

## Characterization of the First Molluscicidal Lipopolysaccharide from *Moraxella osloensis*

Li Tan† and Parwinder S. Grewal\*

Department of Entomology, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, Ohio 44691

Received 20 November 2002/Accepted 24 February 2003

***Moraxella osloensis* is a bacterium that is mutualistically associated with *Phasmarhabditis hermaphrodita*, a nematode that has potential for the biocontrol of mollusk pests, especially the slug *Deroceras reticulatum*. We discovered that purified *M. osloensis* lipopolysaccharide (LPS) possesses a lethal toxicity to *D. reticulatum* when administered by injection but no contact or oral toxicity to this slug. The toxicity of the LPS resides in the lipid A moiety. *M. osloensis* LPS was semiquantitated at  $6 \times 10^7$  endotoxin units per mg. The LPS is a rough-type LPS with an estimated molecular weight of 5,300. Coinjection of galactosamine with the LPS increased the LPS's toxicity to the slug two- to four-fold. The galactosamine-induced sensitization of the slug to the LPS was reversed completely by uridine.**

*Moraxella osloensis* is a gram-negative opportunistic human pathogen that has been found to cause several human diseases (1, 2, 6, 14, 15, 23). The bacterium is also associated with *Phasmarhabditis hermaphrodita*, a parasitic nematode that is lethal to slugs and which has potential for the biocontrol of mollusk pests, especially the gray garden slug *Deroceras reticulatum* (9, 10, 24). *P. hermaphrodita* has been found to be associated with different bacterial species (25, 26). *M. osloensis* was finally selected as the preferred associated bacterium to mass-produce *P. hermaphrodita* in a monoxenic culture (25). NemaSlug, a commercial product which is based on the nematode-bacterium complex, was launched in England in 1994 (9).

We previously discovered that *P. hermaphrodita* is a vector that transmits *M. osloensis* into the shell cavity of *D. reticulatum* and that the bacterium is the main killing agent (17). We further discovered that *M. osloensis* produces an endotoxin(s) to kill the slug and indicated that lipopolysaccharide (LPS) from *M. osloensis* is an active endotoxin (18). The LPS was found to have lethal toxicity against the slug when administered by injection (18). However, for direct application of the endotoxin against slugs, it is important to determine whether the LPS possesses lethal contact or oral toxicity. LPS is usually composed of a lipid A moiety and a polysaccharide moiety (8). The *Limulus* amoebocyte lysate (LAL) assay is a sensitive and powerful test for the semiquantitation of endotoxins (13). We reported recently that *M. osloensis* LPS is a rough-type LPS (19). However, the toxic moiety, LAL activity, and molecular weight of the LPS are not yet available. Thus, the present study was conducted to further characterize *M. osloensis* LPS.

Treatment of rabbits, rats, and mice with galactosamine (namely, D-galactosamine-HCl) increased their sensitivity to the lethal effect of an LPS from the bacterium *Salmonella*

*enterica* serovar Abortusequi several thousandfold. The mechanism of enhanced sensitization was thought to be associated with the liver injury caused by galactosamine, and this enhanced sensitization could be reversed by the administration of uridine (7). Since *D. reticulatum* is a lower-order animal that does not have a liver (5), we wanted to test whether galactosamine or the galactosamine-uridine combination would have an effect on the susceptibility of *D. reticulatum* to *M. osloensis* LPS that is similar to its effect on mammals. We hypothesized that galactosamine induces the susceptibility of *D. reticulatum* to *M. osloensis* LPS and that this galactosamine-induced sensitization is blocked by uridine.

