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# The structures of the cell wall teichoic acids from the thermophilic microorganism *Geobacillus thermoleovorans* strain Fango

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**Abstract**—The structures of two teichoic acid fractions (**TA1** and **TA2**) isolated from the thermophilic Gram-positive bacterium *Geobacillus thermoleovorans* strain Fango were investigated by means of chemical and NMR spectroscopic methods. The most abundant species (**TA1**) exhibited a rather regular structure comprising two different repeating units of 1,3-glycerol phosphate non-stoichiometrically substituted by terminal-α-D-Gal*p* (t-α-D-Gal*p*). The second molecular species (**TA2**) presented a higher structural variability and t-α-D-Glc*p* and the disaccharides t-α-D-Glc*p*NAc-(1→2)-α-D-Glc*p* and t-α-D-Glc*p*NAc-(1→3)-α-D-Glc*p* were also present as minor substituents at O-2 of the glycerol phosphate residues. Minor substitution by alanine could also be detected.


*Keywords:* Bacterial cell wall; Teichoic acids; *Geobacillus thermoleovorans*; NMR spectroscopy

In addition to lipoteichoic acids,[1] teichoic acids (TA) represent essential cell wall constituents of Gram-positive bacteria.[2] They are polyanionic molecules, of which, based on the composition of the main chain, four structural types can be distinguished, that is, poly(polyol phosphates), poly(glycosylpolyol phosphates), poly(polyol phosphate–glycosyl phosphates) and poly(polyol phosphate–glycosylpolyol phosphates).[3] Of these, the first two are most often occurring. Poly(polyol phosphates) contain as polyol moieties glycerol, erythrol, ribitol, arabinitol or mannitol. Teichoic acids comprise between 20% and 50% of the Gram-positive cell wall. While lipoteichoic acids are embedded in the cytoplasmic membrane through a lipid anchor (acylated glycerol), TA are linked directly to muramic acid of the peptidoglycan through a phosphodiester bond.[1,3] In this study, the structure of two different teichoic acids isolated from the thermophilic bacterium *Geobacillus thermoleovorans* strain Fango (ATCC BAA-872) was investigated. This microorganism is aerobic, endospore forming, and thermophilic, and was isolated from a geothermal soil sample in Italy (Stufe di Nerone, Lucrino, Na). *G. thermoleovorans* is characterized by phenotypic heterogeneity and genotypic homogeneity. The cell wall structure is Gram-positive, but the Gram-stain reaction may vary between negative and positive depending on the strain.[4] This unusual behaviour prompted us to study the chemical composition of the cell wall. After growth of the bacteria at 70 °C, the extraction of TA was performed with hot phenol–water.[5] The water phase collected after the extraction was enzymatically digested to remove nucleic acids and proteins, and subsequently purified by size exclusion chromatography. A first purification on Sephacryl S-400 yielded two fractions, of

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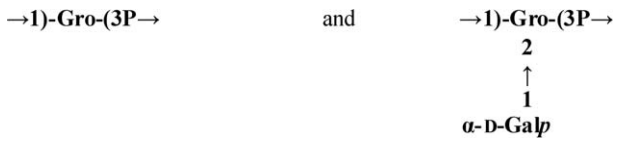
which the most retained fraction was entirely constituted by TA (**TA1**). The first and high molecular mass fraction was mainly composed by lipid components that eluted as micelle aggregates. From this fraction, a second and different teichoic acid fraction (**TA2**) was purified by size exclusion HPLC on TSK G-5000. Both TA fractions were analyzed for their monosaccharide content. GC–MS analysis of acetylated *O*-methyl glycosides derivatives identified in both fractions a high amount of glycerol (Gro) and glycerol phosphate (GroP). Moreover, **TA1** contained D-galactose (D-Gal) while **TA2** possessed, in addition, minor amounts of D-glucose (D-Glc) and D-glucosamine (D-GlcN).

Methylation analysis identified t-Gal*p* in **TA1**, and minor amounts of t-Glc*p*, t-Glc*p*N, 2-substituted Glc*p* and 3-substituted Glc*p* in **TA2**. After treatment with 48% aqueous HF and methylation, additional Hex-O-Gro fragments could be detected.

