ISOLATION AND PARTIAL CHARACTERIZATION OF A WATER STRESS PROTEIN OF THE DESICCATION-TOLERANT CYANOBACTERIUM *NOSTOC COMMUNE* UTEX 584 EXPRESSED IN *ESCHERICHIA COLI*.

By

Brian J. Sines

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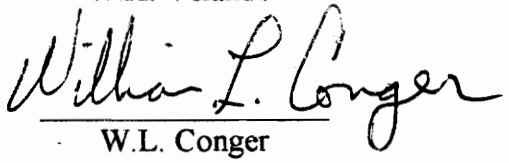
APPROVED:

D.R. Bevan, Chairman

M. Potts

W.H. Velander



W.L. Conger

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ISOLATION AND PARTIAL CHARACTERIZATION OF A WATER STRESS PROTEIN OF THE DESICCATION-TOLERANT CYANOBACTERIUM *NOSTOC COMMUNE* UTEX 584 EXPRESSED IN *ESCHERICHIA COLI.*

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ABSTRACT

A desiccation-tolerant cyanobacterium *Nostoc commune* accumulates a novel group of water stress proteins (Wsp) in response to cycles of repeated drying and rehydration. Antibodies, specific for Wsp, were used to screen a lambdafix II library of *N. commune* UTEX 584 Bam H1 DNA fragments and an 8.5-kb fragment, containing a gene cluster that synthesized a 59-kDa cross-reactive protein. The cloned fragment comprised five ORF's. The ORF's 59, 24, 22, 36, and 70, each potentially encode products of molecular weights of 59, 24, 22, 36, and 70-kDa, respectively. The 59 and 24 ORF products were found to be expressed in *E. coli.* The 59-kDa product of this fragment gives the strongest cross-reaction with the Wsp antiserum. The 59-kDa protein was partially purified. The 24-kDa product was successfully purified to homogeneity and partially characterized.

This study used *E. coli.* strain DH10B transformed with the pTrc 99A plasmid. The pTrc 99A contains the 8.5-kb gene cluster fragment of interest. The products of

ORF 24 and 59 were isolated using an initial 40-60 % ammonium sulfate precipitation of a clarified *E. coli* cell lysate. The clarified cell lysate was then subjected to streptomycin sulfate precipitation. The cell lysate was then dialyzed extensively. The cell lysate was then applied to a Mono Q HR 5/5 anion exchange column using a 2 M KCl gradient elution procedure. The Mono Q column yielded a fraction containing both ORF products which eluted with approximately 400 mM KCl. This fraction was then applied to a Superose 12 HR 10/30 gel filtration column. The eluent fraction containing the ORF 24 product was then reapplied to the Superose 12 to yield the final fraction containing only the ORF 24 product. The final fraction of ORF 24 was purified to homogeneity as determined by SDS-PAGE analysis. Approximately 750 μg of ORF 24 was isolated. This preparation was used for characterization studies.

Characterization studies of ORF 24 consisted of an amino-terminal sequence analysis, an estimation of the molecular weight using gel filtration chromatography and SDS-PAGE analysis, and an analysis of enzymatic activity as suggested by amino acid sequence homologies. The amino-terminal sequence of ORF 24 is P V E Q R S H D. The molecular weight of ORF 24 using gel-filtration chromatography and SDS-PAGE analysis is 26-kDa and 23-kDa, respectively. From gene sequence analysis, the molecular weight of ORF 24 is known to be 24,340-Da. These data indicate that ORF 24 is a monomer. ORF 24 was found to have amino acid sequence homologies with a pectate lyase (E 4.2.2.2) periplasmic precursor from *Erwinia caratovora* subspecies and a dextransucrase (EC 2.4.1.5) precursor from *Streptoccocus mutans* GS-5. However,

pectate lyase activity was not detected in cellular extracts over a 24 hour period. In addition, ORF 24 was not found to interact with 10 % substrate solutions of N-acetylglucosamine, pectin, UTEX 584 sheath material, DRH1 sheath material, sucrose, or glucose using thin layer chromatography. These studies indicate that the enzymatic activities proposed from amino acid sequence homologies have not been detected. The suggestion that ORF 24 is a water stress protein with a protective function on a structural level with regards to desiccation-tolerance requires further study.

ACKNOWLEDGMENTS

I would like to thank the members of my committee for their support throughout this project. The technical assistance of the research groups of Dr. M. Potts and Dr. W.H. Velander is greatly appreciated. I would especially like to give thanks to Dr. M. Potts and Dr. D.R. Bevan for this research opportunity.

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LIST OF ABBREVIATIONS

CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
CBB	coomassie brilliant blue
DFP	diisopropyl fluorophosphate
DTT	dithiothreitol
E. Coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FPLC	fast protein liquid chromatography
HRP	horse radish peroxidase
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton
LB	Luria-Bertani culture media
N. Commune	*Nostoc commune*
ORF	open reading frame
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 0.05 % Tween 20 (polyxyethylenesorbitan monolaurate)
TEMED	N-N-N'-N'-tetramethylethylene
Tris	tri(hydroxymethyl)aminomethane
UTEX	University of Texas culture collection
UV	ultraviolet
Wsp	water stress protein

LIST OF FIGURES

INTRODUCTION

It has been an accepted principle of modern biology that living organisms require an aqueous internal environment and that water comprises an essential constituent of cells (Crowe et al. 1973). In general, this concept is assuredly true, though there are many exceptions. Water accords structural order upon cells and is an indispensible component in reaction mechanisms. Water is also involved in the stability and regulation of proteins, lipids, and DNA. In addition, water plays a critical role in membrane structure and function. Therefore, the dehydration and rehydration of cells, and the storage of dried cells, imposes physiological constraints which few organisms can endure (Kuntz et al. 1977, Wiggins 1990, Crowe et al. 1992, and Potts 1994). Certain organisms can survive the complete, or nearly complete, removal of cellular water for extended periods of time without any apparent irreversible harm. This ability is referred to as desiccation tolerance. Antony Leeuwenhoek (1632-1732) made the first recorded account of this phenomenon in 1702 involving rotifers (Crowe et al. 1973). The mechanism for desiccation tolerance is currently inadequately understood, although mechanisms which bestow structural integrity of membranes seem significant (Hill et al. 1994). The role of water in cells that exhibit desiccation tolerance has been inferred from investigations of purified proteins (Carpenter 1993, and Carpenter et al. 1987, 1989, and 1990). Ecological studies provide an inadequate understanding of the mechanisms involved in desiccation tolerance (Potts 1994).

Desiccation tolerance is a primitive feature which is more prevalent in prokaryotic rather than in eukaryotic organisms. This prevalence in prokaryotes could be related to simpler internal organization of the prokaryotic cells, such as lack of cellular compartmentalization, and less complex membrane structures (Crowe et al. 1973, Bewley 1979, and Hill et al. 1994). Potts provides an extensive review of desiccation tolerance in prokaryotes (Potts 1994). Desiccation tolerance has also been observed in insects, yeasts, fungi and their spores, plants and their seeds, and crustacea (Antheunisse et al. 1979 and 1981, Kosanke et al. 1992, Koshi et al. 1977, Chen et al. 1973, Potts 1994, Crowe et al. 1992, and Leopold 1986).

