Isolation of Lipoteichoic Acids from *Butyrivibrio fibrisolvens*

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SUMMARY

Lipoteichoic acid (LTA) and deacylated lipoteichoic acid have been isolated from the bovine-rumen Gram-negative anaerobe *Butyrivibrio fibrisolvens* by phenol extraction. Lipoteichoic acid (21.8 μ mol phosphorus/g cells) consisted of a conventional 1,3-phosphodiester-linked chain of glycerol phosphate units joined covalently to a glycolipid. It was not substituted with glycosyl or D-alanyl ester groups. Deacylated lipoteichoic acid (57.5 μ mol phosphorus/g cells) was similar in constitution but lacked fatty acid esters. Lipoteichoic acid reacted serologically with antisera to the glycerol phosphate backbone of known lipoteichoic acids. The presence of similar teichoic acid polymers has also been demonstrated in some other strains of *B. fibrisolvens* and this is of significance in demonstrating that teichoic acids can occur in Gram-negative bacteria.

INTRODUCTION

Teichoic acids have generally been considered as uniquely Gram-positive bacterial components and, in the form of membrane lipoteichoic acids (LTA), are common to practically all Gram-positive bacteria (Wicken & Knox, 1975). The use of specific antisera to the common polyglycerol phosphate backbone of lipoteichoic acids has made it easy to detect this antigenic determinant in extracts of a wide range of Gram-positive bacteria (Sharpe *et al.*, 1973; Wicken & Knox, 1975). Recently it has been reported that extracts of some strains of the Gram-negative rumen anaerobe, *Butyrivibrio fibrisolvens*, show a strong positive serological reaction for the polyglycerophosphate antigenic determinant: material which had chemical degradation products typical of glycerol teichoic acids and gave specific serological reactions with antisera to the polyglycerophosphate backbone, was isolated in very low yield – of the order of 0.02 % dry wt of the cells – from strain Nor37 (Sharpe, Brock & Phillips, 1975a). This was the first evidence for the hitherto unreported occurrence of teichoic acids in Gram-negative bacteria.

This paper describes the isolation, by conventional procedures, and partial characterization of lipoteichoic acid from strain Nor37; and indicates that similar polymers are present in some other strains of *B. fibrisolvens*. Some of these results were reported in a preliminary communication (Sharpe *et al.*, 1975b).

METHODS

Strains. Three strains of *B. fibrisolvens* (Nor37, 1L6-31 and 49), which showed a positive serological reaction for polyglycerophosphate, and two strains (DI and 7A), which did not react with antisera to polyglycerophosphate (Sharpe *et al.*, 1975a), were chosen.

Cultivation. Bacteria were cultured using strictly anaerobic techniques (Bryant & Robinson, 1961) in a modified medium M10 (Caldwell & Bryant, 1966) containing (% w/v): yeast extract, 0.4; acetic acid, 0.06; glucose, 0.5; and Trypticase (BBL), mineral solutions I and II, resazurin, cysteine-HCl and sodium carbonate in the same concentrations as originally described. To obtain a suitable cell mass, each strain was grown in three 5 l portions of medium to which the glucose had been added as a separate sterile solution after autoclaving. Each 5 l portion was inoculated with 10 ml of overnight culture and gassed continuously with CO₂ during 20 h incubation at 39 °C. Microscopic examination of stained preparations showed the cultures to be uniformly Gram-negative. Bacterial cells were harvested by centrifuging at 21 000 g, and lyophilized.

Yields of bacterial cells (g dry wt/15 l culture) were: Nor37, 12.0; 1L6-31, 11.8; 49, 5.2; DI, 11.0; 7A, 4.0.

Extraction and purification of lipoteichoic acids. Freeze-dried organisms were suspended in distilled water and extracted with hot aqueous 90 % (w/v) phenol as previously described (Wicken, Gibbens & Knox, 1973). Dialysed aqueous-phase extracts were fractionated on columns of 6 % agarose or Sephadex G75 and monitored for organic phosphorus, and extinction at 280 nm.

Chemical and serological methods. All chemical, chromatographic, analytical and serological procedures were as detailed in previous publications (Knox, Hewett & Wicken, 1970; Knox & Wicken, 1970; Knox & Wicken, 1972; Wicken *et al.*, 1973; Wicken & Knox, 1970; Wicken & Knox, 1971).

RESULTS AND DISCUSSION

Fractionation of the aqueous phase of hot phenol extracts of *B. fibrisolvens* Nor37 on columns of 6 % agarose yielded two peaks of material containing organic phosphorus. The higher molecular weight fraction (I) contained 21.8 µmol phosphorus/g dry wt of organisms and the lower molecular weight fraction (II) contained 57.5 µmol phosphorus/g dry wt organisms. The elution volumes of fractions I and II corresponded closely to those of LTA and deacylated LTA, respectively, extracted by the same procedure from other organisms (Knox & Wicken, 1973; Markham *et al.*, 1975). Preparations of fractions I and II contained varying amounts of protein. Fraction II was also contaminated with nucleic acid material, but this was reduced to less than 1 % (w/w) by further chromatography on Sephadex G75.

