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Inhibition of *Rhizobium etli* Polysaccharide Mutants by *Phaseolus vulgaris* Root Compounds

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Crude bean root extracts of *Phaseolus vulgaris* were tested for inhibition of the growth of several polysaccharide mutants of *Rhizobium etli* biovar phaseoli CE3. Mutants deficient only in exopolysaccharide and some mutants deficient only in the O-antigen of the lipopolysaccharide were no more sensitive than the wild-type strain to the extracts, whereas mutants defective in both lipopolysaccharide and exopolysaccharide were much more sensitive. The inhibitory activity was found at much higher levels in roots and nodules than in stems or leaves. Inoculation with either wild-type or polysaccharide-deficient *R. etli* did not appear to affect the level of activity. Sequential extractions of the crude root material with petroleum ether, ethyl acetate, methanol, and water partitioned inhibitory activity into each solvent except methanol. The major inhibitors in the petroleum ether and ethyl acetate extracts were purified by C_{18} high-performance liquid chromatography. These compounds all migrated very similarly in both liquid and thin-layer chromatography but were distinguished by their mass spectra. Absorbance spectra and fluorescence properties suggested that they were coumestans, one of which had the mass spectrum and nuclear magnetic resonances of coumestrol. These results are discussed with regard to the hypothesis that one role of rhizobial polysaccharides is to protect against plant toxins encountered during nodule development.

Deficiencies of certain bacterial polysaccharides prevent development of functional symbiosis between legumes and bacteria of the genus *Rhizobium*. Two of the types of rhizobial polysaccharides that have received attention with regard to symbiosis are exopolysaccharide (EPS) and lipopolysaccharide (LPS).

LPS consists of a lipid portion that anchors it to the bacterial outer membrane and a polysaccharide portion composed of an inner (core) region linked to the lipid and an outer region, the O-polysaccharide or O-antigen (OPS), which extends into the environment. LPS mutants of various *Rhizobium* and *Bradyrhizobium* species are unable to complete the infection process with their respective host plants (9, 10, 22, 24, 27, 28). For example, *Rhizobium etli* biovar phaseoli mutants deficient in OPS (Ops$^-$) elicit fewer nodules than a wild-type strain on bean roots. In the nodules that are elicited, infections are arrested such that the nodules do not differentiate normally and bacteria are not released into plant cells (24).

EPSs are secreted beyond the outer membrane (22). Studies with EPS-deficient rhizobial mutants have led to the generalization that production of the major acidic EPS by the microsymbiont is required for infection in hosts that have indeterminate nodule development but not in hosts with determinate nodule development (1, 11, 22). Indeed, in bacteroids on the determinate-nodulating bean, the production of EPS appears to be repressed (18).

A number of roles in symbiotic development have been proposed for these polysaccharides, ranging from serving as molecular signals to aiding in attachment to plant surfaces. In order to prevent arrested infection on bean, *Rhizobium etli* must have abundant OPS; i.e., even a mutant having 40% of the normal amount of OPS is defective in nodulation (6, 22). The OPSs that are effective vary considerably in structure (2, 5). Although other hypothetical roles of OPS certainly are not ruled out, one hypothesis that is consistent with these observations is that the OPS protects the bacteria from host compounds encountered during infection. In this role, specific polysaccharide residues might not be required, but OPS abundance could be critical in providing an unbroken barrier to the entry of toxic compounds into the bacteria.

A number of studies appear to support such a role for the polysaccharide portion of LPS in enteric bacteria. *Salmonella typhimurium* mutants deficient in most of the saccharide residues of the LPS are much more sensitive than the wild type to hydrophobic antibiotics and dyes. The truncated LPS and accompanying rearrangements of outer membrane components are believed responsible for the increased permeability toward hydrophobic substances in these mutants (21). *Escherichia coli* K-12 mutants defective in the core oligosaccharide of LPS are more sensitive than the wild type to antibiotics and lysozyme (33). Likewise, increased penetration of gentian violet in *E. coli* K-12 mutants has been attributed to loss of glucose and galactose residues from the outer portion of the LPS core oligosaccharide (13).

Plants defend themselves against herbivores and pathogens in part by synthesizing toxic compounds. Well-known examples are phytoalexins, compounds with low molecular weights produced by plants as part of defense responses to potential pathogens, including at least some fungi (30) and bacteria (19).

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TABLE 1. *R. etli* biovar phaseoli strains

