

## Cell Surface Properties of *Pseudomonas syringae* pv. *phaseolicola* Wild-type and *hrp* Mutants

WILLIAM F. FETT, STANLEY F. OSMAN, MICHAEL F. DUNN  
and NICKOLAS J. PANOPOULOS

Authors' addresses: W. F. FETT and S. F. OSMAN, USDA, ARS, Eastern Regional Research Center, Plant Science Research Unit, Philadelphia, PA 19118, USA; M. F. DUNN, Department of Plant Pathology, University of Missouri, Columbia, MO 65211; N. J. PANOPOULOS, Department of Plant Pathology, University of California, Berkeley 94720, USA.

### Abstract

The cell surface hydrophobicity and charge as well as surface polysaccharides of eight independent prototrophic *hrp*::Tn5 mutants (LINDGREN *et al.*, J. Bacteriol. 168, 512—522, 1986) were compared to the wild-type parent strain NPS3121 of *Pseudomonas syringae* pv. *phaseolicola*. No significant differences were found in cell surface charge, but mutant strain NPS4005 exhibited significantly lower cell surface hydrophobicity than the wild-type and the other mutant strains. The mutant strains all retained the ability to produce the exopolysaccharides (EPS) levan, a neutral fructan, and alginate, an acidic polymer. Relative amounts of EPS produced *in vitro* was dependent on culture conditions. Lipopolysaccharide (LPS) chemotypes were similar for all nine strains. Chemical as well as <sup>13</sup>C-NMR analyses of the O-antigens from four wild-type strains of *P. s.* pv. *phaseolicola* representing two physiological races as well as the O-antigens of two strains of *P. s.* pv. *syringae* which belong to the same serogroup as *P. s.* pv. *phaseolicola* indicated that all of the O-antigens were very similar if not identical. LPS of three strains of *P. s.* pv. *phaseolicola* produced *in vitro* or *in planta* were also compared and no significant differences were detected. The altered phenotype of the Tn5 mutants of *P. s.* pv. *phaseolicola* does not appear to be due to changes in the ability to produce exopolysaccharides or to an altered composition of cell surface polysaccharides (LPS and EPS). However, a change in an unidentified cell surface component(s) leading to lowered cell surface hydrophobicity of mutant strain NPS4005 may be important.

### Zusammenfassung

#### Zelloberflächeneigenschaften von *Pseudomonas syringae* pv. *phaseolicola*-Wildtypen und *hrp*-Mutanten

Verglichen wurden die Zelloberflächenhydrophilie und deren Ladung sowie die Oberflächenpolysaccharide acht unabhängiger prototrophischer *hrp*::Tn5-Mutanten (LINDGREN *et al.* J. Bacteriol. 168, 512—522, 1986) mit dem Wildtyp-Urstamm NP3121 von *Pseudomonas syringae* pv.

*phaseolicola*. Es wurden keine signifikanten Unterschiede bei der Zelloberflächenladung festgestellt, bei dem mutanten Stamm NPS4005 jedoch wurde eine signifikant niedrigere Zelloberflächenhydrophilie ermittelt als bei dem Wildstamm und den anderen Mutantenstämmen. Alle Mutantenstämme behielten die Fähigkeit, die Exopolysaccharide (EPS) Levan, ein neutrales Fructan, und Alginat, ein Säurepolymer, zu produzieren. Die relativen Mengen der *in vitro* produzierten EPS war abhängig von den Kulturbedingungen. Lipopolysaccharidchemotypen waren bei allen neuen Stämmen ähnlich. Chemische sowie C-NMR-Analysen der O-Antigene von vier Wildtypstämmen von *P. s. pv. phaseolicola*, die zwei physiologische Rassen vertraten, sowie die O-Antigene zweier *P. s. pv. syringae*-Stämme, die zur gleichen Serogruppe wie *P. s. pv. phaseolicola* gehören, zeigten, daß alle O-Antigene sehr ähnlich, wenn nicht identisch sind. Verglichen wurden auch die LPS von drei *P. s. pv. phaseolicola*-Stämmen, die *in vitro* oder *in planta* produziert worden waren und es konnten keine signifikanten Unterschiede festgestellt werden. Der veränderte Phenotyp der Tn5-Mutanten von *P. s. pv. phaseolicola* scheint nicht durch Änderungen in der Fähigkeit, Exopolysaccharide zu produzieren oder durch eine veränderte Zusammensetzung der Zelloberflächenpolysaccharide (LPS und EPS) verursacht zu sein. Eine Änderung eines oder mehrerer Oberflächenkomponenten, die zu einer niedrigeren Zelloberflächenhydrophilie des Mutantenstammes NPS4005 führte, könnte jedoch wichtig sein.

Bacterial cell surface polysaccharide such as exopolysaccharide (EPS) and lipopolysaccharide (LPS) may be important components of recognition phenomena between microbes and plants and as determinants of microbial pathogenicity. Plant host receptors may recognize surface polysaccharides or their degradation products in a highly specific manner leading to selective attachment to plant surfaces and/or triggering a cascade of events culminating in either resistance or susceptibility. Alternatively, bacterial surface polysaccharides may mask the presence of other bacterial surface components which elicit host defense responses.

To date the majority of studies in this area have centered on the role of bacterial surface polysaccharide in the interaction of rhizobia with their legume hosts. Even though a role for such bacterial surface polysaccharides in the highly complex nodulation process has not been proven, many EPS-deficient and LPS-defective mutants of rhizobia have been generated which either fail to form nodules or form pseudonodules that do not contain bacteria and do not fix nitrogen (for reviews, see JOHNSTON 1989 and ROLFE *et al.* 1989). Bacterial cell surface components have also been implicated as playing a role in plant-bacterial pathogen interactions as virulence or recognition factors (for a review see CHATTERJEE and VIDAVER 1986).