Of the isolated fractions, a complete 1D and 2D NMR spectroscopical analysis was performed. $^{1}$H NMR on **TA1** (Fig. 1) showed a highly homogeneous product. A single signal (H-1**A**) appeared in the anomeric region of the spectrum at 5.150 ppm. On the basis of TOCSY and DQF-COSY spectra, all resonances were assigned for t-Gal*p* (Table 1), on the basis of chemical shift and the small $^{3}J_{3,4}$ and $^{3}J_{4,5}$ values. A coupling constant of 3.4 Hz for $^{3}J_{1,2}$ identified, together with the typical chemical shifts for anomeric proton and carbon, the α-configuration. $^{13}$C NMR resonances were deduced from a $^{1}$H,$^{13}$C HSQC spectrum (not shown). In this spectrum, signals for at least two magnetically different glycerol units (**B** and **C**) were identified. In particular, residue **B** presented down-field displacement of the C-2 resonance (75.1 ppm), compared to residue **C**, likely due to glycosylation. This hypothesis was confirmed by a 2D ROESY spectrum, in which a strong dipolar correlation between H-1**A** and H-2**B** was found. This data were coherent with the occurrence of two different repeating units, one composed of glycerol phosphate and the other one by glycerol phosphate substituted by t-α-D-Gal*p*:




It was not possible to evaluate the exact ratio between the two units due to the overlapping of the signals of the glycerol units between 4.200 and 3.500 ppm.

The second teichoic acid fraction (**TA2**), isolated after HPLC purification, gave a rather complex NMR spectrum. At least four different anomeric signals, designed **D**, **E**, **F** and **F′**, were visible in the region between 5.200 and 4.700 ppm (Fig. 2). These signals were present in non-stoichiometric amounts, suggesting the occurrence of more than one type of repeating unit within the TA structure. A complete assignment of proton resonances was possible for the major spin systems, on the basis of DQF-COSY and TOCSY spectra, while $^{13}$C resonances were obtained from a $^{1}$H,$^{13}$C HSQC spectrum (Table 1, Fig. 3). Spin system **D** was identified as t-α-D-Gal*p*, on the basis of the same considerations applied for residue **A** in **TA1**. The signal at 5.031 ppm accounted for the presence of at least two major magnetically different glucose units (H-1**E**/**E′**), identified on the basis of the small $^{3}J_{1,2}$ as α-D-Glc*p* units. In particular, residue **E** was identified as t-α-D-Glc*p*, while the down-field displacement of the C-2 resonance of residue **E′** (78.6 ppm) identified it as 2-substituted α-D-Glc*p*. In the TOCSY spectrum, an alternative and very minor spin system (3-substituted α-D-Glc*p* **E″**) was identified on the basis of the H-3 signal at 3.776 ppm that correlated in the $^{1}$H,$^{13}$C HSQC spectrum with a carbon signal at 81.3 ppm. The two minor anomeric signals at 4.894 and 4.845 ppm (H-1**F**/**F′**) were identified as the anomeric signals of two t-α-D-Glc*p*NAc units, in accordance with chemical analysis and on the basis of the C-2 chemical shift values. The N-acetylation was proven by the down-field shift of H-2 and by the occurrence of acetyl methyl group resonances around 2.000 ppm. The full assignment of proton and carbon ring signals could not be achieved owing to the complete overlap with the more abundant spin systems.

With the help of a $^{1}$H,$^{13}$C HSQC spectrum, three magnetically different glycerol phosphate units could be identified (**G**, **H** and **I**). In particular, residue **G** was

