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Note

A novel teichoic acid from the cell wall of *Streptomyces* sp. VKM Ac-2275

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Dedicated to the memory of Professor Nikolay K. Kochetkov

Abstract—The cell wall of a pathogenic strain *Streptomyces* sp. VKM Ac-2275 isolated from potato tubers infected by scab contains a teichoic acid related to poly(glycosylpolyol phosphate) with a repeating unit established by chemical and NMR spectroscopic methods. About 60% of L-rhamnose residues bear an *O*-acetyl group at O-2 and 20% of the internal glucose residues contain an additional phosphate at C-4. The polymer is built of 5–6 units. This structure is found in bacteria for the first time. The strain is phylogenetically closest to the scab-causing species *Streptomyces scabiei* and *Streptomyces europaeiscabiei*, but differs from both these species in morphological and physiological characters and does not produce thaxtomin A, the main phytotoxin produced by *S. scabiei*.

Keywords: Streptomycetes; Teichoic acid; NMR spectroscopy

Cell walls of potato pathogenic streptomycetes studied so far were shown to contain different polymers related to teichoic acids, viz., poly(glycerol phosphates),[1,2] poly(ribitol phosphates),[3] teichuronic acids,[3] oligomers and polymers of Kdn[1–3] as well as neutral polysaccharides.[4] These streptomycetes with weak, medium or high pathogenicities are characterised by the presence of several cell wall polymers.[1–4]

In the present communication, we report the structure elucidation of a teichoic acid from the cell wall of an isolate from potato tubers infected by scab lesions.

The 16S rDNA-based phylogenetic analysis confirmed that the strain belongs to the genus *Streptomyces* and was the closest (99.3% 16S rDNA binary sequence similarity, 831 identical nucleotides out of 837) to *Streptomyce scabiei*[5] and *Streptomyces europaeiscabiei*,[6] which are also causative agents of potato scab. Among the peptidoglycan diagnostic amino acids, LL-diaminopimelic acid and glycine, typical of members of the genus *Streptomyces*,[7,8] were present. The strain differs from the phylogenetically closest species by spiral spore chains, by production of a melanoid pigment on tyrosine agar and by utilisation of trehalose and melibiose as sole sources of carbon and energy. All the data obtained suggest that the strain *Streptomyces* sp. VKM Ac-2275 represents a new species in the genus *Streptomyces*.

Acid hydrolysis of the cell wall of *Streptomyces* sp. VKM Ac-2275 (2 M HCl, 100 °C, 3 h) afforded glycerol monophosphate as the major phosphorus-containing component, as well as inorganic phosphate and monosaccharides, viz., glucose, rhamnose and an amino sugar, which was indicative of the presence of a teichoic acid.

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Three consecutive extractions of the cell wall with 10% trichloroacetic acid (4 °C, 24 h each) afforded teichoic acid-containing preparations with identical chemical compositions. The combined preparation was used for the structural analysis of the polymer.

The components of the acid hydrolysate of the preparation were identical with those of the cell wall. The amino sugar identified as glucosamine in the hydrolysates of both the cell wall and the preparation was shown to be a constituent of the teichoic acid.

Alkaline hydrolysis of the preparation (1 M NaOH, 100 °C, 3 h) afforded inorganic phosphate and a phosphate with $E_{GroP} = 0.2$ as the major product. Its acid hydrolysis resulted in glucose, rhamnose, glucosamine, glycerol and inorganic phosphate.

The absolute configurations of glucose and rhamnose were determined as D- and L-, respectively. The absolute configuration of glucosamine was established using ^{13}C NMR spectroscopic data[9] (see below). The absence of glycerol bisphosphate in the acid hydrolysates of either the cell wall or the preparation was indicative of the poly(glycosylpolyol phosphate) nature of the cell wall polymer of the streptomycete under study.[10]

The decisive evidence for the structure of the teichoic acid was gained by NMR spectroscopy. The NMR experiments were carried out for the intact polymer preparation (**1**) (Table 1), the O-deacetylated teichoic acid (this was effected by treatment with dilute ammonia) (**2**) (Table 2) and oligomer obtained upon HF-induced depolymerisation (48% HF, 4 °C, 16 h) of the O-deacetylated polymer (**3**) (Table 3).

