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Agrobacterium tumefaciens is a plant pathogenic bacteria which incites crown gall tumors. Although virulence has been associated with a 97-156 Mdalton "tumor-inducing" (Ti) plasmid, few potential plasmid gene products have been identified. The objective of this study was to identify plasmid-coded gene products through a comparison of protein fingerprints of virulent and avirulent isogenic strains. This required development of techniques for cell fractionation which could provide comparable protein samples from the various strains of bacteria and a reproducible high resolution method of protein characterization. The analytical system developed consisted of isoelectric focusing in the first dimension followed by SDS-polyacrylamide gel electrophoresis in the second dimension and was capable of reproducibly resolving nearly 500 proteins using this two-dimensional system. A. tumefaciens strain C58, which contains a nopaline Ti plasmid, produced five proteins detected neither in plasmid-free isogenic strains nor in A277. Strain A277, which contains the octopine plasmid, pTiB6-806, produced three proteins detected neither in plasmid-free isogenic strains nor in C58. Strain A136, a plasmid-free derivative of C58, was deficient in two

proteins found in the other three strains examined. The implications behind the presence of these proteins and their possible biological roles are discussed.

Ti PLASMID CODED PROTEIN IN *AGROBACTERIUM TUMEFACIENS*

by

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Ti PLASMID CODED PROTEIN IN *AGROBACTERIUM TUMEFACIENS*

I. INTRODUCTION

Crown gall is a non-self limiting neoplastic disease infecting at least 93 different families of dicotyledonous and gymnospermatous plants (DeCleene and DeLay, 1976). While rarely killing the host, tumors will reduce general growth and vigor, making infected plants more susceptible to other diseases, and diminishing the quality of fruit, as well as the overall appearance of the plant. A common mesophilic, gram negative rod, soil bacterium, *Agrobacterium tumefaciens* was first identified as the causative agent by Smith and Townsend in 1907. Forty years later, Braun introduced the concept of a "tumor-inducing principle" (TIP), a hypothetical material that passed from tumor-inducing bacteria to the plant cells during transformation of the latter. A fresh wound site and the plant's response to wounding were required to yield a "competent" state in which the plant cells were susceptible to infection and tumor formation upon inoculation with live bacterial cells (Braun, 1952). Earlier reports suggesting bacterial DNA (Kado, *et al.*, 1972) or RNA (Swain and Rier, 1972) alone incited tumors could not be substantiated by others (Nester, *et al.*, 1977), thus emphasizing the requirement of live cells for infection.

After transformation, tumors require neither the continued presence of bacteria (Braun, 1952), nor exogenous phytohormones - auxins and cytokinins (Braun, 1956) - to maintain growth. Usually, tumors are found to contain "opines", an unusual class of amino acid derivatives (Biemann, *et al.*, 1960). The most predominant opines found in tumors are octo-

pine (N^2-(D-1-carboxyethyl)-L-arginine) and nopaline (N^2-(1,3-dicarboxypropyl)-L-arginine). Typically, tumors will have one or the other, but not both.

More current investigations have revealed a large 96-156 Mdalton plasmid (a covalently closed, extrachromosomal DNA molecule), referred to as a tumor-inducing (Ti) plasmid, which is connected with strain oncogenicity (Zaenan, et al., 1974). Necessity of the plasmid for virulence has been demonstrated by cultures of virulent strains grown at elevated temperatures (37-40°C) whereupon the plasmid is lost and the bacteria becomes avirulent. Upon reintroduction of the plasmid, virulence is regained (Watson, et al., 1975).

Early work showed large amounts of the Ti plasmid in plant DNA of transformed cells (Schilperoort, et al., 1967). These results, however, were not supported by results in other laboratories (Chilton, et al., 1974). It is generally believed that improper controls and misinterpretation of the results occurred. Recent attempts to hybridize whole plasmids to transformed plant cell DNA yielded negative results (Gordon, et al., 1978). However, hybridization of transformed plant DNA with Ti plasmid restriction endonuclease fragments showed stable incorporation of a 3.5 Mdalton portion of the plasmid (Chilton, et al., 1977). This piece of DNA is referred to as T-DNA.

Morel's group showed there was good correlation between the production of an opine by tumors and the ability of the inciting bacteria to utilize it as a sole carbon and nitrogen source (Goldmann, et al., 1968; Petit, et al., 1970). Induction of synthesis in tumor cells and bacterial utilization was shown to be Ti plasmid mediated (Bomhoff, et al., 1976; Montoya, et al., 1977). This provided a convenient classi-

fication system, therefore, plasmids were divided into three types: octopine type, nopaline type, and null type which was found associated with cryptopine, an uncharacterized opine.

Plasmids of different bacterial strains of the same opine type have been compared. Octopine type plasmids showed a high degree of homology within their type by DNA-DNA hybridization (Currier and Nester, 1976) and restriction endonuclease cleavage patterns (Sciaky, et al., 1978). Nopaline type plasmids showed only moderate degrees of homology by DNA-DNA hybridization (Currier and Nester, 1976) and Sma I restriction endonuclease patterns (Sciaky, et al., 1978).

Different opine types of Ti plasmids were compared by hybridization (Drummond and Chilton, 1978; Depicker, et al., 1978). This study revealed regions of high, moderate, and no homology between different octopine and nopaline type plasmids. There appeared to be a highly conserved region contiguous to the T-DNA region in all the plasmids surveyed.

Ti plasmids determine a number of tumor and bacterial characteristics and are intimately associated with tumorigenesis. Opines present in tumor tissue are related to the Ti plasmid involved. Dehydrogenases which synthesize octopine are found in tumor tissue incited by bacteria harboring octopine type plasmids (Otten, et al., 1977). Likewise, enzymes capable of nopaline synthesis are found in tumors incited by bacteria containing nopaline type plasmids (Sutton, et al., 1978).

Transformed plant cells become auxin and cytokinin autonomous (Braun, 1956). These phytohormones are required by in vitro cultures of normal tissue (Jablanski and Skoog, 1954). The auxin, indoleacetic acid (IAA), can be synthesized by the same enzymatic pathway in virulent and avirulent

strains of bacteria (Hasan, et al., 1979). Tumor and normal tissue synthesize IAA by the same mechanism which is different from the bacterial pathway. While the elevated levels of auxins in tumors is not understood, they are attributed to enhanced expression of normal plant pathways, utilization of plasmid-coded pathways not expressed in bacteria, or by a decrease in auxin catabolism. Achievement of cytokinin autonomy is not fully understood either, but several important observations have been made. Modified adenine derivatives are found adjacent to the 3' side of the anticodon triplet in animal, plant, and procaryotic t-RNA. In plants, cytokinin nucleotides are found in this position. Bacteria that live in close association with plants, either as symbionts or pathogens, contain modified bases in this position which are cytokinins and normally found only in plants. Plants have cis-ribosylzeatin [the cis isomer of 9-β-D-ribofuranosyl-6-(4-hydroxy-3-methylbut-2-enylamino) purine] in this position (Hall, et al., 1967), while cytokinins occurring free in plants are mostly in the trans configuration of this modified base (trans-ribosylzeatin) and the free base, trans-zeatin. Investigations show trans-ribosylzeatin and 2-methylthio-ribosylzeatin in t-RNA of A. tumefaciens strain C58 (Chapman, et al., 1976). Also, in the culture medium of this virulent strain, cis and trans - zeatin as well as ribosylzeatin are found (Kaiss-Chapman, and Morris, 1977).

Multiple copies of the T-DNA segment of the Ti plasmid have been found in tumor tissue cultured for several years (Chilton, et al., 1977). The importance of this DNA fragment in opine synthesis and phytohormone autonomy has been suggested but no definite role has been experimentally demonstrated.

Virulent strains of A. tumefaciens generally are able to catabolize

a certain class of opine depending on their Ti plasmid type. Opine catabolism is related to a permease and an oxidase (Petit and Tempe, 1978), which are induced by the presence of the specific opine. Interestingly, stimulation of the opine catabolizing enzymes by opines also induces the conjugative system for Ti plasmid transfer (Petit and Tempe, 1978). Therefore, in a sense, conjugative transfer of plasmids is a nutritionally mediated event stimulated in the tumor tissue environment. In view of this, the in planta "Kerr Cross" (Kerr, 1969) can be understood in which an avirulent strain becomes virulent upon conjugative acquisition of the Ti plasmid (Van Larebeke, et al., 1975; Watson, et al., 1975).