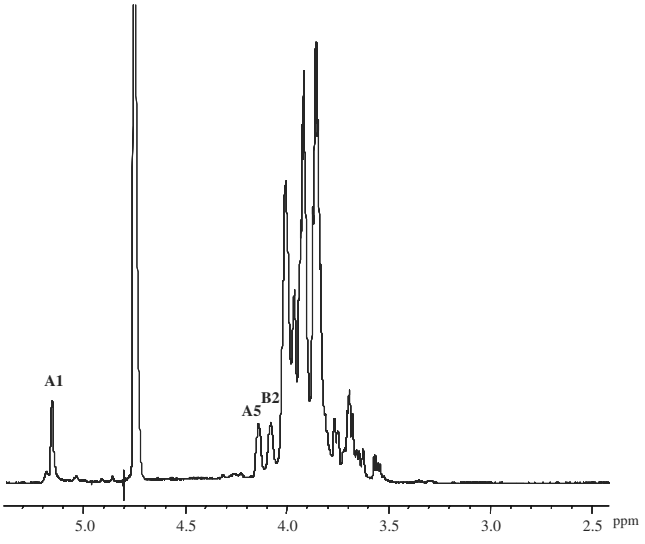



**Figure 1.** $^{1}$H NMR spectrum of **TA1** from *Geobacillus thermoleovorans* strain Fango. Capital letters refer to the spin systems described in Table 1, and chemical shifts refer to internal acetone (2.225 ppm).

**Table 1.** Proton and carbon chemical shifts for the cell wall teichoic acids **TA1** and **TA2** from *Geobacillus thermoleovorans* strain Fango

| | Residue | H-1/C-1 | H-2/C-2 | H-3/C-3 | H-4/C-4 | H-5/C-5 | H-6/C-6 |
|---|---|---|---|---|---|---|---|
| **TA1** | t-α-Gal | 5.150 | 3.762 | 3.859 | 3.956 | 4.137 | 3.684 |
| | **A** | 97.9 | 67.9 | 69.5 | 69.2 | 70.7 | 61.3 |
| | GroP | 4.006 | 4.074 | 3.973 | | | |
| | **B** | 65.2 | 75.1 | 65.0 | | | |
| | GroP | 3.930 | 4.013 | 3.851 | | | |
| | **C** | 66.2 | 69.1 | 66.3 | | | |
| **TA2** | t-α-Gal | 5.155 | 3.751 | 3.859 | 3.964 | 4.135 | 3.675 |
| | **D** | 97.8 | 68.1 | 70.5 | 69.1 | 70.8 | 61.0 |
| | t-α-Glc | 5.031 | 3.263 | 3.545 | 3.347 | 3.679 | 3.631 |
| | **E** | 98.0 | 71.0 | 72.3 | 68.4 | 71.0 | 62.0 |
| | 2-α-Glc | 5.028 | 3.827 | 3.757 | 3.511 | 3.679 | 3.631 |
| | **E′** | 97.1 | 78.6 | 69.4 | 68.5 | 71.0 | 62.0 |
| | 3-α-Glc | 5.031 | 3.263 | 3.776 | 3.511 | 3.679 | 3.631 |
| | **E″** | 98.0 | 71.0 | 81.3 | 68.5 | 71.0 | 62.0 |
| | t-α-**GlcNAc** | 4.894 | 4.202 | 3.832 | 3.950 | 3.728 | n.a. |
| | **F** | 99.6 | 51.5 | 70.6 | n.a. | n.a. | n.a. |
| | t-α-GlcN | 4.845 | 4.292 | 3.838 | n.a. | n.a. | n.a. |
| | **F′** | 99.2 | 50.8 | n.a. | n.a. | n.a. | n.a. |
| | GroP | 3.393 | 4.077 | 3.977 | | | |
| | **G** | 65.3 | 75.1 | 66.0 | | | |
| | GroP | 3.918/3.848 | 4.001 | 3.635/3.560 | | | |
| | **H** | 66.1 | 69.4 | 61.9 | | | |
| | GroP | 3.808 | 3.853 | 3.635 | | | |
| | **I** | 66.4 | 69.4 | 61.9 | | | |
| | GroP | | 5.182 | 4.012 | | | |
| | **L** | | 71.8 | 64.7 | | | |
| | Ala | — | 4.245 | 1.250 | | | |
| | | n.a. | 53.3 | 16.5 | | | |

Chemical shifts refer to internal acetone ($\delta_H$ 2.225 and $\delta_H$ 31.45 ppm). n.a.: not assigned.

