Glycerol Teichoic Acid as an Antigenic Determinant in a Gram-negative Bacterium *Butyrivibrio fibrisolvens*

By M. ELISABETH SHARPE, J. H. BROCK* AND B. A. PHILLIPS

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT

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SUMMARY

An antigenic determinant isolated from a strain of the Gram-negative bacterium *Butyrivibrio fibrisolvens* reacted with specific antisera to the polyglycerophosphate backbone of membrane teichoic acids of lactobacilli. It gave a reaction of identity with membrane glycerol lipoteichoic acid and glycerol teichoic acid preparations from lactobacilli, and with phenol extracts of other Gram-positive bacteria. The antigen-antibody reaction was strongly inhibited by glycerol-phosphoryl-glycerol-phosphoryl-glycerol and the chemical composition was consistent with glycerol teichoic acid. It was concluded that this Gram-negative bacterium contained a glycerol teichoic acid whose polyglycerophosphate backbone was acting as antigenic determinant. Extracts of 33 out of 52 other strains of butyrivibrios examined gave similar reactions.

INTRODUCTION

Membrane glycerol teichoic acids occur in most Gram-positive bacteria (Baddiley, 1970). Their polyglycerophosphate backbone may act as a common antigenic determinant, and allow their presence to be detected serologically (Sharpe *et al.* 1973). There have not been any fully authenticated reports of teichoic acids in Gram-negative bacteria. During an attempt to detect serologically the presence of the polyglycerophosphate backbone of membrane glycerol teichoic acid in Gram-variable anaerobic cocci, a strain of the Gram-negative anaerobe *Butyrivibrio fibrisolvens* Nor 37 was also examined. A phenol extract of this bacterium gave an unexpected strong positive precipitin reaction against the antiserum to polyglycerophosphate determinant, indicating the possible presence of a glycerol teichoic acid as an antigenic factor. This organism was therefore further investigated and other strains of butyrivibrios were also examined serologically for the presence of glycerol teichoic acid antigenic determinant.

METHODS

Strains of bacteria examined. Most of the 19 laboratory strains of butyrivibrios examined (Table 1) have been described previously (Bryant & Small, 1956; Shane, Gouws & Kistner, 1969; van Gylswyk & Roché, 1970; Sharpe & Reiter, 1972; Leatherwood & Sharma, 1972; Roché *et al.* 1973). Shane *et al.* (1969) divided butyrivibrios into two groups: group 1 which utilized acetate and produced large amounts of lactate, and group 2 which produced acetate but little lactate. The two groups also had different nutritional requirements. Of the laboratory strains, CE65, CE66, 6B, 6D, 7A and 11 belonged to group 2, strain CE74 was a borderline strain eventually assigned to group 1 by nutritional requirements, whilst the other 12 strains all belonged to group 1. Strain JL originally grew atypically as long twisted chains forming a

* Present address: Fundacion F. Cuenca Villoro, Gasconde Gotor 4, Zaragoza, Spain.

Table 1. *Strains of butyrvibrios examined*

Species	No. of strains	Strains	Source	Received from
<i>Butyrvibrio fibrisolvens</i>	4	DI (ATCC12560), 49, A38, C14	Bovine rumen	M. P. Bryant
	2	NOR 37, IL6-31	Bovine rumen	NIRD
	4	7, 12, 15, 17	Ovine rumen	N. O. van Gylswyk
	1	834	Ovine rumen	P. N. Hobson
<i>Butyrvibrio</i> spp.	7	CE65, CE66, CE74, 6B, 6D, 7A, 11	Ovine rumen	N. O. van Gylswyk
	1	JL	Bovine rumen	J. M. Leatherwood
	31	Fresh isolates	Bovine rumen	NIRD
	2	Fresh isolates	Bovine caecum	NIRD

ATCC, American Type Culture Collection;
NIRD, isolated at the National Institute for Research in Dairying.

double helix (Leatherwood & Sharma, 1972) but on our media reverted to morphology typical of butyrvibrios. Strains NOR 37 and IL6-31 have now been identified as *B. fibrisolvens* (Bryant & Small, 1956).