Recently, LINDGREN *et al.* (1986) reported on a set of eight independent Tn5-generated prototrophic mutants (designated *hrp* mutants) of *P. s. pv. phaseolicola* which are either attenuated in virulence or nonpathogenic on their host plant, common bean (*Phaseolus vulgaris* L.) and which do not induce the hypersensitive response (HR) on nonhost plants. For the majority of these mutants the defect(s) leading to the Hrp-phenotype have not been identified. GRIMM and PANOPOULOS (1989) showed that one of these mutants is affected in a regulatory gene (*hrpS*) which controls the expression of another *hrp* gene (*hrpD*). Subsequent studies (MINDRINOS *et al.* 1990, RAHME, MINDRINOS and PANOPOULOS, unpublished data) showed that this gene also regulates the expression of several additional *hrp* genes. The products of the *hrpS*-regulated genes and their biochemical functions have not been identified. EL-KADY *et al.* (1986) analyzed a set of phenotypically similar

mutants (which they designated HRi<sup>-</sup>) of another strain of *P. s. pv. phaseolicola* by crossed immunoelectrophoresis and showed that each of them lacked one or more proteinaceous antigens. The role of these antigens in pathogenesis or in the induction of the HR is not known. Finally, in a study of *hrp* mutants generated from a third strain of *P. s. pv. phaseolicola*, DEASEY and MATTHYSSE (1988) did not detect alterations in the amount of EPS produced or in the electrophoretic patterns given by LPS produced *in vitro*, but the chemical nature of these polymers was not examined. They also reported no differences in mobility.

We undertook a detailed study to determine if alteration of cell surface properties could be responsible for the altered plant reactions given by the *hrp* mutants generated by LINDGREN *et al.* (1986). Previous studies in our laboratory (FETT *et al.* 1986) as well as those by GROSS and RUDOLPH (1987 a) have established that *P. s. pv. phaseolicola* is capable of producing both levan (a neutral fructan) and the acidic polymer alginic acid (polymannuronic acid with varying amounts of guluronic acid) as EPS's. These studies also indicated that the relative amounts of these EPS's produced *in vitro* is strain-dependent as well as dependent on the available source of carbon (levan produced only when sucrose is available). During growth in compatible leaves of bean, *P. s. pv. phaseolicola* produces solely alginate or a mixture of alginate and levan, again dependent on the particular strain studied (FETT and DUNN 1989, GROSS and RUDOLPH 1990). We have also previously determined the O-antigen structure of the LPS of parent strain NPS3121 to consist of a repeating unit of alternating 2- and 3-0-substituted  $\alpha$ -D-rhamnopyranosyl residues with every second 3-0-substituted D-rhamnose also substituted at 0-4 with a terminal  $\alpha$ -D-fucofuranosyl group (OSMAN *et al.* 1988).

In this study we compared the cell surface charge and hydrophobicity, the ability to produce EPS and its composition, as well as LPS composition of parent strain NPS3121 to that of its Tn5 mutants. We also examined the relationship of LPS chemotype with physiological race of *P. s. pv. phaseolicola* and its relationship with serological relatedness of *P. s. pv. phaseolicola* and *pv. syringae*. Two strains of *P. s. pv. syringae* which belong to the same *P. syringae* serogroup as *P. s. pv. phaseolicola* (SAUNIER and SAMSON 1987) were used for the latter purpose.

## Materials and Methods

### Bacterial strains

A list of bacterial strains, their source and relevant phenotypic information is given in Table 1. Bacteria were maintained on Difco (Detroit, MI) *Pseudomonas* agar F (PAF) at 4 °C. When culturing *P. s. pv. phaseolicola* wild-type strain NPS3121, rifampicin (100 µg/ml) was added to the media. The transposon mutants were cultured in the presence of rifampicin (100 µg/ml) and kanamycin (10 µg/ml). Long term storage was by broth culture impregnated filter paper discs stored at 4 °C and by lyophilization in double-strength skim milk.

### Plant inoculations

Virulence and physiological race of *P. s. pv. phaseolicola* strains were determined using bean cvs. Red Kidney (susceptible to race 1, 2 and 3); Improved Tendergreen (susceptible to races 1 and 2, resistant to race 3) and Red Mexican UI34 (susceptible to races 2 and 3, resistant to race 1). Red

Table 1  
Pseudomonas strains used in this study

Designation	Relevant characteristics <sup>a</sup>	Host of origin	Reference or source
<i>P. syringae</i> pathovars pv. <i>phaseolicola</i>			
NPS3121	race 2, rif <sup>r</sup> , Hrp <sup>+</sup>	<i>Phaseolus vulgaris</i>	LINDGREN <i>et al.</i> 1986, this study
NPS4000, NPS4001, NPS4002, NPS4004, NPS4006, NPS4007	NPS3121::Tn5, rif <sup>r</sup> , km <sup>r</sup> , Hrp <sup>-</sup>		LINDGREN <i>et al.</i> 1986
NPS4003, NPS4005	NPS3121:Tn5, rif <sup>r</sup> , km <sup>r</sup> , Hr <sup>-</sup> p <sup>±</sup>		LINDGREN <i>et al.</i> 1986
R1	race 1	<i>Phaseolus vulgaris</i>	FETT and DUNN 1989
HB35	race 1	<i>Phaseolus vulgaris</i>	FETT and DUNN 1989
R2	race 2	<i>Phaseolus vulgaris</i>	FETT <i>et al.</i> 1986
At	race 2	<i>Phaseolus vulgaris</i>	FETT <i>et al.</i> 1986
pv. <i>syringae</i>			
CFBP1147		<i>Pyrus communis</i>	R. SAMSON
CFBP1542		<i>Evonymus japonicus</i>	R. SAMSON

<sup>a</sup> rif, rifampacin; km, kanamycin; <sup>r</sup>, resistant; Hrp<sup>+</sup>, induces a hypersensitive response on tobacco and is fully virulent on bean; Hrp<sup>-</sup>, unable to induce a hypersensitive response in tobacco and nonpathogenic on bean; Hr<sup>-</sup>p<sup>±</sup>, unable to induce a hypersensitive response in tobacco and attenuated in virulence on the bean cultivar Red Kidney.

Kidney and Improved Tendergreen seed were obtained from W. Atlee Burpee Co., Warminster, PA. Ability to induce the hypersensitive response was determined using tobacco cv. Turk. Plants were cultivated in a mix of Baccto potting soil (Michigan Peat Co., Houston, TX) and vermiculite (2 : 1) in clay pots (for virulence assays) or plastic flats (for isolation of *in planta* grown cells). Plants were maintained in a growth chamber at 24 to 26 °C day, 20 °C night, 75 % RH. Fluorescent and incandescent bulbs provided 1.1 × 10<sup>4</sup> lux on a 13 h photoperiod.