The cyanobacterium *Nostoc commune* exhibits desiccation tolerance and has therefore become an effective model for study due to its prokaryotic cell organization and ability for higher plant type of photosynthesis (Hill et al. 1994, Eickmeier et al. 1993, Hochachka et al. 1987, Oliver et al. 1984 and 1992, Scherer et al. 1989, and Potts 1994). The effects of desiccation tolerance in *N. commune* have been studied on the biochemical (Angeloni et al. 1986, Olie et al. 1986, Potts et al. 1986, and Scherer et al. 1986), physiological (Coxson et al. 1983, Scherer et al. 1984, and Potts et al. 1985), and structural levels (Potts et al. 1985, and Peats et al. 1987). *N. commune* is a filamentous, heterocystous, obligate autotroph capable of nitrogen-fixation (Potts 1985). In karst regions throughout the world, desiccated colonies *of N. commune* grow over exposed limestone, nutrient poor soil, and accumulate in shallow depressions in exposed limestone rock (Potts 1992). Vegetative cells of *N. commune* can endure desiccation for years

without the formation of spores and then promptly recover their metabolic capabilities upon rehydration (Taranto et al. 1993). The foundation for desiccation tolerance in *N. commune* demonstrates a complex orchestration of interactions at the molecular, physiological, biochemical, and structural levels (Potts 1993). *N. commune* strain UTEX 584 has been found to exhibit reproducible and sequential recovery of metabolic capabilities upon rehydration commencing with respiration, followed by photosynthesis, and then nitrogen fixation. A possible explanation for this recovery is a step-wise, highly regulated insertion of proteins into membranes (Taranto et al. 1993).

The effects of desiccation on the synthesis of novel proteins remains unknown (Scherer et al. 1989). Studies of laboratory grown cultures of *N. commune* UTEX 584 exposed to various water stresses identified no novel classes of protein synthesis in response to desiccation (Potts 1985 and 1986). However, similar studies with *N. commune* collected in situ expressed a class of acidic proteins upon response to desiccation. These water stress proteins were determined to be the most abundant soluble proteins in desiccated colonies of *N. commune* and are very stable (Potts 1994). These water stress proteins (Wsp) were initially theorized to contribute a structural role in cell stability due to their abundance of serine, threonine, and tyrosine amino acid residues which can provide hydrogen bonding in polar and nonpolar environments (Potts 1994). It has been hypothesized that these water stress proteins provide some structural or protective role during desiccation, although there is no evidence of this theory that has been forthcoming (Potts 1994).

The genes for these water stress proteins were isolated and subcloned into *E. coli*. A lambdafix II library of *N. commune* UTEX 584 Bam H1 DNA fragments was constructed. The upstream and downstream regions of an 8.5-kbp fragment were successfully cloned into *E. coli*. This fragment contains a gene cluster which comprises five ORF's (Potts 1995). These ORF products may comprise part of one or more operons involved in the modification of carbohydrate and polymer secretion. This study presents the isolation and partial characterization of the product of a 24-kDa ORF and the partial isolation of a 59-kDa ORF product.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Culture, and Induction Conditions

This study used *E. coli* strain MAX EFFICIENCY DH10B™ (Life Technologies, Bethesda, MD). This strain was previously transformed with the pTrc 99A plasmid (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). This plasmid contains the *N. commune* Wsp genes of interest.

E. coli strain DH10B (pVJ18+) is stored at –70 °C in 30 % (v/v) glycerol and LB liquid media. LB liquid media consisted of 10 g L^{-1} bacto-tryptone, 5 g L^{-1} bacto-yeast extract, 10 g L^{-1} sodium chloride, pH 7.0 (Maniatis et al. 1989). Culture plates inoculated with cells from frozen cultures were incubated overnight at 37 °C. LB media for culture plates was the same as that for the liquid media with the exception that 15 g L^{-1} agar was added as the solidifying agent (Maniatis et al. 1989). Individual, isolated colonies were subcultured twice on culture plates. LB 3 mL liquid cultures inoculated with individual colonies from culture plates were incubated overnight at 37 °C on a rotor drum. These cultures were used as a 1 % (v/v) inoculum for 250 mL LB liquid cultures. These cultures were incubated overnight at 37 °C with shaking at 200 rpm. LB/agar culture plates and LB liquid cultures contained 0.2 mg mL^{-1} ampicillin (sodium salt, Sigma Chemical Co., St. Louis, MO). Culture growth was monitored at an absorbance of 550 nm using a Shimadzu spectrophotometer using 1 mL cuvettes. Culture induction occurred in the mid-logarithmic phase of cell growth. The mid-logarithmic phase of cell growth occurred after

approximately four hours of growth when the culture reached an absorbance of 1.0. Cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, MO) added to a final concentration of 0.5 mM. Cells were collected after 8 hours of growth.

Cell Disruption and Isolation of Cellular Protein

Cells were pelleted by centrifugation at 9,000 × g for 30 minutes at 4 °C using an SS-34 rotor (Sorvall, Wilmington, DE). The supernatant was discarded. Cell pellets were lyophilized overnight. Lyophilized cell pellets were suspended in a minimal amount of ice-cold grinding buffer (50 mM Tris-HCl pH 7.8, 10 mM magnesium chloride, 20 mM potassium chloride, 1 mM sodium azide, and 1 mM β-mercaptoethanol). Grinding buffer contained the following protease inhibitors at the specified final concentrations: 1 mM PMSF, 0.1 mM DFP, 1 μM leupeptin, 0.07 μM benzamidine hydrochloride, 0.1 μM DTT, and 1 mM EDTA.

The cell suspension was poured into a chilled mortar. Alumina (Sigma Chemical Co., St. Louis, MO) was added in a 2:1 ratio of alumina to cell suspension and mixed thoroughly. Liquid nitrogen was poured on top of this mixture and permitted to evaporate. The frozen mixture was then ground and pulverized with a pestle. Grinding continued until the cell paste completely thawed. This procedure was repeated several times. The mortar and pestle were autoclaved prior to use. The cell suspension was centrifuged at 9,000 × g for 30 minutes at 4 °C. The lysate supernatant was collected.

Precipitation of Cellular Protein

Streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) was added slowly to the lysate to a final concentration of 0.2 mg mL^{-1} with gentle stirring for 1 hour at 4 °C. The resulting precipitate was removed by centrifugation at 9,000 × g for 30 minutes at 4 °C. The supernatant fraction was collected and subjected to ammonium sulfate precipitation.

Enzyme grade ammonium sulfate (Fisher Scientific, Pittsburgh, PA) was ground to a fine powder with a mortar and pestle. The mortar and pestle were autoclaved prior to use. Ammonium sulfate powder was added slowly with gentle stirring to the supernatant fraction at 4 °C to attain 40 % saturation (0.242 mg mL^{-1}) overnight. The resulting precipitate was removed by centrifugation at 9,000 × g for 30 minutes at 4 °C. The supernatant fraction was collected. Ammonium sulfate powder was added slowly with gentle stirring to the supernatant fraction at 4 °C to attain 60 % saturation (0.13 mg mL^{-1}) overnight. This solution was subjected to centrifugation at 9,000 × g for 30 minutes at 4 °C. The supernatant fraction was discarded. The pellet was dissolved in a minimal amount of buffer (50 mM Tris-HCl pH 7.4, containing 10 mM potassium chloride), and dialyzed against this same buffer overnight at 4 °C. The dialysis method used Spectropor™ cellulose membranes and prepared according to the manufacturers instructions (12-14-kDa molecular weight cut-off, Spectrum, Houston, TX). After completion of dialysis, the resulting precipitate was removed by centrifugation at 9,000 ×

g for 30 minutes at 4 °C. The dialyzed supernatant fraction was collected and concentrated by ultrafiltraton using Centriprep-10 cartridges (10-kDa molecular weight cut-off, Amicon Inc., Beverly, MA).

