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Structure of an antigenic teichoic acid shared by clinical isolates of *Enterococcus faecalis* and vancomycin-resistant *Enterococcus faecium*

Ying Wang [a,*], Johannes Huebner [a], Arthur O. Tzianabos [a], Gajane Martirosian [a], Dennis L. Kasper [a,b], Gerald B. Pier [a]

[a] *Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115-5804, USA*

[b] *Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA*

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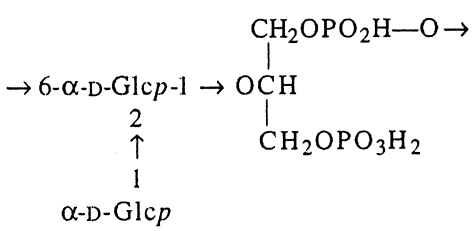
Abstract

A shared antigenic teichoic acid, previously found to be a surface capsule-like polysaccharide, was isolated from clinical isolates of *Enterococcus faecalis* and vancomycin-resistant *E. faecium*. It was composed of glucose, glycerol, and phosphate as determined by chemical and GC–MS analysis. The repeating-unit structure was elucidated by a series of ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy to be the following:



Keywords: Teichoic acid; Polysaccharide; *Enterococcus faecalis*; *Enterococcus faecium*; Antigen

1. Introduction

Enterococci have emerged among major pathogens in hospital intensive care units during the past few years and as one of the most common causes of infections in newborns and severely immunocompromised patients [1,2]. Enterococcal infections contribute significantly to mortality, as enterococci become highly resistant to a number of antimicrobial agents, such as penicillin, aminoglycosides, and most recently vancomycin [3,4]: crude mortality rates of up to 100% have been reported for patients infected with vancomycin-resistant enterococci [3–5]. Thus, treatment of enterococcal infections has become a major concern. As part of our ongoing search for carbohydrate virulence factors and development of alternative approaches such as glycoconjugate vaccines to combat enterococcal

* Corresponding author. Tel.: +1-617-732-8585; fax: +1-617-731-1541.

E-mail address: ywang@channing.harvard.edu (Y. Wang)

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infections, we are attempting to characterize surface antigens of enterococci.

Enterococci are Gram-positive bacteria previously classified as group D streptococci [6]. The cell-wall group antigen for enterococci has been identified as an intracellular glycerol teichoic acid containing various carbohydrate components that contribute to serological specificity [7–11]. We isolated two surface polysaccharides from a strain of *Enterococcus faecalis* [12]. ^{1}H NMR spectroscopy showed one polysaccharide to have a tetrasaccharide repeating unit [13,14] and the other to have a disaccharide repeating unit [12]. This latter antigen was also isolated from a vancomycin-resistant *E. faecium* strain. The shared polysaccharide antigen was found to be a unique glycerol teichoic acid containing a kojibiosyl [α-D-glucopyranosyl-(1→2)-D-glucose] disaccharide component also found in *E. faecalis* intracellular teichoic acid and lipoteichoic acid [15–17]. In this paper we report the structural characterization of this antigen.

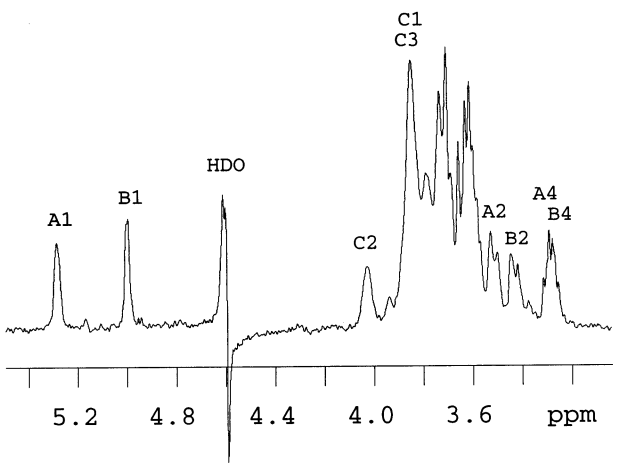


Fig. 1. ^{1}H NMR spectrum of the teichoic acid antigen from *E. faecalis*, recorded in D_2O at 50 °C. The signals were partially labeled. **A** and **B** denote the two glucose residues, and **C** denotes the glycerol residue.

