JOURNAL OF BACTERIOLOGY, Mar. 1989, p. 1755–1759
0021-9193/89/031755-05$02.00/0

Vol. 171, No. 3

Two Chromosomal Loci Involved in Production of Exopolysaccharide in *Agrobacterium tumefaciens*

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Received 2 September 1988/Accepted 9 December 1988

The chromosomal locus *pscA* (*exoC*) of *Agrobacterium tumefaciens* LBA4301 has been cloned by complementation of the avirulent and exopolysaccharide (EPS)-deficient mutant LBA4301 *pscA*. We have also identified a new locus, termed *psdA* (polysaccharide depression) and located 16 kilobases from *pscA* in the *A. tumefaciens* chromosome, that negatively affects EPS production when it is present in more than one copy in *A. tumefaciens* LBA4301. Subcloning, transposon mutagenesis, and transcriptional analysis have been conducted for both loci and indicate that *pscA* and *psdA* are transcribed in the same orientation. Acidic-EPS assays showed that *psdA* depresses succinoglycan production and that its negative effect increases with the copy number of the gene. Virulence tests of *psdA* transconjugants on *Datura stramonium* showed no visible alteration in virulence, while LBA4301 *pscA* was totally avirulent.

Agrobacterium tumefaciens causes crown gall tumors on a wide range of plants. Large (approximately 200-kilobase [kb]), tumor-inducing (Ti) plasmids are essential for pathogenicity and are present in all pathogenic strains. Tumorigenesis involves the transfer and integration of a segment of the Ti plasmid, the T-DNA, in the host plant genome. The genes of the T-DNA responsible for synthesis of opines and plant hormones are then expressed in the transformed plant cell. Another portion of the Ti plasmid, the *vir* region, is required for the processing and transfer and possibly the integration of the T-DNA (for a recent review, see reference 8). There are also chromosomal loci that play a role in the pathogenic process. These include the following. (i) The two linked loci *chvA* and *chvB* (4) seem to be required for virulence. Strains carrying mutations at these loci are defective in plant cell attachment and thereby avirulent. The *chvB* mutant is defective in the synthesis of the cellular and extracellular polysaccharide β-1,2-glucan (15). (ii) The *cel* mutants are defective in cellulose synthesis and are weakly virulent (12). (iii) Mutants defective in plant cell attachment (*att*) are avirulent and are altered in outer membrane protein composition (13). The *cel* and the *att* mutants were mapped in the same region of the *A. tumefaciens* chromosome (16). (iv) The pleiotropic mutant *ros* lacks succinoglycan, the major acidic exopolysaccharide (EPS) of *A. tumefaciens*. It is fully virulent and derepresses the expression levels of *virC* and *virD* (3); an actual role of *ros* in pathogenicity has not yet been determined (2). (v) The pleiotropic mutant *pscA*, which can be complemented by the *exoC* locus of *Rhizobium meliloti* (1, 20), lacks both β-1,2-glucan and succinoglycan; it is avirulent, nonmotile, and deficient in plant cell attachment.

By Tn*5* mutagenesis, we have isolated a *pscA* mutant of *A. tumefaciens* LBA4301 Rec$^-$ Rif^r (from R. A. Schilperoort; designated the wild-type strain in this note) that is complemented by an *R. meliloti exoC* clone, pD15 (10), and exhibits characteristics similar to those previously described (1, 20). We report here the cloning and genetic characterization of *pscA* and a genetically linked new locus that we termed *psdA*, for polysaccharide depression. *psdA* depresses EPS production when it is present in more than one copy in the parental strain of *A. tumefaciens*.