Chromatography

Analytical liquid chromatography was performed using a Pharmacia FPLC system (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). This study used a 5 mL Mono Q HR 5/5 anion exchange column, and a 25 mL Superose 12 HR 10/30 gel filtration column.

The dialyzed supernatant was initially applied to the Mono Q HR 5/5 column. The Mono Q HR 5/5 column was prequilibriated with 10 mM Tris-HCl pH 6.8. A 2 M potassium chloride gradient was used for the elution of bound proteins. Relevant elution fractions that contained the protein of interest were combined and concentrated by ultrafiltration using Centriprep-10 cartridges (10-kDa molecular weight cut-off, Amicon Inc., Beverly, MA), then applied to the Superose 12 HR 10/30 column. All solutions used were vacuum-filtered using a 0.2 μm filter (Millipore Corporation, Bedford, MA) and degassed prior to use. The absorbance was monitored at 280 nm.

The Superose 12 HR 10/30 column was prequilibriated with 10 mM Tris-HCl pH 6.8, containing 20 mM potassium chloride. The column was eluted with the same buffer and fractions collected and concentrated by ultrafiltration using Centriprep-10 cartridges (10-kDa molecular weight cut-off, Amicon Inc., Beverly, MA). All solutions used were

vacuum filtered using a 0.2 μm filter (Millipore Corporation, Bedford, MA) and degassed prior to use. The absorbance was monitored at 280 nm. The flow rate was 0.5 ml min^{-1}. The chart speed was 0.5 cm min^{-1}.

Total Protein Determination

A colorimetric dye-binding assay was used to estimate total protein concentration (Pierce Chemical Co.). Bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) was used as the standard.

SDS-PAGE Analysis

SDS-PAGE analysis was done using the procedure of Laemmli (1970). SDS-PAGE analysis was conducted using a Pharmacia PhastSystem™ PhastGel™ Unit with Gradient 4-15 gels following the manufacturers instructions (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), and 18 % (w/v), 1.5 mm slab minigels.

SDS-PAGE analysis was conducted using 18 % (w/v) acrylamide, 1.5 mm slab minigels using a Mighty Small II SE 250 apparatus (Hoefer Scientific Instruments, San Francisco, CA). The stacking gel was 5 % (w/v) acrylamide. The electrophoresis running buffer contained 25 mM Tris-HCl, 200 mM glycine, and 3.5 mM sodium dodecyl sulfate. The samples were diluted in a 1:1 ratio in SDS-buffer containing 62.5 mM Tris-HCl pH 6.8, 2 % (v/v) sodium dodecyl sulfate, 20 % (v/v) glycerol, and 5 % (v/v) β-mercaptoethanol. Prestained low molecular range markers were used for the molecular

weight determination of proteins (Bio-Rad laboratories). The standard markers included phosphorylase B (112-kDa), BSA (84-kDa), ovalbumin (53.2-kDa), carbonic anhydrase (34.9-kDa), soybean trypsin inhibitor (28.7-kDa), and lysozyme (20.5- kDa). The gels ran for approximately 1 hour at 50 mA. The protein bands were stained by treating the gel with a solution containing 0.2 % (w/v) coomassie brilliant blue R-250, 10 % (v/v) acetic acid, and 45 % (v/v) methanol. The gel was destained with a solution consisting of 10 % (v/v) methanol and 10 % (v/v) acetic acid.

Western Blotting

After the extract proteins were resolved using SDS-PAGE analysis, the proteins were transferred to PVDF Immobilon P membranes (Millipore Corporation, Bedford, MA) and immediately subjected to immunoanalysis. The western blotting procedure was a modification of the technique used by Matsudaira (1987). The PVDF membranes were prewashed with 100 % (v/v) methanol for 5 seconds and then with water for 10 minutes. The PVDF membranes were then prequilibriated in thoroughly degassed 10 mM CAPS, pH 10.3, containing 10 % (v/v) methanol for 15 minutes prior to use. The proteins were transferred using a Hoeffer Scientific TE 22 Transphor Electrophoresis Unit (Hoefer Scientific Instruments, San Fransisco, CA). The SDS-PAGE minigels were placed onto gel blotting paper preequilibriated with 10 mM CAPS, pH 10.3, containing 10 % (v/v) methanol in a transfer cassette. The PVDF membrane was then placed onto the SDS-PAGE minigel. Additional prequilibriated gel blotting paper was placed onto the PVDF

membrane. The transfer cassette was then closed and submerged in 10 mM CAPS, pH 10.3, containing 10 % (v/v) methanol. The transfer cassette was then placed into the transfer chamber containing 10 mM CAPS, pH 10.3, containing 10 % (v/v) methanol. The PVDF membrane was positioned facing the anode. The transfer process was conducted at 450-500 mA for 30 minutes. Cooling water circulated through the transfer unit during the transfer process. After completion of the transfer process, the PVDF membrane was treated 1 % (w/v) BSA in TBST for 30 minutes with shaking. The membrane was then washed 3 times with TBST for 5 minutes each with shaking. The membrane was then incubated with a 1:2,000 dilution of rabbit-Wsp primary antibody in TBST with gentle shaking. The membrane was then washed 3 times with TBST for 5 minutes each with shaking. The membrane was then incubated for 2 hours with a 1:1,000 dilution of blotting grade affinity purified goat anti-rabbit IgG (H+L) HRP conjugate secondary antibody in TBST with gentle shaking. The membrane was then washed 3 times with TBST for 5 minutes each with gentle shaking. The color development process was initiated with the simultaneous addition of a solution of 50 mL TBS containing 30 μL of 30 % hydrogen peroxide, and a 10 mL ice-cold solution of 100 % (v/v) methanol containing 30 mg HRP color development reagent (4-chloro-1-napthol). The color development process was terminated by rinsing the membrane with water. The membrane was then dried and photographed immediately. All steps were conducted at room temperature.

Molecular Weight Determination using Gel Filtration Chromatography

An estimation of the apparent molecular weight of native ORF 24 protein was determined using a Superose 12 HR 10/30 gel filtration column (Darbre 1986, and Pharmacia Fine Chemicals AB 1983) (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The column was prequilibriated with 10 mM Tris-HCl pH 6.8, containing 20 mM potassium chloride. The column was eluted with the same buffer. Calibration standards comprised ribonuclease A (13.7-kDa), carbonic anhydrase (30-kDa), ovalbumin (43-kDa), bovine serum albumin (67-kDa), and blue dextran 2000 (2×10^6-kDa) (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Each standard protein sample was applied twice individually to the column in a 50 µL injection. Each standard sample application consisted of 3 mg of protein dissolved in 200 µL of buffer (10 mM Tris-HCl pH 6.8, containing 20 mM potassium chloride). Samples of purified ORF 24 were applied twice to the column in a 50 µl injection containing approximately 78 µg protein. The absorbance was monitored at 280 nm. The AUFS was 1.0. The flow rate was 0.5 mL min^{-1}. The chart speed was 0.5 cm min^{-1}.

