LIPOTEICHOIC ACIDS OF BIFIDOBACTERIUM SUBSP PENNSYLVANICUM

STRUCTURE, BIOSYNTHESIS AND BIOLOGICAL PROPERTIES

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LIPOTEICHOIC ACIDS OF BIFIDOBACTERIUM SUBSP. PENNSYLVANICUM STRUCTURE, BIOSYNTHESIS AND BIOLOGICAL PROPERTIES

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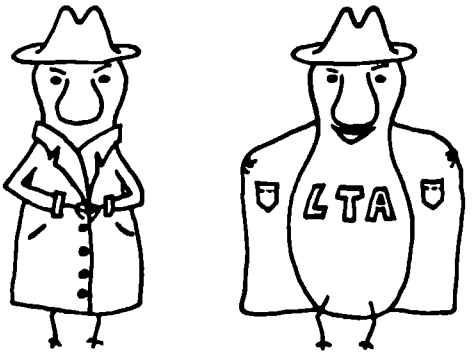
General introduction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1.1 History of Bifidobacterium bifidum subsp. pennsylvanicum

The genus Bifidobacterium

Bifidobacteria are Gram-positive, non-motile, nonsporeforming, catalase negative, anaerobic bacteria. The bacteria appear as irregularly shaped rods often with branches or knobs. They can be isolated from faeces of infants, human adult and several animals, human vagina and oral cavity, bovine rumen, sewage and intestine of honeybees (Mitsuoka & Kaneuchi, 1977; Scardovi, 1981). For many years bifidobacteria were included in the genus *Lactobacillus*, although in 1924 Orla-Jensen had already recognized the existence of the genus *Bifidobacterium* as a separate taxon. On basis of their characteristic morphology, biochemistry, cell wall constituents and DNA-composition, 11 species of the genus *Bifidobacterium* Orla-Jensen are listed in the 8th edition of Bergey's Manual (Rogosa, 1974) and the genus is included in the family *Actinomycetaceae* Buchanan. Recently Scardovi (1981) presented a list of 24 species and a key for their identification based on fermentation pattern, G+C mol %, DNA-homology and zymogram. For an excellent review of bifidobacteria see Râsić & Kurmann (1983).

The intestinal flora of breast-fed infants consists largely of the species *B. bifidum* (Neut & Beerens, 1980). Bifidobacteria appear in the stools from 2 to 5 days after birth in breast-fed babies with the establishment of a relatively stable microflora in the colon within several days (Beerens *et al.* 1980; Râsić & Kurmann, 1983). By the end of the first week bifidobacteria account for 85-99% of the faecal flora (counts ranging from 10^6 to 10^{11} bacteria/g of faeces)(Râsić & Kurmann, 1983). The difference between the faecal bacterial flora of infants who are exclusively breast-fed and those receiving formula has long been a subject of great interest. Breast-fed infants appear better protected against gastrointestinal disorders (Mata & Urrutia, 1971; Mata *et al.*, 1969). It was first considered that the "bifidus flora" of breast-fed infants directly inhibited the growth of pathogens in the large bowel. A more plausible mechanism for the antagonistic action of bifidobacteria is that these bacteria maintain the pH of the large bowel at acid level (pH about 5.0) due to production of acetic, lactic and formic acid (Bullen & Tearle, 1976; Bullen *et al.*, 1976, 1977). The faeces of breast fed infants contain acetic acid as the main organic acid and have a pH of 5.1 to 5.4. Bottle fed infants have stools with higher pH values.

Bifidobacterium bifidum subsp. pennsylvanicum

B. bifidum subsp. *pennsylvanicum* was first isolated from stools of infants and vaginal secretions of pregnant women (Gyorgy *et al.*, 1954). This subspecies of *B. bifidum* requires specific factors found in human milk for growth. These compounds are lacking in bovine milk (Gyorgy & Rose, 1955). Many investigators have attempted to isolate the specific factors from human milk (bifidus factor). They appear to be oligosaccharides (Gyorgy & Rose, 1955; Gyorgy *et al.*, 1974) as well as glycoproteins (Bezkorovainy *et al.*, 1979; Nichols *et al.*, 1974) containing N-acetyl-D-glucosamine saccharides which are used for cell wall synthesis. Labeled N-acetylglucosamine derivatives are incorporated into the peptidoglycan (O'Brien *et al.*, 1960; Lambert & Zilliken, 1965; Lambert *et al.*, 1965). Lack of these growth-promoting factors results in the formation of bacteria with bizarre forms ranging from bulbous to knobbed (Glick *et al.*, 1960).

The cytoplasmic membrane of this organism contains 70% protein and 8% lipid material (Exterkate *et al.*, 1970). The lipid composition was studied extensively (Exterkate & Veerkamp, 1969, 1971; Veerkamp, 1972; Veerkamp & van Schaik, 1974). The membrane lipids consist predominantly of phospholipids and glycolipids. Eighteen phospholipids were detected, the major ones being glycerophosphorylgalactosyldiacylglycerol, phosphatidylglycerol and diphosphatidylglycerol. The glycolipids of the bacterium were all galactolipids accounting for about 50% of total lipids. Comparison of the fatty acid patterns showed no large differences between total lipids, cytoplasmic membrane lipids and phospho- and glycolipids. The major fatty acids are palmitic and octadecenoic acid.

The chemical composition of the cell wall was reported by Veerkamp *et al.* (1965). The isolated cell wall contains in addition to the peptidoglycan components large amounts of rhamnose and glucose together with minor amounts of galactose, glycerol, phosphorus and protein. The structure of the peptidoglycan was elucidated by Veerkamp (1971). Structural investigations on the polysaccharide part of the cell wall revealed the presence of a glucosylated rhamnose polymer and a mannitol phosphate teichoic acid (Hoelen, 1981; Veerkamp *et al.*, 1983) which were linked to each other and/or to the peptidoglycan.

1.2 Occurrence and structure of lipoteichoic acids

The presence of teichoic acids as cell wall components was well established by 1960, but a further ten year passed before the lipid-teichoic acid complexes (lipoteichoic acids) were discovered (Toon *et al.*, 1972; Wicken & Knox, 1970). In comparison to wall teichoic acids, lipoteichoic acids are more wide-spread among the Gram-positive bacteria (Knox & Wicken, 1973; Wicken & Knox, 1975, 1980). Organisms of *Micrococcus* species however contain amphiphatic lipomanans instead of lipoteichoic acid (Powell *et al.*, 1975). Untill now at least one Gram-negative species containing lipoteichoic acid has been described (Hewett *et al.*, 1976). The main structural feature which discriminates lipoteichoic acid from teichoic acid is the lipid portion that is cova-

lently linked to the polar chain. The lipid part may consist of a glycolipid (Nakano & Fischer, 1978; Wicken & Knox, 1970, 1975), a phosphoglycolipid (Ganfield & Pieringer, 1975) or simply diacylglycerol (Button & Hemmings, 1976). The lipid moiety is generally also a constituent of the bacterial membrane (Fischer *et al.*, 1978a,b). Due to the presence of a covalently linked lipid, lipoteichoic acids will form micelles in aqueous solutions. This is a common feature of amphiphatic molecules (Wicken & Knox, 1980).

Thus far only three types of lipoteichoic acids have been described, this in contrast with teichoic acids which show a great structural divergence (Knox & Wicken, 1973). The first and main type consists of a 1,3-phosphodiester-linked glycerol phosphate backbone which is variously substituted with glycosidic or D-alanyl ester groups (Knox & Wicken, 1973; Lambert *et al.*, 1977). Fig. 1.1 shows five lipoteichoic acids of this main type. Glycosylation ranges from unglyco-

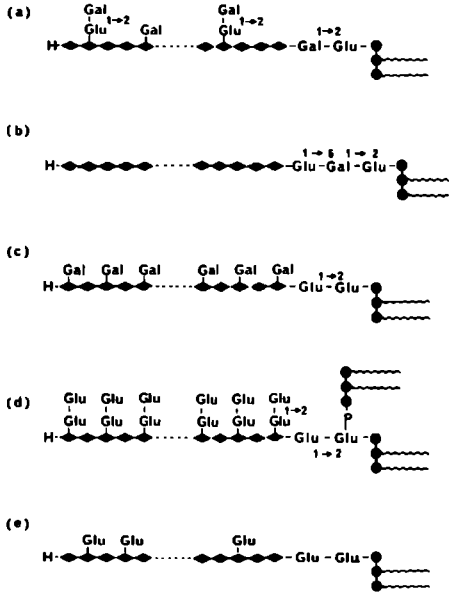


Fig. 1.1: Proposed structures of some lipoteichoic acids. (a), *Lactobacillus fermentum*; (b), *L.casei*; (c), *Streptococcus lactis*; (d), *S.faecalis*; (e), *L.helveticus*. ◆ glycerol phosphate unit, ●●● glycerol residue, —— fatty acid. Taken from Wicken (1980).

sylated in *Lactobacillus casei* to heavy glycosylated in *Streptococcus faecalis*. A second type of lipoteichoic acid was described by Arakawa *et al.* (1981). They isolated a lipoteichoic acid from *Staphylococcus aureus* H in which some ribitol phosphate residues are linked to the terminal glycerol of the poly(glycerol phosphate) chain. The third type isolated from *Streptococcus lactis* Kiel 42172 (Koch & Fischer, 1978) is characterized by the presence of galabiosyl residues as integral parts of the hydrophilic chain.