Cloning of *pscA* and *psdA*. A genomic library from *A. tumefaciens* LBA4301 *ros* (3) was constructed by partial *Sau*3A digestion of total DNA, treatment with alkaline phosphatase, and ligation to the 11.5-kb *Bam*HI fragment of the cosmid vector pUCD5 (5). DNA was then packaged in vitro into bacteriophage lambda (in vitro packaging kit; Amersham Corp., Arlington Heights, Ill.) and transduced into *Escherichia coli* HB101 (from H. W. Boyer). The library was used to complement LBA4301 *pscA* dark phenotype on medium 523–1.5% agar plates (6) supplemented with calcofluor (300 μg/ml; fluorescent brightener 28; Sigma Chemical Co., St. Louis, Mo.). Most of the transconjugant colonies appeared dark, but several bright colonies were observed. Two of the bright colonies, harboring the overlapping cosmids pUCD1810 and pUCD1811, were chosen for further analysis (Fig. 1). pUCD1810 fully restored bright green-yellow fluorescence and mucoid colony characteristics in LBA4301 *pscA*, while pUCD1811 gave rise to somewhat dry colonies with an intermediate dull blue fluorescence. Interestingly, the wild-type parent LBA4301 containing pUCD1811 formed the same type of dry colonies with dull blue fluorescence on calcofluor plates, whereas LBA4301 (pUCD1810) formed colonies similar to those formed by the plasmid-free wild-type strain, i.e., mucoid with green-yellow fluorescence. Therefore, besides containing the *pscA* locus, pUCD1811 was suspected to possess another locus that is able to depress EPS production in *A. tumefaciens* when it is present in more than one copy in the cell. The presence of this locus, designated *psdA*, for polysaccharide depression, was further verified in the studies described below.

*Bam*HI and *Kpn*I restriction enzyme maps were established for both pUCD1810 and pUCD1811; the two cosmids overlapped by approximately 17 kb (Fig. 1). All the *Bam*HI fragments were then subcloned into the *Bam*HI site of pUCD615 (18), and all the *Kpn*I fragments were subcloned into the *Kpn*I site of pUCD2001 (5). The *pscA* locus was confined to the 16.1-kb *Bam*HI fragment since pUCD1938 fully complemented LBA4301 *pscA*, while no apparent complementation was observed with the *Kpn*I subclones pUCD1933 and pUCD1934, suggesting that the *Kpn*I site might be within *pscA* (Fig. 1). Further mapping with *Eco*RI

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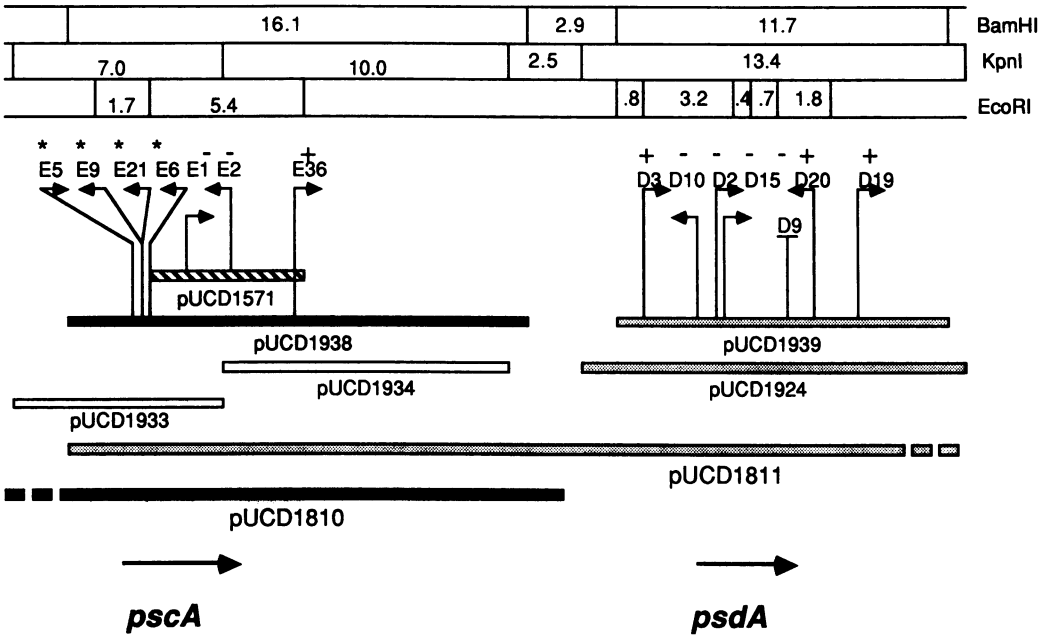

