Molecular Microbiology (1997) **25** (2), 211–218

Spontaneous duplication of a 661 bp element within a two-component sensor regulator gene causes phenotypic switching in colonies of *Pseudomonas tolaasii*, cause of brown blotch disease of mushrooms

Bin Han, Arnab Pain† and Keith Johnstone*
Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK.

Summary

Spontaneous sectoring of *Pseudomonas tolaasii* colonies results in a phenotypic switch from the smooth, pathogenic form (designated 1116S) to the rough non-pathogenic form (designated 1116R). This phenotypic switch can also be induced by mutation of the *pheN* master regulatory locus, which encodes a 99 kDa protein with homology to the conserved family of sensor regulator proteins. Southern blot analysis of genomic DNA from 1116S and 1116R probed with a 3.4 kb *Xho*I–*Bam*HI fragment containing the *pheN* gene has revealed restriction fragment length polymorphisms in the *pheN* locus of 1116R. In order to characterize the genetic basis of this variation, the *pheN* locus (designated *pheN′*) was cloned from 1116R and its nucleotide sequence determined. A 661 bp duplication was identified within *pheN′* introducing a frameshift mutation in the predicted *pheN* open reading frame (ORF). A resulting predicted ORF of *pheN′* designated ORF2 encodes a polypeptide of 706 amino acid residues, with a predicted molecular weight of 77 kDa, and which lacks part of the PheN sensor domain. Southern blot analysis of genomic DNA using a probe within the duplicated sequence revealed the presence of two bands in 1116R but only one band in the 1116S form. Polymerase chain reaction (PCR) analysis of 25 independently isolated 1116R sectors using primers flanking the duplication site in *pheN* confirmed the presence of the duplicated 661 bp sequence within this region in all of the sectors and the absence of the duplicated sequence in spontaneous revertants from 1116R to 1116S. Northern blot analysis of RNA from 1116S and 1116R using a *pheN* probe showed that ORF2 was transcribed in the 1116R form. The presence of a truncated PheN protein in 1116R was verified by Western blot analysis of total cell protein using a LemA antiserum, which revealed the presence of 99 kDa and 77 kDa cross-reactive bands in 1116S and 1116R respectively. It is concluded that the spontaneous colony-sectoring event that results in the 1116R phenotypic variant form of *P. tolaasii* arises owing to a 661 bp DNA duplication within the 5′ end of the *pheN* gene, which results in loss of the periplasmic sensor domain of PheN and elimination of normal PheN function.

Received 7 February, 1997; revised 30 April, 1997; accepted 6 May, 1997. †Present address: Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, UK. *For correspondence. E-mail kj10@cam.ac.uk; Tel. (1223) 333933; Fax (1223) 333953.

Introduction

Pseudomonas tolaasii is the causal agent of the economically important brown blotch disease of the cultivated mushroom *Agaricus bisporus* (Lange) Imbach (Tolaas, 1915). The primary pathogenicity determinant of *P. tolaasii* is the 1975 Da lipodepsipeptide toxin tolaasin (Brodey *et al*., 1991), which has properties both as an ion channel-forming agent (Brodey *et al*., 1991; Rainey *et al*., 1991) and as a biosurfactant (Hutchison and Johnstone, 1993). The wild-type strain of *P. tolaasii* (designated 1116S) is opaque, mucoid, pathogenic and non-fluorescent (Goor *et al*., 1986; Grewal *et al*., 1995). Relatively stable sectors of a phenotypic variant form (designated 1116R) arise from the margins of colonies of 1116S grown on solid media, are translucent, non-mucoid, non-pathogenic and fluorescent (Grewal *et al*., 1995). A 3.4 kb DNA fragment containing the *pheN* gene has been shown to be able to restore the phenotypic characteristics of 1116R to those of 1116S (Grewal *et al*., 1995). In addition, Tn5*lacZ* mutagenesis of the *pheN* locus in the wild-type strain showed that a functional copy of the *pheN* gene is required to maintain the 1116S phenotype and that loss of the *pheN* gene or its function results in conversion of the characteristics of 1116S to those of 1116R (Grewal *et al*., 1995). The *pheN* locus encodes a 99 kDa protein, the predicted amino acid sequence of which shows homology to the sensor and regulator domains of the conserved family of two-component bacterial sensor regulator proteins (Grewal *et al*., 1995), including *lemA*

of *Pseudomonas syringae* pv. *syringae* (Hrabak and Willis, 1992; Rich *et al*., 1994). It was therefore concluded that, in 1116S, PheN acts as a positive regulator required for the expression of a number of phenotypic traits including toxin synthesis, protease secretion and opacity protein production, and as a repressor for chemotaxis and fluorescent pigment synthesis (Grewal *et al*., 1994; 1995).

Phenotypic variation is a strategy frequently used by bacteria to survive disparate environmental conditions and may often be detected as spontaneous and reversible differences in colony morphology (Shapiro, 1986). The ability of bacterial cells to aggregate to form a colony may be influenced by changes in gene expression, altering, for example, cell surface structure and the synthesis of extracellular compounds such as toxins. The resulting changes in the aggregation of bacteria may alter the appearance of parts of the colony as a result of differences in light transmission (Shapiro *et al*., 1993). Phenotypic variation has been shown in bacteria to arise owing to a number of genetic mechanisms, including frameshift mutation as well as DNA duplication, inversion and deletion (Dybvig, 1993). Such events have been observed in structural genes resulting in alteration of gene function, e.g. homologous recombination in the *pilE* and *pilS* loci of *Neisseria gonorrhoeae* (Haas and Meyer, 1986; Stern and Meyer, 1987) and deletions and insertions in the *opa* loci of *N. gonorrhoeae* (Mills *et al*., 1992). In addition, regulatory loci may also be mutated to cause co-ordinated changes in gene expression, e.g. translational frameshift of the poly C tract in the *bvgS* locus of *Bordetella pertussis* (Stibitz *et al*., 1989).