1.3 Cellular location of lipoteichoic acids

The cell envelope of Gram-positive bacteria consists of a cytoplasmic membrane and a cell wall (Fig. 1.2). The latter is a cross-

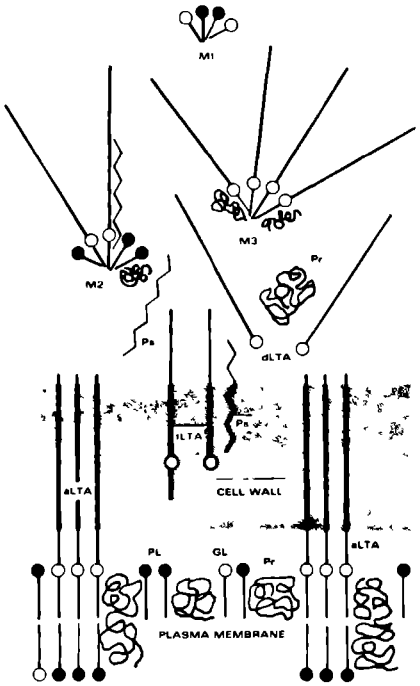


Fig. 1.2: Diagramatic representation of a generalized Gram-positive cell envelope. Pr, protein; PL, phospholipid; GL, glycolipid; Ps, polysaccharide; aLTA, acylated lipoteichoic acid; dLTA deacylated lipoteichoic acid; tLTA, lipoteichoic acid molecules in the process of excretion. M1, M2 and M3 are various micellar complexes present in the external milieu. Taken from Wicken & Knox (1980).

linked network of peptidoglycan to which polysaccharides and wall teichoic acids are covalently bound. Due to its amphiphilic character lipoteichoic acid is a part of both cell wall and cell membrane. The fatty acid residues of the lipid part (anchor) intercalate with the upper halve of the cytoplasmic membrane bilayer and the hydrophilic chain penetrates the peptidoglycan network and may even be detectable as surface antigen (Van Driel *et al.*, 1973; Joseph & Shockman, 1975a). The observations that lipoteichoic acids are excreted even under normal growth conditions in both their acylated and deacylated form (Joseph & Shockman, 1975b; Markham *et al.*, 1975) have made clear that the Gram-positive cell envelope in vivo must be considered as a dynamic rather than as a rigid system. The external milieu (Fig. 1.2) is therefore depicted as being composed of excreted polysaccharide, protein, deacylated lipoteichoic acid monomers and several micellar complexes containing protein, membrane lipids, polysaccharides and lipoteichoic acid.

1.4 Biosynthesis of lipoteichoic acids

Evidence that the glycerol phosphate backbone of the main type of lipoteichoic acid is synthesized from phosphatidylglycerol was obtained in both *in vivo* (Emdur & Chiu, 1974; Glaser & Lindsay, 1974; Koga *et al.*, 1984) and *in vitro* (Emdur & Chiu, 1975; Ganfield & Pieringer, 1980) experiments. Chain elongation occurs through transfer of the *sn*-glycerol 1-phosphate moieties from phosphatidylglycerol. The lipid anchor serves as point of initiation for this process (Ganfield & Pieringer, 1980) and the unacylated *sn*-glycerol 1-phosphate of phosphoglycolipids found in several Gram-positive bacteria may represent the first unit of the polymer (Fischer, 1981). Cabacungan & Pieringer (1981) showed that the glycerol phosphate polymer was synthesized via an external elongation mechanism. CDP-glycerol, the precursor for synthesis of glycerol phosphate wall teichoic acid (Ward, 1981), is a poor substitute for phosphatiydylglycerol in the synthesis of lipoteichoic acid (Pieringer *et al.*, 1981). CDP-glycerol would more likely donate a *sn*-glycerol 3-phosphate unit instead of a *sn*-glycerol 1-phosphate unit.

The interrelationship between lipoteichoic acid biosynthesis and membrane lipid metabolism in growing *Staphylococcus aureus* is shown in Fig. 1.3 (Koch *et al.*, 1984). The biosynthetic routes presented may be

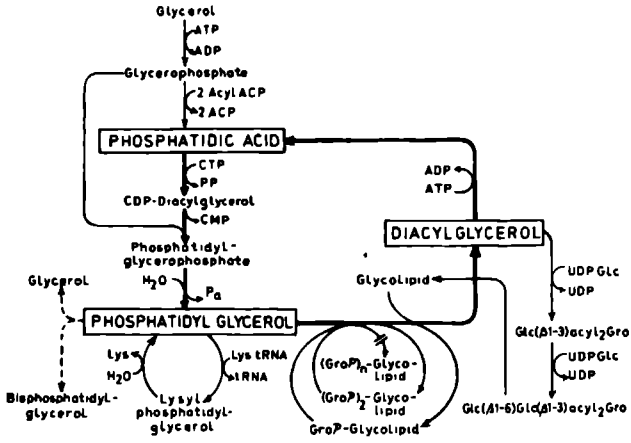


Fig. 1.3: Biosynthetic routes concerning lipoteichoic acid and membrane lipid metabolism in *Staphylococcus aureus*. Taken from Koch *et al.* (1984).

common for several Gram-positive bacteria (Koga *et al.*, 1984; Taron *et al.*, 1983). Phosphatidylglycerol serves as biosynthetic precursor in at least three reactions: (i) formation of aminoacylphosphatidylglycerol; (ii) synthesis of diphosphatidylglycerol, phosphatidylethanolamine and phosphoglycolipids; (iii) transfer of an unacylated sn-glycerol 1-phosphate group to non-lipid substances in the lipoteichoic acid synthesis. The resultant diacylglycerol from the latter reaction can be reutilized for the synthesis of phosphatilglycerol or other lipids.

Glycosylation of the glycerol phosphate units of the backbone of the lipoteichoic acid molecules occurs through transfer of a glycoside from UDP-glycoside to the poly(glycerol phosphate) (Arakawa *et al.*, 1981; Emdur & Chiu, 1975; Mancuso *et al.*, 1979). Recently, evidence was obtained for the presence of a undecaprenollipid as a direct glycosyl-donor in the synthesis of lipoteichoic acid in Streptococcus sanguis (Mancuso & Chiu, 1982). Fig. 1.4 shows the proposed biosynthetic routes leading to the glycosylation of this lipoteichoic acid.

The incorporation of D-alanine into lipoteichoic acid is mediated by a two step reaction. In the first reaction D-alanine is activated in the presence of ATP and the D-alanine-activating enzyme to form an enzyme.AMP-D-alanine complex. In the second reaction a ligase donates the D-alanine moiety from the complex to the backbone of the lipoteichoic acid in the (Brautigan *et al.*, 1981; Childs & Neuhaus, 1980).

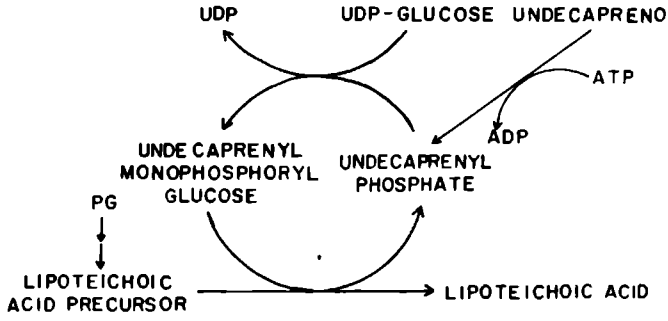


Fig. 1.4: Glycosylation of the lipoteichoic acid of *Streptococcus sanguis*. Taken from Mancuso & Chiu (1982).

1.5 Biological activities of lipoteichoic acids

Interaction of lipoteichoic acid with mammalian cells

Lipoteichoic acids of Gram-positive bacteria bind spontanously to mammalian cell membranes (Beachey *et al.*, 1977, 1979; Courtney *et al.*, 1981; Chiang *et al.*, 1979; Simpson *et al.*, 1980a). In all studies the lipid part of the lipoteichoic acid molecule appeared to be essential for binding. Deacylated lipoteichoic acid exhibited no binding activity. Beachey *et al.* (1979) demonstrated that 50% of the binding could be restored by esterification of the deacylated molecule with stearoyl-chloride. Binding of lipoteichoic acid was reversible since an excess of unlabeled lipoteichoic acid was able to displace 45% to 80% of bound labeled lipoteichoic acid. Mammalian cells possess single populations of lipoteichoic acid-binding sites ranging from 6.6×10^6 binding sites per cell for human polymorphonuclear leukocytes (Courtney *et al.*, 1981) to 5.1×10^9 for human adult oral epithelial cells (Simpson *et al.*, 1980a). The putative receptor is thought to be a membrane protein or glycoprotein with fatty acid-binding sites. This is based on the finding that albumin, which contains several fatty acid-binding sites, effectively inhibited the binding of lipoteichoic acid to mammalian cells (Simpson *et al.*, 1980b,c). Recently (Beachey *et al.*, 1983; Simpson &

Beachey, 1983), it was suggested that fibronectin associated with oral epithelial cells serves as a lipoteichoic acid-sensitive receptor. In addition Courtney *et al.* (1983) demonstrated binding of streptococcal lipoteichoic acid to the fatty acid-binding sites on human plasma fibronectin.

Lipoteichoic acids and the bacteria-eukaryotic cell interaction

Binding characteristics of lipoteichoic acids to various mammalian cells are of special importance because lipoteichoic acids have been proposed to play a role in the adherence of bacteria to these cells. Lipoteichoic acid effectively inhibited the binding of streptococci (Beachey, 1981; Beachey *et al.*, 1983; Nealon & Mattingly, 1984) and staphylococci (Carruthers & Kabat, 1983) to epithelial and other mammalian cells. The importance of lipoteichoic acid in the adhesion of bacteria to eukaryotic cells and the role of the lipid part in the binding of purified lipoteichoic acid to these cells seems to be incompatible with the location of the lipoteichoic acid in the bacterial

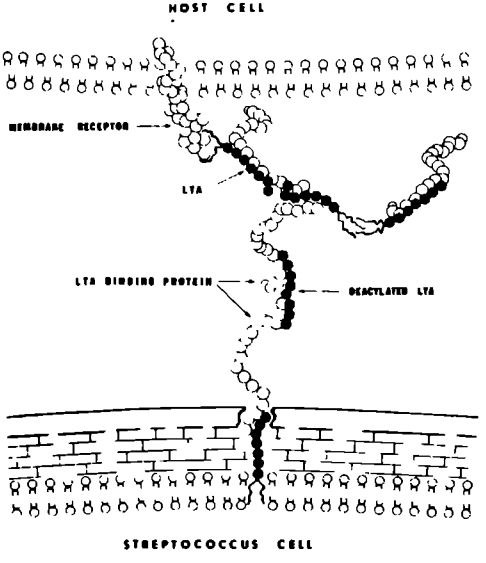


Fig. 1.5: Hypothetic model of the interaction between bacteria (group A streptococci) and eukaryotic cells. LTA, lipoteichoic acid. Taken from Beachey (1981).

cell envelope (section 1.3). However, lipoteichoic acid and its deacylated derivative are excreted into the surrounding medium and are capable of forming macromolecular complexes with protein and polysaccharides (Doyle *et al.*, 1975; Ofek *et al.*, 1982). A structurally defined streptococcal cell wall protein (M-protein) formed complexes by binding to the poly(glycerol phosphate) backbone of both acylated and deacylated lipoteichoic acid (Ofek *et al.*, 1982). On basis of these observations Ofek *et al.* (1982) presented a model for a lipoteichoic acid-mediated interaction between streptococci and mammalian cells (Fig. 1.5). Formation of a fibrillar network of lipoteichoic acid, deacylated lipoteichoic acid and lipoteichoic acid-binding protein would permit the interaction of the lipid parts of firmly anchored lipoteichoic acid molecules with receptors in the mammalian cell membrane.