Ti plasmids in A. tumefaciens confer sensitivity to a bacteriocin produced by Agrobacterium radiobacter strain 84 (Engler, et al., 1975) and also confer resistance to the phage API (Genetello, et al., 1977).

The initial exposure of "competent" plant cells at a fresh wound site to virulent strains of A. tumefaciens results in bacterial attachment to cell walls (Matthysse, et al., 1978). This process is dependent on plant cell wall characteristics (Lippincott, et al., 1977), and bacterial cell walls containing Ti plasmid coded lipopolysaccharide (Whatley, et al., 1976). When strains are cured of their plasmids they generally lose their ability to attach (or block virulent strains from attaching), however, some exceptions occur where cell wall preparations from avirulent strains can block tumor initiation (Watson, et al., 1975). Recently, a very large plasmid (over 200 Mdaltons) has been found in all strains surveyed and it appears to affect membrane protein composition and may give insight to these exceptions (Schell, personal communication).

During transformation, the ability to synthesize opines is acquired

by plant cells and the utilization of opines induced in the bacteria. While these abilities are both Ti plasmid-coded, they are attributed to separate genetic entities (Montoya, et al., 1977) since virulent mutants occur that are deficient in opine utilization, but incite tumors which can produce it.

The ability of A. tumefaciens to transfer genetic information into plant cells makes it unique to the plant kingdom. It is not known if the T-DNA is actually integrated into the plant genome (or where in the genome, if it is) but evidence for its expression and consequential effect on plant cells is strong. This makes the Ti plasmid a potential vehicle for genetic engineering in plants. Techniques for its transfer and manipulation are rapidly developing. The Ti plasmid can be mobilized by formation of a cointegrate with the promiscuous RP4 plasmid and can be transferred into E coli (Holsters, et al., 1978), Rhizobium trifolii (Hooykaas, et al., 1977) and between different strains of A. tumefaciens (Levin, et al, 1976). These transfers occur with the same frequency as the RP4 plasmid alone (Holsters, et al., 1978). By use of these cointegrate plasmids which spontaneously dissociate under certain conditions, and use of the Tn7 drug resistant transposons which can insert themselves at numerous sites in the plasmid, definitive studies of plasmid function are now possible (Hernalsteens, et al., 1978). Numerous Ti plasmid insertion and deletion mutants are now becoming available for study. Genetic expression of Ti plasmids is of prime interest since this should reveal factors responsible for genetic transfer into plants. Enzymatic assays and bacterial morphological and protein content changes may be useful in studying this. Comparative experiments with plasmid containing and plasmidless isogenic strains (with respect to their chromosomal

background) might provide a means of elucidating expression of Ti plasmid genes and possibly give insight to what proteins are involved in tumorigenesis.

The objective of this study is to identify Ti plasmid gene products in A. tumefaciens. This could be done by a comparative survey of bacterial proteins in a plasmid containing and plasmid-free isogenic strains. Availability of isogenic bacterial strains with and without Ti plasmids would be required along with development of a cell fractionation procedure and a reproducible separating technique capable of resolving a large number of proteins. The cell fractionation technique should be able to provide comparable protein samples that could be analyzed by a two-dimensional gel electrophoresis system similar to that of O'Farrell (1975), but modified as necessary to resolve the maximum number of proteins possible by the system.

II. MATERIALS AND METHODS

Organisms and Culture Conditions

Strains used in this study are defined in Table I. They were stored at -20°C in agar stabs. For inoculations into liquid media, single colonies were used from streaks on Petri plates of Medium 523 (Kado and Heskett, 1970) with agar (Table II). Defined media modified from that of Valera and Alexander (1965) was used in growing cultures for analysis (Table III). For this, stock solutions of $FeCl_3$ and biotin were made and autoclaved separately then added under sterile conditions to the medium just before inoculation. Cells were agitated on a rotary shaker at 150 cycles per minute at 23-30°C and growth monitored with a Gilford spectrophotometer (Gilford Inc., Oberlin, OH) at 660 nm. Fifty ml cultures in 125 ml Erlenmeyer flasks were inoculated from plates. All or part of these were used to inoculate 1600 ml in 2.8 liter Fernbach flasks. For labelled cells, 50 ml cultures were monitored. When cell density reached 0.100 A_{660} units, 10 ml was transferred to a 25 ml Erlenmeyer flask and 100 µCi of [^{35}S]methionine (907 Ci/mmole New England Nuclear) added. Cell growth for the labelled culture was followed by monitoring the parent 50 ml culture.

Protein Preparation

Unlabelled Cultures: Large cultures were harvested during log phase growth by cooling in ice followed by centifugation in 250 ml centrifuge bottles at 3000xg (4°C) for 25 minutes. Once harvested, all work with cells was done at 4°C or in an ice bath. Cells were placed in 15 ml Corex centrifuge tubes and washed three times in 10 ml

TABLE I. STRAINS OF AGROBACTERIUM TUMEFACIENS USED IN THIS STUDY

Strain	Plasmid	Virulence	Genetic Characterization[a]	Source
C58	TiC58	+	wild type (pTiC58) onc^+ noc^+ nos^+	Nester
NT1	--	-	heat cured C58 onc^- noc^- nos^-	Nester
A136	--	-	nal^r, rif^r spontaneous mutant of NT1 $onc^-noc^-nos^-$	Nester
A277	TiB6-806	+	A136 (pTiB6-806) onc^+ occ^+ ocs^+	Nester

[a]onc = oncogenic, noc = nopaline catabolizing, nos = nopaline synthesizing, occ = octopine catabolyzing, ocs = octopine synthesizing, nal = nalidixic acid, rif = rifampicin

TABLE II. COMPONENTS OF MEDIUM 523 (KADO AND HESKETT, 1970)

Component	Grams/Liter
Casein hydrolysate	8
Yeast Extract	4
$K_2HPO_4 \cdot 7H_2O$	0.3
Sucrose	10
Agar	11

Medium autoclaved 30 minutes at 120°C

TABLE III. COMPONENTS OF DEFINED MEDIUM MODIFIED FROM VALERA AND ALEXANDER (1965)

Component	Grams/Liter	mg/Liter
Sucrose	15	
$(NH_4)_2SO_4$	2	
KH_2PO_4	.2	
K_2HPO_4	.8	
$MgCl_2 \cdot 6H_2O$		33
NaCl		44
*$FeCl_3$		3
$CaNO_3$		26
$Na_2MoO_4 \cdot 2H_2O$		.2
Zn Acetate		.2
H_3BO_3		.2
$MnCl_2$		.2
$CuCl_2$		.001
$CoCl_2$		.015
*Biotin		1

pH was adjusted to 7.2 before autoclaving
Media autoclaved 30 minutes at 120°C
* Autoclaved separately

ice-cold sonication buffer (See Solution 2 Appendix) with centrifugation at 5900xg for 20 minutes between each wash. Fresh cells were used in all cases. About 1.5 g was suspended in ice-cold sonication buffer (4 ml) in a 10 ml beaker and 125 µl RNase solution was added (Solution 3). This was sonicated with six 20 second pulses, allowing at least two minutes between pulses, at a 140-160W setting on an Ultra Tip Sonicator (Wave Energy System, Newton, PA). These ruptured cells were transferred to a 15 ml Corex tube, 250 µl of DNase solution (Solution 4) was added, and this mixture was spun at 5900xg for 20 minutes. The pellet of whole cells was discarded. The supernatant was collected and then centrifuged at 31,000xg for two hours. The pellet from this step was defined as the membrane fraction and the supernatant, the cytosol fraction.

Lowry protein determinations (Lowry, et al., 1951) were performed on all samples. Cytosol samples were concentrated by pervaporation in dry Sephadex G25 (Pharmacia Fine Chemicals, Piscataway, NJ) for 3-5 hours to obtain concentration of approximately 20-30 mg/ml. The cloudiness which appeared in samples upon concentration was removed by spinning at 132,000xg for one hour in an Airfuge (Beckman Instruments, Palo Alto, CA). The protein solutions, in 20 µl aliquots, were stored at -20°C until needed.

The membrane pellet was washed in 10 ml sonication buffer three times, with centrifugation at 31,000xg for one hour between each wash, resuspended in sonication buffer (about 35 mg/ml protein), and stored at -20°C till solubilization (within two days). To solubilize membrane protein, an equal volume of solubilization buffer (Solution 14) (Ames, 1976) was added to a suspension of membrane and incubated at 70°C for

one hour. The solubilized membrane proteins were spun at 31,000xg for one hour to pellet solid material. The supernatant, divided into 20 µl aliquots, was stored at -20°C.