Southern blot analysis of genomic DNA from 1116S and 1116R revealed restriction fragment length polymorphisms (RFLPs) in the *pheN* locus of the phenotypic variant 1116R (Grewal *et al*., 1995). The aim of this study was to determine the molecular basis of this phenotypic switching event. Cloning and sequencing of the *pheN* locus from 1116R as well as polymerase chain reaction (PCR) analysis of genomic DNA from wild-type and phenotypic variant forms were used to demonstrate that the colony-sectoring event is caused by a reversible duplication event in the *pheN* locus. As far as we are aware, this is the first demonstration of such a reversible mutation of a global regulatory locus associated with phenotypic variation of bacteria.

Results

Cloning of the pheN *locus from the phenotypic variant 1116R*

In a previous study, data from Southern hybridization analysis of chromosomal DNA isolated from the wild-type (1116S) and phenotypic variant (1116R) forms of *P. tolaasii* suggested that a chromosomal rearrangement had occurred within the *pheN* locus in 1116R (Grewal *et al*., 1995). This was confirmed by hybridization of a 3.4 kb *Bam*HI–*Xho*I probe containing the *pheN* gene to *Bam*HI genomic digests by which a 17 kb fragment in 1116S and an 18 kb fragment in 1116R was detected (data not shown).

In order to determine the mechanism of DNA rearrangement that had occurred in 1116R, the *pheN* locus from 1116R (designated *pheN′*) was cloned by colony hybridization of a genomic library of the 1116R. A *Bam*HI partial digest genomic library of 1116R was constructed in the cosmid vector pLAFR3 and screened with a 3.4 kb *Bam*HI–*Xho*I fragment containing *pheN*. One positive clone, designated pHBI (Table 1), containing an 18 kb insert was identified, which, unlike the *pheN* locus, when introduced into 1116R, was unable to restore the phenotypic characteristics to those of 1116S. In addition, introduction of pHBI into 1116S did not affect toxin tolaasin production and protease activity. These results suggest that *pheN′* is the result of a mutation in *pheN* that inactivates its function rather than stimulating a transdominant repressor-like activity.

The restriction map of pHB1 was determined (Fig. 1) and compared with the previously published map of the *pheN* locus (Grewal *et al*., 1995). The restriction sites within the 18 kb *Bam*HI fragment mapped on pSISG29 and pHBI were identical except for the presence of a 0.7 kb *Pst*I fragment in pHBI, which was not present in pSISG29 and suggested that a rearrangement had occurred in the *pheN* locus.

Sequence analysis of pheN′ *from 1116R and evidence for a duplication event*

In order to examine the gene rearrangement in the *pheN* locus further, the sequence of the three *Pst*I fragments (Fig. 1) and the 3.5 kb *Eco*RI fragment containing *pheN′* (Fig. 1) from 1116R was determined (GenBank Accession Number U95300) and compared with that of *pheN*. This analysis showed the two loci to be identical, except for an additional 661 bp duplication in *pheN′*, located near the amino-terminus of PheN and 132 nucleotides from the *pheN* start codon, which results in the formation of a new *Pst*I site (Fig. 1). Analysis of the *pheN′* DNA sequence revealed the presence of two putative ORFs. The *pheN′* ORF1 is terminated after 284 amino acid residues (Fig. 2). Two putative translational start codons are present downstream of the *pheN* start codon at GTG 1378 and GTG 1532. Since only the latter of these is preceded by a purine-rich putative ribosome-binding site, it is a probable start codon. Examination of the G + C bias in the third position of each codon suggested that this is an authentic ORF (Bibb *et al*., 1984). Putative consensus −35 and −10 sites (Deretic *et al*., 1989) start at bases 1385 and 1414

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Genotype or description	Reference or source
E. coli strains		
DH5α	*endA1, gyrSA96, hrdR17* ($r_k^- m_k^-$), *supE44, recA1*	Bethesda Research Labs, Gaithersberg, MD, USA
P. tolaasii strains		
1116S	Wild type, Phe$^+$, *met*, Am^r	Grewal *et al.* (1995)
1116R	Spontaneous phenotypic variant Phe$^-$, *met*, Am^r	Grewal *et al.* (1995)
P. reactans strains		
NCPPB387	Am^r, Nx^r	National Collection of Plant Pathogenic Bacteria, Harpenden, UK
Plasmids		
pLAFR3	IncP, *mob*$^+$, *cos*$^+$, Tc^r	Staskawicz *et al.* (1987)
pRK2013	ColE1, *tra*$^+$, Km^r	Figurski and Helinski (1979)
pSISG29	pLAFR3 containing 29.9 kb *Sau* 3 A NCPPB1116S chromosomal DNA cloned into *Bam*HI site	Grewal *et al.* (1995)
pHB1	pLAFR3 containing 18 kb *Bam*HI fragment of *P. tolaasii* 1116R chromosomal DNA cloned into *Bam*HI site	This study
pKSE3	pBluescript-II KS+ containing 3.5 kb *Eco*RI fragment from pHBI cloned into *Pst*I site	This study
pKSP4	pBluescript-II KS+ containing 1.9 kb *PstI* fragment from pHBI cloned into *Pst*I site	This study
pKSP7	pBluescript-II KS+ containing 1.0 kb *Pst*I fragment from pHBI cloned into *Pst*I site	This study
pKSP8	pBluescript-II KS+ containing 0.7 kb *Pst*I fragment from pHBI cloned into *Pst*I site	This study