Immunogenicity of lipoteichoic acids

Lipoteichoic acids are only very poor immunogens unless complexed with protein (Fiedel & Jackson, 1976; Knox & Wicken, 1973). For instance, hot water-extracted lipoteichoic acid, containing up to 8% complexed protein (by weight) appeared to be more immunogenic then phenol-extracted lipoteichoic acid (Wicken & Knox, 1971, 1977). The role of the associated protein is unknown. Since lipoteichoic acids spontaneously adhere to eukaryotic cells they may function as carrier for other bacterial antigens or proteins to target organs in which these complexes may provoke cytotoxic reactions (Knox & Wicken, 1973; Fiedel & Jackson, 1979).

Antibodies raised to lipoteichoic acid appeared to be of IgM and IgG antibody classes (Wicken & Knox, 1977). Men and several animals showed low levels of natural immunity to lipoteichoic acid as a result of dietary stimulation (Bolton, 1981; Wicken & Knox, 1977). For detection of antibodies to lipoteichoic acid several methods can be applied, such as passive haemagglutination (using the spontanous absorption of lipoteichoic acid to erythocytes), counter immunoelectrophoresis, rocket immunoelectrophoresis, quantitative precipitation and enzyme-linked immunosorbent assay (ELISA) (Knox & Wicken, 1973; Kessler & Thivierge, 1983).

Lipoteichoic acids of the main type (section 1.2) contain three

components that could be immunodominant: (i) the carbohydrate constituents (mono-, di- or trisaccharides); (ii) the glycerol phosphate moieties of the backbone of the molecule; (iii) the D-alanyl ester substituents. In the case of carbohydrate specificity the lipoteichoic acid may be used for serological classification. Antibodies to the group-specific lipoteichoic acids of group D and N streptococci and group F lactobacilli are generally specific for the carbohydrate substituents (Wicken & Knox, 1975). Evidence for the presence of carbohydrate-specific antibodies came from quantitative precipitin inhibition experiments in which structurally related carbohydrates and methylglycosides appeared to be potent inhibitors (Knox & Wicken, 1973; Knox *et al.*, 1970).

Whether the antibodies are specific for the glycerol phosphate backbone or the carbohydrate substituents depends on the degree of substitution. Lipoteichoic acids in which the poly(glycerol phosphate) backbone is devoid of or has a low degree of carbohydrate substituents will give rise to antibodies with backbone-specificity (Knox *et al.*, 1976; Wicken & Knox, 1970). These antibodies account for most of the observed cross-reactions between different lipoteichoic acids (Sharpe *et al.*, 1973). Cross-reactivity will be affected by the degree of substitution of the tested lipoteichoic acid (Kessler & Thivierge, 1983). Glycerol-phosphoryl-glycerol-phosphoryl-glycerol, prepared from diphosphatidylglycerol by mild alkaline deacylation, appeared to be a very effective inhibitor of the quantitative precipitin reaction for poly-(glycerol phosphate)-specific antibodies (Sharpe *et al.*, 1973; Wicken & Knox, 1971). For immunochemical studies on isolated lipoteichoic acids the quantitative precipitin method enables an accurate comparison of reactions and cross-reactions and of the ability of various compounds to inhibit these reactions.

Compared to antibodies against the glycosidic substitution, antibodies against D-alanyl ester substituents are rather rare. This lack of immunogenicity may be related to the lability of the D-alanyl ester linkage (Knox & Wicken, 1973).

Influence on autolytic activity

Lipoteichoic acids are proposed to play a role in the regulation of autolytic enzymes since they appeared to inhibit the autolysis of

several Gram-positive bacteria (Cleveland *et al.*, 1975, 1976a,b; Suginaka *et al.*, 1979b). Furthermore, Suginaka and coworkers (1979a) showed inhibition of penicillin-induced lysis of *Staphylococcus aureus* by cellular lipoteichoic acid. Lipoteichoic acids loose their anti-autolytic activity on deacylation or in the presence of detergent, suggesting that lipoteichoic acid interacts with the autolytic enzymes to form micelles (Fischer & Koch, 1983). The exact mechanism of inhibition of autolysis has not yet been established. Recently, Fischer and coworkers (1981) demonstrated the regulatory effect of D-alanine ester substitution. The inhibitory activity of lipoteichoic acid exponentially decreased with increasing alanine content. On basis of these results it can be concluded that the anti-autolytic activity of lipoteichoic acid is influenced both by deacylation and esterification with alanine.

Some other functions

Lipoteichoic acid was suggested to function as a lipoteichoic acid carrier in the polymerization of wall teichoic acid (Fiedler & Glaser, 1974a,b; Mauck & Glaser, 1972). However, studies from Fischer and coworkers (1980a,b) appear to provide convincing evidence against a role of lipoteichoic acid carrier in this process. The observations that the linkage unit, which links wall teichoic acid covalently to the peptidoglycan, is synthesized on a polyprenol lipid make it more likely that this lipid-linkage unit molecule serves as an acceptor for the synthesis of wall teichoic acid (for review see Fiedler, 1981).

As described for wall teichoic acids (Hughes *et al.*, 1973), lipoteichoic acids may also function as a reservoir of bound bivalent cations (especially Mg^{2+}) for enzymes present in the bacterial cell envelope (Lambert *et al.*, 1975, 1977). Lipoteichoic acid-induced regulation of bacterial enzymes, not depending on cations, were described for glucosyltransferases of *Streptococcus mutans* (Kuramitsu *et al.*, 1980) and streptolysin S of *Streptococcus pyogenes* (Theodore & Calandra, 1981). Lipoteichoic acid inhibited the glucosyltransferase activity in contrast to its deacylated form which had no effect. However, the deacylated molecules interact with the enzymes since they partially protected the enzyme activity against heat inactivation and induced the formation of high molecular weight enzyme complexes. There-

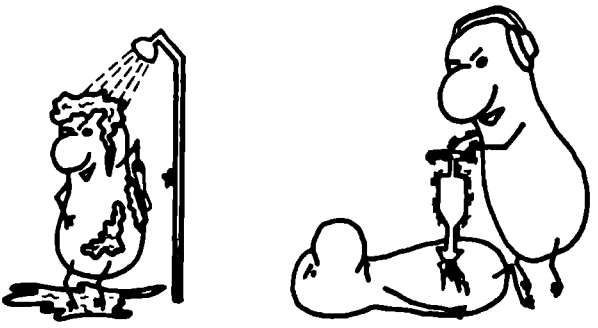
fore both acylated and deacylated lipoteichoic acids may play a role in aggregation and binding of glucosyltransferases to the bacterial surface (Kuramitsu *et al.*, 1980). In the case of streptolysin S from group A streptococci lipoteichoic acid may function as the physiological carrier of the enzyme to host tissues. The lipid part of the lipoteichoic acid molecule is neccesary for this activity (Theodore & Calandra, 1981).

1.6 Outline of this thesis

The aim of this study was to extend our knowledge about the composition and structure of the bacterial cell envelope of *Bifidobacterium bifidum* subsp. *pennsylvanicum*. Previously, studies from this laboratory were reported concerning composition and structure of the bifidobacterial cell membrane (Exterkate, 1970; Molenkamp, 1974; van Schaik, 1974) and cell wall (Hoelen, 1981; Veerkamp, 1971; Veerkamp *et al.*, 1983). The present investigations deal with the lipoteichoic acids of this bacterium. These components are part of both cell wall and cytoplasmic membrane. The isolation and purification of the lipoteichoic acids is described in chapter 3, together with the elucidation of their structure. The biosynthetic routes leading to the synthesis of these molecules are presented in chapter 4. The following chapters describe investigations of some biological properties of these bifidobacterial amphiphiles. Chapter 5 describes their binding affinity for mammalian cells. Some immunological properties are reported in chapter 6. Chapter 7 deals with the relationship between bacterial hydrophobicity and lipoteichoic acid. General conclusions and a summary will be given in chapter 8.

Chapter 2

Materials and Methods



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2.1 Growth of bacteria

Bifidobacterium bifidum subsp. *pennsylvanicum* was grown in a chemically-defined medium containing 2% (v/v) skimmed human milk (Norris *et al.*, 1950; Table 2.1). under a N_2/CO_2 (90%/10%) atmosphere for 16 h at 37°C. The medium was adjusted to pH 6.8 with NaOH. When $^{32}P_i$ was used for labeling (1.0 mCi/l) the K_2PO_4 concentration of the medium was lowered from 2.5 to 0.25 g/l. When [1-^{14}C]oleic acid was included in the medium (250 µCi/l) the Tween-80 concentration was lowered from 0.5 to 0.1 g/l. The medium modifications did not affect growth. When necessary, growth was monitored turbidimetrically at 550 nm (A_{550}). During growth the pH of the medium dropped from 6.8 to 5.0.