Sources of *M. osloensis* and *D. reticulatum* as well as the method of *M. osloensis* LPS purification were the same as those described previously (18). The LPS toxicity against *D. reticulatum* was determined by using three different methods. The injection toxicity of the LPS was tested by injecting a 50- $\mu$ l portion of a high concentration of LPS solution (2 mg/ml) into the shell cavity as described previously (16). Slugs injected with distilled water served as controls for the injection toxicity experiment. The contact toxicity of the LPS was determined by applying 5  $\mu$ l of the LPS solution to the hind dorsal portion of each slug. Slugs receiving 5  $\mu$ l of distilled water served as controls for the contact toxicity experiment. To test the oral toxicity of the LPS, slugs were placed in a petri dish and fed two pieces of half-dry carrot tubers (2 cm in diameter and 0.5 cm in thickness) that had been treated evenly with the LPS solution around the tops of the tubers (600  $\mu$ g of LPS per six slugs per dish). The average uptake of the LPS per slug in the oral-toxicity experiment was estimated to be 100 times the percentage of tuber consumption during the experiment, expressed in micrograms. The volume of each tuber was measured before and after the experiment after immersion of the tubers in a 25-ml cylinder containing water, and the increase in volume was recorded. Slugs fed tubers treated with the same amount of distilled water served as controls for the oral-toxicity experiment. Slugs were fed pieces of fresh carrots and cabbage leaves in the injection and contact toxicity experiments. There were three replicate petri dishes, each containing six slugs. All

\* Corresponding author. Mailing address: Department of Entomology, Ohio State University, OARDC, Wooster, OH 44691. Phone: (330) 263-3963. Fax: (330) 263-3686. E-mail: grewal.4@osu.edu.

† Present address: Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294-2170.

slugs were incubated at 18°C. The numbers of dead slugs were recorded after 4 days.

Lipid A was obtained as a precipitate by hydrolysis of *M. osloensis* LPS (2 mg/ml) with 1% acetic acid at 100°C for 2 h, followed by centrifugation (16,000 × g, 5 min) and successive washes with distilled water and acetone (12). The supernatant from the LPS hydrolysate containing the polysaccharide moiety was centrifuged (150,000 × g, 3 h, 4°C) to remove lipid A and unhydrolyzed LPS and then lyophilized. A 50-μl portion of the obtained lipid A or polysaccharide (dissolved in the same amount of distilled water as that in the original LPS solution) was injected into the shell cavity. Slugs injected with distilled water served as controls. The rest of the experimental design was as described above for the injection toxicity experiment.

*M. osloensis* LPS was diluted serially with endotoxin-free water (Sigma Chemical Co., St. Louis, Mo.). Equal volumes (0.1 ml) of each LPS dose and LAL (Sigma Chemical Co.) were mixed and incubated at 37°C for 1 h. The gelation of LAL at each LPS dose was determined by inverting the mixture. A firm gel was considered a positive reaction (11). *Escherichia coli* O55:B5 LPS (Sigma Chemical Co.) was purchased as a standard endotoxin to semiquantitate *M. osloensis* LPS. The endotoxin activity in the lipid A or polysaccharide moiety was also analyzed by the LAL assay. The sensitivity of the LAL assay is 0.05 to 0.1 endotoxin unit (EU) per ml.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (19). LPSs were then visualized by the classic silver staining method (21). Commercially available rough-type LPSs from *E. coli* J5 ( $M_r = 4,200$ ) and EH100 ( $M_r = 5,500$ ) (22) were used as LPS controls and  $M_r$  markers (Sigma Chemical Co.).

In preliminary experiments, we determined the lethal and sublethal doses of galactosamine and uridine for slugs. A dose of 0.3 mg of galactosamine per slug or 1 mg of uridine per slug did not cause any slug mortality, whereas 1 mg of galactosamine per slug and 3 mg of uridine per slug caused about 30 and 80% mortality, respectively, at day 4 after injection into the shell cavity. The weight of each adult slug used in the experiments was in the range of 0.6 to 0.8 g. Furthermore, a mixture of 3 mg of uridine and 1 mg of galactosamine per slug had no effect on slug mortality. Thus, we used the following treatments: (i) 0.3 mg of galactosamine plus 0.01 mg of LPS with or without 1 mg of uridine per slug and (ii) 1 mg of galactosamine plus 0.03 mg of LPS with or without 3 mg of uridine per slug. Each mixture (in a 50-μl solution) was injected into the shell cavity. Slugs injected with endotoxin-free water (as a solvent in the experiment), galactosamine alone (0.3 or 1 mg per slug), or LPS alone (0.01 or 0.03 mg per slug) served as controls. The rest of the experimental design was as described above for the injection toxicity experiment.