Cultivation. Butyrvibrios were cultured anaerobically using the Hungate (1950) technique and the medium M10 described by Sharpe, Latham & Reiter (1969). Other bacteria were cultured in appropriate conventional media.

Preparation of extracts. (i) Phenol extracts. Bacteria were grown in 100 ml broth, harvested by centrifugation, and 0.4 g wet wt bacteria was taken up in 2 ml distilled water, extracted twice with an equal volume of 90% phenol at 68 °C for 15 min, and the aqueous phase dialysed (Wicken, Gibbens & Knox, 1973). (ii) Autoclaved extracts. Butyrvibrios centrifuged from 10 ml medium M10 broth were suspended in 0.2 ml of 0.02 M-phosphate-0.15 M-NaCl at pH 7.2 (PBS), autoclaved for 10 min at 10 lb/in² and the debris removed by centrifugation.

Preparation of glycerol teichoic acid from B. fibrisolvens NOR 37. During this preparation samples were monitored serologically by ring precipitin test, with an antiserum to the membrane glycerol teichoic acid antigenic determinant found in Gram-positive bacteria (Sharpe *et al.* 1973). Strain NOR 37 (50 g wet wt) was grown under anaerobic conditions in 15 l medium M10 broth, harvested by centrifugation, washed in PBS and disrupted in a Braun homogenizer (Shandon) for 3 min. Whole bacteria were separated at 2000 g for 4 min and the soluble fraction separated from the walls by centrifugation at 17000 g for 30 min. The walls were washed three times with distilled water which removed all serologically-active material, and the washings and soluble fraction were pooled, dialysed against four daily changes of distilled water, and then reduced from approximately 600 ml to 100 ml by rotary evaporation. Molar KH₂PO₄ (8 ml) was added, the pH adjusted to 5.0, and the solution incubated overnight at 37 °C with 5 mg DNAase (Koch-Light) and 10 mg RNAase (Koch-Light). A precipitate formed, and was sedimented by centrifugation at 40000 g for 30 min. This precipitate was then extracted with five successive 40 ml portions of phosphate-buffered saline (0.15 M-NaCl+0.03 M-phosphate, pH 7.0). Each extraction was performed for 18 h at 37 °C, merthiolate (0.01%) being added to prevent bacterial growth. These extracts had titres of 64, 32, 16, 4 and 2 respectively, and were combined with the supernatant from the 40000 g centrifugation which had a titre of 64. After dialysing four times against distilled water the solution was concentrated to 17 ml and then dialysed overnight against 0.02 M-phosphate pH 6.85.

The material was then chromatographed on DEAE cellulose and Sepharose 4B, as described by Sharpe *et al.* (1973), except that a second passage of the serologically-active material through DEAE cellulose was included before Sepharose 4B chromatography. The yield of material from the Sepharose 4B column was 22 mg, but as this still contained some protein, 18 mg was treated with trichloroacetic acid (TCA) and ethanol (Sharpe *et al.* 1973). The final yield of material was only 1.9 mg.

Membrane glycerol teichoic acid preparations. Membrane teichoic acid from *Lactobacillus casei* RO94 (NCD0151) was prepared as described by Sharpe *et al.* (1973). Membrane lipoteichoic acid from *L. fermentum* NCTC6991 (Wicken *et al.* 1973) was kindly provided by Dr K. W. Knox, Institute of Dental Research, United Dental Hospital, Sydney, Australia.

Inhibitor of precipitin test. Glycerol-phosphoryl-glycerol-phosphoryl-glycerol (G_3P_2) was prepared from cardiolipin (General Biochemicals, Chagrin Falls, Ohio, U.S.A.) by mild deacylation (Wilkinson, 1968).

Extraction of Nor 37 walls. Washed walls, obtained during the preparation of the glycerol teichoic acid, were extracted with phenol as described for extraction of whole bacteria, or with 10% (v/v) TCA overnight at 4 °C.

