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Structure of the glycocalyx polysaccharide of *Pseudomonas fragi* ATCC 4973*

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ABSTRACT

The structure of the exocellular glycocalyx polysaccharide of *Pseudomonas fragi* ATCC 4973, a bacterium implicated in the spoilage of meat, has been determined using hydrolysis, methylation analysis and 1D- and 2D-n.m.r. spectroscopy. The polysaccharide, which aids in the adhesion of the cells to each other and to the meat tissue, has the regular repeating unit →4)-3-*O*-[(*R*)-1-carboxyethyl]-α-D-Glc*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→. Random partial *O*-acetylation occurred in some preparations of the polysaccharide.

INTRODUCTION

Pseudomonas fragi, which are motile, aerobic, rod-shaped, Gram-negative bacteria, are members of the group of bacteria[1] responsible for the spoilage of meat in moist, aerobic conditions at temperatures of ~20°. The production of slime by the bacteria is implicated in the spoilage process. The attachment of bacterial cells to surfaces involves reversible sorption associated with hydrophobic and van der Waals forces[2], and then permanent adhesion which involves the formation of a glycocalyx between the bacteria and the substrate[3]. Costerton *et al.*[4] demonstrated that the micro-organisms adhere to substrates by means of a mass of tangled fibres of polysaccharide with the formation of a "felt-like glycocalyx". *P. fragi* exudes such a polysaccharide[5] that aids in its adhesion to the meat surface and to its neighbours. We now report the structure of the polysaccharide obtained from *P. fragi* ATCC 4973.

RESULTS AND DISCUSSION

Isolation, composition, and n.m.r. spectra of the polysaccharide. — *P. fragi* ATCC 4973 bacteria were grown on trypticase-soy agar, harvested, and suspended in aqueous

* Dedicated to Professor Grant Buchanan on the occasion of his 65th birthday.
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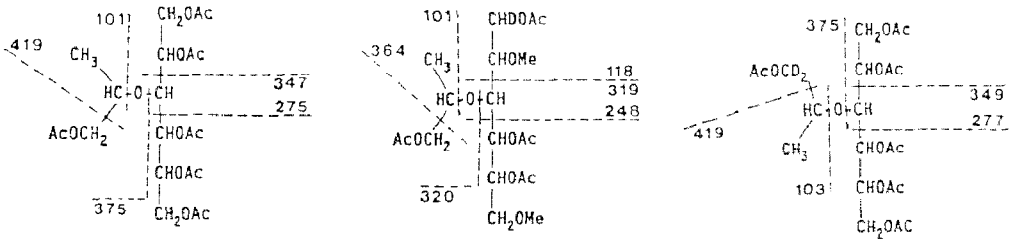
1% phenol. After ultracentrifugation of the cells, the polysaccharide was isolated by precipitation from the supernatant solution with ethanol. The crude polysaccharide was treated with trypsin, dialysed, decationised, and purified further by gel-permeation chromatography. Some preparations of the polysaccharide showed random partial *O*-acetylation, whereas others were devoid of OAc groups. Hydrolysis of the polysaccharide, conversion of the products into alditol acetates, and g.l.c.–m.s. revealed the derivatives of Glc, ManN, and a small proportion of a 3(or 4)-*O*-(2-hydroxy-1-methylethyl)Hex (T_{Glc} 2.56 on DB-225), the partial mass-spectral fragmentation of which is shown in **1**. Methanolysis of the polysaccharide followed by carboxyl reduction, hydrolysis, borohydride reduction, and acetylation gave **1** and glucitol hexa-acetate in approximately equimolar proportions (see below). The Glc and ManN were shown to be D by g.l.c. of their acetylated (−)-2-octyl glycosides[6].



