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Isolation and Partial Analysis of Proteins and Polysaccharides of Rhizobium trifolii

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Isolation and Partial Analysis of Proteins and Polysaccharides of *Rhizobium trifolii*

(TITLE)

BY

Manuelito M. Ordonez

THESIS

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Isolation and Partial Analysis of Proteins and Polysaccharides of *Rhizobium trifolii*

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Dr. Richard L. Keiter

Abstract

The outer membrane and periplasmic proteins of *Rhizobium trifolii* ANU 843 and its mutants ANU 851 and ANU 845(pRt150) were isolated and analyzed by polyacrylamide gel electrophoresis (PAGE). ANU 843 is the wild type strain which possesses all the characteristics necessary for nitrogen fixation. ANU 851 has a transposon (Tn5) inserted into the *nodD* gene of the root hair curling region of the symbiotic plasmid. It has lost its ability to cause root hair curling and therefore can not form nodules and can not fix nitrogen. ANU 845(pRt150) harbors only the 5.5 kb root hair curling region of the pSym which is comprised of the genes *nodD*, A, B, C. The band profile of the outer membrane proteins from the three strains are similar. These proteins have molecular weights of 129, 48, 42, 38, 34,21, and 20 kDa. In the case of the periplasmic proteins, ANU 843 and 851 exhibit the same protein profile except for a 23.4 kDa protein present in 851 but missing in 843. ANU 845(pRt150), on the other hand, has a 38 kDa protein which is absent in both 843 and 851. Also it is missing the 22 and 28 kDa protein found in both 843 and 851.

A strain which contains the 14 kb nodulation region, ANU 845(pRt032), was exposed to inducing materials in order to study the expression of the nodulation genes. The optimum conditions for induction were determined by using a mu-lac mutant. ANU 845(pRt032)121 has a mu-*lac* inserted

in *nodA*. When the root hair curling genes are expressed, mu-*lac* is simultaneously expressed, which leads to the production of B-galactosidase. Induction is thus measured by the extent of B-galactosidase activity. The maximum induction occurs at early log phase of bacterial growth.

The outer membrane, periplasmic and cytoplasmic proteins, extracellularpolyssacharides (EPS), and lipoplysaccharides (LPS) of induced and noninduced ANU 845(pRt032) were isolated and analyzed. The PAGE results indicate induction of the 30, 29, 19, 17, and 16 kDa outer membrane proteins. Several periplasmic proteins seemed to be induced by apigenin, with the major differences noted in the 87, 65, 56, 48, 32, and 20 kDa proteins. The profile of the cytoplasmic proteins of the induced and noninduced are similar except that there is an additional 19 kDa protein and an enhancement of the 18.6 kDa protein in the induced.

The EPS compositions of the induced and noninduced are similar, suggesting that EPS may not play a role in the root hair curling process. Some small differences were noted on the neutral components of the LPSs. Further work involving structural analysis needs to be done to learn the significance of those differences.

Acknowledgement

I am deeply indebted to Dr. Carlson whose sincere guidance has made the attainment of this endeavor possible. The experience I gained in his laboratory is most rewarding.

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INTRODUCTION

Various microorganisms in the soil are able to convert the dinitrogen from the air to ammonia. Some bacteria such as those belonging to the genera Clostridium and Bacillus fix nitrogen anaerobically while others such as the Azotobacter and Rhizobium do so aerobically. The process involving Rhizobium is widely studied since it involves the legumes which are useful to agriculture. It is estimated that in peas and beans, the amount of nitrogen which can be fixed range from 70 to 100 kg/ha/yr to over 300 kg/ha/yr for clover and lucerne (56).

A symbiotic relationship exists in the nitrogen fixing system of the Rhizobium and the legumes: the bacterium (microsymbiont) provides the host plant (macrosymbiont) with nitrogen as ammonia, while the plant supplies them with carbon as products of photosynthesis. Further, the interaction is specific, such that a particular Rhizobium species will only infect and form nodules on the roots of a certain member of the Leguminosae. For example, R. trifolii associates with clover while R. meliloti interacts selectively with alfalfa.

The establishment of an effective symbiosis is determined by the following stages of infection: (1) attachment of bacteria to the root hairs; (2) marked curling of the root hairs (commonly called shepherd's crook

formation); (3) invagination of the cell wall and consequent formation of the infection thread (this serves as a conduit for bacterial migration); (4) penetration of the cortical cells by the infection thread and release of the bacteria, encased in a peribacteroid membrane, into the cytoplasm; (5) reorganization of the cortical cells and differentiation of the bacteria into bacteroids which produce the nitrogenase enzyme that catalyzes the reduction of atmospheric N_2 to NH_3 (5,50,70).

The specificity of infection and nodulation, as in other plant-microbe interactions, is determined by a recognition event. This event was postulated as the interaction of a protein of one organism with a carbohydrate-containing molecule of the other (1). The lectin mediated recognition hypothesis has received much attention throughout the years. Lectins are proteins that bind selectively to carbohydrates and are capable of agglutinating cells by cross-linking the receptors on adjacent cells (26). In general, they have a higher affinity for oligosaccharide structures than they do for monosaccharide structures. Thus, it can be inferred that the binding sites for most lectins *in vivo* consist of several sugar residues linked together in a specific configuration (4).

Lectins isolated from either the seeds or roots of the

legume host have been reported to bind to their respective rhizobial symbionts. A partially purified soybean seed lectin was shown to combine with the infective (able to nodulate) R. japonicum, the symbiont of soybean but not to noninfective heterologous Rhizobia (11). In the R. trifolii-clover system, lectin isolated from white clover binds to the common or cross-reactive antigens of the roots and the surface of the nodulating bacteria (19).

On the other hand, there are some data downplaying the role of lectin recognition in specificity. For example, soybean lectin was also found to have an affinity for R. leguminosarum, R. phaseoli, and R. trifolii, all of which are nonsymbionts of soybean (36). Moreover, there are lectins which fail to bind to the symbiont bacteria, and lectinless varieties of soybean which are still nodulated by R. japonicum. Other experiments have also provided additional evidence for a negative correlation between specificity and lectin mediated recognition (55,75).

Rhizobia, which are gram-negative bacteria, possess surface molecules which are largely implicated as recognition sites for lectins and as participants in the molecular basis of the infection process. The surface components that have drawn much attention for research are the surface polysaccaharides, namely, the lipopolysaccharides (LPS), the exopolysaccharides (EPS), the capsular polysaccharides (CPS), and the glucans (5,14).

The LPSs isolated from four different species of Rhizobium (R. japonicum, R. leguminosarum, R. phaseoli, and R. spp.) exhibited interactions with the lectins isolated from their respective symbionts, while no interaction was observed with the nonsymbionts (74). If LPS is the determinant of specificity for lectin recognition, then strains belonging to a certain species should have similar LPS characteristics. However, compositional and immunochemical analyses of the LPSs from strains of three species revealed differences in compositions and immunodominant sites among strains of one species, as well as among species (13). It was suggested that this finding does not rule out the possibility of host specificity since alteration of the polysaccharide may occur during symbiosis.

The CPS and EPS have been emphasized heavily as the receptors for white clover lectins. The lectin, trifoliin A, is thought to anchor the rhizobial cells to the root hairs by cross-bridging the common antigenic sites in the CPS/EPS and that on the roots (22). The binding is inhibited by the hapten sugar 2-deoxyglucose, suggesting that the receptor molecule possesses a similar structure capable of accomodating the trifoliin. Since lectin is implicated in the recognition, such a model implies that attachment and recognition are a unified process. Whether this is so remains an open question.

Reports indicate that the lectin binding ability of the polysaccharides is a function of the culture age. The LPS isolated from R. trifolii 0403 in the early stationary phase binds greater amounts of trifoliin A than the one derived from cells in the exponential phase (21). Increases in the relative composition of some glycosyl components were also noted, and that an unusual sugar, quinovosamine (2-amino-2,6-dideoxyhexose) was present. The CPSs were also shown to undergo changes in composition and lectin affinity as the culture aged. R. trifolii 0403 CPS isolated from a 5 day old culture had the highest trifoliin binding ability compared to 3, 17, and 14 day old cultures (68). Changes in the levels of uronic acids, pyruvate, and O-acetyl groups were also observed. Two strains of R. japonicum (strains 110 and 138), displayed a similar transient receptivity for lectins (3). Compositional analysis revealed that the galactose units of the CPS and the EPS become methylated at the 4-position of galactose in the late exponential phase.

The role of CPS and EPS in the other stages of infection such as root hair curling, shepherd's crook formation, and nodulation has also been examined. An R. leguminosarum mutant, called Exo-1, which has lost its ability to produce copious amounts of EPS, failed to nodulate peas, its normal host (64). Characterization of three new mutants indicated that a decrease in the EPS

production and in the number of encapsulated cells caused a reduction in the efficiency of root hair curling and nodulation, and blocked the formation of the infection thread (49). An interesting phenomenon was observed in the case of *R. phaseoli* mutants which are deficient in EPS as shown by its inability to bind Calcofluor (a fluorescent stain for beta-linked polysaccharides) (69). These mutants elicited nodule formation but did not form infection threads. The nodules, however, were ineffective and did not contain symbiont bacteria. Presumably, the event is the result of incomplete recognition between the host and the bacterium due to alterations in the cell surface (69).

The possibility that outer membrane proteins may also be involved in the host-bacterial cell surface interaction has received only meager attention. Little is known about the proteins of the *Rhizobia* , in contrast to the mass of information that has been accumulated regarding those of other Gram-negative bacteria. Several outer membrane proteins of *E. coli* and other enteric bacteria have been identified, characterized, and their functions determined. Among these are the lipoproteins, enzymes, phage receptors, the ompA, the general diffusion pore proteins ompF and ompC, and various others (43).

Autoradiography done on the cell envelope of *E. coli* revealed that the ompA proteins are concentrated at

the poles (8). A look into the attachment of the rod-shaped rhizobial cells on the roots of the host plant indicates that it occurs in a polar fashion (22). Thus, one may speculate that if analogous proteins are present in the Rhizobium, they may similarly be concentrated at the poles, and can therefore play a role in the attachment process by itself or in cooperation with other molecules. This is presumably the case for those mutants which are deficient in CPS and yet were able to bind to the roots and be infective. The function ascribed to the ompA protein is that of maintaining the structural integrity of the outer membrane as well as the rod shape of the cell.

The general diffusion pore proteins (porins), ompC and ompF, also called matrix proteins due to their association with the peptidoglycan, are water-filled pores which allow the passage of nutrients and other solutes of M_r up to approximately 600 (48). These proteins, as well as the ompA protein, require LPS for biological activity (43). Alterations in the LPS may therefore hamper the normal functioning of these porins and consequently affect the viability of the cell. Maintenance of the surface structure has also been attributed to ompC and ompF proteins. E. coli cells lacking these components were found to be unstable as shown when treatment with 20 mM Tris buffer caused surface deformation (47). If the Rhizobium possesses similar proteins, this may suggest that viability

is also a consequence of the cooperative activity of the LPS and the porins, and that invasiveness is abeted by stabilization of the surface.

Another porin, called protein K, has been linked to the translocation of CPS in E. coli K1 (73). Its appearance was coincidental with the initial expression of the capsule. It was suggested that insertion of protein K facilitates the translocation of the CPS by stabilizing the zones of adhesion - the discrete sites where the inner and outer membrane come into contact during export of LPS and possibly CPS.

Between the outer and the inner membrane is the periplasmic space which contains proteins and oligosaccharides (43,48). One class of proteins has the ability to degrade or modify harmful substances such as antibiotics and heavy metals. Similar proteins may be intrinsic to the Rhizobium, and be partly responsible for overcoming the host's defenses by modifying the toxins released as a response to infection. The other two classes are the binding proteins which have affinity for nutrients like sugars, amino acids or ions; and proteins which have catabolic function: they convert solutes for which no transport system is available into a form that is transportable into the cytoplasm.

A thorough understanding of the infection process

requires a knowledge of how the genetic make-up of both the host and the bacteria control the attendant events. The tools of molecular genetics have made it possible to manipulate the symbionts to give a characteristic perturbed response affecting the events of infection. Introduction of mutations to a particular region of the genome yields phenotypes that are distinct from the parents. Thus, correlation is established among genetic functions and symbiotic phenotypes. Mutations in the bacteria can be obtained using chemical mutagens such as ethylmethane sulfonate (EMS), and N-methyl-N'-nitro-N-nitrosoguanidine; by insertion mutagenesis through integration of the phage Mu and the transposons: Tn5, Tn7, etc.; and by deletions. Heat curing and treatment with acridine dyes are also employed to give a severe mutation by eliminating extrachromosomal DNA, known as plasmids (33).

Transposon mutagenesis has been very useful and considered accurate since it produces a genetically well defined mutant (59). Another advantage is that the mutants are conferred with an antibiotic resistance carried by the transposon which facilitates their selection. The most widely used transposon in Rhizobium genetic studies is the Tn5. This transposable element is 5.4 Kb, confers kanamycin resistance, (K^r), and is stable once inserted into chromosomes or plasmids (9). Tn5 transposition gives mutants that are affected in their ability to form nodules

(Nod^-) and to fix dinitrogen (Fix^-). Its insertion can arrest the infection process at an early stage if it destroys the bacteria's ability to cause root hair curling (Hac^-). Symbiotic defects that are located between the stage of root hair curling and nitrogen fixation can also be created. Tn5 mutants that can induce slow nodule development (Ndv) and early development of superficially normal nodules lacking nitrogenase activity (Sna^-) have been reported for *R. phaseoli* (51).

One way of obtaining a transposon mutation in a desired location is by site-directed mutagenesis (62). The DNA region to be mutated is isolated from the bacteria and combined with a vector plasmid. The recombinant plasmid is then cloned in *E. coli*. Phage lambda tagged with Tn5 (λ::Tn5) is allowed to infect *E. coli* cells and the plasmids where the Tn5 was integrated are isolated and treated with restriction enzymes such as *Eco*RI, *Hind*III, *Bgl*III, *Bam*HI, etc. To locate the site of insertions, fragments are hybridized with radioactively-labelled Tn5 and seperated by gel electrophoresis. The fragments bearing the transposon are recloned into a conjugative cloning plasmid that confers resitance to some other antibiotic, say tetracycline (Tc^r). The conjugative recombinant plasmid is mobilized into the *Rhizobium* and a process called homology-dependent recombination occurs. This is a crossover between the normal DNA and that flanking the Tn5

and its homolog in the genome, resulting in the transfer of Tn5 at a low frequency. The colonies are screened for both kanamycin and tetracycline resistance. Those that are K^r and Tc^r are mated with another plasmid incompatible with the first conjugative vector, to check whether the Tn5 is actually incorporated in the genome. Production of colonies that are K^r but are tetracline sensitive (Tc^s), indicates that the first conjugative vector was destroyed and that resistance to kanamycin is due to the Tn5 inserted into the genome.

A similar method is by the "reverse genetic approach" (60). The region of interest is subcloned into a multicopy plasmid (carbenicillin resistant, C^r) and replicated in E. coli. The recombinant plasmid is mobilized into another strain of E. coli which is resistant to low levels of kanamycin due to the presence of Tn5 in its chromosome. The multicopy plasmid causes the Tn5 to be disintegrated from the chromosome and be inserted into itself. Such insertion gives rise to a high level of resistance to kanamycin and becomes the basis of selection. The plasmid carrying the Tn5 is then mobilized from E. coli to the wild-type Rhizobium where homologous recombination and destruction of the cloning vector occurs. Kanamycin resistant transconjugants are tested for carbenicillin sensitivity to ensure that the vector is destroyed and therefore K^r is only due to Tn5 insertion

into the wild-type genome. The mutants generated by both methods are assayed on the hosts to determine the symbiotic phenotype.

In the *Rhizobium*, host specificity, nodulation, and nitrogen fixation genes are encoded on the symbiotic plasmid (pSym). Transfer of the pSym or a segment of it from a species to the other may result in the broadening of host-range (10,30). It has also been shown that the nodulation (*nod*) genes can be expressed when transferred to strains belonging to different genera such as *E. coli*, *Lignobacter*, and *Pseudomonas* (54). Although symbiosis seems to be regulated in the pSym, some data point out the involvement of chromosomal genes as well (51). The *nod* and the nitrogenase (*nif*) genes are both on the pSyms of the fast-growing *Rhizobia* *R. trifolii* (66), *R. meliloti* (34), and *R. leguminosarum* (24). Sequence homology in the *nod*, *fix*, and *nif* genes has been established among rhizobial species (31). Homology is also present in the *nif* genes of *Rhizobia* and the free-living nitrogen fixer *Klebsiella pneumoniae*. This has been the basis for the identification and isolation of pSym.