2.2 Isolation and purification of the lipoteichoic acids

Bacteria were harvested in the early stationary phase in a water-cooled Sharples continuous-flow centrifuge. The yield was about 6 g wet weight cells/l. Cells were washed twice with 0.1 M phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$ and suspended in the same buffer containing

Table 2.1 *Composition of Norris-medium*

Component	Amount per liter
Adenine sulphate	17.4 mg
Alanine	200.0 mg
p-Aminobenzoic acid	10.0 mg
Ascorbic acid	2.0 g
Asparagine	100.0 mg
Biotin	10.0 µg
Caseine hydrolysate (enzymatic)	5.0 g
L-Cystine	200.0 mg
Ferrous sulphate ($FeSO_4.7H_2O$)	10.0 mg
Folic acid	10.0 µg
Guanine hydrochloride	12.4 mg
Lactose	35.0 g
Magnesium sulphate ($MgSO_4.7H_2O$)	200.0 mg
Manganese sulphate ($MnSO_4.H_2O$)	6.7 mg
Nicotinic acid	0.6 mg
Pottasium phosphate (K_2HPO_4)	2.5 g
Pyridoxine hydrochloride	1.2 mg
Riboflavine	0.2 mg
Sodium acetate, anhydrous	25.0 g
Sodium chloride	10.0 mg
Sorbitan monooleate (Tween-80)	0.5 g
Thiamine hydrochloride	0.2 mg
Tryptophan	200.0 mg
Uracil	1.0 mg
Xanthine	10.0 mg
Human milk (defatted)	20.0 ml

0.7 M sucrose and 5 mM $MgSO_4$ (1 g wet weight cells/5 ml). Lysozyme (EC 3.2.1.17) was added (1 mg/g wet weight cells) and the bacteria were incubated at 37°C with shaking. Protoplast formation was checked by microscope (Leitz-Wetzlar Dialux phase contrast) and was found to be complete after 30-60 min. The protoplasts were sedimented by centri-

fugation at 8,000 x g for 20 min and sonicated in 0.1 M actetate buffer (pH 6.0) with a Branson Sonifier 70W (4 times for 5 s at maximal output with intervenient cooling). DNase (EC 3.1.4.5) was added (0.1 mg/g wet weight cells) and the mixture was incubated for 10 min at 37°C. Membranes were sedimented by ultracentrifugation (100,000 x g for 45 min), washed twice with 5 mM $MgSO_4$ and lyophilized. Cytoplasmic membranes were suspended in 0.1 M acetate buffer (pH 6.0) and an equal volume of 88% phenol (w/v) was added. The mixture was stirred at 4°C for 16 h and centrifuged (10,000 x g for 30 min). The aqueous layer was collected. The organic layer and the insoluble residue were washed with an equal volume of the acetate buffer and centrifuged as described before. The aqueous layers were combined, dialyzed against several changes of water and lyophilized. Crude phenol extract, obtained from 60 g bacteria (wet weight) was supended in 30 ml of 0.1 M acetate buffer (pH 6.0) containing 5 mM $MgSO_4$. DNase (5 mg), RNase (EC 2.7.7.16) (20 mg) and α-amylase (EC 3.2.1.1) (1 mg) were added and the mixture was incubated at 20°C for 24 h. Subsequently the mixture was extracted with two volumes of chloroform/methanol (1:1, v/v). The water layer was collected, dialyzed and after removal of insoluble material by centrifugation, lyophilized.

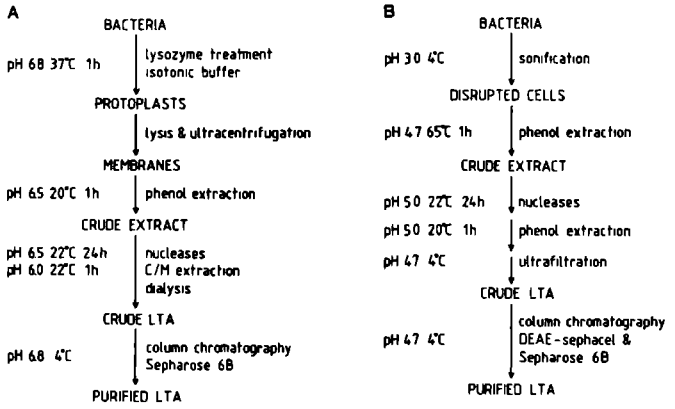


Fig. 2.1: Flow sheet of extraction and purification of lipoteichoic acid. A, our own procedure; B, procedure according to Fischer *et al.* (1983).

The crude lipoteichoic acid material was dissolved in 0.2 M ammonium acetate (pH 7.0) and loaded on a Sepharose 6B column (1.7 x 50 cm). The column was eluted with 0.2 M ammonium acetate (pH 7.0) containing 0.02% NaN_3. Fractions of 5.5 ml were collected. ^{32}P- and [^{14}C]oleic acid-labeled lipoteichoic acids were isolated using the same procedure.

For comparison lipoteichoic acid was isolated and purified according to the procedure recently described by Fischer *et al.* (1983) with the modification that we used sonication (15 times for 45 s with intervenient cooling) to disrupt the bacteria. Purification of the crude extract was achieved by ion-exchange chromatography on DEAE-Sephacel (1.7 x 45 cm) followed by Sepharose 6B chromatography. A flow sheet of both procedures is presented in Fig. 2.1.

2.3 Deacylation of lipoteichoic acid

Lipoteichoic acid was deacylated in 0.1 M NaOH in ethanol for 30 min at 37°C. After incubation the mixture was passed through a Dowex-50 H^+ column and dried under reduced pressure. The deacylated lipoteichoic acid was recovered by extraction of the residue with a mixture of 1 ml water and 2 ml chloroform/isopropanol (2:1, v/v). The water layer was concentrated and chromatographed on Sepharose 6B or Sephadex G75 (1.7 x 100 cm; fractions, 6 ml). The columns were eluted with 0.2 M ammonium acetate (pH 7.0).

2.4 General analytical procedures

Total hexose was determined by the method of Spiro *et al.* (1972); phosphate by the method of Bartlett (1959); protein according to Lowry *et al.* (1951) with bovine serum albumin as standard; glucose with a Glucostat test set (Boehringer); galactose with galactose dehydrogenase (EC 1.1.1.48) (Wallenfels & Kurtz, 1966); glycerol with an enzymatic assay (Boehringer); formaldehyde according to Nash (1953) with serine as standard and glycerol 3-phosphate with glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) as described before (Veerkamp & van Schaik, 1974). Labeled compounds were located after chromatography or electrophoresis by autoradiography using Kodak X-ray films (RP/P_2). Radioactivity was

determined by liquid scintillation counting in Opti-Fluor. Amino acid analysis was performed after hydrolysis of samples in 6 M HCl for 22 h at 100°C in vacuo with norleucine as internal standard.

2.5 Electrophoresis and thin-layer chromatography

Electrophoresis was carried out on Whatman 3 MM paper in acetic acid/pyridine/water (10:1:89, by vol., pH 3.5) during 2.5 h (40 V/cm, 4°C). For quantitative analysis paper was pretreated with 1% acetic acid before use. Spots were eluted with water. Thin-layer chromatography was performed on Silicagel 60 in solvent system; (A) chloroform/methanol/7 M ammonia (70:20:2, by vol.); (B) chloroform/methanol/acetic acid/water (125:37:9.5, by vol.); (C) propanol/pyridine/water (7:4:3, by vol.).

2.6 Materials

The specific reagents and materials used in this study are listed below together with the suppliers.

[1-^{14}C]oleic acid, [U-^{14}C]glucose, $K_2H^{32}PO_4$, UDP[^{14}C]glucose, UDP[^{14}C]-galactose, *sn*-[1-^{14}C]glycerol 3-phosphate: Amersham International, U.K.

Opti-Fluor scintillation fluid: Packard Instrument Company, Inc., Downers Grove, IL, U.S.A.

Sepharose 6B, DEAE-Sephacel, Sepharose G75 and Protein A-Sepharose CL 4B: Pharmacia Fine Chemicals, Uppsala, Sweden.

Biogel P2: Biorad Laboratories, Richmond, CA, U.S.A.

Deuterated water (99.96 atom %): Aldrich, Milwaukee, WI, U.S.A.

Lysozyme (EC 3.2.1.17), pepsin (EC 3.4.4.1), trypsin (EC 3.4.4.4), DNase (EC 3.1.4.5), RNase (EC 2.7.7.16), α-glucosidase (EC 3.2.1.20), β-glucosidase (EC 3.2.1.21), α-galactosidase (EC 3.2.1.22), β-galactosidase (EC 3.2.1.23), galactose dehydrogenase (EC 1.1.1.48), CTP, UDP-glucose and UDP-galactose: Boehringer, Mannheim, F.R.G.

Alkaline phosphatase (EC 3.1.3.1), phosphatidylglycerol, bovine serum albumin and methylated serum albumin, O-phenyl diamine: Sigma Chemical Company, St. Louis, MO, U.S.A.

CDP-dioleylglycerol: Serdary Research Laboratories, London, Ontario, Canada.

Silicagel 60 thin-layer plates: Merck, Darmstadt, F.R.G.

Freund's Incomplete Adjuvant and Agar-agar: Difco Laboratories, Detroit, U.S.A.

Swine anti-rabbit immunoglobulin conjugated peroxidase: Dako, Copenhagen, Denmark.

Anti-rabbit IgM and anti-rabbit IgG: Nordic, Tilburg, The Netherlands.

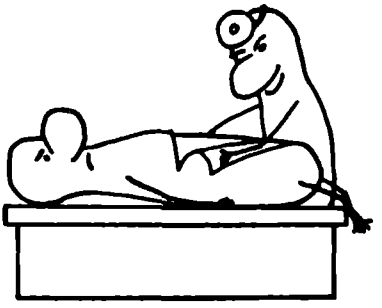
Crude extract from *Aspergillus niger* was a gift from Dr. E. Kennedy, Harvard Medical School, Boston, MA, U.S.A.

All other chemicals were of analytical grade.

Chapter 3

Structure of the lipoteichoic acids from Bifidobacterium bifidum subsp. pennsylvanicum

Adapted from Op den Camp *et al.* (1984)



3

3.1 Introduction

Lipoteichoic acids are membrane-associated amphiphilic molecules found in many Gram-positive bacteria (Knox & Wicken, 1973; Lambert *et al.*, 1977; Wicken & Knox, 1975a, 1980). They are structurally rather uniform and usually consist of a poly(1,3)glycerol phosphate backbone in some cases substituted by alanyl ester or glycosidic groups, covalently linked to a glyco- or phosphoglycolipid (Wicken & Knox, 1975) which is identical or closley related to one of the membrane lipids (Button & Hemmings, 1976b). A poly(glycosylglycerol phosphate) chain was observed as a unit of lipoteichoic acid in *Streptococcus lactis* Kiel 42172 (Koch & Fischer, 1978).