For relative virulence assays, trifoliolate leaves of bean were inoculated using two methods. In the first, abaxial surfaces were forcibly sprayed with bacterial inoculum until the tissues appeared water-soaked. In the second method, abaxial and adaxial surfaces were gently sprayed with bacterial inoculum until run-off and plants were kept covered with plastic bags for 2 days to maintain high relative humidity. Bacterial inoculum was prepared from overnight cultures grown on PAF at 28 °C. Cells were suspended in sterile water to give an OD<sub>600nm</sub> of 1.0 (approximately 7 × 10<sup>8</sup> colony-forming units [cfu]/ml based on standard dilution plating techniques). This suspension was used as is or after a 1 : 100 or 1 : 1000 dilution with sterile water. For isolation of cells grown *in planta*, unifoliolate or trifoliolate leaves of bean cv. Red Kidney plants were forcibly sprayed with bacterial suspensions prepared at OD<sub>600nm</sub> of 0.10 (approximately 7 × 10<sup>7</sup> cfu/ml). Reaction of bean cv. Red Kidney to selected bacterial strains was also determined. Pods were surface sterilized by soaking for 2 min in commercial bleach diluted 1 : 10 with water, rinsed twice with sterile water and let to air-dry under a sterile transfer hood. Bacterial suspensions containing approximately 10<sup>8</sup> cfu/ml were prepared in sterile water using overnight cultures grown on PAF at 28 °C. Droplets of inoculum were placed on the pods, three per pod, and a 26 gauge sterile needle was jabbed once through the droplets into the underlying tissue. Inoculated pods were incubated at room temperature under high humidity.

To determine the ability of strains to induce a hypersensitive response, leaf panels of tobacco (*Nicotiana tabacum* L.) cv. Turk were injected with bacterial suspensions containing approximately 7 × 10<sup>8</sup> cfu/ml (prepared as described above) using a 1 cc syringe fitted with a 26 gauge, 3/8 inch needle.

## Analytical methods

All reagents were supplied by Sigma Chemical Co. Protein was determined by a modified Lowry method (MARKWELL *et al.* 1978) with bovine serum albumin as standard. Total carbohydrate was determined by the method of DUBOIS *et al.* (1956) with D-glucose as standard, and uronic acid content was determined by the procedure of BLUMENKRANTZ and ASBOE-HANSEN (1973) with D-mannurono-3,6-lactone as the standard. Acetyl content was determined by the method of MCCOMB and MCCREARY (1957) with glucose pentaacetate as the standard. 3-deoxy-D-manno-2-octulosonic acid (KDO) was determined by the method of YORK *et al.* (1985) with commercial KDO as standard, and the method of WRIGHT and REBERS (1972) was used for heptose determination with sedoheptulose anhydride monohydrate as standard. Total phosphorus was determined by the method of BARTLETT (1959) with  $\text{KH}_2\text{PO}_4$  as the standard. Hexosamine content was determined by the method of JOHNSON (1971) after acid hydrolysis (6 M HCl, 100 °C, 3 h) and neutralization (10 N NaOH) with D-glucosamine as the standard.

Identification of amino sugars and amino acids was determined using a Beckman 119CL amino acid analyzer. Samples were acid-hydrolyzed (4 M HCl, 4 h, 100 °C) in sealed evacuated tubes. After hydrolysis of free lipid A samples, fatty acids were removed by extraction with diethyl ether. Hydrolyzates were taken to dryness at 40 °C with a rotary evaporator and residues dissolved in sodium citrate starting buffer at pH 2.2.

Monosaccharide composition was determined by gas-liquid chromatography (GLC) using a Hewlett-Packard 5880 gas chromatograph fitted with a 15-m SP-2330 (Supelco) capillary column. The column was temperature programmed from 125 to 225 °C at 4 °C per min. Samples were acid hydrolyzed (1 M  $\text{H}_2\text{SO}_4$ , 100 °C, 1.5 h), neutralized with barium carbonate, and the aldonitrile acetate derivatives prepared according to VARMA *et al.* (1973).  $^{13}\text{C}$  nuclear magnetic resonance spectroscopy ( $^{13}\text{C}$ -NMR) analyses were done as previously described (OSMAN *et al.* 1988).

For total fatty acid analysis samples of free lipid A were hydrolyzed and volatile methyl ester derivatives prepared as described by MAYER *et al.* (1985). The fatty acid methyl esters were separated by GLC using a 15-m SPB-1 column (Supelco Inc.) as described by WELLS and MOLINE (1990) and were identified by comparison of retention time with standards in commercial (Hewlett-Packard, Avondale, PA and Supelco Inc., Bellefonte, PA) mixtures of fatty acid methyl esters.

## Cell surface hydrophobicity and charge

Cell surface hydrophobicity was measured by hydrophobic interaction chromatography, adherence to nitrocellulose filters, and adherence to xylenes. Cell surface charge was determined by adherence to hydroxylapatite as previously described (FETT 1985). Strains were grown for 24 h on nutrient dextrose yeast extract agar (NDY) (Difco nutrient broth, 8 g; Difco dextrose, 10 g; Difco yeast extract, 5 g; Bacto agar, 15 g; per L) plus the appropriate antibiotics at 28 °C.

Cells of strains NPS3121 and NPS4005 prepared as for hydrophobic interaction chromatography assays were also examined for the presence of fimbriae by transmission electron microscopy. Bacterial cells in suspension were fixed by addition of glutaraldehyde to 1 %. Drops of fixed cell suspensions were deposited on Formvar coated copper grids. After 30 seconds, grids were washed by addition of 5 to 10 drops of 1 % uranyl acetate solution. Grids were then air dried and examined with a Zeiss 10B transmission electron microscope operating at 60 kV at a magnification of 20,500.