Table 1
^{1}H chemical shifts[a] of the antigenic teichoic acid from *E. faecalis*

Residue[b]	H-1	H-2	H-3	H-4	H-5	H-6
A	5.29	3.52	3.71	3.30	3.74	3.80, 3.74
B	5.01	3.43	3.64	3.28	3.74	3.71, 3.61
C	3.86	4.04	3.87			

[a] In ppm relative to the HDO signal at 4.60 ppm.
[b] **A** = →6)-[→2)]-α-D-Glc*p* (1→, **B** = α-D-Glc*p*-(1→, **C** = glycerol.

2. Results and discussion

The purified antigens [12] were first hydrolyzed completely with trifluoroacetic acid to yield monosaccharide and small nonsugar components. In order to identify the sugar residues, the components from the hydrolyzed antigens were converted to alditol acetate derivatives. GC–MS analysis of the derivatives identified glucose as the only sugar component. The absolute configuration of the glucose was determined to be D by GC–MS analysis of the derived butyl glycosides. In order to identify the nonsugar components, the components of the hydrolyzed antigen were converted to trimethylsilyl (Me_3Si) derivatives by treatment with bis(trimethylsilyl) trifluoroacetamide/pyridine. GC–MS analysis of the Me_3Si derivatives revealed that the antigen contained glucose, glycerol, and phosphate in an approximate 2:1:2 ratio. These components are characteristic for teichoic acids, thus suggesting that the antigen was a glycerol teichoic acid.

The ^{1}H NMR spectrum of the antigen (Fig. 1) showed two anomeric proton signals at 5.29 (singlet) and 5.01 ppm (J 3.6 Hz), respectively. These signals clearly indicated that there were two glucosyl residues in each repeating unit of the polymer, and their downfield chemical shifts and small coupling constants also indicated that the two D-glucose residues had α-configurations at their anomeric centers. The rest of the resonances from the glucoses and glycerol appeared between 3.2 and 4.1 ppm and were not well resolved. A series of 2D homonuclear and heteronuclear NMR experiments were performed to assign all the resonances for the glucose and glycerol components. Their complete ^{1}H and ^{13}C chemical shift assignments (Tables 1 and 2) were obtained from a combination of ^{1}H–^{13}C HMQC (Fig. 2), ^{1}H–^{1}H TOCSY (data not shown), and DQF-COSY (data not shown), and NOESY (data not shown) spectra. Since the chemical shifts of the glucosyl residues were typical for glucopyranoses, other forms such

Table 2
^{13}C chemical shifts[a] of the teichoic acid from *E. faecalis*

Residue[b]	C-1	C-2	C-3	C-4	C-5	C-6
A	95.31	74.82	71.90	70.07	71.93	67.06
B	96.48	71.86	73.04	70.07	71.93	61.26
C	65.89	75.87	65.89			

[a] In ppm relative to external CH_3I standard at 22.5 ppm.
[b] **A** = →6)-[→2)]-α-D-Glc*p* (1→, **B** = α-D-Glc*p*-(1→, **C** = glycerol.

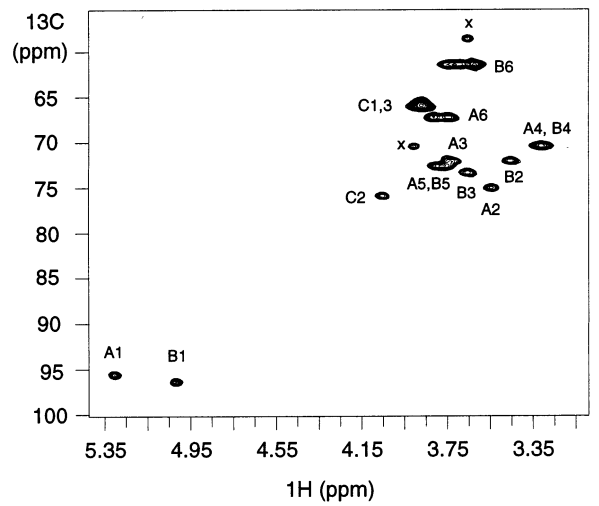


Fig. 2. $^{1}H-^{13}C$ HMQC spectrum of the antigen from *E. faecalis*. Note that signals marked as **A**, **B**, and **C** are from the two glucoses and the glycerol residue, respectively; signals marked X are from an impurity.