Strain	Characteristics[a]	Reference
CFN42	Wild isolate Ndv$^+$ Fix$^+$	29
CE3	*str-1* Ops$^+$ Exo$^+$ Ndv$^+$ Fix$^+$	23
CE109	*str-1 lps-109*::Tn*5* Ops$^-$ Ndv$^-$	23
CE113	*str-1 lps-113*::Tn*5* Ops$^-$ Ndv$^-$	23
CE121	*str-1 lps-121*::Tn*5* Ops$^-$ Ndv$^-$	6
CE125	*str-1 lps-125* Ops$^-$ Ndv$^-$	6
CE166	*str-1 lps-166*::Tn*5* Ops$^{+/-}$ Ndv$^-$	6
CE309	*str-1 lps-309*::Tn*5* Ops$^-$ Ndv$^-$	6
CE320	*str-1 exo-320*::Tn*5* Exo$^-$ Ops$^-$	11
CE338	*str-1 exo-338*::Tn*5* Exo$^-$ Ndv$^+$ Fix$^+$	11
CE339	*str-1 exo-339*::Tn*5* Exo$^-$ Ndv$^+$ Fix$^+$	11
CE343	*str-1 exo-343*::Tn*5* Exo$^-$ Ops$^-$ Ndv$^-$ Fix$^-$	11
CE358	*str-1 lps-2*::Tn*5* Ops$^-$ Ndv$^-$	7
CE359	*str-1 lps-359* Tn*5*[b] Ops$^-$ Ndv$^-$	34
CE388	*str-1 lps-125 exo-339*::Tn*5* Ndv$^-$	This work

[a] Exo$^+$, mucoid colony phenotype; Exo$^-$, nonmucoid; Ops$^-$, lacking the OPS of strain CFN42; Ops$^{+/-}$, producing 40% of the normal number of LPS molecules that carry OPS; Ndv$^+$, normal nodule development; Ndv$^-$, eliciting underdeveloped nodules; Fix$^+$, providing nitrogenase activity in nodules; *str-1*, conferring streptomycin resistance.

[b] It has not been demonstrated that the Tn*5* insertion is responsible for the *lps* mutation of this strain.

In the Leguminosae, most known phytoalexins are isoflavonoid derivatives (17, 39). Some isoflavonoids isolated from legumes have been found to inhibit the growth of *Rhizobium* strains (25), but variation in sensitivity was found, depending on the compound and bacterial species tested.

If *R. etli* OPS serves as a barrier against phytoalexins or constitutive toxins encountered during symbiosis, then it might be possible to extract the toxins from roots or nodules in concentrations high enough to inhibit the growth of bacteria tested in vitro, and one would predict that mutants deficient in OPS would exhibit greater sensitivity than the wild type. The work reported herein tested this prediction and analyzed the chemical complexity of the inhibitory activity found in crude extracts of bean roots. This led to the purification of at least three apparently similar inhibitors and the identification of one as coumestrol.

MATERIALS AND METHODS

Bacterial strains and growth media. Bacterial strains are listed in Table 1. Strain CE3 (23) is a spontaneous streptomycin-resistant derivative of *R. etli* wild isolate CFN42 (29). Strain CE388 was constructed by homologous recombination that substituted the *exo-339*::Tn*5* allele carried on pCOS338.1 (11) for the corresponding *exo*$^+$ locus in Ops$^-$ strain CE125. All other strains were derived from strain CE3 by localized or random Tn*5* mutagenesis as described previously (6, 7, 11, 23, 34). *R. etli* cultures were grown at 30°C in TY (rich) medium (37), YGM (glucose minimal) medium at pH 4.8 (34), or Y (succinate minimal) (37) adjusted to pH 5.5. Agar plates contained Bacto-agar (Difco Laboratories, Detroit, Mich.).

Plant growth. Plastic growth pouches (Vaughan's Seed Co., Downer's Grove, Ill.) were used for the growth of *Phaseolus vulgaris* cv. Midnight Black Turtle Soup (Johnny's Selected Seeds, Albion, Maine). Seeds were surface sterilized by immersion in commercial bleach (5% hypochlorite), rinsed several times in sterile water to remove the bleach, and germinated in sterile glass petri plates on water-moistened filter paper at 30°C for 2 days. Four seedlings were placed in each autoclave-sterilized pouch, containing 40 ml of nitrogen-free plant nutrient (38). Unless otherwise stated, the seedlings were then inoculated with 0.5 ml of bacterial mutant strain CE166 grown in TY and diluted 1:1 with water. This mutant exhibits the infection defect typical of OPS-deficient mutants (6). The plants were grown at 27°C in a chamber under warm fluorescent lights illuminated at approximately 2,800 lx for 16 h, followed by 8 h of darkness per day.

Extraction of inhibitory compounds from bean roots. The roots of 5-day-old bean plants were harvested, rinsed in deionized water, and wrung free of excess water. The roots were weighed, frozen in liquid nitrogen, ground with a mortar and pestle, and extracted as described by Stossel (32), with extensive modifications. Boiling 95% ethanol (4 ml/g of wet root) was added to the ground root and allowed to reflux for 2 h. The resulting suspension was cooled, filtered through Whatman no. 2 filter paper, concentrated under vacuum, and suspended in water (1 ml/30 g of wet root tissue extracted).

For assays of inhibition of bacterial growth, except as noted, 1 ml of this aqueous suspension was lyophilized and reconstituted to 250 μl with 100% methanol. The methanol-soluble portion was used to measure inhibition by crude material.