Mutations induced by Tn5 can be corrected by introducing cloned genes from the wild-type strain. For example, non-nodulating mutants of *R. meliloti* restore their ability to nodulate alfalfa when regions of the

wild-type pSym containing the nodulation genes are mobilized into them (37). Genes from one species can also correct for the phenotypic defects of another species (23,34,39). A Nod$^-$ mutant of *R. meliloti* regains its nodulating ability when a 14-Kb *Hind*III fragment from *R. trifolii* pSym, which contains all the information necessary to nodulate clover, is used to complement the defect. Such genes are designated as common *nod* genes (34,39).

A region of the *nod* genes that is shown to be functionally conserved among species is the one responsible for root hair curling (39). This region consists of four genes designated as *nod*A, B, C, and D. *Nod*A, B, and C are adjacent to each other and are oriented in the same direction. They may be transcribed into messenger RNA as a unit. The *nod*D gene is next to *nod*A but is oriented in the opposite direction and is transcribed separately (45)

A method has been devised to study the expression and regulation of *nod* genes (71). Phage Mu fused with the lac gene from *E. coli* is inserted into either *nod*A, B, C, or D. The insertion induces mutations by inactivating the genes, and in *R. trifolii*, phenotypes were similar to those obtained by Tn5 transposition (29). In this case, the expression of *lac*Z (production of B-galactosidase) is now under the control of the upstream promoters, and so the levels of expression of *nod*A, B, C, or D can be measured

indirectly by assaying for the B-galactosidase activity. It has been shown that exposure to legumes or to their exudates stimulates the expression of the nodulation genes (i.e. increases B-galactosidase activity). NodD, is always expressed at high levels even when the bacteria are not interacting with the legume host i.e. those that are grown in standard laboratory media (29). In contrast, the expression of nodA, B, and C genes requires the plant factor and the product of nodD (29,40).

The work described in this thesis is a comparative study of the outer membrane proteins, periplasmic proteins, acidic EPS, and LPS of R. trifolii strains (Table 1). The parent strain ANU 843 and two mutant strains - ANU 851 and ANU 845(pRt150) - were analyzed for their outer membrane and periplasmic proteins. ANU 843 is the wild-type (Nod^+ Fix^+), ANU 851 has a Tn5 insertion in nodD of the root hair curling region (Hac^-, Nod^-), and ANU845(pRt150) is the wild-type cured of its symbiosis plasmid ($pSym^-$) but with 5.5-kb BglII fragment (the root hair curling region) integrated in the multicopy plasmid pRt150 (Fig. 1). This strain causes intensive root hair curling (Hac^{++}) but does not nodulate (Nod^-). One strain, ANU 845 (pRt032), also $pSym^-$, and contains the 14-kb HindIII nodulation region in the multicopy plasmid pRt032 (Fig. 1), was exposed to extracts from white clover. The periplasmic and outer membrane proteins, acidic EPS, and LPS were compared to one

grown without white clover extracts. Our aim was to harvest the bacteria at a point where the nodulation genes are expressed at their maximum. In this regard, ANU 845 (pRt032)121, with mini-Mu-lac transposition in nodA (nodA::Mu dI1734) was used to measure levels of gene expression via B-galactosidase activity. ANU 845(pRt032) was then harvested at an optical density similar to that of ANU 845(pRt032)121 where B-galactosidase activity is at the maximum.

Table 1. Relevant characteristics of the *Rhizobium* strains

Strain	Genotype	Phenotype	Antibiotic Resistance
ANU 843	wild type	Hac^{+}, Nod^{+}	kan^{s}, $strep^{s}$
ANU 851	Tn5::*nodD*	Hac^{-}, Nod^{-}	kan^{r}, $strep^{r}$
ANU 845(pRt150)	5.5 kb Hac region of pSym	Hac^{++}, Nod^{-}	kan^{r}, $strep^{r}$
ANU 845(pRt032)	14 kb Nod region of pSym	Hac^{++}, Nod^{++}	$carb^{r}$,
ANU 845(pRt032)121	Mu dI1734::*nodA*	Hac^{-}, Nod^{-}	kan^{r}

kan=kanamycin; strep=streptomycin; carb=carbenicllin; Hac=root hair curling; Nod=nodulates

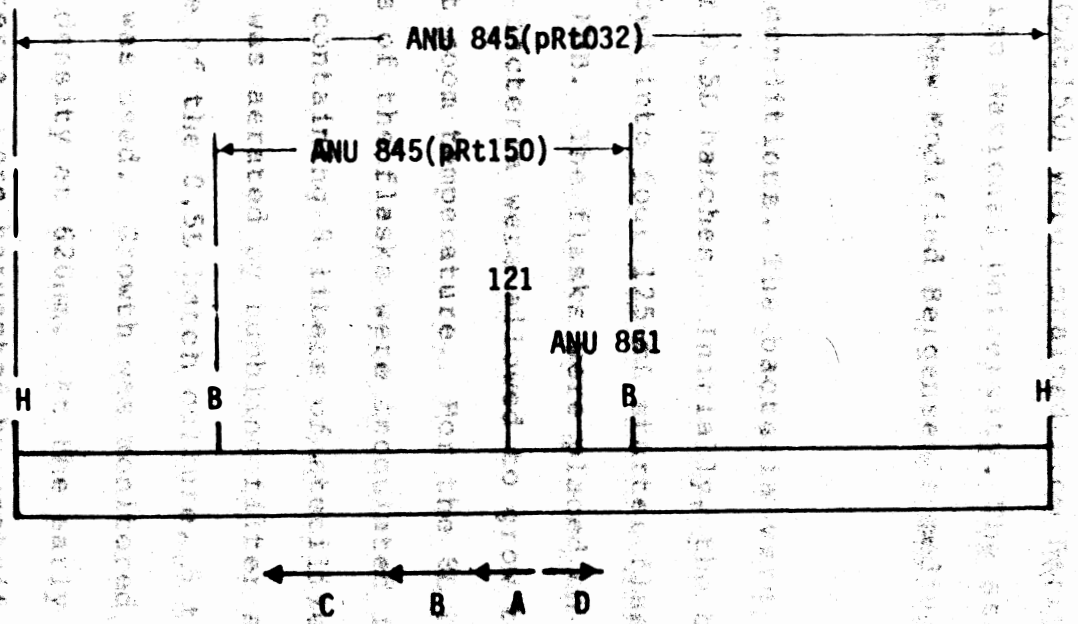

Figure 1. The 14 kb HindIII fragment of the R. trifolii pSym. ANU 845(pRt032) contains the entire nodulation region attached to a multicopy plasmid pRt032. ANU 845(pRt150) harbors the root hair curling region in a multicopy plasmid pRt150. ANU 851 has a Tn5 insertion in nodD. ANU 845(pRt032)121 is ANU 845(pRt032) with Mu dI1734 inserted in nodA. H=HindIII, B=BglII.

EXPERIMENTAL

I. Bacteria without exposure to plant material

Bacterial strains. ANU 843 and its mutants ANU 851 and ANU 845(pRt150) were obtained from Barry G. Rolfe of Australian National University. The strains were maintained on solid New Modified Bergensen's medium (NMB, Appendix 1) at 4°C.

Growth conditions. The bacteria were grown in NMB, either as 8L or 0.5L batches. Initially, the bacteria were introduced into four 125-mL starter flasks containing 40 mL sterile NMB. The flasks were placed on a shaker (125 rpm) and the bacteria were allowed to grow to stationary phase (4 days) at room temperature. For the 8L batch culture, the contents of the flasks were inoculated into a glass jar (Pyrex) containing 8 liters of sterilized medium and the culture was aerated by bubbling filter sterilized air. In the case of the 0.5L batch culture, 5 to 8 mL of the starter culture was used. Growth was monitored by measuring the optical density at 620nm. At the early stationary phase, the bacteria were harvested by centrifugation in either a Sorvall SS-3 or SS-4 centrifuge at 16,000 x g (10,000 rpm, Sorvall GSA rotor) for 20 minutes at 4 °C. The bacterial pellets were collected into a vial and stored in the freezer (-20°C) until use.

Isolation of Cell Membrane. The freezing and thawing procedure(65) was used with minor modifications. The cells were frozen in liquid nitrogen and were ground into a powder using mortar and pestle. The frozen powder was placed in 0.02 M Tris buffer, pH 7.8, containing 5mM EDTA , 0.25M sucrose, and 0.5 mg/ml of lysozyme such that cell weight was 30% of final volume. Next, the suspension was shell frozen in liquid nitrogen and was then thawed in warm water until the ice had just melted. The thawed material was mixed with ten volumes of cold 0.02M Tris buffer, pH 7.8, containing 0.50mM $MgCl_2$, and 0.03-0.05 mg deoxyribonuclease per ml. To shear the DNA and disperse the cells, the material was treated immediately in a laboratory blender for about 20s. Removal of unbroken cells was done by centrifugation at 5,000 x g for 5 min. The supernatant was decanted and saved in the coldroom (4^{o}C) while the pellet (unbroken cells) was again ground and later suspended in the original supernatant. The resulting suspension was treated in the laboratory blender and centrifuged. This treatment of the unbroken cells was repeated two times. After the second treatment the supernatant was centrifuged at 27,000 x g for 20 min. The pellet consisted of the bacterial cell envelope.

Separation of the outer membrane from the inner membrane. A modified sucrose gradient centrifugation (53,65) was used to isolate the outer membrane from the inner membrane. The

cell envelopes were suspended in 25% sucrose (w/w) solution containing 3.3mM Tris, 5mM EDTA, pH 7.5. One milliliter was layered on top of sucrose gradient steps consisting of 3 mL each of 35%, 40%, 45%, 50%, and 55% sucrose solutions (w/w) all containing 5mM EDTA, pH 7.5. Centrifugation was carried out in a Beckman Model L3-50 ultracentrifuge at 82,000 x g, 2-4°C for 30-42 hrs. The bands were collected using a pasteur pipette. The materials were put into Spectrapor tubing (M.W. cutoff:12,000-14,000), dialyzed through four water changes, and then lyophilized.

Colorimetric Assays. Protein content was determined by the method of Lowry (42, Appendix 9) using bovine serum albumin as the standard. The percentage KDO in the membranes was analyzed as described in Appendix 8 (72). Authentic KDO purchased from Sigma Chemicals, was used as the standard.

Isolation of periplasmic membrane. A modified method of Ames et al. (2, Appendix 4) was used to release the periplasmic proteins. The original procedure uses 2mL of bacteria grown to saturation. Our experiments involved the use of 500 ml of bacteria and so to correct the discrepancy, reagents were scaled up accordingly. In this method chloroform was used as a 'shocking' agent which permiabilizes the cells and releases the periplasmic proteins.

SDS-gel electrophoresis. Samples equivalent to 150 microgram outer membrane proteins and 200 microgram periplasmic proteins were dissolved respectively in 20 microliters SDS buffer (0.16 M Tris, pH 6.8; 0.03 M sucrose; 2% SDS (w/v); 0.025% (w/v) bromphenol blue; 5% (v/v) mercaptoethanol). The samples were boiled for either 5 min (periplasmic) or 2 min (outer membrane) and then introduced into a slab gel formed in a Hoeffer electrophoresis unit. Electrophoresis was carried out by the method of Laemmli (35). Proteins were stained with coomassie blue. The details of the procedure are given in Appendix 6.

II. *R. trifolii* Exposed to Plant Material

Bacterial Strains. ANU 845(pRt032) and the *lac* mutant ANU 845(pRt032)121 were obtained from Roger Innes of the University of Colorado. The former is resistant to carbenicillin and the latter to kanamycin. Both strains were maintained on NMB slants at 4°C.

Growth conditions. Bacteria [ANU 845(pRt032)] were grown in NMB, in 0.5 L or 8 L batches. The growth conditions were identical to those of ANU 845(pRt032)121, and were determined by several experiments using white clover extracts or apigenin for induction (discussed below).

Plant Extract. White clover seeds were surface sterilized (58) prior to germination. The seeds were first

washed with sterile deionized water and then with 80% ethanol for 3-5 min in sterile 125-mL flasks. They were rinsed 3 times with deionized water and treated with 40% bleach (40 mL in 60 mL deionized water) for 15 min. The flasks were frequently swirled to assure sufficient seed surface contact with the liquids. Finally, the seeds were washed 3 times with sterile deionized water until the odor of the bleach was no longer evident.

The seeds were spread on moistened paper towels laid in a plastic container, and then covered with moistened paper towels. The cover of the container was replaced and the assembly was put in a Sherer controlled environment chamber. The light/dark schedule was 18 hr/6 hr and the day/night temperature was 22°C/19°C (58).

After seven days, one-half of the clover plants (ca 134g) were frozen in liquid nitrogen and ground into a powder. The powder was suspended in 100 mL deionized water, poured into Spectrapor dialysis tubing (M.W. cut off: 12,000-14,000) and dialyzed against deionized water for 26 hrs. The dialysate was concentrated by roto-evaporation and then freeze-dried. This material was called WCE-D. The other half of the plants (ca 138g) was suspended in 1,000 mL of 40% ethanol and was stirred at room temperature for 2.5 hrs. Clover plants were separated from the liquid by filtering on cheesecloth. The filtrate was passed through a qualitative filter paper to remove any debris. The liquid

(containing the clover extract) was concentrated by rotory-evaporation and then freeze-dried. This extract was named WCE-E1.

In another case, seeds were surfaced sterilized and allowed to sit in sterile deionized water overnight. They were rinsed 4 times before spreading on wet paper towels as before. This time the seeds were allowed to germinate for 2 days only in the dark. Approximately 210 g of sprouts were recovered and extracted in 40% ethanol for 4 hrs. The ethanol was used such that it was enough to cover the sprouts (ca 405 mL) during extraction. Filtration procedures were as before. The extract was concentrated almost to dryness (until syrupy), diluted in 125 mL of water, and centrifuged to remove debris. It was passed successively through 0.8 and 0.45 um millipore filters before being filter sterilized in a Nalgene filtration unit (0.22um). This material was labelled WCE-E2.

Test for inducing ability of the extracts. Solutions (1 mg/mL) of both WCE-D and WCE-E1 extracts were made in deionized water and TM media (Appendix 2) respectively. The solutions were filter sterilized through Nalgene disposable filterware, and then stored in the freezer until use. No further handling was done with WCE-E2.

ANU 845(PRt032)121 was grown to stationary phase ($O.D._{620}$ 1.7) in 40 mL NMB supplemented with 2 mg

kanamycin. Four tenths (0.4) mL was transfered into 40 mL of TM and the bacteria were allowed to grow until the $O.D._{620}$ was about 0.40. To 1.8 ml of WCE-D and WCE-E1 solutions (i.e. WCE-D in TM and deionized water; WCE-E1 in TM and deionized water), 0.2 mL of bacteria was added and incubated for periods of 6.5 or 8.5 hrs. Induction was determined using the B-galactosidase assay (46, Appendix 3).

For WCE-E2 induction anaylsis, ANU 845(PRt032)121 from the stationary phase was grown in TM/Kan overnight ($O.D._{620}$ 0.1-0.2), and to 2.5 mL was added 20 uL of WCE-E2. The cultures were allowed to sit at room temperature and assayed for induction after 3 and 6 hrs. The controls (bacterial samples without plant material added) were treated in the same manner.

Optimum conditions for maximum expression of the nod genes. The goal of the following experiments was to establish the proper growth conditions for growing ANU 845(pRt032), such that the bacteria can be harvested at a time when the nodulation genes are expressed to the fullest extent.

Two types of inducing materials were used. The first one was WCE-E2 which was found to have the highest inducing ability among the white clover extracts (discussed in the 'Results'). The second one was apigenin (4',5,7-trihydroxyflavone) purchased from Sigma Chemicals,

which is similar to the main inducing compound isolated from white clover(4',7-dihydroxyflavone) (61).