Molecular Weight Determination using SDS-PAGE Analysis

An estimation of the molecular weight of purified native ORF 24 protein was determined using Pharmacia PhastSystem PhastGel Gradient 10-15 gels (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and the method of Weber and Osborn (Weber et al. 1969). Standard markers included phosphorylase B (112-kDa), bovine serum albumin (84-kDa), ovalbumin (53.2-kDa), carbonic anhydrase (34.9-kDa), soybean trypsin inhibitor (28.7-kDa), and lysozyme (20.5-kDa) (Bio-Rad laboratories).

Pectate Lyase Activity Assay

The capacity of native extracts of ORF 24 to hydrolyze pectin from citrus fruits (Sigma Chemical Co., St. Louis, MO) was examined. A 1 ml experimental sample consisted of 5 mg ml^{-1} pectin, 1 mM magnesium chloride, and 100 µg ml^{-1} purified ORF 24. The composition of the control sample was the same as the experimental sample, but with the exception that ORF 24 was excluded. The buffer used consisted of 10 mM Tris-HCl pH 6.8, containing 20 mM potassium chloride. The absorbance was monitored at 235 nm using a Shimadzu spectrophotometer with 1 ml cuvettes.

Enzyme Activity Assay

The capacities of crude extracts containing ORF 24 to interact with 10 % solutions of N-acetylglucosamine, pectin, UTEX 584 sheath material, DRH1 sheath material, sucrose, glucose were examined using thin layer chromatography. The crude

extracts examined included rehydrated *N. commune* field material, *E. coli* DH10B, *E. coli* DH10B (pVJ18+), and *E. coli* DH10B (pVJ18+) IPTG induced strains.

LB 3 ml liquid cultures of *E. coli* DH10B, and *E. coli* DH10B (pVJ18+) incubated at 37 °C on a rotator for 6 hours. An *E. coli* DH10B (pVJ18+) culture was induced after 3 hours of growth by adding IPTG to a final concentration of 0.5 mM. The induced *E. coli* DH10B (pVJ18+) culture was incubated at 37 °C on a rotator for 3 hours. The cultures were then sonicated (Fisher model 300 operated at 35 % of maximum setting) for 10 seconds, then chilled on ice for 2 minutes. Sonication was repeated for 5 cycles. Samples were subjected to centrifugation at 12,000 × g for 5 minutes at 4 °C to pellet cellular debris. The supernatant was then recovered. Desiccated *N. commune* was rehydrated with distilled and deionized water for 20 minutes, then the supernatant was recovered. Test extracts were incubated overnight at 37 °C with each 10 % substrate solution. Samples (10 µL) were then applied to 20 × 20 cm, 250 µm layer thickness, 150 Å silica gel plates (Whatman Inc.). Samples were resolved by development of plates in 1-isopropanol/water (6:1, v/v). The plates were then charred at 120 °C for 20 minutes. The plates were observed under a UV light.

Amino-Terminal Sequence Analysis

Purified ORF 24 product was subjected to Edman degradation for amino-terminal sequencing using an Applied Biosystems model 477A gas phase sequenator. A ProSpin ™ Sample Preparation Cartridge (Applied Biosystems, Foster City, CA) was used to prepare

the sample for sequence analysis. Immediately prior to use, the PVDF membrane of the cartridge was briefly moistened with 25 µL of methanol. Approximately 100 µl of purified ORF 24 product at 150 µg/mL was added to the cartridge. The cartridge was then subjected to centrifugation at 5600 × g to dryness for 2 hours. The prepared sample was then subjected to sequence analysis.

RESULTS

Isolation Method

Culture growth of *E.coli* strain DH10B (pVJ18+) is shown in figure 1. A flow diagram for the method of isolation of the ORF 24 product is shown in figure 2. The SDS-PAGE analysis of fractions obtained in the isolation of the ORF 24 product is shown in figure 3. The western blot and SDS-PAGE analysis of a 40-60% ammonium sulfate fraction and of a Mono Q fraction is shown in figure 4.

Cells were induced to synthesize Wsp after approximately four hours of culture growth, and were collected approximately eight hours after induction. The use of minimal media was found to provide no advantage in the synthesis of the ORF 24 and 59 products as indicated from SDS-PAGE analysis. The 40-60 % ammonium sulfate fraction of the clarified cell lystate was found to contain the largest amount of ORF 24 and ORF 59 products based on SDS-PAGE analysis. Dialysis of this ammonium sulfate fraction resulted in further precipitation of other cellular protein which was removed by centrifugation. The dialyzed ammonium sulfate fraction was then applied to a Mono Q HR 5/5 anion exchange column. The fraction containing both ORF 24 and ORF 59 products eluted from the Mono Q column in fraction A with an elution volume of approximately 10 mL with 400 mM KCl as shown in figure 5. This concentrated fraction was then applied onto a Superose 12 gel filtration column. This fraction, containing both ORF 24 and trace ORF 59 products, eluted in fraction B with an elution volume of approximately 13

mL as shown in figure 6. This fraction was concentrated by ultrafiltration and then applied again to the gel filtration column. The ORF 24 product fraction eluted in fraction C with an elution volume of approximately 15 mL as shown in figure 7. The final fraction obtained was determined to be homogeneous when examined by SDS-PAGE analysis. Approximately 750 μg of ORF 24 was isolated. This preparation was used for further characterization of ORF 24.

Molecular Weight Determination using SDS-PAGE Analysis

The molecular weight of the ORF 24 product was estimated from SDS-PAGE analysis. Relative migration distances were calculated for the calibration standards. Phosphorylase B (112-kDa), bovine serum albumin (84-kDa), ovalbumin (53.2-kDa), carbonic anhydrase (34.9-kDa), soybean trypsin inhibitor (28.7-kDa), and lysozyme (20.5-kDa) had calculated relative migration distances (R_f) of 0.24, 0.32, 0.47, 0.62, 0.68, and 0.82, respectively. These data are shown in figure 8. The ORF 24 product had a relative migration distance of 0.75. From the plot in figure 8, the molecular weight of the ORF 24 product is estimated to be approximatley 24-kDa.

Molecular Weight Determination using Gel-Filtration Chromatography

The molecular weight of the ORF 24 product was estimated from gel filtration chromatography. Elution volumes were determined for the calibration standards. Ribonuclease A (13.7-kDa), carbonic anhydrase (30-kDa), ovalbumin (43-kDa), bovine

serum albumin (67-kDa), and blue dextran 2000 (2×10^6-kDa), had elution volumes of 18.5, 14.2, 12.4, 11.9, and 8.9 mL, respectively. The elution volume for blue dextran 2000 was determined to be the void volume of the gel-filtration column used for this study. The average distribution coefficients (K_{av}) for ribonuclease A, carbonic anhydrase, ovalbumin, and bovine serum albumin were calculated to be 0.59, 0.33, 0.22, and 0.19, respectively. These data are shown in figure 9. The ORF 24 product had an elution volume of 16 mL and an average distribution coefficient of 0.44. From the plot in figure 9, the molecular weight of the ORF 24 product is estimated to be approximatley 26-kDa.

