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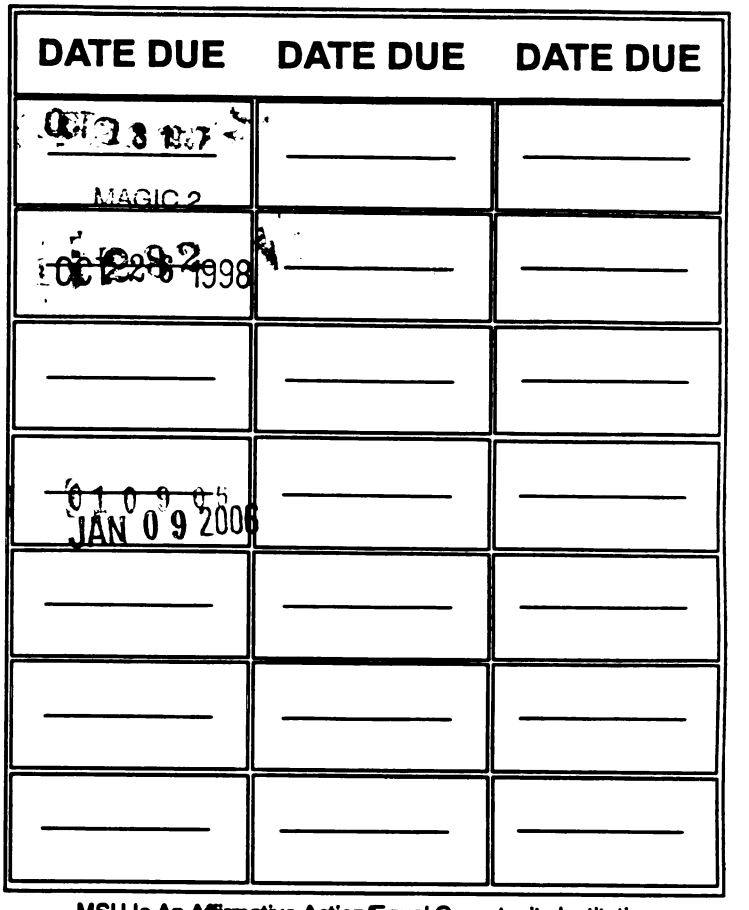

CHARACTERIZATION OF A VIRULENCE LOCUS AND A HIGHLY MUTABLE LOCUS OF *AGROBACTERIUM TUMEFACIENS*, EACH INVOLVED IN EXOPOLYSACCHARIDE EXPRESSION

By

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ABSTRACT

CHARACTERIZATION OF A VIRULENCE LOCUS AND A HIGHLY MUTABLE LOCUS OF AGROBACTERIUM TUMEFACIENS, EACH INVOLVED IN EXOPOLYSACCHARIDE EXPRESSION

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Mutants of Agrobacterium tumefaciens, both spontaneous and transposon-induced, were investigated with respect to their virulence capabilities and exopolysaccharide expression. Two genetic loci, pscA and pscB, with roles in EPS expression, were studied. The pscA sequences were found to be structurally and functionally related to the exoC locus of Rhizobium meliloti, and to have homology with DNA sequences found in several soil bacteria. The pscB locus was found to be the site of a high rate of spontaneous mutation in two strains of A. tumefaciens. pscB mutants were found in two differentstrains of A. tumefaciens and were characterized with respect to their genetic stability and fitness, and the sites of lesions responsible for the mutant phenotype were localized.

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Chapter 1

Introduction

Agrobacterium tumefaciens is a gram-negative, rod-shaped bacterium commonly found in most soils (1). Virulent agrobacteria are pathogens of most dicotyledonous and a few monocotyledonous plants, as well as some gymnosperms (for review, see (2)). A. tumefaciens is a unique pathogen in that, during the course of the infection, a specific portion of extrachromosomal bacterial DNA is transferred to the host plant; it is the expression of genes carried on this DNA which is responsible for the disease symptoms (for review, see (3)), commonly referred to as crown gall.

A. tumefaciens has proved to be a valuable tool in the genetic engineering of plants due to its ability to deliver DNA to the plant genome, and intense research over the past ten years has resulted in the elucidation of many of the mechanisms by which agrobacteria bring about genetic exchange with plants. It has been found that many gene products, both chromosomal and extrachromosomal, act in a concerted manner to elicit the disease response.

A. tumefaciens virulence functions

The Ti plasmid

All virulent agrobacteria possess a large plasmid (tumor-inducing, or Ti) on which resides the DNA (T-DNA) which is transferred to the host. When T-DNA is transferred to plant cells, the resultant transformed plant cells produce novel sugar and amino acid compounds, opines, which can be used both as carbon and nitrogen sources for agrobacteria, and as signal molecules which induce Ti plasmid transfer between agrobacteria. The genes encoding the proteins which catabolize opines are also located on the Ti plasmid, and are not part of the genetic information transferred to the plant. Exactly which opines are expressed in the plant is dependent on the type of Ti plasmid harbored within the bacterium; for example, the agrobacteria used mainly in this study harbor the plasmid pTiA6, which encodes octopine production and utilization, while other agrobacteria harbor the plasmid pTiC58, which encodes nopaline production and utilization.

T-DNA also carries genes to the host plant which encode the biosynthesis of the plant hormones auxin and cytokinin; the accumulation of these hormones is responsible for the proliferative, undifferentiated growth characteristic of crown gall (4). The relative levels of expression of these hormones determines the character of the gall; for example,

production of higher concentrations of auxin relative to cytokinin favors "rooty" tumors.

The left and right edges of the T-DNA, referred to as "borders", define the region of the Ti plasmid which is transferred. The borders are 24 basepair (bp) imperfect repeat sequences that flank all T-DNA regions. The right border is absolutely required for transfer, and sequences to the right of the natural border, including overdrive and gene products encoded by pinF, influence the efficiency of T-DNA processing.

In addition to T-DNA, the Ti plasmid encodes six genetic loci whose gene products are responsible for the transfer of the T-DNA from the bacterium to the host plant cell. These loci, referred to as virulence (vir) loci, are clustered in a region separate from the T-DNA and the other defined regions of the Ti plasmid, the latter being responsible for bacterial conjugation, opine catabolism, and incompatibility of replication. Four of the vir loci, virA, virB, virD and virG, are absolutely required for virulence; the remaining two, virC and virE, are related to host specificity, affecting tumor formation on some plants but not others. Although carried together on the same plasmid as the T-DNA, the vir genes can function in trans; T-DNA with borders, in the same cell with functional vir genes but on a different plasmid, can be successfully transferred to plant cells. The vir loci sequences and organization differ little among Ti plasmids, and are particularly well-conserved for the

four required loci.

Expression of the vir genes is induced by plant molecules which are elicited in response to wounding. The inducing molecules include acetosyringone and other related phenolic compounds. The loci virA and virG appear to encode the receptors of the plant signal and the molecules which transduce the signal. It has been postulated that virA serves to recognize the inducing plant compounds and that virG is the positive regulator of the remaining vir loci (see, for example, (5)). The virA gene product has recently been shown to traverse the cytoplasmic membrane (6), and is thus well-suited to the role of sensing signal molecules in the environment and transmitting this information to the cytoplasm, where other vir genes can respond.

The T-DNA is excised from the Ti plasmid following vir region induction, in a concerted action of vir gene products and the cis-acting border sequences. Nicking of the T-DNA region of the Ti plasmid to generate the single-stranded T-DNA molecule (the "T strand") which is to be transferred is effected by the virD gene products, among which one is a site-specific endonuclease that cleaves at a unique site within the 24 bp direct repeats that flank the T-DNA (8). virB mutants of Agrobacteria are incapable of inciting tumors, but the precise reason for this (and hence the precise functions of the virB gene products) is not known, although the virB gene product is thought to be involved in pH and osmotic adaptation (7).

It has been suggested (9) that the transfer of T-DNA to the plant is analogous to bacterial conjugation. Like that system, the transforming DNA is thought to be transferred as a single strand to the recipient cell; like that system, the transferred molecule is coated with a single-stranded binding protein, and virE encodes such a protein (10).

The process by which transferred T-DNA is integrated into the plant genome is not well understood; there appears to be little specificity of integration besides that of a strong preference for plant nuclear DNA. Once integrated, genes encoded on the T-DNA begin expression of gene products including those for the synthesis of auxin and cytokinin, as well as opines. The bacteria have thus created a niche for themselves, utilizing the plant's metabolic energy to produce compounds, opines, which they, but not the plant, can utilize.

Chromosomal Virulence Loci

In addition to the extrachromosomal sequences detailed above, several chromosomal loci are required for virulence of agrobacteria. To date four such loci have been defined: chvA, chvB, pscA, and a region linked to metR.