The ^{1}H-n.m.r. spectrum (Fig. 1) of a solution of the base-treated polysaccharide in D_2O at 85° contained, *inter alia*, signals for H-1 at δ 5.26 (unresolved d), 4.88 (unresolved d), 4.60 (partially resolved dd), 4.46 (d, ^3J 7.7 Hz), and 4.33 (q, ^3J 6.5 Hz), together with signals at δ 2.06 (3 H, NAc) and 1.40 (d, 3 H, ^3J 5.9 Hz). The ^{13}C-n.m.r. spectrum contained twenty-three resonances, three of which (102.49, 99.98, and 99.89 p.p.m.) occurred in the region (95–105 p.p.m.) for C-1 signals. Other signals observed were at 19.51 (CH_3), 22.94 ($NCOCH_3$), 53.39 (C-2 of a 2-amino-2-deoxy sugar), and 175.35 and 182.71 p.p.m. (2 C=O). A DEPT[7] spectrum confirmed that the resonances at 19.51 and 22.94 p.p.m. arose from CH_3 groups, that at 53.39 p.p.m. arose from CH, and those at 60.45, 61.16, and 61.45 p.p.m. arose from CH_2 groups. The above results suggested the polysaccharide to be composed of an acidic trisaccharide repeating unit that contained an acetamido sugar.

Methylation analysis. — The polysaccharide was methylated[9] and hydrolysed, and the products were converted ($NaBD_4$) into the alditol acetates. G.l.c.–m.s. then revealed derivatives of 2,3,6-tri-*O*-methylglucose and 2-deoxy-4,6-di-*O*-methyl-2-methylacetamidomannose, whereas, after carboxyl reduction, methylation analysis gave the acetylated 2,6-di-*O*-methylated alditol **2**. Thus, the repeating unit of the polysaccharide was composed of 4-linked Glc, 3-linked ManNAc, and a 4-linked acidic sugar that carried an ether substituent at O-3.

Characterisation of the acidic sugar. — The acidic sugar was isolated from a hydrolysate of the polysaccharide by ion-exchange chromatography. Treatment with

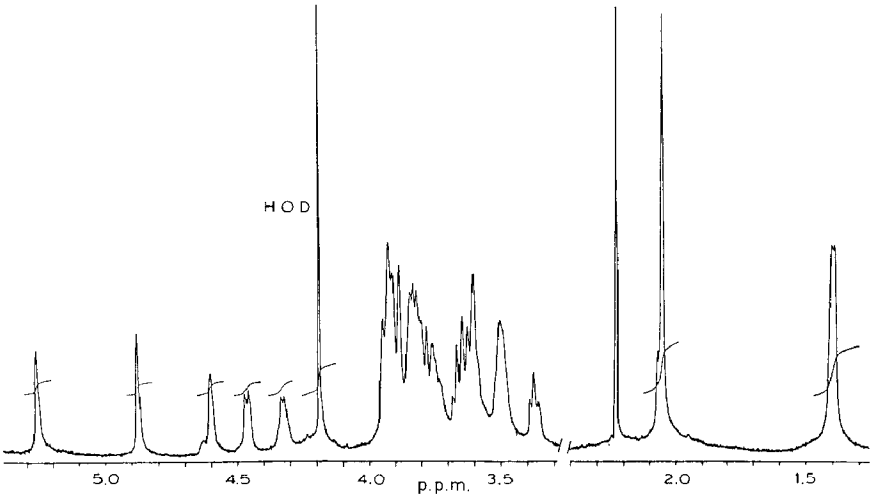


Fig. 1. 500-MHz ^{1}H-n.m.r. spectrum of a solution of the *P. fragi* polysaccharide (acid form) in D_2O at 85°.

aqueous 48% hydrogen bromide[10] and g.l.c. of the alditol acetate derived from the product showed that cleavage to glucose had occurred, and established that the acidic function resided in the ether substituent.

^{1}H-N.m.r. and ^{13}C-n.m.r. spectroscopy (1D and 2D) of the acidic sugar allowed the assignments shown in Table I. The distinct downfield shift of the C-3 resonances of the anomers, with respect to those of glucose[11], confirmed the 3-substitution, whereas the chemical shifts of the ^{1}H resonances and the J values accorded with the *gluco* configuration. These results, together with data from the hydrolysis, methylation, and de-etherification experiments, identified the acidic sugar as 3-*O*-(1-carboxyethyl)glucose (3-*O*-lactylglucose, 3LacGlc). The ^{1}H signals at δ 1.41 and 4.35 given by the polysaccharide may now be assigned to the CH_3 and CH groups, respectively, of the lactyl substituent; likewise, the signal at 19.51 p.p.m. in the ^{13}C spectrum can be assigned to the CH_3 group.