For fractionation by solvent extraction, a 2-ml portion of the crude extract suspended in water (containing the material from 60 g of wet roots) was sequentially extracted three times with 5 ml of petroleum ether (boiling point, 37.4 to 50.0°C) in a separatory funnel. The remaining crude suspension was similarly extracted with ethyl acetate. Material not extracted into ethyl acetate was dried and extracted three times with 500 μl of methanol. The residue not extracted into petroleum ether, ethyl acetate, or methanol was suspended in 500 μl of water prior to assay of inhibition. The petroleum ether, ethyl acetate, and methanol fractions were dried, and each was reconstituted with 500 μl of 100% high-performance liquid chromatography (HPLC)-grade methanol (Curtin Matheson Scientific, Houston, Tex.) prior to assay of inhibition. All fractions were stored at −20°C.

Purification of major inhibitors in petroleum ether and ethyl acetate extracts. Material extracted into petroleum ether or ethyl acetate was dried under vacuum, dissolved in methanol, and separated by HPLC on a Phenomenex Ultracarb 7 ODS30 reverse-phase column (250 by 10 mm) eluted at 1.6 ml/min with the following methanol-water gradient (given in percent methanol [vol/vol]): 45% for 2 min, 45 to 63% for 4 min, 63 to 100% for 24 min, 100% for 5 min, 100 to 45% for 5 min, and 45% for 5 min. Fractions were collected at 30-s intervals, lyophilized, and reconstituted with 100 μl of methanol. Inhibition assays were performed with 10 μl of these reconstituted fractions. Further purification of the material in the main inhibitory fractions was achieved by HPLC on the same column, with the sample being applied as a 100-μl solution in methanol and eluted with a more shallow methanol gradient: 70% for 2 min, 70 to 90% for 3 min, and 90 to 100% methanol for 25 min. The material in the main resulting HPLC peak was purified by isocratic elution with a 60:40 (vol/vol) mixture of acetonitrile and a 5 mM solution of acetic acid in water.

Agar diffusion assay of inhibition. Three milliliters of YGM soft (0.7%) agar, inoculated with 10 μl of fully grown rhizobial wild-type or mutant strains in TY liquid, were overlaid on a YGM (1.5% agar) plate (pH 5.0). In some experiments, Y medium adjusted to pH 5.5 was used instead of YGM. Three sterile paper discs, 0.6 cm in diameter (BBL Microbiology Systems, Cockeysville, Md.), were placed at a distance from each other on the soft agar overlay, and 5 or 10 μl of bean root extract was applied to one of the sterile discs. Tetracycline (0.50 μg) was added to a second disc as a standard to normalize for physical differences between different plates. A third disc

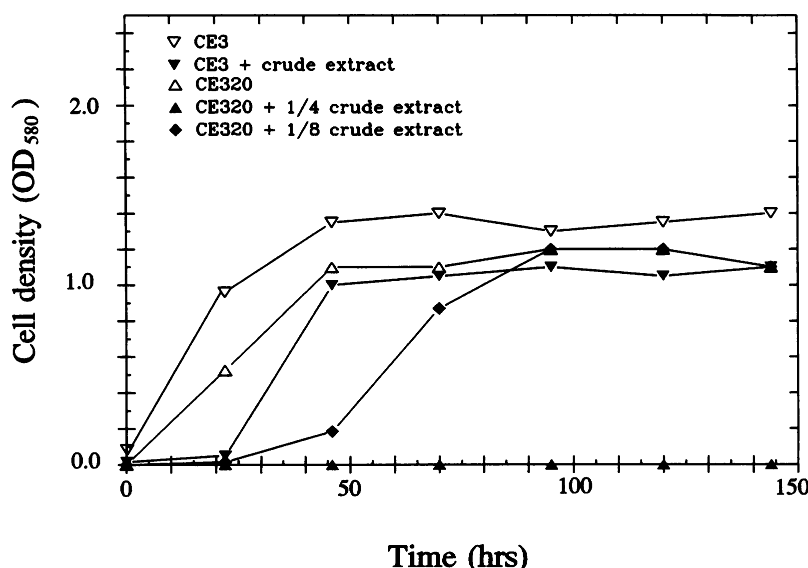

FIG. 1. Representative growth of strains CE3 and CE320 exposed to a crude extract of bean roots. The CE3 culture was exposed to the material extracted from 1.2 g of roots that had been suspended in 10 μl of methanol. CE320 cells were treated with extract at 1/4 and 1/8 of this concentration. (The undiluted and one-half-diluted extract inhibited strain CE320 completely throughout the experiment [data not shown].) The relative cell density in liquid YGM medium was measured as the optical density at 580 nm (OD_{580}).

received 10 μl of methanol to act as a control. Plates were incubated at 30°C for several days. The diameter of the zone of inhibition of each disc was measured daily and recorded. Inhibitory activities were compared by calculating the ratio of the inhibition zone caused by root extract to the inhibition zone formed by 0.50 μg of tetracycline. To test the effect of bacterial density, the fully grown culture was diluted in TY before additions of 10-μl aliquots to the soft agar.