## Screening for exopolysaccharide (EPS) production

*P. s. pv. phaseolicola* strain NPS3121 and the Tn5 mutants were examined for their ability to produce EPS both on solid and in liquid media. For assays using solid media, strains were streaked onto the surface of NDY, modified Vogel and Bonner medium (MVBM) (VOGEL and BONNER 1956) prepared according to CHAN *et al.* (1984) and containing D-gluconate (214 mM) as the sole carbon source, 3 mM  $\text{Mg}^{2+}$  and 15 g/l of agar, Difco nutrient agar plus sucrose (50 g/l) (NSA), Difco MacConkey agar, PAF, and a semi-synthetic medium described by BRUEGGER and KEEN (1979) with glucose at 24 g/l (B-K + G) as the primary carbon source and potassium phosphate in place of yeast extract. Cultures were incubated at 20 °C or 28 °C and were examined daily for 7 days. For assays using liquid medium, MVBM with either D-gluconate or D-glucose (both at 214 mM) as the carbon

*Table 2*  
Cell surface hydrophobicity of *Pseudomonas syringae* pv. *phaseolicola*  
as determined by hydrophobic interaction chromatography

Strain	Mutant class <sup>a</sup>	<i>hrp</i> locus	% absorption to gel <sup>b</sup>
NPS3121	Wild-type	Wild-type	58 <sup>ab</sup>
NPS4000	I	<i>hrpA</i> <sup>d</sup>	67 <sup>a</sup>
NPS4001	I	<i>hrpE</i> <sup>d</sup>	57 <sup>ab</sup>
NPS4002	I	<i>hrpD</i> <sup>d</sup>	55 <sup>ab</sup>
NPS4003	II	<i>hrpF</i> <sup>d</sup>	48 <sup>b</sup>
NPS4004	I	<i>hrpD</i> <sup>d</sup>	58 <sup>ab</sup>
NPS4005	III	<i>hrpM2</i> <sup>c</sup>	30 <sup>c</sup>
NPS4006	I	<i>hrpS</i> <sup>df</sup>	54 <sup>ab</sup>
NPS4007	I	<i>hrpE</i> <sup>d</sup>	51 <sup>b</sup>

<sup>a</sup> Phenotypic classes based on plant reactions according to LINDGREN *et al.* 1986.

<sup>b</sup> Values are means of data from three separate experiments with three repetitions per experiment.

<sup>c</sup> Means followed by different letters are significantly differed ( $P = 0.05$ ) as determined by the Banferroni t-test (MILLER 1981).

<sup>d</sup> According to RAHME *et al.* 1991.

<sup>e</sup> According to FREDERICK, R. D., 1990. Ph.D. Thesis, University of California-Berkeley.

<sup>f</sup> According to GRIMM and PANOPOULOS 1989.

source was dispensed into 300 ml sidearm flasks (Belco Glass, Inc.) with 75 ml per flask. Inoculum was prepared by suspending bacteria grown overnight on PAF at 28 °C in sterile water to give an optical density at 600 nm of 0.10, and 2 ml of this suspension was added per flask. Duplicate flasks per strain were shaken at 200 rpm at room temperature and growth was monitored by measuring the optical density at 600 nm. EPS produced on solid or in liquid medium was isolated after removal of cells by centrifugation. All samples were partially purified by dialysis against water at 4 °C followed by repeated acetone precipitation (3 volumes of ice-cold acetone, 3 X). Some EPS samples were further purified by the method of SUTHERLAND (1981). Before analysis by GLC, EPS samples high in uronic acid were first reduced with sodium borohydride via their carbodiimide adduct (OSMAN *et al.* 1986). Samples were then acid hydrolyzed, the appropriate derivatives prepared and neutral sugar composition determined by GLC (FETT and DUNN 1989).

#### Isolation, purification, and characterization of lipopolysaccharides from cells grown *in vitro*

Bacteria were grown to early stationary phase in B + K broth containing glucose (1 %). As excessive foaming and poor growth occurred in this medium for *P. s. pv. phaseolicola* strains HB-35 and R1, these two strains were cultured in the completely defined medium as described by ANDERSON (1984), with glucose (1 %) as the carbon source. Bacteria were grown in 1 L batches of liquid media contained in 2800 ml Fernbach flasks at room temperature with shaking (200 rpm). Cells were collected by centrifugation, washed once with distilled water or saline (adjusted to pH 7.0), and lyophilized. Lipopolysaccharide (LPS) was extracted from the dried cells with hot, aqueous phenol, and purified by repeated ultracentrifugation (100,000 × g; three times) (WESTPHAL and JANN 1965).

Lipid A was separated from polysaccharide (core and O-antigen) by hydrolysis in 1 % acetic acid for 2 h at 100 °C. The liberated lipid A was removed by centrifugation and CHCl<sub>3</sub> extraction and the water soluble and insoluble materials were lyophilized separately. Free lipid A was examined for the presence of hexosamines by colorimetric assay and by use of the amino acid analyzer as previously described.

The polysaccharide-containing water-soluble materials were fractionated on a column (2.5 × 49 cm) of Sephadex G-50 (Pharmacia) eluted with pyridine-acetate buffer pH 4.25 at 0.4 ml per min.

Fractions (2 ml) were analyzed for hexose and phosphorus content and fractions corresponding to peaks were combined and lyophilized. The neutral sugar composition of the carbohydrate containing peaks was determined by GLC.

### Lipopolysaccharide of *in planta* grown cells

Five to six days after inoculation of leaves of bean cv. Red Kidney with *P. s. pv. phaseolicola* strain At, inoculated leaves were detached, mid-ribs removed, and the remaining leaf tissue weighed and then vacuum infiltrated with distilled water. The excess fluid was collected and bacterial cells present sedimented by centrifugation. Additional bacteria still present in the intercellular leaf spaces were obtained by centrifugation as described by KLEMENT (1965). Sedimented cells were combined, put through a glass sintered funnel (medium grade) to remove contaminating plant material, washed three times with distilled water and lyophilized. LPS was extracted as described above.

### SDS-PAGE

SDS-PAGE analysis of purified LPS preparations followed the procedures of UCHIDA and MIZUSHIMA (1987) using a mini-gel system (Bio-Rad) except that the separation gels contained 4 M urea in order to increase the sharpness of the bands due to high molecular weight O-antigen repeating units (TSAI and FRASCH 1982). Also, stacking gel concentrations were sometimes increased from 4 to 5 %. One  $\mu\text{g}$  sample of LPS (based on dry weight) were loaded into sample wells. The buffer system used was according to LAEMMLI (1970). Electrophoresis was carried out at 30 mAmp. Silver staining was done as described by TSAI and FRASCH (1982) except LPS was oxidized in periodic acid solution for 15 to 20 min instead of 5 min. All reagents were prepared just before use. Staining was stopped by washing gels with 5 % acetic acid solution. Commercial (Calbiochem-Behring) LPS preparations from *Escherichia coli* 0111 : B4 (smooth LPS type) and *Salmonella minnesota* Re595 (deep rough LPS type) were also run for comparison.