In order to determine the absolute configuration of the 3LacGlc, the *R* and *S* isomers of 3-*O*-lactyl-D-glucose were prepared from *S*-(−)- and *R*-(+)-2-chloropropionic acid[12], respectively, and 1,2:5,6-di-*O*-isopropylidene-D-glucofuranose (see Experimental). The n.m.r. data for the sodium salts of the two sugars, shown in Table II, reveal, as expected, downfield shifts for the C-3 resonances, compared with the data for glucose, and confirmed 3-substitution by the lactyl group. The two sets of n.m.r. data are similar and no major differences between the *R* and *S* isomers are apparent. Minor differences include the slightly lower chemical shift (0.063 p.p.m. downfield) of the α-*R* H-6 resonance and the downfield chemical shifts of the C-2 resonances of the *S* isomer (0.891 p.p.m. for α-*S* and 1.384 p.p.m. for β-*S*). The differences in the two sets of n.m.r. data do not allow the configuration of the lactyl ether to be assigned unambiguously. However, the $[\alpha]_D$ values (+69° for *R* and −1.7° for *S*) of the two synthetic sugars and the retention times of their derived alditol acetates in g.l.c. were quite different (2.57 min

TABLE I

N.m.r. data for the *P. fragi* lactyl sugar

Anomer		*Proton or carbon*							*Lactyl*		
		1	*2*	*3*	*4*	*5*	*6*	*6'*	*CH*	CH_3	*COOH*
α	H^a	5.228	3.614	3.643	3.510	3.833	3.762	3.835	4.435	1.462	
	^3J (*Hz*)	3.1	9.5	8.9	9.6		2.1,12.3	5.7	7.0		
	C^a	92.829	72.047	82.830	70.086	72.243	61.459			19.301	182.86
α-Glc[b]	*H*	5.20	3.52	3.72	3.40	3.83	3.74	3.83			
	C	92.9	72.5	73.8	70.6	72.3	61.6				
β	*H*	4.634	3.328	3.448	3.512	3.458	3.720	3.889	4.435	1.455	
	^3J	7.8	9.1	8.8	8.7		2.1,12.3	5.6	7.0		
	C	96.750	74.399	85.379	70.086	76.556	61.459			19.301	182.86
β-Glc[b]	*H*	4.62	3.24	3.48	3.41	3.46	3.71	3.86			
	C	96.7	75.1	76.7	70.6	76.8	61.7				

[a] Chemical shift in p.p.m. downfield from acetone (δ 2.23 for ^1H and 31.07 for ^{13}C). Spectra recorded for the acid form in D_2O at 30° and 500 MHz. [b] Data from ref. 11.

TABLE II

N.m.r data for 3-*O*-(*R*)- and 3-*O*-[(*S*)-1-carboxyethyl]-D-glucose

Isomer	*Anomer*		*Proton or carbon*							*Lactyl*		
			1	*2*	*3*	*4*	*5*	*6*	*6'*	*CH*	*CH_3*	*COOH*
S	α	*H*[a]	5.13	3.611	3.560	3.478	3.819	3.964	3.826	4.289[b]	1.410	
		^3J (Hz)	3.6	9.5	9.1	9.6		1.4,12.5	5.7	7.0		
		C[a]	93.103	72.562	82.460	69.662[c]	72.492	61.448		79.510[c]	19.581	182.956[d]
	β	*H*	4.642	3.319	3.384	3.480	3.463	3.701	3.878	4.248[b]	1.410	
		^3J	7.8	9.2	9.0	9.1		1.9,12.3	5.5	7.0		
		C	96.675	75.372	85.159	69.618[c]	76.745	61.618		79.435[c]	19.581	182.856[d]
R	α	^3H	5.252	3.599	3.569	3.502	3.820	3.757	3.828	4.283	1.411	
		^3J	3.2	9.5	9.5	8.4		1.6,12.5	5.4	7.0		
		C	92.603	71.671	82.412	70.688	72.422	61.257		79.551	19.590	182.863
	β	*H*	4.642	3.302	3.382	3.5017	3.425	3.713	3.883	4.306	1.411	
		^3J	7.8	9.5	8.9	9.9		2.1,12.3	5.6	7.0		
		C	96.918	73.988	85.121	70.688	76.642	61.427		79.551	19.590	182.863

[a] Chemical shift in p.p.m. downfield from acetone (δ 2.23 for ^1H and 31.07 for ^{13}C). Spectra were recorded for the sodium salt form in D_2O at 30° and 300 MHz.
[b, c] Values may be interchanged.