ANU 845(pRt032)121 was grown to stationary phase in NMB supplemented with kanamycin. In the first experiment, 80 mL of stationary phase bacteria were added to 8 L of TM medium contained in two pyrex glass jars. The cultures were allowed to incubate overnight ($O.D._{620}$ .251 and .224). To 1 jar, 60 mL of WCE-E2 were added as the inducing material.

In the second experiment, 80 mL of stationary phase bacteria was added to 8L of NMB in two jars. Apigenin (30 mg), dissolved in 1 mL of deionized water and 1 mL of ethanol with 1 drop of NaOH, was added to one of the two batches of bacteria. At various time periods, 2 mL of induced and noninduced bacteria was assayed for B-galactosidase activity.

The last experiment which was designed for 0.5 L batches, also used apigenin as the inducer. Since apigenin dissolves in mildly basic solutions, 6 N NaOH was added to the resulting 1 mg/ml aqueous solution. This solution was then passed through a Sweeny filter (0.22 um) for sterilization. To 0.5 L of NMB just inoculated with 5 ml of stationary phase bacteria, 1.5 ml of apigenin was added. The control (noninduced) was incubated simultaneously and B-galactosidase activity measured at various time periods.

Apigenin as the inducer. Eighty (80) mL of stationary phase bacteria were added to 8 L of NMB. Apigenin (30mg) dissolved in a mixture of 1 mL of ethanol, 1 ml of water, and 1 drop of NaOH was added for induction. The control was grown simultaneously and both were harvested when the $O.D._{600}$ was 0.330-0.450. To prevent further growth, the bacteria were placed in an ice bath and pelleted by centrifugation at 7,970 x g - 13,200 x g for 20 min. For the 0.5 L batches, 5 mL of stationary phase bacteria were added and 1.5 mL of a 1 mg/mL of a basic solution of apigenin was used to give a final concentration of 3 microgram per mL.

Isolation of outer membrane and cytoplasmic membrane. The freezing and thawing procedure was used to obtain the cell envelope. This was followed by sucrose gradient centrifugation to separate the two membranes (these procedures were dicussed in part I).

Isolation of periplasmic proteins. Two methods were used to obtain the periplasmic proteins. One is that of Ames et al., as previously mentioned, and the other is by Lugtenberg (44, Appendix 5). In the chloroform shock method of Ames, the amount of each reagent was also adjusted with respect to growth conditions. The original procedure uses bacteria in the stationary phase, while our experiment used bacteria in the early log phase. These discrepancies were compensated

as follows:

$$\text{vol. } CHCl_3 \text{ (mL)} = \frac{20 \text{ uL}}{2 \text{ mL}} \times 500 \text{ mL} \times \frac{O.D._{\text{early log}}}{O.D._{\text{sat}}}$$

$$\text{vol. Tris buffer (mL)} = \frac{0.2 \text{ mL}}{2 \text{ mL}} \times 500 \text{ mL} \times \frac{O.D._{\text{early log}}}{O.D._{\text{sat}}}$$

where $O.D._{\text{early log}}$, the optical density of the bacteria at early log phase of growth; and $O.D._{\text{sat}}$, the optical density at the stationary phase.

The method developed by Lugtenberg is one used for isolating the periplasmic proteins of R. leguminosarum strains (44, Appendix 5). Cells were suspended in Tris buffer containing 0.53 M sucrose and lysozyme. Incubation for 30 min releases the periplasmic materials. After centrifugation, the supernatant was treated with trichloroacetic acid (TCA) to precipitate the proteins.

Isolation of cytoplasmic proteins. Cells recovered after periplasmic extraction by the method of Lugtenberg were suspended in 50 mM Tris, pH 8.0, and treated in the Warring blender for 10 s. The homogenized suspension was sonicated for 1 min using Biosonik III (Bronwill) at 70% maximum power. Sonication was executed in 30 s bursts, with 30 s rest period in between. To remove unbroken cells, the suspension was centrifuged at 12,100 x g for 20 min. The supernatant was diluted with 50 mM Tris and made 0.2 M in KCl. It was then centrifuged in a Beckman L3-50 ultracentrifuge at 96,500 x g for 4hrs at 4°C, to remove

the cell envelope. The supernatant containing the cytoplasmic proteins was decanted and the proteins precipitated with 2 volumes of 5% TCA, which was added slowly in the cold room. The resulting cloudy solution was stirred for 1 hr and then centrifuged at 12,100 x g for 1 hr. The white protein precipitate was washed with ethyl ether, lyophilized, and later dissolved in SDS-buffer.

SDS-gel electrophoresis. The periplasmic and the cytoplasmic proteins obtained by the method of Lugtenberg were analyzed directly by polyacrylamide gel electrophoresis (PAGE) while the periplasmic materials isolated by the chloroform 'shock' method were dissolved in deionized water (1 mg/ml) and then assayed for protein content. Material equivalent to 100 ug protein was then dissolved in the SDS-buffer and analyzed by PAGE. The PAGE methods described above were used.

Isolation of EPS. After centrifuging the cells, the supernatant was concentrated in a rotory-evaporator and treated with 3 volumes of ethanol to precipitate the EPS. The EPS was collected (fished out with rubber policeman), drained, dissolved in deionized H_2O, poured into Spectrapor dialysis tubing (M.W. cut off: 12,000-14,000), and dialyzed against 3 changes of deionized water. After dialysis, the solution was diluted with deionized H_2O and was made 10 mM in Na_2SO_4. Three percent

cetyltrimethylammonium bromide (CTAB) was added dropwise, and the material was allowed to stir for 2 hrs at room temperature. The precipitate was recovered by centrifugation for 15 min at 13,200 x g. It was washed 3 times with deionized H_2O, dissolved in 10% NaCl, and reprecipitated with 2 volumes of acetone. The acetone precipitate was redissolved in 10% NaCl and put in dialysis tubing (as previously mentioned). Dialysis was done against 1% NaCl through 3 changes, followed by dialysis against water through 3 changes. The material was freeze-dried, weighed, and stored in the freezer. This was labeled acidic EPS.

Isolation of LPS. Bacterial pellets were suspended in deionized water preheated to 65°C. The volume was brought to 100 mL. Phenol (100 mL, usually preheated) was added and the mixture was heated to 65°C. It was left at this temperature for 15 min., and then plunged into an ice bath and allowed to cool for 15 min. The material was centrifuged for 20 min at 13,200 x g to separate the water layer from the phenol layer. Using a pasteur pipette, the water layer (containing the LPS) was collected, and the phenol layer was subjected to a second extraction by adding water (100 mL, preheated to 65°C). Extraction conditions were as before. The water layers from both extractions were combined, poured into a Spectrapor dialysis tubing (M.W. cut of: 12,000-14,000) and dialyzed through 4 water changes.

For every 100 mL of the dialyzed water layer, 0.1 mL of each solution of RNase and DNase (1 mg/mL in 0.1 M Tris and 0.1 M $MgSO_4$) was added. Also 10 mL of 0.1 M Tris and 0.1 M $MgSO_4$ per 100 mL of water layer was added. If floating particles were noted, materials were centifuged at 13,200 x g before RNAse and DNAse were added. The materials were allowed to stand at room temperature for 24 hours, and then dialyzed through 4 water changes in Spectrapor tubing. After dialysis, this solution was lyophilized and stored in the freezer until it was loaded onto the chromatography column (described below).

Column Chromatography. The lyophilized water layer in the LPS extraction was dissolved in column buffer (per liter: EDTA, 2.922 g; TEA, 4.3 mL; NaN_3, 200 mg). If the resulting solution was too viscous, it was sonicated for 10 min. Otherwise, samples were loaded directly onto the Sepharose 4B colum (void volume - 130 ml, included volume - 385 ml, column height - 51 cm, column diameter - 3 cm). The fraction collector was set to collect 100 drops (or 5 ml fractions) with each drop falling at a 10-15 s interval. Alternate fractions starting from fraction 21 through 95 were assayed for hexose and KDO. Fractions containing KDO were pooled, dialyzed in Spectrapor tubing against deionized water, and then lyophilized.

Colorimetric assays. Hexose was determined using the anthrone assay. Amounts of KDO were assayed as described in

Appendix 8. Uronic acid was quantitated by the method of Blumenkrantz and Asboe-Hansen (7, Appendix 11). The substituents, pyruvyl and acetyl, were also quantitated colorimetrically (32,28, Appendices 10 and 7).

Gas chromatography. The alditol acetates of the polysaccharides (200 ug in duplicates; see Appendix 12 for acetylation procedure) were dissolved in 50 microliters of CH_2Cl_2, while the standard sugars were dissolved in 100 microliters. One microliter of the standard sugars and 1.5-2 microliter of the polysaccharides were injected into a 15 m SP2330 fused silica capillary column (Supelco) in a Hewlett Packard 5890A gas chromatograph. The instrument is provided with a flame ionization detector fueled with hydrogen. The detector, injector, and oven temperatures were set to 240, 220, and 240 ^{o}C respectively. Helium was used as the carrier gas (mobile phase). The oven temperature was varied from 190^{o}C to 240^{o}C at 10^{o}C/min and held at 240^{o}C for 10 min.

RESULTS

Inducing ability of the extracts. ANU 845(pRt032)121 has a mu-*lac* inserted in the *nodA* of the root hair curling region. Therefore, expression of the mu-*lac* (production of B-galactosidase enzyme) is dependent on the induced expression of *nodA*. The clover extracts displayed variations in inducing ability as determined by the B-galactosidase activity assay (Table 2).

Table 2. Inducing ability of the clover extracts.

Inducing material	Enzyme Units		Incubation
	Control	Induced	
WCE-D in water	378	879	6.5 hr
WCE-D in TM	394	716	6.5 hr
WCE-E1 in water	350	1,174	8.5 hr
WCE-E1 in TM	300	1,201	8.5 hr
WCE-E2	341	1,018	3 hr
WCE-E2	334	1,219	6 hr

WCE-E2, one of the clover materials extracted by ethanol, gives the highest induction; 1,219 enzyme units in 6 hr. WCE-E1, another ethanol extracted clover material averages 1,188 enzyme units in 8.5 hr. The dialysate of the powdered clover roots suspended in water, WCE-D, gives the lowest induction, an average of 798 in 6.5 hr. The results suggest that WCE-E2 contains more of the clover material that

induces nod gene expression and that this material is more soluble in relatively nonpolar liquids such as ethanol.

Conditions for maximum expression of nod genes. WCE-E2 was shown to turn on the nod gene at a higher level than the other white clover extracts. This then was the most likely inducing material to use in determining the point at which the nodulation genes are expressed to the maximum. However, when the experiment was in progress, apigenin was indicated by B. G. Rolfe to be an effective inducer and so it was used as an alternative.

ANU 845(pRT032)121 grown in 8 L batches was treated with WCE-E2 and apigenin respectively and the B-galactosidase activity was followed through time (Fig. 2). For the apigenin induced bacteria, induction is highest at 14-25 hr. This corresponds to an $O.D._{600}$ of 0.33-0.36. The WCE-E2 induced bacteria show a fairly similar induction starting at 6 hr ($O.D._{600}$ 0.52) to 18 hr ($O.D._{600}$ 0.87). Maximum induction appears to occur at an earlier stage of bacterial growth ($O.D._{600}$ 0.33-0.36) with apigenin than with WCE-E2. Also, it is evident that apigenin is more potent in causing nod gene expression, about 65% higher than WCE-E2.

Since apigenin was found to be highly effective in turning on the nod genes, it was the sole material adopted for use in the 0.5 L batches. Enzyme activity was highest during the 16th hr ($O.D._{600}$ .122) and steadily decreases

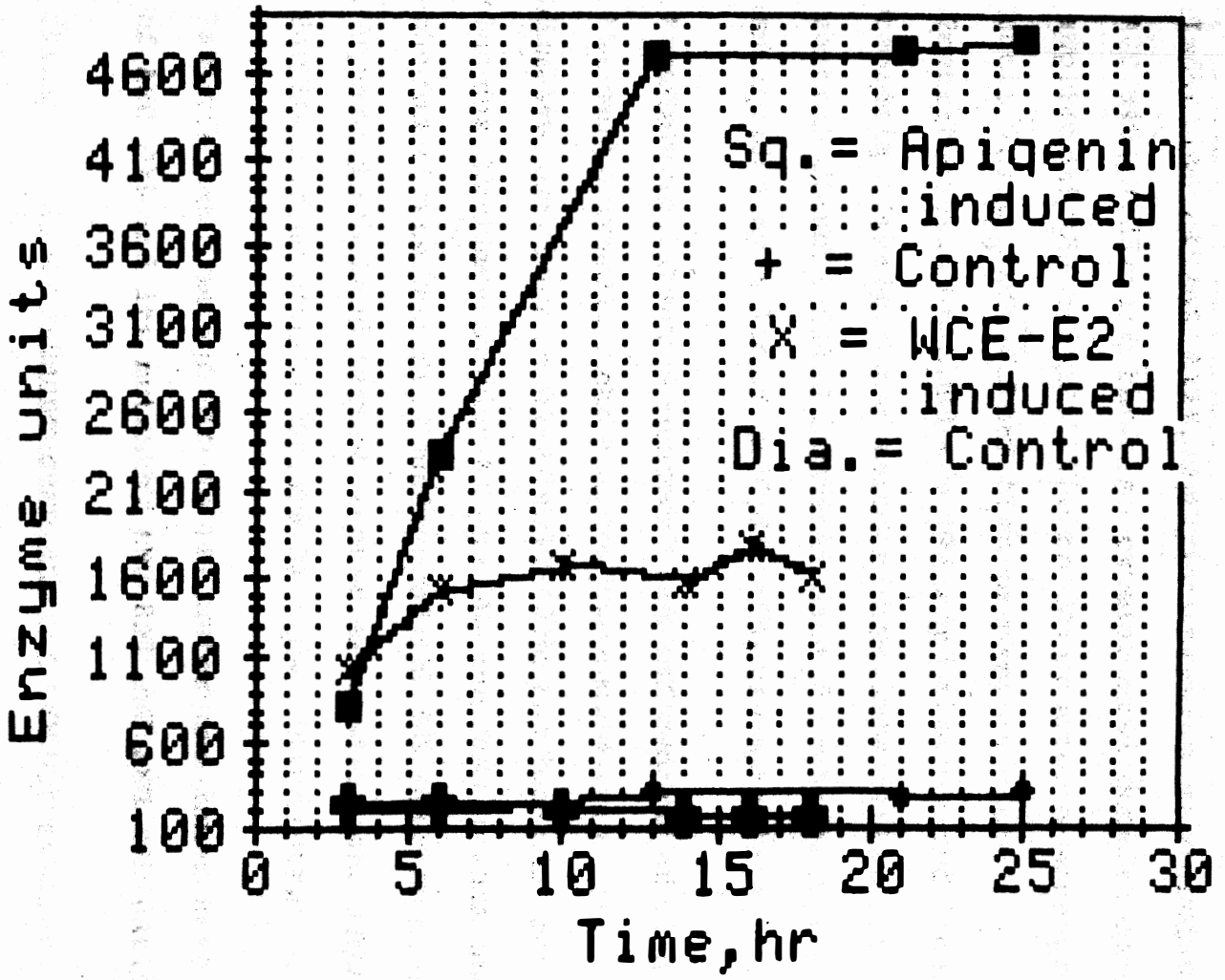

Figure 2. B-galactosidase activity in ANU 845(pRt032)121 (8 L batch) exposed to WCE-E2 and apigenin. Enzyme units= $O.D._{420}$ x 1000 / (v x t x $O.D._{600}$). v=volume of bacteria (0.5 mL); t=reaction time; $O.D._{600}$=cell density before assay. See Appendix 3 for assay procedure.

thereafter (Fig. 3). The reason for the abrupt decrease after 10 hr is unknown. Maximum induction occurs earlier ($O.D._{600}$ around .120), than for the 8 L batches.

Induction of ANU 845(pRt032). Apigenin was used to induce ANU 845(pRt032), following the same experimental conditions applied on ANU 845(pRt032)121. The bacteria were grown until $O.D._{600}$ was 0.35-0.45, and then harvested. Centrifugation at 8,000-16,000 x g pelleted the induced bacteria tightly. On the other hand, the pellets of the control or noninduced bacteria have a tight center surrounded by a loose mass of cellular materials.

