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Proteins conferred by the virulence-specifying plasmid of *Agrobacterium tumefaciens* C-58

(periplasmic proteins/two-dimensional gel electrophoresis/transconjugant/deletion plasmid)

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ABSTRACT **Membrane-associated and periplasmic proteins of *Agrobacterium tumefaciens* C-58 were compared with those from avirulent (nontumorigenic) derivative strains by slab and two-dimensional gel electrophoresis. Two proteins (Per-1 and Per-2), with a molecular weight of 37,500 and 37,300, respectively, were detected in the supernatant fraction of cells of strain C-58 treated with EDTA and lysozyme in which a 117-megadalton plasmid confers virulence on the organism. The same proteins are missing in an avirulent plasmid-free derivative of C-58. When this derivative is mated with C-58, the resulting transconjugants regain the large C-58 plasmid together with the restoration of virulence and the expression of Per-1 and Per-2 proteins. When the transconjugants were cured of their plasmid, they concomitantly lost their virulence and Per-1 and Per-2. The functional roles of these proteins are unknown, but they are associated with the outer membrane and periplasmic fraction of the *Agrobacterium* cell. If directly involved in tumorigenesis, these proteins are not the sole determinants of tumorigenicity because they are synthesized in an avirulent derivative of C-58 that carries a deletion in the plasmid in the region conferring the tumorigenic phenotype. These results strongly suggest that the Per-1 and Per-2 proteins are plasmid-coded gene products. The possible roles of these proteins in specifying host range and host-cell attachment are also discussed.**

A large plasmid (about 100–120 megadaltons) in virulent strains of *Agrobacterium tumefaciens* confers on the bacterial cell properties which allow them to transform higher plant cells into tumor cells during infection. The precise mechanism of this transformation process remains to be explored. One means of studying this aspect of tumorigenesis is to identify the products coded by the plasmid and determine if they have a direct function in tumor induction. Certain plasmid-mediated phenotypic functions are already known: the conferring of virulence (1–3), utilization and biosynthesis of guanido amino acids (2, 4–7), sensitivity to a phage (6), and sensitivity to a bacteriocin (6, 8). Apart from these phenotypically measurable functional roles, no plasmid-coded products have been directly studied. We now present evidence for the identification and isolation of two *Agrobacterium* plasmid-mediated proteins.

MATERIALS AND METHODS

Chemicals. Acrylamide (Matheson, Coleman and Bell) was purified by recrystallization from chloroform. *N,N'*-Methylenebisacrylamide (Eastman Organic Chemicals) was purified by recrystallization from acetone. The crystals were washed several times with the respective ice-cold solvents and collected by filtration onto filter paper. They were dried under reduced pressure at room temperature. Crystalline bovine serum albumin, egg white lysozyme, and *Bacillus subtilis* α-amylase were purchased from Sigma Chemical Company. Ovalbumin and trypsin were purchased from Worthington Biochemical Corporation.

Bacterial Strains. Type virulent strain C-58 of *A. tumefaciens* was obtained from R. S. Dickey, Cornell University. It carries a plasmid of 117 ± 4 megadaltons based on contour length measurements of electron micrographs of 14 molecules and the use of ColE1 DNA as an internal standard. Strain NT1, a avirulent plasmidless derivative of C-58 (2), was obtained from M.-D. Chilton. Strain 1D1173 is an avirulent derivative of C-58 that harbors a plasmid with a 39-megadalton deletion obtained by ethidium bromide curing (9). Strain 1D1274 is a progeny from a cross made *in planta* (10) between C-58 and a neomycin-, rifampicin-resistant mutant of NT1, the transconjugants of which were obtained by counter selections on a medium containing neomycin and rifampicin. All strains harboring the plasmid were tested for virulence on sunflower, cowpea, tobacco, and tomato.