The ^{13}C NMR spectrum of the polymer **1** contained several signals of different intensities in the region of resonances of anomeric carbon atoms (δ 101–105), which suggested the presence of several sugar constituents and an irregular structure of the repeating unit. Analysis of other characteristic regions of the spectrum revealed the presence of an amino sugar (δ 56.9 is typical of a carbon atom bound to nitrogen) and 6-deoxy sugar constituents of the polymer (δ 17.8 and 17.9 for CH_3, (C-6)). Signals at δ 23.8 and 21.4 (less intense) corresponded to *N*- and *O*-acetyl groups, respectively. In the APT (attached proton test) spectrum,[11] signals for three CH_2OH groups (δ 62.2, 62.4 and 63.8) and three substituted hydroxymethyl groups (δ 67.9, 70.4 and 72.6) were observed.

The ^{31}P NMR spectrum contained an intense signal at δ −0.5 and two minor signals at δ −0.7 and +0.5.

In the low-field region of the ^{1}H NMR spectrum (δ 4.5–5.0), several signals of different intensities for anomeric protons and, probably, for the protons at the carbon atoms carrying acetoxy groups were present. The high-field region of the spectrum contained two doublets at δ 1.23 and 1.25 (C–CH_3) with an integral intensities ratio of 2:3 and two singlets at δ 2.15 (CH_3COO) and 2.11 (CH_3CON) with the same intensity ratio.

Table 1. NMR data for the native polymer (**1**) from the cell wall of *Streptomyces* sp. VKM Ac-2275

Residue	Proton	δ_H	Carbon	δ_C
→1)-Gro-(3-*P*-	H-1,1′	3.93; 3.88	C-1	72.6
	H-2	4.12	C-2	70.6
	H-3,3′	4.03; 3.94	C-3	67.9 (63.8)[a]
	H-1	4.57	C-1	102.6
↓ 6)	H-2	3.94 (3.69)	C-2	76.8 (76.2)
-3)-β-D-Glc*p*-(1→1)[b]	H-3	4.29 (4.36)	C-3	79.7 (79.5)
↑ 2)	H-4	4.14 (4.25)	C-4	69.2 (76.9)
	H-5	3.90	C-5	75.0
	H-6,6′	4.04; 3.92	C-6	70.4
→3)-β-D-Glc*p*NAc-(1→2)	H-1	4.96	C-1	101.4
	H-2[c]	3.85	C-2[d]	56.9
	H-3	3.63	C-3	83.9
	H-4	3.50	C-4	70.3
	H-5	3.40	C-5	77.8
	H-6,6′	3.96; 3.77	C-6	62.4
α-L-Rha*p*2OAc-(1→3)	H-1	4.89	C-1	100.1
	H-2[e]	4.95	C-2[f]	74.2
	H-3	3.93	C-3	70.0
	H-4	3.48	C-4	73.7
	H-5	4.05	C-5	70.3
	H-6	1.25	C-6	17.8
β-D-Glc*p*-(1→6)	H-1	4.52	C-1	104.2
	H-2	3.30	C-2	74.6
	H-3	3.50	C-3	77.1
	H-4	3.39	C-4	71.1
	H-5	3.46	C-5	77.4
	H-6,6′	3.92; 3.73	C-6	62.2

^{31}P NMR spectrum, δ −0.5 (major), minor signals at δ −0.7 and +0.5.

[a] Terminal glycerol unit.

[b] The chemical shifts for the residue bearing phosphate groups at both C-3 and C-4 are in parentheses.

[c] CH_3CON at δ 2.11.

[d] CH_3CON at δ 23.8 and 175.5, respectively.

[e] CH_3COO at δ 2.15.

[f] CH_3COO at δ 21.4 and 174.2, respectively.

This fact allowed us to suggest that the irregular character of the repeating unit of the polymer might result from nonstoichiometric O-acetylation of one of the carbohydrate residues. Therefore, the original preparation was subjected to mild alkaline hydrolysis, and further investigations were carried out for the O-deacetylated polymer and the oligomer derived therefrom upon HF-induced depolymerisation.