Membrane isolation. Sucrose gradient centrifugation of the cell envelope results into two bands (Fig. 4). Equilibrium was attained in 30-42 hrs. The top band is located at the boundary of the 40 and 50% sucrose layers and the lower band at the boundary of the 50 and 55% sucrose layers. Each band was collected separately and then assayed for KDO. The results (Table 3) show a higher KDO content

Table 3. Percentage KDO in the outer and inner membranes of ANU 843 and its mutants, ANU 851 and ANU 845(pRt150)

Strain	Inner membrane	Outer membrane
843	0.09	0.16
851	0.10	0.26
845(pRt150)	1.3	3.4

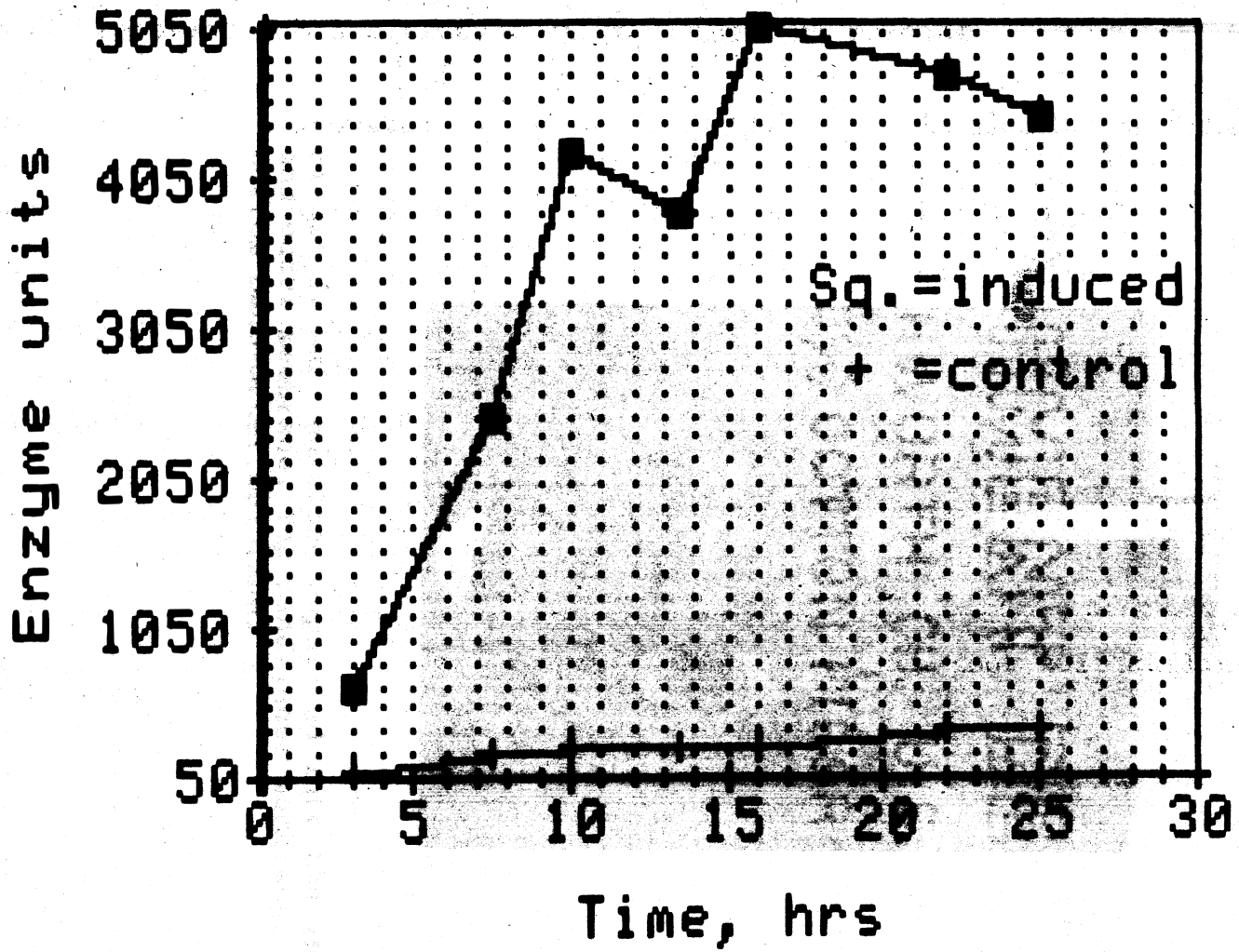

Figure 3. B-galactosidase activity in ANU 845(pRt032)121 (0.5 L batch) exposed to apigenin. Control are bacteria not exposed to apigenin.

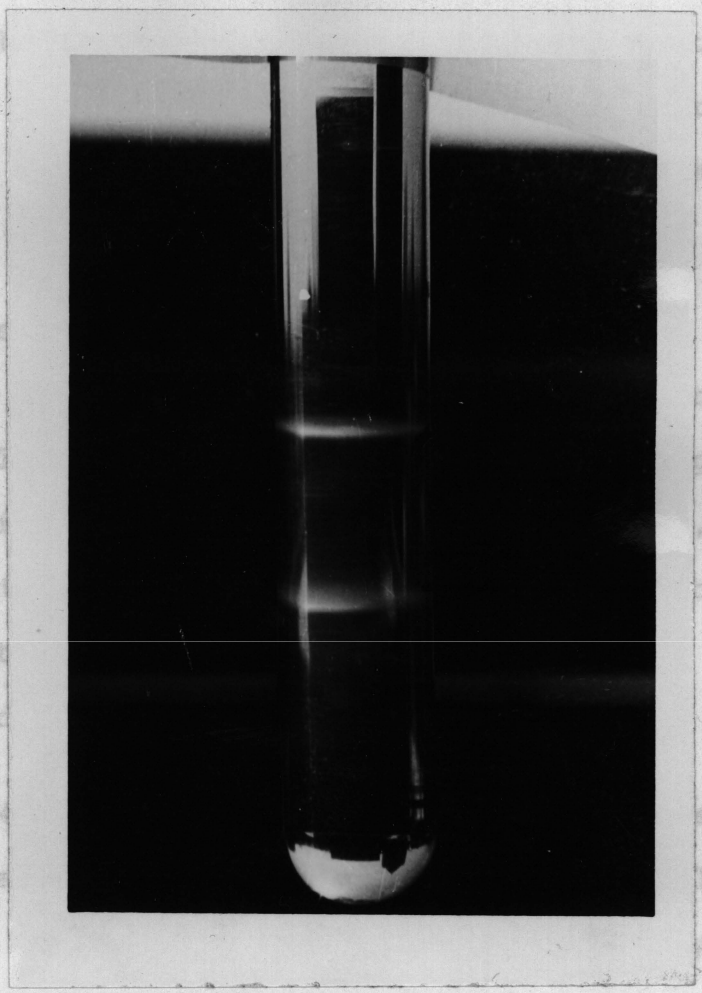

Figure 4. Cell envelope separated into the inner and outer membrane. Top band=inner membrane. Bottom band=outer membrane.

for the top band, suggesting that this is the outer membrane. Experiments involving membrane isolation of other Gram-negative bacteria using sucrose gradient centrifugation have indicated that the top band is the outer membrane and that the lower band is the inner or cytoplasmic membrane (27,63).

Electrophoresis of the outer membranes. The outer membranes were assayed for proteins by the method of Lowry and subsequently run through a 10% polyacrylamide gel. The proteins of the parent ANU843, and the mutants, ANU851 and ANU 845(pRt150) exhibited similar banding patterns (Fig. 5). These proteins have molecular weights of 129, 48, 42, 38, 34, 21, and 20 kDa.

For ANU 845(pRt032) treated with apigenin, there appears to be an enhancement of five bands with molecular weights of 30, 29, 19, 17, and 16 kDa respectively (Fig. 6). The electrophoretic mobilities of these proteins are different than those from the parent and the two mutant strains mentioned above.

Electrophoresis of Periplasmic proteins. Treatment of cells with chloroform or solution with 0.58 M sucrose and lysozyme released the materials in the periplasmic space. Samples containing equivalent amounts of protein were introduced into either a 10 or 12%, or a gradient gel (10-17% polyacrylamide). The band profile (Fig. 7) of ANU

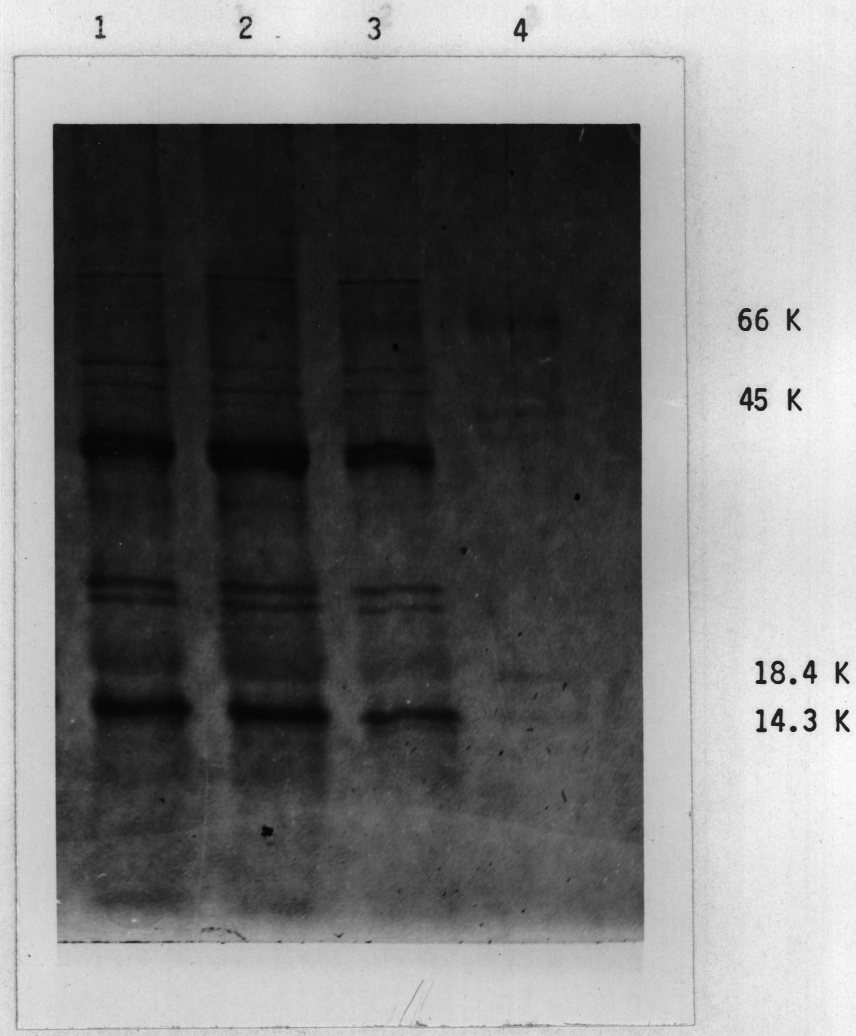

Figure 5. Gel profile of the outer membrane proteins in 10% polyacrylamide. Lane 1=ANU 845 (pRt150); Lane 2=ANU 851; Lane 3= ANU843; Lane4= Lane 4= Standards: BSA (66K), ovalbumin (45K), B-lactoglobulin (18.4K), lysozyme (14.3K).

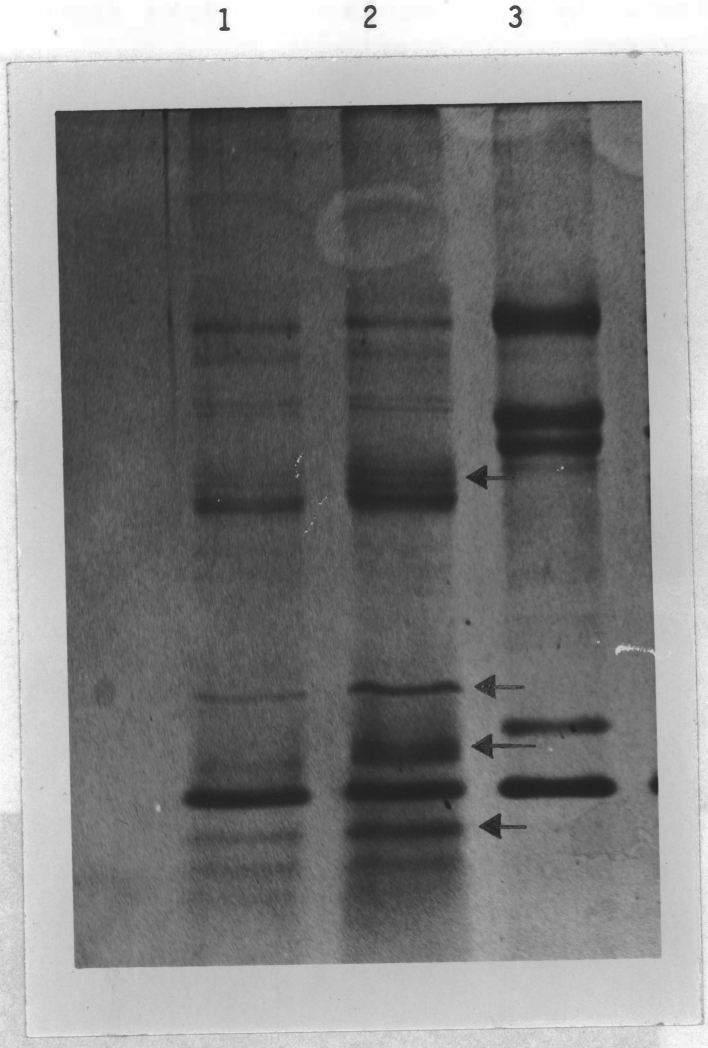

Figure 6. The outer membrane proteins of ANU 845(pRt032) control (lanel) and induced (lane 2). Lane 3=standards, as in Fig. 5.

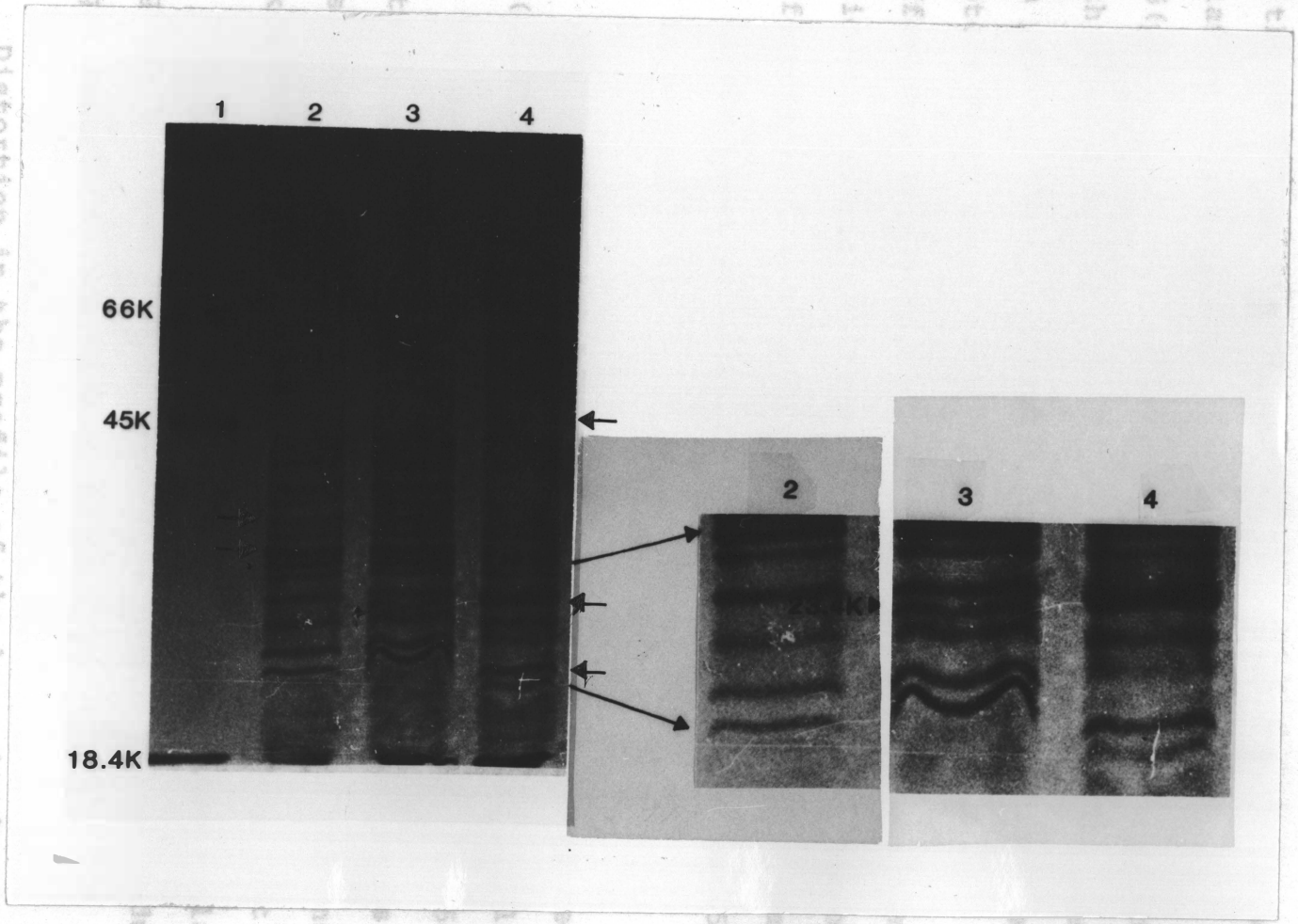

Figure 7. Periplasmic proteins of ANU843(lane 2), ANU 851(lane 3), ANU 845 (pRt150)[lane 4]. The standards are in lane 1. Gel is 12% polyacrylamide.