Preparation of Subcellular and Membrane Proteins. Membrane (inner and outer) and subcellular proteins of *A. tumefaciens* strains were prepared by modifications of the procedures described by Miura and Mizushima (11) and Osborn *et al.* (12). Bacterial cells were cultured in 200 ml of medium 523 [contains, per liter, 10 g of sucrose, 8 g of casein enzymatic hydrolysate (CalBiochem), 4 g of yeast extract (Difco), 2 g of K_2HPO_4, and 0.3 g of $MgSO_4 \cdot 7H_2O$] for 20 hr at 30° with aeration, harvested by centrifugation (6000 × *g*, 5 min, 4°), and washed once with ice-cold distilled water. For labeling studies, cells were grown in 10 ml of minimal medium containing 0.5% glucose to a density of 150 Klett units and then 100 μCi of [^{35}S]sulfate or [^{3}H]leucine was added. Growth was continued for 3 hr at 30°. The cells were resuspended in 17.5 ml of ice-cold distilled water and the following chilled solutions were added gradually over the indicated time with gentle stirring: 10 ml of 0.1 M Tris·HCl, pH 8.3 (2.5 min); 9 ml of 2 M sucrose (2.5 min); 1.75 ml of 1% Na_2EDTA, pH 7.0 (1 min); and 1.75 ml of 0.5% lysozyme. The mixture was stored for 60 min at 4°. The resulting spheroplasts were removed by centrifugation (27,000 × *g*, 20 min, 4°) and the supernatant (Sup-1) was dialyzed exhaustively overnight against glass-distilled water at 4°. The dialyzed solution was then concentrated by lyophilization and stored at −20°. The spheroplasts were resuspended in 80 ml of ice-cold 1.5 mM Na_2EDTA, pH 7.6, and homogenized with a Sorvall Omnimixer at a speed setting of 3 for 30 sec. The homogenate was stirred for 20 min at 4°. Any unbroken cells were removed by centrifugation (500 × *g*, 20 min). The membrane fraction in the supernatant was then collected by centrifugation at 35,000 rpm for 2 hr in a Spinco Beckman 40 rotor and the resulting pellet was stored at −20°. The high-speed supernatant (Sup-2) was dialyzed exhaustively overnight, concentrated by lyophilization, and stored at −20°.

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

Gel Electrophoresis. Proteins were separated by electrophoresis on slab gels made of 12.5% polyacrylamide gels containing 0.8% *N,N'*-methylenebisacrylamide (unless stated otherwise) and 1% sodium dodecyl sulfate ($NaDodSO_4$) under the conditions described by Laemmli (13). The electrophoresis apparatus used is described by O'Farrell (14). Protein samples (50–100 μg of protein in 30–50 μl) in 62.5 mM Tris·HCl, pH 6.8/1% $NaDodSO_4$/10% sucrose/25 mM dithiothreitol were placed in boiling water for 2 min. The samples were placed into gel slots measuring 14 × 7 × 1 mm and electrophoresis was carried out at 100 V for 8 hr. The proteins were stained for 2 hr or overnight with 0.1% Coomassie brilliant blue R-250 in 50% (wt/vol) trichloroacetic acid. The gels were destained by repeated washings in 10% acetic acid at 33°C.

Two-dimensional gel electrophoresis was performed as described by O'Farrell (14). The ^{35}S- and ^{3}H-labeled proteins were detected by autoradiography as described by O'Farrell (14) or by the fluorographic method described by Bonner and Laskey (15) for polyacrylamide slab gels crosslinked with 0.12% *N,N'*-methylenebisacrylamide. Gels were placed between a sheet of Whatman 3MM filter paper and one thin mylar plastic and dried under reduced pressure under an infrared lamp. These gels were exposed to Kodak RP Royal X-Omat film at −70° for 1 day for ^{35}S-labeled proteins and 8 days for ^{3}H-labeled proteins.

Molecular Weight Estimation. Protein molecular weights were determined by the procedures of Weber and Osborn (16) in $NaDodSO_4$/amide slab gels. *Bacillus subtilis* α-amylase (96,000), ovalbumin (44,000), and trypsin (23,300) were used as molecular weight standards.

RESULTS

Search of Proteins Coded by Plasmid Genes of *A. tumefaciens*. Plasmid pCK1145 of *A. tumefaciens* C-58 can accommodate between 100 and 150 genes, based on its molecular weight and on the assumption that average-size polypeptides are coded. Some of these genes are undoubtedly expressed in the bacterial cell. A systematic search was therefore made to detect some of the products coded by this plasmid. Various subcellular fractions were examined for proteins that were present in the virulent strain and absent in the avirulent plasmidless derivative. Soluble proteins extracted from spheroplast membranes and the cytoplasmic fraction (Sup-2) did not reveal any distinct and obvious differences when examined by electrophoresis in acrylamide gels containing $NaDodSO_4$.