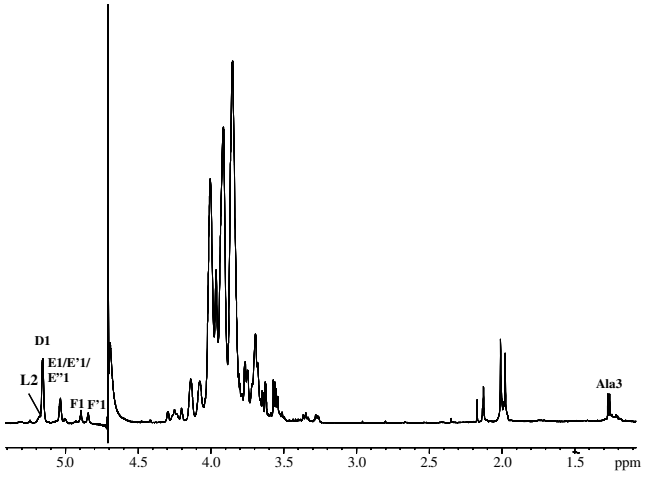



**Figure 2.** $^1$H NMR spectrum of **TA2** from *Geobacillus thermoleovorans* strain Fango. Capital letters refer to the spin systems described in Table 1, and chemical shifts refer to internal acetone (2.225 ppm).

recognized as glycosylated glycerol phosphate, on the basis of the C-2 chemical shift value (75.1 ppm), while residues **H** and **I** did not show substitution at O-2. Minor signals were also observed for a glycerol residue substituted at O-2 by an alanine residue (**L**), previously undetected. The presence of an alanine residue in non-stoichiometric amounts was suggested by a methyl doublet signal at 1.25 ppm in the $^1$H NMR spectrum. In the COSY spectrum, this signal correlated with a proton at 4.245 ppm which in turn correlated in a $^1$H,$^{13}$C HSQC spectrum with a carbon resonance at 53.3 ppm.

On the basis of dipolar correlations observed in the 2D ROESY spectrum (Fig. 4), residues **D**, **E**, **E′** and **E″** appeared to be directly linked to glycerol phosphate units, since their anomeric signals all gave NOE connectivity with H-2**G**. Residues **F** gave a strong NOE contact with H-2**E′**, thus providing evidence for the occurrence of a disaccharide unit bound to a glycosylated glycerol phosphate unit. An *inter*-residual dipolar correlation was also visible between H-1**F′**/H-3**E″**, showing that minor amounts of the terminal D-Glc*p*NAc residue could be attached to O-3 of the glucose residue. On the other hand, the occurrence of substitution by Ala to the glycerol residue **L** was proposed by the comparison with data previously reported for other TA structures.[6,7]

In summary, **TA2** contained a blend of structures, where several different repeating units were simultaneously present. In particular, the most abundant repeating units were identical to the ones previously defined for **TA1**, namely, glycerol phosphate and glycerol phosphate substituted by t-α-D-Gal*p*. Also,