851 is similar to ANU 843 except for a low molecular weight protein (23.4 kDa) that is present in the mutant and missing in the parent. This is located between two proteins with relative molecular weights of 24 and 22.9 kDa. ANU 845(pRt150) is missing two bands of 26 and 28 kDa, which are both present in 843 and 851. On the other hand, it has a 38 kDa protein that is absent in both 843 and 851, and a 24 kDa protein which seems to be produced in larger amount. These differences in the 26, 28, and 38 kDa proteins are also noticeable in the 10-17% gradient gel (Fig. 8). Another difference that is evident in both the 10% and the gradient gel is the presence of 21 and 22 kDa proteins in pRt150 but not in 843 and 851.

The periplasmic proteins of induced and noninduced ANU 845(pRt032) also exhibit some differences (Figs. 9 & 10). For the proteins obtained by chloroform "shock", a 55 kDa protein, present in minute amount in the induced cells is missing in the noninduced. On other hand, a 74 kDa and a 17 kDa protein, are produced by the noninduced but not by the induced pRt032. Also, two proteins having molecular weights of 43 and 20 kDa respectively seem to be produced in larger amounts in the induced bacteria.

Distortion in the profile of the lower bands was thought to be due to the presence of contaminating polysaccharides (52) interfering with the mobility of the proteins. To eliminate this problem, the periplasmic

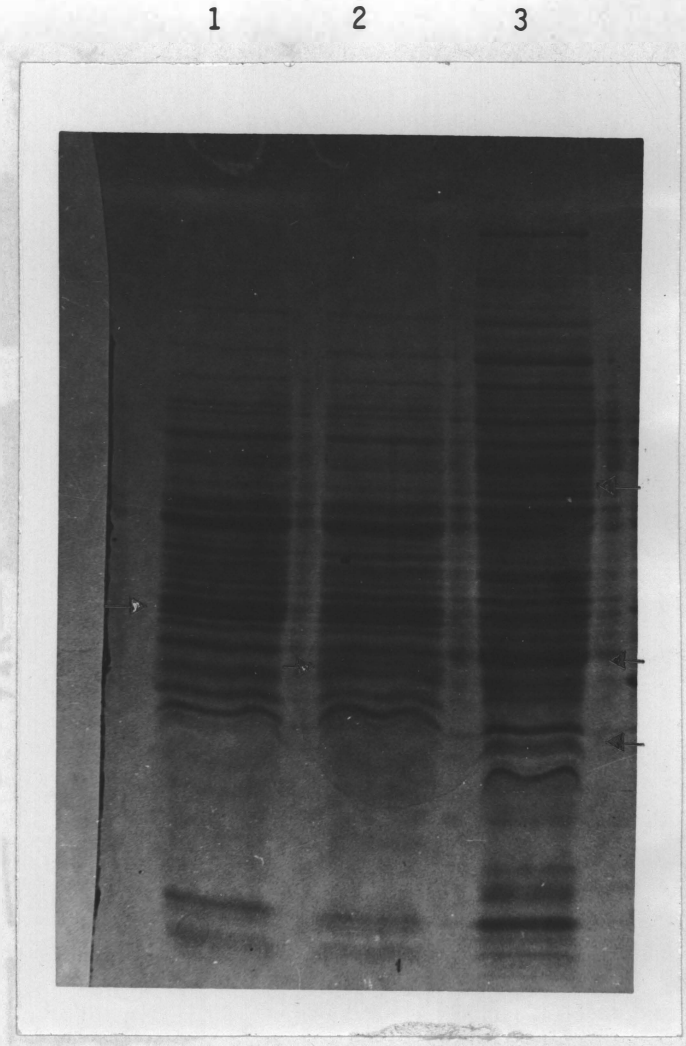

Figure 8. Gradient gel (10-17% polyacrylamide) of the periplasmic proteins of ANU 843 (lane 1), 851 (lane 2), 845(pRt150) (lane3).

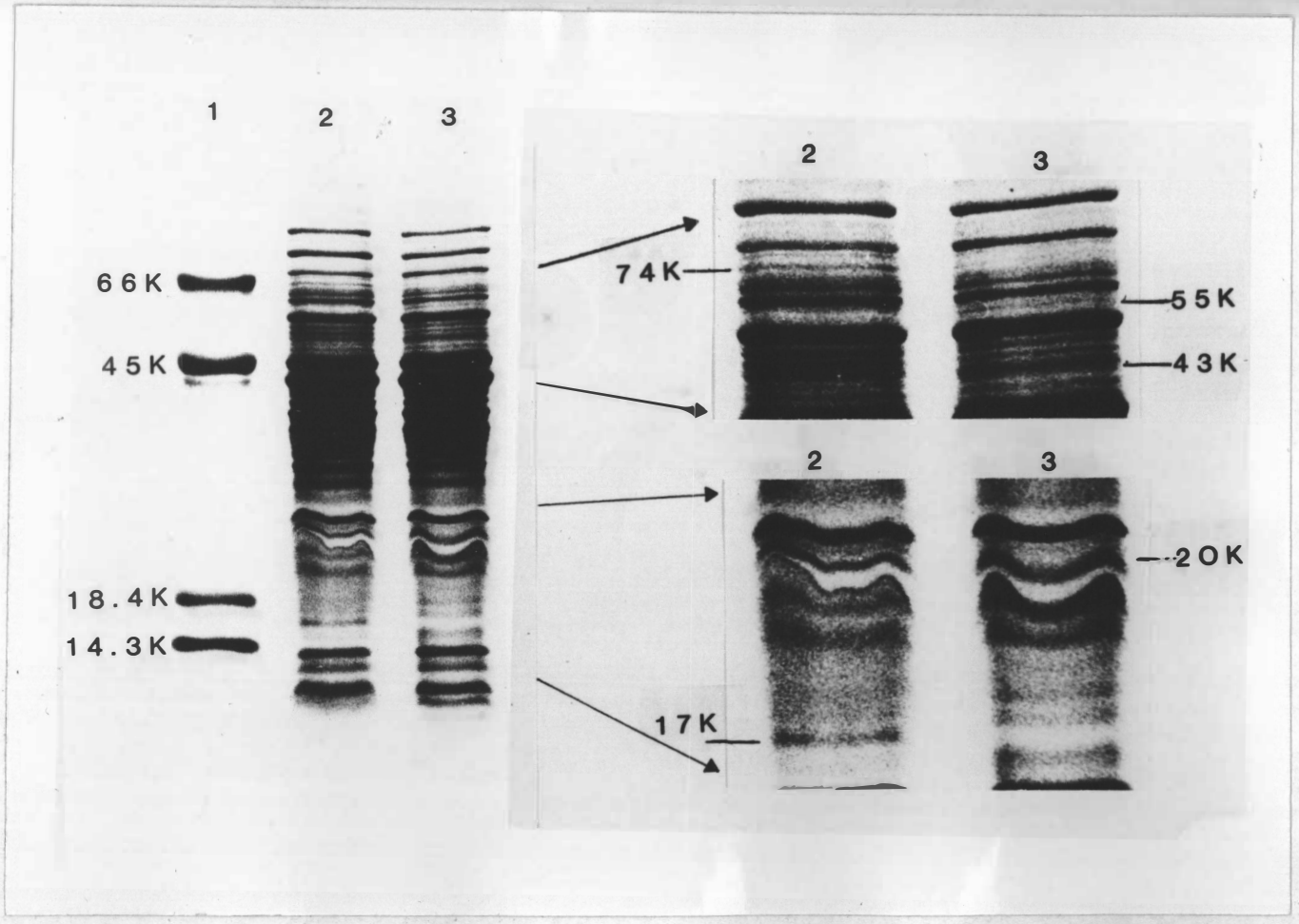

Figure 9. Periplasmic proteins of ANU 845(pRt032) released by chloroform shock. Gel is 10-17% in polyacrylamide. Lane 1=Standards. Lane 2= noninduced pRt032. Lane 3=induced pRt032.

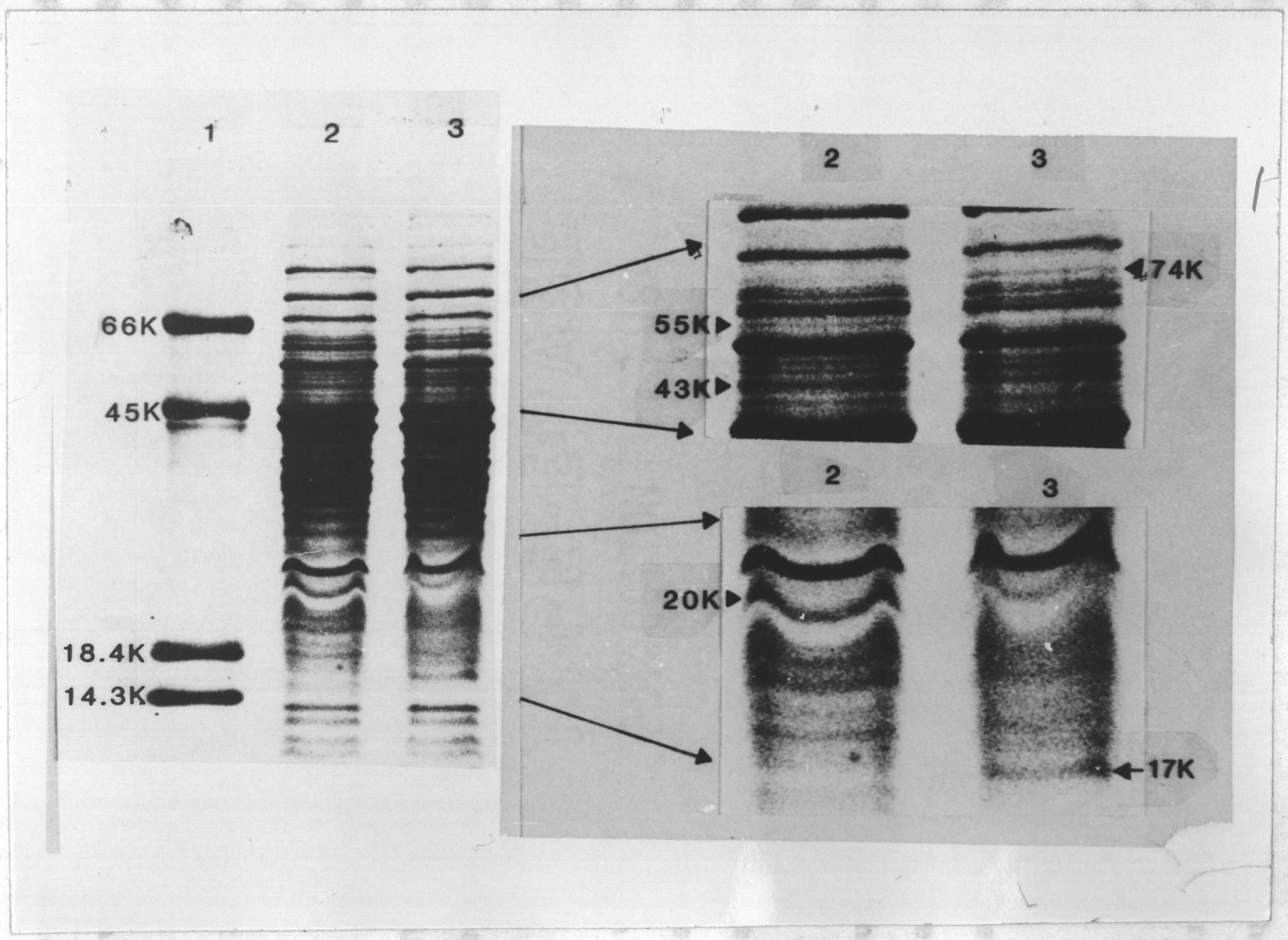

Figure 10. Gradient gel (10-17%) of the TCA precipitated periplasmic proteins of ANU 845(pRt032) obtained by chloroform shock. Lane 1= standards lane 2= induced pRt032, lane 3= noninduced pRt032.

materials were treated with trichloracetic acid to precipitate the proteins. The treatment does not alter the band profile: the periplasmic proteins migrate in the same manner and the differences are the same as before treatment with TCA (Fig. 10).

When periplasmic proteins are isolated by the Lugtenberg shock method, a number of differences are noticeable (Fig. 11). The major differences are the considerable increases in the band intensities of the proteins with molecular weights of 87, 65, 56, 48, 32 and 20 kDa for the bacteria exposed to apigenin. To a lesser extent, the 141, 44, 35, and 23 kDa, proteins also show an increase in intensity. In contrast, one protein (22 kDa) in the control seems to be repressed by apigenin.

Cytoplasmic proteins of ANU 845 (pRt032). A comparison of the profiles of the cytoplasmic proteins of the noninduced and induced bacteria reveals a striking similarity (Fig.12), except that in the induced bacteria there is an additional 19 kDa and the 18.6 kDa protein looks more prominent. The presence of the 19 kDa protein is questionable. After the cells were treated to release the periplasmic proteins, they were then broken by sonication to release the cytoplasmic proteins. It is possible that this 19 kDa protein is due to contamination by the 20 kDa, protein identified in the periplasm. But it is also likely that the protein is synthesized in the cytoplasm and then

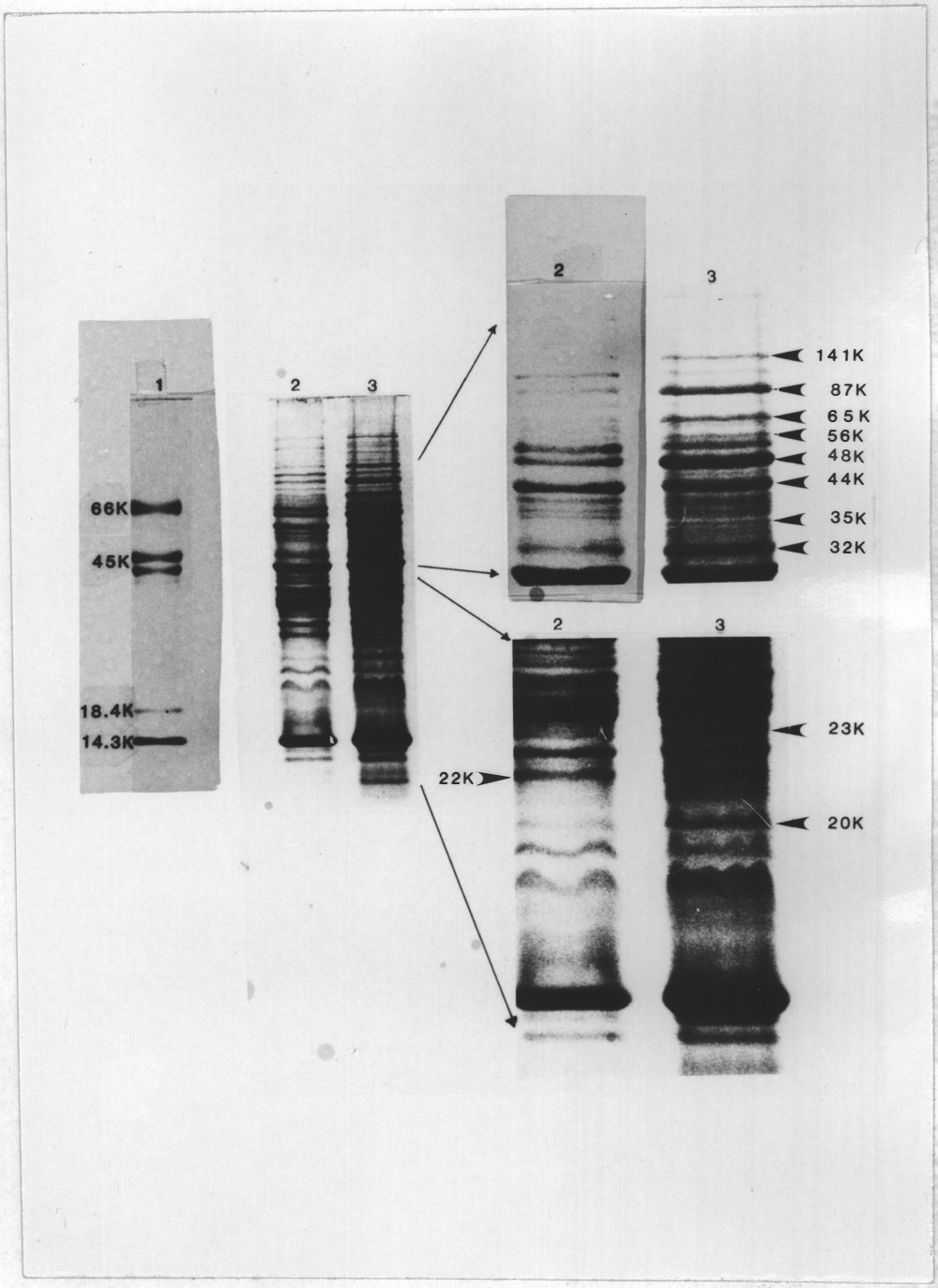

Figure 11. Periplasmic proteins of ANU 845(pRt032) released by Lugtenberg's method. Lane 1=standard. lane2=noninduced lane3=induced.