Proteins Associated with Outer Membrane and Periplasmic Fraction of Virulent Strain C-58. Cells of virulent strain C-58, when treated with lysozyme and EDTA, released into the supernatant fraction (Sup-1) proteins which when analyzed by slab gel electrophoresis showed a distinct heavily stained protein band (Fig. 1, gel a). This band was not detected when the Sup-1 fraction of the avirulent plasmidless C-58 derivative, NT1, was identically treated and examined in the same way (Fig. 1, gel b). The protein band was not an artifact due to the contribution of added lysozyme for derivation of Sup-1 because cells labeled with [^{35}S]sulfate and treated identically gave the same results (Fig. 2). Sup-1 fraction from cells labeled with [^{3}H]leucine or [^{35}S]methionine also gave identical results (data not shown).

All of the proteins released by lysozyme and EDTA were further analyzed by two-dimensional gel electrophoresis, in which the proteins were separated by isoelectric focusing in the first dimension followed by $NaDodSO_4$ slab gel electrophoresis in the second dimension (Fig. 3). Two distinct ^{35}S-labeled proteins can be seen in the protein preparation of strain C-58

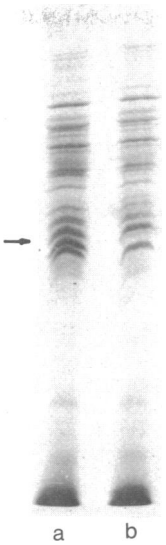

FIG. 1. Slab gel electrophoresis of periplasmic and outer membrane proteins (Sup-1) of virulent and avirulent *A. tumefaciens*. Proteins were stained with Coomassie blue R-250. Migration of proteins was from top to bottom (anode). (Gel a) Strain C-58 harboring virulence plasmid; (gel b) avirulent plasmidless strain NT1. Arrow denotes the position of the stainable protein band in C-58.

which are missing in strain NT1. Identical results were obtained when proteins were labeled with [^{3}H]leucine. These proteins (designated Per-1 and Per-2) have molecular weights in the range of 37,500 and 37,300, respectively (Fig. 4).

The presence of Per-1 and Per-2 in the virulent plasmid-containing strain C-58 and concomitant absence of these proteins in plasmidless strain NT1 suggest that these proteins may be the result of a plasmid-specified gene expression in C-58. The heavy band designated by the arrow in gel a of Fig. 2 was dissected from the electrophoretic gel and the protein was eluted from the gel piece, washed free of $NaDodSO_4$ and Coomassie blue stain by chromatography on Dowex 1, concentrated, and subjected to isoelectric focusing. The analysis revealed that the band is composed of two major polypeptide species (Fig. 5) and represents those observed in strain C-58 in Fig. 3.

Analysis of Transconjugant Harboring Virulence Plasmid. To test whether Per-1 and Per-2 were plasmid-borne products, the plasmid in strain C-58 was transferred conjugatively to the avirulent plasmidless strain NT1. The virulence of the transconjugant, now harboring the plasmid (as determined by electron microscopy of specimens taken from CsCl/ethidium

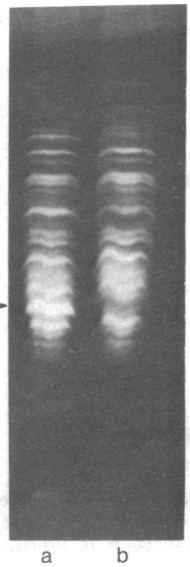

FIG. 2. Slab gel electrophoresis of periplasmic and outer membrane (Sup-1) proteins labeled with [^{35}S]sulfate of virulent and avirulent *A. tumefaciens*. Migration of proteins was from top to bottom (anode). Proteins were visualized by autoradiography. (Gel a) Strain C-58 harboring the virulence plasmid; (gel b) avirulent plasmidless strain NT1. Arrow denotes the position of ^{35}S-labeled protein band.

