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Exopolysaccharide-Deficient Mutants of *Rhizobium* sp. Strain CIAT899 Induce Chlorosis in Common Bean (*Phaseolus vulgaris*)

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Mutants of *Rhizobium* sp. strain CIAT899 deficient in exopolysaccharide (EPS) were generated with Tn*5* and ethyl methanesulfonate. The EPS-deficient mutants produced nonmucoid colonies on solid media and released 8.1–41.0% of the amount of EPS produced by CIAT899 in broth culture. All eight mutants tested induced an interveinal chlorosis in the first trifoliolate leaf of their symbiotic host, common bean (*Phaseolus vulgaris*), whereas CIAT899 caused slight chlorosis on a small proportion of inoculated plants. Two other bean-nodulating strains, *R. leguminosarum* bv. *phaseoli* CE3 and KIM5s, did not induce chlorosis, and neither did EPS-deficient mutants derived from these two strains. CIAT899 and its EPS-deficient mutants all induced pink, effective nodules, indicating that the chlorosis was not due to a lack of nitrogen resulting from an ineffective symbiosis. One EPS-deficient mutant, CT9005, was applied to *Leucaena leucocephala*, and it was found to induce foliar chlorosis and retained the ability to induce nodules on that host. Cultures of chlorosis-inducing strains lost the ability to induce chlorosis upon dilution, while retaining sufficient cells to nodulate abundantly, suggesting that nodulation is not sufficient for chlorosis induction. These data suggest that EPS-deficient mutants of CIAT899, and CIAT899 to a lesser extent, produce a chlorosis-inducing factor either in broth culture or in the rhizosphere.

Additional keywords: cell surface, extracellular polysaccharide, glycocalyx, toxin.

Bacterial exopolysaccharides (EPSs) influence many cellular functions, including permeability of the cell to toxic substances, adhesion to surfaces and other bacteria, and pathogenicity in diseases of animals and plants (Costerton *et al.* 1981). In some rhizobia and bradyrhizobia, EPS-binding proteins (lectins) have been shown to influence nodulation and host specificity (Dazzo and Hubbel 1975; Halverson and Stacey 1985; Dazzo *et al.* 1987; Philip-Hollingsworth *et al.* 1989a, 1989b), and in many of the fast-growing rhizobia, EPS production is required for normal nodule development (Leigh *et al.* 1985; Borthakur *et al.* 1986; Chen *et al.* 1985). However, EPS production does not appear to be required for normal infection of common bean (*Phaseolus vulgaris* L.) by strains of *Rhizobium leguminosarum* bv. *phaseoli*, since EPS-deficient mutants nodulate and fix nitrogen normally (Diebold and Noel 1989; Borthakur *et al.* 1986).

Although EPS is not required for normal nodulation and nitrogen fixation by *R. l.* bv. *phaseoli*, it may play a role in nodulation competitiveness. In the course of testing this hypothesis, we produced EPS-deficient mutants of *Rhizobium* sp. strain CIAT899, which is a broad host range strain that effectively nodulates common bean, *Leucaena leucocephala* (Lam.) de Wit., and *L. esculenta* (Moçiño & Sessé) Benth. (Martinez *et al.* 1985; Brom *et al.* 1988), is resistant to aluminum and low pH, and is resistant to high levels of spectinomycin (Sp) and streptomycin (Graham *et al.* 1982). CIAT899 has not been reported to induce chlorosis in any of its hosts; however, we report here that EPS-deficient mutants of CIAT899 induce severe interveinal chlorosis in bean plants and in *L. leucocephala* and that CIAT899 occasionally causes a slight chlorosis in the leaves of a small proportion of inoculated plants of common bean. Although certain strains of bradyrhizobia are known to induce chlorosis in soybean (Eaglesham and Hassouna 1982; LaFavre and Eaglesham 1986), this is the first report of induction of host chlorosis by fast-growing rhizobia.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. l.* bv. *phaseoli* KIM5s is a spontaneous Sp-resistant mutant of strain KIM5 (Beattie *et al.* 1989). Strain CIAT899 was obtained from F. Bliss, Department of Pomology, University of California, Davis, and strains CE3 and CE301 were obtained from K. D. Noel, Department of Biology, Marquette University, Milwaukee, WI. Strains of *Rhizobium* (Table 1) were grown for inoculation and carbohydrate isolation in yeast extract-mannitol (YM) medium (Wacek and Brill 1976) and maintained on solid YM medium containing 15.0 g of agar per liter. Strains of *Rhizobium* used in bacterial conjugations were grown in tryptone-yeast extract medium (Beringer 1974). Transconjugants containing transposon Tn*5* were selected on Bergersen's mannitol-salts medium (Bergersen 1961). Antibiotics were added to the solid medium at the following concentrations: Sp, 200 μg/ml; kanamycin (Km), 200 μg/ml. Strains of *Escherichia coli* were grown on either solid Luria-Bertani (Maniatis