Labelled Cultures: Upon harvesting, the 10 ml cultures were chilled in ice, transferred to 15 ml Corex centrifuge tubes, pelleted at 5900xg for 20 minutes, and washed three times in sonication buffer containing 10 mM methionine (Sigma, St. Louis, MO). Cells were then suspended in 0.5 ml sonication buffer (containing methionine), mixed with 12.5 µl DNase solution, and then spun at 123,000xg (one hour) in an Airfuge. The supernatant was stored at -20°C. No membrane was solubilized from these cells.

Electrophoresis

Apparatus: A vertical slab gel electrophoresis apparatus was constructed in this laboratory from plexiglas (Rohm and Haas, Philadelphia, PA) using a design similar to that of Reid and Bieleski (1968) but scaled to fit 10 x 11.5 inch plates and to accommodate two gels concomitantly. Isoelectric focusing (IF) was done in a Canalco Model 6 tube gel electrophoresis apparatus. Glass plates used for slab gels were constructed similar to those described by O'Farrell (1975) with this exception: the back plate was of solid glass with no bevel. Lucite spacers of appropriate thickness were used with vaseline to seal the plates. A Buchler Instruments Voltage and Current Regulated Power Supply Model #3-1155 (Searle, Fort Lee, NJ) was used for isoelectric focusing and a Spinco Con-stat Constant Current Supply Model CPO (Beckman Instruments, Palo Alto, CA) was used for slab gels.

One-Dimensional Analysis of Cytosol and Membrane Proteins: Proteins were analyzed by polyacryamide slab gel electrophoresis in the presence of SDS as described by Laemmli (1970), making use of the stacking system of Ornstein (1964) and Davis (1964). This system has more recently been used for fractionated bacterial proteins (Ames, 1974).

SDS (0.1%) slab gels 1 mm thick consisting of a 2.5 cm stacking gel (4.5% acrylamide) and a separating gel (12.5% acrylamide) were poured as follows: 29.2 ml 30% stock acrylamide (Solution 5), 17.6 ml separating gel buffer (Solution 6), and 23.0 ml distilled water were added to a 500 ml filter flask. To this was added 235 µl 10% (w/v) ammonium persulfate (APS) (J.T. Baker Chemical Co., Phillipsburg, NJ) followed immediately by 35 µl N,N,N',N' tetramethylethylenediamine (TMED) (Eastman Organic Chemicals, Rochester, NY). The solution was degassed (1.5 minutes), poured between the plates, and overlayed with 0.1% SDS solution sprayed onto the back plate and allowed to trickle down with minimal disturbance of the gel surface. Polymerization lasted eight hours. The overlay was then removed, the gel surface blotted dry with filter paper, and a stacking gel poured one to two hours prior to a run. A stacking gel was poured as follows: 1.1 ml 30% stock acrylamide (Solution 5), 1.9 ml stacking gel buffer (Solution 7), and 4.5 ml distilled water were added to a 125 ml filter flask. To this was added 23 µl 10% APS, and eight µl TMED, the solution was then degassed (1.5 minutes) and poured onto the separating gel. A 0.9 mm thick comb with 16 teeth, each 0.5 cm wide spaced 0.4 cm apart, was inserted into the unpolymerized gel and overlayed with 0.1% SDS solution. Before a run, the comb was taken out and the overlay removed with a syringe. Then the bottom spacer was

removed, SDS running buffer (Solution 10) added to the lower reservoir, and the slab gel fitted to the apparatus, assuring the gel contact with the reservoir was bubble-free.

Cytosol, membrane, and molecular weight standard proteins were run on the same gel. Cytosol samples and molecular weight standards (bovine serum albumin, ovalbumin, trypsinogen, lactoglobulin, and lysozyme, Sigma Chemical Co., St. Louis, MO) were prepared by adding an equal volume of equilibration buffer (Solution 9) and heating in a boiling water bath (two minutes) (Ames, 1974). To a well 75 to 150 µg was applied. Solubilized membrane protein samples were made 2.5% 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) and 5% glycerol (J.T. Baker Chemical Co., Phillipsburg, NJ) and heated in boiling water (two minutes). Between 50 and 100 µg was applied per well. The remainder of the well was filled with SDS running buffer. The top reservoir was then filled and the gel run at 30 ma till the buffer front had migrated 17.5 cm into the separating gel.

Two-Dimensional Analysis: Proteins were analyzed by two-dimensional polyacrylamide electrophoresis similar to O'Farrell (1975) and Ames (1976). This system consists of an isoelectric focusing (IF) first dimensions followed by SDS electrophoresis in the second dimension.

Isoelectric focusing (IF) gels were poured in 130 x 2 (id) mm glass tubes sealed with parafilm at the bottom. When pouring four gels, 120 mm in length, a 3 ml aliquot of stock IF gel solution (Solution 11) with 20 µl 1.5% APS was placed in a 125 ml filter flask, the solution degassed (30 seconds) and poured into the tubes as described by O'Farrell (1975). Gels were overlayed with 20 µl water (O'Farrell, et al., 1977) and allowed to polymerize at least one hour.

It should be noted, IF gels did not have polarity when poured. Polarity was determined by placement of the electrodes in the gel apparatus. The basic end of the gel upon completion of a run was at the cathode (-) which contained 0.02M NaOH solution (pH 12.0 ± .1) (made fresh before each run from a standardized 2 M stock) and the acidic end was at the anode (+) containing a 0.01 M H_3PO_4 solution (pH 2.5 ± .1) (made fresh before each run from a standardized 2 M stock).

Protein samples were stored at -20° till just before a run. At this time, the IF gels were placed in a Canalco Model 6 tube gel electrophoresis unit with the appropriate lower reservoir solution, 0.01 M H_3PO_4 for cytosol samples and 0.02 M NaOH for membrane samples. Cytosol samples were made 9 M in urea (deionized, charcoal treated, recrystallized in water) and diluted with one volume cytosol dilution buffer (Solution 1). To the basic end (cathode) 100-250 µg of unlabelled protein, or 0.75 x 10^6 to 1 x 10^6 cpm of labelled protein was applied. Membrane samples were made 9 M in urea, diluted with two volumes membrane sample dilution buffer (Ames, 1976) (Solution 12) and 50-100 µg was applied to the acidic end (anode). IF sample overlay (25 µl) (Solution 8) was layered over the sample and the remaining space in the tubes filled with reservoir solution. Gels were run (without prefocusing) at 350V (0.5 hour) then 800V (three hours).

Upon completion of a run, gels were either stained (See "Staining, Drying, and Autoradiography"), run directly on the second dimension, frozen, or used for pH gradient measurements. Extrusion from the tubes was performed with a water-filled syringe fitted to the gel tube with Tygon tubing. Cytosol gels were equilibrated in 10 ml equilibration buffer (Solution 9) in a bench-top shaker (Eberbach, Ann Arbor, MI) at low

speed for 30 minutes, then placed on the second dimension. Membrane gels were placed directly on the second dimension with no equilibration. Gels to be frozen were extruded into 10 ml equilibration buffer and stored at -70°C (no longer than two days). Upon thawing, cytosol gels were equilibrated while membrane gels were put directly on the second dimension.

The pH gradient formed in IF gels was determined by direct placement of a Corning Flat Surface Combination Electrode (Corning Science Products, Medfield, MA) onto 4 mm sliced sections of a gel extruded on a parafilm strip.

The second dimension consisted of a slab gel as described in the "One-Dimensional Analysis" section with these exceptions: It was 0.75 mm thick and no comb was inserted into the stacking gel; rather, the stacking gel, 2.5 cm for equilibrated IF gels and 5.0 cm for nonequilibrated gels, was poured so that it came just above the bevelled edge of the front plate. The slab was placed in the apparatus as described above.

IF gels were placed on a strip of parafilm (140 x 30 mm) and transferred to the notch formed by the bevel and back plate. About 1 ml of melted 1% agarose in equilibration buffer was poured over the IF gel. This was allowed to set (three minutes) and then the plates clamped to the apparatus. For equilibrated gels, the top reservoir was filled with SDS running buffer (Solution 10) and run at 20 ma per slab. For non-equilibrated gels, high SDS running buffer (Solution 13) was placed in the top reservoir and the gel run at 20 ma for 30 minutes. At this point, the run was stopped. The high SDS running buffer was pulled off and replaced with SDS running buffer. The run then was continued at 20 ma until the buffer front migrated 17.5 cm into the separating portion of the gel.