In previous studies of the cell envelope of *Bifidobacterium bifidum* subsp. *pennsylvanicum* we elucidated the structure of the galactosyldiacylglycerols, phospholipids and phosphogalactolipids present in the membrane (Veerkamp, 1972; Veerkamp & van Schaik, 1974) and of the peptidoglycan (Veerkamp, 1971), a glucosylated rhamnose polymer and a mannitol teichoic acid (Hoelen, 1981; Veerkamp *et al.*, 1983) present in the cell wall. The occurrence of glycerol, galactose and phosphate together with fatty acids in a fraction of the cell wall which was not

solubilized with lysozyme, suggested the presence of a lipoteichoic acid (Veerkamp *et al.*, 1983). The unusually high content of glycerophosphogalactosyldiacylglycerol in the membrane of *B. bifidum* (Veerkamp, 1976; Veerkamp & van Schaik, 1974) and the parallel in occurrence of glycerophosphoglycolipids and lipoteichoic acids in many Gram-positive bacteria (Fischer, 1981) initiated our studies on the lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum*. This chapter describes their isolation and structural analysis.

Indications were obtained for the presence of a diacyl derivative of glycerophosphogalactosyldiacylglycerol as the lipid anchor. As structural units of the polar chain we identified a poly(1,2)glycerol phospate backbone and two terminal polysaccharides. The latter consisted either of glucose or of galactose units. The presence of a 1,2-phosphodiester linked glycerol phosphate backbone together with terminal polysaccharide units discriminates the lipoteichoic acids of *B. bifidum* from all lipoteichoic acid structures described up to now.

3.2 Methods

Acid hydrolysis of lipoteichoic acid

Lipoteichoic acid (1-2 mg) was hydrolyzed in 0.5 ml 2 M HCl for 2.5 h at 100°C. For enzymatic analysis the hydrolysate was neutralized with NaOH. For gas liquid chromatography (GLC) HCl was evaporated in vacuo by several additions of water. The products were dephosphorylated with 0.5 unit alkaline phosphatase for 3 h at 37°C in 0.5 ml water, brought to pH 9.0 with diluted ammonia, and converted into alditol acetates as described before (Veerkamp *et al.*, 1983). For GLC deoxyglucose was used as internal standard.

^{32}P-labeled lipoteichoic acid (100,000 dpm) was hydrolyzed in the same way. The HCl was evaporated and ^{32}P-labeled compounds were analyzed by paper electrophoresis.

Alkaline hydrolysis of lipoteichoic acid

Unlabeled (5-25 mg) or ^{32}P-labeled lipoteichoic acid (100,000 dpm) was dissolved in 1 ml of 1 M NaOH and heated for 1 h at 100°C. The

hydrolysates were passed through a Dowex-50 H^+ column to remove Na^+. ^{32}P-labeled compounds were analyzed by paper electrophoresis. The hydrolysate derived from unlabeled lipoteichoic acid was applied to a Biogel P2 column (2.0 x 80 cm) and eluted with water. Fractions of 3.0 ml were collected. Mild alkaline treatment was performed in 1 M NaOH for 24 h at 37°C.

Treatment with HF

Unlabeled (5-25 mg) or ^{32}P-labeled lipoteichoic acid (100,000 dpm) was hydrolyzed in 48% (w/w) HF (1 ml) at 4°C for the periods indicated in section 3.3. After freezing, HF was removed in vacuo over NaOH pellets. Lipid and polar compounds were separated by extraction of the residue with chloroform/methanol/water (1:1:0.9, by vol.). The lipid fraction was analyzed by thin-layer chromatography in systems A and B. The extent of hydrolysis was determined from the ratio of inorganic phosphate to total phosphate in the aqueous layer. The sugar content of the aqueous layer was determined before and after HCl hydrolysis. The aqueous layer was also concentrated and separated on a Biogel P2 column by elution with water.

Periodate oxidation

Smith degradation of lipoteichoic acid was performed in 50 mM $NaIO_4$-0.1 M Na-acetate (pH 6.0) for 24 h at 22°C in the dark. The oxidation was terminated with ethylene glycol. After reduction with KBH_4, hydrolysis with HCl or NaOH and another reduction step, phospate-containing compounds were analyzed by paper electrophoresis at pH 3.5. In another experiment the oxidation products were reduced with KHB_4, hydrolyzed with 2 M HCl (2.5 h at 100°C), dephosphorylated, reduced, converted to alditol acetates and analyzed by GLC (Veerkamp *et al.*, 1983).

Mild periodate oxidation was performed in 5 mM $NaIO_4$-0.1 M Na-acetate (pH 6.0) for 15 min at 22°C. The products were analyzed after reduction, mild acid hydrolysis (0.2 M HCl, 1 h, 100°C) and again reduction as their alditol acetates (Veerkamp *et al.*, 1983)

Hydrazinolysis was carried out as described by Fischer *et al.* (1982).

Enzymatic hydrolysis procedures

Native lipoteichoic acid and water-soluble products derived from HF treatment were incubated with 5 units of glycosidases in appropriate buffers at 37°C. The following buffers were used: 0.1 M acetate buffer (pH 6.0) containing 2.7 mM EDTA for α-glucosidase (EC 3.2.1.20); 0.1 M acetate buffer (pH 5.0) for β-glucosidase (EC 3.2.1.21); 0.1 M phosphate buffer (pH 6.5) for α-galactosidase (EC 3.2.1.22) and 0.1 M phosphate buffer (pH 7.0) containing 2 mM $MgSO_4$ for β-galactosidase (EC 3.2.1.23). Liberated sugars and glycerol were determined enzymatically. The lipoteichoic acid and the product after periodate oxidation and hydrazinolysis were also treated with a phosphodiesterase-phosphomonoesterase preparation from *Aspergillus niger* (Schneider & Kennedy, 1978), according to the procedure of Fischer *et al.* (1980).

NMR study of deacylated and alkaline-hydrolyzed lipoteichoic acid

Lipoteichoic acid derived samples for NMR investigations were exchanged against D_2O by fivefold lyophilization, finally using 99.96% deuterated water. For ^{13}C-NMR measurements, 25 mg lipoteichoic acid derived material was used, while for ^{1}H-NMR spectroscopy 1 mg was ample. Acetone served as an internal standard. ^{13}C chemical shifts were measured relative to the methyl carbon singlet of acetone (δ 31.40, relative to external tetramethylsilane at 25°C), with an estimated precision of 0.05 ppm. ^{1}H chemical shifts were measured relative to the methyl proton singlet of acetone (δ 2.225, relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate at 27°C), with an accuracy of 0.002 ppm.

The ^{13}C-NMR spectra were recorded on a Bruker WM-200 WB spectrometer (SON hf-NMR facility, University of Nijmegen, The Netherlands), operating at 50.3 MHz under control of an Aspect 2000 computer. 10 mm sample tubes containing 1.4 ml D_2O were used (pD=7). The applied pulse width was 15 μs corresponding to a flip angle of about 75°. The spectral width was 10,000 Hz. The probe temperature was 25°C. To avoid line-broadening due to temperature fluctuations as much as possible, a minimal proton-decoupling power (0.5 W) was employed during aquisition. During the 1.5 s relaxation delay, a lower decoupling level (0.1 W) was applied in order to maintain the Nuclear Overhauser Enhancement. For

additional ^{31}P-decoupling a continuous broadband decoupling level of 0.4 W was applied. Spectra were recorded on 8 K datapoints; free inductions decays were multiplied by an exponential window and zero-filled to 16 K datapoints. To determine the ^{13}C-^{31}P coupling constants more accurately, ^{13}C-NMR spectra were also recorded on a Bruker HX-360 spectrometer (SON hf-NMR facility, University of Groningen, The Netherlands), operating at 90.5 MHz under control of an Aspect 2000 computer. Sample tubes: 5 mm; solvent 0.4 ml D_2O; pulse width: 30 µs (about 90°); spectral width: 7500 Hz; pD=7.

The ^{1}H-NMR spectra were recorded at 500 MHz with a Bruker WM-500 spectrometer (SON hf-NMR facility, University of Nijmegen), operating under control of an Aspect 2000 computer. The pulse width was about 12 µs corresponding to a flip angle of 90°. The spectral width was 3000 Hz. Probe temperature was 27°C. Spectra were recorded on 16 K datapoints. Resolution enhancement was achieved by Lorentzian-to-Gaussian transformation followed by zerofilling to 32 K datapoints.

Analytical assays

Methylation of native lipoteichoic acid and of preparations after treatment with NaOH or HF and subsequent analysis of methylated hexoses were performed according to Jansson *et al.* (1976) with some modifications (Veerkamp *et al.*, 1983).

For fatty acid analysis, lipoteichoic acid (1 mg) was methanolyzed in 0.5 ml hexane with 1 ml 14% BF_3 in methanol (w/v) for 10 min at 100°C. After cooling 2 ml water was added. The fatty acid methylesters were extracted twice with 4 ml pentane. Gas chromatographic analysis was performed on a Packard 428 gas chromatograph, equiped with a 15% diethyleneglycol succinate (on Gaschrom P, 60-80 mesh) column, at 167°C. Nonadecanoic acid was used as internal standard.

Chromium trioxide oxidation was performed according to Pazur *et al.* (1978).