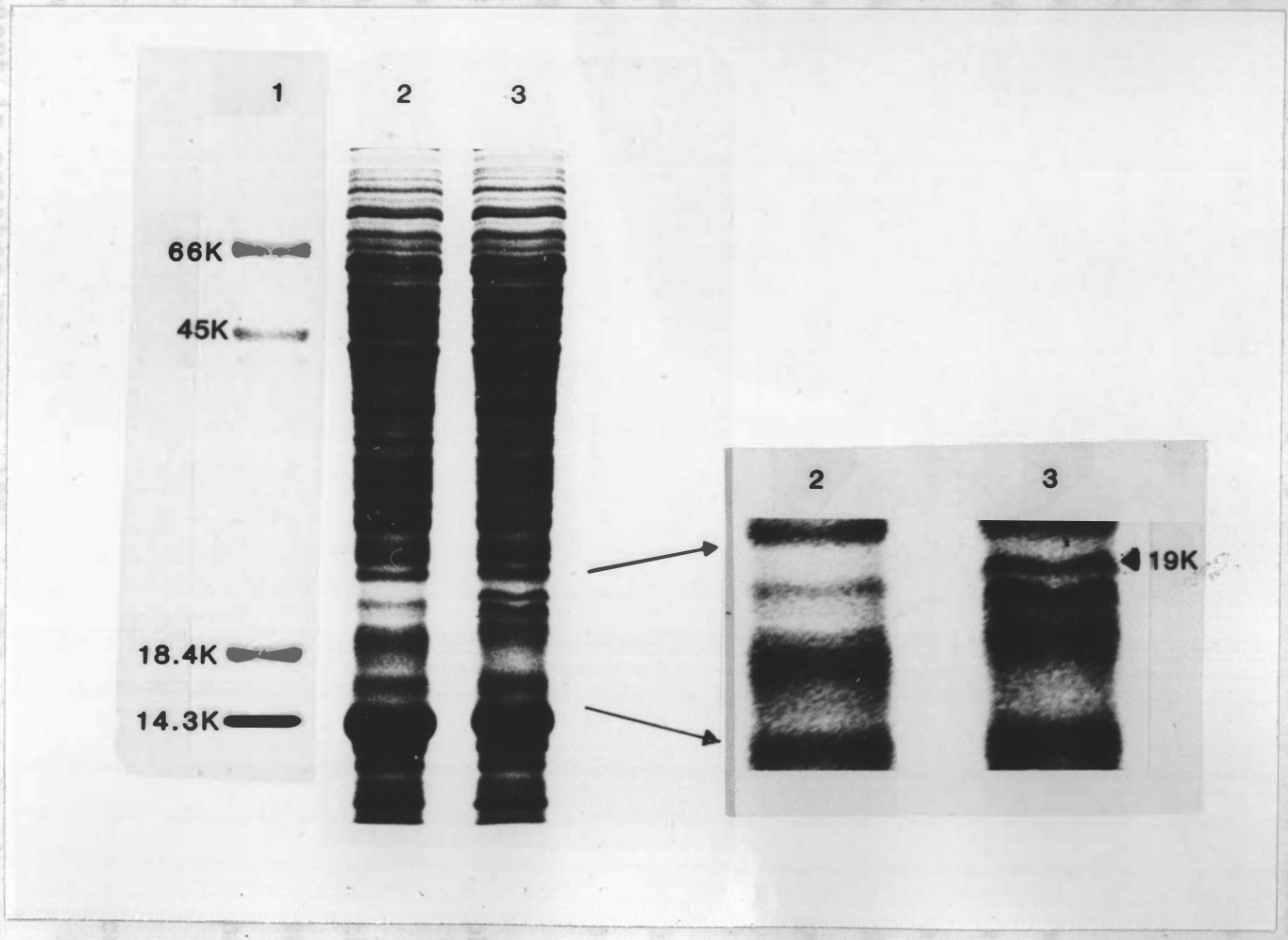

Figure 12. Gradient gel (10-17%) of the cytoplasmic proteins of ANU 845(pRt032). Lane 1=standards, lane 2=noninduced, lane 3=induced.

ransported to the periplasmic space.

Purification of LPS. The polysaccharides in the water layer from the hot phenol-water extraction were separated into component polysaccharides by the Sepharose 4B gel-filtration columnn. Three peaks were revealed by the hexose assay (Figs. 13 & 14). The middle peak contains the KDO, and is therefore the LPS. Since there was an overlap in the KDO peak and the third peak (small molecular weight polysaccharides) of the noninduced water layer, the KDO peak was combined and reloaded onto the column. A single peak was obtained after rechromatography (Fig. 15).

Chemical compositions. Colorimetric assays reveal similar amounts of KDO, acetate, pyruvate, and uronic acids for the induced and noninduced ANU 845(pRt032) LPSs and EPSs (Tables 4 & 5). The small amounts of KDO in the EPS's are probably due to contamination from the LPS that is released inadvertently into the medium, or to cellular debris that was not removed during centrifugation and precipitated along with the EPS when the supernatant was treated with ethanol. Both the LPS and the EPS contain large quantities of carboxyl groups reported as uronic acids, and their acetate compositions are very close (1.1-1.8 %). Pyruvate is present in greater amounts (10%) in EPS than in the LPS (1.0%).

The EPSs and the LPSs were hydrolyzed into component sugars, acetylated, and the compositions determined by gas

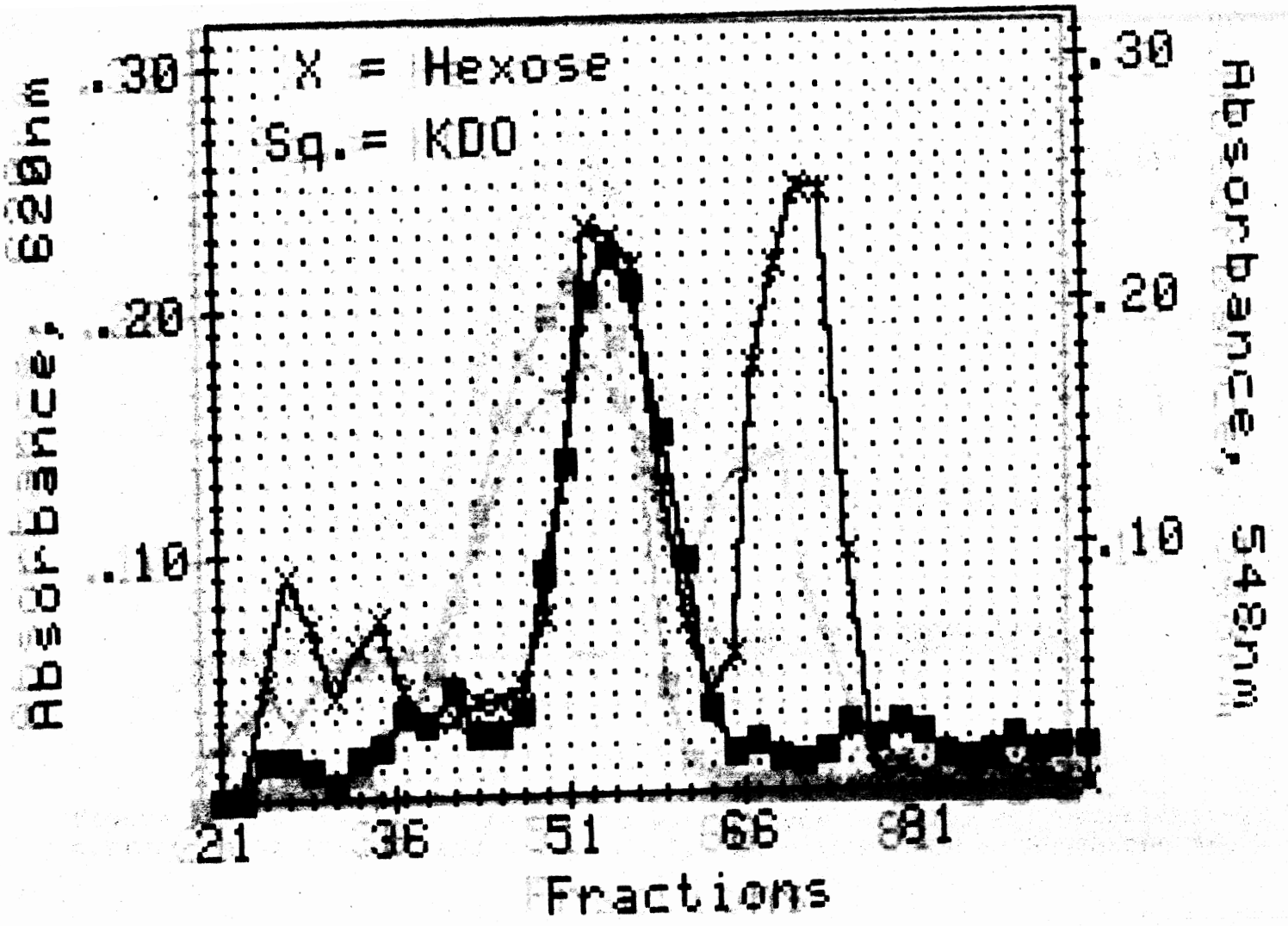

Figure 13. Elution profile of the water layer from the hot phenol/water extraction of noninduced ANU 845(pRt032). The column used is Sepharose 4B.

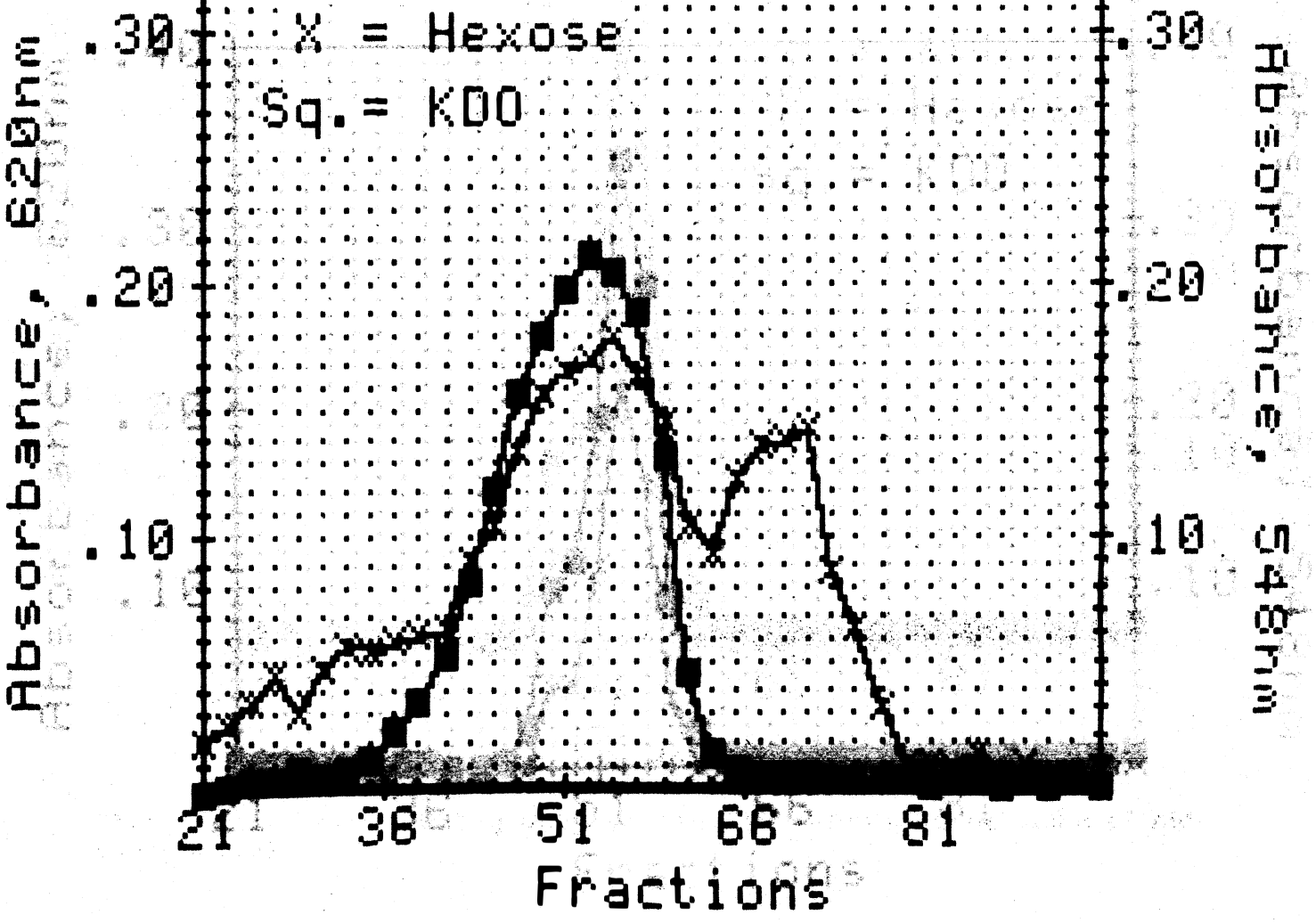

Figure 14. Elution profile of the water layer from the hot phenol/water extraction of induced ANU 845(pRt032). The column used is Sepharose 4B.