The ^{13}C NMR spectrum of the polymer **2** (Table 2) contained four signals of virtually equal intensities in the region of resonances of anomeric carbon atoms (δ 101.4, 102.7, 103.0 and 104.2). The high-field region of the spectrum contained a signal typical of CH_3CON (δ 23.8), whereas no signal for the CH_3COO (δ 21.4) was observed. The region of resonances of CH_3 groups of 6-deoxy sugars was characterised by the presence of a single signal at δ 17.9. The APT spectrum (Fig. 1) con-

Table 2. NMR data for the O-deacetylated polymer (**2**) from the cell wall of *Streptomyces* sp. VKM Ac-2275

Residue	Proton	δ_H	Carbon	δ_C	Phosphorus	δ_P
1)-Gro-(3-*P*-	H-1,1′	3.92; 3.87	C-1	72.5		
	H-2	4.10	C-2	70.6		
	H-3,3′	4.04; 3.95	C-3	67.9	P-3	−0.5 (+0.6)
↓	H-1	4.56	C-1	102.7		
6)	H-2	3.93 (3.68)	C-2	76.7 (76.2)		
-3)-β-D-Glc*p*-(1→1)[a]	H-3	4.28 (4.36)	C-3	79.7 (79.5)	P-3	−0.5 (+0.6)
2)	H-4	4.13 (4.25)	C-4	69.2 (76.4)	(P-4)	(−0.7)
↑	H-5	3.89	C-5	75.0		
	H-6,6′	4.04; 3.92	C-6	70.3		
→3)-β-D-Glc*p*NAc-(1→2)	H-1	4.96	C-1	101.4		
	H-2[b]	3.84	C-2[c]	56.9 [−0.1]		
	H-3	3.63	C-3	83.5		
	H-4	3.47	C-4	69.9 [−1.3]		
	H-5	3.49	C-5	77.8		
	H-6,6′	3.94; 3.76	C-6	62.4		
α-L-Rha*p*-(1→3)-	H-1	4.85	C-1	103.0		
	H-2	3.77	C-2	72.2		
	H-3	3.73	C-3	71.6		
	H-4	3.41	C-4	73.4		
	H-5	3.98	C-5	70.3		
	H-6	1.23	C-6	17.9		
β-D-Glc*p*-(1→6)-	H-1	4.51	C-1	104.2		
	H-2	3.29	C-2	74.5		
	H-3	3.49	C-3	77.1		
	H-4	3.38	C-4	71.1		
	H-5	3.44	C-5	77.4		
	H-6,6′	3.91; 3.72	C-6	62.2		

The effects of glycosylation[9] are given in square brackets.
[a] The chemical shifts for the residue bearing phosphate groups at both C-3 and C-4 are in parentheses.
[b] CH_3CON at δ 2.11.
[c] CH_3CON at δ 23.8 and 175.5, respectively.

tained the signals of CH_2OH groups and substituted hydroxymethyl groups as in the APT spectrum of the polymer **1**.

The ^{31}P NMR spectrum was identical with that of the polymer **1**.

The low-field region of the ^1H NMR spectrum contained four intense signals at δ 4.51, 4.56, 4.96 (each as a doublet with $^3J_{1,2} = 8$ Hz) and at 4.85 (a broad singlet with $^3J_{1,2} < 2$ Hz) (Table 2). Two three-proton signals were present in the high-field region, viz., a singlet at δ 2.11 and a doublet at δ 1.23 ($^3J_{5,6} = 6$ Hz).

The assignment of the signals in the 1D ^1H, ^{13}C and ^{31}D NMR spectra was carried out using 2D techniques (^1H, ^1H COSY and TOCSY) and heteronuclear experiments (^1H, ^{13}C HSQC, HMQC-TOCSY and ^1H, ^{31}P HMQC). The COSY and TOCSY spectra revealed closed spin systems typical of protons for two β-glucopyranose residues (β-Glc*p*[1] and β-Glc*p*[2]), a 2-acetamido-2-deoxy-β-glucopyranose residue (β-Glc *p*NAc), an α-rhamnopyranose residue (α-Rha*p*) and glycerol (Gro). The linkage sequence of these residues was inferred from the analysis of ROESY and ^1H, ^{31}P HMQC spectra. The following cross-peaks were identified in the ROESY spectrum: H-1 (α-Rha*p*)/H-3 (β-Glc*p*NAc), 4.85/3.63; H-1 (β-Glc*p*NAc)/H-2 (β-Glc*p*[1]), 4.96/3.93; H-1 (β-Glc*p*[2])/H-6′ (β-Glc*p*[1]), 4.51/3.92; H-1 (β-Glc*p*[1])/H-1,1′ (Gro), 4.56/3.92, 3.87. The most abundant peak in the ^{31}P NMR spectrum (δ_P −0.5) correlated with the H-3 protons of β-Glc*p*[1] (δ_H 4.28) and H-3,3′ protons of Gro (δ_H 4.04 and 3.95) (Fig. 2).