## Results

### Plant reactions

*P. s. pv. phaseolicola* wild-type strain NPS3121 was virulent on leaves of cvs. Red Kidney, Improved Tendergreen and Red Mexican UI34. Water-soaked lesions and localized chlorosis resulted using the two inoculation procedures at  $7 \times 10^5$  and  $7 \times 10^8$  cfu/ml. This strain produced more symptoms on leaves of cvs. Red Kidney and Improved Tendergreen than on cv. Red Mexican UI34. The results indicate that this strain belongs to physiologic race 2 and not race 1 as initially reported (LINDGREN *et al.* 1986). The Tn5 mutant strains were only tested against cvs. Red Kidney and Red Mexican UI34. Strains NPS4003 and NPS4005 were of reduced virulence on cv. Red Kidney as reported by LINDGREN *et al.* (1986) with NPS4005 causing the formation of water-soaked lesions only at the higher inoculum concentration and only when forcibly sprayed into the leaf intercellular spaces. However, on cv. Red Mexican, NPS4003 was of similar virulence as NPS3121. Once again, NPS4005 only caused water-soaked lesions when the higher inoculum concentration was forcibly sprayed into the leaf intercellular spaces. The other Tn5 mutants were not virulent on either cultivar under all the experimental conditions tested in agreement with the earlier results of LINDGREN *et al.* (1986). These strains caused either no symptoms or slight chlorosis except for NPS4006 which caused slight browning of the infiltrated area on leaves of cv. Red Kidney, but only when the inoculum of higher concentration was forcibly sprayed. The ability of the additional *P. s. pv. phaseolicola* strains to

cause disease on bean was determined only on leaves of cv. Red Kidney and all were found to be virulent.

The two *P. s. pv. syringae* strains (1147 and 1542) were tested for virulence on leaves and pods of bean cv. Red Kidney. Inoculum containing approximately  $7 \times 10^5$  cfu/ml was forcibly sprayed into leaves. Both strains caused the formation of brown, non-watersoaked areas on inoculated leaves by 48 h after inoculation. On pods, the tissue at the inoculated sites was reddish-brown and sunken by 24 h after inoculation. No change occurred in the additional 8 day incubation period. As controls, *P. s. pv. phaseolicola* strains At and HB-35 induced the formation of water-soaked lesions on pods first evident by 48 h after inoculation.

Only *P. s. pv. phaseolicola* strain NPS3121 and the Tn5 mutants were tested for ability to induce an HR on tobacco. NPS3121 caused an HR on leaves of tobacco cv. Turk with significant collapse of tissue in the infiltrated area evident by 24 h after injection. None of the Tn5 mutants induced an HR on tobacco. The results agreed with those of LINDGREN *et al.* (1986).

Reaction of the strains on the test plants was tested periodically throughout the experimental period and no changes were noted.

#### Cell surface properties

The *P. s. pv. phaseolicola* NPS strains were examined for cell surface hydrophobicity and charge by a variety of assays. Hydrophobic interaction chromatography indicated that Tn5 mutant strain NPS4005 had a less hydrophobic surface than the wild-type parent strain NPS3121 and the other Tn5 mutants examined (Table 2). The lower cell surface hydrophobicity of strain NPS4005 was confirmed by the xylene adherence and nitrocellulose filter assays (data not shown). No significant differences among the NPS strains in cell surface charge were demonstrated by measuring adherence to hydroxylapatite (data not shown).

Cells of strains NPS3121 and NPS4005 prepared as for hydrophobic interaction chromatography assays were examined for the presence of pili by electron microscopy. No pili were noted for either strain.

#### EPS production

The wild-type parent *P. s. pv. phaseolicola* strain NPS3121 and all eight of the Tn5 mutants were first screened for the ability to produce the neutral EPS levan on a sucrose-containing agar medium. All NPS strains produced domed, mucoid colonies on NSA medium indicative of the production of levan (FETT *et al.* 1989). After two to three days incubation at 28 °C colonies of NPS4005 were more mucoid than colonies of NPS3121 and the additional Tn5 mutants and this held constant for the rest of the 7 day incubation period. EPS produced under these conditions by strains NPS3121, NPS4000, NPS4003 and NPS4005 was isolated and partially purified by dialysis followed by acetone precipitation. EPS produced by strain NPS4005 gave a stringy precipitate after addition of acetone, while the EPS produced by the other three strains gave a granular precipitate. EPS of NPS4005 contained 10 % uronic acid while the EPS samples from the other

three strains had 4 % or less based on colorimetric assay. Analysis of oxalic acid-hydrolyzed EPS samples by GLC indicated that all samples had a levan content of 70 % or higher based on comparison with commercial levan except for the preparation from NPS4005 which contained only 59 %. Samples did not contain other neutral sugars based on GLC analysis of H<sub>2</sub>SO<sub>4</sub>-hydrolyzed samples. Neutral and acidic EPS in the NPS4005 sample were separated by selective precipitation of acidic EPS with cetyltrimethylammonium bromide (CTMAB) (Aldrich) (FETT and DUNN 1989). The non-CTMAB precipitable material accounted for 44 % (by weight) of the original sample and CTMAB precipitable 34 % with the other 22 % unaccounted for. The non-CTMAB precipitable material was shown to consist of levan by GLC analysis. The CTMAB-precipitable material was reduced and shown to be composed of a polymannuronan (alginate). This indicates that strain NPS4005 produced both levan and alginate as EPS's when grown on NSA.

The NPS strains were screened for ability to produce mucoid growth on a variety of solid media, all of which did not contain sucrose. All of the NPS strains gave mucoid growth only on MVBM agar medium with gluconate as the carbon source. We previously demonstrated that this medium promotes alginate production by *P. syringae* pathovars (FETT *et al.* 1986). *P. s. pv. phaseolicola* strains NPS3121, NPS4000, NPS4003, NPS4005 and NPS4006 were also tested for ability to produce EPS in liquid MVBM with either gluconate or glucose as carbon source. All five strains had doubling times of approximately 2.5 h. Yields of purified EPS from cultures grown in MVBM plus gluconate ranged from 0.35 to 0.73 mg/ml with strain NPS4005 giving the highest yield. EPS production was much reduced for all five strains when grown with glucose as carbon source. Under these conditions, strain NPS3121 gave the highest yield (0.26 mg/ml) of purified EPS with yields for the other four NPS strains ranging from 0.06 to 0.13 mg/ml. All purified samples had uronic acid values exceeding 40 % based on colorimetric assay. The EPS produced in MVBM with gluconate was examined further. Analysis of reduced samples by GLC indicated that the samples were alginates composed primarily of mannuronic acid with low levels of guluronic acid (2 to 5 % based on relative peak areas of mannose and gulose). The alginates were acetylated containing 8 to 10 % acetate by weight as determined by colorimetric assay.