FIG. 1. Restriction map of the *A. tumefaciens pscA* and *psdA* regions. The numbers in the map proper indicate the sizes of the fragments in kilobases. The bars below the map represent the restriction fragments present in subclones, with the plasmid names indicated. Fragments represented include those that complement the *pscA* mutant (■), those that partially complement the mutant (▧), those that do not complement the mutant (□), and those that cause the *psdA* effect (▨). Note that pUCD1811 also complements the *pscA* mutant. The arrows represent TnCAT insertions and indicate the orientation of the promoterless *cat* gene, except for that in pUCD1939-D9, for which the orientation has not been determined. Insertion designations are above the arrows. Symbols: −, no complementation; *, partial complementation; and +, full complementation for the *pscA* locus and no effect (−) and depression (+) of EPS for the *psdA* locus. The minimum sizes and the directions of transcription of *pscA* and *psdA* are shown at the bottom.

subclones showed that pUCD1571 carrying the 5.4-kb *Eco*RI fragment was able to partially complement the *pscA* mutant; i.e., LBA4301 *pscA*(pUCD1571) showed the bright green-yellow fluorescence typical for full complementation but formed smaller colonies than the wild-type strain did. The *psdA* locus was located in the 11.7-kb *Bam*HI fragment, since both pUCD1939 (11.7-kb *Bam*HI fragment) and pUCD1924 (13.4-kb *Kpn*I fragment) were able to depress EPS production when introduced in the wild-type strain, while none of the other subclones had any effect.

Analysis of *pscA* locus. A transposon, TnCAT, that allows transcriptional and translational fusions with a promoterless chloramphenicol acetyltransferase (CAT) gene (*cat*) was constructed by a method like that previously described for Tn1736Tc (21). The transposition of TnCAT on pRK2013 (from D. Helinski), a helper plasmid for conjugal transfer, was obtained, and a chloramphenicol-sensitive strain of HB101(pRK2013::TnCAT) was selected for the mutagenesis experiments. Strain DH1 (from D. Hanahan) was transformed with plasmid pUCD1571 or pUCD1938 and mated with HB101 (pRK2013::TnCAT) at 30°C. DH1 transconjugants were selected on 1.5% LB agar plates (14) containing nalidixic acid (20 μg/ml), tetracycline (20 μg/ml), and kanamycin (20 μg/ml) for the presence of both plasmids and were subsequently mated with LBA4301 *pscA*. *A. tumefaciens* transconjugants harboring TnCAT insertions in the respective plasmids were selected on medium 523 agar plates containing rifampin (50 μg/ml), gentamicin (15 μg/ml), and tetracycline (5 μg/ml) and screened on calcofluor plates. LBA4301 *pscA* transconjugants with pUCD1571::TnCAT showed hundreds of bright (complemented) colonies and two dark (noncomplemented) colonies harboring plasmids pUCD1571-E1 and pUCD1571-E2, whereas those with pUCD1938::TnCAT showed only bright (complemented) colonies on calcofluor plates. Four strains containing plasmids pUCD1938-E5, pUCD1938-E9, pUCD1938-E21, and pUCD1938-E6 were found to contain closely linked TnCAT insertions and complemented the *pscA* mutant only partially, in a manner like that of pUCD1571 (bright but small colonies). Mutant plasmid pUCD1938-E36 fully complemented the *pscA* mutant, suggesting that TnCAT had inserted outside the *pscA* locus (Fig. 1).

Spectrophotometric CAT assays (19) were conducted with representative fusion plasmids in both the wild-type strain and the *pscA* mutant to determine the direction of transcription of *pscA* (Table 1). Plasmid pUCD1938-E5 showed relatively high CAT activity in both genetic backgrounds, whereas pUCD1571-E1 showed low CAT activity only in the

TABLE 1. CAT expression for *pscA-cat* and *psdA-cat* fusions in wild-type *A. tumefaciens* and the *pscA* mutant

Mutagenized locus and plasmid	CAT sp act (nmol/min per mg of protein)	
	Wild type	*pscA* mutant
pscA		
pUCD1938-E5	25.2	34.9
pUCD1938-E6	<1.0	<1.0
pUCD1571-E1	<1.0	4.5
pUCD1571-E2	<1.0	<1.0
psdA		
pUCD1939-D3	<1.0	<1.0
pUCD1939-D10	<1.0	<1.0
pUCD1939-D2	79.6	146.0
pUCD1939-D15	83.8	220.0
pUCD1939-D9	<1.0	<1.0
pUCD1939-D19	361.0	541.0

