few codons of the coding sequence of *astR* are shown in bold. (b) Primer extension analysis of *astR* mRNA and 16S rRNA abundance following exposure to various environmental conditions and at various times during *P. luminescens* growth at 30 °C in Schneider medium. Lanes: 1, exponential growth; 2, linear growth; 3, early stationary phase; 4, stationary phase; SO<sub>4</sub><sup>2-</sup>, supplemented with 30 mM MgSO<sub>4</sub>; Nic, supplemented with 10 mM nicotinic acid; 15 °C, grown at 15 °C for 3 h. As *P. luminescens* synthesizes a variety of secondary metabolites that interfere with optical density measurement during the late exponential phase, correlations between c.f.u. and OD<sub>600</sub> measurements were used to define the various growth stages. Equal amounts of each RNA (50 µg) were used. Experiments were done in triplicate and the data presented are from one representative experiment. In the graphical representation of the relative *astR* mRNA abundances, the lowest mRNA level was taken as 1.

A mutant strain, PL2106, was constructed with a plasmid harbouring a kanamycin-resistance cassette inserted into *astR* (see Methods). In this construct, the insert is unlikely to exert a polar effect on *astS* because of its convergent transcription. The *astR* mutation had no effect on exponential growth rate or on cell morphology. Exponential-phase cells were mainly rod-shaped, although they became increasingly pleiomorphic with the appearance of coccoid bodies during the stationary phase. Stationary-phase cells harboured one or two crystal inclusions (visualized by phase-contrast microscopy).

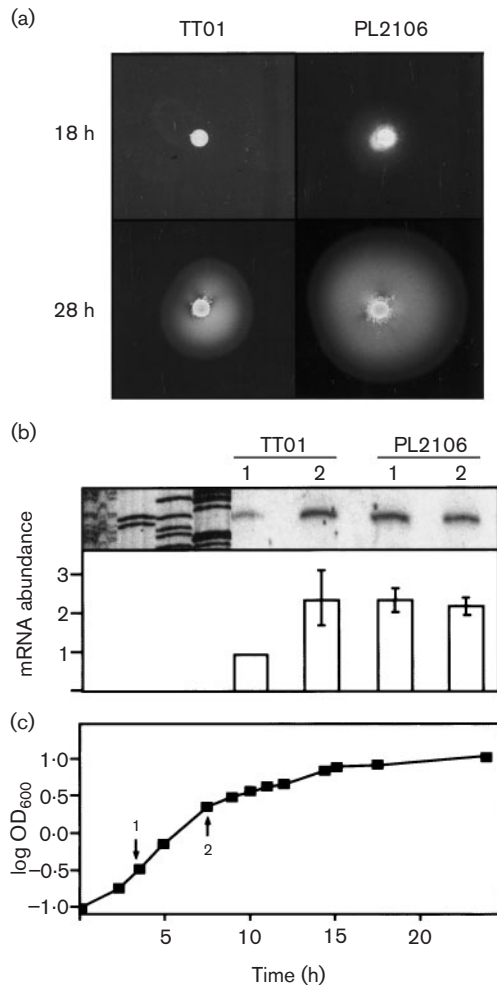
We then examined several phenotypic traits of PL2106. In our assays, both PL2106 and TT01 adsorbed the blue dye



**Fig. 3.** (a). Analysis of the effect of the *astR* mutation on the ability of *P. luminescens* to produce antibiotics. Antibiotic production was visualized by the antibiosis of lawns of *Enterobacter cloacae* as indicator strain. Three-day-old LB plates spotted with 5 µl of both TT01 (wild-type) and PL2106 were inoculated with 100 µl of indicator strain culture (OD<sub>600</sub> 0.2) mixed in soft agar. Growth inhibition around a spot indicates the production of antibiotics to which the indicator strain is sensitive. Experiments were repeated at least three times. (b) Comparative morphology of *P. luminescens* TT01 and PL2106 spotted colonies grown on LB solid medium after 5 days at 30 °C.

when incubated on NBTA (nutrient agar supplemented with 25 mg bromothymol blue l<sup>-1</sup> and 40 mg triphenyl-tetrazolium chloride l<sup>-1</sup>), were bioluminescent and pigmented, although pigmentation was less intense in the mutant. We also compared antibiotic production by the mutant and wild-type (Fig. 3a) using various clinical bacterial isolates as indicator strains (Derzelle *et al.*, 2002). In similar conditions, the inhibition haloes caused by PL2106 on each indicator strain tested were smaller than those produced by the wild-type strain, demonstrating that the mutant produces less antibiotics than does TT01.