The chvA and chvB loci (11), 1.5 and 5.0 kilobases (kb), respectively, are each single transcriptional units which are constitutively expressed and are physically linked on the chromosome. Agrobacteria with mutations at either or

both of these loci are defective in their abilities to attach to plant cells, attachment being the necessary first step in the infection process, and those which do attach are unable to inhibit tumor formation by virulent strains (12). The phenotype of chvA and chvB mutants is not total avirulence but rather a dramatic attenuation of virulence; chvA and chvB mutants produce, on average, 50% and 7%, respectively, the number of tumors incited by an equivalent number of wild type bacteria, leading to the conclusion that the mutants are prevented from forming an efficient interaction with the host plants.

chvA and chvB mutants exhibit pleiotropic effects, making it difficult to assign to a particular defect the cause of the attenuated virulence phenotype. chvB mutants are non-motile, resistant to specific bacteriophage, non-flagellated, and do not produce ß-1,2-D-glucan, a cyclic glucose polymer found in the periplasm as well as extracellularly in wild type A. tumefaciens cells.

chvA mutants also fail to express ß-1,2-D-glucan, suggesting a role for this polymer in the infection process. It has been shown (13) that chvA mutants produce wild type amounts of ß-1,2-D-glucan, but fail to export it from the cytoplasm, and sequence homology of the chvA gene to bacterial and eukaryotic export protein-encoding genes strengthens the hypothesis that the role of the chvA gene product is that of an exporter of ß-1,2-D-glucan from the cytoplasm to the periplasm and surface of the agrobacterium.

The closely related soil microorganism Rhizobium meliloti, of the same bacterial family but a symbiont of specific plants rather than a pathogen, has been shown to possess two genetic loci, ndvA and ndvB, which are structurally and functionally related to chvA and chvB, respectively (14). R. meliloti cells mutant at these loci are unable to invade root hairs and form ineffective nodules which lack infection threads and are devoid of bacteroids. This finding of relatedness between two species of bacteria which form such close contacts with plants, albeit toward different ends, is of particular interest, and led to many of the experiments described here.

A third chromosomal virulence locus of A. tumefaciens is the pscA locus (17). Agrobacteria mutant at the pscA locus are severely attenuated in their abilities to attach to plant cells in culture and to induce crown gall. The pscA mutation is also pleiotropic; mutants make essentially no exopolysaccharide (EPS), being deficient in the production of cellulose, succinoglucan, and ß-1,2-D-glucan.

This pleiotropism has made it difficult to draw firm conclusions about the role of the pscA gene product in attachment and virulence. Again, as with chvA and chvB, a defect is seen in the expression of ß-1,2-D-glucan, but no specific ß-1,2-D-glucan mutants have been described, leaving the role of this polysaccharide in the infection process uncertain. Some researchers (16) have ascribed to ß-1,2-D-glucan the function of osmotic adaptation both in

agrobacteria and rhizobia; how or if this relates to the infection process is unclear.

The only other locus with a demonstrated role in virulence was described by Matthysse (15), who found, through transposon mutagenesis, mutants which were unable to attach to carrot cells in culture, and were avirulent. These mutants mapped to an area of the A. tumefaciens chromosome closely linked to metR. Although the surface polysaccharide chemistry of the mutants appears to be unaltered, they were shown to lack polypeptides present in wild type spheroplast preparations and which, by implication, may be involved in the attachment, and hence infection, process.

The factors permitting a successful infection by A. tumefaciens of a susceptible plant have proven to be complex and interrelated. With at least five extrachromosomal and four chromosomal stretches of DNA absolutely required for virulence, the specific molecular interactions between bacterium and plant remain elusive. No one has yet identified a specific bacterial determinant which is absolutely required for the organism to bind plant cells, nor has a specific plant cell determinant been identified which must be present for agrobacteria to adsorb.

In addition to the characteristics detailed above, A. tumefaciens exhibits other phenotypes which are of interest in the study of microbial genetics. One such phenotype is its propensity to spontaneously give rise to variants which

are altered in surface chemistry. This study focuses on the expression of surface polysaccharide moieties of the bacterium, their potential roles in virulence, and explores the phenomenon of this spontaneous variation of surface polysaccharide determinants.

Chapter 2

The pscA Locus of Agrobacterium tumefaciens is structurally and functionally related to the exoC locus of Rhizobium meliloti, and has homology with the genomes of a number of soil bacteria.

Introduction

A. tumefaciens and R. meliloti are both members of the Rhizobiaceae family. Both are phytobacteria which establish intimate cell-cell interactions with plants, and although these interactions differ, A. tumefaciens being a pathogen and R. meliloti a symbiont of plants, they have been shown to possess genetic loci which are structurally and functionally related. As has already been stated, the chvA and chvB loci of A. tumefaciens are related to the ndvA and ndvB loci, respectively, of R. meliloti.

The pscA locus of A. tumefaciens plays an important role in EPS expression, and this finding suggested that perhaps it, too, might be related to a R. meliloti locus; pscA mutants do not produce succinylglycan, a phenotype also associated with the exo mutants of R. meliloti (18). R. meliloti exo mutants induce the formation of ineffective nodules similar to those produced by the ndvA and ndvB mutants (14), which lack infection threads, are devoid of bacteroids, and do not fix nitrogen. Like the pscA mutants, exo mutants differ from the wild type parent strain in that they do not fluoresce on medium containing Leucophor. It

Like the pscA mutants, exo mutants differ from the wild type parent strain in that they do not fluoresce on medium containing Leucophor. Here I show that the A. tumefaciens locus is indeed related, structurally and functionally, to one of the exo loci, exoC.

Materials and Methods

Bacterial strains, plasmids, and media. The strains of A. tumefaciens, R. meliloti, and E. coli are listed in Table 1. Bacteria were grown on LB medium. Antibiotics used in solid media for A. tumefaciens and R. meliloti were: rifampicin (100 ug/ml); tetracycline (2 ug/ml); streptomycin (500 ug/ml); kanamycin (50 ug/ml). Antibiotics used for E. coli were: tetracycline (12.5 ug/ml); kanamycin (50 ug/ml); and spectinomycin (50 ug/ml). Leucophor liquid was used at a concentration of 1.5 ml/L. Antibiotics used in liquid media were halved in concentration. Agrobacteria and rhizobia were grown at 30°C, and E. coli at 37°C.

Restriction endonucleases and chemicals. Restriction endonucleases were purchased from New England Biolabs, Beverly, Massachusetts. Radiolabeled chemicals were purchased from New England Nuclear Corp., North Billerica, Mass. Leucophor BSB liquid was a gift of Sandoz Chemicals, Charlotte, NC.

Table 1. Bacterial strains and plasmids used in the study of the *pscA* locus

Bacterial strain or plasmid	Relevant properties	Source or reference
A. tumefaciens		
A6	wild type	A. Binns
A6.1	rif^R A6 derivative	17
A6.1d	A6.1::Tn*5* avir	17
$A6.1d_3$	A6.1::Tn5 avir	17
R. meliloti		
1021	$strep^R$ wild type	18
7020	1021::Tn*5* exoC	18
E. coli		
HB101	*recA*, *hsdR*, *specR*, *strepR*	19
Plasmids and Cosmids		
pLAFR1	IncP, tet^R	20
pRK2073	ColE1::pRK2 Tra^+ $spec^R$	21
pRK2013	ColE1::pRK2 Tra^+ kan^R	22
pD15	*exoC* in pLAFR1; tet^R	18
pJ4.0	*pscA* in pLAFR1; tet^R	18
pJ4.0-4C	active *pscA*::Tn*3HoHo1* in pLAFR1; Tet^R	17
pJ4.0-8A	inactive *pscA*::Tn*3HoHo1* in pLAFR1; Tet^R	17
pJOYe5.1	*pscA* in pBR325; Tet^R	17
pSUP2021	Tn*5* mob^+ ap^R,cm^R,kan^R	22

abbreviations: rif=rifampicin
strep=streptomycin
tet=tetracycline
kan=kanamycin
cm=chloramphenicol
ap=ampicillin
avir=avirulent

Bacterial transformations and conjugations. E. coli was transformed as described (23). Bacterial conjugations were performed on LB agar incubated overnight at 30°C. Triparental matings were performed using either pRK2013 or pRK2073 to supply the mobilizing functions. All cultures grown in media containing antibiotics were washed twice in growth medium without antibiotics before conjugation.

DNA isolations. Plasmid DNA was isolated by the procedure of Birnboim and Doly (24). Total DNA was isolated as described (25). In some cases, DNA preparations were further purified by using cesium chloride-ethidium bromide density gradients.