et al. 1982) or solid tryptone-yeast extract media containing Km (50 μg/ml).

Transposon mutagenesis. Tn*5* was introduced into CIAT899 by triparental matings according to the method of Triplett and Barta (1987). Matings were interrupted by suspending the bacteria in sterile distilled water, and aliquots of the mating mixture were spread on solid Bergersen's medium containing Sp and Km. Nonmucoid colonies arose at a frequency of approximately 8×10^{-3} per Tn*5* recipient from CIAT899 and were identified by visual inspection.

Chemical mutagenesis. Late-log-phase cells of CIAT899 were pelleted in an Eppendorf microcentrifuge, washed once with phosphate-buffered saline (containing per liter of H_2O: 8.76 g NaCl, 5.22 g K_2HPO_4, 1.36 g KH_2HPO_4), and resuspended to the original volume in phosphate-buffered saline. Ethyl methanesulfonate (Sigma Chemical Co., St. Louis, MO) was added to the cells at a final concentration of 4% (v/v). The cells were mixed gently with a vortex mixer and incubated at room temperature for 1 hr. They were then diluted and spread on either solid YM or Bergersen's medium containing Sp and incubated at 28° C. Nonmucoid colonies were identified visually after 4–5 days, purified by restreaking on solid Bergersen's medium, and tested for EPS production and the ability to nodulate and induce chlorosis in bean plants. Nonmucoid mutants arose at a frequency of approximately 5×10^{-4} per survivor.

Quantification of EPS. Cells were grown in YM broth for 3 days at 28° C and then removed from cultures by centrifugation. EPS was precipitated from the supernatants by adding four volumes of 95% ethanol and storing the mixtures overnight at −20° C. Precipitates were collected by centrifuging the mixtures at 15,300 × *g* for 30 min and dried with filtered air to remove the ethanol. The precipitated material was redissolved in water and centrifuged to remove insoluble material, and the resulting supernatant was lyophilized and weighed. EPS was identified as material in culture supernatants that was ethanol-precipitable and composed substantially of hexose material as determined by the phenol-sulfuric acid assay (Ashwell 1966). Glucose equivalent was defined as the mass of crude EPS that could be accounted for by the presence of hexoses, as compared with a glucose standard assayed by the same method.

Plant growth conditions. Seeds of bean cultivars Puebla 152 and 22-034 were surface-sterilized, planted in glass test tubes, and maintained as described previously (Araujo *et al.* 1986; Beattie *et al.* 1989). Plants were watered when they became slightly wilted. Seeds of *L. leucocephala* were surface-sterilized by immersion in 18 M sulfuric acid for 10 min, followed by extensive washing in sterile distilled water. *L. leucocephala* seeds were planted and maintained in glass test tubes in the same manner as the bean plants. Bacterial inocula were grown in YM broth at 28° C for 3 days with shaking. One milliliter of a 3-day-old culture, containing $2\text{–}4 \times 10^9$ bacteria per milliliter, was added to each seed at planting, except where noted.