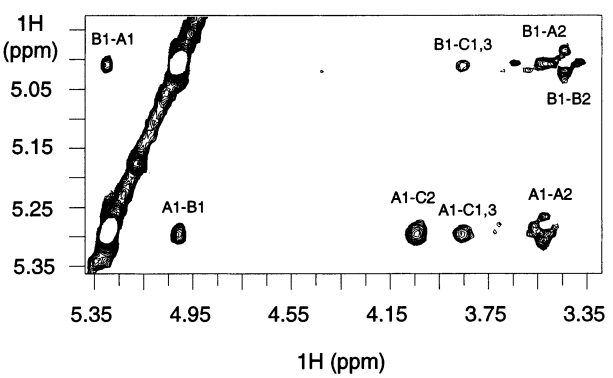


Fig. 3. A section of the $^{1}H-^{1}H$ NOESY spectrum of the antigen from *E. faecalis*.

as glucofuranose and acyclic glucose were excluded.

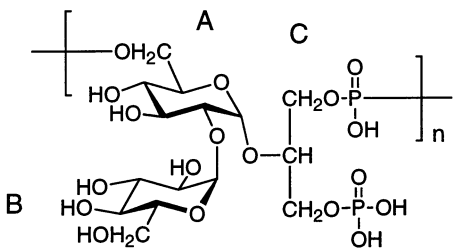
Further NMR analysis revealed that the two glucose residues were linked together to form an α-D-glucopyranosyl-(1→2)-α-D-glucopyranose or kojibiose structure. In the $^{1}H-^{13}C$ HMBC spectrum (not shown) of the antigen, the H-1 (δ 5.01 ppm) of a glucose residue (**B**) showed a cross peak to the C-2 (δ 74.82 ppm) of the other glucose residue (**A**). This long-range $^{1}H-^{13}C$ correlation due to H-1(B)–C-1(B)–O-1(B)–C-2 (**A**) directly showed the glycosidic linkage between the two Glc residues, thus establishing a **B**-(1→2)-**A** structure. This structure was further supported by the NOESY spectrum of the antigen (Fig. 3), where the H-1 of **B** showed strong NOEs to the H-1 and H-2 of Glc **A**. These inter-residue NOEs could only arise from an α-D-Glc-(1→2)-α-D-Glc moiety, as confirmed by molecular models calculated with Quanta/CHARM force field. Other possible disaccharides, e.g., α-D-Glc-(1→*X*)-α-D-Glc (where *X* = 3, 4, 6), would not give rise to these inter-residue NOEs.

The kojibiose component was attached to the glycerol component directly to give rise to a 2-*O*-kojibiosyl-glycerol component. This fragment was established from the inter-residue NOEs. In the NOESY spectrum of the antigen (Fig. 3), the H-1 of **A** showed NOEs to the H-2 (strong), H-3, and H-1 of the glycerol (residue **C**). These NOEs demonstrated that the H-1 of **A** was closer spatially to the H-2 than to the H-1 or H-3 of the glycerol, thus suggesting that the **A** residue was linked through its anomeric position to the 2-hydroxy of the glycerol residue.

The kojibiose was also substituted at the C-6 of glucose residue **A**. This substitution was obvious since its ^{13}C resonance appeared at 67.06 ppm, which was characteristic for substituted 6-hydroxymethyl carbons. It was clear from this analysis that glucose residue **A** was connected at three positions: 1-, 2-, and 6-. In a polymer sequence, the residue with sites connecting to three residues must be a branching point, and the polymer is, therefore, a branched polymer. Thus, the antigen is a branched polymer with glucose **A** located at the backbone to form a branching point that bears a side chain. Glucose **B**, which was linked to A in the kojibiose, had no substitution site other than its anomeric position; it was, therefore, a side-chain residue. Thus far, a Glc-(1→2)-[→6]-Glc-(1→2)-glycerol fragment was constructed.