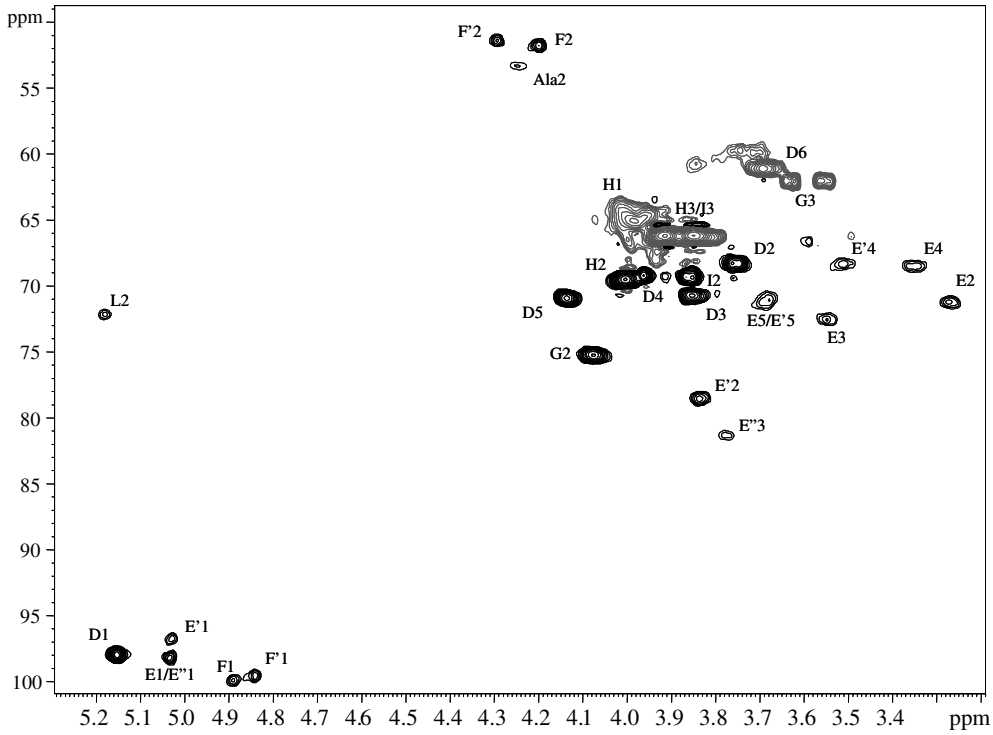



**Figure 3.** Section of the $^{1}H,^{13}C$ HSQC spectrum of **TA2** from *Geobacillus thermoleovorans* strain Fango. The spectrum was edited for the detection of signal multiplicity. Methylene groups are shown in grey. Letters refer to the identified major spin systems (Table 1).

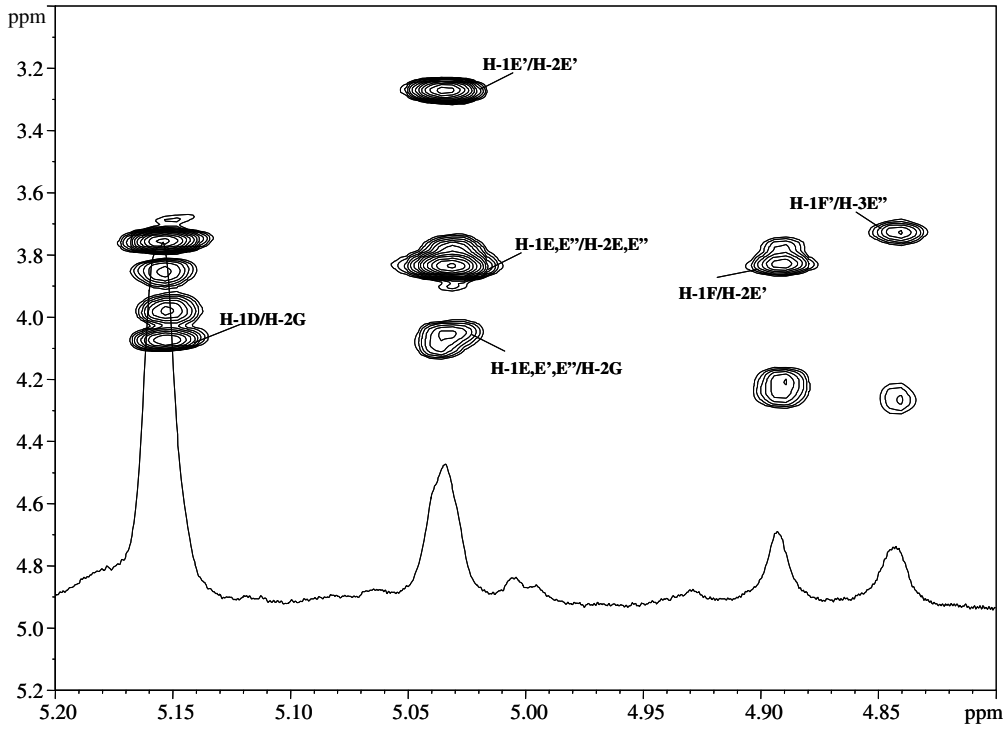
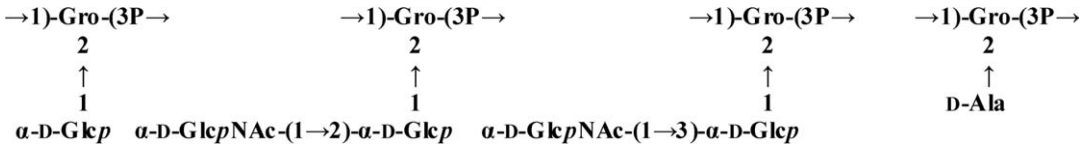