All data presented in percentage values were arcsine transformed and subjected to one-way analysis of variance by using the statistical software STATISTICA Kernel release 5.5 (StatSoft Inc., Tulsa, Okla.). Significant differences among treatment results were determined by using Tukey's honestly significant difference tests at a *P* value of 0.05.

Compared to the results with the controls, *M. osloensis* LPS caused significant slug mortality (*P* < 0.05) only when injected into the shell cavity (Table 1). In the oral-toxicity experiment, the slugs were found to eat the top halves of the tubers (data

TABLE 1. Percentage mortality of *D. reticulatum* slugs following injection, contact, or oral administration of purified LPS from 3-day *M. osloensis* cultures<sup>a</sup>

Treatment group	% Mortality (mean ± SE) <sup>b</sup>		
	Injection	Contact	Oral
Control	5.6 ± 5.6A	5.6 ± 5.6A	5.6 ± 5.6A
Treated	77.8 ± 5.5B	5.6 ± 5.6A	11.1 ± 5.6A

<sup>a</sup> *n* = 3.

<sup>b</sup> Petri dishes differed significantly at a *P* value of <0.05 as indicated by different letters for each type of LPS administration.

not shown); thus, the average uptake of the LPS was estimated to be 50 μg per slug. Therefore, *M. osloensis* LPS possesses a lethal toxicity when administered by injection but no contact or oral toxicity against *D. reticulatum*, which implies that it is not feasible to directly apply the LPS for slug control. It is suggested that *P. hermaphrodita* is a necessary natural vector of *M. osloensis* for the biological control of slugs. The lipid A moiety resulted in 61.1% ± 5.6% (mean ± standard error; *n* = 3 petri dishes) slug mortality, which differed significantly (*P* < 0.05) from the results for the controls, but the polysaccharide moiety did not (data not shown). Therefore, the toxicity of *M. osloensis* LPS resides in the lipid A moiety but not in the polysaccharide moiety. In addition, the slugs killed by the LPS or lipid A were found to lie on their sides, and their bodies and tentacles were contracted. Their skin surfaces were sticky because they secreted a lot of slime before death. Their mouths occasionally were prolapsed.

A 0.1-pg amount of *M. osloensis* LPS (in 0.1 ml of the LPS dilution) induced the gelation of LAL, but 0.05 pg of LPS did not. Thus, the minimal dose of *M. osloensis* LPS required for the gelation was estimated to be 1 pg per ml. Moreover, 0.06 EU per ml was the minimal dose for the standard endotoxin (*E. coli* O55:B5 LPS) to cause a positive reaction. Therefore, *M. osloensis* LPS was semiquantitated to be  $6 \times 10^7$  EU/mg by the LAL assay. Furthermore, the lipid A moiety also caused a positive reaction, but the polysaccharide moiety did not. As with the results with the rough-type LPS controls, only one main band was detected in the gel for *M. osloensis* LPS. The LPS  $M_r$  was about 5,300, as estimated from a comparison of the electrophoretic mobility of the band representing *M. osloensis* LPS with that of bands representing the two  $M_r$  markers (Fig. 1).

The lethal (1-mg/slug) or sublethal (0.3-mg/slug) dose of galactosamine enhanced the susceptibility of *D. reticulatum* to *M. osloensis* LPS (0.03 or 0.01 mg/slug, respectively), but uridine (3 or 1 mg/slug, respectively) inhibited the sensitization effect completely (Table 2). A mixture of galactosamine and LPS caused slug mortality that differed significantly (*P* < 0.05) from the mortality caused by treatment with galactosamine alone or LPS alone. However, coinjection of uridine with the galactosamine-LPS mixture caused slug mortality similar only to that caused by the treatment with LPS alone.