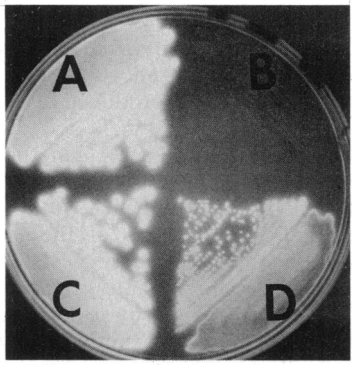

FIG. 2. Fluorescence of the *pscA* mutants on medium 523 agar plates supplemented with calcofluor (300 μg/ml) and photographed under UV light. (A) LBA4301(pUCD615); (B) LBA4301 *pscA* (pUCD615); (C) LBA4301 *pscA*(pUCD1938); (D) LBA4301 *pscA* (pUCD1938-E5).

pscA mutant and no detectable activity in the wild type. Plasmids pUCD1938-E6 and pUCD1571-E2 carrying TnCAT insertions in the opposite orientation showed no detectable CAT activity in either strain. These results suggest that *pscA* of LBA4301 *ros* is transcribed from left to right, as previously proposed for *A. tumefaciens* A6.1 (20).

The *pscA* mutants of A6.1 are deficient in two exopolysaccharides, a high-molecular-weight acidic succinoglycan and a low-molecular-weight neutral β-1,2-glucan (1, 20). We conducted qualitative (Fig. 2) and quantitative (Table 2) acidic-EPS assays with LBA4301 or LBA4301 *pscA* harboring various plasmids. For the quantitative EPS assay, cultures were grown to early stationary phase (optical density at 600 nm, 0.5 to 0.8) under selective pressure and identical conditions. Then 1.4 ml of each culture was harvested, and 1 ml of the supernatant was transferred to a separate glass tube, precipitated with 0.3 ml of 1% cetyltrimethylammonium bromide, and centrifuged for 15 min at 1,250 × *g*. The pellet was dissolved in 1 ml of 10% (wt/vol) NaCl and precipitated in 4 volumes of 95% ethanol, centrifuged for 15 min at 1,250 × *g*, and dissolved in 1 ml of distilled H_2O. The material was precipitated one more time with 4 volumes of ethanol, and the final amount of polysaccharides was determined by the anthrone assay (17), with glucose solutions used as a standard. The cloning vehicle pUCD615 was introduced into the wild-type or *pscA* mutant strains. As expected, the *pscA* mutant showed extremely low contents of acidic EPS relative to content in the wild type or the *pscA* mutant complemented with pUCD1938. The quantitative assay revealed that LBA4301 *pscA*(pUCD1571-E1), which is indistinguishable from LBA4301 *pscA* on calcofluor plates, produced some acidic EPS (about threefold more than the mutant did). It also showed that both LBA4301 *pscA* (pUCD1571) and LBA4301 *pscA*(pUCD1938-E5), which appeared as bright as the wild type on calcofluor, had a threefold-lower content of acidic EPS than the wild type did (Table 2).

TABLE 2. Acidic-EPS content in *A. tumefaciens pscA* and *psdA* strains

LBA4301 or *pscA* strain containing plasmid:	Acidic EPS (μg/10^9 cells)[a]
pUCD615	64
pUCD1939	36
pUCD2519	9
pUCD1939-D2	71
pUCD1939-D9	68
pscA(pUCD615)	6
pscA(pUCD1938)	72
pscA(pUCD1938-E5)	27
pscA(pUCD1571)	24
pscA(pUCD1571-E1)	11

[a] Average results for two experiments.