Another trait affected by the mutation was the ability to swim on agar surfaces. We examined motility on 0.3% (w/v) agar and found that the *astR* mutant reproducibly migrated farther from the point of inoculation than did the wild-type, whatever the growth phase of cultures spotted into the swim plates (OD<sub>600</sub> values ranging from 0.8 to 5 were tested). This was caused by an early onset of the swarming behaviour: in early to mid-exponential phase, the mutant started to swarm 14 h after inoculation, whereas the wild-type strain did so after 20 h (Fig. 4a). At the entry into stationary phase, the mutant and wild-type strains did so after 11 h and 16 h, respectively. Swarming velocity was similar for both strains. In *Bordetella*, BvgA is known to repress motility through an analogue of *flhDC*, the flagellar master operon. Analysis of *flhDC* mRNA levels in *P. luminescens* by primer extension indicated that negative regulation by AstR occurs at the level of transcription during the exponential growth phase (Fig. 4b, c). The twofold induction of this operon at mid-exponential



**Fig. 4.** (a) Motility of the *P. luminescens* TT01 and PL2106 strains on plates of semi-solid (0.3%, w/v, agar) medium. Plates were incubated for 18–28 h at 30 °C. (b) Primer extension analysis of *flhDC* mRNA abundance in TT01 and PL2106 strains at various times during *P. luminescens* growth at 30 °C in Schneider medium. Lanes: 1, exponential growth; 2, late exponential growth. Equal amounts of each RNA sample (50 µg) were used. The relative *flhD* mRNA abundances at different times during *P. luminescens* growth, with the lowest mRNA level taken as 1, are represented graphically. Each bar represents the mean  $\pm$ SD of results from three independent experiments. (c) *P. luminescens* TT01 growth curve indicating the times when mRNA was extracted.

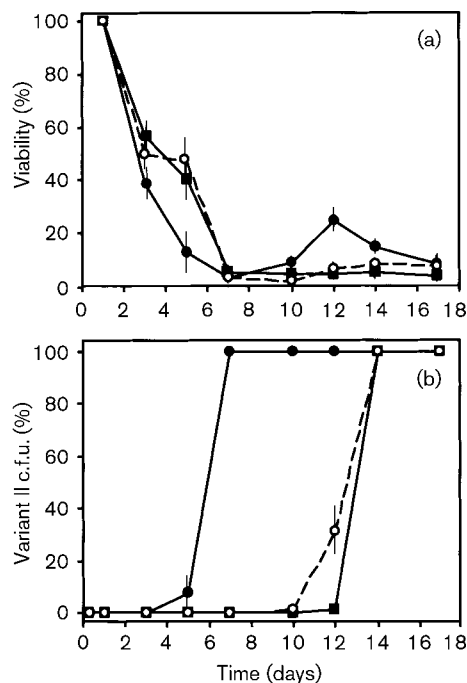
growth phase in the mutant probably explains the early onset of swarming observed in the mutant.

To assess the effect of the *astR* mutation on virulence in insects, wild-type or mutant cells were injected into the haemocoel of the lepidopteran *Spodoptera littoralis*. Insect mortality was subsequently monitored for 3 days. Both strains killed almost all injected larvae within 36 h and septicaemia was observed in every case (data not shown), showing that AstR is not required for virulence.

### The *astR* mutation induces early phenotypic variation in stationary-phase culture

When analysing PL2106, we noticed that some of the spotted colonies grown on solid medium displayed at their periphery a different morphology and pigmentation after several days of incubation at 30 °C (Fig. 3b), suggesting that part of the population had undergone phenotypic variation. Spontaneous switching is known to occur with the wild-type strain after long-term growth in artificial media (Boemare *et al.*, 1997). Analysis of the cells found on the periphery demonstrated that these cells were true secondary variant cells, as they were altered in all the primary-specific properties examined. They did not produce any crystal inclusions, and were impaired in bioluminescence and antibiotic production. They exhibited no evidence of adsorption of neutral red from MacConkey agar and were red when grown on NBTA (Boemare *et al.*, 1997). The colonies also lost the ‘stickiness’ of variant I cell colonies and were translucent with irregular edges (Forst & Clarke, 2002), and the cells resumed growth faster than did variant I cells following periods of starvation (Bleakley & Nealson, 1988; Boemare *et al.*, 1997). Moreover, the cells displayed a haemolytic activity at least twice that of the wild-type variant I, as recently described for variant II of the same strain TT01 (Brillard *et al.*, 2002). Finally, their phenotype was stable and they were never found to revert to variant I.