Inhibition assayed in liquid medium. Rhizobia (15 μl of a culture fully grown in TY liquid overnight) were added to 1.5 ml of YGM liquid at pH 5.0, 10 μl of root extract diluted in methanol was added, and the tube was shaken at 30°C on a gyratory shaker (180 rpm). The optical density at 580 nm was measured to monitor growth. In control incubations, 10 μl of methanol was added in place of root extract. To measure the effect of inoculum density, fully grown cultures were diluted in TY; 15 μl of the diluted culture was tested as described above. Relative MICs for two strains were calculated from the ratio of the dilutions of an extract or solution that inhibited growth to the same extent.

Commercial sources of phytoalexins. Coumestrol, daidzein, and genistein were obtained from Eastman Kodak, Roth Chemical Co., and ICN Biochemicals, respectively. Scopeletin, umbelliferone, and naringenin were obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wis.).

Analytical methods. (i) HPLC. Complexity of crude extracts was analyzed by chromatography on a Phenomenex Ultracarb 5 ODS20 reverse-phase column (250 by 4.6 mm) eluted at 0.6 ml/min with a gradient of methanol and water as described by Dorr and Guest (12), with modifications. The runs were programmed and monitored by an Isco Chemresearch 150 system, utilizing a Linear UVIS-201 dual-wavelength detector with scanning capabilities. Water purified by a Modulab Polisher I HPLC Laboratory Reagent Grade Water System and methanol (Chempure; HPLC grade) were filtered through 47-mm nylon membrane filters (ChromTech, Minneapolis, Minn.) and degassed under vacuum prior to use. The methanol-water gradient for routine analysis was the same as that used in the first step of purification of the ethyl acetate extract.

(ii) Thin-layer chromatography. Thin-layer chromatography plates (20 by 20 cm) coated with silica gel 150A (Whatman) were eluted with chloroform (Baker)-methanol (Curtin Matheson Scientific) (10:1) and visualized with UV light.

(iii) UV spectroscopy. Spectrophotometric analysis of purified inhibitory compounds dissolved in 100% methanol was performed with a Pharmacia LKB Ultraspec II Spectrophotometer. Absorbance was scanned from 220 to 400 nm.

(iv) Mass spectrometry. High-resolution electron ionization mass spectral analysis was performed at the Midwest Center for Mass Spectrometry at the University of Nebraska-Lincoln, Department of Chemistry, Lincoln, Nebr. 68588-0362. The sample was shipped in a dried state and then dissolved in methanol immediately prior to analysis.

(v) NMR spectroscopy. The ^{1}H nuclear magnetic resonance (NMR) spectra were obtained with a General Electric 500-MHz NMR spectrometer. Samples were equilibrated with three additions of methyl-d_3 alcohol 99.8 atom%d (Aldrich Chemical Company, Inc.) and dissolved in this solvent for analysis. The ^{1}H NMR resonances were referenced to the middle resonance of the methoxy signal (3.35 ppm).

RESULTS

Inhibition of bacterial growth by crude bean root extract. Bean plants were harvested 5 days after 2-day-old seedlings had been inoculated with OPS-deficient mutant CE166. The crude extracts obtained from the roots of these plants were tested for inhibition of growth of *R. etli* bacteria cultured in liquid (Fig. 1) or dilute agar (Fig. 2). In either succinate (Y) or glucose (YGM) minimal medium, the MIC of the crude extract was two- to fourfold lower against all strains at pH 5 than at pH 7 or higher. Except as noted (Fig. 2), growth inhibition data presented in this report were obtained with liquid cultures in YGM medium at pH 5.

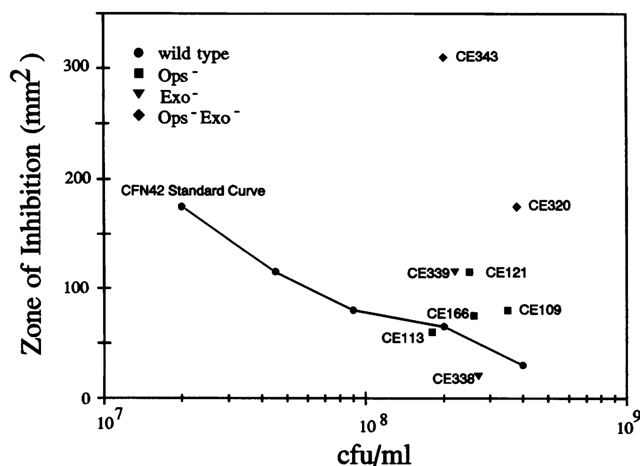

FIG. 2. Area of zone of inhibition after 48 h as a function of inoculum cell density (measured as CFU on TY agar) in agar diffusion assay on Y succinate medium. Each disc received 5 μl of the same crude extract (from 20 g of roots reconstituted in 2.5 ml of methanol). This is one representative experiment; data from numerous repetitions of this type of assay in YGM and Y succinate media showed that there was no significant difference in sensitivity between CE339, CE166, CE113, and the wild-type strains CFN42 and CE3.