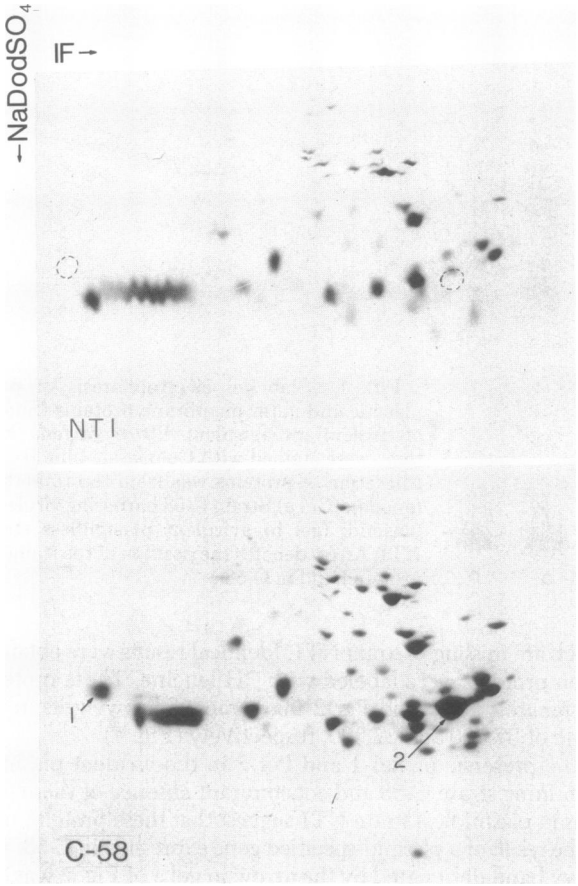

FIG. 3. Two-dimensional gel electrophoretograms of Sup-1 proteins of *A. tumefaciens* strains labeled with [^{35}S]methionine as indicated in the figure. Proteins (200,000 cpm in 60 μl) were subjected to isoelectric focusing (IF) in the first dimension followed by Na-DodSO$_4$ gel electrophoresis in the second dimension and were detected by autoradiography. Arrows indicate the position of Per-1 and Per-2 proteins in strain C-58; broken circles, their relative positions in strain NT1. The C-58 protein (Per-2) on the right partially overlaps a weakly labeled protein of strain NT1.

bromide density gradients), was restored, and also the capacity to synthesize the heavy band (Fig. 6), which contained Per-1 and Per-2, as checked by two-dimensional slab gel electrophoresis. When the transconjugant was again freed of its plasmid by a curing treatment at 36° (9), it lost its virulence and its ability to synthesize Per-1 and Per-2 (Fig. 7). Thus, these results strongly suggest that Per-1 and Per-2 are coded by plasmid pCK1145 of *A. tumefaciens* C-58.

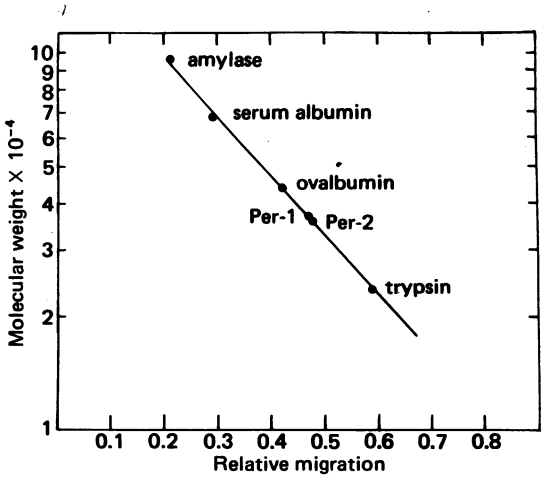

FIG. 4. Molecular weight estimation of Per-1 and Per-2 proteins by NaDodSO$_4$ gel electrophoresis (0.12% crosslinkage) with protein molecular weight standards as indicated. Amylase is *B. subtilis* α-amylase.