This observation prompted us to examine the survival of *P. luminescens* in starvation conditions in liquid medium. For this purpose, we monitored the survival of the *astR* mutant and the wild-type during long-term culture in Schneider medium (Fig. 5a). Every 2 days, samples of the stationary-phase cultures were diluted, plated onto nutrient agar and c.f.u. counted 72 h later. The number of variant II colonies was simultaneously counted (Fig. 5b). The number of c.f.u. decreased steadily over 1 week for both strains, although initially the viability of the *astR* mutant decreased more rapidly. A plateau was then reached and about 3–5% of both wild-type and *astR* populations were able to survive for longer periods. At the end of the first week, the number of c.f.u. in the mutant culture started to increase for a few days (Fig. 5a); at this time, all mutant c.f.u. were composed of variant II cells (Fig. 5b). As previously described, the colonies were secondary-like in all phenotypes tested and remained stable when subcultured. The growth observed is therefore probably due to the ability of the secondary variants to grow further in media that have been partially depleted by primary variants, as reported by Boemare *et al.* (1997). As illustrated in Fig. 5(b), the *astR* mutation induced a phenotypic shift to secondary variant of all cells in the population a few days after the culture entered stationary phase. Similar phenotypic switching did not occur in the wild-type population until the culture had been incubated at 30 °C for at least 10 days. Complementation of PL2106 with pDIA608, an intermediate copy number plasmid carrying *astR*, restored a wild-type behaviour (Fig. 5). Phenotypic switching occurred at the same



**Fig. 5.** (a) Viability during long-term culture: TT01 (■), PL2106 (●) and PL2106 complemented for *astR* with pDIA608 (○, dashed lines). Results are expressed as the number of viable c.f.u. as a percentage of the total number of c.f.u. counted on day 1 (i.e. about  $3 \times 10^6$  c.f.u.). Means of three independent experiments are shown  $\pm$ SD. (b) The appearance of secondary-type cells in the previous long-term cultures over time. The number of variant II colonies is expressed as a percentage of total viable c.f.u.

time in the complemented mutant culture and in the wild-type strain culture, i.e. after at least 10 days in stationary phase. The effect on phenotypic variation is therefore specific to *astR*.

Interestingly, we noticed that the time at which the variant II shift occurred varied simultaneously for both strains from one experiment to another, indicating the presence of a subtle systematic bias in the experimental conditions. Depending on the experiment, PL2106 cultures completely shifted to variant II after between 2 and 9 days of culture, whereas the wild-type cultures completely shifted after between 11 and 18 days. However, phenotypic switching always took place about 1 week earlier in PL2106 than in TT01. One reason for this variation could be change in oxygen availability from one set of experiments to another. Indeed, less aerated cultures (4 ml medium and 12 ml flasks) shifted much more rapidly than well-aerated cultures (10 ml medium and 250 ml flasks) (data not shown). This is consistent with previous data showing that stress conditions, such as lack of oxygen, induce the formation of secondary cells (Boemare *et al.*, 1997). In addition, the phenotypic switching was found to be independent of the