The present study demonstrated that galactosamine enhances the susceptibility of *D. reticulatum* to *M. osloensis* LPS and that the induced sensitization can be blocked completely by uridine. Galanos et al. (7) reported that the administration of galactosamine in experimental mammals increases their sus-

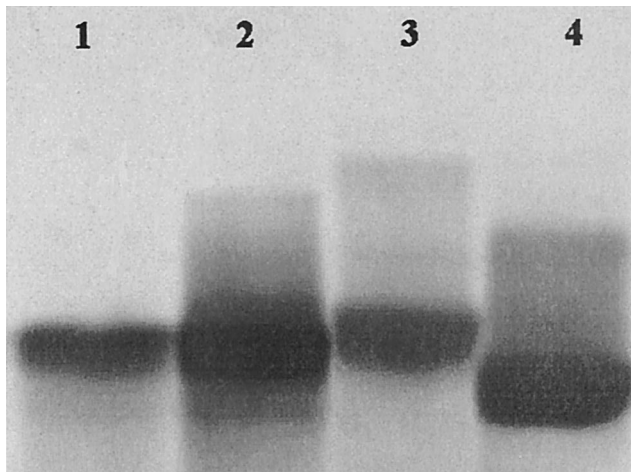


FIG. 1. Detection of LPS by SDS-PAGE following silver staining. One-microgram samples were analyzed by SDS-PAGE except as noted otherwise. Lanes 1 and 2, *M. osloensis* at 1 and 5  $\mu$ g, respectively; lane 3, *E. coli* EH100; lane 4, *E. coli* J5. The figure was created with Photoshop 5.5 software (Adobe Systems Inc.).

ceptibility to the LPS from *Salmonella* serovar Abortusequi several thousandfold and that the sensitization effect is inhibited completely by uridine. They suggested that the mechanisms leading to the sensitization effect are associated with the early metabolic alterations in the liver that are elicited by galactosamine (7). Galactosamine causes a high level of accumulation of UDP-galactosamine derivatives and leads to a depletion of hepatic UTP in the liver, thus inhibiting the biosynthesis of macromolecules and eventually resulting in the damage and death of hepatocytes (4). However, the hepatic lesions elicited by galactosamine can be blocked completely by uridine (3).

It is not fully clear why the galactosamine-induced sensitization also exists in *D. reticulatum* organisms, which do not have livers. Compared with mammals such as rabbits, rats, and

TABLE 2. Mortality of *D. reticulatum* slugs following injection into the shell cavity of galactosamine alone, *M. osloensis* LPS alone, galactosamine and *M. osloensis* LPS, or a mixture of galactosamine, *M. osloensis* LPS, and uridine<sup>a</sup>

Substance injected	% Mortality (mean $\pm$ SE) with indicated level of treatment <sup>b</sup>	
	Lethal <sup>c</sup>	Sublethal <sup>d</sup>
Endotoxin-free water	0A	0A
Galactosamine	33.3 $\pm$ 9.6B	0A
LPS	27.8 $\pm$ 11.1B	5.6 $\pm$ 5.6A
Galactosamine + LPS	77.8 $\pm$ 5.5C	27.8 $\pm$ 5.5B
Galactosamine + LPS + uridine	27.8 $\pm$ 5.5B	0A

<sup>a</sup> n = 3 petri dishes.

<sup>b</sup> Values in the same row differed significantly at a *P* value of <0.05 as indicated by different letters.

<sup>c</sup> Lethal treatments were the following: galactosamine (1 mg/slug) alone, *M. osloensis* LPS (0.03 mg/slug) alone, 1 mg of galactosamine plus 0.03 mg of LPS per slug, and 1 mg of galactosamine plus 0.03 mg of LPS plus 3 mg of uridine per slug.