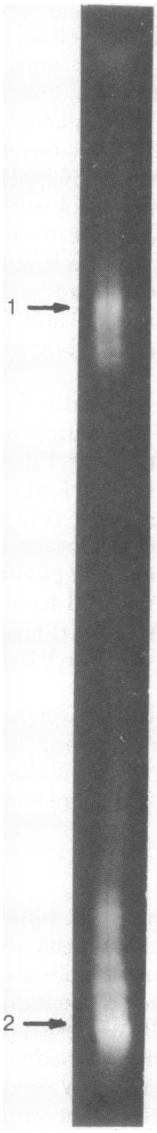

FIG. 5. Autoradiogram of isoelectric gel electrophoretogram of the C-58 [^{35}S]protein band eluted from a NaDodSO$_4$/acrylamide gel. The NaDodSO$_4$ and Coomassie blue stain were removed before isoelectric focusing. Arrows indicate positions of Per-1 and Per-2.

Location of Per-1 and Per-2 Loci. Both proteins and the virulence properties were shown above to be cotransferred by conjugation, thereby indicating plasmid-mediating functions. However, the location of the Per-1 and Per-2 loci on the plasmid relative to the virulence locus was unknown. One means of determining the location of these loci would be to analyze C-58 mutants harboring plasmids with deletions. Analyses made of one such mutant (strain 1D1173) showed that the Per-1 and Per-2 loci are located at some distance from the virulence locus. At least one of these proteins may be found in the C-58 avirulent derivative 1D1173, which harbors a plasmid with a deletion of 39 megadaltons (Fig. 8).

DISCUSSION

It is established that the large plasmid in *A. tumefaciens* C-58 confers several phenotypically identifiable features: (*a*) virulence (tumorigenicity) (1–3), (*b*) octopine or nopaline utilization (2, 4–7), (*c*) octopine/nopaline biosynthesis in tumors (2, 4–7), and (*d*) sensitivity to certain *Agrobacterium* phages (6) and bacteriocins (6, 8). So far, no gene product of the large plasmid

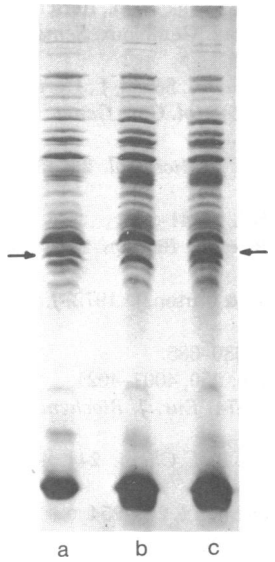

FIG. 6. NaDodSO$_4$ slab gel electrophoretogram of Per-1 and Per-2 proteins (seen as a single heavy Coomassie blue-stained band) from (gel a) *A. tumefaciens* virulent strain C-58; (gel b) avirulent plasmidless strain NT1; and (gel c) virulent transconjugant strain 1D1274, a strain resulting from a cross between strains C-58 and NT1. Arrows indicate the positions of Per-1 and Per-2 that were isolated from C-58 and 1D1274 Sup-1 fraction.

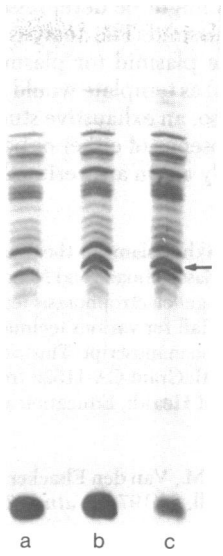

FIG. 8. NaDodSO$_4$ slab gel electrophoretogram as in Fig. 6. (Gel a) Plasmidless avirulent strain NT1; (gel b) virulent strain C-58 harboring a 117-megadalton plasmid; (gel c) avirulent strain 1D1173 harboring a plasmid with a 39-megadalton deletion. Arrow indicates the protein band consisting of Per-1 and Per-2.