RESULTS

Nonsymbiotic properties. Characteristics of the three parent strains CIAT899, KIM5s, and CE3, and their EPS-deficient mutants are described in Table 1. All mutants were chosen for their apparent lack of EPS based on their nonmucoid colony morphology on solid YM medium, and then EPS deficiency was confirmed by measuring the EPS

Table 1. Properties of parent strain CIAT899 of *Rhizobium* sp. and parent strains KIM5s and CE3 of *R. leguminosarum* bv. *phaseoli* and their mutants that are deficient in exopolysaccharide (EPS)

Strain	Mutagen	Colony morphology[a]	EPS production[b] (mg/ml)	Glucose equivalent[c] mg/ml	Glucose equivalent[c] Wild type (%)
CIAT899	···	Mucoid, with clear halo	1.86 ± 0.56[d]	1.25	100.0
CT9001	Tn*5*	Nonmucoid	0.77 ± 0.14[e]	0.38	30.4
CT9002	Tn*5*	Nonmucoid	0.55 ± 0.16[d]	0.13	10.4
CT9003	Tn*5*	Nonmucoid	0.23 ± 0.05	0.12	9.6
CT9005	Tn*5*	Nonmucoid	0.15 ± 0.03	0.03	2.4
CT9009	Tn*5*	Nonmucoid	0.73 ± 0.18[d]	0.20	16.0
CT9010	Tn*5*	Nonmucoid	0.30 ± 0.05	0.07	5.6
CT8014	EMS[f]	Nonmucoid	0.22 ± 0.07	0.07	5.6
CT8032	EMS	Nonmucoid	0.37 ± 0.20	0.04	3.2
KIM5s	···	Mucoid	0.75 ± 0.11	0.29	100.0
KM5005	Tn*5*	Nonmucoid	0.10 ± 0.04	0.02	7.9
KM5021	Tn*5*	Nonmucoid	0.13 ± 0.01	0.02	16.3
CE3	···	Mucoid	1.72 ± 0.44	0.76	100.0
CE301	Tn*5*	Nonmucoid	0.10 ± 0.02	0.02	6.2

[a] Appearance of colonies on solid yeast extract-mannitol medium.
[b] Average measurement of EPS from two separate cultures (except where noted), ± SE.
[c] Milligrams per milliliter of ethanol-precipitable hexoses in culture supernatants; wild type (%) indicates amount of EPS produced by mutant compared with amount produced by wild type.
[d] Average measurement of EPS from three separate cultures.
[e] Average measurement of EPS from four separate cultures.
[f] Ethyl methanesulfonate.

Table 2. Symbiotic properties of parent strain CIAT899 of *Rhizobium* sp. and parent strains KIM5s and CE3 of *R. leguminosarum* bv. *phaseoli* and their exopolysaccharide-deficient mutants on plants[a]

Strain	Chlorosis rating[b]	Acetylene reduction activity[c]	Nodules per plant[d]
CIAT899	1.3 ± 0.1	435 ± 85	39 ± 4
CT9001	3.1 ± 0.3	454 ± 81	35 ± 3
CT9002	4.1 ± 0.3	460 ± 79	39 ± 5
CT9003	3.9 ± 0.2	388 ± 60	31 ± 2
CT9005	4.6 ± 0.1	575 ± 75	47 ± 5
CT9009	3.8 ± 0.2	598 ± 91	48 ± 6
CT9010	3.9 ± 0.3	413 ± 101	52 ± 5
CT8014	3.5 ± 0.3	485 ± 114	37 ± 3
CT8032	3.7 ± 0.3	341 ± 80	16 ± 2
KIM5s	1.0 ± 0.0	242 ± 33	82 ± 8
KM5005	1.0 ± 0.0	244 ± 22	64 ± 4
KM5021	1.0 ± 0.0	172 ± 22	73 ± 5
CE3	1.0 ± 0.0	347 ± 64	33 ± 5
CE301	1.0 ± 0.0	463 ± 51	27 ± 3
None	1.0 ± 0.0	4 ± 4	0 ± 0