Acid degradation of fractions I and II produced glycerol and its mono- and di-phosphates together with small amounts of glucose and a range of fatty acids from fraction I. Both fractions were completely hydrolysed in alkali to a mixture of glycerol, glycerol mono- and di-phosphates and small amounts of diglycerol triphosphate. The latter product was characterized by converting it with alkaline phosphomonoesterase to diglycerol phosphate [R_{glycerol} was 0.70 in n-propanol-ammonia ($d = 0.88$)-H₂O; 6:3:1, by vol.]: this has a characteristic fast reaction with the periodate + Schiff's chromatographic spray reagent, and was further hydrolysed in alkali to glycerol and glycerol phosphate. Such an hydrolysis pattern is indicative of a conventional 1,3-phosphodiester-linked polymer of glycerol phosphate units. Phosphomonoesterase digestion of a total alkali hydrolysate of either

fraction failed to yield detectable quantities of 2-substituted glycerol glycosides. However, small quantities of a glycoside having a fast reaction with the periodate + Schiff's reagent, which indicates a 1-substituted glycerol glycoside, were detected in alkali hydrolysates of both fractions. Acid hydrolysis of this material yielded glucose and glycerol only and the low chromatographic mobility of the glycoside (R_{glucose} was 0.35 in n-butanol-pyridine-water; 6:4:3, by vol.) suggests that it is possibly a trisaccharide-substituted glycerol. Ester-linked D-alanine was not detected in either fraction and ammonolysis failed to release significant quantities of ninhydrin-reactive material from either polymer. Quantitative analyses of several preparations of fractions I and II showed molar ratios of phosphorus:glucose varying between 1.00:0.02 and 1.00:0.04. The absence of any sugar other than glucose in acid hydrolysates of these preparations indicates the absence of a conventional lipopolysaccharide in phenol extracts of strain Nor37.

These findings are consistent with fraction I being a conventional LTA having an unsubstituted 1,3-phosphodiester-linked polyglycerophosphate chain linked to a glycosyl-1-glyceride, and fraction II being a non-micellar and lower molecular weight deacylated form of fraction I. The presence of two forms of LTA in extracts of other bacteria, as well as in the external media used for their growth, has been reported (Markham *et al.*, 1975; Wicken & Knox, 1975; Joseph & Shockman, 1975). In these latter studies, the existence of deacylated LTA does not appear to be an artefact of the extraction procedures employed but rather it is related to the excretion of LTA in a deacylated form. The total amount of teichoic acid phosphorus extracted from strain Nor37 is comparable to that found in Gram-positive organisms and is some 50 to 100 times greater than the amount previously isolated from this strain (Sharpe *et al.* 1975*a*). This previously isolated material probably relates to fraction II, fully acylated LTA being expected to be largely retained by two passages through columns of DEAE-cellulose (Knox & Wicken, 1973). The material obtained by Sharpe *et al.* (1975*a*) also had a low glucose content (molar ratio phosphorus:glucose, 1:0.08) and was almost devoid of ester-linked amino acids.

Extracts of *B. fibrisolvens* 1L6-31 gave essentially similar results. Lipoteichoic acid and deacylated LTA fractions showed identical degradation products to those from strain Nor37. Phenol extraction of strain 49 also yielded two organic phosphorus-containing fractions from 6% agarose columns. Both fractions were contaminated with polysaccharide material, and were not characterized further other than to demonstrate the presence of typical glycerol teichoic acid degradation products in acid hydrolysates. Extracts of strains DI and 7A, which did not react with anti-polyglycerol phosphate antisera, did not show any LTA or deacylated LTA on subsequent chromatography.

Fraction I (LTA) from *B. fibrisolvens* Nor37 was further characterized serologically. The reaction between Nor37 LTA and antiserum (217) to *Lactobacillus casei* NCTC6375 LTA, which also lacks 2-glycosyl substituents (Wicken & Knox, 1975), is shown in Fig. 1: 95% of the antibody precipitated in the homologous reaction was precipitated by Nor37 LTA. The reaction between Nor37 LTA and serum 217 was also strongly inhibited by triglycerol diphosphate (Fig. 2), which indicates that the antiserum reacts with the polyglycerophosphate backbone of the polymer, and glycerol phosphate units in the LTA are joined by 1,3-phosphodiester linkages (Wicken & Knox, 1971). Injection of Nor37 LTA into rabbits in Freund's complete adjuvant gave only weakly reacting sera (0.3 mg precipitating antibody/ml serum), the specificity of which did not appear to be directed at the backbone of the polymer in that the presence of 2 μmol triglycerol diphosphate failed to inhibit the reaction between 50 μg LTA and 0.4 ml serum.

This study confirms the earlier observations that glycerol teichoic acids are present in

Fig. 1

Fig. 2

Fig. 1. Precipitation of lipoteichoic acids from *B. fibrisolvens* NOR37 (○) and *Lactobacillus casei* NCTC6375 (●) by antiserum 217 (0.1 ml) to *L. casei* lipoteichoic acid.

Fig. 2. Inhibition of the precipitin reaction between *B. fibrisolvens* NOR37 lipoteichoic acid (10 μg) and antiserum 217 (0.1 ml) by triglycerol diphosphate.

some strains of *B. fibrisolvens* (Sharpe *et al.*, 1975*a*). It also shows that in this Gram-negative genus, apart from the lack of alanine substituents, teichoic acid can occur as a conventional LTA, as well as in the deacylated form, in amounts comparable to that found in most Gram-positive bacteria.

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