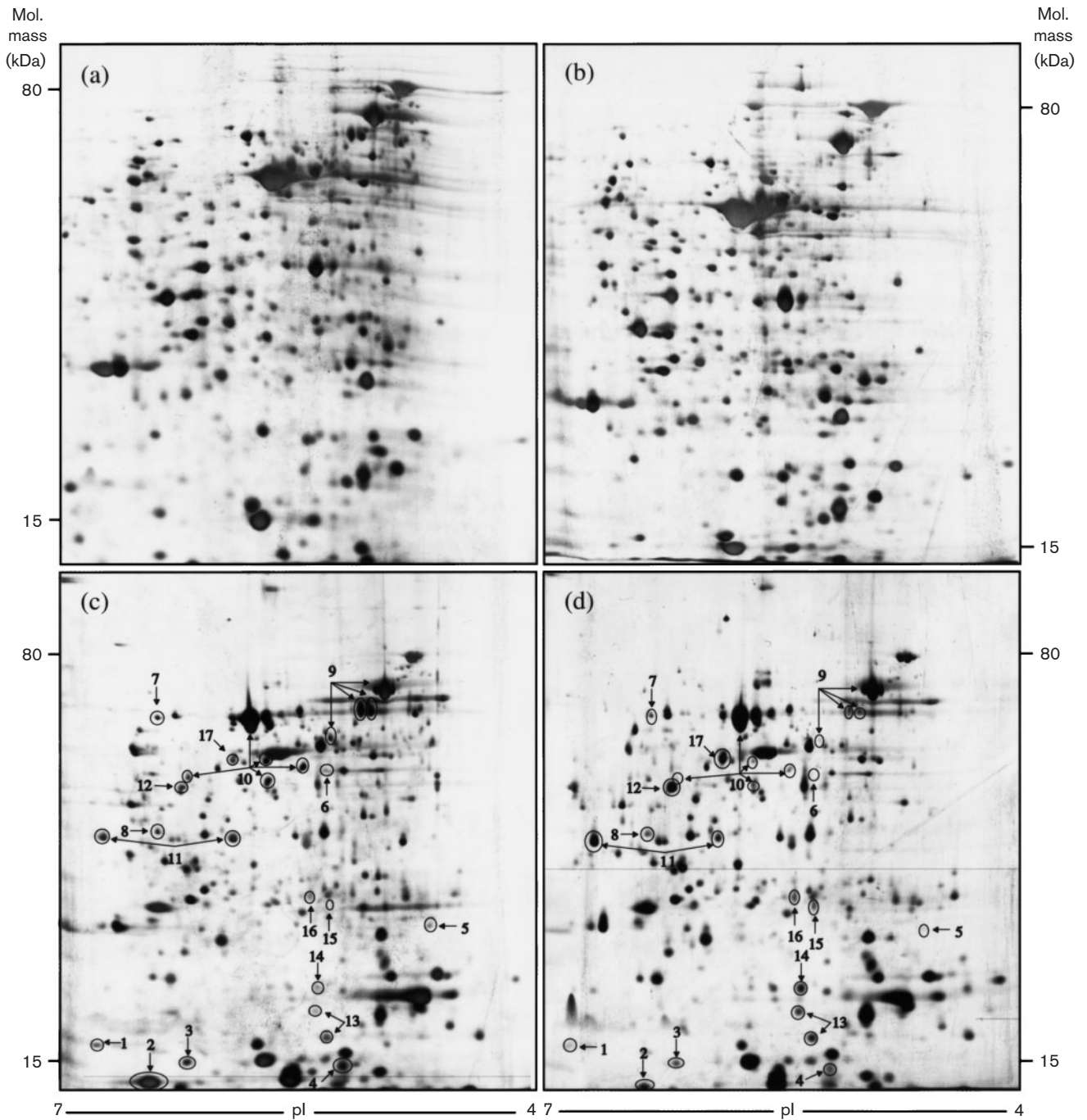
growth medium used, as similar observations were made on LB and Schneider media.

### Comparative analysis of the 2D protein pattern associated with the *astR* mutation

To obtain insight into the role that AstRS plays in adaptation and survival in stationary phase, and phenotypic variation processes, we next attempted to identify the proteins that are produced under the control of the AstRS system. For this purpose, proteome modifications generated by the disruption of *astR* were explored using 2D electrophoresis to visualize and to identify the AstR targets. Strains PL2106 and TT01 were grown in Schneider medium to exponential or stationary phase. After disrupting the cells, proteins were separated on a 2D SDS-PAGE gel, silver stained, scanned and analysed. Representative patterns of silver-stained proteins are shown in Fig. 6. The overall profile of total soluble proteins in both strains was found to be identical during the exponential growth (Fig. 6a, b), whereas some differences were observed in the stationary phase (Fig. 6c, d). Several polypeptides were affected (by at least a factor of two) when *astR* was inactivated. Some were up-regulated, others were down-regulated. Using the whole-genome sequence data (Duchaud *et al.*, 2003) and MALDI-TOF MS (Shevchenko *et al.*, 1996), we identified these spots without ambiguity (Table 1).