Staining, Fixing, and Autoradiography: IF gels were extruded directly into 0.05% Coomassie Blue G (Sigma, St. Louis, MO), 45% ethanol, 9% acetic acid, and 46% water (similar to Radola, 1973) and stained one hour. Slab gels were taken from the apparatus, the spacers removed, and the plates pried apart with a microtome. These gels were stained (eight hours), then transferred to a circulating destaining solution (10% ethanol, 7.5% acetic acid, 82.5% water) at 40°C. About 30 minutes was sufficient.

Slab gels with labelled protein were fixed in 10% trichloracetic acid (TCA) (Mallinckrodt, St. Louis, MO) for one hour, soaked in 10% glycerol (one hour) and dried under vacuum in a Slab Gel Dryer Model SE540 (Hoefer Scientific Instruments). Gels were exposed to Kodak X-Omat X-ray film (2-8 days) at -70°C. Film was developed according to manufacturer's recommendations.

III. RESULTS

Cultures and Protein Preparations

Unlabelled Cultures: Cells cultured in minimal medium modified extensively from Valera and Alexander (1965) (See Figure I) had a doubling time near 2.2 hours and upon reaching a density of 0.350 A_{660} units began to clump subsequently giving density readings which suggested a slowed growth rate (but not stationary phase). Harvest from late log phase produced 1 g cells per liter of culture.

Labelled Cultures: Near a density of 0.100 A_{660} units, 10 ml of the 50 ml parent culture was transferred to a 25 ml Erlenmeyer flask and mixed with 100 μCi (^{35}S)- methionine. These cultures were grown 4.5 hours (two doubling times) and incorporated 40-80% of the label into protein.

Cell Fractionation: It was important to prepare equivalent samples for comparative analysis. These steps were taken to ensure comparable samples: 1) lysates were freed of unruptured cells by centrifuging at 5900xg for 20 minutes (Ames, 1974); 2) membrane was freed of cytosol by taking care to completely suspend it during washes; and 3) cytosol was freed of membrane (a problem encountered by Chai and Foulds, 1974) by centrifuging at 132,000xg for 60 minutes. Preparations yielded approximately 20 mg cytosol protein and 25 mg membrane protein per gram of cells.

Solubilization: Under the conditions used here, Ames (1976) showed approximately 70% of the protein in the membrane fraction was solubilized with 10 to 20% of the remaining insoluble protein being peptidoglycan-bound lipoprotein. More than 90% was solubilized with

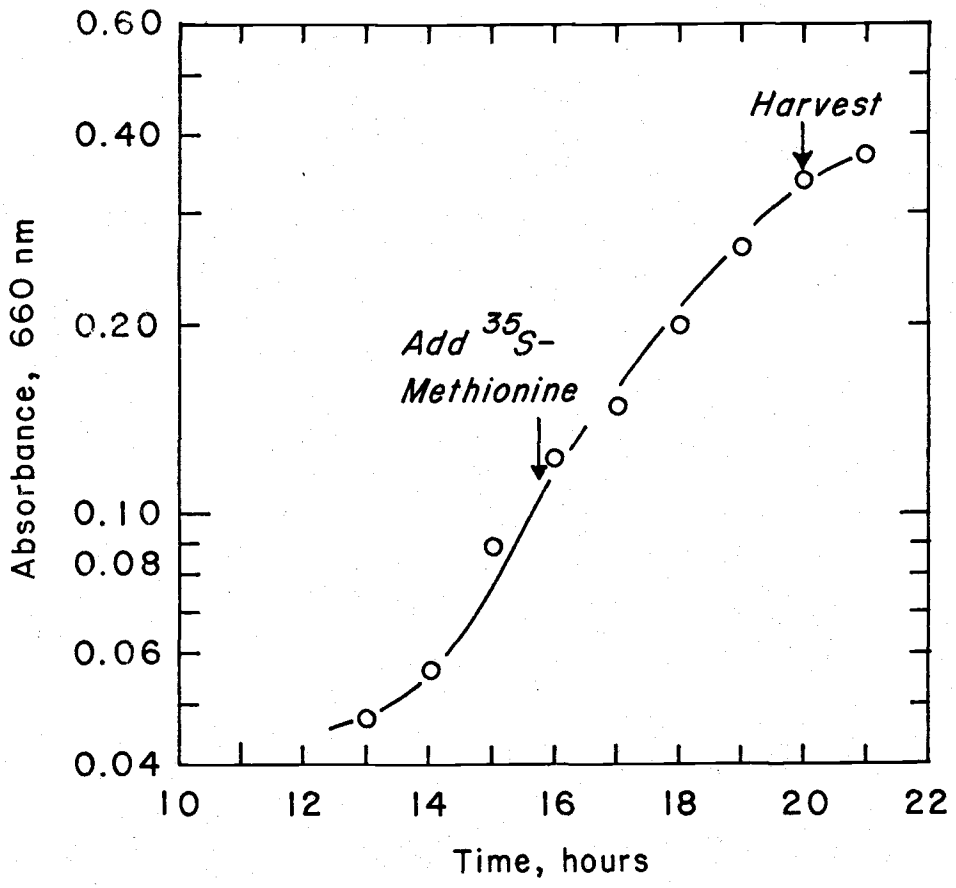

Figure I. Growth curve for Agrobacterium tumefaciens cultured on minimal medium modified extensively from Valera and Alexander (1965). Cell density was monitored by absorbance at 660 nm. Addition of [^{35}S]-methionine to labelled cultures and the harvest points are noted.

higher SDS concentrations and higher temperature (100°C). However, higher NP-40 concentrations were then required to displace SDS and this significantly reduced protein concentration.

One-Dimensional Analysis

One-dimensional SDS electrophoresis has demonstrated the capability of detecting the presence (or absence) of a single protein in mutants (Chai and Foulds, 1974) or nutritionally-induced protein profile changes (Koplow and Goldfine, 1974). Therefore, it was employed for comparative analysis of cytosol and membrane protein fractions from virulent and avirulent strains.

Sample Preparation: To prevent ambiguities in protein banding (Schnaitman, 1973) and to saturate proteins resistant to SDS binding (Nelson, 1971) all samples were placed in a boiling water bath (two minutes) just prior to application to the gel. While band arching has been attributed to SDS concentration and polymerization conditions (Fairbanks, et al., 1971) the experimental conditions used here were such that the majority of the protein did not behave this way (See Figure III, membrane samples).

Molecular Weight Standards: Proteins used for molecular weight standards were bovine serum albumin (66,000), egg albumin (45,000), trypsinogen (24,000), lactoglobulin (18,400), and lysozyme (14,300). Figure II is a graph of their relative migrations into gels.

Cytosol and Membrane Protein Analysis: Arrows denote regions of interest in Figure III. The strain C58 cytosol fraction show a heavier band at 32,000 daltons indicative of either extra proteins or a higher expression level than in other strains. Strain A277 cytosol

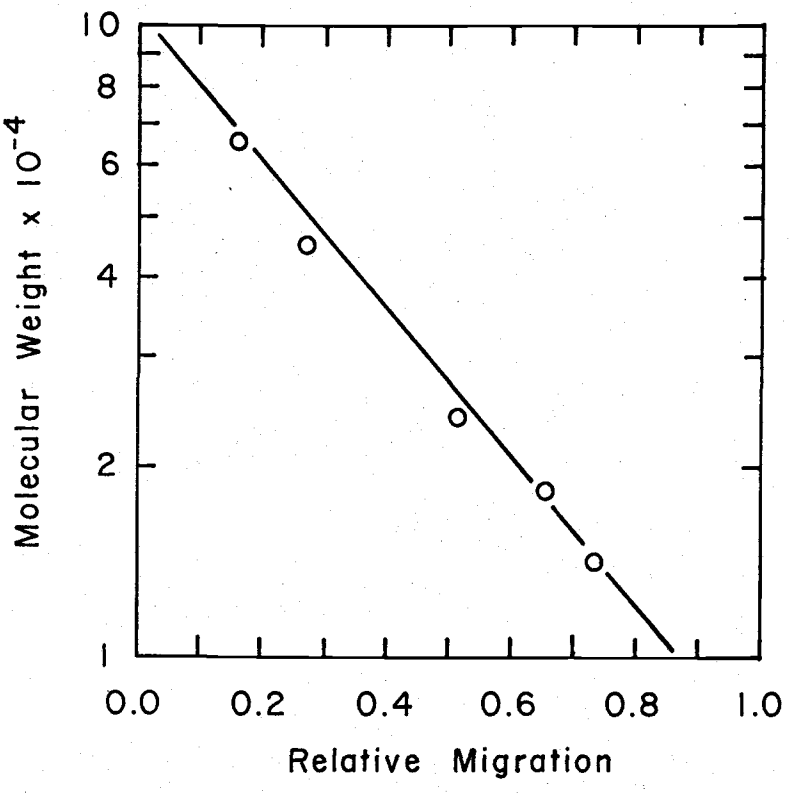

Figure II. Relative migration of molecular weight standards in a one-dimensional SDS polyacrylamide gel. Proteins used and their molecular weights were bovine serum albumin (66,000), egg albumin (45,000), trypsinogen (24,000), lactoglobulin (18,400), and lysozyme (14,300). A 2.5 cm stacking gel was used and the proteins were run 17.5 cm into the separating gel.