#### Lipopolysaccharide composition

None of the Tn5 mutants of *P. s. pv. phaseolicola* strain NPS3121 exhibited an altered colony morphology on solid media. However, *a priori* this did not rule out the possible absence of O-antigen or an alteration of O-antigen composition (KOVAL and MEADOW 1977). The Tn5 mutants grew equally well in the semi-synthetic B + K liquid medium with doubling times of approximately 3 h. Yields (based on cell dry weight) of whole LPS of *P. s. pv. phaseolicola* strain NPS3121 and the Tn5 mutants grown *in vitro* ranged from 3.3 to 6.5 % (Table 3). All LPS samples contained amino sugar, heptose, KDO and phosphorus. Lipid A content ranged from 10.6 to 27.3 % and showed considerable variability when two LPS preparations from a single strain were examined (Table 3). Protein levels were

Table 3

Composition of lipopolysaccharides from *Pseudomonas syringae* pv. *phaseolicola* wild-type strain NPS3121 and its Tn5 mutants

Component	% by weight								
	NPS 3121	NPS 4000	NPS 4001	NPS 4002	NPS 4003	NPS 4004	NPS 4005	NPS 4006	NPS 4007
Yield of whole LPS <sup>a</sup>	6.5 ± 0.4 <sup>b</sup>	5.6	4.6	3.7	3.3	6.2	6.0 ± 1.1	4.3	6.4
Hexose	25.5 ± 2.1	23	22	26	23	29	27.8 ± 2.8	21	27
Amino sugar	5.9 ± 2.3	6.0	11.7	12.2	11.3	10.4	7.0 ± 2.8	6.3	8.1
Heptose	2.1 ± 0.4	1.7	2.2	2.4	1.2	3.4	1.7 ± 0.1	1.2	3.4
KDO	2.5 ± 0.1	2.3	2.6	2.7	3.2	1.7	3.0 ± 1.5	2.1	1.7
Phosphorus	4.0 ± 0.5	3.9	4.0	4.6	3.9	3.3	3.9 ± 0.6	3.4	3.6
Lipid A	18.6 ± 4.7	20.0	19.0	27.3	11.1	18.5	17.2 ± 8.1	10.6	22.1

<sup>a</sup> Based on cell dry weight.

<sup>b</sup> Values are for single preparations except where average values followed by standard deviations are given.

4 % or less with uronic acid values less than 2 %. No nucleic acid contamination was present as determined by UV spectroscopy.

Yields of LPS obtained from the other *P. s.* pv. *phaseolicola* strains grown *in vitro* ranged from 2.7 to 7.5 % (based on cell dry weight). Total hexose was 22 to 37 %, lipid A content 12 to 27 %, with protein and uronic acid contents of less than 2 %. No nucleic acid contamination was present. For the two *P. s.* pv. *syringae* strains, yields of purified LPS ranged from 1.6 to 2.3 %. Total hexose ranged from 49 to 52 %, lipid A content was 15 % and uronic acid was less than 2 %. No contamination with protein or nucleic acid was detected. KDO, heptose, amino sugar and phosphorus contents of these preparations were not determined.

Results of GLC analyses for determining the neutral sugar composition of the polysaccharide released from LPS by mild acetic acid hydrolysis indicated that the samples from *P. s.* pv. *phaseolicola* strain NPS3121 and the Tn5 mutants, had similar rhamnose : fucose : glucose ratios (1 : 0.22 to 0.31 : 0.10 to 0.21). The LPS polysaccharide samples from the additional strains of *P. s.* pv. *phaseolicola* and from *P. s.* pv. *syringae* all showed neutral sugar composition similar to NPS3121.

All LPS polysaccharide samples, except those from strains NPS4000, NPS4003 and NPS4006, were fractionated by gel filtration. An elution profile which was typical for all LPS polysaccharide preparations from *P. s.* pv. *phaseolicola* samples tested is shown in Figure 1. Three carbohydrate- and phosphorus-containing peaks were detected. Material comprising Peak I was composed primarily of carbohydrate with low amounts of phosphorus. Materials comprising Peaks II and III were higher in phosphorus and lower in carbohydrate. LPS polysaccharide from the two *P. s.* pv. *syringae* strains did not exhibit peak II. To determine if a very low amount of material was actually present in this region for LPS polysaccharide of *P. s.* pv. *syringae* strain CFBP1542, fractions

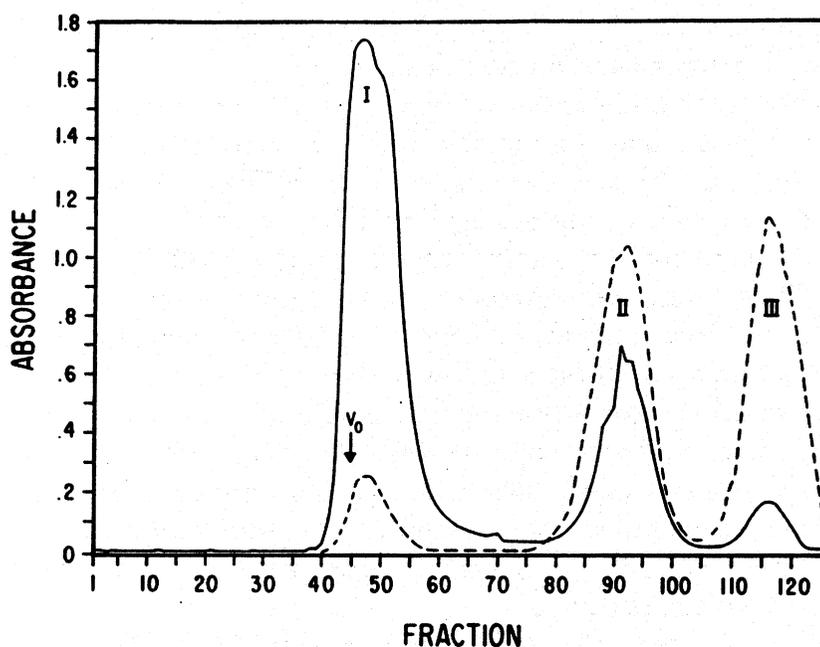


Fig. 1. Fractionation of polysaccharides obtained after acetic acid hydrolysis of lipopolysaccharide of *P. syringae* pv. *phaseolicola* strain NPS3121. Material was eluted from the column of Sephadex G-50 ( $2.5 \times 49$  cm) with pyridine-acetate buffer, pH 4.25. Fractions (2 ml) were analyzed for hexose ( $A_{490 \text{ nm}}$ ) (—) and phosphate ( $A_{830 \text{ nm}}$ ) (---) content

corresponding to those found to constitute Peak II for the *P. s.* pv. *phaseolicola* strains were combined and lyophilized. A low amount of carbohydrate-containing material was found.