<sup>d</sup> Sublethal treatments were the following: galactosamine (0.3 mg/slug) alone, *M. osloensis* LPS (0.01 mg/slug) alone, 0.3 mg of galactosamine plus 0.01 mg of LPS per slug, and 0.3 mg of galactosamine plus 0.01 mg of LPS plus 1 mg of uridine per slug.

mice, slugs are lower-order animals. However, the slug hepatopancreas has been found to have some primitive liver-like functions (e.g., production of digestive enzymes and detoxification of dietary metals) (5, 20). Thus, the hepatopancreas may be the potential target of galactosamine in the slug. Compared with the several-thousandfold increase in the sensitization of experimental mammals to LPSs induced by galactosamine, the galactosamine-induced increase in the sensitization of *D. reticulatum* to *M. osloensis* LPS is only two- to fourfold. Since the liver is an important organ and the main one for clearance of endotoxins in mammals (7), it is understandable that liver damage caused by galactosamine dramatically increases the susceptibility of mammals to LPSs. Although galactosamine was thought to be a specific hepatotoxic agent (4), the involvement of other target organs cannot be excluded. Since important organs, including the kidney, lung, and heart, are located in the mantle region of *D. reticulatum* (24), it is also possible that galactosamine damages another important organ(s) in the slug, leading to the induced sensitization of *D. reticulatum* to *M. osloensis* LPS. This appears to be the first report of galactosamine-induced sensitization to an LPS in an animal without a liver.

This work was supported by a graduate research competitive grant from the Ohio Agricultural Research and Development Center and by a presidential fellowship from Ohio State University to L. Tan.

#### REFERENCES

- Berrocal, A. M., I. U. Scott, D. Miller, and H. W. Flynn. 2002. Endophthalmitis caused by *Moraxella osloensis*. Graefes Arch. Clin. Exp. Ophthalmol. **240**:329–330.
- Buchman, A. L., M. J. Pickett, L. Mann, and M. E. Ament. 1993. Central venous catheter infection caused by *Moraxella osloensis* in a patient receiving home parenteral nutrition. Diagn. Microbiol. Infect. Dis. **17**:163–166.
- Decker, K., and D. Keppler. 1972. Galactosamine-induced liver injury, p. 183–199. In H. Popper and F. Schaffner (ed.), Progress in liver diseases, vol. 4. Grune & Stratton, New York, N.Y.
- Decker, K., and D. Keppler. 1974. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. Rev. Physiol. Biochem. Pharmacol. **71**:77–103.
- Dimiriadis, V. K. 2001. Structure and function of the digestive system in Stylommatophora, p. 237–257. In G. M. Barker (ed.), The biology of terrestrial mollusks. CABI Publishing, New York, N.Y.
- Fijen, C. A. P., E. J. Kuijper, H. G. Tjia, M. R. Daha, and J. Dankert. 1994. Complement deficiency predisposes for meningitis due to nongroupable meningococci and *Neisseria*-related bacteria. Clin. Infect. Dis. **18**:780–784.
- Galanos, C., M. A. Freudenberg, and W. Reutter. 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA **76**:5935–5943.
- Galanos, C., O. Luderitz, E. T. Rietschel, and O. Westphal. 1977. Newer aspects of the chemistry and biology of bacterial lipopolysaccharides with special reference to their lipid A component, p. 239–335. In T. W. Goodwin (ed.), Biochemistry of lipids II. International review of biochemistry, vol. 14. University Park Press, Baltimore, Md.
- Glen, D. M., and M. J. Wilson. 1997. Slug-parasitic nematodes as biocontrol agents for slugs. Agro Food Ind. Hi-Tech **8**:23–27.
- Grewal, P. S., S. K. Grewal, R. A. J. Taylor, and R. B. Hammond. 2001. Application of molluscicidal nematodes to slug shelters: a novel approach to economic biological control of slugs. Biol. Control **22**:72–80.
- Hochstein, H. D., R. J. Elfin, J. F. Cooper, and S. M. Wolff. 1973. Further development of *Limulus* amebocyte lysate test. Bull. Parenter. Drug Assoc. **27**:139–148.
- Masoud, H., M. B. Perry, and J. C. Richards. 1994. Characterization of the lipopolysaccharide of *Moraxella catarrhalis*: structural analysis of the lipid A from *M. catarrhalis* serotype A lipopolysaccharide. Eur. J. Biochem. **220**:209–216.
- Roth, R. I., J. Levin, and S. Behr. 1989. A modified *Limulus* amebocyte lysate test with increased sensitivity for detection of bacterial endotoxin. J. Lab. Clin. Med. **114**:306–311.
- Stryker, T. D., W. J. Stone, and A. M. Savage. 1982. Renal-failure secondary to *Moraxella osloensis* endocarditis. Johns Hopkins Med. J. **151**:217–219.
- Sugarman, B., and J. Clarridge. 1982. Osteomyelitis caused by *Moraxella osloensis*. J. Clin. Microbiol. **15**:1148–1149.
- Tan, L., and P. S. Grewal. 2001. Infection behavior of the rhabditid nema-