TABLE III

N.m.r. data for the *P. fragi* polysaccharide

Residue		*Proton or carbon*							*Lactyl*			*NAc*	
		1	*2*	*3*	*4*	*5*	*6*	*6'*	*CH*	CH_3	*COOH*	CH_3	*COOH*
a	*H*[a]	5.30	3.61	3.61	3.82	4.01	3.78	3.88	4.35	1.41			
	^3J (Hz)	3.5							7.0				
→4)-α-LacGlc	*C*[a]	99.89	71.48	79.60	76.33	72.63	61.51			19.51	182.71		
b	*H*	4.89	4.57	3.96	3.78	3.48	3.83	3.90				2.06	
	^3J		4.5										
→3)-β-ManNAc	*C*	99.98	53.39	77.83	67.69	76.95	60.45					22.94	175.35
c	*H*	4.42	3.38	3.65	3.66	3.51	3.86	3.92					
	^3J	7.7	8.0										
→4)-β-Glc	*C*	102.49	74.39	74.74	80.04	75.82	61.16						

[a] Chemical shift in p.p.m. downfield from acetone (δ 2.23 for ^{1}H and 31.07 for ^{13}C). Spectra were recorded for the acid form in D_2O at 45° and 500 MHz.

for *R* and 2.45 min for *S*, relative to that of glucitol hexa-acetate on DB-225) and indicated the acidic sugar from *P. fragi* to be 3-*O*-[(*R*)-1-carboxyethyl]-D-glucose.

2D-N.m.r. spectroscopy of the polysaccharide. — The ^{1}H- and ^{13}C-n.m.r. spectra of the polysaccharide were assigned using COSY[13], RELAY COSY[14], and HETCOR[15] experiments, and the data are presented in Table III. The sugar residues are labelled **a**–**c** in order of the decreasing chemical shifts of their H-1 resonances. The *J* values were measured from a high-temperature 1D ^{1}H-n.m.r. spectrum that had been resolution-enhanced. The data indicated the 3LacGlc to be α, and the Glc and ManNAc to be β. The β-ManNAc assignment was confirmed by the absence of the signal at δ 4.89 from the ^{1}H-n.m.r. spectrum of the deaminated polysaccharide and by the appearance of a new *gluco*-type[16] signal for H-1β at δ 4.5 (^3J 8 Hz). The glycosylation shifts observed for C-4 of 3LacGlc and Glc and C-3 of ManNAc accorded with the substitution patterns deduced from the methylation analysis.

The 1D- and 2D-^{1}H-n.m.r. spectra, which were all acquired on the acid form of the polysaccharide, were complicated by the twinning of some of the signals, *e.g.*, H-1 of **a** and H-2 of **b** and **c**. The extent of the twinning varied with different samples of the polysaccharide, and with different acquisition times and temperatures. At 85°, there was no twinning but, on lowering the temperature, twinning was detectable. This effect was ascribed to partial lactonisation of the carboxylic group of the lactyl moiety at the lower temperatures. Lactone formation was demonstrated by reducing a hydrolysate of the polysaccharide with $NaBD_4$ and conversion of the products into the alditol acetates. G.l.c.–m.s. then revealed the derivative **3** and the characteristic fragment with *m/z* 103. This result also explains the small proportion of "hydroxypropylhexitol" derivative described above.