DNA filter hybridizations. DNA fragments were size-fractionated and transferred to nitrocellulose (0.45 m; Schleicher and Schuell Inc., Keene, NH) by the method of Southern (26). DNA hybridization probes were radiolabelled in vitro with [Á-^{32}P]dCTP by the nick translation procedure (27). Hybridization and washing conditions were as described in (25) or as stated in the text.

Virulence testing. Bacterial strains were grown in liquid LB to early stationary phase, washed and resuspended in sterile water. Plants were wounded with a sterile pipet tip and wounds were inoculated with approximately 20 l of the bacterial suspension. Tumor production was scored after about 6 weeks.

Preparation of EPS. Bacterial strains were grown in liquid M9-glucose medium (27) to an O.D. of about 0.75, washed in the growth medium and resuspended in nitrogen-free M9-glucose medium, then shaken two days at 30°C. Tubes were centrifuged at 11,600xg and the pellets discarded; supernatants were added to 0.3 parts (wt/vol) cetrimide (hexadecyltrimethylammonium bromide) and assayed by the anthrone method (28).

Assay of nitrogen reduction. Alfalfa seedlings were grown in nitrogen-free medium, inoculated with bacteria, and scored after six weeks for the ability to reduce acetylene to ethylene as described (29).

Maxicell analysis. Maxicell analysis was performed, and labeled protein products resolved, as previously described (30).

Results

The pscA locus is structurally and functionally related to the exoC locus. Wild-type colonies of R. meliloti and A. tumefaciens fluoresce blue-green under UV light when grown on medium supplemented with Leucophor, a stilbene brightener which binds carbohydrates possessing specific ß linkages (31). Both the pscA and exo mutants, of A. tumefaciens and R. meliloti, respectively, fail to exhibit this phenotype, and are thus referred to as "dark" mutants. The first experiment was an attempt to complement this phenotype in trans by conjugating into the mutants the wild type sequences from the other species. Wild type exo sequences from R. meliloti were transferred into the pscA mutants of A. tumefaciens, and wild type pscA sequences (pJ4.0) were introduced into exo mutants of R. meliloti; the transconjugants were scored on medium containing Leucophor for the restoration of the blue-green fluorescence, or "brightness". Cosmids representing five of the exo loci, exoA, exoB, exoC, exoD and exoF were used in the complementation experiment. It was found that, while neither the cloning vector (pLAFR1) nor any of the other exo loci had any effect on the pscA mutant, the wild type exoC sequences (encoded on cosmid pD15) were able to restore brightness to the A. tumefaciens pscA mutants (Table 2). Consistent with this was the finding that the wild type pscA sequences were able to complement the exoC mutant with

respect to brightness (Table 2). In addition, a cosmid containing pscA loci rendered inactive by Tn3Hoho1 insertion (pJ4.0-8A) was incapable of complementing the dark phenotype of the R. meliloti exoC mutant, whereas an insertion outside the coding sequences (pJ4.0-4C) had no effect and produced bright exconjugants (Table 2).

The pscA mutants, after receiving exoC sequences, were tested for virulence on Kalanchoe plants (Table 2). Those which were rendered bright by the sequences received in conjugation were restored to virulence, while those which received the other exo loci or the cloning vector alone and were still dark were still avirulent.

These data suggested that the pscA locus of A. tumefaciens was indeed related to the exoC locus of R. meliloti, and DNA hybridization experiments were performed to add further evidence. Total genomic DNA was extracted from R. meliloti wild type and exoC mutant strains, digested with restriction enzymes, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and probed with either pJOYe5.1, a cosmid representing the pscA locus, or pSUP2021, representing Tn5 (both the exo mutants and the pscA mutants were generated by Tn5 transposon mutagenesis). The experiment was also performed using A. tumefaciens DNA and with the cosmids.

When R. meliloti DNA was digested with EcoRI and hybridized with pJOYe5.1, two fragments, of 2.7 and 8.1 kb, were detected (Figure 1). The 2.7 kb band hybridized also

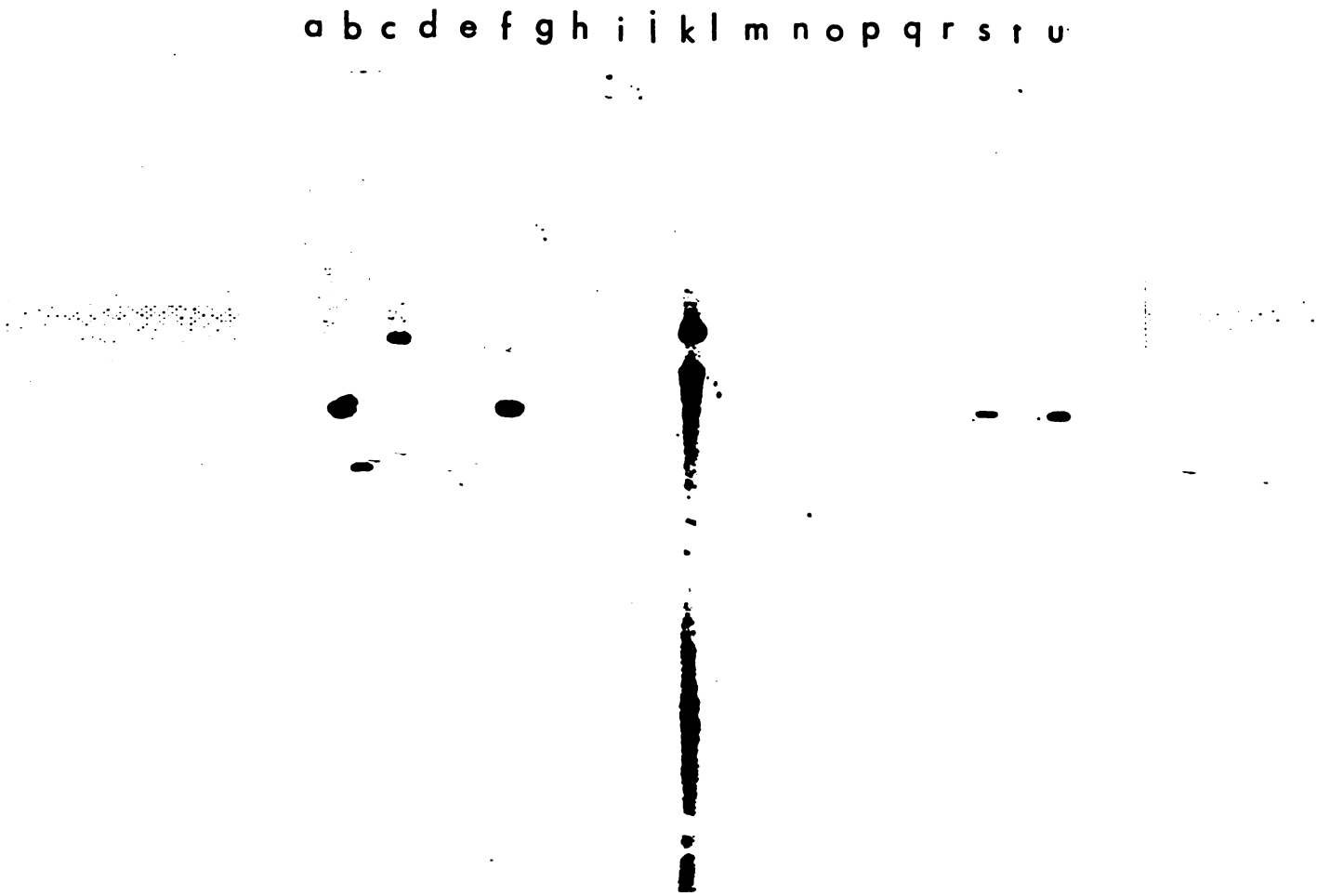
Table 2. Complementation of *exoC* mutants by wild type *pscA* sequences and of *pscA* mutants by wild type *exoC* sequences

Strain	Leucophor fluoresence	Virulence	EPS production[2]	Acetylene reduction
R.m.WT	+		ND[1]	+
R.m.*exoC*	-		ND	-
R.m.WT(pLAFR1)	+		155	+
R.m.*exoC*(pLAFR1)	-		2	-
R.m.*exoC*(pD15)	+		105	+
R.m.*exoC*(pJ4.0)	+		70	+
R.m.*exoC*(pJ4.0-4C)	+		ND	+
R.m.*exoC*(pJ4.0-8A)	-		2	-
A6.1 WT	+	+		
A6.1*pscA*	-	-		
A6.1 WT(pLAFR1)	+	+		
A6.1*pscA*(pLAFR1)	-	-		
A6.1*pscA*(pJ4.0)	+	+		
A6.1*pscA*(pJ4.0-4C)	+	+		
A6.1*pscA*(pJ4.0-8A)	-	-		
A6.1*pscA*(pD15)	+	+		

abbreviations: R.m.= *R. meliloti*

[1]--ND=not determined
[2]--measured as ug/ml precipitable material

Figure 1. DNA hybridization indicating homology between the A. tumefaciens pscA locus and the R. meliloti exoC locus