Amino-Terminal Sequence Analysis

The amino-terminal sequence of ORF 24 is: P V E Q R S H D. The complete amino acid sequence of the ORF 24 product is shown in figure 10 (Potts 1995).

Pectate Lyase Activity Assay

The absorbance at 235 nm remained constant throughout a 24 hour period. Therefore, pectate lyase activity was not detected in the crude cellular extracts.

Enzyme Activity Assay

The crude extract cultures did not interact with the 10 % substrate solutions. By examining the thin layer chromatography plates under a UV light, the IPTG induced *E.*

coli DH10B (pVJ18+) culture was found to have no indication of interaction with the 10 % substrate solutions.

Figure 1. Culture growth of *E. coli* strain DH10B (pVJ18+) in 250 ml LB liquid media. Each data point is an average of three experiments under equivalent growth conditions.

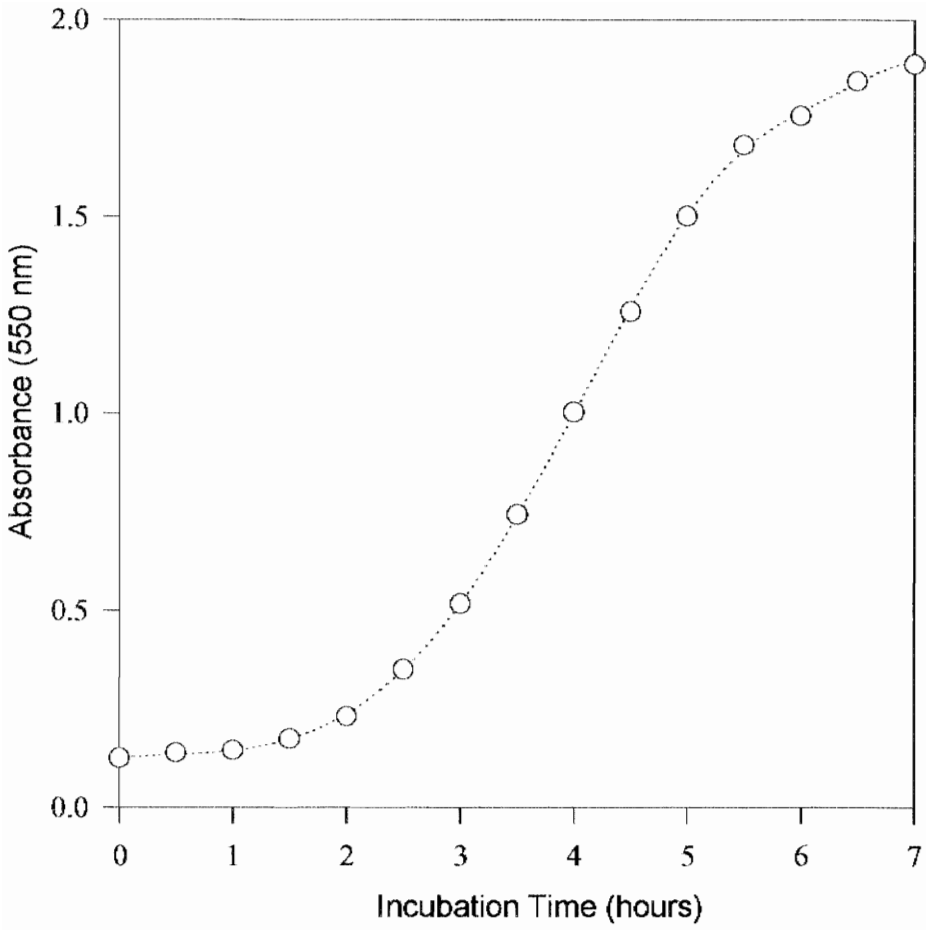
2.0
1.5
1.0
0.5
0.0
Absorbance (550 nm)
0
1
2
3
4
5
6
7
Incubation Time (hours)

Figure 2. Isolation method for ORF 24 used in this study.

clarified cell lysate

↓

streptomycin sulfate

↓

dialyzed 40-60 % ammonium sulfate fraction

↓

Mono Q HR 5/5 anion exchange chromatography

↓

Superose 12 HR 30/30 gel filtration chromatography

↓

Superose 12 HR 30/30 gel filtration chromatography

Figure 3. SDS-PAGE analysis of fractions obtained in isolation scheme of ORF 24.Lane A: cell lysate (24 μg total protein), Lane B: dialyzed 40 - 60 % ammonium sulfate fraction (35 μg total protein), Lane C: Mono Q fraction (12 μg total protein), Lane D: Superose 12 fraction (8 μg total protein), Lane E: final Superose 12 fraction (6 μg total protein) (from top to bottom, arrows indicate 112, 84, 53.2, 34.9, 28.7, and 20.5-kDa markers, respectively, Bio-Rad Laboratories)

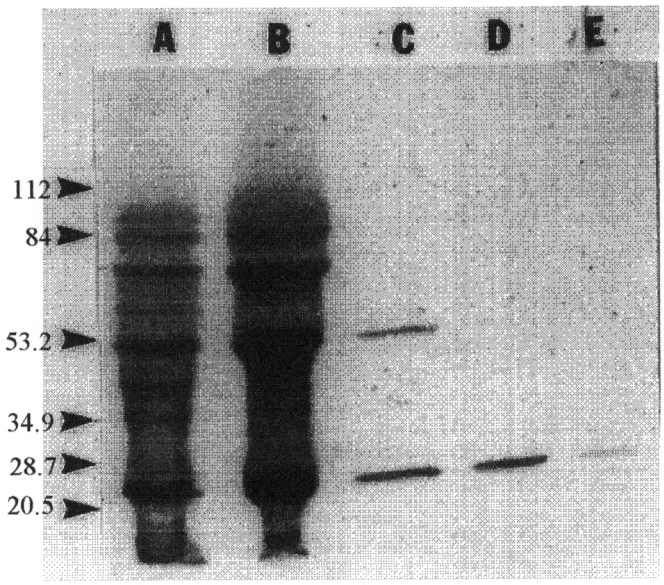
A
B
C
D
E
112
84
53.2
34.9
28.7
20.5

Figure 4. Western blotting analysis of a Mono Q elution fraction and of a 40-60 % dialyzed ammonium sulfate fraction with Wsp antiserum. Lane A: SDS-PAGE analysis of the 10.5 mL elution fraction from the Mono Q column corresponding to approximately 400 mM potassium chloride (refer to figure 5) (9 μg total protein), Lane B: Corresponding western blot of sample in lane A (9 μg total protein), Lane C: dialyzed 40-60 % ammonium sulfate fractionation of clarified cell lysate (21 μg total protein), Lane D: Corresponding western blot of sample in lane C (21 μg total protein). (from top to bottom, arrows indicate 112, 84, 53.2, 34.9, 28.7, and 20.5-kDa markers, respectively, Bio-Rad Laboratories).