respectively. The second *pheN′* open reading frame (ORF2) encoded a polypeptide of 706 amino acid residues, with a predicted molecular mass of 77 kDa, which is identical to the corresponding amino acid sequence of PheN. Thus, the *pheN′* ORF2 is smaller than that of *pheN*, which is predicted to contain 910 amino acid residues with a molecular mass of 99 kDa (Grewal *et al*., 1995).

The nature of the DNA rearrangement that switches 1116S to 1116R is illustrated in Fig. 3. As a result of the DNA duplication event, the truncated *pheN′* ORF (ORF1) contains only the sensor domain. In addition, the second putative *pheN′* ORF (ORF2) encodes a protein product lacking 204 amino acids of the N-terminus of PheN.

PCR and Southern hybridization analysis of DNA duplication within the pheN *locus*

In order to confirm that DNA duplication within the *pheN* locus is associated with the 1116S to 1116R phenotypic switch and not an artefact of the cloning or sequencing procedures, PCR analysis using primers flanking the site of the duplication of the *pheN* sequence was performed. The locations of the primers (Phen1 and Phen2) are shown in Fig. 3. PCR analysis was performed on independently isolated 1116R forms as well as on independently isolated 1116R to 1116S revertants in comparison with

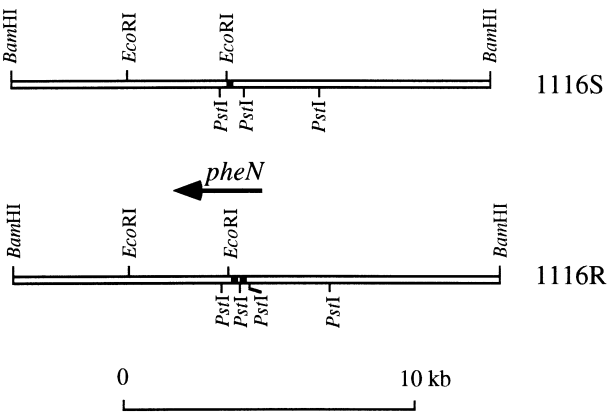

Fig. 1. Deduced map of the *pheN* region in 1116S (Grewal *et al*., 1995) and 1116R. The location and orientation of the *pheN* locus is indicated by an arrow. The additional 700 bp fragment within the *pheN* locus in 1116R is indicated as a solid bar.

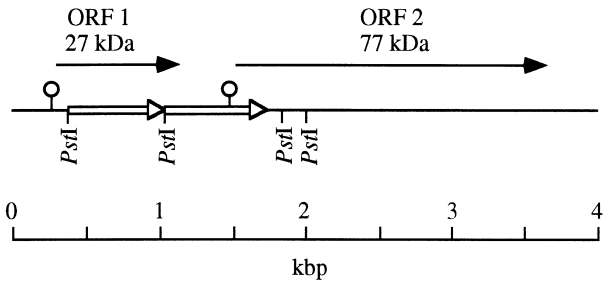

Fig. 2. DNA sequence analysis of *pheN′*. Base numbers refer to Genbank accession number U95300. Putative ORF1 encoding 284 amino acids and corresponding to *pheN* of 1116S starts at base 259, terminates at base 1113 and is preceded by a GGAGAG putative ribosome-binding site at bases 249–2254 (indicated by a circle). Putative ORF2 is preceded by a CAAGCA putative ribosome-binding site at bases 1522–1527, starts at base 1532, ends at base 3652 and encodes a 77.3 kDa protein. Amino acids 1–204 of ORF1 are identical to amino acids 1–204 of PheN, and amino acids 1–706 of ORF2 are identical to amino acids 205–910 of PheN (Grewal *et al*., 1995; GenBank accession number U25692). The 661 bp duplicated sequence is indicated by open arrows.

the wild-type strain. The results showed that the predicted 1.8 kb PCR product was produced from 1116R forms, whereas a smaller 1.2 kb PCR product was observed in the 1116S wild type and in the 1116R to 1116S revertants (Fig. 4). Identical results were obtained for 25 independently isolated 1116R forms and for five independently isolated 1116R to 1116S revertants (data not shown). Southern hybridization of genomic DNA of 1116S and 1116R using the 0.7 kb *Pst*I fragment as probe confirmed that only a single copy of this sequence is observed in 1116S and that two copies are present in 1116R (data not shown). These results therefore confirm that a 661 bp DNA duplication fragment was present in *pheN′* in the 1116R forms and that loss of this 661 bp fragment results in reversion to the 1116S phenotype.