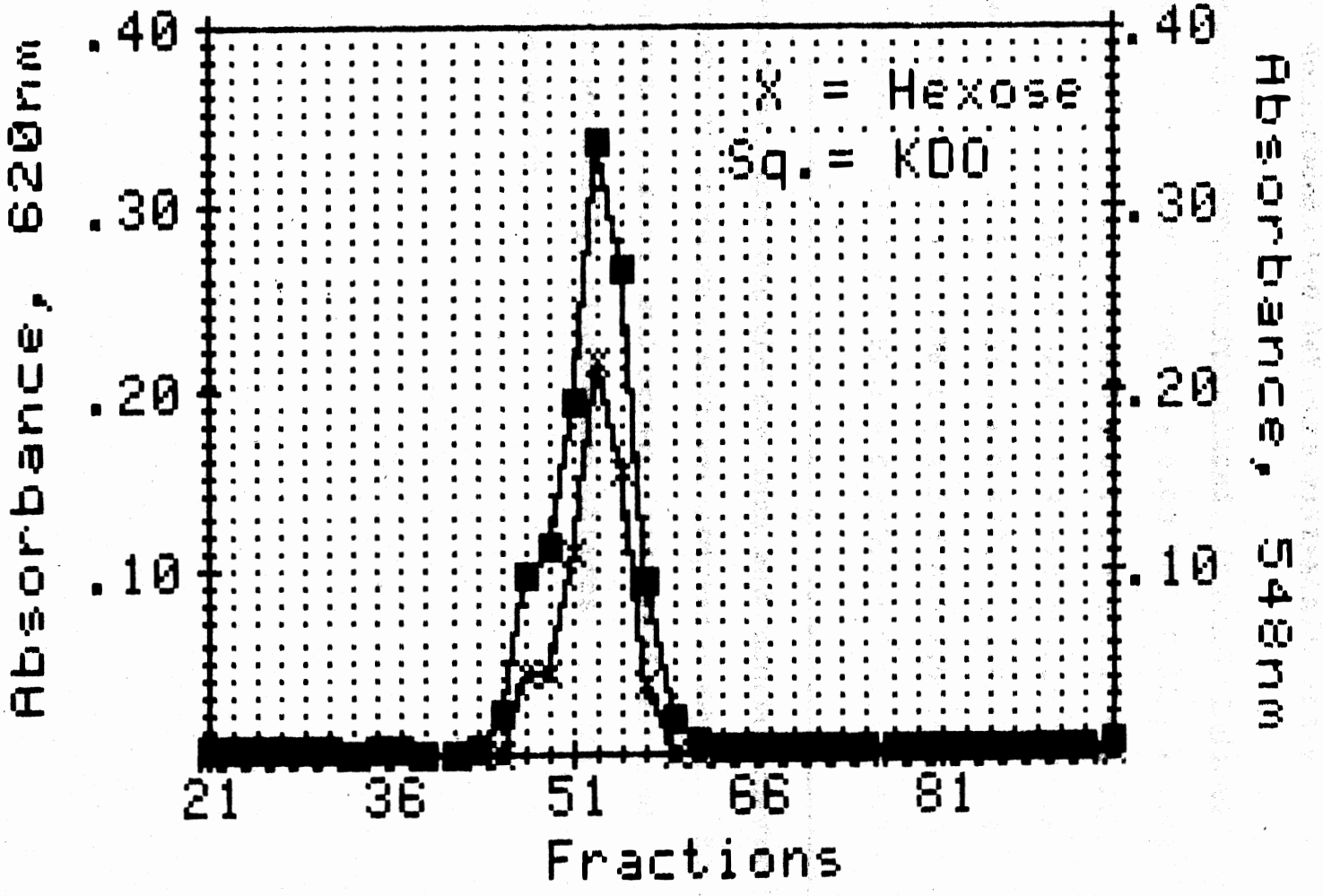

Figure 15. Rechromatography of the LPS fractions of induced ANU 845(pRt032).

Table 4. Percentage compositions of induced and noninduced ANU 845(pRt032) EPS's

Component	Noninduced	Induced
KDO	0.20 ± 0.02	0.18 ± 0.02
Acetate	1.5 ± 0.1	1.8 ± 0.1
Pyruvate	9.8 ± 0.9	9.8 ± 0.2
Uronic acid	23.3 ± 0.4	22.2 ± 1.4
Glucose	58.1 ± 4.5	56.8 ± 2.0
Galactose	11.3 ± 0.9	13.3 ± 0.5

The percentages are the averages and standard deviations for either duplicate or triplicate samples analyzed. KDO, acetate, pyruvate, and uronic acid are determined colorimetrically; glucose and galactose by gas cromatography.

Table 5. Percentage compositions of induced and noninduced ANU 845(pRt032) LPS's

Component	Noninduced	Induced
KDO	5.6 ± 0.4	7.0 ± 0.6
Acetate	1.1 ± 0.2	1.3 ± 0.2
Pyruvate	0.8 ± 0.2	1.0 ± 0.1
Uronic acid	13.2 ± 1.3	13.5 ± 1.3
2-O-methyl-dideoxyhexose	3.0 ± 0.1	5.0 ± 0.2
Fucose	1.3 ± 0.0	2.0 ± 0.1
Mannose	1.7 ± 0.0	2.6 ± 0.2
Galactose	2.0 ± 0.0	2.4 ± 0.2
3-N-methyl-3-amino-3,6-dideoxyhexose	2.9 ± 0.1	1.2 ± 0.0
Glucose	3.0 ± 0.0	4.4 ± 0.3
2-amino-2,6-dideoxyhexose	1.4 ± 0.0	2.3 ± 0.5
Heptose	4.9 ± 0.2	6.5 ± 0.8

The percentages are the averages and standard deviations for either duplicate or triplicate samples analyzed. KDO, acetate, pyruvate, and uronic acid are determined colorimetrically; the rest by gas chromatography.

chromatography. The EPSs are comprised of galactose and glucose, which are present in similar amounts in the induced and noninduced bacteria (Table 4). Either or both of these sugars may contain the acetyl or pyruvyl subtituents. There seem to be some differences among the neutral components of the LPSs from the noninduced and induced bacteria (Table 5). For example, there are larger amounts of 2-O-methyl-dideoxyhexose, glucose, and heptose in the LPS of the induced than that of the noninduced bacteria. Whether this differences are significant may be determined by analyzing the structure of the LPSs. The following neutral components are also present: galactose, fucose, mannose, and the methyl or amino substituted dideoxy sugars 3-N-methyl-3-amino-3,6-dideoxyhexose, and 2-amino-2,6-dideoxyhexose.

DISCUSSION

Introduction. It was shown that white clover root extracts induce the expression of the root hair curling genes (*nodA, B, C*) (29). Apigenin, an analog of the main inducing compound in the white clover extract (7,4'-dihydroxyflavone) causes a large induction of *nod* genes as measured by the B-galactosidase activity. It was observed that the growth of the bacteria was retarded to a small extent by apigenin. The retardation of growth was confirmed by an experiment involving the application of the compound at a concentration higher than 3 ug/mL, the concentration we used for induction. Apigenin was also found to induce fast-growing strains of *R. japonicum*, the symbiont of soybean, although at very low levels (57). Another flavone, luteolin (3',4',5,7-tetrahydroxy flavone) has been isolated and purified from alfalfa extract, and was shown to have the ability to induce the *nodA*, B, and C genes of *R. meliloti* (41). Since the root hair curling genes are common to *Rhizobium* species, the flavones present in the legumes can be assumed to act interchangeably as inducers, though not as effective as the normal interaction (41) (i.e. 7,4'-dihydroxyflavone::*R. trifolii*, luteolin::*R. meliloti*).

Protein Analysis. The outer membrane proteins of the parent strain ANU 843 are similar to those from the mutants ANU 851 and ANU 845(pRt150). These three strains were not

exposed to plant material. It may be interesting to see in the future the effect of apigenin on pRt150, since this strain harbors only the root hair curling genes, nod D, A, B, C. In the case of ANU 845(pRt032), which has the 14 kb HindIII fragment of the pSym, containing the genes for nod A-D as well as other symbiotic genes, enhancement of some bands may suggest induced expression of the nodA, B, and C, leading to increased production of outer membrane proteins.

Treatment of the bacterial cells with $CHCl_3$ releases the periplasmic proteins by an unkown mechanism (2). ANU 843 and 851 periplasmic proteins display similar band patterns except for an additional 23.4 kDa protein present in the latter. It is possible that this is the product of Tn5 inserted in the nodD region. The nodD gene product is constitutive and is required in addition to the flavones to induce the nod genes (40).

The chloroform shock and the Lugtenberg method applied to pRt032 give some differences in the electrophoretic profile of the periplasmic proteins. Both methods show a fairly heavy enhancement (induction) of the 20 kDa protein. The 55 kDa protein released in minute amounts from the apigenin-exposed pRt032 using the chloroform shock method is possibly similar to the 56 kDa protein identified in the Lugtenberg method. The same may be true for the 43 kDa and the 44 kDa respectively. An explanation for the release of

the 141, 87, 65, 35, 32, and 23 kDa protein by Lugtenberg's method but not during chloroform treatment can not be offered at this time. Also, the reasons for the presence of the 74 kDa and 17 kDa proteins from noninduced bacteria obtained by chloroform shock but not by Lugtenberg's method are not known. Perhaps the action of lysozyme (used in Lugtenberg's method) on the peptidoglycan causes the release of these additional proteins. At this moment, we can say that apigenin induces periplasmic proteins with molecular weights of 141, 87, 65, 56 (or 55), 48, 44 (or 43), 35, 32, 23, and 20 kDa, and represses the 74, 22, and 17 K protein. At the present time we do not know the function of these induced proteins.

The protein products of nodD, A, B, and C, have been synthesized in vitro and in E. coli maxi-cells (38). The molecular weights reported are: nodA, 21 kDa; nodB, 28 kDa; nodC, two polypeptides, 44 and 45 kDa; and nodD, 33 kDa. Some of the periplasmic proteins and the cytoplasmic protein we have identified by gel electrophoresis have molecular weights similar to these. It is likely that some of our proteins are the same as those synthesized in vitro and in E. coli-maxicells.

Polysaccharide analysis. No significant differences were found in the EPS compositions of pRt032 exposed to apigenin and the control. Both EPSs contain the same sugars in similar quantities. Thus synthesis of this macromolecules

does not seem to be controlled in the nodulation region and therefore they may not play a role in the root hair curling process. A study of the EPSs of ANU 843, 851, pRt150, and several other mutants including one cured of its pSym revealed similarities in compositions (15). These strains grown to early stationary phase in a laboratory media, were also found to have the same sugar linkages (15). This further suggests that the EPS is not a determinant of root hair curling. It is highly likely that when ANU 845(pRt032) is grown to early stationary phase, the structure of its EPS will be similar to that of the strains just mentioned. At the early logarithmic phase ($O.D._{600}$ 0.33-0.45, the stage we harvested our bacteria), the acetyl composition is about three times lower than it is for the bacteria grown to early stationary phase. This data agrees with the findings that changes in EPS composition occurs as a function of growth phase (3).

The differences noted between the LPSs of the control and induced bacteria is at the present time inconclusive. Additional work on the structure of the LPSs has to be made in order to determine whether the differences are significant.

It is interesting to note that both the EPSs and LPSs of pRt032 at early logarithmic phase contain high amounts of uronic acids. One of the uronic acids in the LPS of the parent and those of the three of its mutants (including one

with the root hair curling region deleted) was identified as galacturonic acid (16). This acidic sugar has been recently determined as one of the two main components of the core region of R. trifolii. This oligosaccharide is very distinct from that found in the core of other Gram-negative bacteria (17).

Oligosaccharides containing galacturonic acids (pectic oligomers) are reported to be released from the plant cell walls by the pathogen's endogalacturonase; the oligomers then elicit the production of phytoalexins (18). Thus, if the same biological property is possessed by the LPS, these molecules may aid in the production of phytoalexins that destroy other invading microorganisms but not the rhizobia. The effect of phytoalexins on rhizobia is not known.

ANU 845(pRt032) when grown in NMB medium gives loose bacterial pellets upon centrifugation. When the medium is supplemented with apigenin, the pellets are tight. Such change in the nature of the pellet, aside from a decrease in the production of capsular polysaccharides, may suggest a change in the composition of the outer membrane. Reports have indicated that the product of the nodA, B, and C genes are hydrophobic proteins located in the cell membranes. An experiment determining the hydrophobic nature of the outer membrane of the control and induced pRt032 has been started in our lab. Two trials have somewhat indicated that the outer membrane of the induced bacteria is more

hydrophobic. These results are currently under further investigation.

Future work. In the future, it will be necessary to perform induction experiments on ANU 845(pRt150); this will determine which proteins are truly the products of nodA, B, and C genes. An attempt will be made to isolate from among the periplasmic, cytoplasmic and outer membrane proteins the product of nodA (38). This will determine if the 20 kDa periplasmic protein identified in this work is identical to the 21 kDa synthesized *in vitro*. It may also determine if the 19 kDa cytoplasmic protein is the same as the 20 kDa protein in the periplasm. The hydrophobicity experiments will be continued in order to better understand the hydrophobic nature of the outer membrane and relate this to the root hair curling process. The *R. trifolii* will be investigated for the presence of proteinaceous pili which, in *B. japonicum*, may play a role in the attachment of the bacteria to the roots (6).

Appendix 1

New Modified Bergensen's Media

Components	Amount per liter
K_2HPO_4	0.25 g
$MgSO_4 \cdot 7H_2O$	0.10 g
Glutamic acid	1.10 g
Mannitol	10.00 g
Calcium panthothenate	0.004 g
$CaCl_2 \cdot 2H_2O$	6.62 mg
H_3BO_3	0.145 mg
$FeSO_4 \cdot 7H_2O$	0.125 mg
$CoCl_2 \cdot 6H_2O$	0.059 mg
$CuSO_4 \cdot 5H_2O$	0.005 mg
$MnCl_2 \cdot 4H_2O$	0.0043 mg
$ZnSO_4 \cdot 7H_2O$	0.108 mg
$Na_2MoO_4 \cdot 2H_2O$	0.125 mg
Riboflavin	0.020 mg
p-aminobenzoic acid	0.020 mg
Nicotinic acid	0.020 mg
Biotin	0.020 mg
Thiamine-HCl	0.020 mg
Pyridoxin-HCl	0.020 mg

Notes:

1. The pH is adjusted to 6.9 using 6N NaOH.
2. Stock solution of vitamins are made in 0.05M Na_2HPO_4 buffer pH 7.0

3. When making stock solutions of the trace elements, adjust pH of water to 5.0 before adding 7.0 g of Nitriolo triacetate (NTA), in order to prevent precipitation of elements added in order given above.

4. For solid media, add Bacto-agar at 15 g/L.

Appendix 2

TM Media (as modified by B. Innes)

Composition (per liter):

$MgSO_4$	0.200 g
$CaCl_2$ $2H_2O$	0.050 g
$FeCl_3$	0.03 g
Sucrose	10.0 g

TM Salts

K_2HPO_4	1.04 g
KH_2PO_4	0.44 g
NaCl	0.10 g
$(NH_4)_2SO_4$	0.50 g

Vitamins*

Thiamine HCl	1 mg
Biotin	0.5 mg
Nicotinic acid	1 mg
Pyridoxine HCl	1 mg

Trace elements

Same as in New Modified Bergensen's Media (as a substitute for Gamborg's trace elements required in the original formulation).

pH is approximately 7.0 without adjustment.

For plates, add 15 g/L Bacto-agar.

*For liquid media, add after autoclaving.

Appendix 3

B-galactosidase activity assay

Reagents

1. Sample buffer (per liter):

   16.1 g $Na_2HPO_4 \cdot 7H_2O$

   5.5 g $NaH_2PO_4 \cdot H_2O$

   0.75 g KCl

   0.246 g $MgSO_4 \cdot 7H_2O$

   2.7 ml B-mercaptoethanol

   pH 7.0

2. Chloroform
3. 0.1% Sodium Dodecyl Sulfate (SDS)
4. o-nitrophenyl-B-D-galactopyranoside (4 mg/mL in 0.1 M phosphate buffer, pH 7.0), always used fresh
5. 1 M Na_2CO_3

Procedure

1. Measure the $O.D._{600}$ at the time of sampling.
2. Add 0.5 mL of sample buffer to 0.5 mL of bacteria.
3. Add 0.1 mL of chloroform and 0.05 mL of SDS. Vortex for 10 s.
4. Add 0.2 mL of o-nitrophenyl-B-D-galactopyranoside to start the reaction. Vortex briefly to mix. Incubate preferably at 27°C.
5. When sufficient yellow color has developed, stop the reaction by adding 0.5 ml Na_2CO_3. Vortex.
6. Centrifuge the material on a table top centrifuge for 15

min. Take out the clear liquid and measure the O.D. at 420 nm.

