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Note

Structure of the exocellular polysaccharide produced by the fungus, *Nomuraea rileyi*

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The fungus, *Nomuraea rileyi*, is an important mycopathogen of various insect defoliators of several agriculture commodities[1]. In light of its specificity and virulence to noctuid larvae, research on this fungus has been directed at studying its po tential as a microbial control agent. Observations on its *in vivo* growth in host insects and its *in vitro* growth in submerged fermentation has revealed that, during its development, it produces excessive amounts of exocellular slime material[2,3]. The ability to precipitate large amounts of this slime with ethanol suggested it to be mainly comprised of polysaccharides. In light of its relative abundance and probable role in the host infection process, we characterized its chemical composition by use of a combination of chemical and enzymic methods.

Nomuraea rileyi produced an exocellular slime comprised exclusively of D-glucans. Besides D-glucose, no other monosaccharide, 2-amino-2-deoxyhexose, or glycuronic acid was detected in the ethanol precipitate of the culture broth. After methanolysis of the methylated glucan, six compounds were identified with retention times corresponding to methyl 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-α,β-D-glucopyranosides (Table I). These results indicated a (1→3)-linked backbone with (1→6)-linked branches. The molar ratio calculated from both the methyl and methyl-*O*-acetyl derivatives was 1:2:1 (Table I). After acetolysis, gel filtration gave only two peaks. The void-volume peak was essentially comprised of (1→3)-linked sugar residues with very few *O*-6-linked residues. The second peak was comprised of only glucose monomers, suggesting the presence of single glucosyl groups as side chains. Increasing the time of acetolysis to 36 or 46 h resulted in the cleavage of the

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TABLE I

G.L.C. ANALYSIS OF METHY ETHERS OBTAINED FROM METHYLATED D-GLUCANS OF THE EXOCELLULAR POLYSACCHARIDE EXTRACTED FROM CULTURE BROTH OF *Nomuraea rileyi*[a].

	Molar ratio	
Methyl-α,β-D-glucosides	*O-Methyl derivatives*	*O-Acetyl-O-methyl derivatives*
2,3,4,6-Tetra-*O*-methyl	1	
2,4,6-Tri-*O*-methyl	2.3	2.3
2,4-Di-*O*-methyl	[b]	1

[a]Similar molar ratios were found for the methylated sclerotan (Actigum CS6, CECA 78140 Velizy-Villacoublay) produced by *Sclerotium rolfsii*. [b]Not calculated because of incomplete methylation.

(1→3) backbone. On methylation analysis, the permethylated, periodate-resistant polysaccharide gave exclusively methyl 2,4,6-tri-*O*-methylglucopyranoside, indicating a linear (1→3)-D-glucan. This result was in agreement with a (1→3)-linked backbone having one D-glucose unit as the side chain.

The *N. rileyi* polysaccharide was hydrolyzed only with combinations of various enzyme fractions (Table II). Individually, the exo- and endo-(1→3)-, and exo-(1→6)-β-D-glucanase did not hydrolyze the *N. rileyi* polysaccharide. However, the exo-(1→3)-β-D-glucanase was able to hydrolyze the polysaccharide after periodate oxidation, demonstrating that the polysaccharide is a branched glucan with a (1→3)-β-D-glucan backbone. A mixture of both endo- and exo-(1→3)-β-D-glucanases degraded the slime at a rate similar to that of the complex enzymic mixture

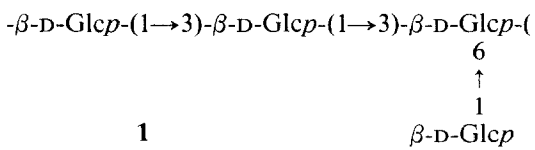
TABLE II

ENZYMIC HYDROLYSIS[a] OF *Nomuraea rileyi* EXOCELLULAR POLYSACCHARIDE AND OTHER D-GLUCANS

	Substrates			
			Nomuraea rileyi polysaccharide	
Enzymes	*Laminarin*	*Pustulan*	*Original*	*Smith degraded*[b]
Laminarinase (Sigma)	+	−	−	+[c]
exo-(1→3)-β-D-Glucanase (G_1)	+	−	−	[d]
exo-(1→3)-β-D-Glucanase (G_{2a})	+	−	−	+
exo-(1→6)-β-D-Glucanase (G_{2b})	−	+	−	−
endo-(1→3)-β-D-Glucanase (G_3)	+	−	−	[d]
$G_1 + G_{2a} + G_{2b}$	+	+	+[e]	[d]
$G_1 + G_3$	+	[d]	+	[d]
$G_1 + G_{2a} + G_{2b} + G_3$	+	+	+	[d]