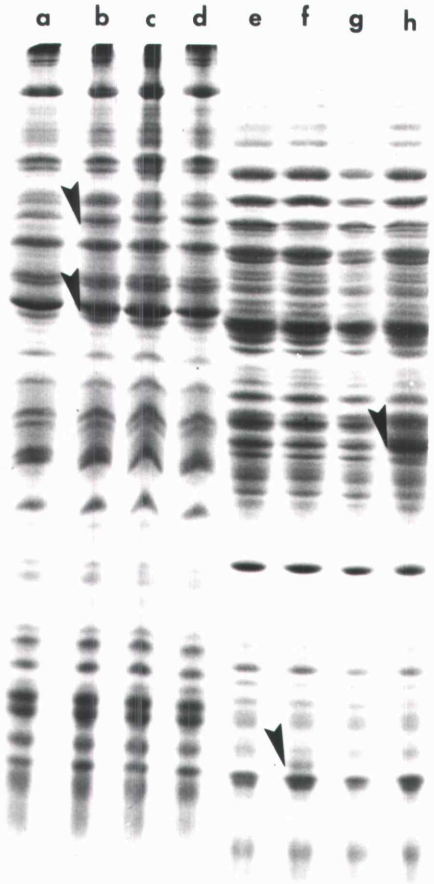

Figure III. One-dimensional SDS polyacrylamide gel electrophoresis of membrane and cytosol proteins from virulent and avirulent strains of Agrobacterium tumfaciens. Migration of proteins was from top to bottom. Membrane samples from strains NT1 (a), A277 (b), A136 (c) and C58 (d) as well as cytosol samples from strains NT1 (e), A277 (f), A136 (g), and C58 (h) are shown. For all samples 75 µg of protein was applied to the gel. Arrows denote extra proteins resolved in plasmid-containing strains A277 and C58.

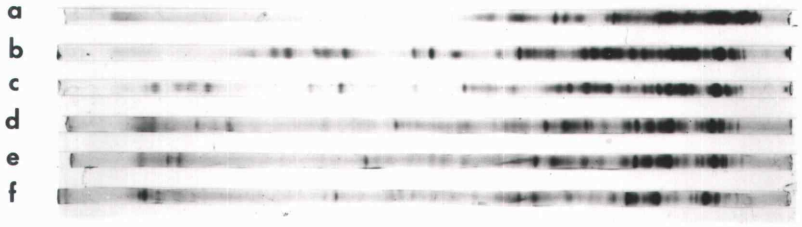

Figure IV. Optimization of run time for isoelectric focusing. Strain A277 cytosol protein (125 µg) was applied to the anode of all gels. Electrophoresis was done at 350V (0.5 hr) then 800V. Gels were removed from the run at 500V•hr (a), 875 V•hr (b), 1250 V•hr (c), 1625 V•hr (d), 2000 V•hr (e), 2750 V•hr (f). The basic end is on the left.

shows one protein, at 14,000 daltons, not apparent in the other three strains. The membrane fraction profiles show proteins at 61,000 and 48,500 daltons in strain A277 which are absent in the other strains.

Isoelectric Focusing

Reproducibility: Isoelectric focusing was very sensitive to subtle changes in experimental conditions. To ensure the most reproducible and comparable protein pattern, these steps were taken: 1) large stock solutions of gel mixture (excluding APS) were made as suggested by Righetti, et al., (1973); 2) the same gel tubes were used throughout work to avoid tube diameter variation; 3) gels were poured to the specified length (120 mm) within 1 mm (there was some shortening upon polymerization but this was uniformly seen in all gels); 4) electrode solutions were made fresh from standardized stocks and were pH monitored before each run; and 5) the run time was accurately defined to standardize time dependent changes in pH gradients noted by Chrambach, et al. (1973).

Optimal Run Time: O'Farrell et al., (1977) developed a nonequilibrium pH gradient electrophoresis (NEPHGE) system that was capable of resolving 15-30% more protein than equilibrium isoelectric focusing he previously used in his two-dimensional system (O'Farrell, 1975). This NEPHGE system was identical to equilibrium isoelectric focusing except that a shorter run time was used which did not allow proteins to reach equilibrium in the pH gradient. This allowed separation of acidic as well as basic proteins which had isoelectric point values beyond pH values actually attained by the gel in which they were resolved. This dynamic system was used in this analysis since it had a large protein-separating

capacity and yielded reproducible banding. To determine an optimal electrofocusing time, a time course was run (See Figure IV). When samples were applied to the anode, acidic proteins rapidly focused while basic proteins focused more slowly and tended to migrate off the cathodic end of the gel. This can be noted by comparing basic ends of the gels as the volt•hour (V•hr) values increase. Between 2000 and 2750 V•hr proteins were optimally banded and distributed across the gel. Therefore, 2575 V•hr (0.5 hours at 350V followed by 3 hours at 800V) was selected for analysis. The short period at 350V minimized visible convection in the sample and the overlay, an effect observed if the run was started at 800V. Figure V shows the pH gradient in cytosol and membrane gels.

Sample Application: Cytosol protein samples showed some precipitation when applied to the anodic end of the gel, but not when applied to the cathodic end. Membrane protein samples streaked when applied to the cathode, but not at the anode. For analysis, therefore, cytosol samples were applied to the cathode and membrane samples were applied to the cathode.

Cytosol and Membrane Protein Isoelectric Focusing Patterns: Isoelectric focusing analysis of cytosol and membrane proteins are shown in Figure VI. Cytosol patterns show no consistantly reproducible difference between strains, although minor difference in resolution can be noted. Membrane samples appear identical except for one or two acidic proteins missing in strain A136.

Equilibration

After isoelectric focusing, the gels contained no SDS (except

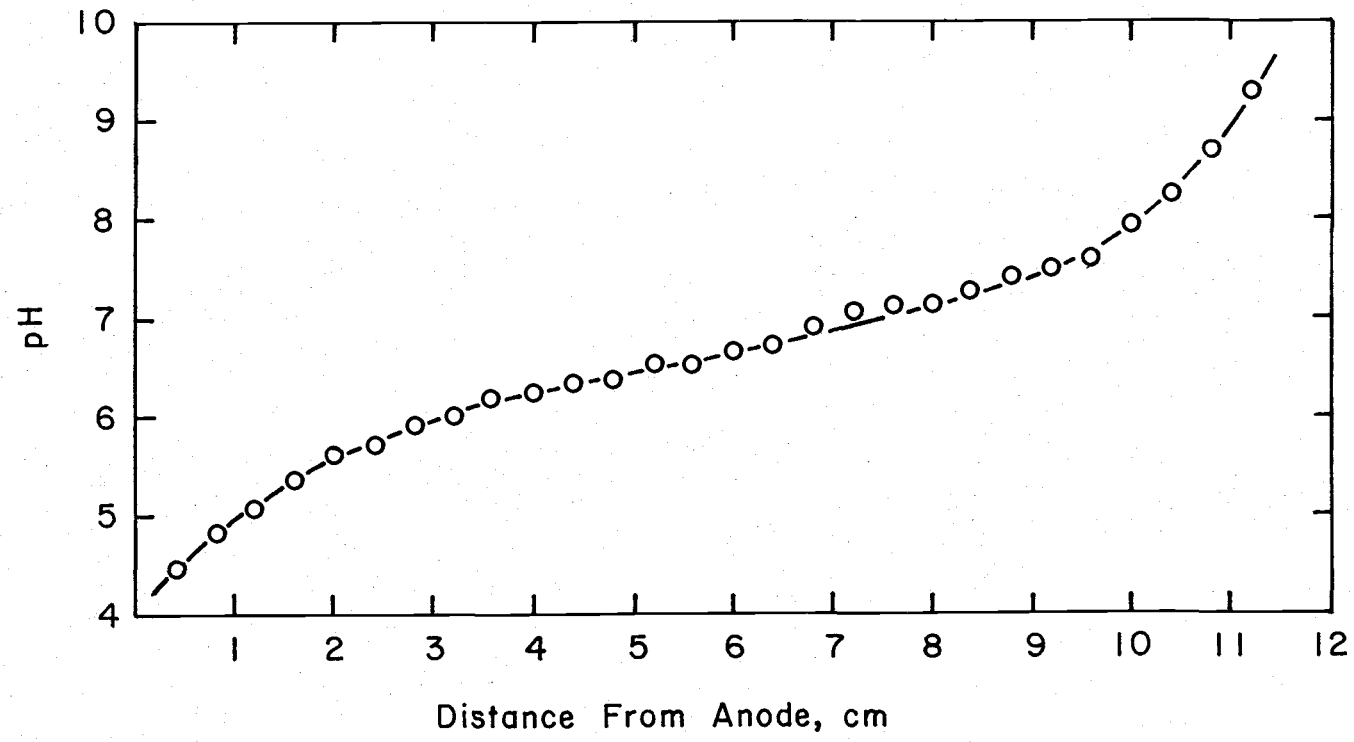

Figure V. The pH gradient found in isoelectric focusing gels run under optimized conditions (2575 V•hr).