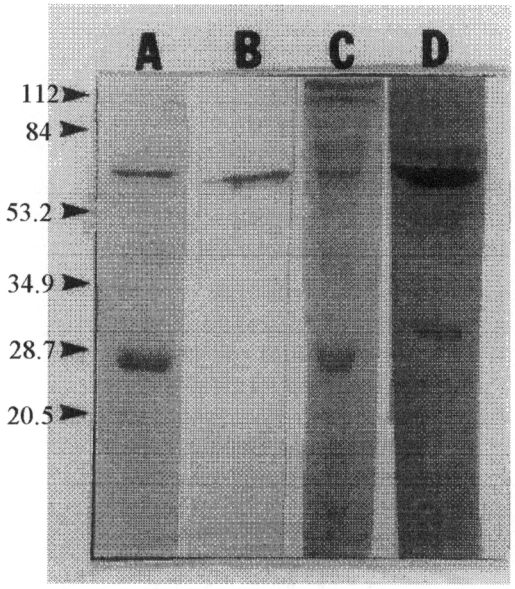
A
B
C
D
112
84
53.2
34.9
28.7
20.5

Figure 5. Elution profile of 200 μL (~75 μg total protein) of dialyzed 40-60 % ammonium sulfate fraction from Mono Q HR 5/5 column equilibriated with 10 mM Tris-HCl pH 6.8 (buffer A) with a flow rate of 1 mL min^{-1}. Profile was developed with a gradient buffer B (buffer A, 2 M KCl). 1 mL fractions were collected. Absorbance was monitored at 280 nm. Aliquots from fraction A were subjected to Western blotting and SDS-PAGE analysis. Fraction A contained both products of ORF 24 and 59.

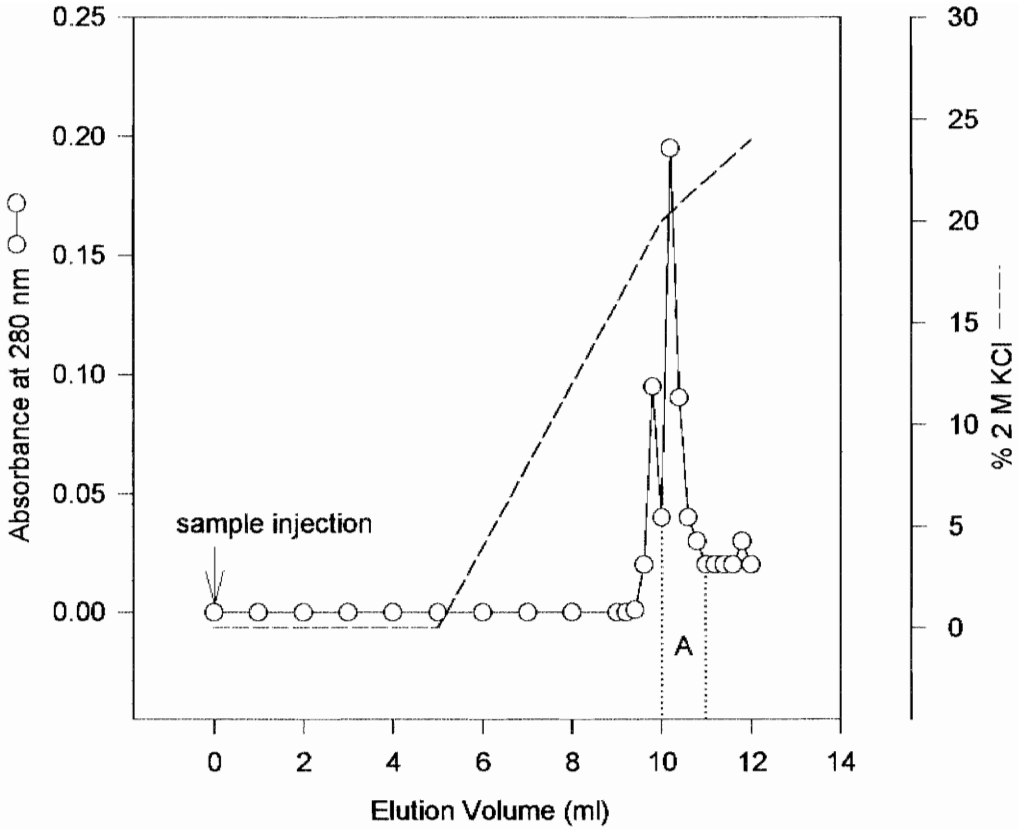
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance at 280 nm
30
25
20
15
10
5
0
% 2 M KCl
sample injection
A
0
2
4
6
8
10
12
14
Elution Volume (ml)

Figure 6. Elution profile of 200 μL (~60 μg total protein) of fraction A collected from Mono Q column from a Superose 12 HR 10/30 gel filtration column equilibriated with 10 mM Tris-HCl pH 6.8, containing 20 mM KCl. The absorbance was monitored at 280 nm. The flowrate was 0.5 mL min^{-1}. Aliquots from fraction B were subjected to Western blotting and SDS-PAGE analysis. Fraction B contained ORF 24 product and trace ORF 59 product.

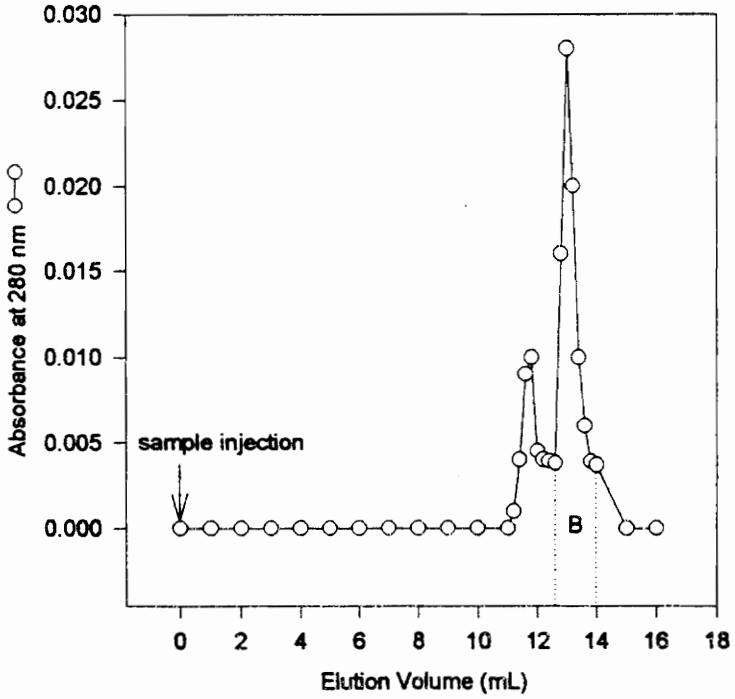
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Absorbance at 280 nm
sample injection
B
0
2
4
6
8
10
12
14
16
18
Elution Volume (mL)

Figure 7. Elution profile of 200 μL (~55 μg total protein) of reapplication of fraction B from Superose 12 HR 10/30 gel filtration column equilibriated with 10 mM Tris-HCl pH 6.8, containing 20 mM KCl. The absorbance was monitored at 280 nm. The flowrate was 0.5 mL min^{-1}. Aliquots from fraction C were subjected to Western blotting and SDS-PAGE analysis. Fraction C contained purified ORF 24 product.