Sugar sequence. — The sequence of the sugar residues in the repeating unit was determined by 2D-n.O.e. spectroscopy (NOESY[17]), and the observed inter- and intra-residue n.O.e.'s are listed in Table IV. The intra-residue n.O.e.'s are consistent with the assigned configurations and conformations of the sugars. The inter-residue n.O.e.'s observed between H-1 of **a** and H-3 of **b**, and between H-1 of **b** and H-4 of **c**, indicated the sequence **a**–**b**–**c**. N.O.e.'s were also observed between the lactyl C*H* and H-2 and H-3 of **a**; however, because the lactyl C*H* and H-1 of **c** resonated so closely together in the

TABLE IV

N.O.e. data for the *P. fragi* polysaccharide

Proton	*n.O.e. to*
a1 →4)-α-LacGlc	3.95(**b**3), 3.60 (**a**2)
b1 →3)-β-ManNAc	4.58(**b**2), 3.95(**b**3), 3.66(**c**4), 3.48(**b**5)
c1 →4)-β-Glc + CH of Lac	3.65(**c**3), 3.51(**c**5), 3.60(**a**3, **a**2)

NOESY spectrum, the n.O.e. to H-3 of **a** could also have arisen from H-1 of **c**, which is consistent with the sugar sequence.

Thus, the repeating unit of the *P. fragi* polysaccharide may be written as

→4)-3-*O*-[(*R*)-1-carboxyethyl]-α-D-Glc*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→
a **b** **c**

The occurrence of a Glc residue 3-substituted by a lactyl ether is unusual in bacterial polysaccharides. Most lactyl ethers of glucose described hitherto have been found at position 4.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer 141 polarimeter and a 1-cm cell. G.l.c. was performed using a Hewlett–Packard 5890A gas chromatograph fitted with flame-ionisation detectors and a 3392A recording integrator. Fused-silica bonded-phase capillary columns (30 m × 0.25 mm, 0.25-μm film) of DB-17, DB-225, and DB-Wax (J & W Scientific) were used with helium as the carrier gas and operated at 220° (DB-225) or with the temperature programmes: *A*, 180° for 1 min, then 2°/min to 250° (for methylated alditol acetates, DB-17); *B*, 165° for 5 min, then 3°/min to 240° (for acetylated octyl glycosides, DB-17); *C*, 180° for 2 min, then 5°/min to 240° (for alditol acetates, DB-17); and *D*, 80° for 1 min, then 20°/min to 180°, for 1 min, then 4°/min to 210°, for 1 min, then 10°/min to 240° (for *O*-methyloximes, DB-Wax). The identity of each derivative was confirmed with a Hewlett–Packard 5988A g.l.c.–mass spectrometer, using the above columns, an ion-source temperature of 200°, and an ionisation energy of 70 eV. Gel-permeation chromatography of the polysaccharide was performed on a column (1.6 × 65 cm) of dextran-calibrated Sephacryl S-400 with 0.1M sodium acetate (pH 5.0) as eluent. Fractions were analysed by differential refractometry.

The polysaccharide was hydrolysed[18] with 4M trifluoroacetic acid at 125° for 1 h. The hydrolysate was co-concentrated with water under reduced pressure at ≤40° (bath). Alditol acetates were prepared conventionally by reduction of aqueous solutions of hydrolysates with sodium borohydride (or deuteride) followed by acetylation with 1:1 acetic anhydride–pyridine (100°, 1 h). Samples were carboxyl-reduced by treatment with refluxing methanolic 3% hydrogen chloride (16 h), and the methyl esters were reduced with sodium borohydride in anhydrous methanol. The identity of the ManNAc was confirmed by analysis of the derived *O*-methyloximes[18] on DB-Wax. Methylation was carried out on the acid form of the polysaccharide, using potassium dimsyl and methyl iodide in methyl sulphoxide[19].

Preparation of the polysaccharide. — *P. fragi* ATCC 4973 was grown in trypticase-soy broth (200 mL, 21°, 24 h), and the liquid culture was used to inoculate trays of trypticase-soy agar (30 × 38 cm) which were incubated at 21° for 3 days. The lawn of bacteria was harvested, phenol was added to 1%, the suspension was stirred at 4° for 24

h and then ultracentrifuged (60 000*g*, 3.5 h), and the polysaccharide was precipitated from the supernatant solution with ethanol. A solution of the precipitate in water was centrifuged and freeze-dried. A solution of the residue in 50mM phosphate buffer (pH 7) was treated with trypsin (37°, 16 h). After destruction of the enzyme by heat (1 h, steam bath), the solution was dialysed against tap water (3 days), decationised, and freeze-dried. Gel-permeation chromatography yielded purified polysaccharide (1.6 g) that had a fairly broad distribution of molecular weights. Prior to n.m.r. studies, the polysaccharide was *O*-deacetylated with 0.1M NaOH for 4 h at 40°.