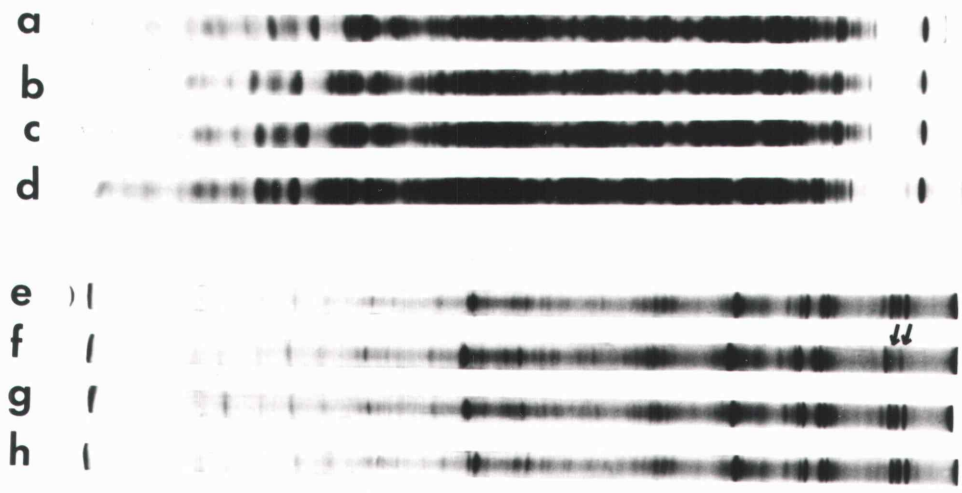

Figure VI. Isoelectric focusing protein profiles from cytosol and membrane samples. For cytosol profiles, 100 µg protein from strains C58 (a), A136 (b), A277 (c), and NT1 (d) was applied to the cathode. For membrane profiles, 75 µg protein from strains C58 (e), A136 (f), A277 (g), and NT1 (h) was applied to the anode. Runs were 2575 V•hr for all samples. The arrows denote proteins in strain A136 that were in lower levels or missing when compared to the other strains. The basic end is on the left.

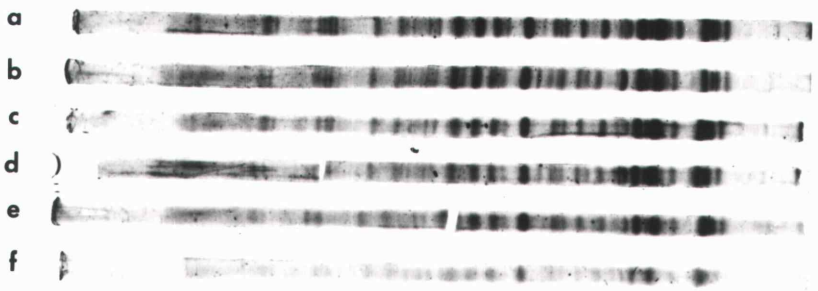

Figure VII. SDS equilibration time course of isoelectric focusing gels. Strain A277 cytosol protein (150 µg) was isoelectric focused as before. Upon completion of the run, gels were extruded into 10 ml equilibration buffer and were shaken at low speed in a bench top shaker for 5 (a), 10 (b), 20 (c), 40 (d), 80 (e), and 120 (f) minutes then stained as described in "Materials and Methods". The basic end is on the left.

the small amount from the solubilized membrane protein samples). It was desirable to equilibrate the gel (protein) in SDS to prevent vertical streaking in the SDS second dimension.

Two hours was suggested optimum for SDS equilibration of IF gel samples (O'Farrell, 1975) to prevent streaking problems. However, protein losses of 5-25% were reported with this length of SDS exposure. To observe losses during equilibration, a time course experiment (Figure VII) was performed. Bands became quite diffuse after 40 minutes and protein loss was noticeable. For analysis, therefore, a 30 minute equilibration was used. Monitoring labelled protein gels showed around 10% of the radioactivity was lost during this treatment.

Unlike cytosol samples, membrane samples were run without equilibration to get the maximum amount of protein possible into the SDS gel. Samples equilibrated as described above displayed low protein levels in the second dimension and were difficult to analyze. High SDS runnig buffer and a 5 cm stacking gel compensated for the lack of SDS equilibration and minimized vertical streaking.

Two-Dimensional Analysis

While surveying gels to determine the presence (or absence) of a protein, low level inconsistencies should be cautiously interpreted. Vertical streaking may also introduce ambiguities.

Cytosol proteins: Figure VIII shows two-dimensional characterization of strains with unlabelled cytosol protein. Strain C58 shows three basic proteins, one at 56,000 daltons (1) and two near 35,000 daltons (2,3), and one acidic protein at 34,500 daltons (4), that were not apparent in the other three strains. Strain A277 shows an acidic protein