The HSQC spectrum confirmed the absence of substituents in the α-Rha*p* and β-Glc*p*[2] residues and the presence of substituents at O-2, O-3 and O-6 of β-Glc*p*[1], at O-3 of β-Glc*p*NAc and O-1 and O-3 of Gro when comparing with the spectra of the corresponding non-substituted pyranoses and glycerol.

The small β-effects of glycosylation of glucosamine at O-3 with L-rhamnose on the chemical shifts of C-2 and C-4 ($\Delta\delta$ −0.1 and −1.3 ppm, respectively) suggest[9] that the absolute configurations of these monosaccharide residues are different; hence, 2-acetamido-2-deoxyglucopyranose is D.

Analysis of the 2D spectra gave also a rationale for the presence of minor peaks in the 1D spectra of the deacetylated polymer. Thus minor signals at δ_P −0.7 and +0.6 in the ^{31}P NMR spectrum are due to the presence of an additional phosphate at C-4 of some of the β-Glc*p*[1] residues (∼20%) (Fig. 2). This substitution

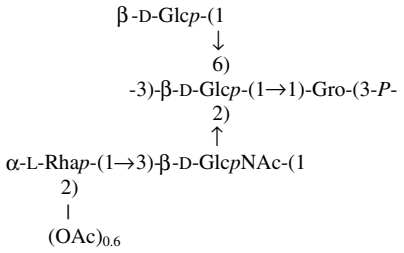
Table 3. NMR data for the oligomer (**3**) of the cell wall from *Streptomyces* sp. VKM Ac-2275

Residue	Proton	δ_H	Carbon	δ_C
→1)-Gro	H-1,1′	3.89; 3.83	C-1	72.8
	H-2	3.95	C-2	71.9
	H-3,3′	3.69; 3.62	C-3	63.8
	H-1	4.51	C-1	103.1
↓	H-2	3.69	C-2	80.1
6)	H-3	3.71	C-3	74.4
P-4-β-D-Glc*p*-(1→1)	H-4	3.88	C-4	75.2
2)	H-5	3.68	C-5	76.5
↑	H-6,6′	4.04; 3.91	C-6	70.5
→3)-β-D-Glc*p*NAc-(1→2)	H-1	4.87	C-1	102.6
	H-2[a]	3.83	C-2[b]	57.3
	H-3	3.63	C-3	82.8
	H-4	3.51	C-4	69.9
	H-5	3.46	C-5	77.4
	H-6,6′	3.95; 3.77	C-6	62.4
α-L-Rha*p*-(1→3)	H-1	4.88	C-1	102.8
	H-2	3.81	C-2	72.2
	H-3	3.75	C-3	71.7
	H-4	3.44	C-4	73.3
	H-5	3.99	C-5	70.4
	H-6	1.24	C-6	17.9
β-D-Glc*p*-(1→6)	H-1	4.53	C-1	104.2
	H-2	3.30	C-2	74.5
	H-3	3.50	C-3	77.1
	H-4	3.39	C-4	71.1
	H-5	3.48	C-5	77.6
	H-6,6′	3.92; 3.73	C-6	62.2

[a] CH_3CON at δ 2.07.
[b] CH_3CON at δ 23.7 and 176.0, respectively.

produces a predictable effect on the chemical shifts of the adjacent protons and carbon atoms (see Note to Table 2). A comparison of the respective 1D and 2D NMR spectra of the polymers **1** and **2** made it possible to localise the *O*-acetates in the intact polymer. A downfield displacement of the signal for H-2 in the major part (~60%) of α-Rha*p* residues in the polymer **1** is an unambiguous evidence for the presence of the acyl substituent at *O*-2 in these residues. Characteristic α- and β-effects due to acylation could be revealed from the analysis of the ^{13}C NMR spectrum: the signal for C-2 is shifted downfield and the signals for C-1 and C-3 are shifted upfield.