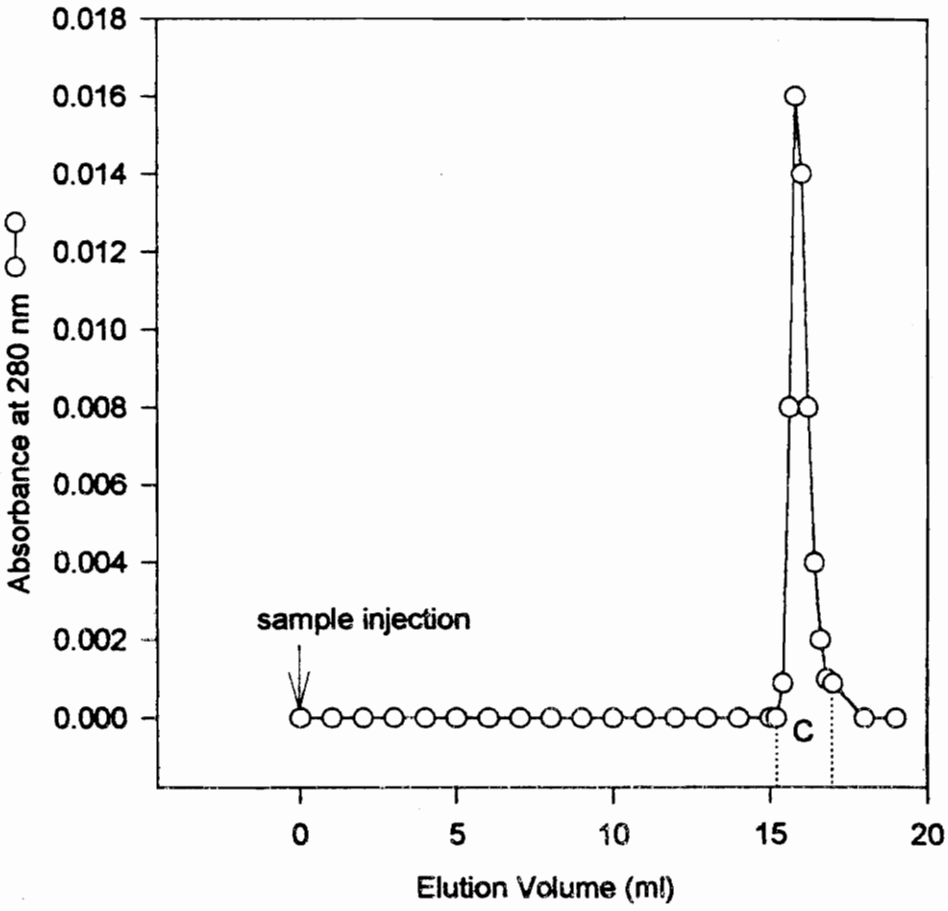
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Absorbance at 280 nm
sample injection
C
0
5
10
15
20
Elution Volume (ml)

Figure 8. Molecular weight estimation of ORF 24 using SDS-PAGE analysis. Each data point is an average of two experiments.

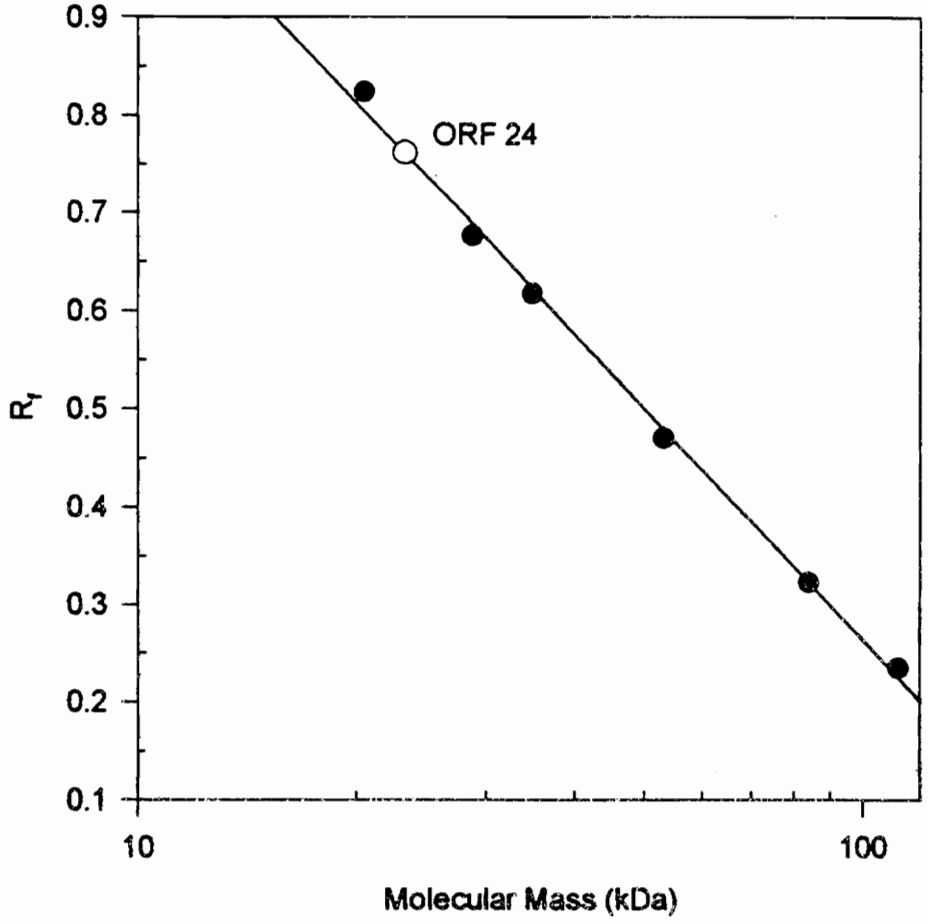
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
R_f
ORF 24
10
100
Molecular Mass (kDa)

Figure 9. Molecular weight estimation of ORF 24 using a Superose 12 HR 10/30 gel filtration column. Each data point is an average of two experiments.

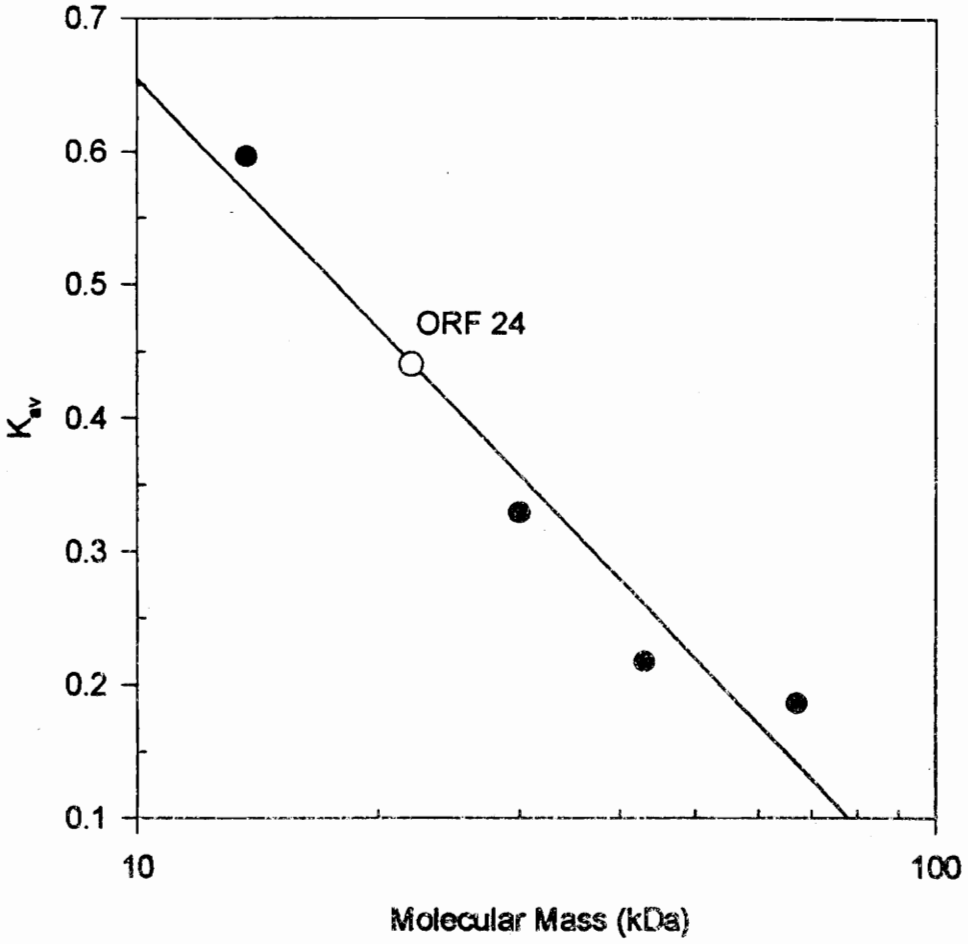
0.7
0.6
0.5
0.4
0.3
0.2
0.1
K_{av}
ORF 24
10
100
Molecular Mass (kDa)