Chemical analysis of glycerol teichoic acid from Nor 37. Estimations of total phosphorus, hexose and ester-linked amino acid, and methods for acid hydrolysis and paper chromatography were as described by Sharpe *et al.* (1973). Alkaline hydrolysis was carried out with 2 M-NaOH for 2 h at 100 °C in a sealed tube, and the hydrolysate passed through a column of Dowex 50 resin (NH_4^+ form) before evaporating to dryness.

Glycerol was estimated by the following procedure: the sample (400 µg) was hydrolysed with 4 ml of 2 M-HCl for 2 h at 100 °C in a stoppered tube. The acid was neutralized by cautious addition of solid $(NH_4)_2CO_3$ and the pH adjusted to 9.0 with 1 M-NaOH. The solution was incubated for 18 h at 37 °C with 0.5 mg alkaline phosphatase (Sigma) and then heated at 100 °C for 15 min to inactivate the enzyme. Glycerol in the hydrolysate was estimated by the method of Wieland (1965) in which glycerol is phosphorylated with glycerokinase in the presence of ATP, and the L-glycerol 3-phosphate thus formed is oxidized to dihydroxyacetone phosphate by α -glycerophosphate dehydrogenase and NAD. Corrections were applied for extinction at 340 nm of reagents, and of a solution containing the hydrolysate, NAD and ATP but no enzymes. All enzymes and coenzymes were obtained from Boehringer.

Incubation with phosphatase. Samples (1 ml) were buffered to pH 9.0 with $(NH_4)_2CO_3$, incubated for 36 h with 0.5 mg alkaline phosphatase and then neutralized with HCl, and a portion was titred serologically by ring precipitin test.

Treatment with periodate. Samples (1 ml) were incubated with 1 ml of 1% (w/v) $NaIO_4$ for 18 h at room temperature in the dark. Periodate was then removed by adding 0.03 ml ethylene glycol, and a portion titred serologically by ring precipitin test.

Serological tests. Antisera to the membrane glycerol teichoic acid antigenic determinant common to Gram-positive bacteria were prepared using *L. casei* subsp. *rhamnosus* ATCC-7469. Washed bacteria suspended in PBS were disrupted in a Braun homogenizer and, without any separation, stored in 1 ml amounts at -20 °C. One ml of this preparation was inoculated into rabbits intravenously or subcutaneously for a course of inoculations. Other rabbits were inoculated with whole heat-killed organisms (Sharpe *et al.* 1973). Antisera to *B. fibrisolvens* Nor 37 were prepared similarly. Antiserum to purified lipoteichoic acid obtained from *L. casei* subsp. *rhamnosus* ATCC7469 (antiserum 217) was kindly provided by Dr K. W. Knox.

Other serological methods were those described by Sharpe *et al.* (1973).

Electron microscopy. To prepare sections, centrifuged bacteria were fixed for 1 h in 2.5%

Table 2. *Chemical analysis of teichoic acid from Butyrivibrio* NOR 37

	Percentage (w/w)	Molar ratio (P = 1)
Phosphorus (P)	9.1	1.00
Glycerol	27.6	1.02
Hexose	4.0	0.08
Ester-linked amino acids	0.016*	0.05

* $\mu\text{mol/mg}$.Table 3. *Serological precipitin reaction of three antisera tested against phenol extracts of different micro-organisms*

The antisera tested were C16/B, 217 and Nor 37; see text for details.

Strain	Ring precipitin and immunodiffusion reaction with the antisera
<i>Lactobacillus casei</i> ATCC7469	+
<i>L. acidophilus</i> A22	+
<i>Streptococcus bovis</i> NCD0243	+
<i>Streptococcus cremoris</i> 924	+
<i>Staphylococcus aureus</i> BB	+
<i>Escherichia coli</i> NCTC8603	—
<i>E. coli</i> B	—
<i>E. coli</i> H509a	—
<i>Salmonella typhimurium</i>	—
<i>Serratia marcescens</i>	—
<i>Pseudomonas</i> sp.	—
<i>Vibrio</i> sp. ATCC4711	—
<i>Bacteroides ruminicola</i> B1/18	—
<i>Bacteroides amylophilus</i> 78	—
<i>Butyrivibrio fibrisolvens</i> 49, NOR 37 and IL6-31	+