[a] All values are the mean observation from 12–14 bean plants, ± SE. LSD values were calculated with the harmonic mean of the number of plants per treatment. Plants were inoculated with cultures containing $2\text{–}4 \times 10^9$ bacteria per milliliter.
[b] $a = 0.05$; LSD = 0.5.
[c] Nanomoles of C_2H_2 reduced per hour per plant, ± SE. ($a = 0.05$; LSD = 202.1)
[d] Values are rounded off to the nearest integer. One of 14 plants in the water treatment control had three nodules. ($a = 0.05$; LSD = 13.0).

content of culture supernatants. Every nonmucoid mutant produced significantly less ethanol-insoluble, hexose-containing material in broth culture than did its parent strain, and the proportion of this material that was accounted for by hexose was less in all mutants than in their parent strains (Table 1).

Chlorosis induction by EPS-deficient mutants. All EPS-deficient mutants of CIAT899, isolated after mutagenesis with either Tn*5* or ethyl methanesulfonate, caused chlorosis in the first trifoliolate leaves of bean plants within 3 wk after planting, at which time the plants were inoculated with 1 ml of a 3-day-old culture containing 2–4 $\times$ 10^9 bacteria per milliliter (Table 2). All mutants, including both those in Table 1 and those mentioned below, were isolated in independent mutageneses. Chlorosis was usually evident as soon as the first trifoliolate leaves were expanded. The timing of the appearance of chlorosis did not vary among the mutants. The severity of chlorosis was rated on a scale from 1 to 5, with a rating of 1 indicating no chlorosis and 5 indicating severe chlorosis (Fig. 1). The average chlorosis ratings for plants treated with cultures of EPS-deficient mutants were significantly higher than the average ratings for plants inoculated with CIAT899 (Table 2). Neither strain CE3 nor KIM5s, nor any of their EPS-deficient mutants, caused chlorosis in bean plants. KM5021, KM5005, and CE301 are representative of the 17 EPS-deficient mutants derived from KIM5s and the 20 derived from CE3. All of the EPS-deficient mutants of CE3 and KIM5s that we isolated were tested, and none were observed to cause chlorosis.

Chlorosis was usually limited to the interveinal spaces except in the most severely affected leaflets, in which the veins also became chlorotic. Severely chlorotic leaflets, which received ratings of 4 or 5, were often elongated and stunted when compared with normal leaflets. Plants with severely chlorotic leaflets of the first trifoliate often showed stunting and witches'-broom-like symptoms, including shoot growth from the cotyledon scars, and such new shoots were also chlorotic. Chlorosis was not reversible by spotting solutions of iron (5 mM $FeSO_4 \cdot 7H_2O$, 5.5 mM disodium EDTA) or quarter-strength Hoagland's micronutrients (Hoagland and Arnon 1938) on leaflets.

Strain CIAT899 also induced slight chlorosis in a small proportion of plants, but the resulting average chlorosis rating, 1.3 $\pm$ 0.1 ($\pm$ SE), was significantly lower than that of the EPS-deficient mutants, such as CT9005, which had an average chlorosis rating of 4.6 $\pm$ 0.1. Mutant strain CT9005 also caused interveinal chlorosis in the leaves of *L. leucocephala* approximately 3 wk after seeds were planted and inoculated with 1 ml of culture. Only mutant CT9005 was tested on *L. leucocephala*. Seedlings of *L. leucocephala* were not stunted, however, and did not develop witches'-broom-like symptoms, as did the bean plants.

Dilutions of cultures were applied to plants to determine the relative amounts of chlorosis-inducing activity in the cultures of representative strains (Fig. 2). Cultures were serially diluted fourfold, and 1 ml of each dilution was applied to several plants; the number of plants represented by each point varied from 3 to 10 due to the poor germination rate of the seed lot used in this experiment. Cultures of the EPS-deficient mutants of CIAT899 had dilution endpoints for chlorosis induction approximately 10-fold lower than CIAT899, which is able to cause chlorosis, but much less severely and less consistently than its EPS-deficient mutants.