As discussed earlier, GC–MS revealed that the antigen contained phosphate in addition to glucose and glycerol. The presence of phosphate was also confirmed by ^{31}P NMR spectroscopy. There were two ^{31}P signals at -0.64 and -0.51 ppm, respectively. The connections between phosphates and the rest of the polymer residues were established from ^{1}H–^{31}P TOCSY spectroscopy (Fig. 4). The phosphorous resonance at -0.64 ppm showed a strong correlation to the H-3 resonance of glycerol at 3.86 ppm and a weak correlation to the H-2 of glycerol at 4.04 ppm. These correlations indicated that the 3-position of the glycerol was substituted by a phosphate group. The phosphorous resonance at -0.51 ppm showed cross peaks to both the H-1 (3.87 ppm) of glycerol and the H-6s (3.80 and 3.74 ppm) of **A**, respectively. These correlations demonstrated that this phosphate group was linked to the 1-position of the glycerol as well as the 6-position of **A** and thus bridged the two residues.

The structure of the antigen isolated from *E. faecalis* was thus far determined by NMR spectroscopy in conjunction with chemical and GC–MS analysis. The antigen is a teichoic acid with the repeating unit as below.



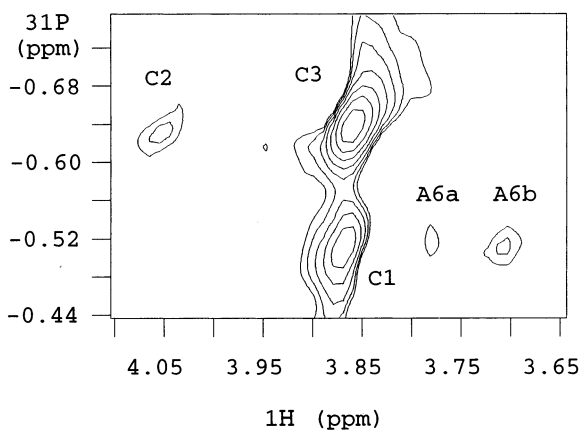


Fig. 4. ^{1}H–^{31}P TOCSY spectrum of the antigen from *E. faecalis*.

This same antigen, also isolated from a vancomycin-resistant *E. faecium* strain as confirmed by ^{1}H NMR spectroscopy, seems to be a common antigen among different strains of *E. faecalis* and *E. faecium*.

A similar structure for a reported intracellular teichoic acid from enterococci was proposed by Wicken and Baddiley in 1963 [8]. Their proposed structure, based on chemical analysis of the antigen components, is a poly-(1→3)-glycerol phosphate teichoic acid in which all or nearly all glycerol units are substituted at the 2-position with sugar residues including glucose, kojibiose, and kojitriose [α-D-Glc-(1→2)-α-D-Glc-(1→2)-D-Glc]. They identified kojibiose, glycerol, and phosphate by acid hydrolysis and TLC analysis. The linkage or the sequence of their proposed structure was largely speculative. Undoubtedly, it is difficult to establish a complex polymer sequence without modern NMR spectroscopy. In addition they had evidence to indicate that their material was intracellular [9], whereas ours is clearly a surface polysaccharide, as determined by immunoelectron microscopy [12]. Alternatively, the discrepancy between their proposed structures and ours may simply be due to the isolation of these teichoic acids from different strains. Although alanine has been reported to be an alkalilabile residue in some teichoic acids [10], it was not identified in our teichoic acid preparations.

3. Experimental

Bacterial growth and antigen purification.— *E. faecalis* and *E. faecium* were isolated from patients in various hospitals between 1994 and 1996. *E. faecalis* strain 12030 was grown in Columbia broth (Difco Laboratories) with 5% glucose and 0.005% hemin added. The antigen was isolated and purified as described [12]. Briefly, the bacterial cells were recovered and suspended in sucrose, and digested with mutanolysin and lysozyme, and then treated with nucleases and either proteinase K or pronase. The remaining insoluble cell-wall fragments and cell bodies were removed by centrifugation. The supernatant was collected, filtered,

and size-fractionated on a Sephacryl S400 or S500 column (Pharmacia Biotech, Uppsala, Sweden), and further purified on an anion-exchange column (Bio-Rad Laboratories, Richmond, CA). Fractions containing antigenic polysaccharides were identified by both UV adsorption at 206 nm and immuno-dot-blots with rabbit antisera to *E. faecalis* strain 12030.