Northern blot and Western blot analysis

In order to establish whether *pheN′* ORF2 is transcribed in 1116R, Northern blotting analysis of total RNA from 1116S and 1116R with the probe of 3.4 kb *Xho*I–*Bam*HI fragment containing *pheN* was performed. The results demonstrated that *pheN′* is transcribed in 1116R to generate a single transcript, which is smaller than that of *pheN* in 1116S and corresponds to ORF2 of *pheN′* (data not shown). This result is in agreement with the conclusions based on nucleotide sequence analysis of *pheN′*. It is not known why a transcript corresponding to ORF1 was not seen. Since the predicted PheN protein showed strong homology (87.5%) to LemA from *Pseudomonas syringae* pv. *syringae,* a polyclonal antiserum raised against LemA sensory α-kinase domain was used to identify the PheN product in 1116S and the pheN′ product in 1116R. The results (Fig. 5) showed that the anti-LemA antiserum identified a 99 kDa cross-reactive protein in the wild-type 1116S, which is assumed to be the PheN protein. In the 1116R form, a 77 kDa cross-reactive protein was detected, which is deduced to be the product of *pheN′* ORF2. The same cross-reactive bands were observed in Western blots using antibodies raised against the LemA response regulator domain (results not shown). No cross-reactive protein was identified that corresponded to the predicted 27 kDa product of *pheN′* ORF1, which corresponds with the results of Northern analysis. These results are therefore consistent with the results of DNA sequencing and Northern blotting experiments for the *pheN* gene in 1116S and the *pheN′* gene in 1116R.

Discussion

Phenotypic variation of bacteria may be regulated by a range of mechanisms including sensory transducers (e.g. two-component sensor regulator protein pairs), which respond to specific signals in a changing environment (Miller *et al*., 1989) or through the generation of genetic variants that provide the diversity needed to ensure survival (Robertson and Meyer, 1992; Wise, 1993). Phenotypic variation in *Pseudomonas tolaasii*, which is apparent as spontaneous changes in colony morphology, was originally reported by Lucas and Grogan (1969) and

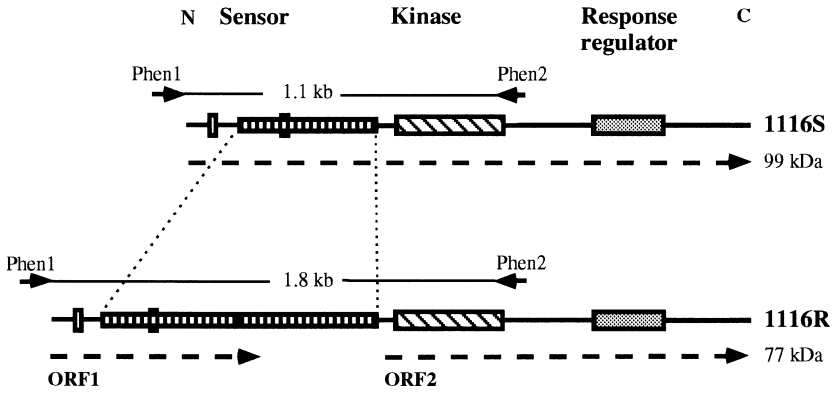

Fig. 3. Diagrammatic representation of the DNA duplication event in the *pheN* locus. Small black squares, hydrophobic domains; hatched boxes, regions with homology to the conserved histidine protein kinase domain; stippled boxes, regions with homology to the conserved regulator domain; vertical striped boxes, 661 bp duplicated sequence. Translation products are indicated by horizontal dotted lines. Phen1 and Phen2 indicate the position of PCR primers used in Fig. 4.

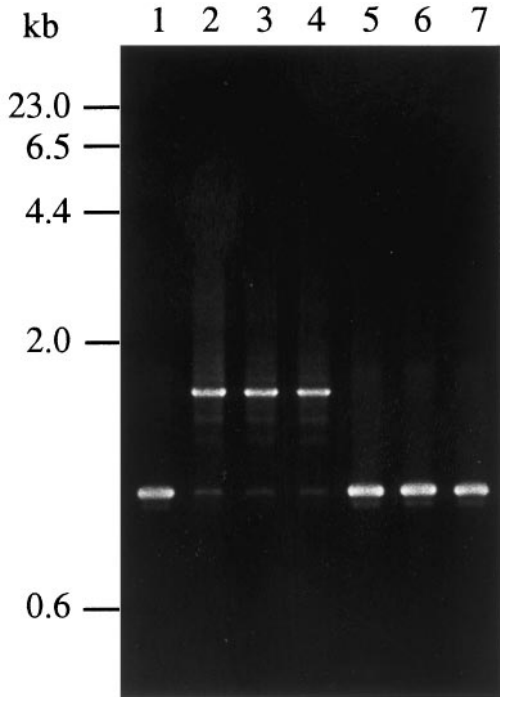

Fig. 4. Agarose gel electrophoresis of PCR products using Phen1 and Phen2 primers with *P. tolaasii* genomic DNA. Lane 1, 1116S; lanes 2–4, independent 1116R isolates; lanes 5–7, independent wild-type revertants from 1116R.

Cutri *et al.* (1984). The environmentally responsive two-component sensor regulator protein designated PheN provides *P. tolaasii* with a mechanism for regulating factors associated with pathogenicity in mushrooms (Grewal *et al*., 1995).