Thus, the polymer has the following repeating unit:



The teichoic acid-derived oligomer (**3**) was studied using 2D NMR techniques described above. It was shown that the structure of the oligomer corresponds to that of the polymer repeating unit and differs from the latter in the absence of phosphate groups at C-3 of glycerol and C-3 of β-Glc*p*[1] (Table 3); however, the phosphate residue at C-4 of this glucose residue is retained. This result could be explained either by

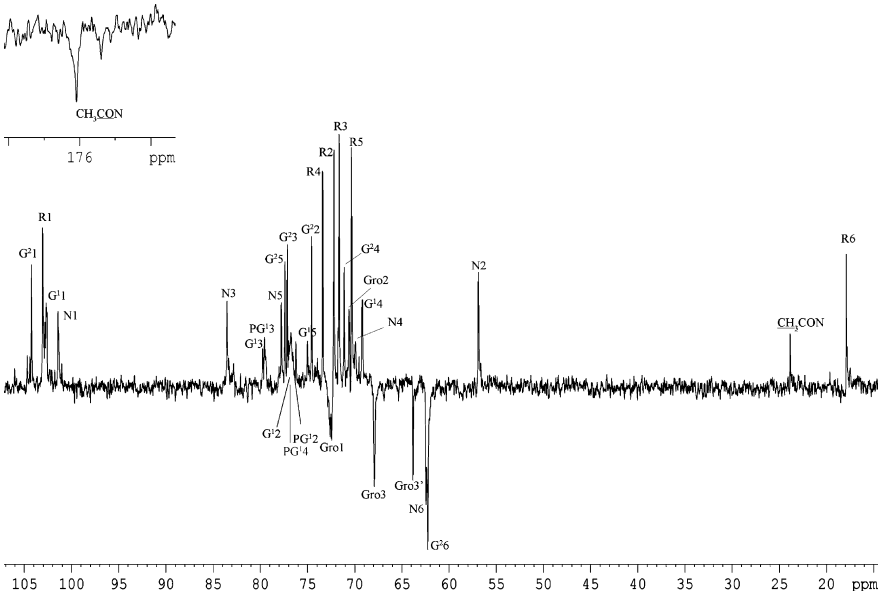


Figure 1. APT spectrum of the O-deacetylated teichoic acid (**2**) from the cell wall of *Streptomyces* sp. VKM Ac-2275. Abbreviations: R1–R6, carbon atoms C-1–C-6 of Rha; N1–N6, carbon atoms C-1–C-6 of GlcNAc; $G^1$1–$G^1$6, carbon atoms C-1–C-6 of substituted Glc; $G^2$1–$G^2$6, carbon atoms C-1–C-6 of nonsubstituted Glc; $PG^1$2–$PG^1$4, carbon atoms C-2–C-4 of 3,4-bisphosphorylated Glc; Gro1–Gro3, carbon atoms of glycerol, Gro3′, –CH_2OH group of the terminal glycerol unit.

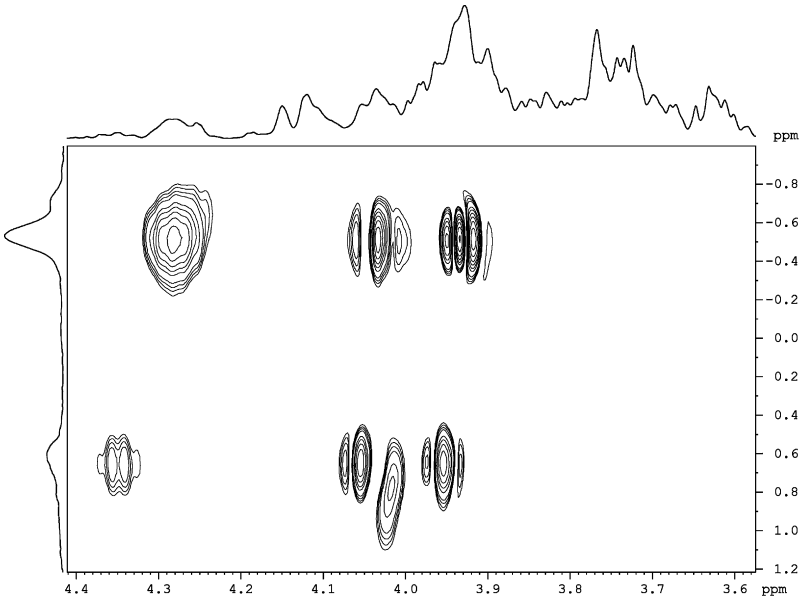


Figure 2. ^{1}H, ^{31}P HMQC spectrum of the O-deacetylated teichoic acid (**2**) from the cell wall of *Streptomyces* sp. VKM Ac-2275. The ^{1}H and ^{31}P NMR spectra are shown along the horizontal and vertical axes, respectively.

successful isolation of repeating fragments initially phosphorylated at C-4 of β-D-Glc*p* residues or by migration of phosphate group from C-3 to C-4 during depolymerisation under HF hydrolysis conditions.