DNA isolation, restriction, and hybridization was done as described in Materials and Methods. The filters were hybridized with either pJOYe5.1 (A) or pSUP2021 (B) washed at either (A) low or (B) high stringency. DNA from A. tumefaciens A6.1 (lanes 1), pD15 (lanes 2), wild type R. meliloti 1021 (lanes 3,5 and 7), and R. meliloti exoC mutant 7020 (lanes 4,6 and 8). DNA was digested with either EcoRI (lanes 1 through 4), HindIII (lanes 5 and 6), or BglII (lanes 7 and 8).

to pD15 (the exoC-encoding cosmid), indicating that the hybridization was with the exoC sequences. The data also showed that the Tn5 insert in the R. meliloti exoC mutant was into the 2.7 kb EcoRI fragment, as evinced by the fact that this band was missing from exoC mutant total DNA probed with pSUP2021 or pJOYe5.1 and was replaced by a band about 5 kb larger (Tn5 is approximately 5 kb in size). HindIII and BglII digests of total DNA from the exoC mutant also indicated that the exoC locus is related to the pscA locus. Both of these enzymes cleave within the Tn5 sequences; in each case, the major fragment showing homology with the pscA probe in the wild type strain was missing in the mutant and in its place were two fragments showing hybridization with both the pscA probe and the Tn5 probe.

The amounts of EPS produced by the R. meliloti exconjugants and wild type were measured directly by precipitation with cetrimide and compared with each other. As expected, exoC mutants harboring either the cloning vector or inactive pscA sequences produced only a fraction of wild type EPS, whereas those harboring functional pscA sequences produced amounts of EPS approximating those of wild type strains (Table 2).

Finally, various rhizobia were tested for their nitrogen-fixing capabilities. Alfalfa seedlings were grown axenically on medium lacking nitrogen, inoculated with the various rhizobia, and scored for overall appearance and ability to fix nitrogen. Alfalfa seedlings inoculated with

wild type R. meliloti, or exoC mutants harboring intact pscA sequences, throve, having dark green leaves, thick stems, and large, cylindrical nodules present on their roots. The exoC mutants, alone or harboring inactive pscA sequences or the cloning vector, were spindly, chlorotic, and exhibited small, round nodules on their roots. The former were capable of reducing acetylene to ethylene, an assay of nitrogen-fixing ability, while the latter were not (Table 2).

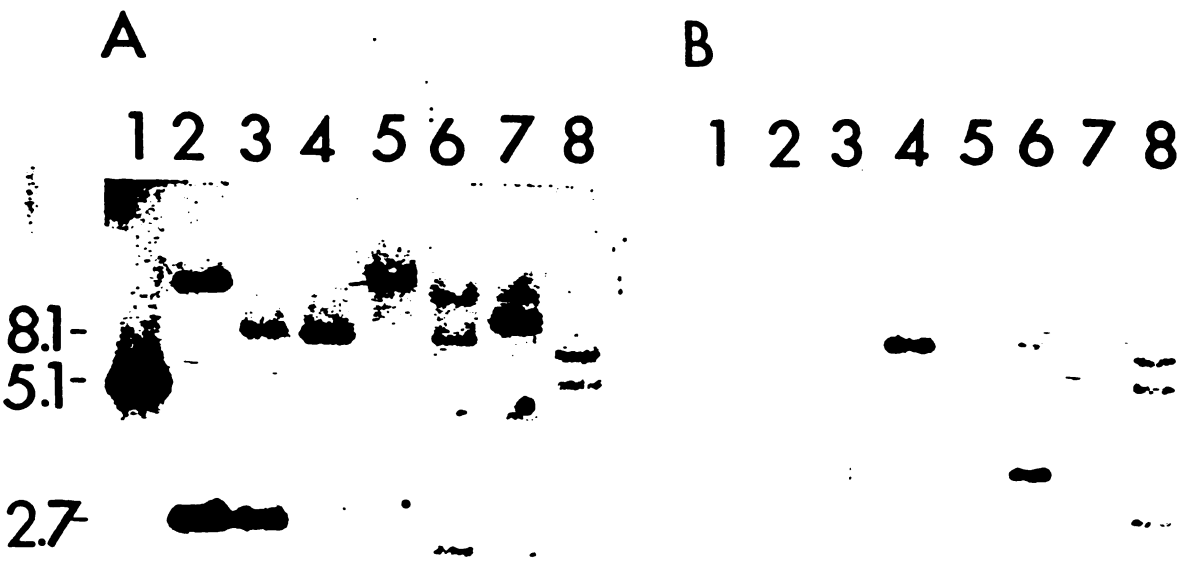
The pscA locus has homology with DNA sequences found in other soil bacteria. One further experiment was conducted, the results of which suggest that the pscA/exoC gene may be important in other plant/microbial interactions. Total genomic DNA was extracted from the plant pathogenic bacterial strains listed in Table 3, digested with restriction enzymes, fractionated by agarose electrophoresis, transferred to nitrocellulose, and probed with pJOYe5.1, on which reside the pscA sequences as well as some other flanking sequences. The filter was washed at low stringency (6X SSC at approximately 50°C), and the resulting autoradiogram (Figure 2) indicated that significant homology exists between pJOYe5.1 and DNA present in the following strains: Rhizobium leguminosarum, R. trifolii, Xanthomonas campestris pv. campestris, and Clavibacter michiganensis. Of note is the fact that no homology was detected between the pscA sequences and the agrobacteria isolated from grape plants (Table 3), even though those strains are bright on

Table 3. Hybridization of pscA sequences with total DNA of various plant pathogenic bacteria.

Strain	Host	Homology with pscA[1]	Source
A. tumefaciens Ag57	grape	-	R. Carlson
A. tumefaciens Ag63	grape	-	R. Carlson
A. tumefaciens SM4R	grape	-	R. Carlson
Rhizobium leguminosarum	pea	+[2]	F. Dazzo
R. trifolii	clover	+[2]	F. Dazzo
Pseudomonas solanacearum Race 1	tomato	-	R. Carlson
P. syringae pv. syringae	wheat	-	R. Carlson
Xanthomonas campestris pv. campestris	Brassica sps.	+	D. Fulbright
X. campestris pv. pelargonii	geranium	-	D. Fulbright
Clavibacter michiganense pv. michiganense	tomato	+	D. Fulbright
E. coli S17-1	non-plant pathogen "control"	-	22

[1]-homology measured as detectable hybridization on Figure 2.
[2]-homology not detected on all strains tested (see Figure 2).

Figure 2. DNA hybridization indicating homology between the A. tumefaciens pscA locus and sequences in other soil bacteria



All lanes digested with EcoRI except lane b (HindIII) and lane c (BamHI). Lane a, E. coli S17-1; lanes b and c, Clavibacter michiganensis; lane d, Erwinia carotovora; lane e, Xanthomonas campestris pv. pelargonii; lane f, X. campestris pv. campestris; lane g, Pseudomonas solanacearum; lane h, P. syringae; lane i, P. syringae 2009; lane j, Rhizobium trifolii 128663; lane k, R. trifolii 0403; lane l, R. leguminosarum 843; lane m, R. leguminosarum 300.3; lane n, Bradyrhizobium japonicum 109j; lane o, A. tumefaciens SM4R; lane p, A. tumefaciens SM6S; lane q, A. tumefaciens Ag63; lane r, A. tumefaciens Ag57; lane s, A. tumefaciens A6.1; lane t, pBR325; lane u, pJOYe5.1.

leucophor plates and virulent on their respective plant hosts. It must be noted, however, that no control blot was done using just the cloning vector, pBR325, and that the construct used for the blot, pJOYe5.1, contains sequences in addition to the pscA sequences from Agrobacterium; the possibility remains, therefore, that the homology detected between the pscA sequences and sequences present in the other soil microorganisms is due to homology with either vector or non-pscA sequences.

The pscA locus encodes a protein product of approximately 55 kilodaltons. Maxicell analysis was performed as described (30) using the cosmid pJOYe5.1, which contains the pscA sequences cloned into pBR325, as well as additional flanking sequences, and a control lane with pBR325 alone. The resultant, labeled protein products were resolved on a polyacrylamide gel (Figure 3). A comparison of the pJOYe5.1 lane with the pBR325 lane indicated that the pscA locus may encode a protein or family of proteins with a molecular weight of approximately 55 kilodaltons.