(w/v) glutaraldehyde in 0.2 M-cacodylate buffer pH 7.2 at room temperature. After several 1 h washes in cacodylate-HCl buffer, pH 7.4, the bacteria were fixed in cacodylate-buffered 1% (w/v) osmium tetroxide for 2 h and then transferred to 1% (w/v) uranyl acetate in 25% (w/v) ethanol for 1 h. Dehydration in a series of ethanol-water mixtures and absolute ethanol was followed by treatment with propylene oxide and propylene oxide-Araldite mixtures before embedding in Araldite. Thin sections were stained with uranyl acetate and lead citrate and examined in an Hitachi HU-11E electron microscope.

RESULTS

Paper chromatography

Because of the low yield of material in the final preparation from *B. fibrisolvens* NOR 37, paper chromatography was carried out using the material from the Sepharose 4B column. A sample (2 mg) hydrolysed in 2 M-HCl contained glycerol, glycerol monophosphates and diphosphates, and inorganic phosphate as major components. All except glycerol diphosphate were identified by direct comparison with authentic samples. Only traces of material reacting with AgNO_3 in alkali were detected, indicating that hexoses and pentoses were present only as minor contaminants. Alkaline hydrolysis gave glycerol monophosphate as the major product, plus some glycerol and glycerol diphosphate. Insufficient material was

Fig. 1. Reactions between antisera to glycerol teichoic acid determinants and bacterial extracts. Centre wells: A, antiserum to purified lipoteichoic acid from *Lactobacillus casei* subsp. *rhamnosus* 7469; B and C, antisera to *L. casei* subsp. *rhamnosus* disrupted cells (C, after a long course of inoculations); D, antiserum to *B. fibrisolvens* disrupted cells. Outer wells: 1. purified lipoteichoic acid isolated from *L. fermentum*; 2. membrane teichoic acid from *L. casei* R094; 3. phenol extract of *Staphylococcus aureus* Mexi; 4. phenol extract of *Streptococcus bovis*; 5. phenol extract of *B. fibrisolvens* 49; 6. teichoic acid from *B. fibrisolvens* Nor 37.

available to allow detection and characterization of diglycerol triphosphate, which is a characteristic but minor alkaline hydrolysis product of 1 $\rightarrow$ 3-linked glycerol teichoic acids. These results are consistent with the presence of a non-glycosylated glycerol teichoic acid. A sample (2 mg) hydrolysed in 6 M-HCl revealed several ninhydrin-positive components which were presumably amino acids derived from contaminating protein. One component had an R_f similar to alanine, but this was not prominent.

Chemical analysis

The results of chemical analysis of the final product after TCA treatment are shown in Table 2. The molar ratio of glycerol to phosphorus is close to 1 : 1, as would be expected in a glycerol teichoic acid. The low molar proportions of hexose and ester-linked amino acids indicate that the teichoic acid is largely non-glycosylated and also devoid of ester-linked alanine or other amino acids, and is in agreement with the results of paper chromatography. The chemical analysis does, however, indicate that some material remains unaccounted for in the analysis; some insoluble material was present even after hydrolysis in 2 M-HCl but the very low yield of material precluded any attempts at further purification.

Serology

Antisera. Of six rabbits inoculated with the disrupted organisms of *L. casei* subsp. *rhamnosus* ATCC7469, three produced antibodies reacting with the glycerol teichoic acid antigenic determinant; the other three, and the three rabbits inoculated with heat-treated cultures, produced antibodies only to the group C antigen of lactobacilli (Sharpe & Wheater, 1957) and/or to type antibodies. One of the three rabbits inoculated with disrupted organisms of *B. fibrisolvens* Nor 37 produced antibodies reacting weakly with the glycerol teichoic acid antigenic determinant (antiserum Nor 37). Harvests from the most potent antisera to *L. casei* subsp. *rhamnosus* reacting with the glycerol teichoic acid antigenic determinant were pooled and used as antiserum C16/B.