Judging from the relative intensity of the signal for C-3 of the terminal glycerol residue (δ 63.4), the polymer is built of 5–6 repeating units.

The presence of an additional phosphate group at C-4 of β-Glc*p*[1] in the polymer increases its negative charge density. This is characteristic of cell wall teichoic acids of actinomycetes, which were shown to often contain various acid groups.[12–17]

Streptomyces sp. VKM Ac-2275 differs from all other potato pathogenic streptomycetes described earlier in that it contains only one cell wall anionic polymer, viz., poly(glycosylpolyol phosphate) teichoic acid, with the structure hitherto unknown in bacteria. Determination of plant pathogenicity on the microtubers showed that the strain VKM Ac-2275 induced rough, corky lesions such as those resulting from natural infections. Compared with *S. scabiei* and some other scab-causing streptomycetes, the strain, however, did not produce thaxtomin A, the nitrated dipeptide phytotoxin, which inhibits cellulose biosynthesis in expanding plant tissues.[18]

All the data obtained suggest that the strain *Streptomyces* sp. VKM Ac-2275 represents a new species in the genus *Streptomyces*.

1. Experimental

The strain 2275 was isolated from common scab lesions from potatoes *Solanum tuberosum*, *cultivar* '*Detskosel-sky*' as reported earlier.[19] For studying phenotypical characteristics, the methods and media are those as described.[20] Production of thaxtomin was determined according to the known protocol.[21] The 16S rRNA gene was amplified and analysed as described previously.[3]

The culture of streptomycete was grown on a peptone–yeast medium[22] for 18–20 h on a shaker at 28 °C. The biomass was collected at the logarithmic phase of growth. The cell wall was obtained by centrifugation after disruption of mycelium by sonication in 2% sodium dodecyl sulfate, washed several times with water and freeze-dried. The polymers were isolated from the cell walls by repeated extraction with 10% trichloroacetic acid at 4 °C for 24, 48 and 72 h, the extracts were separated from cell debris, dialysed against distilled water and freeze-dried.

The peptidoglycan preparation and hydrolysis was also carried out as described previously.[23] The ammonolysis was carried out as described previously.[24]

Descending paper chromatography and electrophoresis were carried out on a Filtrak FN-3 paper (Germany). Electrophoresis was run in a pyridinium acetate buffer, pH 5.6, to separate phosphates. Paper chromatography was performed in the solvent systems 3:1:5:3 pyridine–benzene–BuOH–water to separate glycerol and monosaccharides and 4:1:5 BuOH–AcOH–water to separate the amino sugar and amino acids. Phosphate esters were detected with the molybdate reagent; reducing sugars, with aniline hydrogenphthalate; glycerol and monosaccharides, with 5% $AgNO_3$ in aqueous ammonia; amino sugar and amino acids were detected with a ninhydrin reagent. Acid hydrolysis of the cell wall and the polymer were carried out with 2 M HCl at 100 °C for 3 h and alkaline hydrolysis, with 1 M NaOH at

100 °C for 3 h. Partial hydrolysis with 48% HF was carried out at 4 °C for 16 h followed by freeze-drying through a trap with NaOH. The hydrolysis products were separated on a column (90 × 1.5 cm) with TSK-gel HW-40S (Toyopearl, Japan), elution with 1% AcOH being monitored with a differential refractometer (Knauer, Germany).

The absolute configuration of glucose and rhamnose was determined according to a published procedure.[25]

To evaluate the pathogenic activity of the strain, the aseptically cultured potato microtubers in vitro were used.[26] The latter were immersed for 5–10 min in a suspension of 14-day old agar culture (mainly spore mass) followed by incubation at 100% relative humidity for 5 days at 22–24 °C in the darkness.

NMR spectra were recorded with a DRX-500 spectrometer (Bruker, Germany) for 2–3% solns in D_2O at 30 °C with TSP (δ_H 0.00) and acetone (δ_C 31.45) as the internal standards and 80% phosphoric acid (δ_P 0.0) as the external standard. 1D ^1H NMR specthoricra were obtained with a presaturation of the HDO signal for 1 s. 2D spectra were obtained using standard pulse sequences from the Bruker software. A mixing time of 150 and 200 ms was used in 2D TOCSY and ROESY experiments, respectively.

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