3.3 Results

Isolation and purification

Lipoteichoic acids were isolated and purified by two different procedures (Fig. 2.1). In the usual procedure protoplast formation was

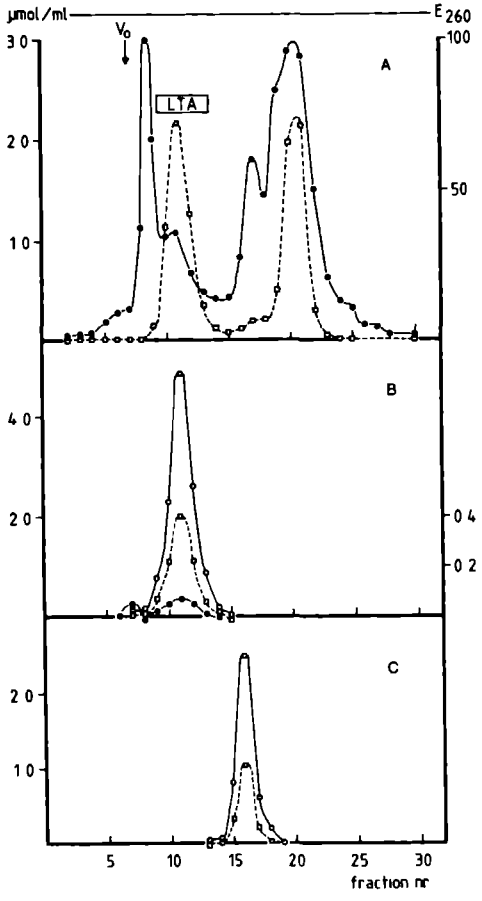


Fig. 3.1: Sepharose 6B chromatography of lipoteichoic acid and its deacylation product. Load; 100 µmol phosphate. Elution was performed with 0.2 M ammonium acetate (pH 7.0) containing 0.02% NaN_3. Fractions of 5.5 ml were collected and assayed for phosphate (□), hexose (○) and nucleic acid material (●, E_{260}).
(A) Crude phenol extract; (B) Rechromatography of lipoteichoic acid-containing fractions from A; (C) Lipoteichoic acid after mild alkaline treatment (deacylation).

included to avoid contamination with cell wall mannitol teichoic acid and α-amylase was used to split contaminating glycogen (Veerkamp *et al.*, 1983). Sepharose 6B column chromatography of the crude phenol extract is shown in Fig. 3.1A. The lipoteichoic acid eluted near the void volume. After rechromatography of the lipoteichoic acid-containing (Fig. 3.1B), the resulting purified lipoteichoic acid was characterized by a high hexose content and contained negligible amounts of nucleic acids (absorbance at 260 nm). The high hexose content is not due to a contamination of the preparation with free cell wall polysaccharides, since the carbohydrate and phosphate elution profiles from the gel permeation chromatography were coincident and symetrical. The yield was about 85 mg lipoteichoic acid/100 g bacteria (wet weight).

For comparison we also used the method recently described by Fischer and coworkers (1983). Phenol extraction was performed on disrupted cells without any pretreatment. The crude lipoteichoic acid preparation was applied on DEAE-Sephacel. The elution profile is shown in Fig. 3.2. The lipoteichoic acid (peak II) was well separated from

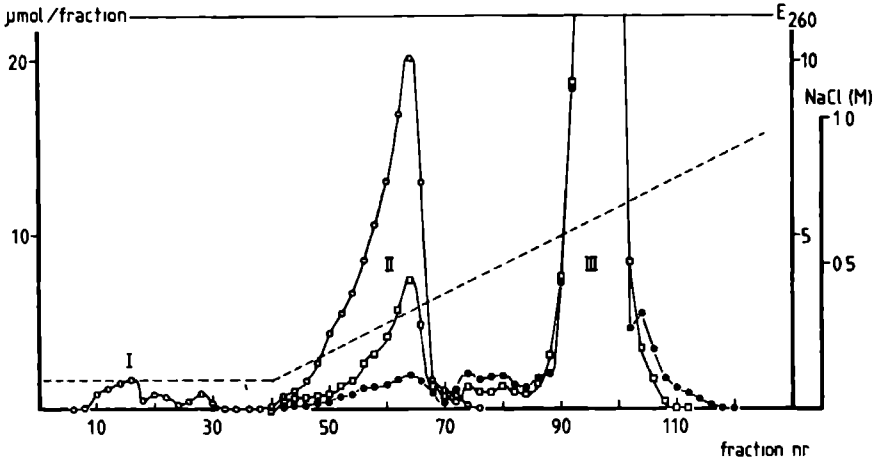


Fig. 3.2: Chromatography of crude phenol extract from desintegrated bacteria on DEAE-Sephacel. Load; 250 µmol phosphate. Elution was performed with 0.1 M Na-acetate (pH 4.7) containing 0.05% Triton X-100 followed by a gradient of 0 - 1 M NaCl in the same buffer (----). Fractions of 6.0 ml were collected and assayed for phosphate (□), hexose (○) and nucleic acid material (●, E_{260}).

contaminating polysaccharide (peak I), and nucleic acid (peak III). Although the lipoteichoic acid peak was rather broad, suggesting a heterogeneous composition, chromatography of this peak on Sepharose 6B gave an elution profile identical to the first procedure (Fig. 3.1B). The yield obtained by applying the Fischer procedure was about 220 mg/100 g bacteria (wet weight). For both procedures, free lipids, included in the micelles, could be removed by extraction of the pure lyophilized lipoteichoic acid preparations with chloroform/methanol (2:1, v/v).

We also tried to purify the lipoteichoic acid using Octyl-Sepharose (Fischer *et al.*, 1983). This purification step was not useful, since only a minor amount of the lipoteichoic acid bound to the column, probably due to the high hexose content.

The lipoteichoic acid did not absorb to phosphatidyl choline vesicles as was described for intra- and extracellular lipoteichoic acids from *Streptococcus mutans* (Silvestri *et al.*, 1978), so this technique could not be used for purification of the bifidobacterial lipoteichoic acid.

Deacylation of the lipoteichoic acid preparation

Mild alkaline hydrolysis resulted in deacylation of the lipoteichoic acid. The deacylated lipoteichoic acid (dLTA) eluted on Sepharose 6B near the inner volume (Fig. 3.1C) indicating a micellar composition of the native material. Elution of the deacylated molecule on Sephadex G-75 indicated a molecular weight of about 10,000. The deacylated product however eluted as a rather broad peak.

Chemical analysis

The chemical composition of the purified lipoteichoic acid preparations is given in Table 3.1. The two purification procedures gave comparable results. Glycerol, galactose and glucose were identified and quantified as their alditol acetates or as their trimethyl silyl ethers by gas chromatography. Analysis was also performed by enzymatic assay. The latter assays proved that galactose and glucose possess the D-configuration. The galactose/glucose ratio was 0.94 ± 0.28, indicating a rather great divergence between different preparations. Glycerol and

Table 3.1 *Chemical composition of the lipoteichoic acid and its deacylation product*

Component	LTA (μmol/mg)	Molar ratio to phosphate (LTA)	dLTA (μmol/mg)
Galactose	1.33 ± 0.19	1.29 ± 0.29	1.55 ± 0.21
Glucose	1.24 ± 0.38	1.20 ± 0.39	1.29 ± 0.39
Glycerol	1.03 ± 0.11	1.00 ± 0.06	1.28 ± 0.15
Phosphate	1.05 ± 0.14	1.00	1.25 ± 0.11
Fatty acids	0.31 ± 0.08	1.00 ± 0.05	-

Values are means ± S.D. of 7 and 5 separate preparations of lipoteichoic acid (LTA) and deacylated lipoteichoic acid (dLTA), respectively.

phosphate are present in equimolar amounts. No ribitol was detected. This corroborated the absence of nucleic acid material in the lipoteichoic acid preparation and excluded the presence of ribitol as structural unit of the lipoteichoic acid (Arakawa *et al.*, 1981).

The fatty acid composition is given in Table 3.2. The major fatty acids are palmitic and octadecenoic acid. The protein content of the purified lipoteichoic acid preparations, determined as Lowry-positive material or by amino acid analysis, varied from 1.3 - 4.8%. Amino acid analysis showed the presence of only a slight amount of the peptidoglycan amino acids (Veerkamp, 1971) with alanine in a proportional amount. This excludes the presence of alanyl ester substituents in the lipoteichoic acid. No other constituents of the cell wall (rhamnose, mannitol) were observed.

Structural analysis

On HCl hydrolysis of ^{32}P-labeled lipoteichoic acid about 68% of the phosphate was released as glycerol monophosphate, 7% as inorganic phosphate and 12% as hexose phosphate, as determined by paper electrophoresis. The hexose phosphate could be identified as galactose monophosphate. HCl hydrolysis of unlabeled lipoteichoic acid liberated

Table 3.2 *Fatty acid composition of the lipoteichoic acid*

Fatty acid	% of total fatty acids
12:0	1.9 ± 0.8
14:0	9.3 ± 1.0
15:anteiso	1.2 ± 0.9
16:0	31.9 ± 3.0
16:1	4.2 ± 0.4
18:0	9.6 ± 1.2
18:1	39.8 ± 2.6
19:0 cyclo	2.7 ± 0.6

Values (means ± S.D. of 5 preparations) are given as mol %

glycerol (23% of total amount), galactose (85% of total amount), and glucose (100% of total amount). Phosphatase treatment released additional glycerol and some galactose. Strong alkaline hydrolysis (1 M NaOH, 1 h, 100°C) of ^{32}P-labeled lipoteichoic acid resulted in the release of 95% of total phosphate as glycerol monophosphate. The remaining 5% appeared as a rather diffuse spot with M_{Pi} 0.20–0.40. On milder hydrolysis conditions these products accounted for 81 and 19%, respectively. Enzymatic analysis, after acid hydrolysis and dephosphorylation, of the rather immobile compound showed the presence of a small amount of glycerol together with almost all of the galactose and glucose. No glycerol diphosphate, diglycerol phosphate or phosphoryl-glyceryl-phosphoryl-glycerol phosphate were detected in the acid nor in the alkaline hydrolysate.

In another experiment we applied the strong alkaline hydrolysate from lipoteichoic acid on a Biogel P2 column (Fig. 3.3B). The peak eluting in the void volume contained galactose, glucose and glycerol in a ratio of 1.0 : 1.0 : 0.1. Almost the total amount of galactose and glucose present in the lipoteichoic acid preparation eluted in this peak. The second peak eluting near the inner volume of the column could be identified as glycerol monophosphate together with a small amount of galactosylglycerol. No free galactose, glucose or glycerol was detected.