Although it has been shown that inactivation of *pheN* in the genome of *P. tolaasii* by Tn5*lacZ* mutagenesis resulted in a switch from 1116S to 1116R, the mechanism underlying the spontaneous formation of 1116R sectors in colonies of *P. tolaasii* remained unknown. The results presented here show that this phenotypic switch arises because of a 661 bp duplication within the *pheN* gene. Since an increase in the same PCR fragment size was observed in 25 independent 1116R sectors, it is concluded that DNA duplication is responsible for this phenotypic switch. This duplication event results in termination of the PheN ORF and the synthesis of a protein lacking 204 amino acids from the N-terminus of PheN. Since it was deduced that the N-terminal periplasmic domain of PheN is the environmental sensor (Grewal *et al*., 1994), loss of this domain is predicted to result in loss of PheN function. The similarity of phenotype between 1116S (*pheN*::Tn5-*lacZ)* and 1116R supports this conclusion.

High-frequency phenotypic switching in *Candida albicans* has been found to be involved in the regulation of phase-specific genes. These genes have also been shown to be regulated at the level of gene transcription (Soll *et al*., 1993; Srikantha and Soll, 1993). The results of Northern and Western blot analysis presented here show that, in the 1116S to 1116R phenotypic switch, the *pheN* gene was not regulated at the level of gene transcription.

The process of frameshift induced in the two-component sensor regulator gene *pheN* by DNA duplication differs from that in other frequent mispairings in spontaneous mutants (Miller, 1985; Streisinger and Owen, 1985). It also differs from the processes of variation observed in other bacteria (for reviews see Robertson and Meyer, 1992; Dybvig, 1993). These include general homologous recombination in pilus variation of *N. gonorrhoeae* (Meyer, 1990; Ende *et al*., 1995), variation via repetitive domains in lipoprotein of *M. hyorhinis* (Citti and Wise, 1995), site-specific recombination in flagellin of *S. typhimurium* (Glasgow *et al*., 1989) and *recA*− independent recombination via short repeats in the *bvgS* locus of *B. pertussis* (Stibitz *et al*., 1989). The mechanism of the DNA duplication event in *P. tolaasii* is unknown, but whether the DNA rearrangement in *P. tolaasii* is RecA dependent is currently being analysed in our laboratory.

The biological significance of the phenotypic switch described here remains to be established. The observation that the switch between the 1116S and 1116R forms of *P. tolaasii* is reversible may, however, have important implications for the study of the epidemiology of brown blotch disease. It has been concluded on the basis of their phenotypes that the 1116S and 1116R forms of *P. tolaasii* are adapted to different environmental niches (Grewal *et al*., 1995). Conventional methods for detection of *P. tolaasii* only detect the 1116S form (Wong and Preece, 1979), and no account has been taken of the population size of the 1116R form in previous epidemiological studies (Wong and Preece, 1980). At the present time, nothing is known of the environmental or other cues that induce the switch between these forms, although we have noted that

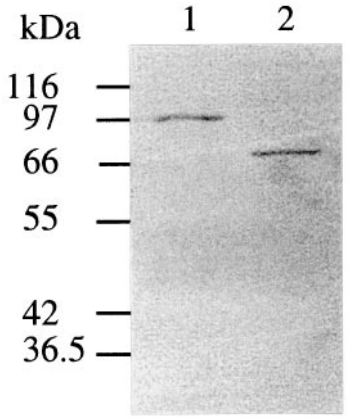

Fig. 5. Western blot analysis of soluble proteins separated by 10% SDS–PAGE from *P. tolaasii* 1116 S (lane 1) and 1116R (lane 2) using a polyclonal anti-LemA antiserum raised against LemA sensory α-kinase domain (Rich *et al*., 1994).

1116R forms arise on agar plates in colonies plated at high cell density.

In addition to the 1116S to 1116R phenotypic switch observed as sectors in colonies of *P. tolaasii*, other phenotypic variant forms can be identified from liquid cultures of *P. tolaasii* (Rainey, 1989). We have isolated at least seven distinct phenotypes from such cultures (unpublished data), and work is currently in progress to identify the molecular basis of these phenotypic variants.

Experimental procedures

Bacterial strains, media and culture conditions

The *Escherichia coli* and *Pseudomonas tolaasii* strains used here are described in Table 1. *E. coli* strains were grown in LB media at 37°C, and *P. tolaasii* strains were maintained on Pseudomonas agar F (PAF) or in Pseudomonas broth (PB) medium at 25°C. Recombinants were grown in the same media containing appropriate antibiotics at the following concentrations: kanamycin 25 μg ml^{-1}; ampicillin 100 μg ml^{-1} and tetracycline 20 μg ml^{-1}. The wild-type strain of *P. tolaasii* (NCPPB 1116S) was obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, UK. A spontaneous ampicillin-resistant strain of *P. tolaasii* was selected after overnight growth of approximately 1×10^8 cells on PAF, supplemented with ampicillin. A relatively stable typical phenotypic variant (designated 1116R) appearing in the form of translucent greenish yellow sectors at the margins of wild-type colonies was isolated from a 5-day-old colony of 1116S grown on PAF at 25°C. This variant was subcultured 2–3 times from single colonies and was used for further studies. For isolation of the wild-type revertants of the phenotypic variant form, a single colony isolate of the phenotypic variant was inoculated into PB liquid medium and grown overnight at 25°C on a rotary shaker. Smooth form revertants were screened on 250 skimmed milk plates (14 cm diameter) inoculated with $5–6\times10^3$ colonies per plate. After incubation for 48 h at 25°C, revertants were identified by the presence of clearing zones resulting from proteolytic activity. Eighteen revertants were isolated from 10^6 rough colonies. The terms 1116S and 1116R are used throughout to refer to the wild-type and phenotypic variant forms of *P. tolaasii* respectively.