Discussion

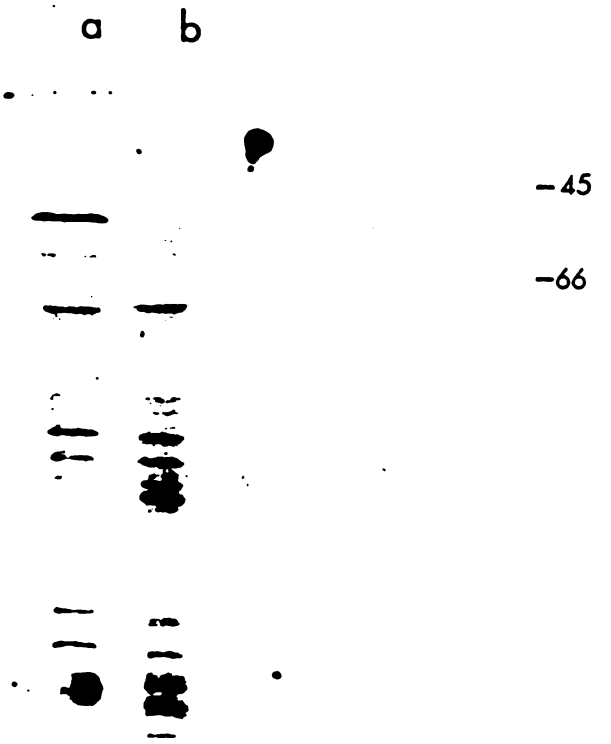
From the data presented it was possible to conclude that the pscA locus of A. tumefaciens is structurally and functionally related to the exoC locus of R. meliloti.

These findings are in agreement with those of Cangelosi, et al (32), who, working with a different A. tumefaciens isolate, also concluded that A. tumefaciens had DNA sequences capable of suppressing the exoC mutations of R. meliloti. Thus, A. tumefaciens and R. meliloti harbor at least three sets of related genes that have crucial roles in establishing the interactions that each bacterium has with its respective plant host.

Maxicell analysis suggests that the pscA locus encodes a protein or family of proteins with a molecular weight of approximately 55 kilodaltons. This implies that the locus is a structural one, and that the phenotype of the pscA mutants is not the result of lesions in regulatory elements but is more likely due to the loss of a functional protein.

The gene products encoded by pscA and exoC are clearly important in the expression of EPS. The roles of these gene products could be direct, such as coding for a synthetic step in EPS synthesis. They could, alternatively, function indirectly, perhaps encoding a membrane protein which imparts a general membrane structure required for EPS synthesis, or a scaffolding to hold EPS moieties in place. In addition, the links between the EPS defects of the mutants and the inability of A. tumefaciens to attach to plant cells and form tumors, and the links between the exoC mutation and the inability of R. meliloti to form effective

Figure 3. Maxicell analysis of the gene product encoded by the A. tumefaciens pscA locus



Maxicells were prepared and proteins labeled and separated as described in Materials and Methods. Lane A represents pJOYe5.1, which is pBR325 harboring the pscA sequences. LaneB represents the cloning vector, pBR325. Molecular weights are given in kilodaltons.

nodules, remain to be established.

The finding of sequences with apparent homology to the pscA locus in the genomes of soil bacteria is of interest on several levels. First, it appears that pscA sequences are found in some closely related species and not others. While A. tumefaciens A6.1 absolutely requires functional pscA sequences for virulence, DNA from A. tumefaciens strains Ag63 and Ag57, which are virulent on grape plants but exhibit reduced virulence, relative to A6.1, on carrot discs (25), showed no detectable hybridization with the pscA probe. It is possible that homologs of pscA exist in these species, but that the sequences have so diverged as to be undetectable under the conditions of the experiment as performed here.

Secondly, the data suggest that the presence of pscA sequences may be related to host range. In the case of the Xanthomonas campestris strains investigated, DNA from one pathovar, campestris, which infects a Brassica species, has sequences which showed homology to the pscA probe, while another pathovar, pelargonii, a pathogen of geraniums, showed no such homology. Why the pscA gene products might be required for infection of some plants and not others is not clear; perhaps pscA reflects a general strategy of some pathogens which is efficacious only with some hosts.

Finally, detection of pscA sequences in the Clavibacter isolate is of interest, for this species, unlike all the

others tested, which are gram negative bacteria, is a gram positive. That homology with pscA sequences apparently exists in such a diverse set of genetic backgrounds implies that the role of the pscA gene products is a general one.

This finding illustrates what appears to be a fundamental contradiction in these data. The fact that the pscA mutants of A. tumefaciens produce essentially no EPS (17) suggests that the pscA gene products are involved in some essential step in EPS expression, perhaps an early step in EPS biosynthesis or a general pathway by which most EPS molecules are transported or anchored. This generality of function is also suggested by the diverse genetic backgrounds in which pscA sequences seem to be found. Yet it is hard to reconcile this presumed fundamental role of the pscA function with the fact that no pscA sequences appear to be present in strains such as A. tumefaciens Ag57 or Xanthomonas campestris pv. pelargonii, which are virulent and make Leucophor-positive EPS (J. Marks, unpublished results).

In the species where pscA mutants have been identifed and characterized, the pscA locus has proved to be of crucial importance in the interactions of the bacteria with their plant hosts. It remains to be seen whether this importance extends to all isolates in which pscA sequences have been detected. It is clear that the demonstrated role of the pscA sequences in virulence and its presence in the genomes of several, distantly related bacterial species render the

pscA locus of more than passing interest in the understanding of plant/bacterial interaction.

Chapter 3

Characterization of spontaneous variants of *Agrobacterium tumefaciens* which are altered in expression of surface polysaccharides.

Introduction

At the time of the isolation of the transposon-induced mutants which led to the discovery of the *pscA* locus (17), it was necessary to determine whether the dark mutants produced resulted from Tn*5* insertion, or were due to some other cause. A control experiment was performed in which wild type *A. tumefaciens* cultures were plated, without exposure to Tn*5*, on medium containing Leucophor. Surprisingly, dark colonies were found to arise from the non-mutagenized cultures, and to do so at the high frequency of 10^{-3} to 10^{-4} (M. Thomashow and J. Marks, unpublished results). Further experiments established that most of the spontaneous, dark mutants themselves spontaneously gave rise to apparent wild type colonies at approximately the same frequency.

While the *pscA* mutants proved to be the result of transposon insertion, the spontaneous mutants were of

interest in themselves, due to the high frequency at which they arose and were seen to revert. A study of these mutants was undertaken in order to determine what implications this seeming instability of EPS expression might have for A. tumefaciens physiology.

Many microorganisms give rise, in pure culture, to spontaneous colony variants. The phenomenon is referred to as "dissociation", a term coined by de Kruef in 1921 to describe the reversible change from "smooth" to "rough" colony morphologies of Streptoccus pneumoniae (31). Many other examples of dissociation have since been described, and for many of these the molecular basis of this genetic instability has been elucidated. For some examples, a distinct teleological advantage seems to explain such instability; for others, the reason why the microoganisms display variability remains obscure.

Perhaps the best-studied cases of dissociation involve antigenic variation. Antigenic variation is important as a means by which many pathogens evade the host immune system. In animals, which respond to microbial invasion with specific antibodies which react with specific surface determinants on the microorganisms, it is a great advantage to the microbial population as a whole to present a variety of different antigens so as to evade the host immune response. A number of genetic mechanisms have arisen to effect this variation. The chromosome of Neisseria gonorrhoeae, a human pathogen, contains several complete

expression genes encoding variant surface determinants referred to as opacity genes (34). Changes in opacity gene expression are associated with production of a variety of serologically distinct proteins. This variation is a function of repeated pentameric units of nucleotides in the opacity structural genes. Addition or removal of these pentameric units results in placing the entire gene into or out of frame for translation and thus determines whether or not a given gene product will be expressed.

There are many other examples. Candida albicans, another human pathogen, switches reversibly and at high frequency between seven different colony morphologies. While the genetic basis of this dissociation has not yet been elucidated, it has been proposed that the high frequency and reversibility of these changes may suggest changes in the location of mobile genetic elements (35). Slutsky, et al postulate that this variability may allow the organism to invade diverse body locations, evade the immune system, or change antibiotic resistance. The causative agent of whooping cough, Bordetella pertussis, regulates expression of various virulence-related parameters in what is termed phase variation, the mechanism for which has been attributed to programmed frameshift mutations (36). Other mechanisms, similar and diverse, have been shown to be responsible for: flagellar variation in Serratia marcescens (37), antigenic variation in Trypanosoma equipiderum (38), and phase variation in Salmonella typhimurium (39).