A significant increase in the synthesis of the following seven proteins was observed. (i) A probable amidinotransferase that is highly similar (71% amino acid identity) to an L-arginine : lysine amidinotransferase from *Pseudomonas syringae* pv. *phaseolicola* and to L-arginine : glycine amidinotransferases found in man and animals (48–50% identity). (ii) A protein similar to carbonic anhydrases. Carbonic anhydrases are zinc-containing enzymes that catalyse the interconversion of carbon dioxide and bicarbonate. *P. luminescens* contains multiple carbonic anhydrase genes, as do many other prokaryotes. The one identified in the *AstR* background is a  $\beta$ -class carbonic anhydrase, showing similarity with gene products identified in *Mycobacterium tuberculosis* (40% identity), *Sulfolobus solfataricus* (34%) and *Methanothermobacter thermoautotrophicus* (32%). (iii) The peptidyl-prolyl *cis-trans* isomerase B (PpiB). (iv) Two spots were identified as being the bacterioferritin comigratory protein (Bcp), a thioredoxin-dependent hydroperoxide peroxidase that belongs to the peroxiredoxin (Prx) family of enzymes. (v) The molybdopterin biosynthesis protein (MoeB), which is implicated in the synthesis of the cofactor molybdopterin (MPT). (vi) A protein showing a very strong level of identity with a putative periplasmic substrate-binding transport protein of *Yersinia pestis* (69%) and some similarities with iron(III)-binding protein ABC transporter from *Agrobacterium tumefaciens* (38%), the ferrisiderophore receptor Irp6A of *Corynebacterium diphtheriae* (37%) and the iron(III) dicitrate ABC transporter of *Methanosarcina acetivorans* (35%). (vii) Two spots identified as a protein similar to ferrichrome ABC transporter of *Bacillus subtilis* (38% identity).





**Fig. 6.** Comparison of the protein synthesis patterns of *P. luminescens* TT01 (a, c) and PL2106 (b, d). Cells were grown in Schneider medium at 30 °C for 6 h (exponential growth phase) (a, b) and 24 h (stationary phase) (c, d). Proteins were separated in IPG pH 4–7 gels in the first dimension and in 11.5% (w/v) polyacrylamide gels in the second dimension. After silver staining, proteins regulated by AstRS were submitted to MALDI-TOF MS. Identified proteins are labelled with arrows. 2D gels were repeated at least three times for each strain and condition.

The amounts of ten proteins decreased: (i–ii) two proteins similar to several universal stress proteins and highly similar to the nucleotide-binding proteins of the *uspA* family (we have accordingly named the two proteins UspB and UspC); (iii) UspA, the universal stress protein A, a

general responder to growth inhibitory conditions, which accumulates following a large number of different environmental insults, including depletion of essential nutrients (Nyström & Neidhardt, 1994); (iv) a protein weakly similar to a hypothetical protein from *Streptococcus pneumoniae*

**Table 1.** Proteins with altered level of synthesis in the *astR* mutant PL2106

No.	AA*	pI <sub>th</sub> †	pI <sub>ob</sub> †	MM <sub>th</sub> ‡	MM <sub>ob</sub> ‡	Sequence coverage (%)	Induction ratio TT01/PL2106§	Homology
1	146	6.98	6.80	15.91	15.70	62	5 ×	Similar to conserved universal stress protein, UspB
2	210	5.57	5.90	23.71	13.80	11	5 ×	Weakly similar to hypothetical protein from <i>Streptococcus</i>
3	143	6.43	5.80	15.28	13.80	82	4 ×	Similar to conserved universal stress protein, UspC
4	144	5.41	5.10	15.77	15.80	37	3 ×	UspA, universal stress protein A
5	258	5.13	4.80	27.34	27.50	23	5 ×	ArgB, arginine biosynthesis
6	373	6.51	5.10	41.55	40.20	16	5 ×	Weakly similar to triacylglycerol lipase from <i>Candida</i>
7	477	6.40	5.80	50.47	51.30	45	5 ×	LpdA, dihydrolipoamide DH
	477	6.29	5.80	51.37	51.30	31	5 ×	Aldehyde DH, putative betaine-aldehyde DH
8	328	6.57	5.75	35.19	35.20	31	3 ×	Qor, quinone oxidoreductase
9	549	5.08	4.90	57.43	59.50	34	5 ×	GroEL, molecular chaperone
10	495	5.73	5.50	54.18	53.60	33–61	5 ×	Aldehyde DH
11	317	8.43	6.40	34.18	34.70	25	1/3	Probable ferrichrome ABC transporter
12	375	6.86	5.70	40.65	38.80	68	1/3	Iron(III) ABC transporter, iron-binding protein
13	157	5.48	5.20	17.62	16.60	74	1/2	Bcp, hydroperoxide peroxidase
14	165	5.44	5.20	18.26	17.70	41	1/2	PpiB, peptidyl-prolyl <i>cis-trans</i> isomerase
15	194	5.38	5.2	21.07	28.90	27	1/5	Carbonic anhydrase
16	251	6.58	5.4	27.32	29.55	15	1/2	MoeB, molybdopterin biosynthesis
17	367	5.85	5.6	42.16	42.00	54	1/3	Probable amidinotransferase (L-Arg:Gly/L-Arg:Lys)

\*Number of amino acid residues.

†pI<sub>th</sub>, pI<sub>ob</sub>, theoretical and observed isoelectric point.