DNA manipulation

Procedures for DNA manipulation, including extraction of chromosomal DNA from *P. tolaasii* strains, restriction enzyme digestions, agarose gel electrophoresis, plasmid isolation, DNA ligation and transformation of *E. coli* cells, have been described previously (Rainey *et al*., 1993; Sambrook *et al*., 1989). Transformation of *P. tolaasii* cells by electroporation was as described by Grewal (1991). Recombinant cosmids in *E. coli* (DH5α) were mobilized *en masse* into *P. tolaasii* strains 1116S and 1116R grown from a freshly subcultured single isolated colony by triparental mating with the aid of the helper plasmid pRK2013 (Figurski and Helinski, 1979).

DNA sequencing and sequence analysis

Double-stranded DNA was sequenced by the dideoxy chain termination method (Sanger *et al*., 1977), using mostly the Sequenase Kit (Version 2.0) of USB. The translated protein sequences were tested for homology to sequences in the NEWEMBL database.

Southern blot analysis

Restriction endonuclease-digested DNA was separated on a 0.7% agarose/TBE gel and capillary transferred onto positively charged nylon membranes (Boehringer Mannheim). Southern hybridization was performed using random ^{32}P-labelled DNA fragments in the hybridization buffer in the presence of sonicated single-stranded salmon sperm DNA as a competitor. The blots were washed twice in 2× SSC with 0.1% SDS at 37°C, followed by two washes in 0.1× SSC with 0.1% SDS at 65°C before exposure to a radiographic film with intensifying screens at −70°C.

Northern blot analysis

Total RNA of *Pseudomonas tolaasii* was prepared by a modified hot phenol protocol (O'Reilly *et al*., 1994). The cells were grown in 100 ml of PAF broth at 25°C and harvested at an OD_{600} of 0.2–0.3. The pellet was resuspended in 3 ml of 0.1% SDS and 1 mM EDTA. Three millilitres of phenol (redistilled; pH 5.5, equilibrated in 0.02 M NaCl) was added, and the mixture was incubated at 60°C for 5 min with gentle shaking. The mixture was centrifuged and the aqueous phase re-extracted with phenol at 65°C. The RNA was precipitated by adding 300 μl of 7.5 M ammonium acetate and three volumes of ethanol, followed by chilling to −70°C for 30 min. The RNA was centrifuged, redissolved in 3 ml of the same acetate/SDS buffer and the ethanol precipitation repeated twice. The final precipitate was dissolved in 1 ml of dH_2O and the RNA treated with RNase-free DNaseI. The RNA concentration was determined by measuring the absorbance at 260 nm. Equal amounts of RNA were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis was completed, the RNA from the gel was transferred to a nitrocellulose filter. Northern hybridization was performed using the same random ^{32}P-labelled DNA fragment in the hybridization buffer as that used for Southern blotting.

SDS–PAGE and Western blotting

Cells (1.5 ml) from overnight broth cultures were pelleted, washed with phosphate-buffered saline (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.5) and then resuspended in 200–400 μl of lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM PMSF, 10% sucrose). Lysozyme was added at 1 mg ml^{-1} and, after incubation on ice for 10 min, Triton X-100 was added to a final concentration of 0.1%, and the cell lysates were incubated on ice for a further 10 min. The lysate was centrifuged (20 000 × *g*) for 30 min at 4°C. Protein samples were mixed with 2× Laemmli sample buffer (20% glycerol, 10% β-mercapthoethanol, 6% SDS, 0.125 M Tris-HCl, pH 6.8, 0.1% bromophenol blue) and heated for 3 min at 100°C before loading onto 12.5% polyacrylamide gels containing 0.1% SDS. Proteins were detected by Coomassie blue staining or transferred to nitrocellulose membranes

using a semi-dry Western transfer apparatus (Atto) according to the manufacturer's instructions and blotted in transfer buffer (25 mM Tris, 102 mM glycine, 20% methanol) at 1–2 mA cm^{-2}. Polyclonal anti-LemA antisera prepared by injection of a purified LemA fusion protein into New Zealand White Rabbits (Rich *et al*., 1994) were used for immunoblotting. The secondary antibody was anti-rabbit IgG (whole molecule) alkaline phosphate conjugate (Sigma), and the detection was done using NBT-BCIP tablets (Sigma) according to the manufacturer's instructions. A blocking solution of 3% skimmed milk in TBS (50 mM Tris-HCl, pH 7.0, 150 mM NaCl) was used to dilute the primary and secondary antibodies in order to reduce non-specific hybridization, and the membranes were treated for 1 h in the blocking solution before adding the primary antibody.

PCR reactions

PCR reactions were performed in a 50- to 100-μl volume containing 1 mM Tris-HCl (pH 8.3), 50 mM KCl (pH 5.5), 100 μM dNTP, 1.5–2.5 mM $MgCl_2$, 100 pmol of each primer, 200 ng of genomic or plasmid DNA and 0.5 units of Biotime *Taq* polymerase (Bio-line). PCR was performed for the first cycle of 40 s at 94°C, 1 min at 56°C and 5 min at 72°C, then for 30 cycles of 40 s at 94°C, 40 s at 56°C and 3 min at 72°C. The sequences of the oligonucleotides used in the PCR experiments were Phen1: 5′-AGATTGCCCGTGTCATTA-3′ (Grewal *et al*., 1995, Fig. 3, residues 141–158) and Phen2: 5′-GTTCGAGCTGCTTGGCAT-3′ (Grewal *et al*., 1995, Fig. 3, residues 1402–1385).