Table 4. *Presence of glycerol teichoic acid (GTA) determinant in strains of butyrivibrios, as determined by ring precipitin and immunodiffusion reactions*

	Serological reaction showing GTA in strain	
	Present*	Absent
<i>B. fibrisolvens</i>	49, 7, 12, 15, 17, NOR 37, IL6-31	DI, A38, C14, 834
<i>Butyrivibrio</i> spp.	JL, CE74	CE65, CE66, 6B, 6D, 7A, 11
Fresh isolates	22 rumen strains, 2 caecum strains	9 rumen strains

* Positive ring precipitin test; positive (all giving a reaction of identity) immunodiffusion test.

Ring precipitin and immunodiffusion tests. Table 3 shows the precipitin reactions of phenol extracts of strains from different bacterial species against antiserum C16/B. Only Gram-positive and butyrivibrio strains showed a positive reaction. In addition, purified teichoic acid preparations of *L. casei* R094 (15 µg/ml), *B. fibrisolvens* NOR 37 (15 µg/ml) and a lipoteichoic acid preparation of *L. fermentum* 6991 (15 µg/ml) reacted strongly with undiluted antiserum C16/B (titres 256, 256 and 128, respectively) and also with antiserum to lipoteichoic acid (antiserum 217). Immunodiffusion tests with antisera C16/B and 217 showed reactions of identity between the phenol extracts of all organisms giving a positive ring precipitin reaction and the three teichoic acid preparations. An extra precipitin line was observed with both antisera against the lipoteichoic acid preparation (Fig. 1). An antiserum against *L. casei* subsp. *rhamnosus* 7469 from a rabbit inoculated over a long period, showed spurring of extracts of the three butyrivibrio strains with phenol extracts or with purified teichoic acid preparations of lactic acid bacteria. The antiserum to *B. fibrisolvens* NOR 37 also gave spurred reactions (Fig. 1).

Quantitative precipitin tests. The amount of antibody in antiserum C16/B reacting with the different teichoic acid preparations ranged from 3.1 to 4.0 mg/ml.

Absorption of antiserum C16/B. After removal of antibodies by absorption with *L. casei* R094 teichoic acid, or with *L. fermentum* 6991 lipoteichoic acid, the antiserum no longer reacted with the NOR 37 teichoic acid, whilst removal of antibodies reacting with *B. fibrisolvens* NOR 37 abolished the reaction with *L. casei* R094 teichoic acid. However, when the antiserum was absorbed with either *B. fibrisolvens* NOR 37 or *L. casei* R094, it still showed 15% activity on reaction with *L. fermentum* 6991 lipoteichoic acid. This was in agreement with the extra precipitin line seen with this preparation in the immunodiffusion test.

Inhibitory effect of G₃P₂ on precipitin reaction. The reaction between the *B. fibrisolvens* NOR 37 teichoic acid preparation (2.5 µg) and antiserum C16/B (50 µl) was inhibited 60% by 23 µg G₃P₂.

Extracts of NOR 37 walls. Neither the phenol nor the TCA extract of the walls gave a positive ring precipitin reaction with antiserum C16/B.

Treatment of phenol extract of strain NOR 37 with phosphatase and periodate. Treatment of a phenol extract with phosphatase or periodate did not reduce the precipitin titre. Treatment with phosphatase followed by periodate was also without effect.

Precipitin reactions of antiserum C16/B with extracts of other butyrivibrios. Extracts of further strains of butyrivibrios were reacted serologically with antiserum to the glycerol teichoic acid determinant but this determinant could not be detected in all strains (Table 4). Using phenol and autoclaved bacterial extracts, positive ring precipitin reactions and immunodiffusion reactions of identity occurred with only nine of the laboratory strains and could not be detected in the other ten. None of the six strains belonging to group 2 of Shane *et al.* (1969) reacted, but with group 1 strains eight out of 12 reacted. The borderline strain CE74

Fig. 2. Electron micrograph of sections of *B. fibrisolvens* NOR 37.

was also positive. Extracts of 24 of the 33 fresh isolates (72%) gave positive ring precipitin and immunodiffusion reactions but it was not determined if they were group 1 or group 2. All 33 positive strains gave a reaction of identity with the glycerol teichoic acid prepared from NOR 37.