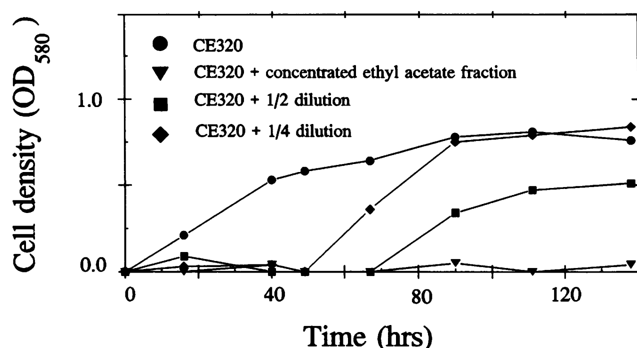

FIG. 3. Growth of strain CE320 in liquid cultures (1.5 ml) exposed to the portion of an extract from 0.90 g of roots that partitioned into ethyl acetate and two serial dilutions of this concentrate. OD_{580}, optical density at 580 nm.

The relative sensitivities of wild-type *R. etli* CE3 and various polysaccharide-deficient mutants were assayed in both liquid and agar culture, by using numerous separately prepared extracts. Experiments conducted independently by five of the investigators corroborated each other. EPS-deficient (Exo^-) mutants CE338 and CE339 were no more, or only slightly more, sensitive than wild-type strain CE3 and its streptomycin-sensitive parent, CFN42 (Fig. 2 and Table 2). Strains deficient in OPS were more variable in sensitivity. Strain CE166, which carries OPS on 60% fewer LPS molecules than does the wild type (6), and strain CE358, which lacks OPS completely (Ops^-) (34), exhibited wild-type sensitivity (Table 2). When cultured in liquid, Ops^- strains CE109 and CE309 were appreciably more sensitive than the wild type, whereas Ops^- mutant CE359 was intermediate in sensitivity (Table 2). Exo^- Ops^- mutants CE320 and CE343 were consistently more sensitive than any of the other strains tested (Fig. 1 and 2; Table 2). Likewise, strain CE388, which had been constructed to be Exo^- and Ops^-, was inhibited by crude extract at less than one-fourth the concentration needed to inhibit either of its parents, CE339 (Exo^-) and CE125 (Ops^-).

TABLE 2. Relative resistances of *R. etli* strains exposed to crude root extract

Strain	Polysaccharide phenotype	Resistance score[a]
CE3	Wild type	8
CE109	Ops^-	3
CE125	Ops^-	8
CE166	$Ops^{+/-}$	8
CE309	Ops^-	2
CE358	Ops^-	8
CE359	Ops^-	4
CE339	Exo^-	6
CE320	Ops^- Exo^-	1

[a] The higher the number, the greater the resistance. The scores indicate the relative concentrations of the crude extract needed to give equivalent inhibition of these strains. Each value was the median score for a given strain from experiments in which several strains, always including strains CE3 and CE320, were assayed at the same time by serial twofold dilutions of the crude extract in liquid assays. For instance, from the results shown in Fig. 1, strain CE3 would be assigned a score eight times that of CE320 because these two strains were inhibited equivalently when CE3 was exposed to extract eightfold-more concentrated than that applied to CE320.

In either the agar diffusion or liquid assays of inhibition, the MIC of the extract decreased as the density of the bacterial inoculum increased (Fig. 2 and data not shown). However, even when inoculated in the liquid assay at 10-fold higher densities than the wild type, Ops^- Exo^- mutants were sensitive to 2-fold-lower concentrations of the extract than the wild type. Likewise, when top agar was inoculated with 10-fold more mutant bacteria and the mutant bacterial lawn was visible sooner than the wild-type lawn, the zone of inhibition was still larger on the Exo^- Ops^- mutant lawn (Fig. 2 and data not shown).

Mutants deficient in both OPS and EPS also were subject to much greater killing by the crude extract. When exposed for 24 h to a concentration that completely inhibited growth of the wild type, about 50% of the wild-type bacteria remained viable as measured by CFU on TY agar. The same treatment of Exo^- Ops^- mutant CE320 resulted in only 1% survival.

Solvent fractionation. The lyophilized crude bean root extract was suspended in water and extracted sequentially with solvents of increasing polarity. After extraction with petroleum ether and ethyl acetate, the aqueous suspension was lyophilized, and the dried material was extracted with methanol. That which did not dissolve in methanol was dried and resuspended in water.

Inhibitory activity was detected in the petroleum ether, ethyl acetate, and (residual) water fractions (Fig. 3 and data not shown). In the numerous independent preparations of root extracts, the material that partitioned into ethyl acetate or petroleum ether generally inhibited Ops^- Exo^- strain CE320 at one-fifth to one-fourth the concentration needed to inhibit the wild type. The ethyl acetate and petroleum ether fractions each accounted for 40 to 50% of the total inhibitory activity of the crude extract against strain CE320, with the residual water fraction containing the remaining 10%.