6. Calculate the B-galactosidase activity using the formula:

$$\text{Enzyme units} = 1000 \times \frac{O.D._{420}}{O.D._{600} \times t \times v}$$

where t = reaction time

v = volume of culture used in assay (0.5 mL)

$O.D._{600}$ = cell density before assay

Appendix 4

Isolation of periplasmic proteins

Isolation of periplasmic proteins

(Lugtenberg method)

Reagents

1. Chloroform
2. 0.01 M Tris buffer, pH 8.0

Procedure

1. Collect the stationary phase bacteria (500 mL) by centrifuging at 9,000 rpm for 20 min in Sorvall GSA rotor.
2. Wash the cells with phosphate buffered saline, PBS (0.85% NaCl, 0.05 M Na_2HPO_4, pH 7.0).
3. Add 5 mL of chloroform. Vortex briefly and allow to stand at room temperature for 15 min.
4. Add 50 mL Tris buffer. Mix. Centrifuge the material for 20 min at 5,900 x g to separate the cells.
5. Carefully collect the supernanatant with a pasteur pipete. This is the "shock fluid" containing the periplasmic proteins.
6. Pour the shock fluids into Spectrapor tubing (M.W. cut off: 12,000-14,000) and dialyze through 3-4 water changes.
7. Lyophilize.

Note: For bacteria harvested before the stationary phase, adjust the volume propotionately using the O.D.'s.

Appendix 5

Isolation of periplasmic proteins

Discontinuous SDS (Laemmli method) Electrophoresis

Reagents

1. **Incubating solution: 50 mM Tris (pH 8.0), 20% (w/v) sucrose, 2 mM EDTA, 0.2 mg of lysozyme per ml**
2. **5% trichloroacetic acid (TCA)**
3. **Diethyl ether**

Procedure

1. **Suspend the cells in the incubating solution and homogenized by blending in the Warring blender for 10 s.**
2. **Allow to stay at room temperature for 30 min.**
3. **Centrifuge the suspension at 5,900 x g for 20 min to remove the cells.**
4. **Treat the supernatant with TCA (2 vol super./1 vol TCA) Add the acid slowly with stirring in the cold room, 4°C.**
5. **Centrifuge the cloudy solution at 17,000 x g for 1 hr to recover the periplasmic proteins.**
6. **Wash the precipitate three times with diethyl ether and allow to dry at room temperature or lyophilize.**

A. Stacking gel stock buffer:

Tris base: 7.69 g

Deionized water: 75 mL

Adjust pH to 6.8 with HCl

Add deionized water to a total volume of 100 mL

B. Stacking gel stock buffer:

Tris base: 7.69 g

Deionized water: 75 mL

Adjust pH to 6.8 with HCl

Add deionized water to a total volume of 100 mL

C. Stock acrylamide solution

Acrylamide: 25 g

Bis-acrylamide: 0.625 g

Add deionized water to a total volume of 50 mL

D. Sodium dodecyl sulfate (SDS) solution, 10%

SDS: 1.5 g

Deionized water: 25 mL

E. Sample buffer:

Solution B:	5 mL
Sucrose:	2 g
SDS:	0.4 g
Bromphenol blue:	5 mg
B-mercaptoethanol:	1 mL

Appendix 6

Discontinuous SDS-Polyacrylamide Gel Electrophoresis

Stock solutions

A. Running gel stock buffer

Tris base: 22.71 g

Deionized water: 75 mL

Adjust pH to 8.8 with HCl

Add deionized water to a total volume of 100 mL

B. Stacking gel stock buffer

Tris base: 7.69 g

Deionized water: 75 mL

Adjust pH to 6.8 with HCl

Add deionized water to a total volume of 100 mL

C. Stock acrylamide solution

Acrylamide: 25 g

Bis-acrylamide: 0.625 g

Add deionized water to a total volume of 50 mL

D. Sodium dodecyl sulfate (SDS) solution, 10%

SDS: 2.5 g

Deionized water: 25 mL

E. Sample buffer

Solution B: 5 mL

Sucrose: 2 g

SDS: 0.4 g

Bromphenol blue: 5 mg

B-mercaptoethanol: 1 mL

Dilute to 20 mL with deionized water.

F. Running buffer

Tris base: 3.0 g/L

Glycine: 14.4 g/L

SDS: 1.0 g/L

Preparing the running gel

1. Assemble the gel electrophoresis apparatus.
2. For a 25 mL of 12% running gel, mix the following:

Solution A:	5.0 mL
Solution C:	6.0 mL
Solution D:	0.25 mL
Deionized water:	13.75 mL

For a gradient gel, 10%-17%, prepare 12 mL each of 10% and 17% running gel by mixing the following:

	10%	17%
Solution A:	2.0 mL	2.0 mL
Solution C:	2.4 mL	4.08 mL
Solution D:	0.12 mL	0.12 mL
Deionized water:	7.5 mL	5.82 mL

Add a pinch of bromphenol blue to the 17% gel mix to see later how the gradient forms.

3. Pouring the gel

a. Just before pouring the 12% gel, add 0.05 mL of freshly prepared 10% ammonium persulfate solution (in degassed deionized water) and 0.007 mL of TEMED (both from Bio-Rad). Pour into the gel apparatus using a pasteur pipete. Avoid introducing a lot of bubbles.

(Note: the amount of persulfate and TEMED added will depend upon the source of those reagents).

b. For the gradient gel, pour the 17% gel solution into the first tube of the gradient former then add 0.020 mL of ammonium persulfate and 0.005 mL TEMED. To the 10% gel solution, add the same amounts of persulfate and TEMED. Mix, and pour the solution into the second tube. Slowly open the pinch clamp between the tubes to the fullest and then, slowly open the clamp before first tube to give a reasonable flow of liquid into the gel apparatus.

c. Using a glass syringe carefully overlay the gel with about 1.5 mL of deionized water.

d. Allow the gel to polymerize, preferably overnight.

Preparing the stacking gel

1. For 10 mL of 5% stacking gel, mix the following solutions:

Solution B:	2.0 mL
Solution C:	1.0 mL
Solution D:	0.1 mL
Deionized water:	6.9 mL

2. Remove the water overlaying the running gel and blot dry with a paper towel.
3. Just before pouring the stacking gel, add 0.05 mL of a freshly prepared 10% ammmonium persulfate solution and 0.007 mL TEMED and mix.
4. Pour the stacking gel using a pasteur pipete and be

be careful to avoid bubbles.

5. Insert the well former slowly and at a slight angle to avoid trapping air bubbles beneath the wells. Allow 1 cm between well former and running gel.
6. Allow to polymerize at least an hour.

Preparing the samples

1. Place samples in a small tapered tube which can be easily made from a pasteur pipete.
2. Add 20 uL of sample buffer, seal the tubes and place in a boiling water bath for 5 min. (For periplasmic proteins boil for 2 min only)
3. Fill the upper gel reservoir with running buffer (solution F) and carefully remove the well former. Alternative: Prepare a stacking gel solution without acrylamide, with water adjusted to make 10 mL. Introduc this solution to back of well former using a pasteur pipete, and then pull out the well former. Finally, pour solution F.
4. Open the tubes and place one sample in each of the wells.
5. Once the samples are all loaded, place the running buffer in the lower reservoir and assemble all electrical connections.
6. Run at constant current of 20 ma. Electrophoresis is complete when the bromphenol blue reaches the bottom of the gel (3-4 hrs).
7. Turn off the power, empty the reservoirs, carefully

disassemble the apparatus, and remove the gel.

8. Stain the gel for appropriate macromolecules.

Assay for Acetyl groups

Reagents

1. 2% Hydroxylamine hydrochloride. Stored at 4°C.
2. 3.5 N NaOH
3. 0.37 M $FeCl_3 \cdot 6H_2O$ in 0.1 N HCl
4. 4CO. 1 part concentrated HCl + 2 parts H_2O
5. Standard: 0.3 mg/ml D-Dimethylglucose pentaacetate in CH_3OH. 10 microliters = 2.73 microgram acetate. Stored at 4°C.

Procedure

1. Prepare dry 400-600 microliters of a known solution of samples.
2. Prepare different volumes of standard (recommended: 0, 10, 20, 40, 80, 200 microliters), add 30 microliters of H_2O to each and dilute to a final volume of 400 microliters with CH_3OH.
3. To 200 microliters of sample, add 100 microliters of CH_3OH and 100 microliters of H_2O. To the treated samples add 100 microliters of CH_3OH and 300 microliters of H_2O.
4. Mix equal parts of reagents 1 and 2 just before using. This is stable for 1 hour at room temperature.
5. Add 800 microliters of the mixture. Vortex and allow to stand for 1 min.
6. Add 400 microliters of reagent 4. Vortex.

Appendix 7

Assay for Acetyl groups

Reagents

1. 2 M Hydroxylaminehydrochloride. Stored at $4^{o}C$.
2. 3.5 N NaOH
3. 0.37 M $FeCl_3 \cdot 6H_2O$ in 0.1 N HCl
4. HCl, 1 part concentrated HCl + 2 parts H_2O
5. Standard: 0.5 mg/ml B-D(+)-glucose pentacetate in CH_3OH. 10 microliters = 2.75 microgram acetate. Stored at $4^{o}C$.

Procedure

1. Freeze dry 400-600 microliters of a 1mg/mL solution of samples.
2. Pipete different volumes of standard (recommended: 0, 10, 20, 40, 80, 100 microliters), add 300 microliters of H_2O to each and dilute to a final volume of 400 microliters with CH_3OH.
3. To 200 microliters of sample, add 100 microliters of CH_3OH and 100 microliters of H_2O. To the freeze-dried samples add 100 microliters of CH_3OH and 300 microliters of H_2O.
4. Mix equal parts of reagents 1 and 2 just before using. This is stable for three hours at room temperature.
5. Add 800 microliters of the mixture. Vortex and allow to stand for 1 min.
6. Add 400 microliters of reagent 4. Vortex.

7. Add 400 microliters of reagent 3. Vortex

8. Measure absorbance at 540 nm.

Note: If samples contain significant quantities of protein, this will precipitate out upon addition of reagent 3. Centrifuge.

Appendix 9
Appendix 8

Protein Assay
KDO(2-keto-3-deoxyoctonic acid) Assay

Reagents

1. 0.4N H_2SO_4 (Strong acid hydrolysis, use 10N)
2. 0.04N HIO_4 in 0.4N H_2SO_4
3. 2% $NaAsO_2$ (sodium arsenite) in 0.5N HCl
4. 0.3% Thiobarbituric acid

Procedure

The type of material dictates the amount of samples to be used. All samples are diluted to a final volume of 200 uL.

1. Prepare standard samples from a 0.1 mg/mL solution of KDO. Dilute to 200 uL with deionized water.
2. Add 20 uL of H_2SO_4 to the standards and samples. Vortex.
3. Place in a boiling water bath. For mild acid hydrolysis leave for 30 min; for strong acid hydrolysis, leave for 1 hr.
4. Add 250 uL of HIO_4. Vortex, and leave at room temperature for at least 40 min.
5. Add 500 uL of $NaAsO_2$. Vortex. Allow to stand 5 min.
6. Add 2 mL of thiobarbituric acid. Vortex. Place in boiling water bath for 20 min.
7. Read absorbance immediately at 548 nm. Centrifuge any cloudy samples for 1 min in a tabletop centrifuge.

Appendix 9

Protein Assay

Reagents

1. Alkaline Copper Reagent (always used fresh)

1% $CuSO_4 \cdot 5H_2O$		1 mL
2% Sodium Tartrate		1 mL
2% Na_2CO_3 in 0.1N NaOH		98 mL

2. Phenol Reagent Solution, 2N (Fisher Scientific Co.)

3. Stnadard: Bovine Serum Albumin (BSA)

Procedure

1. Prepare six standard samples by pipeting 0, 20, 50, 100, 150, and 200 uLs of BSA solution (1mg/mL) and dilute to a final volume of 500uL using deionized water.

2. Make up triplicate samples for each unknown, usually containing 100, 200, and 500 uLs respectively. All samples should be diluted to 500uL by adding deionized water.

3. Add 3 mL of the alkaline copper reagent to all samples, mix immediately, and allow to stand for 10 min.

4. Add 0.2 mL of phenol reagent, vortex briefly, and allow to stand for 30 min.

5. Measure the absorbance at 650nm.

Appendix 10

Pyruvic Acid Assay

Reagents

1. 2,4-dinitrophenylhydrazine (DNP). Five hundred (500) micromoles in 100 mL 2.0 N HCl. Dissolve at 40°C.
2. 2.2 N NaOH
3. 2.0 N HCl
4. Standard (0.1 mg/mL)
5. Toluene
6. 10% Na_2CO_3.
7. H_2O

Pyruvic acid standard and DNP must be made fresh the day they are used.

Procedure

1. Prepare a set of standards 0-200 microliters with a total volume of 200 microliters. Use small screw cap test tubes.
2. Prepare samples from 200 to 400 micrograms. Use small screw cap test tubes. Add enough H_2O to a total volume of 200 microliters. If samples contain protein, treat with perchloric acid.
3. Add 300 microliters of HCl, vortex, cap the tubes, and heat at 100°C in the heating block for 3 hours.
4. Add 100 microliters of DNP, vortex, and allow to stand at room temperature for 30 min.
5. Add 600 microliters of toluene. Vortex. Remove the

aqueous phase using a Pasteur pipete. To the toluene layer, add 600 microliters of Na_2CO_3 solution. Vortex.

6. Discard the top toluene layer. Add 400 microliters of H_2O and 1.0 mL of NaOH to the bottom layer. Vortex.
7. Centrifuge for at least 1 min in tabletop centrifuge and read absorbance at 416 nm.

Appendix II

Uronic Acid Assay

Reagents

1. 0.0125 M sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) in conc. H_2SO_4. Stored at 4°C.
2. 0.15% m-hydroxybiphenyl in 0.5% NaOH. Stored at 4°C.
3. 0.5% NaOH
4. Standard glucuronic or galacturonic acid (0.1 mg/mL) solution in 1 M ammonium hydroxide.

Procedure

1. Prepare a set of standards having volumes of 0-200 microliters. Dilute to final volume of 200 microliters.
2. Prepare two sets of samples with a total volume of 200 microliters.
3. Add 1.2 mL of tetraborate. Vortex.
4. Heat for 5 min in a boiling water bath.
5. Cool 1-2 min in cold water.
6. To the standards and one set of samples add 20 microliters of m-hydroxybiphenyl. To the other set of samples add 0.5% NaOH. Vortex.
7. Leave at room temperature for 5 min.
8. Read absorbance at 520 nm. Subtract any reading of the samples treated with NaOH from those treated with m-hydroxybiphenyl for the true reading.

Appendix 12

Acetylation

Reagents

1. Inositol (1 mg/mL solution), as internal standard.
2. Standard sugars (1 mg/mL solution): Rhamnose, fucose, ribose, xylose, mannose, galactose, glucose, and heptose
3. 2.0 M trifluoroacetic acid (TFA)
4. Sodium borohydride (or sodium borodeuteride): 10 mg/mL solution in 1 M ammonium hydroxide.
5. Glacial acetic acid
6. 10% glacial acetic acid in methanol
7. Methanol
8. Pyridine
9. Acetic anhydride
10. Chloroform

Procedure

1. Determine the percent hexose in the sample using the anthrone assay.
2. Place sample equivalent to not more than 250 micrograms hexose in screw-cap test tube. In another tube, place 100 microliters of the standard sugars. Add 20 microliters of inositol to the samples and standard. Dry the materials by blowing filtered air or by freeze-drying.
3. Add 500 microliters of TFA to each tube, seal with teflon-lined srew cap, and heat at 121°C for 2 hr.

4. Remove tubes and blow dry with filter air. This may be done in a water bath at 40-50°C. For methylated polysaccharide, keep the temperature at no more than 35°C.

5. Add 250 microliters of sodium borohydride solution, mix and allow to stand at room temperature for 1 hr (can be left overnight with loose caps on). For methylated samples always use sodium borodeuteride, and leave at room temperature for 2 hr.

6. Add 50 microliters of glacial acetic acid. Vigorous bubbling will take place. Repeat two more times.

7. Add 500 microliters of the 10% glacial acetic acid in methanol and blow dry with filtered air. Keep to drying temperatures mentioned previously. Do this four times.

8. Add 500 microliters of methanol and blow dry as in step no. 7.

9. Add 50 microliters of pyridine and 50 microliters of acetic anhydride. Mix, seal tubes with teflon caps and heat at 121°C for 30 min.

10. Cool on ice and extract by adding 500 microliters of water and 500 microliters of chloroform. Mix, centrifuge on table top centrifuge for 5 min, remove chloroform layer (bottom) with a pasteur pipete and transfer to another tube.

11. Extract water layer with another 500 microliters of chloroform and combine chloroform layers from first and second extractions. Blow dry with filtered air.

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