Such variation is not peculiar to human pathogens; many other organisms exhibit genetic dynamism. Rhizobium phaseoli, a nitrogen-fixing plant symbiont, has been shown to undergo spontaneous genomic rearrangement which can lead to phenotypic variation; the mechanism has not been determined, but it is notable that the species contains a large number of reiterated DNA sequences and insertion sequences (40). Two species of the genus Thiobacillus, T. ferrooxidans (41) and T. versutus (42), iron- and sulfur-utilizing bacteria, undergo phenotypic switching leading to variation in carbon source-utilizing capability, and colonial heterogeneity associated with surface fibrils, respectively. Finally, A. tumefaciens has been shown to give off spontaneous variants with different surface chemistries after incubation for extended periods of time in storage stabs (43); these variants were shown to produce relatively less succinoglucan, and more curdlan, than the wild type.

The selective pressures which make such instability an asset to microorganisms which are not under pressure to evade specific host responses can only be speculated upon. Switches in carbon source, as in the case of the Thiobacillus isolates, might be expected to bestow a selective advantage to a microbial population as a whole. The variants in Agrobacterium and Rhizobium strains may have some relation to host range, for polysaccharides may play a role in the interaction of bacterial pathogens with their

plant hosts, as is suggested by the avirulence of A. tumefaciens mutants that do not produce EPS (17) and the inability of EPS mutants of R. meliloti to fix nitrogen (18).

As mentioned at the beginning of this chapter, spontaneous, dark mutants of A. tumefaciens A6.1 were isolated from pure cultures of wild type bacteria. The high frequency at which these variants arose, and the high frequency at which most of them were able to revert to apparent wild type, suggested that the phenomenon of dissociation in A. tumefaciens might be important in understanding the role EPS plays in the interaction of the bacteria with their hosts. Starting with a large set of spontaneous, dark mutants, the following questions were addressed: do all of the spontaneous mutants map to the same region of the genome? do the mutants have greater or lesser fitness than the wild type? is the dissociation phenomenon unique to A6.1, or is it common to other A. tumefaciens strains? and, is the phenomenon peculiar to the genes associated with EPS production, or are other phenotypic characteristics given to high rates of spontaneous mutation?

Materials and Methods

Bacterial strains and plasmids. The strains of A. tumefaciens and E. coli used are listed in Table 4. Media and antibiotics were used in the manner described previously

(Materials and Methods, Chapter 2). Spontaneous mutants of A6.1 were obtained in the following manner: wild type A. tumefaciens cultures were grown overnight at 30°C in LB medium, then diluted in the growth medium, plated on LB agar containing Leucophor, and incubated at 30°C for approximately 48 hours. Plates were then examined under long wave UV light (366nm; Blak-Ray Lamp, UVP, Inc., San Gabriel, CA), and colonies which failed to fluoresce the characteristic blue-green of wild-type colonies were carefully picked, then restreaked at least twice on fresh medium to obtain a pure clonal line. Spontaneous mutants of A. tumefaciens strains A136, A136(pTiA6), and A348(pTiC58) were obtained by growing wild type cultures overnight at 30°C in LB medium, then allowing cultures to sit at least 4 days stationary on the lab bench at room temperature. Cultures were then plated and mutants selected as described above.

Bacterial conjugations and transformations. Selection for transconjugants which harbored deletion constructs in the chromosome was done in the following way: plasmid constructs in E. coli S17-1 were conjugated into various spontaneous mutants. After selection (rifampicin for the agrobacteria, tetracycline for pLAFR3), at least ten individual isolated colonies were picked together with the same sterile toothpick and grown in LB medium containing 2mg/L

Table 4. Bacterial strains and plasmids used in the study of the *pscB* locus.

Strain or plasmid	Relevant properties	Source or reference
A. tumefaciens		
A6.1	wild type	A. Binns
JMc	A6.1 Tn*5*::*pscB*	this study
A136	C58 background; Ti^-	49
A136(A6)	C58; pTiA6	49
A348	C58; pTiC58	49
Plasmids and cosmids		
pLAFR3	IncP, tet^R	43
pPH1JI	IncP, $gent^R$	P. Hirsch
pR/H	WT pscB in pLAFR3	T. Lynch
pR/B	deletion in pLAFR3	T. Lynch
p5-2	deletion in pLAFR3	T. Lynch
p10-4	deletion in pLAFR3	T. Lynch
p15-1	deletion in pLAFR3	T. Lynch
p15-3	deletion in pLAFR3	T. Lynch

abbreviations: tet=tetracycline
gent=gentamycin

tetracycline. These cultures were conjugated with E. coli S17-1 harboring plasmid pPH1JI, which encodes gentamycin resistance and the extrachromosomal exsistence of which is incompatible with pLAFR3. Exconjugants were selected for resistance to rifampicin, tetracycline, and gentamycin. All other manipulations were as described (Materials and Methods, Chapter 2).

Determination of the reversion frequencies of the spontaneous, dark mutants. Reversion frequencies were determined in the following way: overnight cultures of spontaneous, dark mutants were diluted and plated at 10^{-5} and 10^{-8} on LB Leucophor medium on large (150mm diameter) and small (100cm diameter) petri plates, respectively. The total number of colonies from the 10^{-8} plate was used to estimate the total number of colonies on the 10^{-5} plate, and the number of bright revertants on the large plate was then used to arrive at the frequency. M9 medium (27) was used for experiments requiring minimal medium.

Soil persistence assay. Tomato seeds (LA1221 and Ace varieties, a gift from Dr. Hans Kende) were sterilized in ethanol and bleach, rinsed ten times with sterile water, dried, and allowed to germinate on YMB plates (27) for three days in the dark at room temperature. A. tumefaciens strains A6.1 wild type and JMc, a Tn5-derived dark mutant

mapping to the pscB locus, were grown in 2ml LB for two days, shaking, at 30°C, washed once in sterile water, and resuspended in 1ml sterile water. Baccto soil mix (Michigan Peat Co., Austin, TX) was placed in magenta boxes (10cm x 7cm x 7cm), wetted with approximately 50ml water, and autoclaved. Tomato seedlings were imbibed in the agrobacterium suspensions, then transferred to the magenta boxes (one box/seedling) and buried about one-quarter inch in soil; this work was done in a sterile hood. Magenta boxes were then placed on the lab bench (about 26°C) for two days, then transferred to a growth chamber (18 hour day, 22°C) for approximately three weeks, by which time the tomato seedlings reached the tops of the magenta boxes. The tomatoes were then topped in a sterile hood with sterile scissors, soil knocked off the roots, and the roots and remaining soil placed in 2ml sterile water and vortexed vigorously. 10ul of these suspensions was then plated on selective and non-selective media.

Restriction endonucleases and chemicals; Virulence testing. All materials and procedures were as described (Materials and Methods, Chapter 2).

Results

Isolation of spontaneous mutants. Approximately 50 spontaneous, dark mutants of A. tumefaciens A6.1 were isolated as described in Materials and Methods. These mutants were found to arise at the surprisingly high rate of 10^{-3} to 10^{-4}. A subset of those isolated (Table 5) was then used to test for reversion frequencies. It was found that most of the mutants (approximately 75% of the A6.1 mutants tested) did indeed revert to apparent wild type, and this reversion occurred at frequencies approximating those of the forward mutations. The remainder of the mutants did not revert to brightness, or, if they did, the frequency was less than 10^{-7}.

The possibility existed that the particular strain of A. tumefaciens used in this study, A6.1, possessed a genome which was inherently unstable, and that the appearance of spontaneous dark mutants at such a high frequency was a manifestation of this general instability. To establish whether or not this was the case, the following experiment was performed. It was reasoned that a genetically unstable strain would be prone to give rise to mutants unable to grow on minimal medium (wild type cells grow luxuriantly on minimal medium) due to mutations in genes encoding metabolic functions. It was further reasoned that there must be many more genes involved in the biosynthetic processes of metabolism, protein synthesis, and the like than in EPS

expression, and that the spontaneous frequency of mutants unable to grow on minimal medium should then greatly exceed the frequency of EPS mutants, should the entire genome prove to be genetically unstable. One thousand inidividual A. tumefaciens colonies were screened, and all were found to be capable of growth on minimal medium. Thus, the spontaneous, dark mutants did not appear to arise from a general genetic instability. The fact that all of the mutants were complemented by the same fragment of DNA (see below) further established that the genetic variation responsible for the mutant phenotypes were a local, rather than global, phenomenon.

Complementation of the dark phenotype of the spontaneous mutants. The next set of experiments was undertaken to determine if the dark phenotype of the mutants was due to mutations at the same locus in the A. tumefaciens genome. It was discovered that a single cosmid clone from a wild type genomic library was capable of complementing all of the spontaneous dark mutants (H. Malkawi, unpublished results), and that a subclone of the wild type cosmid clone, a 2.5 kilobase (kb) EcoRI-HindIII restriction fragment, could effect the same complementation (T. Lynch, unpublished results). Dark mutants harboring the fragment, the coding sequences of which were termed pscB, appeared bright like wild type colonies on Leucophor plates.