Purification and characterization of main inhibitor of ethyl acetate fraction. After C_{18} HPLC of the ethyl acetate extract in a methanol-water gradient, inhibitory activity was detected mainly in fractions that eluted at approximately 93% methanol (Fig. 4). The material in this peak was resolved into two closely eluting peaks of UV absorbance by applying a shallow gradient

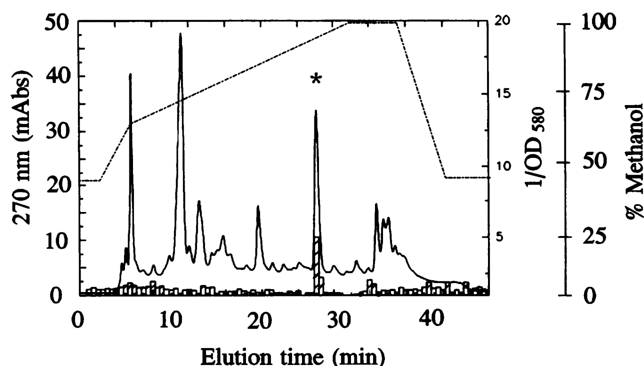

FIG. 4. HPLC purification of major inhibitor extracted into ethyl acetate. To a semipreparative C_{18} column (10 by 250 mm), 100 μl of the ethyl acetate extract in methanol was applied. The relative inhibition (1/optical density at 580 nm [OD_{580}]) of each 30-s fraction, measured 38 h after addition to strain CE320 in the standard assay in YGM liquid, is indicated by the bar graphs. The dashed line represents the methanol gradient used to elute the column. Elution was monitored by relative A_{270}. mAbs, milliabsorbance units.

of 90 to 100% methanol. Both peaks contained inhibitory material, with the substance(s) in the second peak exhibiting greater absorbance at all UV wavelengths. The bulk of the material in this second peak was resolved from a closely eluting minor contaminant by isocratic elution of the same HPLC column with a solution of acetonitrile, water, and acetic acid.

The UV absorbance spectrum exhibited by the purified major inhibitor (Fig. 5) was indicative of a coumestan or 3-arylcoumarin (39). Coumestrol, a coumestan possessing hydroxyls at the 3 and 9 positions, gave a nearly identical spectrum (Fig. 5) that agreed with published values (40).

In thin-layer chromatography, the isolated unknown compound and coumestrol on silica plates each exhibited a single bright blue band of qualitatively the same color, relative intensities under UV light, and similar R_fs (0.560 ± 0.003) (Fig. 6). No other compounds that exhibited fluorescence or absorbed UV light were observed in the lane of purified unknown material.

High-resolution electron impact mass spectral analysis of the isolated compound detected fragments with the following *m/z* values (and relative intensities): 268.0 $[M]^+$ (100), 240.0 (6), 211.0 (3), 184.1 (3), 155.0 (3), and 57.1 (9). These values are

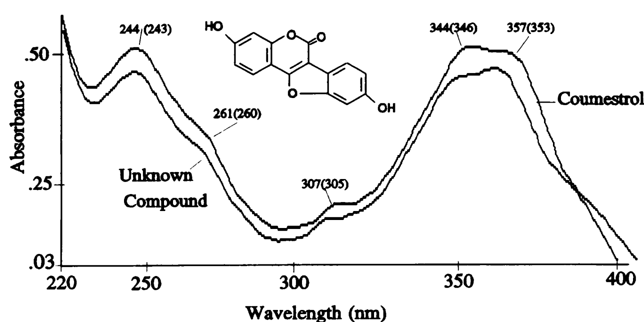

FIG. 5. UV spectra of coumestrol and main inhibitor purified from ethyl acetate extract. Numerical values indicate wavelengths corresponding to spectral peaks or shoulders for the unknown, with the value for coumestrol in parentheses. The structure of coumestrol is shown above the spectra.

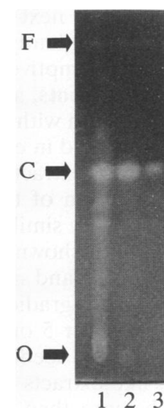

FIG. 6. TLC of ethyl acetate extract (lane 1), main inhibitor purified from this extract (lane 2), and coumestrol (lane 3). Arrows indicate the origin (O), solvent front (F), and the position of coumestrol (C).

consistent with those reported by Koshino et al. (16) for coumestrol. Coumestrol was obtained from Eastman Kodak and, after purification by HPLC, yielded high-resolution electron impact mass spectral fragments with *m/z* values (and relative intensities) of 268.0 $[M]^+$ (100), 240.0 (8), 211.0 (1), 184.1 (3), 155.0 (2), and 57.1 (4). The calculated mass for $C_{15}H_8O_5$ is 268.037. The mass ion of the unknown compound at high resolution was found to be 268.0361, and the mass ion for Eastman Kodak coumestrol was found at 268.0370.

The NMR spectrum of the unknown sample exhibited proton resonances in CD_3OD at δ 7.81 (1 H, d, J = 8.6 Hz), 7.72 (1 H, dd, J = 8.4 Hz, J = 0.3 Hz), 7.07 (1 H, dd, J = 2.0 Hz, J = 0.3 Hz), 6.91 (1 H, dd, J = 8.4 Hz, J = 2.1 Hz), 6.87 (1 H, dd, J = 8.6 Hz, J = 2.2 Hz), and 6.81 (1 H, d, J = 2.2 Hz). Coumestrol from Eastman Kodak had proton resonances in CD_3OD at δ 7.84 (1 H, d, J = 8.6 Hz), 7.73 (1 H, d, J = 8.4 Hz), 7.08 (1 H, d, J = 2.0), 6.906 (1 H, dd, J = 8.6 Hz, J = 2.2 Hz), 6.913 (1 H, dd, J = 8.4 Hz, J = 2.1 Hz), and 6.86 (1 H, d, J = 2.0 Hz). These values were consistent with previously reported values (8, 16).