Figure 10. (A) Complete amino acid sequence of ORF 24. (B) Amino-terminal sequence of ORF 24.

(A)

1 MPVEQRSHDTNNGSLPYLFSPVSDNAKQPA

31 PLVIFLHGGRDRGTDLNVLLKWGLPRFVDL

61 SDSLPYVFAAPQIPAEQTWADRADDVLTLL

91 DELIVSQPVDPARVILAGFSLGSAGIWHIA

121 ALHPDRFAGLVAVSGRVPKTLAESELAALK

151 NIPVQIFQGGQDKNLPIEDTEIFVERLRNV

181 GGKVDLTVLPEGDHFIADEVYGNPKLQQWL

211 ISQNRREISVVA

(B) PVEQRSHD

DISCUSSION

Preservation and maintenance of the integrity of cellular membranes is critical to bacteria that exhibit desiccation tolerance (Potts 1994, and Carpenter et al. 1989). Extracellular polysaccharides accord bacterial cells the capacity to survive desiccation (Potts 1994, and Ernst et al. 1987). There have been many roles attributed to the extracelluar polysaccharide investment of anhydrobiotic bacterial cells in addition to protection against desiccation, such as substrate anchorage, protection against phagocytic predation, inhibition of antibody recognition, and the prevention of lysis by viruses and infectious bacteria (Potts 1994, Fazio et al. 1982, and Tease et al. 1987). The entire role of the bacterial extracellular polysaccharide investment is not yet completely known (Barbosa et al. 1992). The presence of proteins, uronic and pyruvic acids, and O-methyl, O-acetyl, and sulfate moieties within these layers suggests that enzymes would be required to synthesize and degrade the polysaccharide layer (Potts 1994). Although there is a similarity of the amino acid sequence of the ORF 24 product to the aformentioned carbohydrate-modifying enzymes, neither pectate lyase or dextransucrase activity was detected in crude cellular extracts. The homology of the ORF 24 product with dextransucrase from *S. mutans* GS-5 indicates that this product may have a related function similar to the synthesis of water-insoluble and water-soluble glucans from sucrose (Ueda et al. 1988). In addition, the similarity of the ORF 24 product with pectate lyase (E 4.2.2.2) periplasmic precusor from the *Erwinia caratovora* indicates that it may have a related role in the synthesis and/or degradation of extracellular polysaccharides (Hinton

et al. 1989). These subsequent characterization studies were inconclusive as to any inherent enzymatic properties as suggested by sequence homologies.

Due to the proximity of the values obtained for the molecular weight estimation from gel filtration chromatography and SDS-PAGE analysis, 26-kDa and 23-kDa, respectively, ORF 24 is considered a monomer. According to the amino-terminal sequence, an amino-terminal methionine is absent. The distribution of amino-termini of proteins from microorganisms is extremely random (Scherer et al., 1989). An assumption is that methionine is removed by proteolytic cleavage during the maturation of the protein. There is an apparent association between the ORF 24 and ORF 59 products as indicated by the difference in elution volumes of the ORF 24 product from the gel-filtration column steps. In the elution of the ORF 24 product from the gel-filtration column in the presence of the ORF 59 product, the elution volume of the ORF 24 product is approximately 13 mL as shown in figure 6. However, the fraction containing purifed ORF 24 has an elution volume of approximately 16 mL as shown in figure 7.

The deduced amino-terminal and complete amino acid sequence of the ORF 24 product is shown in figure 10. The ORF 24 product consists of 222 amino acids. The known molecular weight is 24,340-Da. The amino acid composition of ORF 24 product comprises 19 A, 12 R, 9 N, 17 D, 0 C, 11 Q, 11 E, 16 G, 5 H, 13 I, 27 L, 7 K, 1M, 9 F, 17 P, 13 S, 7 T, 4 W, 3 Y, and 21 V residues. There are 12 potential glycosylation sites. The theoretical isolectric point is 4.7. If the amino-terminal methionine is conserved in the

mature protein, the half-life is greater than 20 hours, otherwise the second proline will be the amino-terminal. The estimated half-life is approximately 7 minutes (Potts 1995).

There are several other ORF's identified within the gene cluster on the 8.5-kbp DNA cloned from *N. commune* UTEX 584. The first ORF (ORF 59) shares sequence similarity with glucosamine fructose-6-phosphate aminotransferase involved in the formation of N-acetylglucosamine. ORF 59 indicates homologies with ATP carrier proteins aldose reductase and β-lactamase. The third ORF (ORF 22) shares sequence similarity with an exopolysaccharide regulatory protein, and a β-glucoside-specific phosphotransferase. The fourth ORF (ORF 36) shares sequence homology with a 2,5-diketo-D-gluconate reductase involved in ascorbate synthesis and xylose reductase involved in aldoketose reductase. The fifth ORF (ORF 70) shares sequence homology with a β-1,2-glucan export protein, which is involved in inner membrane transport in Agrobacterium and essential for crown-gall tumor formation (Potts 1995). Of these ORF's, only the products of ORF 24 and ORF 59 appear to be expressed to any significant level.

This study presented the partial purification and characterization of the ORF 24 product and partial purification of the ORF 59 product. These ORF's may constitute part of one or more operons involved in the modification of sugars and polymer secretion. The observation that the ORF 59 product cross-reacts with the Wsp antiserum indicates that it may share epitopes with Wsp and may be involved in the modification and synthesis of a common structure (Potts et al. 1994 and Potts 1995). The further characterization of

Wsp from *N. commune* should provide understanding of desiccation tolerance, and the possible protective structural role of Wsp in photosynthetic bacterial cells.

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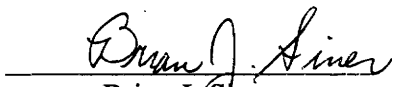
VITA

Brian J. Sines earned the Bachelor of Science degree in Chemical Engineering from the University of Maryland at College Park in May, 1993.



Brian J. Sines