[a]For 60 min. [b]Periodate oxidized prior to enzyme hydrolysis. [c]− No liberation of free hexose; + hydrolysis of 25-50% of the polysaccharide. [d]Not tested. [e]The liberated hexoses were < 25 % of the polysaccharide.

(Table II). These enzymic degradation data and the chemical analysis demonstrated that the polysaccharide of *N. rileyi* is a (1→3)-β-D-glucan with (1→6)-linked sidechains (**1**).



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The exocellular polysaccharide recovered from the culture supernatant of hyphal body fermentation had the same composition as the one produced by the mycelial culture. Several fungi are known to produce exocellular (1→3)-, (1→6)-β-D-glucans which are differentiated by the number of side-chain D-glucose units[4]. The polysaccharide of *N. rileyi* has the same structure as sclerotan which has been isolated from *Sclerotium rolfsii* and *Sclerotinia libertiana*[5]. Related polysaccharide structures have been found[6–8] in *Claviceps purpurea, Botrytis cinerea,* and *Pullularia pullulans*. The role that the exocellular polysaccharide plays in the *N. rileyi* infection process of host insects is presently unclear. Certain microbial polysaccharides, *i.e.,* linear (1→3)-β-D-glucans, which are ubiquitous in many fungi, have been reported to elicit, suppress, or both, the cellular immune response in invertebrates[9]. Alternatively, this component, detected in germ tubes and mycelium, could be playing a role in adhesion to host cuticle, or possibly in the mummification (preservation) of dead, infected larvae, respectively.

EXPERIMENTAL

Cultivation of fungus and extraction of slime material. — Selected strains of the *Nomuraea rileyi* isolate (ATCC 52631), which produce either yeast-like hyphal bodies or mycelium, were propagated on a Sabouraud D-glucose + 2% yeast extract or 3% D-glucose + 1% yeast extract broth under submerged fermentation conditions[3]. Culture filtrations harvested 96 h post-inoculation were separated form cellular material by combined centrifugation (10 000*g*, 30 min) and filtration through fritted glass (12 μm). Exocellular polysaccharide samples were precipitated from the filtrates by addition of 95% ethanol (2 vols.) at 4°, washed with 95% ethanol, suspended in water, and freeze-dried. Up to 5 g of lyophilyzed slime was obtained per liter of 4-day old culture broth.

Structural analysis of the polysaccharide. — (*a*) *Monosaccharide composition.* The hexose content was determined on total, unhydrolyzed slime samples with the phenol and anthrone methods as described by Latgé *et al.*[10]. The absence of 2-amino-2-deoxyglucose, glycuronic acid, and protein was demonstrated by various chemical tests [10]. The monosaccharide composition was determined after methanolysis with 0.5M HCl in methanol for 24 h at 80°. Methyl glycosides were identified as trifluoroacetyl derivatives by g.l.c. according to the procedure of Zanetta *et al.*[11].

(*b*)*Methylation.* Exocellular polysaccharide samples (2 mg) were methylated by the method of Finne[12] using potassium *tert*-butoxyde. The permethylated glucan was purified by elution from a LH-20 column with 1:1 ethanol–chloroform, dried, and then methanolyzed with 0.5M HCl in methanol for 24 h at 80°. The products were identified by g.l.c., before and after acetylation 1:1 pyridine–acetic anhydride for 2 h at room temperature, in a glass column (0.3 x 300 cm) filled with 3% PEG 6000 on WHP 80/100 with a N_2 pressure of 0.15 MPa and a temperature program from 120° to 200° at 2°/min. Identification of the peak was confirmed by g.l.c.–m.s. using standard sugars[13]. The absence of mono-*O*-methyl derivatives or methyl-α,β-D-glucopyranoside demonstrated that methylation was complete.