GLC analysis to determine neutral sugar composition of the peak I material demonstrated the presence of rhamnose and fucose in an approximate 4 : 1 to 5 : 1 ratio for all strains. Glucose was completely absent or present in very low amounts. Peak II material, including the material from *P. s.* pv. *syringae* strain CFBP1542, had glucose to rhamnose ratios of 1 : 0.4 to 1 : 1 with no fucose present. The peak materials from NPS3121 were subjected to further examination. Peak I material contained KDO and hexosamine at 0.2 and 1.8 % by weight, respectively, while peak II material contained 0.3 and 8.1 % by weight, respectively. The peak III material contained 8.1 % KDO, but was not examined for hexosamine content. Analysis of the peak II and peak III materials by use of an amino acid analyzer indicated the presence of the following: peak II — galactosamine (6.2 %), glucosamine (5.5 %) and alanine (2.5 %); peak III — glucosamine-6-phosphate (3.5 %), glucosamine (1.0 %), ethanolamine (0.55 %), galactosamine (0.2 %) and alanine (0.10 %). The peak II fractions from strains R1 and R2 were also found to have a similar composition to the peak II fractions from strain NPS3121. Based on these results, our previous results for LPS of strain NPS3121 (OSMAN *et al.* 1988) and results of others for enteric bacteria (MAYER *et al.* 1985), these three peaks were assumed to correspond to core polysaccharide substituted with O-antigen (Peak I), unsubstituted core polysaccharide (Peak II) and released phosphate, KDO and monomers (Peak III).

$^{13}\text{C}$ -NMR analyses of the peak I fractions from *P. s.* pv. *syringae* strains NPS3121, NPS4005, R1, HB-35, At, R2 and *P. s.* pv. *syringae* strain CFBP1542

were identical indicating that the O-antigen portion of these LPS molecules are highly similar, if indeed not identical.

The composition of isolated lipid A from some of the strains was examined. The free lipid A isolated from the LPS of *P. s. pv. phaseolicola* strains NPS3121 and NPS4005 and *P. s. pv. syringae* strains CFPB1147 and 1542 were similar in fatty acid composition containing 3-OH 10 : 0 (19 to 22 %), 12 : 0 (19 to 23 %), 2-OH 12 : 0 (16 to 17 %) and 3-OH 12 : 0 (35 to 38 %). The free lipid A from strain NPS3121 also was shown to contain glucosamine.

SDS-gel electrophoresis of whole LPS from *in vitro* grown cells gave similar banding patterns for all NPS strains with a rapid moving wide band near the dye front and multiple slower moving narrow bands in a ladder pattern with no bands of intermediate mobility (not shown). Based on previous studies with enteric bacteria (GOLDMAN and LEIVE 1980) the fast moving band of low molecular weight can be assumed to correspond to unsubstituted core and the slower moving bands of higher molecular weight to core substituted with increasing numbers of O-antigen units.

Cells of *P. s. pv. phaseolicola* strains NPS3121, NPS4005 and At were also recovered from inoculated leaves of compatible bean cv. Red Kidney five to six days after inoculation. At this time leaves inoculated with strains NPS3121 and At were showing extensive water-soaked lesions surrounded by yellow-green chlorosis. Strain NPS4005 was less virulent and gave only scattered water-soaked lesions. The amount of cells recovered from leaves inoculated with strain NPS3121, NPS4005 and At were 3.4, 0.02 and 1.6 mg dry wt/g fresh wt of tissue, respectively. LPS was extracted from dried cells of *in planta* grown strain At and purified resulting in a yield of purified LPS of 3.4 %. GLC analysis of the sugar components of LPS polysaccharide obtained from strain At after mild acid hydrolysis indicated the presence of rhamnose, fucose and glucose in the relative ratio 1 : 0.24 : 0.12, respectively, similar to the composition of *in vitro* grown cells of this strain. LPS isolated from *in vitro* and *in planta* grown *P. s. pv. phaseolicola* strain At gave similar banding patterns to each other and to the LPS of strain NPS3121 grown *in vitro* upon SDS-PAGE (not shown).

## Discussion

Examination of the physicochemical properties of the transposon-generated mutants of *P. s. pv. phaseolicola* strain NPS3121 indicated that only mutant strain NPS4005 had an altered cell surface being less hydrophobic. The mutants analyzed in this study have been placed in six different *hrp* genes/operons by complementation analysis (RAHME *et al.* 1990, FREDERICK 1990). It is interesting to note that the NPS4005 mutation maps in a separate locus, *hrpM*, which is unlinked to the other *hrp* genes (LINDGREN *et al.* 1986, FREDERICK and PANOPOULOS 1989). The *hrpM* locus is highly homologous with the *hrpM* locus cloned from *P. s. pv. syringae* by NIEPOLD *et al.* (1985). This locus contains two open reading frames, ORF 1 and ORF 2, the second of which is inactivated by the NPS4005 mutation (FREDERICK 1990). Sequence and Tn*phoA* fusion analysis

of the *hrpM* ORF 2 suggests that the NPS4005 mutation affects a transmembrane protein, possibly involved in the uptake of nitrogenous compounds (MUKHOPADHYAY *et al.* 1988, MILLS and MUKHOPADHYAY 1990, FREDERICK 1990).