Approximately 1.1 μg of this inhibitor was extracted per gram (wet weight) of roots, in agreement with previous estimates of the coumestrol content of bean roots (19).

Purification and characterization of major inhibitor(s) from petroleum ether fraction. The petroleum ether extract was fractionated by HPLC in the same manner as the ethyl acetate extract. The only peak of substantial inhibitory activity eluted in almost exactly the same position as coumestrol and the other inhibitors found in the ethyl acetate extract. The material in this peak was purified further by the same HPLC procedures described above. It migrated on thin-layer chromatography with an R_f similar to that of coumestrol and exhibited qualitatively the same bright blue fluorescence under UV light (data not shown). However, the high-resolution electron impact mass spectrum of this fraction was very different from that of coumestrol. Material desorbed at 275 and 350°C, yielding major ions at 461.4 and at 368.3 *m/z*, respectively. The molecular ion of coumestrol (268.03) was a very minor fragment in each case relative to the presumptive molecular ion of the desorbed substance. Another difference of these substances from coumestrol was the much greater solubility in petroleum ether. HPLC analysis showed that abundant material with the R_f of this inhibitor was extracted from the crude extract in the first volume of petroleum ether added and relatively little of

such material was found in the next two volumes, and then material at this R_f appeared abundantly in the subsequent ethyl acetate extract (mainly as presumptive coumestrol).

Effect of inoculation, age of roots, and plant organ on level of inhibitory activity. Inoculation with mutants CE166 (Ops$^-$) and CE320 (Ops$^-$ Exo$^-$) resulted in essentially the same level of inhibition by the crude root extract as inoculation with the wild-type CE3 or no inoculation of the plants at all. HPLC profiles of the extracts were very similar as well, regardless of the type of inoculant (data not shown).

Equivalent levels of inhibition and similar HPLC C_{18} elution profiles in the methanol-water gradient were obtained with extracts of roots harvested either 5 or 10 days after planting. Extracts of the roots of 2-day-old seedlings were less inhibitory per weight of tissue but, like extracts of older plants, affected Exo$^-$ Ops$^-$ strains much more than the wild type (data not shown).

Leaves, nodules, and stems of the bean plant were extracted by the same method utilized in the root extraction. Inhibitory activity toward the wild-type bacteria and Exo$^-$ Ops$^-$ mutant CE320 was detected in extracts of nodules, but relatively little from leaves and none from stems were observed. Per wet weight of tissue, nodules contained 1.5-fold more material that possessed the HPLC retention of coumestrol than roots had, while stems and leaves had 7 and 1%, respectively, as much (data not shown).

Inhibition by certain phytoalexins and detergents. Exo$^-$ Ops$^-$ strain CE320 was inhibited by the isoflavonoids coumestrol and genistein and slightly by daidzein. The MIC of coumestrol, 1 μM, was approximately fivefold lower than that of genistein. Wild-type strain CE3 was less sensitive to these compounds, with the MIC of coumestrol being about fourfold higher on strain CE3. Although inhibition by the crude extract was pH dependent, inhibition by coumestrol of both the wild type and strain CE320 was the same at pH 5 and pH 7 (data not shown). Scopeletin, umbelliferone, and naringenin were not inhibitory to either the wild type or mutant strain CE320, even at 10- to 20-fold-higher concentrations (data not shown). Tested in an agar diffusion assay, 20 μg of phaseollin or phaseollinisoflavan gave zones of inhibition that were 12 mm in diameter on strain CE320, whereas the zones were 6 to 7 mm on the wild type, strains CE113 and CE166, deficient only in OPS, and strains CE338 and CE339, deficient only in EPS.

The MICs of the detergent Triton X-100 were approximately 2, 30, 30, and 70 μg/ml on Ops$^-$ Exo$^-$ strain CE320, Ops$^-$ strain CE358, Exo$^-$ strain CE339, and wild-type strain CE3, respectively. Likewise, strain CE320 was inhibited by 3 μg of sodium dodecyl sulfate per ml, whereas 30 μg/ml was required to inhibit the wild type and strains deficient in only OPS or only EPS. Thus, OPS and EPS not only protect against the toxins of bean roots but also provide a more general barrier against hydrophobic or amphipathic compounds.

DISCUSSION

Extraction of *P. vulgaris* roots produced a complex mixture that inhibited growth of mutants deficient in both OPS and EPS at 1/16 to 1/8 the concentration required to inhibit the wild type and killed Ops$^-$ Exo$^-$ mutants at concentrations that were biostatic on the wild type. On the other hand, mutants deficient only in EPS and some of the mutants deficient only in OPS were no more sensitive than the wild type to the crude extract. Therefore, either OPS or EPS, individually, is a barrier to host toxic compounds.