Marker exchange data (T. Lynch, unpublished results) was consistent with the hypothesis that the pscB sequences were indeed responsible for the mutant phenotype, and that the restoration of the bright phenotype was not due to pseudocomplementation. That the mutations had occurred at the pscB locus was further demonstrated by the fact that pscB sequences isolated from nine of the spontaneous, dark mutants were unable to complement any of the mutants, whereas the wild type sequences could effect this complementation (T. Lynch, unpublished results).

Experiments were then conducted in order to more precisely define the location of the mutations responsible for the dark phenotype, and to establish if all the independent, spontaneous mutants had suffered the same genetic lesions. A series of deletions of the 2.5kb fragment encoding pscB was generated using the Exonuclease III/Mung Bean nuclease protocol (45), and these deletions were cloned into the broad host range plasmid pLAFR3, which encodes resistance to tetracycline (Figure 4).

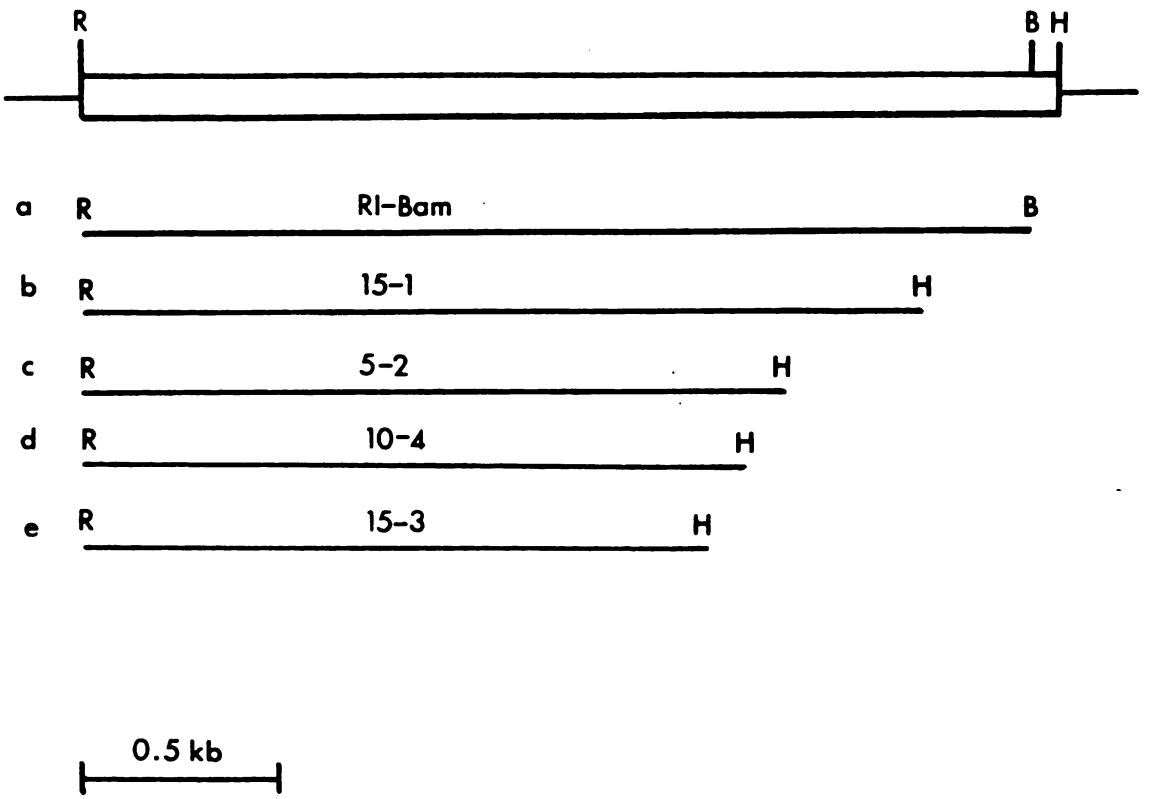
The deletion constructs were used to determine more accurately the site of the pscB sequences on the 2.5kb restriction fragment. The constructs were introduced into various spontaneous, dark mutants of A6.1 which had been shown to be complemented by pscB sequences encoded on the 2.5kb EcoRI-HindIII fragment. The exconjugants were then

assayed for the ability of the deletion constructs to restore the bright phenotype in *trans*. None of the deletions were able to do so. Therefore, the right-hand edge of the *pscB* coding sequences must lie between the *Hin*dIII site which defines the right-hand side of the 2.5kb fragment and the *Bam*HI site just to the left of it (Figure 4).

The next experiments were undertaken to determine if the mutations had occurred at the same site in the *pscB* sequences in all of the spontaneous mutants. The site of DNA lesions in mutant chromosomes can be determined by selecting for single recombinational events between mutant, chromosomal sequences and a series of progressive deletions of cloned, wild type sequences. Those deletions which do not extend into the sequences where the mutation has occurred in the chromosome of the recipient will be capable, through recombination, of forming a functional allele. When the deletions extend past the point where the lesion has occurred on the mutant chromosome, no recombinational event will be able to create an intact, functional gene, and no restoration of wild type phenotype, in this case bright colonies, can occur. Thus, it is possible to determine between which two deletion constructs the lesion in the mutant DNA is located.

Deletions prepared to establish the right-hand border of the *pscB* coding sequences were used for the recombination analysis. Plasmid pPH1JI was conjugated into spontaneous mutants harboring the deletion derivatives in *trans*.

Figure 4. Deletions of the EcoRI-HindIII fragment encoding the pscB sequences of A. tumefaciens



Top: wild type fragment capable of complementing the pscB mutants in trans. a through e: deletions of the wild type fragment incapable of complementing the pscA mutants in trans. R=EcoRI; B=BamHI; H=HindIII; kb=kilobases.

pPH1JI, which encodes gentamycin resistance, is incompatible with pLAFR3. Exconjugants from this mating were selected for resistance to rifampicin, gentamycin, and tetracycline, and plated on medium containing Leucophor. Bacteria resistant to all three antibiotics were agrobacteria which harbored pPH1JI extrachromosomally and had integrated into the genome, via homologous recombination between mutant and wild type pscB sequences, the deletion derivatives, including the cloning vector, pLAFR3. Alternatively, resistance to all three antibiotics could reflect cointegration of pPH1JI and the pLAFR3 constructs; such transconjugants would appear dark.

That deletions extend past the point in the wild type sequences at which the lesion occurred in the mutant chromosome was indicated by the fact that only dark exconjugants were observed. Where the deletions still harbored wild type sequences which were mutant in the chromosome, bright colonies were seen among the exconjugants, at a frequency of 1-5% (Table 5). The fact that not all of the transconjugants were bright is due to crossover events which did not create a functional pscB, or to the formation of cointegrates, as described above. This set of experiments established that the actual site of mutation was not the same for every mutant. None of the mutants could be complemented in cis by p15-3. One group of mutants could be complemented in cis by pR/B, and the rest

Table 5. Spontaneous mutants and their characteristics

Mutant	Reversion frequency[1]	complementation[2] in *trans* by pR/H	in *cis* by pR/B	in *cis* by p15-3	Source
A. tumefaciens A6.1:					
spoC21	0.3×10^{-3}	+	+	-	T. Lynch
spoC24	0	+	-	-	T. Lynch
spoC25	2.5×10^{-3}	+	+	-	T. Lynch
spoC29	0	+	-	-	T. Lynch
D2	47×10^{-3}	+	-	-	this study
D3	1.3×10^{-3}	+	+	-	this study
D4	0.6×10^{-3}	+	-	-	this study
D5	1.5×10^{-3}	+	+	-	this study
A. tumefaciens A136:					
2.8	0.05×10^{-3}	+	+	-	this study
2.9	0.2×10^{-3}	+	-	-	this study
A. tumefaciens A136(A6):					
D1	0.1×10^{-3}	+	-	-	this study
D3	0	-			this study
D5	0.1×10^{-3}	+	+	-	this study
D6	0	+	+	-	this study
A. tumefaciens A348:					
1.2	0	-			this study
1.3	0	+	+	-	this study
1.5	0	-	-	-	this study
1.6	0	+	-	-	this study
1.7	0	-			this study
1.9	0	+	+	-	this study

[1]-reversion frequencies were determined as detailed in Materials and Methods; mutants with a "0" reversion frequency designation did not give rise to any bright revertants at frequencies greater than 10^{-7}.

[2]-"+" indicates complementation and "-" indicates no complementation

of the mutants could only be complemented by the undeleted sequences. Although the mutations do not occur at the same exact place in the pscB locus, all the mutants tested have been found to have suffered mutations within five hundred or so base pairs of the extreme right-hand side of the locus.