‡MM<sub>th</sub>, MM<sub>ob</sub>, theoretical and observed molecular mass.

§Induction ratios were calculated on mean of three values after normalization. In the normalization method used, the raw quantity of each spot in a member gel is divided by the total intensity value of all the pixels in the image.

||The MALDI-TOF MS spectrum of spot 2 did not lead to an unequivocal result. The ion with *m/z* 1553.7389 was chosen for MALDI post-source decay (PSD) analysis.

(27% identity and 50% conservative replacement); (v) ArgB, an acetylglutamate kinase involved in the biosynthesis of arginine; (vi) the quinone oxidoreductase Qor, a protein similar to the  $\xi$ -crystalline protein found in the liver and kidneys of higher organisms, where it acts through a one-electron transfer process to produce the semiquinone radical (Thorn *et al.*, 1995); (vii) two dehydrogenases (DHs) found in the same spot – LpdA, the dihydrolipoamide DH E3 (LPD) component of both pyruvate and 2-oxoglutarate DH complexes, and an aldehyde DH with some homology with putative betaine-aldehyde DH; (viii) a protein showing in its internal region some similarity to triacylglycerol lipase protein of *Arabidopsis thaliana* (40% identity) and *Candida ernobii* (33%); (ix) five spots identified as isoforms of a predicted aldehyde DH showing the highest amino acid identities with aldehyde DH B of *Fusobacterium nucleatum* (54%); and (x) four spots identified as isoforms of the chaperonin GroEL.

In conclusion, the proteomic analysis indicated that the *astR* deletion affected the synthesis in stationary growth phase of proteins involved in electron-transport systems, energy metabolism and iron acquisition, as well as universal stress proteins, antioxidant protein and molecular chaperones.

## DISCUSSION

AstRS is a novel two-component signal transduction system identified in *P. luminescens*. It is somewhat similar to the BvgAS regulatory system from *Bordetella* spp. AstR is composed of an N-terminal receiver domain and a C-terminal helix–turn–helix motif. Its sensor, AstS, contains three domains, the histidine kinase, response regulator and second transmitter domains, which are essential for signal transduction via a four-step His–Asp–His–Asp phosphorylation specific to the BvgS family (Uhl & Miller, 1996). Analysis of this system revealed that AstRS is not an exact homologue of BvgAS. In *Bordetella*, BvgA (Bvg<sup>+</sup> phase) induces the expression of all known virulence factors required for infection and the Bvg<sup>-</sup> phase is characterized by the loss of virulence (Kinnear *et al.*, 2001; Lochter *et al.*, 2001). The situation in *P. luminescens* is quite different as the organism shuttles between two partners: a nematode vector, in which colonization is not pathogenic, and an insect host, which is killed by the bacteria. *P. luminescens* does not require AstR for virulence, but its deletion affects the phenotypic variation process that can prevent the bacterium from colonizing the nematode vector. The *astR* mutant is as virulent as its wild-type parent and proliferates normally when injected

into the haemolymph of insect larvae. In addition, *astRS* expression is not affected by the signals that modulate *bvgAS* expression and activity (i.e. low temperature,  $\text{MgSO}_4$  or nicotinic acid) in *Bordetella* spp. (Locht *et al.*, 2001; Miller *et al.*, 1992), suggesting that AstS may respond to a different, not yet identified, environmental stimulus than BvgS. Sequence data are consistent with this idea, as the N-terminal regions of both sensors are poorly conserved. This region includes the periplasmic domain, which senses the environmental input.

Nevertheless, some functional similarities exist between the two signal transduction systems. Both systems control swarming in their respective species (Akerley & Miller, 1993; Han *et al.*, 1999). A prominent feature of the Bvg<sup>-</sup> phase is the motility phenotype. In *Bordetella* spp., the flagellar genes, which are regulated as a cascade at the top of which is an analogue of *flhDC*, are negatively regulated by BvgAS. Similarly, in *P. luminescens*, the *astR* mutant PL2106 is more motile than its parental strain. Primer extension analysis of *flhDC* mRNA abundance indicates that this operon is negatively regulated by AstRS in *P. luminescens*. In addition to flagella, both systems also control genes involved in iron acquisition. In *B. pertussis*, BvgA activates a TonB-dependent siderophore receptor named BfrD (Antoine *et al.*, 2000), whereas in *B. bronchiseptica* strains, the production of the siderophore alcaligin is induced in the Bvg<sup>-</sup> phase (Giardina *et al.*, 1995). In *P. luminescens*, we identified two putative iron-transport proteins among the proteins up-regulated in the *astR* background: a probable iron(III) ABC transporter and the acidic isoform of a probable ferrichrome ABC transporter, which is likely to be involved in the acquisition of exogenous iron, such as ferrichrome.