Mutants deficient only in OPS are symbiotically defective (6), whereas mutants deficient only in EPS are symbiotically proficient on bean hosts (1, 11). Hence, the generally similar ex planta sensitivities of these mutants to host toxins do not parallel the contrasting symbiotic phenotypes. However, there is evidence to suggest that EPS synthesis is repressed in soybean and bean bacteroids (18, 35). Although there are no data regarding bacteria in infection threads, it is conceivable that rhizobial EPS synthesis could cease during infection before the formation of the bacteroid. If EPS synthesis is terminated during the course of infection, a mutant deficient in OPS in planta would acquire the sensitivity to host antibiotics of a mutant defective in both OPS and EPS. The formal possibility exists that this sensitivity is the basis of, or at least contributes to, the symbiotic defect of Ops$^-$ mutants on bean hosts. Speculating more generally, if EPS synthesis is repressed within infection threads on determinate-nodulating hosts, but not on indeterminate-nodulating hosts, this possibility could explain why infections of Ops$^-$ mutants appear to terminate much earlier during determinate nodule development than during indeterminate development (22).

The Ops$^-$ strains were not equally resistant to the root extract. Those that appeared most sensitive, CE109 and CE309, not only lack OPS but also are missing certain sugar residues of the core oligosaccharides of the LPS (4). However, strain CE358, which lacks OPS and one residue of the core (3), appeared as resistant as the wild type.

The most abundant of the inhibitors of bean roots that extracted into ethyl acetate appears to be coumestrol. However, the presence of additional activity in the petroleum ether and water fractions, as well as the ethyl acetate fraction, suggests that several compounds could contribute to the inhibition of rhizobia that are deficient in planta in both EPS and OPS. On the other hand, except for the minor and elusive activity extracted into water, the other purified fractions containing the major inhibitors all exhibited the absorbance spectrum and fluorescence typical of coumestans (39). Only in mass spectrum and solubility properties did they differ significantly.

Coumestrol is one of the phytoalexins of *P. vulgaris* (19). Phaseollin, phaseollinisoflavan, and genistein, other documented bean phytoalexins, also were more inhibitory to Ops$^-$ Exo$^-$ mutants than to the wild-type rhizobial strain. However, if these compounds were present in the root and nodule extracts, they were relatively insignificant compared with coumestrol and the unidentified inhibitor(s) that was extracted into petroleum ether.

In anticipation that phytoalexins active against Ops$^-$ rhizobia might be induced, plants in this study routinely were inoculated with a mutant deficient in OPS and infection. Significant induction of the soybean phytoalexin glyceollin I has been detected in root hairs and exudates within 10 h after inoculation with very dense suspensions of *Bradyrhizobium japonicum* and in nodules following inoculation with ineffective strains of *B. japonicum* (15, 31). Moderate increases in the levels of coumestrol and more dramatic increases in the relatively weak inhibitors daidzein and genistein have been observed in exudates of 10-day-old bean seedlings in response to inoculation with wild-type *Rhizobium leguminosarum* (8). In the present study exudates were not sampled, but root extracts provided no evidence of induction by bacterial inoculation, either of overall inhibitory activity or of coumestrol.

Routine sampling was done at 5 days postinoculation because that approximates the time in nodulation at which infection by Ops$^-$ mutants obviously has broken down (24, 36). It is possible that by not sampling at earlier times, a transient induction of inhibitory compounds was missed. Furthermore, even at the times sampled, it is conceivable that an induced

inhibitor is deposited at very high concentrations in and along the infection thread, but nowhere else, and therefore escapes detection by the methodology of this study. Recent cytological investigations suggest that rhizobial polysaccharide mutants provoke localized host-defense responses in nodules elicited on pea and alfalfa hosts (20, 26). Locally high production of coumestrol or other phytoalexins could accompany such responses, if they are induced by mutant infection of bean nodules, and increase the potential inhibition of Ops$^-$ mutants in planta.

The coumestrol content per wet weight of root implies an average concentration of at least 4 μM, sufficient to severely retard the growth of an Ops$^-$ mutant that becomes Exo$^-$ in the plant. However, there is no evidence that infecting rhizobia actually encounter it. One type of evidence would be a mutant selected for resistance to coumestrol even though still Ops$^-$ Exo$^-$ and, because of the resistance phenotype, able to achieve more-complete infection. A potential problem with this approach is that other compounds in root extract, besides coumestrol, are sufficiently inhibitory to contribute to the breakdown of infection. However, most of them appear to be structurally related and, hence, possibly act by the same mechanism.

Coumestrol induces *nod* gene activity in *R. leguminosarum* bv. phaseoli (8), but genistein, a more potent *nod* inducer (14), is less inhibitory, with a 5-fold-higher MIC. Perhaps relative inhibitory potency has played a role in the evolution of the particular spectrum of plant compounds that now act to induce *nod* expression. Nevertheless, some isoflavonoids that are important in the plant-*Rhizobium* communication process early in infection are sufficiently inhibitory that they might act as deterrents to mutants lacking the protective barriers of OPS and EPS.

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