(*c*) *Acetolysis.* Acetolysis of the polysaccharide samples was performed as described by Dubourdieu *et al.*[6]. The freeze-dried polysaccharide (10 mg) was suspended in 10:10:1 acetic anhydride–acetic acid–H_2SO_4 (10 mL) for 12, 18, or 24 h in a sealed tube at 25°. The mixture was neutralized on ice with $NaHCO_3$. Acetylated sugars were extracted with chloroform, and the solution was washed with $CaCl_2$ solution and evaporated under vacuum. The residues were dissolved in acetone and 0.2M $NaBH_4$ was added. After 30 min at 4°, the reaction was stopped by addition of Dowex 50 X8 (H^+) cation-exchange resin to bring the pH of the mixture to 5.0. After removal of the resin, the filtrate was concentrated under vacuum and passed through a column of Sephadex G-15. Sugars were detected in the eluent fractions with the phenol reagent[10]. The excluded peak was methylated as described previously.

(*d*) *Smith degradation.* This degradation was performed as described by Dubourdieu *et al.*[6]. The polysaccharide (25 mg) was dissolved in (60 mL) aqueous 100mM $NaIO_4$. The mixture was incubated in darkness for 8–12 d at 4°, and the undissolved material removed by centrifugation. 1,2-Ethanediol (0.6 mL) was added to the supernate and, after a 30-min incubation, the mixture was dialyzed against water for 2–3 d. $NaBH_4$ (500 mg) was added, and the mixture incubated for 24 h at 20°. After dialysis for 24 h, M H_2SO_4 was added to bring the pH to 1.0. After 24 h at 20°, the solution was concentrated with Carbowax, dialyzed for 3 d at room temperature, and freeze-dried. Methylation of the freeze-dried material was performed as described above.

(*e*) *Enzymic analysis.* Exo-, and endo- (1→3)-β-D-glucanase and exo-(1→6)-β-D-glucanase were prepared form a *Trichoderma* extract (Novozym 116-1) according to the general procedures described by Dubourdieu[14]. Laminarin and pustulan (Calbiochem) were used as substrates at a concentration of 0.5 mg/mL in 0.1M sodium acetate at pH 5.0 to monitor the (1→3)- and (1→6)-β-D-glucanase activities during the fractionation of Novozym 116-1. After 15–60 min at 37°, the relative levels of sugars released from these substrates by the enzyme fractions were measured with the Nelson–Somogyi method[15]. Laminarinase (Sigma) was used as an exo-(1→3)-β-D-glucanase standard. The fractionation of the Novozym 116-1 (100 mg) involved initially a desalting through a Sephadex G-25 column. The enzyme-containing eluate was applied in water to a Bio-Gel–DEAE column (2.5 x 11 cm).

This was followed by a step-wise elution with water, 0.2M NaCl and 0.4M NaCl, which allowed the recovery of three peaks of activity, (G_1, G_2, and G_3). A final wash with 1.0M NaCl did not elute any additional (1→3)- or (1→6)-D-glucanase activity. Peak G_1 possessed exo-(1→3)-β-D-glucanase activity, G_2 had both (1→3)- and (1→6)-β-D-glucanase activity, and G_3 had only endo-(1→3)-β-D-glucanase activity. Fraction G_2 was passed through a column of Sephacryl S-200 in 0.15M NaCl and 0.1M sodium acetate buffer, pH 5, to resolve the (1→3)- (Fraction G_{2a}) from the (1→6)-β-D-glucanase activity (Fraction G_{2b}).

The ability of the enzyme fractions, alone and in combination, to hydrolyze both crude slime or the polysaccharide modified by acetolysis or periodate oxidation was assayed as follows. Aliquots of polysaccharide (0.5 mg/mL) in 0.1M sodium acetate, buffer pH 5.1, were incubated with the various enzyme preparations at a concentration of 100 μg of protein/mL, and the relative levels of released reducing sugars were determined as described previously.

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