LBA4301 *pscA*(pTiC58 Tra^c) (7) was found to be totally avirulent in inoculations of *Datura stramonium* (Jimson weed) stems (9). Plasmid pUCD1571-E1 did not complement the virulence deficiency of the *pscA* mutant. However, LBA4301 *pscA*(pTiC58 Tra^c, pUCD1938-E5), which shows an intermediate phenotype for EPS production, was fully virulent.

pscA insertion mutant analysis shows that this locus is at least 3.6 kb in size—similar to the homolog locus of the octopine type strain A6.1 (20). In addition, we observed two different phenotypes during complementation with insertion mutants. The first set of insertions, clustered in a region of about 0.4 kb at the 5′ end of the locus, caused partial complementation of the *pscA* mutant. The complemented colonies were virulent and appeared bright on calcofluor plates, but they showed low acidic-EPS content and formed small, dry colonies. The second set of insertions was located farther downstream (the 3′ region) and apparently did not complement the mutant. These results are inconsistent with an operon model for *pscA*, which would imply that insertions in the 5′ region are polar and affect the whole operon. Moreover, the similar phenotypes caused by *pscA* subclone pUCD1571 and the 5′-region mutants in LBA4301 *pscA* suggest that the 5.4-kb *Eco*RI fragment contains a sequence that causes partial complementation. A plausible model is that there are two overlapping promoters, i.e., a relatively strong promoter upstream (or overlapping insertion E5) and a weak one between insertions E6 and E1. Hence, *pscA* may give rise to two phenotypes, depending on the promoter. The difference in CAT activity observed between fusions with the two regions is consistent with this model.

Analysis of *psdA* locus. Insertion mapping of the *psdA* locus was conducted by TnCAT mutagenesis of pUCD1939 by a method like that used for *pscA* plasmids. A total of seven insertions was obtained in the *psdA* region (Fig. 1). Plasmids pUCD1939-D10, pUCD1939-D2, pUCD1939-D15, and pUCD1939-D9 were unable to alter the typical mucoid colony characteristics of LBA4301 or its green-yellow fluorescence on calcofluor plates. The corresponding insertions are therefore localized within the *psdA* gene. On the other hand, plasmids pUCD1939-D3, pUCD1939-D20, and pUCD1939-D19 had the same effect on LBA4301 as pUCD1939 did, i.e., dry colonies with blue fluorescence on calcofluor plates.

To determine the direction of transcription of *psdA*, CAT assays (19) were conducted on LBA4301 and LBA4301 *pscA* carrying selected mutagenized plasmids. In both strains, plasmids pUCD1939-D2 and pUCD1939-D15 showed high CAT activity, whereas pUCD1939-D10 showed no detectable activity. These results suggest that transcription of *psdA* occurs from left to right (Fig. 1). The highest CAT activity was found in strains carrying pUCD1939-D19. TnCAT in this plasmid is inserted outside *psdA*, and it revealed the presence of relatively strong promoter activity downstream from *psdA* that reads in the same direction (Table 1).

Since the *A. tumefaciens* gene bank had been made from the *ros* mutant of LBA4301, it was not clear whether the

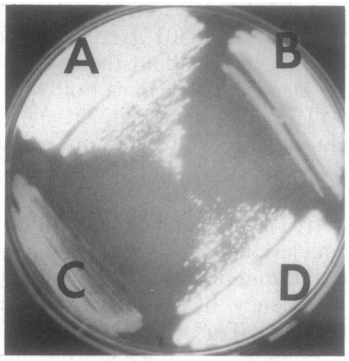

FIG. 3. Fluorescence of the *psdA* strains on medium 523 agar plates supplemented with calcofluor (300 μg/ml) and photographed under UV light. (A) LBA4301(pUCD615); (B) LBA4301(pUCD 1939); (C) LBA4301(pUCD2519); (D) LBA4301(pUCD1939-D2).

psdA effect was due to a dominant *psdA* mutation present on the cosmids or simply to a gene dosage effect caused by extra copies of the wild-type *psdA* allele on the cosmids. To investigate this question, we made a gene bank of LBA4301 in pUCD615; the isolation of a cosmid with the same characteristics as pUCD1811 supported the second possibility (data not shown). To determine the extent to which the effect of *psdA* is dependent on copy number, the 13.4-kb *Kpn*I fragment containing *psdA* was recloned from the low-copy-number plasmid pUCD1924 (approximately 4 copies per cell) (Fig. 1) onto pUCD1002 (approximately 40 copies per cell) (5), yielding pUCD2519. Plasmid pUCD2519 had a more dramatic effect on EPS production by LBA4301, leading to a dark appearance with very dim fluorescence on calcofluor plates, whereas LBA4301(pUCD1939) showed some blue fluorescence (Fig. 3).