Ubiquity of spontaneous mutants. All of the spontaneous, dark mutants thus far discussed were obtained from our standard laboratory strain of A. tumefaciens, A6.1. Concerned that the generation of such mutants might be anomalous to A6.1, we endeavored to obtain similar mutants from another isolate of agrobacteria. As A6.1 harbors the Ti plasmid pTiA6, we looked at agrobacteria harboring a different Ti plasmid, pTiC58, and of separate, distinct chromosomal backgrounds. A. tumefaciens A348(pTiC58), A136(harboring no Ti plasmid), and A136(pTiA6), all of C58 chromosomal lineage, were used. The same regimen by which the spontaneous mutants from A6.1 were obtained was unsuccessful in obtaining dark mutants from the non-A6.1 strains. It was discovered, however, that such mutants could be obtained from these strains if the liquid broth cultures were allowed to sit, stationary, at room temperature, for approximately four days. Liquid cultures thus treated revealed the presence of dark mutants when plated on solid medium containing leucophor. The frequency of the appearance of the mutants approximated that of A6.1

in most cases (Table 5). Liquid broth cultures which were shaken at 30°C instead of being left stationary at room temperature did not give rise to spontaneous dark colonies.

The spontaneous mutants from the C58 background were then tested as to whether they could be restored to brightness by a cosmid encoding the pscB locus. Active pscB sequences, pscB sequences rendered inactive by transposon insertion, as well as the cloning vector were introduced by conjugation into the mutants. The results of these experiments are summarized in Table 5. The majority of the spontaneous, dark mutants from the C58 background were indeed restored to brightness by the pscB sequences. None of the mutants were complemented by a cosmid encoding the pscA sequences (data not shown).

Virulence and fitness of the spontaneous mutants. All of the spontaneous mutants were tested for virulence on Kalanchoe plants and carrot discs, and all formed tumors whose onset, size, and characteristics were indistinguishable from those formed by wild type isolates.

To determine whether the mutants were capable of persisting in liquid medium and in soil to the same degree as the wild type, the following experiments were performed. The wild type strain and JMc, as dark pscB mutant obtained by transposon mutagenesis, were used to compare the fitness of the mutant with that of the wild type in liquid culture. The mutant strain showed a survival rate in continuous

liquid culture which approximated closely that of the wild type. The difference between the number of wild type agrobacteria and the number of JMc mutant bacteria still viable over a period of three weeks was less than an order of magnitude.

The transposon mutant and the wild type were then compared with respect to their persistence in sterile soil. The transposon-induced mutant, JMc, and the wild type were compared both alone and in coinoculations in soil over a period of three weeks (see Materials and Methods) with respect to the number of colonies of each which could be rescued from the soil (JMc is resistant to kanamycin and rifampicin, the wild type to rifampicin, and these resistances were used to calculate the relative numbers of each strain in the soil). Again, both strains showed approximately the same numbers of recoverable bacteria relative to the initial inoculum after the three week interval.

Discussion

It was established that the strains of Agrobacterium tested give rise to spontaneous EPS mutants at the high frequency of 10^{-3} to 10^{-4}, and that most of the mutants gave rise to apparent wild type revertants at approximately the same frequency at which the forward mutations occurred. The mutations were found to occur at a single locus, termed

pscB. In A. tumefaciens strain A6.1, these mutants arise during standard culture procedures, but in the other strain tested such mutants arise only when cultures were allowed to sit, stationary, over a period of several days. It was established that the appearance of spontaneous, dark mutants is a phenomenon not associated with the Ti plasmid; an isolate cured of its Ti plasmid gave rise to such mutants.

These mutants proved to be equally capable as the wild type of survival in liquid culture and sterile soil, and to be fully virulent. It is important to note that the virulence testing was performed under circumstances that are not likely to occur in nature: the creation of a fresh wound and the introduction into that wound of millions of bacteria. The possibility remains that the dark phenotype does have some significance for virulence that transcends our ability to detect it in our virulence assays.

The mutants all mapped to roughly the same region of the pscB sequences, as established by deletion/recombination analysis, but the exact site of mutation differed from mutant to mutant. The vast majority of the spontaneous mutations leading to darkness appeared specifically at the pscB locus, and not at other sites in the genome.

The mechanism by which A. tumefaciens cells generate such mutants has not been established. Comparison of the mutant and wild type pscB alleles indicates that no gross rearrangements have occurred; both alleles are, to the closest approximation, of the same size, and no restriction

fragment length polymorphisms have been detected, even down to the level of restriction enzymes with four base pair recognition sequences (T. Lynch, unpublished results).

Nor is it clear why this phenomenon occurs. It is of particular interest that the non-A6.1 strains require stress to induce mutations; it is possible that some mechanism in these strains recognizes stress and responds by inducing mutations at the pscB locus. Polysaccharide expression is an energetically demanding process to the cell; the addition of every monosaccharide unit requires one molecule of ATP. One could imagine that it would be an advantage to a microbial population under stress to generate mutants whose energy requirements were less than those of the wild type. Perhaps strain A6.1 has lost this stress-sensing mechanism, and expresses the mutant-generating capability in a constitutive manner.

It is not clear whether the pscB mutants of A. tumefaciens A6.1 and A348 have anything in common with the mutants of A. tumefaciens IFO358 described by Hisamatsu, et al (43). Those mutants arose after storage of the wild type strain in agar slants, a situation reminiscent of the procedure required to obtain mutants from the C58 strains. The instability of surface chemistry of A. tumefaciens certainly appears to be a generalized phenomenon.

A number of potential mechanisms by which the pscB sequences are preferentially mutagenized can be envisaged. It is possible that the particular base composition of the

pscB locus renders it a hotspot for mutation; the fact that there are no gross rearrangements, however, argues against large deletion or insertion events as the cause of the mutations, though small insertions or deletions, such as those described in Neisseria gonorrhoeae, could be responsible. Similar logic argues against mobile genetic elements present within the genome being responsible for the mutations. A likely explanation is that the mutations are the result of point mutations, which would generate mutant alleles which are the same size as the wild type and not readily detectable by restriction analysis, unless the mutations were to happen to fall on a restriction site.

Although one of the central dogmas of molecular genetics has been that all mutations occur randomly and are merely selected for by the environment in which a microorganism finds itself, recent findings suggest that microorganisms may possess the ability to adapt, at the DNA level, to their surroundings under certain circumstances.

Cairns, et al (47) investigated the occurrence of Lac+ mutants arising from Lac- cultures of E. coli, and found the distribution of such mutants to be inconsistent with the parameters of a random occurrence of mutations for which there is a subsequent selection. Simply put, cultures in which the occurrence of a particular mutation precedes its selection should give rise, on repeated experiments, to a broad distribution of the number of mutants which arise from any given culture. This broad distribution reflects the

fact that the mutation which is subsequently selected for can occur at any generation in the culture; it may occur in the first generation or the last before selection, and the earlier it occurs the greater the number of its clonal line which will be represented after the final selection. When mutation occurs at the time of selection, however, the distribution of the number of mutants arising from repeated experiments will be much narrower, for all of the directed mutations will have had to occur during the one generation that is exposed to selection. Cairns, et al, found that, when they performed repeated experiments in which Lac+ mutants were selected from a Lac- culture of E. coli, the distribution reflected a composite of these two distributions, which to them suggested that both spontaneous and directed mutation were occurring in their cultures.

The findings of Rosqvist, et al, in a study of virulence determinants in Yersinia pestis (48), suggest that the appearance of hypervirulent variants of that organism may not conform to the precepts of random, undirected mutation. The occurrence of more or less virulent strains of Y. pestis seems to correlate with the greater or lesser availability, respectively, of susceptible hosts; the organisms may be changing the genetic bases of their virulence characteristics not as a result of the whims of random mutation but rather in response to the exigencies of maintaining a population-sustaining balance between transmissibility and virulence.

Whether A. tumefaciens responds to environmental stress by directing mutations in one of the loci responsible for EPS expression is questionable; other, more conventional, mechanisms resulting in the genetic variation which occurs at the pscB locus can easily be imagined. Sequencing of mutant and wild type alleles could establish the mechanism and character of the mutations. It would be of interest to determine if recombination-deficient derivatives of the agrobacteria which require stress to give rise to pscB mutants would do so with a chromosome thus altered.

Thus far, no physiological importance has been attached to the components which are missing or altered in the spontaneous mutants of A. tumefaciens. These components have proven not to be necessary for the virulence or the fitness of the agrobacteria as measured in the laboratory. It is possible that the unique character of the pscB locus is an anomaly or a manifestation of some function required at some time in the evolution of A. tumefaciens but no longer of importance. It is also possible that the ability to give rise to spontaneous variants in its surface determinants lends A. tumefaciens a subtle advantage as a soil bacterium and a plant pathogen in a dynamic and competitive environment.

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