We also studied the configuration of the glycerol monophosphate obtained from acid and alkaline hydrolysis. With stereospecific glycerol 3-phosphate dehydrogenase we determined the amount of glycerol 3-phosphate after alkaline hydrolysis (Veerkamp & van Schaik, 1974). Glycerol 2-phosphate was determined by use of periodate oxidation followed by reduction and dephosphorylation. The resulting glycerol must originate from glycerol 2-phosphate. The value for glycerol 1-phosphate can be derived from the former results. 39, 1 and 60% of the glycerol phosphate

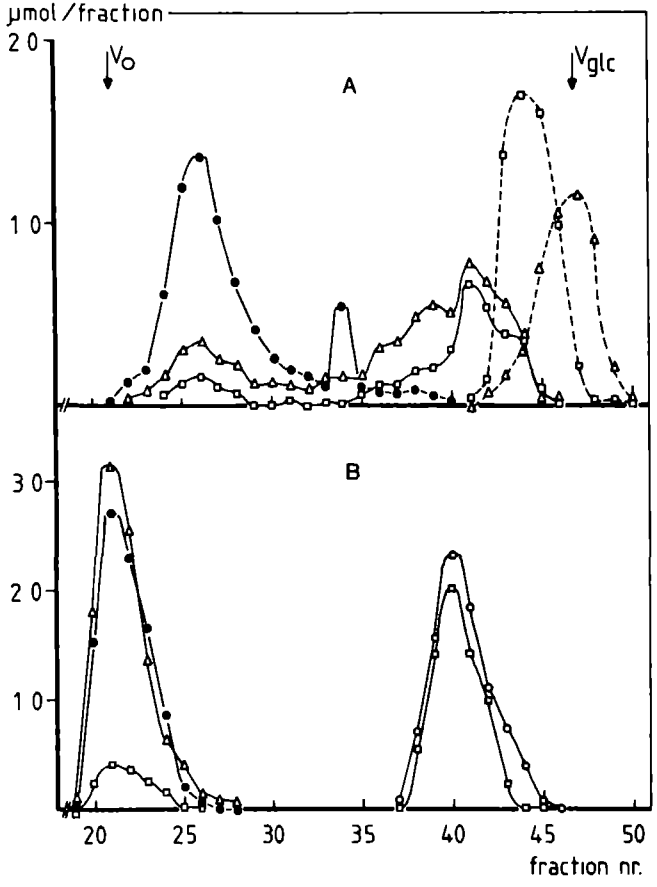


Fig. 3.3: Chromatography of HF (A) and alkaline (B) hydrolysates from lipoteichoic acid on Biogel P2. Elution was performed with water. Fractions of 3.0 ml were collected and assayed for phosphate (○), galactose (△), glucose (●) and glycerol (□). Dotted lines indicate free sugars, solid lines bound components.

released by alkaline hydrolysis appeared to be present as glycerol 1-phosphate, glycerol 3-phosphate and glycerol 2-phosphate, respectively. After acid hydrolysis 63 and 37% of glycerol phosphate was present as α- and β-isomer, respectively. Since no glycerol diphosphate, glycerol diphosphate or phosphoryl-glyceryl-phosphoryl-glycerol phosphate were observed after acid or alkaline hydrolysis, but only glycerol monophosphate, a 1,3-phosphodiester linked glycerol phosphate chain is unlikely (Archibald & Baddiley, 1966). Alkaline treatment does not cause phosphate migration (Baer & Kates, 1950; Fischer & Landgraf, 1974). Therefore, the released α-isomer retains the stereochemical configuration that it had in the parent compound. The presence of glycerol 1-phosphate in the alkaline hydrolysate together with the ^{13}C-NMR data (see below) gave evidence for poly(1,2)glycerol phosphate being a structural unit of the lipoteichoic acid molecule. The high alkaline lability of the glycerol phosphate chain further indicates that C-3 of glycerol is not substituted with glycosidic groups.

Treatment with HF

Table 3.3 shows the influence of exposure time on liberation of water-soluble lipoteichoic acids components during HF hydrolysis.

Table 3.3 *Liberation of water-soluble lipoteichoic acid components during treatment with HF*

Component	Hydrolysis time		
	48 h	96 h	240 h
Galactose	32 ± 6	38 ± 5	73 ± 4
Glucose	2 ± 1	7 ± 5	n.d.
Glycerol	51 ± 5	64 ± 9	75 ± 3
Phosphate	63 ± 9	70 ± 8	79 ± 7

Values are means ± S.D. of 3 - 7 separate experiments and expressed in percents of the total amount present. n.d., not determined.

Glucose is liberated only to a slight extent by HF treatment. Considerable amounts of galactose and glycerol were liberated. The release of glycerol points to its presence as phosphate esters in the lipoteichoic acid. For galactose, another explanation has to be found for cleavage by HF, since its occurrence as only galactose phosphate is not consistent with the results of alkaline hydrolysis. Free galactose increases after longer hydrolysis periods with HF, resulting in the liberation of 73% of total galactose after 240 h. Obviously, HF splits not only phosphomono- and diesters in this case, but also glycosidically bound galactose. This suggests the presence of galactofuranosyl groups since for this ring size, the glycosidic linkage is very acid labile. With the deacylated lipoteichoic acid the same results were obtained.

The elution profile of a HF-hydrolysate (96 h) loaded on Biogel P2 (Fig. 3.3A) is completely different from that of an alkaline hydrolysate (Fig. 3.3B). In the former the galactose is spread over the whole column volume indicating the partial degradation of a galactan into several fragments. The glucose remains bound in a large compound, since it eluted near the void volume.

Enzymatic hydrolysis

Incubation of the lipoteichoic acid preparations or the products from alkaline hydrolysis with glycosidases did not liberate glucose or galactose. This supports the presence of these two sugars in a rather large polysaccharide and not as mono- or oligosaccharide substituents on the glycerol phosphate backbone. Treatment of the water-soluble products after HF hydrolysis (96 h) with galactosidases resulted in the additional liberation of about 15% of total galactose together with some glycerol (5%) after 24 h. α-glucosidase had no effect. β-glucosidase liberated 10 and 20% of glucose after 24 h and 112 h, respectively.

Incubation of the lipoteichoic acid with alkaline phosphatase resulted in the liberation of 0.5% of total phosphate as free phosphate, indicating the absence of phosphate monoesters. Treatment of the lipoteichoic acid with the phosphodiesterase-phosphomonoesterase preparation of *Aspergillus niger* (Schneider & Kennedy, 1978) resulted in the liberation of only 12% of total glycerol after 24 h. Degradation of the polysaccharide part of the lipoteichoic acid by periodate oxidation and hydrazinolysis however, allowed the liberation of 71% of total glycerol

within 24 h. The phosphodiesterase removes the terminal glycerol from a poly(glycerol phosphate) chain leaving a phosphomonoester which in turn is hydrolyzed by the phosphomonoesterase (Fischer *et al.*, 1980; Scheider & Kennedy, 1978). From the former results we may conclude that most, if not all, terminal glycerols of the poly(glycerol phosphate) chains in the bifidobacterial lipoteichoic acids are linked to polysaccharides containing galactose and/or glucose. Furthermore, after degradation of the lipoteichoic acid by periodate oxidation, hydrazinolysis and phosphodiesterase-phosphodiesterase treatment, we could detect a small amount of galactosylglycerol, originating from the lipid anchor, by thin-layer chromatography on Silicagel 60 in system C.

Methylation analysis

In order to define the types of glycosidic bonds in which galactose and glucose are involved in the lipoteichoic acid molecule we carried out methylation analysis on native lipoteichoic acid, its deacylation product as well as on alkaline and HF hydrolysates. The results are compiled in Table 3.4. The native lipoteichoic acid shows the presence of two major methylated alditol acetates, namely 2,3,4-trimethyl-glucitol, indicating the presence of C-6 substituted glucopyranosyl residues, and 2,3,4-trimethylgalactitol, derived from C-5 substituted galactofuranosyl groups. Besides these two main constituents we could also detect 2,3,4,6-tetramethylglucitol originating from terminal gluco-pyranosyl groups; 2,3,5,6-tetramethylgalactitol from terminal galacto-furanosyl groups and 2,3,5-trimethylgalactitol from C-6 substituted galactofuranosyl residues. Methylation analysis of the deacylation product and the alkaline hydrolysate shows the same products as described above, in identical ratios. This confirms that under the alkaline hydrolysis conditions used glycosidic bonds are not split. Analysis of the HF-hydrolysate shows the absence of C-5 substituted galactofuranosyl groups and an increase of the amount of unsubstituted, terminal galactofuranosyl groups. In addition we detected a new peak namely 2,3,4,6-tetramethylgalactitol originating from unsubstituted galactopyranosyl residues which were formed after splitting of some furanosyl linkages by HF.

Table 3.4 *Permethylation analysis of native and deacylated lipoteichoic acid and of products obtained after treatment with NaOH or HF*

Methylated alditol acetate	LTA	dLTA	LTA/NaOH	LTA/HF
2,3,4-Trimethylglucitol	1.00	1.00	1.00	1.00
2,3,4,6-Tetramethylglucitol	+*	+	+	0.28 ± 0.14
2,3,6-Trimethylgalactitol	0.80 ± 0.07	0.75 ± 0.10	0.83 ± 0.10	-
2,3,5-Trimethylgalactitol	+	+	+	0.20 ± 0.05
2,3,4,6-Tetramethylgalactitol	-**	-	-	0.70 ± 0.30
2,3,5,6-Tetramethylgalactitol	+	+	+	0.70 ± 0.25

Values (means ± S.D. of 3 separate experiments) are peak area ratios relative to 2,3,4-trimethylglucitol. LTA, lipoteichoic acid; dLTA, deacylated lipoteichoic acid. * < 0.1; ** not detectable (< 0.01).

Treatment of the lipoteichoic acid with 5 mM $NaIO_4$ followed by mild acid hydrolysis (0.1 M HCl, 90 min, 100°C) resulted in the formation of a small amount of arabinose. This indicates the presence of terminal non-reducing galactofuranosyl groups. Treatment of the water-soluble products after HF hydrolysis (96 h) in the same way resulted in a three times larger amount arabinose. This is consistent with the increase of terminal galactofuranosyl residues after HF hydrolysis shown with permethylation.

Smith degradation of native lipoteichoic acid resulted in the consumption of 3.2 µmol periodate and the production of 0.88 µmol formaldehyde per mg lipoteichoic acid. A part of the oxidized lipoteichoic acid was analyzed after HCl hydrolysis, dephosphorylation and preparation of alditol acetates. All glucose and nearly all galactose appeared to be oxidized. Gas chromatography revealed the presence of glycerol (2.10 µmol/mg), erythritol (0.27 µmol/mg) and threitol (0.22 µmol/mg). The high amount of glycerol is derived from the glycerol phosphate backbone and from the C-6 substituted glucopyranosyl groups. The threitol originated from C-5 substituted galactofuranosyl groups and erythritol may be derived from incomplete periodate oxidation of liberated galactofuranose and C-6 substituted glucopyranosyl residues. The high amount of formaldehyde formed also indicated the degradation of the galactan chain.