Acidic EPS assays were conducted with *psdA* transconjugants strains as described above to identify the nature of the EPS affected by the *psdA* locus (Table 2). The results show that *psdA* caused a roughly twofold decrease in acidic EPS when present in low copy numbers and a more than sixfold decrease when present in high copy numbers. These data are consistent with the phenotypes of these strains on calcofluor plates (Fig. 3). Two insertion mutant plasmids of *psdA*, pUCD1939-D2 and pUCD1939-D9, were also tested for their effect on the acidic-EPS content of LBA4301. On calcofluor plates, the negative effect of *psdA* on EPS was fully abolished by these insertions (Fig. 3).

LBA4301(pJK270) (7), a fully virulent strain, showed no visible alteration in virulence when tested on *D. stramonium* stems in the presence of the low-copy-number *psdA* plasmid pUCD1939.

Transposon mutagenesis of *psdA* revealed a region of at least 2.8 kb, apparently transcribed in the same direction as *pscA*. All inserts completely abolished the *psdA* effect on calcofluor fluorescence and acidic-EPS production of *A. tumefaciens*. Thus, *psdA* may be one unit of transcription, but the number of gene products cannot be inferred from our data.

Our results suggest that *pscA* and *psdA* are involved in the regulation of EPS production in *A. tumefaciens*. These genes were located about 16 kb apart in the *A. tumefaciens* chromosome and were transcribed in the same orientation (Fig. 1). They had opposite effects on EPS production (Fig. 2 and 3): the lack of *pscA* led to a ~16-fold decrease in acidic-EPS production and to loss of virulence, while the presence of extra copies of *psdA* caused a threefold (4 extra copies) to sixfold (20 extra copies) decrease in acidic-EPS production, but no alteration in virulence. The amount of acidic EPS measured probably reflects the amount of succinoglycan, the major acidic EPS produced by *A. tumefaciens* (2).

The function of the *pscA* and *psdA* gene products is still unknown. *pscA* is essential for EPS production and may encode a product involved in either a synthetic or a secretory step. The pleiotropic nature of the *pscA* mutation demonstrated for strain A6.1 (11) suggests that *pscA* is a regulatory gene, but the data in Table 1 indicate that *psdA* is not (or is not strongly) regulated by *pscA*, because there is no significant difference in CAT activity in the *psdA-cat* fusions between wild-type and *pscA* mutant strains. For *psdA* the copy number effect suggests that this gene product may also be a regulatory protein that negatively regulates succinoglycan production. Since several EPS loci (*exo*) have been identified in *A. tumefaciens* as being essential for succinoglycan production (1), it will be interesting to determine whether *psdA* directly affects these genes, including *pscA* (*exoC*). Furthermore, *A. tumefaciens* strains with high *psdA* copy numbers are similar to the *ros* mutant in EPS composition, but no significant effect of *psdA* on *virC* promoter has been observed (data not shown).

Previous studies showed that *A. tumefaciens* and *R. meliloti* have similar genes for polysaccharide production (1, 11). Thus a counterpart of *psdA* is probably present in *R. meliloti*. Plasmid pD56 (*exoB* and *exoF* of *R. meliloti*) (10) was shown to depress EPS production in a manner similar to that of *psdA*, when conjugated in wild-type *A. tumefaciens* strains A6.1 (11) and LBA4301 (data not shown). Further analysis of pD56 may help in the identification of the *R. meliloti* homolog of *psdA*.

ACKNOWLEDGMENTS

We thank Timothy J. Close for help with the construction of the genomic library from *ros* and John A. Leigh for providing us with the *exo* plasmids from *R. meliloti*.

This work was supported by Public Health Service grant CA-11526 from the National Cancer Institute and CRGO grant 87-CRCR-1-2282 from the CSRS/USDA. S.K. was supported by the University Mission of Tunisia, Washington, D.C. M.B.C. was supported by a McKnight fellowship from funds granted by the McKnight Foundation for Interdisciplinary Research Projects in Plant Biology.

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