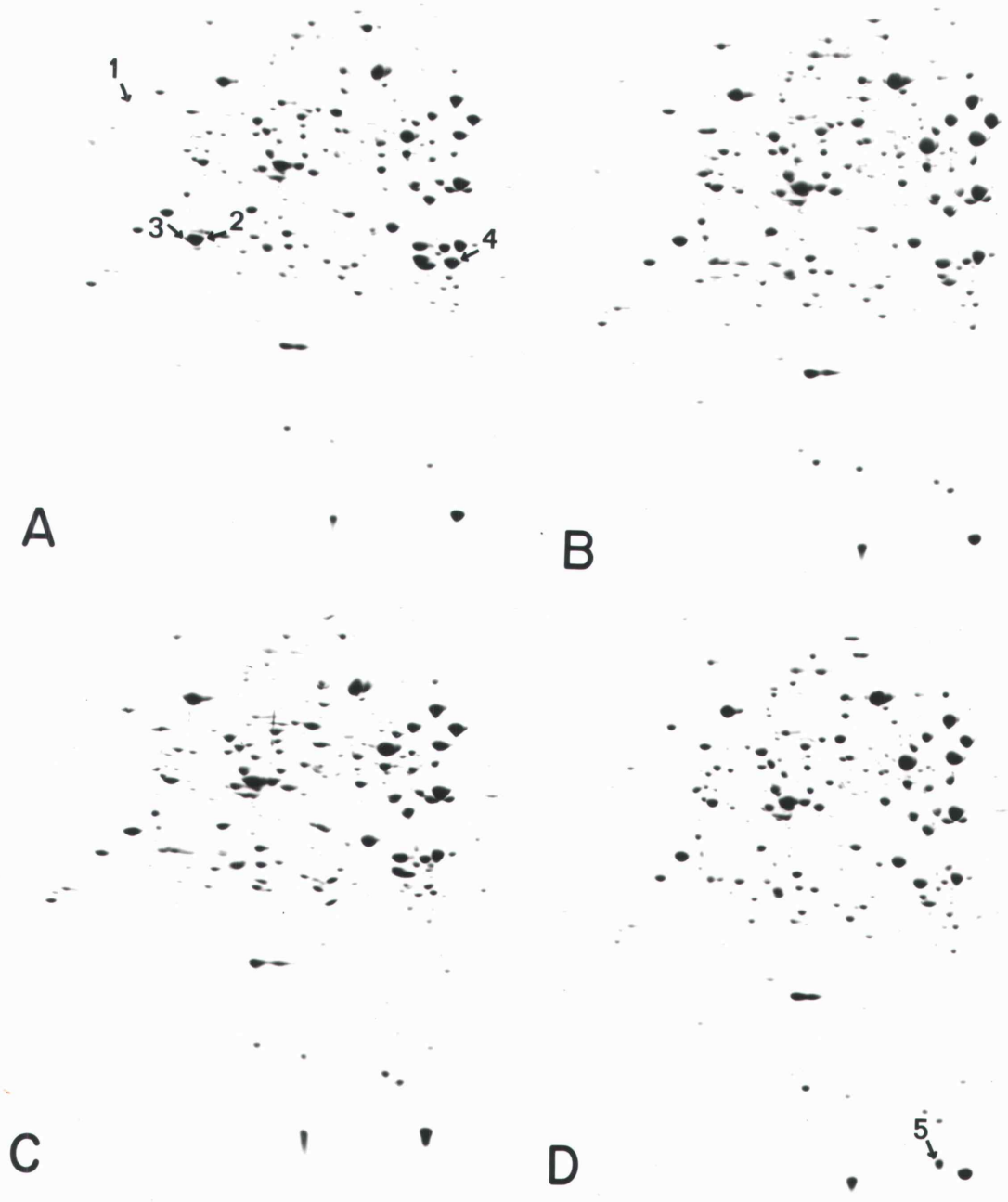

Figure VIII. Two-dimensional analysis of unlabelled cytosol proteins from C58 (A), NT1 (B), A136 (C), and A277 (D). For all samples, 100 µg was applied to the basic end of the IF gels which were run for 2575 V•hr then equilibrated (30 minutes) before transfer to the second dimension. The extra proteins in strain C58 and A277 are noted with arrows. The basic end is on the left.

of 14,000 daltons (5) not seen in the other strains.

Labelled protein analysis (Figure IX) shows slightly different protein patterns than unlabelled since detection is solely related to the methionine content of proteins. Extra proteins noted when strains C58 and A136 were compared were three basic ones, one at 56,000 (1) daltons and two near 35,000 daltons (2, 3), and one acidic protein at 34,500 daltons (4). This pattern thus confirms the extra protein that were noted in strain C58 unlabelled gels.

Membrane Proteins: Figure X shows two-dimensional characterization of the four strains with unlabelled protein. Strain C58 shows a neutral protein near 49,500 daltons (6) not apparent in the other three strains. Strain A277 shows two proteins, a basic one at 47,000 daltons (7) and an acidic protein at 61,000 daltons (8), which are absent in the other three strains. The 61,000 dalton protein (8) may be only a quantitative difference since the other strain show lower protein levels at the same point. Strain A136 appears to lack one or two acidic proteins around 32,000 daltons (possibly just a low expression level of the heavier protein) that are present in the other three strains.

Figure IX. Two-dimensional analysis of [^{35}S]-labelled cytosol protein from strains C58 (A) and A136 (B). For both samples 750,000 cpm was applied to the basic end of the gels which were run for 2575 V•hr then equilibrated (30 minutes) before transfer to the second dimension. The slab gels were fixed, dried, and exposed to X-ray film eight days. The extra proteins in C58 are noted with arrows. The basis end is on the left.

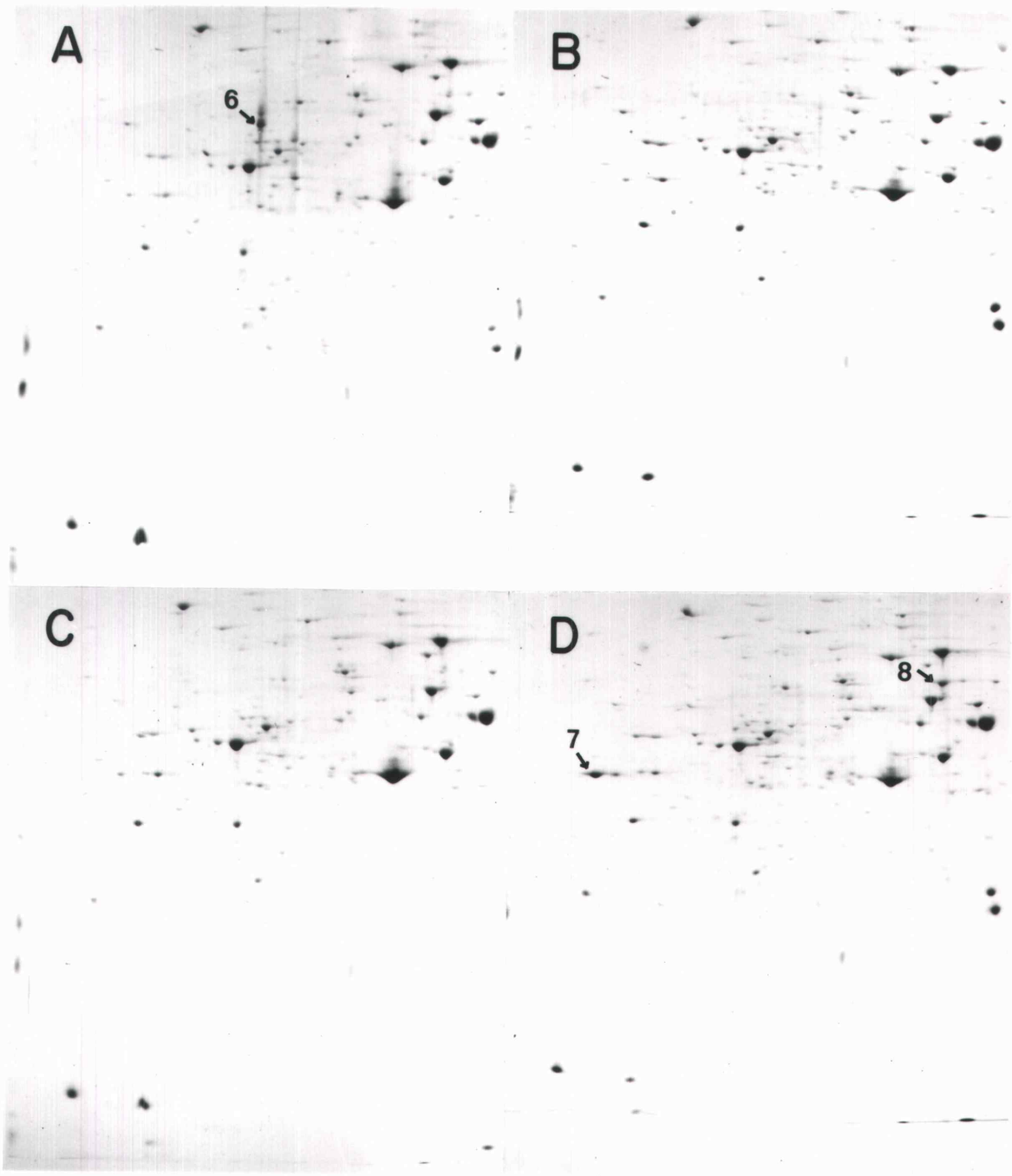

Figure X. Two-dimensional analysis of unlabelled membrane proteins from strains C58 (A), NT1 (B), A136 (C), A277 (D). For all samples, 75 µg was applied to the acidic end of the IF gels which were then run for 2575 V•hr then transferred directly to the second dimensional slab gel which had a 5 cm stacking gel. High SDS running buffer (Solution 13 Appendix) was placed in the top reservoir for the first 30 minutes of the run then exchanged with SDS running buffer. The extra proteins in strains C58 and A277 are denoted by arrows. The basic end is on the left.

followed by an SDS discontinuous gel electrophoretic second-dimension, similar to that described by O'Farrell (1975). This system separated nearly 500 proteins by two independent parameters, net charge (isoelectric point) and molecular weight, and provided "fingerprints" of cell fractions which were useful in comparative analysis. Preparation of IF gels for the second-dimension was optimized to obtain the best resolution with the maximum number of proteins. In the case of membrane gels, no equilibration in SDS was performed, hence no proteins were lost, while cytosol gels were equilibrated in SDS for 30 minutes and lost approximately 10% of their radioactivity. Coomassie Brilliant Blue G was used to stain unlabelled proteins. Detection by this method depended on the mass of a specific protein present, with the approximate lower detection limit reportedly near 10 ng (O'Farrell, 1975). A fairly large load (100 µg) was required to detect 500 proteins in a two-dimensional analysis. The high load resulted in some loss of resolution, especially near major proteins. This stain has been reported to be less efficient in detecting hydrophobic protein also (Wilson, 1979). Autoradiographic detection of [^{35}S]-labelled proteins was more sensitive than staining and consequently required smaller protein loads to detect an equal number of proteins. While this enhanced resolution, detection is limited to proteins incorporating methionine.