Electron microscopy

An electron micrograph of sections of *B. fibrisolvens* NOR 37 (Fig. 2) indicates a multi-layered wall which is atypical of classical Gram-positive or Gram-negative wall membrane profiles.

DISCUSSION

Butyrivibrios are among the most commonly occurring organisms in the rumen of cows on high roughage diets and their original description (Bryant & Small, 1956) and all further work has consistently shown them to be Gram-negative.

The chemical analysis indicates that the serologically-active fraction isolated consists of glycerol and phosphate and is indicative of a glycerol teichoic acid. The serological results confirm the presence of such a teichoic acid determinant, antigenically similar to the poly-glycerophosphate backbone of the membrane teichoic acids in Gram-positive organisms. These teichoic acids consist of 1 → 3-linked glycerol phosphate units (Baddiley, 1970) and the present findings are consistent with such a structure for the teichoic acid of *B. fibrisolvens* NOR 37. The fact that this antigenic determinant was not destroyed by periodate/phosphatase treatment indicates that the reaction could not have been due merely to a glycerophosphate end-group. This is supported by the lack of reaction between antiserum C16/B and a phenol extract of *Escherichia coli* H509a (Table 2). This strain, possessing antigen O:100, has a terminal glycerophosphate group in the K antigen (Jann *et al.* 1970).

The immunodiffusion tests with one of the antisera prepared against *L. casei* subsp. *rhamnosus* 7469 using a very prolonged course of inoculations, and those with antiserum against *B. fibrisolvens* NOR 37 suggest that there may be slight differences in the structure of the glycerol teichoic acid antigenic determinant in the butyrivibrio and in the lactic acid bacteria.

This appears to be the first report of the isolation and chemical characterization of a

glycerol teichoic acid from a Gram-negative organism. Clarke & Lilly (1962) and Lilly, Clarke & Meadow (1963) reported possible teichoic acid precursors in *E. coli* strain 26-26, and Lilly (1962) reported briefly the occurrence of ribitol and phosphate in the hydrolysate of a wall extract of the same organism. However, no teichoic acid was purified from this organism, and no quantitative chemical or serological analysis was made. Traces of glycerol were also reported to be present in hydrolysates of walls of *E. coli* B (Armstrong *et al.* 1959) but no further evidence for a teichoic acid in this strain has been presented, and we found that an extract of this organism did not react with antiserum C16/B (Table 3). Recently, however, Argaman, Liu & Robbins (1974) have shown that the capsular polysaccharide of *Haemophilus influenzae* type b contains a ribitol-phosphate component which gives rise to cross-reactions with Gram-positive organisms containing ribitol teichoic acids in their walls. This is strongly suggestive of a teichoic acid-like structure in the capsule of this Gram-negative organism. Our *Butyrivibrio*, Nor 37, is of even greater interest, as in addition to a teichoic acid, it possesses the antigenic determinant of *Salmonella* O:4 (Sharpe & Reiter, 1972).

The method of isolation and the fact that glycerol teichoic acid was serologically undetectable in extracts of washed walls indicated that the teichoic acid was mainly associated with the intracellular fraction of disrupted organisms, the small amount of wall-associated material being easily removed by washing. Thus this teichoic acid appears to be analogous to the membrane-associated teichoic acid of Gram-positive bacteria rather than to a wall teichoic acid. The exact location of this teichoic acid would be of considerable interest. Subsequent investigations of products extractable by phenol from this strain indicate that it contains a substance with the chemical and serological properties of a lipoteichoic acid (A. J. Wicken, K. W. Knox and M. E. Sharpe, unpublished).

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