Construction of 1116R genomic DNA library

Genomic DNA was extracted from the cells of overnight cultures of *P. tolaasii* 1116R and partially digested with *Bam*HI. After phenol–chloroform extraction and precipitation, the digested genomic DNA was ligated to dephosphorylated pLAFR3 vector digested with *Bam*HI. The ligation reactions were carried out at 16°C for 16 h, and electroporated into *E. coli* strain DH5α after ethanol precipitation. The resulting library contained more than 1×10^4 recombinants, and the size of the main insert was approximately 20 kb after the *Bam*HI digestion. Recombinants were plated out at 2×10^2 colonies per 9 cm Petri dish with LB agar containing 10 μg ml^{-1} tetracycline. Cells were partially stored as glycerol stocks at −70°C, the remaining bacteria being used to isolate plasmid DNA by the alkaline lysis method (Sambrook *et al*., 1989).

Colony hybridization

Individual colonies of the 1116R library were transferred onto circular nitrocellulose filters and then onto master agar plates. The colonies were incubated overnight at 37°C and then lysed by immersion in 10% SDS and denaturing solution (0.5 N NaOH, 1.5 M NaCl). The filters were neutralized in a solution of 1.5 M NaCl and 0.5 M Tris (pH 7.4) and washed twice with 2× SSC. The filters were dried at 80°C and hybridized with a *pheN* probe as described for Southern hybridization.

Acknowledgements

We thank Julia Smith and members of the Molecular Plant Pathology Group for helpful discussions, and Dr Todd Kitten for the generous gift of anti-LemA antibodies. This work was performed under the provisions of licence number PHF 1600/978/44 issued by the Ministry of Agriculture, Fisheries and Food under the Plant Health (Great Britain) Order 1987, supported by the Biotechnology and Biological Sciences Research Council (grant no. PE8/509 and PO6162; B.H.), the Cambridge Commonwealth Trust (A.P.) and conducted within the BBRSC Cambridge Centre for Molecular Recognition.

References

Bibb, M.J., Findlay, P.R., and Johnston, M.W. (1984) The relationship between base compositions and codon usage in bacterial genes and its use for the simple and reliable identification of protein coding sequences. *Gene* **30:** 157–166.

Brodey, C.L., Rainey, P.B., Tester, M., and Johnstone, K. (1991) Bacterial blotch disease of the cultivated mushroom is caused by an ion channel forming lipodepsipeptide toxin. *Mol Plant–Microbe Inter* **4:** 407–411.

Citti, C., and Wise, K.S. (1995) *Mycoplasma hyorhinis vlp* gene transcription: critical role in phase variation and expression of surface lipoproteins. *Mol Microbiol* **18:** 649–660.

Cutri, S.S., Macaulay, B.J., and Roberts, W.P. (1984) Characteristics of pathogenic non-fluorescent (smooth) and non-pathogenic fluorescent (rough) forms of *Pseudomonas tolaasii* and *Pseudomonas gingeri*. *J Appl Bacteriol* **57:** 291–298.

Deretic, V., Konyecsni, W.M., Mohr, C.D., Martin, D.W., and Hibler, N.S. (1989) Common denominators of promoter control in *Pseudomonas* and other bacteria. *Biotechnology* **7:** 1249–1254.

Dybvig, K. (1993) DNA rearrangements and phenotypic switching in prokaryotes. *Mol Microbiol* **10:** 465–471.

Ende, A.V.D., Hopman, C.T.P., Zaat, S., Essink, B.B.O., Berkhout, B., and Dankert, J. (1995) Variable expression of class 1 outer membrane protein in *Neisseria meningitidis* is caused by variation in the spacing between the −10 and −35 regions of the promoter. *J Bacteriol* **177:** 2475–2480.

Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc Natl Acad Sci USA* **76:** 1648–1652.

Glasgow, A.C., Hughs, K.T., and Simon, M. (1989) Bacterial DNA inversion system. In *Mobile DNA*. Berg, D.E., and Howe, M. M. (eds). Washington DC: American Society for Microbiology, pp. 637–672.

Goor, M., Vantomme, R., Swings, J., Gills, M., Kersters, K., and Deley, J. (1986) Phenotypic and genotypic diversity of *Pseudomonas tolaasii* and white line reacting organisms isolated from cultivated mushrooms. *J Gen Microbiol* **132:** 2249–2264.

Grewal, P.S. (1991) Effects of *Caenorhabditis elegans* (Nematoda: Rhabditae) on the spread of the bacterium *Pseudomonas tolaasii* in mushrooms (*Agaricus bisporus*). *Ann Appl Biol* **118:** 47–55.

Grewal, S.I.S., Han, B., and Johnstone, K. (1994) Regulation of toxin synthesis and phenotypic variation in *Pseudomonas*

tolaasii, cause of brown blotch disease of mushroom. In *Advances in the Molecular Genetics of Plant–Microbe Interactions*, Vol 3. Daniels, M.J., Downie, J.A., Osbourn, A.E. *et al.* (eds). Dordrecht, The Netherlands: Kluwer Academic Publisher, pp. 25–32.

Grewal, S.I.S., Han, B., and Johnstone, K. (1995) Identification and characterization of a locus which regulates multiple functions in *Pseudomonas tolaasii,* the cause of brown blotch disease of *Agaricus bisporus*. *J Bacteriol* **177:** 4658–4668.