**Figure 4.** Zoom of the anomeric region of the 2D ROESY spectrum of **TA2** from *Geobacillus thermolevorans* Fango. Letters refer to the diagnostic *intra*- and *inter*-residual dipolar correlations.

minor units could be found, in which substitution at O-2 of glycerol phosphate was effected, in order of decreasing abundance, by t-α-D-Glc*p*, by the disaccharides t-α-D-Glc*p*NAc-(1→2)-α-D-Glc*p* or t-α-D-Glc*p*NAc-(1→3)-α-D-Glc*p*, or by an alanine residue.

These additional minor structures are the following:




Nevertheless, on the basis of our analysis, it was not possible to state whether the structures defined are present in a single, extremely variable glycerol polymer with non-stoichiometric appendages or if they constitute different building blocks of chains with the same chromatographic behaviour. It is noteworthy that **TA2** constitutes only the minor percentage of the total teichoic fraction isolated from *G. thermoleovorans* strain Fango, whereas the most abundant species is represented by **TA1**.

The D configuration of the Ala residue in **TA2** is proposed on the basis of the literature data where the totality of Ala substitution in TAs is accomplished by D enantiomer.[3] In fact, substitution by D-Ala is one of the characteristic features of TA structure, and its presence and abundance play a crucial role concerning the structure of the cell envelope and the toxicity of TA.[3,8–10] It is known that during the biosynthesis of TA, the isomerization of L-Ala into D-Ala is realized by an isomerase that acts prior to the formation of the ester linkage with the glycerol unit.[11,12]

## 1. Experimental

*G. thermoleovorans* strain Fango (ATCC BAA-872) was grown at 70 °C in a 50 L fermenter (Biostat-D, Braun) with a mechanical agitation of 100 rpm and an aeration flux of 56%. Growth was followed turbidimetrically at 540 nm. The standard culture medium (YN medium) contained: (g/L) yeast extract (Oxoid) 6.0, NaCl 2.0; pH 6.8. Cells were harvested in the stationary phase of growth by continuous-flow centrifugation on a Alfa Laval Model LAB 102 B-20 centrifuge. The pellet obtained was lyophilized under vacuum.

Cells (4.71 g) were extracted with hot phenol/water procedure.[5] After dialysis, the water phase was digested with DNase, RNase and Proteinase K and dialyzed again against water (3.5 kDa molecular mass cut-off) and lyophilized obtaining 55 mg of digested water phase. Purification of **TA1** (20 mg after purification) (was achieved with a column of Sephacryl S-400 (1.5 cm × 96 cm, Amersham) in 25 mM $NH_4HCO_3$ buffer whereas **TA2** (5 mg) was purified by HPLC on a column of TSK G-5000 (7.8 mm × 30 cm, Tosoh Bioscience) in 20 mM $NH_4OAc$ buffer (pH 4.7), and monitored with an UV detector at 220 nm and for the phosphate content.

Monosaccharide analyses were performed by GC–MS of acetylated derivatives after methanolysis with 2 M HCl in methanol, 85 °C, 16 h. The absolute configurations of the monosaccharides were established as published.[13] Methylation analysis was realized following the Hakomori procedure modified by Ciucanu and Kerek.[14,15] Methylation analysis of the HF derivatives was performed by treating 0.5 mg of the samples with 50 μL of 48% aqueous HF at 4 °C for 16 h. After evaporation of the HF under a stream of nitrogen and washing with water, the samples were methylated. The reaction was then quenched with water and the methylated derivatives, recovered from the organic layer after $CHCl_3/H_2O$ extraction, were analyzed by GC–MS on an Agilent 6850 Gas Chromatograph equipped with a capillary column RTX-5 (Restek, 30 m × 0.25 mm) and a MS detector (Agilent 5973 Network). The oven temperature was programmed from 150 to 300 °C with a gradient of 5 °C/min.