Symbiotic properties. All of the mutants studied induced pink, effective nodules on bean plants (Table 2). None of the mutants were significantly less effective in acetylene reduction activity than their parent strains. One mutant, CT8032, induced significantly fewer nodules per plant than CIAT899; however, plants inoculated with CT8032 did not reduce significantly less acetylene than plants inoculated with CIAT899 and did not show symptoms of nitrogen deficiency. Similarly, strain KM5021 induced fewer nodules than its parent KIM5s, but it did not reduce significantly less acetylene. The analyses of variance of nodule number

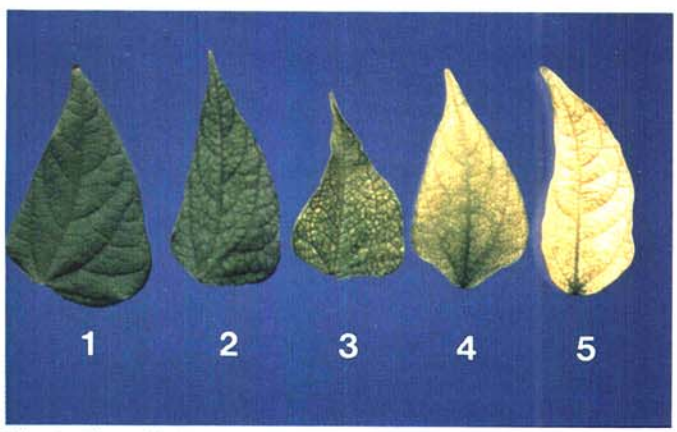

Fig. 1. First trifoliolate leaflets of common bean showing interveinal chlorosis induced by exopolysaccharide-deficient mutants of *Rhizobium* sp. strain CIAT899. Chlorosis was evident as soon as the leaflets were expanded. Numerals under the leaflets indicate the chlorosis severity rating; 1 = no chlorosis and 5 = severe chlorosis. All reported ratings of plants were conducted against this scale.

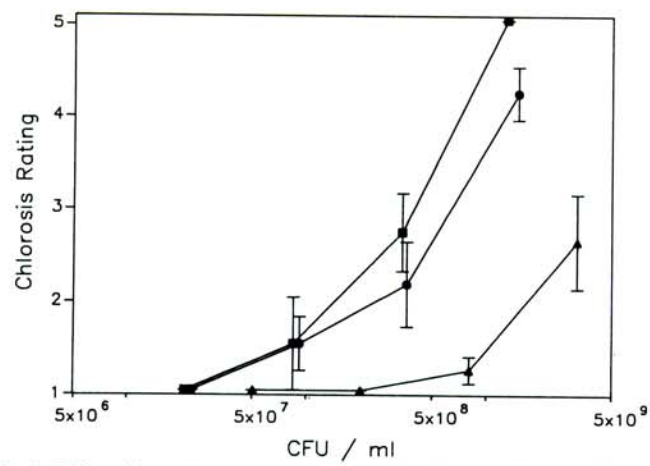

Fig. 2. Effect of inoculum concentration on chlorosis severity in common bean. Yeast extract-mannitol broth cultures of CIAT899 and exopolysaccharide-deficient mutants were incubated at 28° C with shaking for 3 days and serially diluted fourfold. Plate counts were used to determine the concentration of the cultures prior to dilution (the highest concentration in each curve). Error bars indicate the standard error of the mean for 3–10 plants per treatment. Chlorosis was rated as given in Figure 1. Symbols are as follows: ▲, CIAT899; ■, CT9005; and ●, CT8032.

and acetylene reduction were performed without the inclusion of data for the water control. Only one plant in the water control treatment had acetylene reduction activity, presumably induced by a contaminant; this plant had three nodules.

DISCUSSION

We have isolated from a fast-growing, broad host range *Rhizobium* strain a collection of EPS-deficient mutants that induced an interveinal chlorosis in their host plants. The mutants nodulated normally and reduced acetylene, and the chlorosis induced by the mutants did not resemble symptoms of nitrogen deficiency.