Analysis of the AstRS system suggests that AstRS plays a role in stationary-phase adaptation and starvation survival in *P. luminescens*. *astRS* is maximally expressed at the onset of the stationary phase and 2D PAGE analysis demonstrated that the protein pattern is specifically affected. It is however unknown whether AstR, directly or indirectly, regulates the genes encoding the proteins whose abundance is altered by its inactivation. Interestingly, three universal stress proteins are down-regulated in the *astR* mutant background during this period: UspA and its paralogues UspB and UspC. Usp proteins have a general protective function in growth-arrested cells and are required for the management of DNA damage in cells entering stationary phase. They become some of the most abundant proteins in stationary phase (Gustavsson *et al.*, 2002). In the *astR* mutant, Usp proteins are no longer induced in the stationary phase. This lack of induction may account for the more rapid decrease in survival observed for the mutant during long-term cultures. The *E. coli* UspA mutant is impaired in its ability to survive growth arrest.

In addition, some of the secondary metabolism molecules synthesized by *P. luminescens* upon entry into stationary phase, antibiotics, pigments and exoenzymes such as the

putative lipase identified in 2D electrophoresis experiments, are produced in smaller amounts in PL2106. This finding is not surprising if AstRS plays a role in the adaptation of *P. luminescens* to stationary-phase growth. Interestingly, this decrease might be connected to the parallel decrease in several isoforms of the chaperonin GroEL in PL2106. GroEL is responsible for the folding, repair and degradation of proteins, particularly when export and translocation processes are altered (Grallert & Buchner, 2001). Some of the pleiotropic effects observed in the mutant may result from the partial loss of GroEL chaperone activities. Once again this is consistent with the nematode biotope of the bacteria. The above-mentioned molecules are surface associated or secreted; therefore they might be affected by lack of GroEL. They are not the only ones, as some universal stress proteins easily acquire unstable conformations and are very sensitive to proteolysis. In *E. coli*, one of these proteins (termed UP12) is a persistent *in vivo* GroEL substrate (Bochkareva *et al.*, 2002).

Another finding supporting the role of AstRS in stationary-phase-related processes is the fact that several of the proteins that are regulated by AstR are enzymes involved in cellular energetics, as entry into the stationary phase and the induction of the anaerobic respiratory chain often go hand in hand. Enzymes important for the metabolism of aerobic organisms were decreased in the mutant (i.e. LPD, Qor and two unknown aldehyde DHs), whereas enzymes important for fermentative micro-organisms and anaerobic metabolism were increased (MoeB). LPD (*lpdA* gene) is an essential component of two complexes playing a crucial role in the central metabolism of aerobic organisms (pyruvate DH and 2-oxoglutarate DH). LPD is able to transfer electrons from NADH to various redox-active compounds and quinones. Excess LPD (Smith & Neidhardt, 1983) is probably involved in transport of reducing equivalents across the membrane and/or in the reduction of membrane-bound quinones (quinone redox cycling) (Owen *et al.*, 1980; Walker *et al.*, 1997; Youn & Kang, 2000; Schwinde *et al.*, 2001). Qor is an electron-transport-associated component with NAD(P)H-dependent quinone redox activity. Quinones play an essential role in hydrogen transfer reactions, e.g. during aerobic and anaerobic respiration (Thorn *et al.*, 1995). The *moeAB* operon encodes proteins connecting molybdate metabolism, molybdopterin synthesis and apomolybdoenzyme synthesis during anaerobic growth (Hasona *et al.*, 2001). As molybdoenzymes play important roles when oxygen is limited, serving a redox function, the synthesis of Mo-cofactor is essential for optimal growth in these conditions (Wootton *et al.*, 1991). Interestingly, in *E. coli*, the transcriptional regulatory complex that induces the anaerobic respiratory chain (which includes several molybdoenzymes) during the transition to stationary phase is FlhDC (Prüß *et al.*, 2001).