Haas, R., and Meyer, T.F. (1986) The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. *Cell* **44:** 107–115.

Hrabak, E.M., and Willis, D.K. (1992) The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. *J Bacteriol* **174:** 3011–3020.

Hutchison, M.L., and Johnstone, K. (1993) Evidence for the involvement of the surface active properties of the extracellular toxin tolaasin in the manifestation of brown blotch disease symptoms by *Pseudomonas tolaasii* on *Agaricus bisporus*. *Physiol Mol Plant Pathol* **42:** 373–384.

Lucas, L.T., and Grogan, R.G. (1969) Pathogenicity and other characteristics of smooth and rough isolates of *Pseudomonas lachrymans*. *Phytopathology* **59:** 1919–1923.

Meyer, T.F. (1990) Variation of pili and opacity-associated protein in pathogenic *Neisseria.* In *Molecular Basis of Bacterial Pathogenesis*. Iglewski, B.H., and Clark, V. L. (eds). San Diego: Academic Press, pp. 137–153.

Miller, J.F., Mekalanos, J.J., and Falkow, S. (1989) Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science* **243:** 916–922.

Miller, J.H. (1985) Pathways of mutagenesis revealed by analysis of mutational specificity. In *Genetics of Bacteria*. Scaife, J., Leach, D., and Galizzi, A. (eds). London: Academic Press, pp. 25–40.

Mills, J.A., Malabi, M., Venktesan, M., Baron, L., and Buysse, J. (1992) Spontaneous insertion of an *IS*1-like element into *virF* gene is responsible for avirulence in opaque colonial variants of *Shigella flexneri*. *Infect Immun* **60:** 175–182.

O'Reilly, M., Woodson, K., Dowds, B.C.A., and Devine, K.M. (1994) The citrulline biosynthetic operon *argC-F*, and a ribose transport operon, *rbs*, from *Bacillus subtilis* are negatively regulated by *SpoOA*. *Mol Microbiol* **11:** 87–98.

Rainey, P.B. (1989) The involvement of *Pseudomonas putida* in basidiome initiation of the cultivated mushroom *Agaricus bisporus*. PhD thesis. University of Canterbury, Christchurch, New Zealand.

Rainey, P.B., Brodey, C.L., and Johnstone, K. (1991) Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. *Physiol Mol Plant Pathol* **39:** 57–70.

Rainey, P.B., Brodey, C.L., and Johnstone, K. (1993) Identification of a gene cluster encoding three high-molecular-weight proteins, which is required for synthesis of tolaasin by the mushroom pathogen *Pseudomonas tolaasii*. *Mol Microbiol* **8:** 643–652.

Rich, J.J., Kinscherf, T.G., Kitten, T., and Willis, D.K. (1994) Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. *J Bacteriol* **176:** 7468–7475.

Robertson, B.D., and Meyer, T.F. (1992) Antigenic variation in bacterial pathogens. In *Molecular Biology of Bacterial Infection*, Vol 49. Hormaeche, C.W., Penn, C.W., and Smyth, C. J. (eds). Cambridge: Cambridge University Press, pp. 61–73.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-termination inhibitors. *Proc Natl Acad Sci USA* **74:** 5463–5467.

Shapiro, J.A. (1986) Control of *Pseudomonas putida* growth on agar surfaces. In *The Bacteria*, *Vol 10*: *The Biology of Pseudomonads*. Sokatch, J.R. (ed.). London: Academic Press, pp. 27–70.

Shapiro, L., Kaiser, D., and Losick, R. (1993) Development and behaviour in bacteria. *Cell* **73:** 835–836.

Soll, D.R., Morrow, B., and Srikantha, T. (1993) High-frequency phenotypic switching in *Candida albicans*. *Trends Genet* **9:** 61–65.

Srikantha, T., and Soll, D.R. (1993) A white-specific gene in the white-opaque switching system of *Candida albicans*. *Gene* **131:** 53–60.

Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. (1987) Molecular characterization of cloned avirulence genes from race O and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J Bacteriol* **169:** 5789–5794.

Stern, A., and Meyer, T.F. (1987) Common mechanisms controlling phase and antigenic variation in pathogenic *Neisseriae*. *Mol Microbiol* **1:** 5–12.

Stibitz, S., Aaronson, W., Monack, D., and Falkow, S. (1989) Phase variation in *Bordetella pertussis* by frameshift mutation in a gene for a novel two-component system. *Nature* **338:** 266–269.

Streisinger, G., and Owen, J.E. (1985) Mechanisms of spontaneous and induced frameshift mutation in bacteriophage T4. *Genetics* **109:** 633–659.

Tolaas, A.G. (1915) A bacterial disease of cultivated mushrooms. *Phytopathology* **5:** 51–54.

Wise, K.S. (1993) Adaptive surface variation in mycoplasmas. *Trends Microbiol* **1:** 59–63.

Wong, W.C., and Preece, T.F. (1979) Identification of *Pseudomonas tolaasii*: the white line in agar and mushroom tissue block rapid pitting tests. *J Appl Bacteriol* **47:** 401–407.

Wong, W.C., and Preece, T.F. (1980) *Pseudomonas tolaasii* in mushroom crops: a note on primary and secondary sources of the bacterium on a commercial farm in England. *J Appl Bacteriol* **49:** 305–314.