Monosaccharide composition analysis.—The monosaccharide component of the antigen was detected by GC–MS analysis of its alditol acetate derivatives as described previously [18]. Briefly, a 0.5-mg polysaccharide sample was hydrolyzed with 1 mL of 2 M TFA at 110 °C for 2 h and dried under a stream of nitrogen. It was redissolved in 0.2 mL of water and reduced with 5 mg of $NaBH_4$ at room temperature for 2 h. The excess $NaBH_4$ was destroyed by the addition of two drops of 2 M HCl and drying under nitrogen, followed by five additions of four drops of MeOH and evaporation under nitrogen. The alditol acetate derivatives obtained from treatment with Ac_2O/pyridine were subjected to GC–MS analysis with a DB17 column on a Hewlett–Packard 6890/5973 GC–MS instrument. The GC run was started at 180 °C held for 2 min, and increased to 260 °C by 2 °C/min, and held at 260 °C for 20 min.

Antigen composition analysis.—The total compositions including sugar and nonsugar residues were obtained from the Me_3Si derivatives of the hydrolyzed antigen. Briefly, a 0.5-mg sample was hydrolyzed with 2 M TFA at 110 °C for 2 h and then dried under a stream of nitrogen. To the dried product was added 0.1 mL of pyridine and 0.1 mL of bis(trimethylsilyl)trifluoroacetamide; it was left at room temperature for 40 min, and a sample of the derivatives was then subjected to GC–MS analysis. The GC run was started at 100 °C, increased by 5 °C/min to 220 °C, and held at the final temperature for 20 min.

Determination of D *and* L *configuration.*—A 0.5-mg sample was hydrolyzed with 2 M TFA at 110 °C for 2 h. The hydrolyzed sample and a D-glucose standard was butanolyzed with 0.5 mL of (−)-(*R*)-2-butanol and AcCl in a ratio of 10:1 (v/v) at 80 °C for 9 h, and then treated with 0.1 mL $CF_3C(=NSiMe_3)OSiMe_3$ and 0.1 mL pyridine at room temperature for 40 min. The Me_3Si derivatives were then subjected to GC–MS analysis as described above.

NMR spectroscopy.—A 10-mg antigen sample in 0.7 mL of D_2O was used for all NMR experiments. NMR spectra were recorded on a Varian Unity 400 or Unity 500 spectrometer with proton frequencies of 399.98 and 499.95 MHz, respectively. Spectra were recorded at 50 °C unless otherwise indicated, and ^1H chemical shifts were referenced to the HDO resonance at 4.60 ppm as calibrated externally. The water signal was suppressed by presaturation in most experiments. 1D ^{31}P spectra were recorded with a spectral width of 30 ppm both with and without proton decoupling, and chemical shift was referenced to an external H_3PO_4 standard at 0 ppm. ^{13}C chemical shifts were referenced to an external CH_3I standard at 22.5 ppm.

^1H–^1H DQF-COSY [19], TOCSY [20], and NOESY [21] were recorded with standard pulse sequences with a spectral width of 10 ppm at both dimensions. A mixing time of 100 ms was used for the NOESY experiment, and a spin-lock time of 50 ms was used for the TOCSY. ^1H–^{13}C HMQC [22,23] and HMBC [24] were recorded with a carbon spectral width of 180 and 200 ppm, respectively. ^1H–^{31}P TOCSY [20] spectra were recorded with protons as the detected nuclei, and a mixing-time of 50 ms was used. Typically these 2D spectra were obtained in TPPI mode with 512 real points along t_1 and 2048 complex points along t_2; at least 32 scans were acquired for each t_1 value. In the processing of 2D data, zero-filling was applied in the t_1 dimension and shifted squared sine bell or Gaussian functions were applied in both dimensions before Fourier transformation.

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