The amount of EPS produced in YM broth culture by three of the mutants, CT9001, CT9002, and CT9009, was surprising, given the morphology of the colonies they produce on solid YM medium. On YM plates CT9001, CT9002, and CT9009 produced the same small, dry colonies that are produced by mutants such as CT9005 and CT8014, both of which yielded very little ethanol-precipitable material in broth culture. The morphology of the colonies produced by all of the EPS-deficient mutants of CIAT899 was dramatically different from the parent strain. CIAT899 produced large, watery colonies surrounded by halos of translucent EPS. The data suggest that some mutants may be totally defective in EPS production while others produce decreased amounts of EPS, and that this difference is not detectable by visual inspection of colonies. We note that YM broth contains a small amount of ethanol-precipitable material; however, we did not use this data in the calculation of crude EPS yields, because it is not clear that the material present in unused broth is not metabolized or altered during the growth of cultures. It should also be noted that culture supernatants contain other carbohydrate-containing molecules such as lipopolysaccharides and glucans, which may be also present in our EPS preparations.

The correlation between EPS deficiency and severe chlorosis induction is intriguing. Several possible bases for the relationship can be imagined. Precursors of the EPS itself may accumulate in cultures of the mutants and may be toxic to the host plant. Alternatively, it is possible that the parent EPS forms a chemical barrier to the release of a phytotoxin which is produced constitutively under our culture conditions, and thus, in the case of mutants that lack EPS, the toxin might diffuse freely to the plant. The role of EPS as an ion-exchange resin around bacterial cells has been suggested by Durbin and Langston-Unkefer (1988) as a possible strategy for the protection of bacteria from their own phytotoxins.

The symptoms induced in bean plants reported here closely resemble those induced in leaflets of the first trifoliolate leaves of soybeans by rhizobitoxine-producing strains of bradyrhizobia (Johnson *et al.* 1958). Rhizobitoxine inhibits the enzyme β-cystathionase, which is involved in methionine biosynthesis (Giovanelli *et al.* 1971). In some plants ethylene biosynthesis is also affected, most likely because methionine is a precursor (Owens *et al.* 1971). Interestingly, chlorosis induction by the bradyrhizobia is associated with reduced capsule production (LaFavre *et al.* 1988). The cationic nature of rhizobitoxine (Owens *et al.* 1972) may suggest that the acidic EPS is indeed playing a role as an ion-exchange resin by binding rhizobitoxine; capsule-deficient derivatives of *Bradyrhizobium japonicum* USDA76 may simply bind less of the rhizobitoxine they produce and release the rest to the plant. If CIAT899 produces a cationic toxin, EPS deficiency may lead to chlorosis in beans by a similar mechanism. Although the similarity of symptoms and the correlation of severe chlorosis induction with the lack of capsular or extracellular polysaccharide suggest an intriguing parallel between the rhizobitoxine-producing strains and our mutants, it would be premature to suggest the involvement of rhizobitoxine in chlorosis induction in beans by EPS-deficient mutants of CIAT899.

Plants treated with diluted cultures of EPS-deficient mutants did not show chlorosis, although sufficient bacteria were applied to induce abundant nodulation. This may suggest that nodulation is not sufficient for chlorosis induction and that the bacteria may release a chlorosis-inducing factor into the culture medium, or into the rhizosphere, raising the possibility that the production of a chlorosis-causing factor in the rhizosphere may be plant-induced.

Finally, the possibility exists that the ability to induce chlorosis and EPS deficiency are unrelated; a thorough genetic analysis will reveal whether the same mutation is responsible for both phenotypes in each mutant. However, it is unlikely that several independent mutageneses using either a transposon or a chemical mutagen would consistently produce mutants with the same two unrelated phenotypes. We are continuing to characterize these mutants biochemically and genetically to gain an understanding of the mechanism of chlorosis induction in beans, and to understand the relationship between EPS deficiency and the ability to induce chlorosis.

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