1D and 2D $^1H$ NMR spectra were recorded on a solution of 2 mg in 0.6 mL of $D_2O$ at 25 °C. $^1H$ and $^{13}C$ NMR experiments were carried out using a Bruker DRX-600 equipped with a cryogenic probe. Spectra were calibrated with internal acetone ($\delta_H$ 2.225, $\delta_C$ 31.45). ROESY experiments were measured using data sets (t1·t2) of 4096·1024 points, and 16 scans were acquired. A mixing time of 200 ms was used. Double quantum-filtered phase-sensitive COSY experiments were performed with 0.258 s acquisition time, using data sets of 4096·1024 points, and 64 scans were acquired. TOCSY experiments were performed with a spinlock time of 120 ms, using data sets (t1·t2) of 4096·512 points, and 16 scans were acquired. In all homonuclear experiments the data matrix was zero-filled in the F1 dimension to give a matrix of 4096·2048 points and was resolution enhanced in both dimensions by a shifted sine-bell function before Fourier transformation. Coupling constants were determined on a first-order basis from 2D phase sensitive double quantum filtered correlation spectroscopy (DQF-COSY).[16,17] The HSQC experiment was recorded in the $^1H$-detected mode via single quantum coherence with proton decoupling in the $^{13}C$ domain, using data sets of 2048·512 points, and 64 scans were acquired for each t1 value. Experiments were carried out in the phase-sensitive mode according to the described method.[18] The data matrix was extended to 2048·1024 points using forward linear prediction extrapolation.[19]

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## References

1. Fisher, W. In *Handbook of Lipid Research*; Hanahan, D. J., Ed.; Plenum Press: New York, 1990; Vol. 6, pp 123–234.
2. Archibald, A. R.; Baddiley, J.; Blumson, N. L. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1968**, *30*, 223–253.
3. Neuhaus, F. C.; Baddiley, J. *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 686–723.
4. Nazina, T. N.; Tourova, T. P.; Poltaraus, A. B.; Novikova, E. V.; Grigoryan, A. A.; Ivanova, A. E.; Lysenko, A. M.; Petrunyaka, V. V.; Osipov, G. A.; Belyaev, S. S.; Ivanov, M. V. *Int. J. Syst. Evol. Microbiol.* **2001**, *5*, 433–446.
5. Westphal, O.; Jann, K. *Meth. Carbohydr. Chem.* **1965**, *5*, 83–91.
6. Sadovskaya, I.; Vinogradov, E.; Li, J.; Jabbouri, S. *Carbohydr. Res.* **2004**, *339*, 1467–1473.
7. Vinogradov, E.; Sadovskaya, I.; Li, J.; Jabbouri, S. *Carbohydr. Res.* **2006**, *341*, 738–743.
8. Ward, J. B. *Microbiol. Rev.* **1981**, *45*, 211–243.
9. de Vos, W. M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 10763–10764.
10. Steen, A.; Palumbo, E.; Deghorian, M.; Cocconcelli, P. S.; Delcour, J.; Kuipers, O. P.; Kok, J.; Buist, G.; Hols, P. *J. Bacteriol.* **2005**, *187*, 114–124.
11. Perego, M.; Glaser, P.; Minutello, A.; Strauch, M. A.; Leopold, K.; Fischer, W. *J. Biol. Chem.* **1995**, *270*, 15598–15606.
12. Linzer, R.; Neuhaus, F. C. *J. Biol. Chem.* **1973**, *248*, 3196–3201.
13. Leontein, K.; Lönngren, J. *Meth. Carbohydr. Chem.* **1978**, *62*, 359–362.
14. Hakomori, S. *J. Biochem. (Tokio)* **1964**, *55*, 205–208.
15. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
16. Piantini, U.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800–6801.
17. Rance, M.; Sørensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479–485.
18. States, D. J.; Haberkorn, R. A.; Ruben, D. J. *J. Magn. Res.* **1982**, *48*, 286–292.
19. De Beer, R.; van Ormondt, D. *NMR Basic Princ. Prog.* **1992**, *26*, 201.