[designated as the Ti plasmid by the laboratory of J. Schell (8)] has been isolated and identified. The work presented herein reports that two polypeptides, as shown by a protein-specific stain (17) and by labeling with [^{35}S]sulfate, [^{35}S]methionine, and [^{3}H]leucine, are conferred by plasmid pCK1145 (or Ti plasmid). The evidence presented supports the hypothesis that these polypeptides are coded by plasmid genes for the following reasons. (*i*) The two polypeptides are recovered from strain C-58 harboring the large 117-megadalton plasmid but are absent in strain NT1, which is a derivative of C-58 freed of this plasmid (2). (*ii*) These proteins are observed in transconjugants derived from a cross between strain C-58 and strain NT1, that is, when the plasmid is transferred from strain C-58 to NT1 by conjugation. (*iii*) These proteins are again not detected when the above transconjugant is freed of the plasmid by curing. The protein band in NaDodSO$_4$ gels can be resolved by isoelectric focusing into two proteins with similar molecular weights of 37,500 and 37,300, as judged by NaDodSO$_4$ and two-dimensional gel electrophoresis. The intensity of the radioactively labeled proteins relative to other surrounding protein spots in the two-dimensional electrophoretogram suggests that these proteins are synthesized in appreciable amounts by *A. tumefaciens* C-58. The presence of these proteins in the periplasmic fraction and their release from spheroplasts suggest that these proteins are loosely associated with the outer membrane of this bacterium.

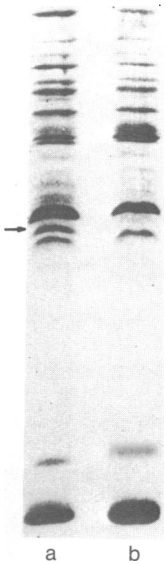

FIG. 7. NaDodSO$_4$ slab gel electrophoretogram as in Fig. 6. (Gel a) Transconjugant strain 1D1274 of Fig. 6 harboring the virulence plasmid; (gel b) transconjugant strain 1D1274 that was freed of its plasmid by curing (9). Arrow indicates the protein band consisting of Per-1 and Per-2.

Attribution of any biological function to these proteins cannot be made at this time. It is not known how many genes are necessary for tumorigenesis. If only one gene product is required for this function, then Per-1 and Per-2 are not responsible because analysis of an avirulent C-58 derivative, 1D1173, that harbors a plasmid with a 39-megadalton deletion showed the presence of at least one of these proteins. However, if several proteins are required for tumorigenesis, then one or both proteins may complement the function that is missing in the deletion mutant. Lippincott *et al.* (18) recently reported enhancing virulence by mixed inoculation with avirulent strain IIBNV6 and C-58 but not with strain NT1 and C-58. Since IIBNV6 was found to contain a plasmid of about 40% the size of that in the usual virulent strain, they have assumed that the IIBNV6 plasmid retains part of the genetic information essential for tumor initiation. If this assumption is substantiated, it would be evidence for the need of a number of plasmid-coded products for tumor initiation and thus would not rule out the need for Per-1 and Per-2 in this role.

Other proteins of potential importance in tumor initiation are those that are functional in DNA metabolism and found in the periplasmic space of *Agrobacterium*. Three of these proteins have been isolated from *Agrobacterium* (19). One is a DNA unwinding protein, a second one (*Atu* II) is specific for superhelical DNA and functions as a nicking enzyme, and the third protein (*Atu* I) is a restriction enzyme isoschizomeric (19, 20) to *Eco*RII, which is coded by a plasmid in *E. coli* R245 (21, 22). Also, Klapwijk *et al.* (23) recently reported that an octopine permease gene and an accessory regulatory gene may likely be carried on the *Agrobacterium* Ti plasmid. Because of the denaturing conditions to which Per-1 and Per-2 are subjected during gel electrophoresis, their possible enzymatic properties could not be tested. Other possible functions of Per-1 and Per-2 would be facilitation of cell attachment to host target cells and maintenance of host range.

The evidence presented in this paper is highly suggestive that proteins Per-1 and Per-2 are coded by *Agrobacterium* plasmid genes. However, the chance of these proteins being the product of chromosomal genes has not been completely ruled out since

there is the possibility that these genes might be derepressed in the presence of the *Agrobacterium* plasmid. The analysis of proteins synthesized *in vitro* using the plasmid (or plasmid segments recombined on EK2 plasmids) as template would be a means of resolving this argument. Also, an exhaustive study of avirulent deletion mutants for the absence of either or both Per-1 and Per-2 proteins would certainly aid in answering this question.

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