The Agrobacterium tumefaciens strains analyzed and their genetic characteristics are shown in Table I. Strain C58 is virulent and carries a nopaline Ti plasmid (pTiC58). Strain NT1 is an avirulent, plasmidless strain, derived from C58. Strain A136 is a spontaneous mutant of NT1 which is resistant to rifampicin (an RNA polymerase inhibitor) and nalidixic acid (an inhibitor of DNA replication). Strain A277 is virulent

and contains an octopine plasmid (pTiB6-806). Therefore, all the strains used contain the C58 chromosomal background with strain C58 harboring a representative nopaline plasmid and strain A277 a representative octopine plasmid. Since Ti plasmid-containing strains confer traits such as: 1) virulence (Watson, et al., 1975); 2) bacterial attachment (Matthysse, et al., 1978); 3) opine utilization (Petit et al., 1970); 4) opine biosynthesis in tumors (Otten, et al., 1977); 5) phage exclusion (Genetello, et al., 1977); and 6) bacteriocin sensitivity (Engler, et al., 1975), the extra proteins noted in the plasmid containing strains, which are not seen in the plasmidless strains, may be related to expression of these traits.

This study demonstrates the presence of proteins which, with further study, may be shown to be plasmid gene products of the pTiC58 and the pTiB6-806 plasmids. Preliminary experiments with SDS one-dimensional electrophoresis display the presence of extra proteins in both plasmid containing strains (Figure III). Two-dimensional analysis shows strain C58 expresses four proteins (1,2,3,4 Figure VIII, Figure IX) in the cytosol fraction, and one protein (6 Figure X) in the membrane fraction that are unique to itself, while A277 expresses one protein (5 Figure VIII) in the cytosol fraction, and two proteins (7,8 Figure X) in the membrane fraction that are unique to it. However, protein (8) is not unambiguously resolved (Figure X), since proteins of lower level expression than (8) co-migrate to the same position in the other three strains. Isoelectric focusing shows low-level expression of one protein and complete absence of another in strain A136 (Figure VI). This is supported by two-dimensional analysis (Figure X). The implications of the absence of certain proteins in this case are unclear. They are not plasmid-

coded proteins since they appear in NT1 and there is a low level expression of one in strain A136. This cannot be attributed to a rifampicin or nalidixic acid resistance-related genetic difference since these proteins are present in strain A277, in comparable levels to that in strains C58 and NT1, which has exactly the same chromosomal background as strain A136. However, if these proteins are resistance-related, coupling of the A136 chromosome with the pTiB6-806 plasmid, may cause derepression of these genes. Recent observations of a large 200 Mdalton plasmid by Schell's group (personal communication) which is closely associated with membrane character may give some insight to this observation. While experimental artifacts from proteolysis, solubilization, or incomplete displacement of SDS by NP40 cannot be ruled out, it is unlikely that they are the cause of only two missing proteins since the profile of the remaining proteins in strain A136 is consistent with the other three strains. Artifacts attributed to these problems would most likely be noted in many proteins from a sample.

The extra proteins observed in strain C58 differ from those in A277. Considering that strain C58 carries a nopaline plasmid and strain A277 an octopine plasmid, this difference might be expected.

A comparative analysis of strains C58 and NT1, similar to this study, has been done by Sonoki and Kado (1978). They reported two major proteins of 37,500 and 37,300 daltons as potentially plasmid-coded proteins which were located in the EDTA-lysozyme susceptible portion of the cell, this being indicative of loosely bound peripheral proteins. Isoelectric points and molecular weights of their proteins place them in positions very similar to the major proteins (2) and (4) of the C58 cytosol fraction. Rupturing the cells by sonication is capable

of dislodging peripheral proteins from the membrane, thus releasing them into the soluble portion of the lysate mixture. In this analysis, these proteins would be, by definition, part of the cytosol fraction. This may explain the appearance of these similar proteins in different fractions.

Because of the denaturing conditions to which these putative gene products are subjected to, no enzymatic properties can be tested. However, characteristics of strains containing plasmids with deletions provide a basis for speculation of biological roles. The avirulent C58 derivative, ID1173, which harbors a plasmid with a 39 Mdalton deletion, shows the presence of at least one of the proteins reported by Sonoki and Kado (1978), suggesting that it may be one of several proteins required for tumorigenesis. Also, the avirulent strain IIBNV6, which contains only 40% of the intact virulent plasmid, has been reported to enhance virulence in mixed inoculations (Lippincott, et al., 1977a). This indicates that the avirulent strain may retain part of the information necessary for virulence, thus suggesting the need for a number of plasmid-coded products to incite tumors and a role for the several proteins reported here. Proteins involved with DNA metabolism (LeBon, et al., 1978) and those connected with phage and bacteriocin sensitivity, along with enzymes involved with opine utilization are also possible functions for these proteins.

This study does not unequivocally demonstrate that the extra proteins observed are plasmid gene products. Further analysis is necessary to rule out the possibility that these are chromosomal genes being derepressed in the presence of the Ti plasmid. A definite genetic origin for these proteins might be located by analyzing proteins synthesized

after ultraviolet-irradiation of cells sufficient to damage chromosomal DNA, making it unable to serve as a transcription template, but leaving plasmid DNA intact. Using whole plasmids or segments of plasmids as templates for an *in vitro* transcription-translation system along with exhaustive studies of Ti plasmid deletion mutants would aid in this search.

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APPENDIX

APPENDIX

Solutions not described in the text are listed below. Reference to solution numbers are made following use. Urea used in solutions was charcoal treated, deionized, and recrystallized from water. All solutions except Solution 9 were stored at -20°C.

1. Cytosol sample dilution buffer: 9 M urea, 2% (w/v) Nonidet P-40 (NP-40) (Particle Laboratories Ltd., Elmhurst, IL), 1.6% (w/v) Biolyte pH 5-7 (Biorad Laboratories, Richmond, CA), 0.4% (w/v) Biolyte pH 3-10, 5% (v/v) 2-mercaptoethanol (Sigma Chemical Co. St. Louis, MO).

2. Sonication buffer: 0.01 M Tris-HCl (Sigma Chemical Co., St. Louis, MO) pH 7.4, 5mM $MgCl_2$.

3. RNase solution: 2 mg bovine pancreatic RNase (Sigma Chemical Co., St. Louis, MO) per ml water.

4. DNase solution: 1 mg bovine pancreatic DNase (Sigma Chemical Co., St. Louis, MO) per ml sonication buffer (Solution 2).

5. 30% stock acrylamide: 29.2% (w/v) acrylamide, .8% (w/v) N,N' methylenebisacrylamide (Eastman Chemical Co., Rochester, NY) recrystallized twice in acetone. This was made, then charcoal treated overnight.

6. Separating gel buffer: 1.5 M Tris-HCl pH 8.8, 0.4% (w/v) SDS (Sigma Chemical Co., St. Louis, MO).

7. Stacking gel buffer: 0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS.

8. Isoelectric focusing sample overlay: 9 M urea, 1% (w/v) Biolytes (0.8% pH 5-7, 0.2% pH 3-10).

9. Equilibration buffer: 0.0625 M Tris-HCl pH 6.8, 10% (w/v) glycerol, 2.3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol.

10. SDS running buffer: 0.025 M Tris base (Sigma Chemical Co., St. Louis, MO), 0.192 M Glycine (Sigma Chemical Co., St. Louis, MO), 0.1% (w/v) SDS.

11. Stock IF gel solution: 33 g urea, 2.26 g acrylamide (Biorad, electrophoresis grade), 0.129 g N,N' methylenebisacrylamide (Eastman Chemical Co., Rochester, NY) recrystallized in acetone twice, 1.20 g NP-40, 2.4 ml Biolyte pH 5-7, 0.60 ml Biolyte pH 3-10, 0.042 ml N,N,N',N' tetramethylethylenediamine and water were mixed to make 60 ml. Aliquots (3 ml) were made and stored at -20°C till needed.

12. Membrane sample dilution buffer: 9.5 M urea, 1.6% (w/v) Biolyte pH 5-7, 0.4% (w/v) Biolyte pH 3-10, 5% (v/v) 2-mercaptoethanol, 8% (w/v) NP-40.

13. High SDS running buffer: 0.025 M Tris base, 0.192 M glycine, 2% (w/v) SDS.

14. Solubilization buffer: 0.10 M Tris-HCl pH 6.8, 4% (w/v) SDS, 1 mM $MgCl_2$.