Several other proteins (Bcp, PpiB, carbonic anhydrase, putative amidinotransferase) that may play a role in stationary-phase survival were also up-regulated in the

*astR* background. Bcp is a probable antioxidant protein that may be important for coping with oxidative damage, especially during long-term culture. PpiB catalyses protein folding. The induction of this specific chaperone may represent an adaptation that allows protein synthesis and folding to continue during the stationary phase. This function may be particularly important in the stationary phase, when protein synthesis is drastically altered and proceeds very slowly. In *B. subtilis*, peptidyl-prolyl *cis-trans* isomerases are important for growth in starvation conditions (Gothel *et al.*, 1998). The carbonic anhydrase enzyme found in PL2106 may also be important for long-term survival. It has long been known that bacteria are dependent on the presence of CO<sub>2</sub> for growth or for overcoming long lag times (Neidhardt *et al.*, 1974; Repaske & Clayton, 1978). At least two roles have been suggested for this enzyme (Smith & Ferry, 2000). It could provide CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> for enzymic reactions or remove them to improve the energetics of the reaction. This role would be particularly important for decarboxylation reactions coupled to energy generation. In fermentative bacteria, the decarboxylation of dicarboxylic acids can serve as the sole energy source for growth. *P. luminescens* could also take advantage of the interconversion of a freely diffusible uncharged species (CO<sub>2</sub>) to a charged species (HCO<sub>3</sub><sup>-</sup>) to perform various physiological functions such as solute transport. A possible example is the transport of acetate into the cell via a H<sup>+</sup>/acetate symport mechanism using the H<sup>+</sup> generated from the catalysed hydration of CO<sub>2</sub> into HCO<sub>3</sub><sup>-</sup>. Finally, it was intriguing to find an increased level of a probable L-arginine:lysine amidinotransferase in PL2106, whereas the amount of ArgB decreased. This might signify that arginine is no longer synthesized but broken down via a new pathway to be used as source of carbon, nitrogen or energy. The identity (48–50%) found between this enzyme and L-arginine:glycine amidinotransferases of animals is in this respect highly suggestive, as these proteins are involved in creatine biosynthesis (Humm *et al.*, 1994).

One interesting result concerning the analysis of AstRS function is that this two-component transduction system is somewhat involved in phenotypic variation, a fairly elusive phenomenon in *Photorhabdus*. In this respect, the AstRS system shows functional similarities with another two-component signal transduction system studied in *Pseudomonas*, the GacA–GacS system (Bull *et al.*, 2001; Sanchez-Contreras *et al.*, 2002). In *Photorhabdus*, deletion of *astR* creates a background that seems to promote the switch to secondary cells earlier (by about 1 week) during stationary phase. The secondary phenotype is supposed to be a response to environmental conditions not favouring nematode host association. Such a decision would have to be tightly regulated, as secondary cells represent terminal and irreversible variants whose ability to reassociate with the nematode, and therefore to persist in the tripartite association, is compromised. Although appearance of variant II is naturally counter-selected by the infective juveniles in the biotope, the *Photorhabdus* genome probably

contains regulators to control the switch. The AstRS system may be one of those. Its *in vivo* functions may be to ensure that the phenotypic shift occurs only when survival in the primary form is no longer likely. The shift from variant I to variant II cells increases stationary-phase cellular capacities which may be useful for survival outside usual hosts. AstR-regulated proteins may be responsible for some of these properties, as they include proteins involved in electron transport systems, energy metabolism, iron acquisition and oxidative stress. Comparative analysis of the 2D protein pattern associated with phenotypic variation adds weight to the putative role of AstRS in controlling phenotypic variation. Indeed, the relative amount of several *astR*-regulated proteins (i.e. UspA, Bcp, PpiB, MoeB, the carbonic anhydrase and the iron(III) ABC transporter) is similar in the wild-type secondary form to that in the primary *astR* mutant, being up- or down-regulated compared to the wild-type primary form (unpublished data). However, we could not exclude that AstRS may actually affect cell survival and not directly affect phenotypic variation regulation. By decreasing the survival ability of variant I cells, the *astR* mutation may affect their competitive advantage, resulting in a rapid takeover of the secondary variant cells under stationary-phase growth conditions. The level at which AstRS might exert its effect and the nature of the signal it perceives therefore need further investigation.

Finally, our results also suggest that stationary-phase growth, phenotypic variation and symbiosis-specific traits are linked. This is consistent with the findings of others (Bintrim & Ensign, 1998; Ciche *et al.*, 2001; Forst & Clarke, 2002; Joyce & Clarke, 2003). During the mutualistic relationship, *P. luminescens* is expected to require functions for attachment to the nematode intestine, nutrient acquisition or synthesis, molecular communication and resistance to host-imposed stresses, as well as regulatory mechanisms to control the expression of these functions. The identification of regulatory systems affecting the phenotypic switching, such as AstRS, may help us to understand the regulatory pathways controlling symbiosis itself and to identify factors important for symbiosis.

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