The biochemical basis of the altered properties of strain NPS4005 remains unknown. The lowered cell surface hydrophobicity of this mutant appears not to be due to an increased ability of strain NPS4005 to produce EPS *in vitro* when grown on dextrose as the primary carbon source. None of the NPS strains exhibited mucoid growth on NDY agar medium, the medium used to culture the strains for use in the hydrophobicity assays, and strain NPS4005 did not produce more EPS (alginate) than did parent strain NPS3121 in liquid medium with glucose. In addition, the presence of increased levels of tightly bound acidic EPS should have led to a higher negative cell surface charge. Such a change was not detected. Altered LPS chemotype also does not appear to be responsible for the decrease in cell surface hydrophobicity of strain NPS4005.

Another cell surface component that affects surface hydrophobicity is pili (FARIS *et al.* 1981). No pili were detected on cells of the NPS strains after growth on NDA for 24 h by transmission electron microscopy. In general, production of pili is favored by growth in broth under static, aerobic conditions (OLD and DUGUID 1970, STEMMER and SEQUEIRA 1987).

Based on recent molecular genetic studies indicating that NPS4005 may be defective in a transmembrane protein, it is possible that the lowered cell surface hydrophobicity of this strain is due either directly or indirectly to the absence of this membrane protein. Outer membrane proteins can be exposed on the bacterial surface (MUTHARIA and HANCOCK 1983) and may contribute to cell surface hydrophobicity (BAR-NESS *et al.* 1988).

The altered *in planta* phenotype of the transposon mutants appears not to be due to alterations in the inherent ability of these mutants to produce EPS. All mutant strains were able to produce both levan and alginate. Relative amounts of EPS produced by the parent and mutant strains *in vitro* was dependent on culture conditions. Alginate samples were uniformly low in guluronic acid content and were acetylated similar to previously characterized alginates from *P. s. pv. phaseolicola* (FETT *et al.* 1986, GROSS and RUDOLPH 1987a). The findings do not rule out mutations in regulatory genes which may affect EPS production solely *in planta*.

Gross differences in LPS composition also do not appear to be responsible for the altered phenotype of the *P. s. pv. phaseolicola* mutant strains. Preparations of polysaccharide from the LPS of mutant strains of *P. s. pv. phaseolicola* were similar to that of parent strain NPS3121. Polysaccharide preparations gave the same elution profiles upon gel filtration as found for a different strain of *P. s. pv. phaseolicola* by GROSS *et al.* (1988). Gel filtration elution profiles obtained from chromatography of LPS polysaccharide preparations from the two strains of *P. s. pv. syringae* were different in that they did not give a detectable peak II, but further analysis of the fractions in this region indicated the presence of a low amount of material containing glucose and rhamnose. This indicates that these two strains have a lower amount of unsubstituted core oligosaccharide than do *P. syringae* *pv. phaseolicola* strains so far examined.

Core oligosaccharide structure may be conserved among *P. syringae* pathovars. Our data, and that of GROSS *et al.* (1988), indicate that the core oligosaccharide of *P. s.* pv. *phaseolicola* contains glucose, rhamnose, galactosamine, glucosamine, alanine, heptose, KDO and phosphorus. The same components were reported for the core oligosaccharide of *P. s.* pv. *morsprunorum* (SMITH *et al.* 1985). Lipid A structure may also be conserved with glucosamine and the fatty acids 3-OH 10 : 0, 12 : 0, 2-OH 12 : 0 and 3-OH 12 : 0 present for *P. s.* pv. *phaseolicola* (GROSS *et al.* 1988; this study) and pv. *morsprunorum* (ZAMZE *et al.* 1985).

O-antigen structure appears to be much more variable among the *P. syringae* pathovars. SAUNIER and SAMSON (1987) examined 27 pathovars of *P. syringae* including 96 strains of *P. s.* pv. *syringae* for the presence of serogroups. Seven serogroups were identified based on antibodies directed against LPS (GUILLORIT and SAMSON 1987). All 31 strains of *P. s.* pv. *phaseolicola* fell into the same serogroup, but the *P. s.* pv. *syringae* strains were very heterogeneous, with individual strains falling into six of the seven serogroups with no correlation with host of origin. Two strains of *P. s.* pv. *syringae* included in this study fell into the *P. s.* pv. *phaseolicola* serogroup. Our results confirm that the O-antigen of these two strains is the same or very similar to that of *P. s.* pv. *phaseolicola*. KNIREL and associates (KNIREL *et al.* 1988a and 1988b) determined the O-antigen structure of three strains of *P. s.* pv. *syringae*. The O-antigen of all three strains has a rhamnan backbone. Two strains contained D-rhamnose and D-fucose in a 3 : 1 ratio and the third strain contained L-rhamnose and 3-acetamido-3-deoxyfucose in a 4 : 1 ratio. KNIREL *et al.* (1986) also reported that strains of *P. s.* pv. *glycinea*, pv. *pisi* and pv. *atofaciens* have identical O-antigens agreeing with the serological groupings of SAUNIER and SAMSON (1987) and indicating that O-antigen structure does not determine host specificity. O-antigen structure also does not appear to be related to host cultivar specificity of physiologic races of *P. s.* pv. *phaseolicola*, as shown in this study, or of *P. s.* pv. *glycinea* (BARTON-WILLIS *et al.* 1987).

A reduction in length or a complete absence of O-antigen for animal and human bacterial pathogens after growth *in situ* or exposure to host components has been reported (HANCOCK *et al.* 1983, KELLY *et al.* 1989, PARSONS *et al.* 1988). However, we observed that LPS of *in planta* grown *P. s.* pv. *phaseolicola* strain At did not exhibit a different banding pattern when compared to LPS from *in vitro* grown cells, similar to results for *Erwinia carotovora* (PIRHONEN *et al.* 1988). Analysis of the composition of the LPS produced *in planta* by strain At also did not indicate any significant alterations in chemotype.

In conclusion, an altered bacterial cell surface leading to a lowered cell surface hydrophobicity may be responsible or may contribute to the unique phenotype of *P. s.* pv. *phaseolicola* mutant strain NPS4005. The altered cell component that is responsible for this change has not yet been identified. Further studies on the comparison of cell surface components of the wild-type and mutant strains are needed. Proteinaceous components may be involved based on the results of EL-KADY *et al.* (1986) who used crossed immunoelectrophoresis to demonstrate antigenic differences among a similar set of *hrp* mutants of *P. s.* pv.

*phaseolicola* and the wild-type. However, DEASEY and MATTHYSSE (1988) did not detect any differences in surface proteins between the wild-type and *hrp* mutants of an additional strain of *P. s. pv. phaseolicola*.

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