Isolation and characterisation of 3-O-*[(*R*)-1-carboxyethyl]*-D-*glucose.* — The polysaccharide (50 mg) was hydrolysed (4M trifluoroacetic acid, 125°, 1 h), the acid was removed under vacuum, and the hydrolysate was applied to a column (1 × 7 cm) of Amberlite IRA-400 (AcO^-) resin. The neutral and amino sugars were eluted with water, and the acidic sugar with M formic acid. The acid was removed under vacuum and a solution of the residue in water was freeze-dried to yield the title sugar (12 mg), $[\alpha]_D$ +81° (sodium salt, *c* 0.64, water). A portion (2 mg) was treated with aqueous 48% hydrogen bromide (0.5 mL) for 6 min at 100°, the acid was neutralised (Ag_2CO_3), the salts were removed by ultracentrifugation, and the supernatant solution was concentrated. The product was converted into the alditol acetate and analysed by g.l.c. The acidic sugar was further analysed by 1D- and 2D-n.m.r. spectroscopy (COSY, HETCOR), and the results are listed in Table I.

Preparation of 3-O-*[(*R*)-1-carboxyethyl]*-D-*glucose and its* S *isomer.* — 1,2:5,6-Di-*O*-isopropylidene-D-glucofuranose (100 mg) was treated[12] separately with *R*-(+)- and *S*-(−)-2-chloropropionic acid (0.3 mL) and NaH in dry tetrahydrofuran. The products were purified by chromatography (1:1 dichloroethane–ether) on silica gel and hydrolysed (2M trifluoroacetic acid, 100°, 2 h). Gel-permeation chromatography on Bio-Gel P-2 (H_2O) of the products yielded 3-*O*-[(*R*)-1-carboxyethyl]-D-glucose (25 mg), $[\alpha]_D$ +69° (sodium salt, *c* 1.25, water) and 3-*O*-[(*S*)-1-carboxyethyl]-D-glucose (30 mg), $[\alpha]_D$ −1.7° (sodium salt, *c* 1.35, water).

A portion (2 mg) of each sugar was methanolysed, carboxyl-reduced, hydrolysed, and analysed by g.l.c. on DB-225 (220°) as the alditol acetate. 1D-N.m.r. and 2D-n.m.r. spectroscopy (COSY, HETCOR) of the two isomers (as sodium salts) gave the data in Table II.

N.m.r. spectroscopy. — Samples were deuterium-exchanged by freeze-drying solutions in D_2O, and then dissolved in 99.99% D_2O (0.45 mL) that contained a trace of acetone as internal reference (δ 2.23 for ^1H, and 31.07 p.p.m. for ^{13}C). Spectra were recorded at 30°, 45°, and 85° with a Bruker WM-500 spectrometer equipped with an Aspect 2000 computer, and/or Bruker AM-400 and AM-300 spectrometers equipped with Aspect 3000 computers.

^1H-Homonuclear shift-correlated experiments (COSY[13] and 1-step RELAY-COSY[14]) and homonuclear dipolar-correlated experiments (NOESY[17]) were performed at 30°, using spectral widths sufficient to include the areas of interest. Data matrices of 512 × 2048 data points were collected for 32 transients for each t_1 delay. The matrices were zero-filled in the t_1 dimension and transformed in the magnitude mode by use of a

non-shifted sine-bell window function in both dimensions, and symmetrised. Digital resolution in the resulting 1024 × 2048 matrices varied between 1.5 and 3.0 Hz/point, depending on the spectrometer used. Relaxation delays of 1.2–1.5 s were used. For the RELAY-COSY experiment, a fixed delay of 0.036 s was used. The mixing delay in the NOESY experiment was 0.3 s. ^{13}C–^{1}H Shift-correlated spectra (HETCOR[15]) were recorded at 30° with proton decoupling in the F_1 domain. The matrices of 128 × 2048 points were zero-filled to 256 × 2048 points, and processed with Gaussian functions and a magnitude calculation to give a final resolution of between 6 and 9 Hz/point in the F_2 domain and between 5 and 7 Hz/point in the F_1 domain, depending on the spectrometer used.

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