- tode *Phasmarhabditis hermaphrodita* to the grey garden slug *Deroceras reticulatum*. J. Parasitol. **87**:1349–1354.
17. **Tan, L., and P. S. Grewal.** 2001. Pathogenicity of *Moraxella osloensis*, a bacterium associated with the nematode *Phasmarhabditis hermaphrodita*, to the slug *Deroceras reticulatum*. Appl. Environ. Microbiol. **67**:5010–5016.
  18. **Tan, L., and P. S. Grewal.** 2002. Endotoxin activity of *Moraxella osloensis* against the grey garden slug, *Deroceras reticulatum*. Appl. Environ. Microbiol. **68**:3943–3947.
  19. **Tan, L., and P. S. Grewal.** 2002. Comparison of two silver staining techniques for detecting lipopolysaccharides in polyacrylamide gels. J. Clin. Microbiol. **40**:4372–4374.
  20. **Triebkorn, R., and H. R. Kohler.** 1996. The impact of heavy metals on the grey garden slug, *Deroceras reticulatum* (Muller): metal storage, cellular effects and semi-quantitative evaluation of metal toxicity. Environ. Pollut. **93**:327–343.
  21. **Tsai, C., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
  22. **Vanechoutte, M., G. Verschraegen, G. Claeys, and A.-P. V. D. Abeele.** 1990. Serological typing of *Branhamella catarrhalis* strains on the basis of lipopolysaccharide antigens. J. Clin. Microbiol. **28**:182–187.
  23. **Vuori-Holopainen, E., E. Salo, H. Saxen, M. Vaara, E. Tarkka, and H. Peltola.** 2001. Clinical “pneumococcal pneumonia” due to *Moraxella osloensis*: case report and a review. Scand. J. Infect. Dis. **33**:625–627.
  24. **Wilson, M. J., D. M. Glen, and S. K. George.** 1993. The rhabditid nematode *Phasmarhabditis hermaphrodita* as a potential biological control agent for slugs. Biocontrol Sci. Technol. **3**:503–511.
  25. **Wilson, M. J., D. M. Glen, S. K. George, and J. D. Pearce.** 1995. Selection of a bacterium for the mass production of *Phasmarhabditis hermaphrodita* (Nematoda: Rhabditidae) as a biocontrol agent for slugs. Fundam. Appl. Nematol. **18**:419–425.
  26. **Wilson, M. J., D. M. Glen, J. D. Pearce, and P. B. Rodgers.** 1995. Monoxenic culture of the slug parasite *Phasmarhabditis hermaphrodita* (Nematoda: Rhabditidae) with different bacteria in liquid and solid phase. Fundam. Appl. Nematol. **18**:159–166.