Chromium trioxide oxidation of lipoteichoic acid preparations resulted in a total oxidation of galactose and glucose. This suggests that glucose molecules are present in β-glycosidic linkages, since α-hexapyranoside bonds are not affected by chromium trioxide (Pazur *et al.*, 1978). About the galactose anomeric form it gives no indication since both galactofuranoside anomers are degraded under the conditions used (Oshima & Ariga, 1976).

^{13}C- and ^{1}H-NMR spectroscopic analysis

The ^{13}C-NMR spectrum of the pure, native lipoteichoic acid was rather complex, owing to the presence of O-acyl groups. The occurrence of the native molecules in micelles gave rise to broad, unresolved signals. Alteration of the amphiphilic character by deacylation resulted

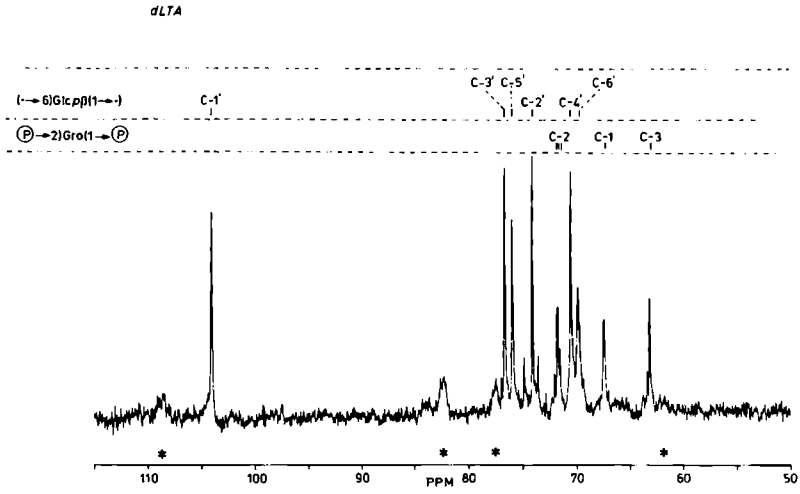


Fig. 3.4: $^{13}C\{^{1}H\}$-NMR spectrum of deacylated lipoteichoic acid (dLTA) in D_2O, recorded at pD 7, 25°C and 90.5 MHz. The minor-intensity, relatively broad signals in the spectrum of dLTA, marked by asterisks, stem from the (•→5)Gal f-β(1→•) carbon atoms (compare Fig. 3.5). For discussion of linebroadening, see text.

in a simplified ^{13}C- and ^{1}H-spectrum; apparently this was due to reduction of aggregation (Batley *et al.*, 1981). Relevant chemical shifts are compiled in Table 3.5.

The ^{13}C-NMR spectrum of deacylated lipoteichoic acid (Fig. 3.4) was dominated by six well-defined singlets stemming from C-1' to C-6' of β-glucopyranosyl residues that bear a substituent monosaccharide at C-6' (Table 3.5). The latter can be concluded from the chemical shift displacements of C-6' (+ 8.2 ppm) and C-5' (- 0.7 ppm) in deacylated lipoteichoic acid as compared to β-methyl-glucopyranoside. The β-anomeric configuration is supported by the H-1 signal in the ^{1}H-NMR spectrum at δ 4.528 ($J_{1,2}$ 7.8 Hz). Three additional signals of lower intensity are observable in the ^{13}C-NMR spectrum which were attributed to C-1, C-2 and C-3 of glycerol forming part of a 1,2-phosphodiester linked poly(glycerol phosphate) chain. Assignments were based on comparison with data for glycerol, glycerol 2-phosphate and glycerol 3-phosphate (Table 3.5). The possibility of a 1,3-linked poly(glycerol phosphate) could be excluded

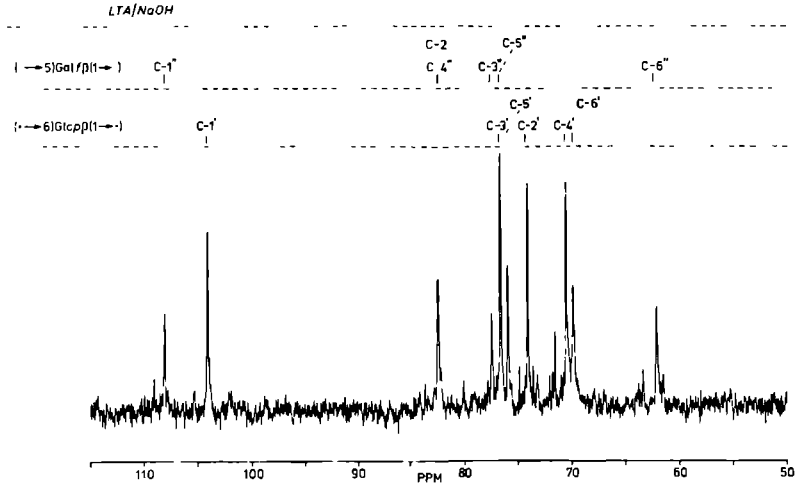


Fig. 3.5: $^{13}C\{^{1}H\}$-NMR spectrum of the Biogel P2 void volume peak of alkaline hydrolyzed lipoteichoic acid (LTA/NaOH) in D_2O, recorded at pD 7, 25°C and 90.5 MHz.

because the C-1 and C-3 signals for deacylated lipoteichoic acid were found to be clearly different in chemical shift (compare poly(1,3) and substituted poly(2,3) compounds, Table 3.5). In principle, ^{13}C-NMR can not distinguish between 1,2- and 2,3-linked poly(glycerol phosphate). However, from the stereochemical analysis of glycerol monophosphate liberated by alkaline hydrolysis (see above) we could rule out the occurrence of 2,3-linked poly(glycerol phosphate). The assignments of the glycerol carbon signals were proved by a ^{31}P-decoupling experiment resulting in complete removal of the ^{2}J and $^{3}J(^{31}P-^{13}C)$ couplings on C-1, C-2 and C-3.

Signals from galactosyl carbons could hardly be observed in the ^{13}C-NMR spectrum of deacylated lipoteichoic acid, although these residues are present (Table 3.1). Apparently, these signals are broadened, most probably due to restricted molecular motion. ^{31}P-decoupling did not affect the line width of these signals. However, the ^{13}C-NMR spectrum (Fig. 3.5) of the Biogel P2 void volume peak of alkaline hydrolyzed lipoteichoic acid (Fig. 3.3B) permitted, besides the confirmation of the presence of (•→6)Glc p-β(1→•) units, to define the

Table 3.5 ^{13}C *Chemical shifts of deacylated lipoteichoic acid, its alkaline hydrolysate and relevant reference substances*

Compound	Chemical shifts of								
	C-1'	C-2'	C-3'	C-4'	C-5'	C-6'	C-1	C-2	C-3
Glycerol	-	-	-	-	-	-	63.8	73.3	63.8
Glycerol 2-phosphate	-	-	-	-	-	-	62.5(3.8)	75.1(5.0)	62.5(3.8)
Glycerol 3-phosphate	-	-	-	-	-	-	62.8	71.6(6.4)	65.2(4.8)
Poly(1,3)glycerol phosphate	-	-	-	-	-	-	66.3(5.6)	69.8(8.1)	66.3(5.6)
Teichoic acid	103.3	73.6	76.1	70.1	76.3	61.2	69.5(4.0)	75.3*	66.1*
β-Methyl glucopyranoside	104.0	74.1	76.8	70.6	76.8	61.8	-	-	-
β-Methyl galactofuranoside	109.2	81.9	77.8	84.0	72.0	63.9	-	-	-
dLTA (glc)	104.2	74.2	76.8	70.7	76.1	70.0	67.7*	71.9(6.5)	63.3*
LTA/NaOH (glc)	104.1	74.2	76.7	70.6	76.0	69.9	-	-	-
(gal)	108.1	82.4	77.5	82.4	76.7	62.2	-	-	-

Chemical shifts are expressed in ppm downfield from external tetramethylsilane (measured relative to internal acetone, at δ 31.40, in D_2O at 25°C). ^{31}P-^{13}C coupling constants (in parentheses) are in Hz. Teichoic acid consists of a 2,3-phosphodiester-linked glycerol phosphate chain, substituted with glucose at C-1 of glycerol. Chemical shifts of reference compounds were derived from De Boer *et al.* (1976, 1978); Bock & Peddersen (1983) and Gorin & Mazurek (1975). LTA, lipoteichoic acid; dLTA, deacylated lipoteichoic acid. * poorly resolved; coupling constants could not be detected.

galactosyl ring form and linkage configuration. A second set of six singlets was observed (Table 3.5) and ascribed to (•→5)Gal f-β(1→•) units. The chemical shift of the C-1' of galactose (δ 108.1) is very typical of β-galactofuranosyl residues (Matsunaga *et al.*, 1981). This is substantiated by the occurrence of a singlet for H-1 of this galactosyl residue in the ^{1}H-NMR spectrum at δ 5.186. The galactofuranosyl residues are substituted at C-5' by another sugar as is evident from the shift increment of C-5' (+ 4.7 ppm) and decrements of C-4' (- 1.6 ppm) and C-6' (- 1.7 ppm), compared to β-methylgalactofuranoside (Table 3.5) (compare Matsunaga *et al.*, 1981). The amount of glycerol present in the void volume peak was too low to obtain further information on its linkage by NMR.

The lipid part of the lipoteichoic acid

HF is usually applied to liberate the lipid anchor from the lipoteichoic acids (Koch & Fischer, 1978; Nakano & Fischer, 1978). Therefore we extracted a HF hydrolysate (96 h) from [^{14}C]oleic acid labeled-lipoteichoic acid with chloroform/methanol/water (1:1:0.9, by vol.). The chloroform-soluble compounds (90% of radioactivity) could be identified by thin-layer chromatography on Silicagel 60 in systems A and B, as monoacylglycerol, diacylglycerol and free fatty acid accounting for 13, 25 and 47% of total radioactivity applied, respectively. These results indicated that HF degraded the lipid anchor. Other hydrolysis conditions using chloroform/methanol/3.8 M HCl (1:1:0.3, by vol.), 0.2 M HCl (Ganfield & Pieringer, 1975), moist acetic acid (Nakano & Fischer, 1978) or ammonia (Ofek *et al.*, 1975) yielded the same products as described above. The results may suggest that diacylglycerol is the lipid anchor as was found in a mutant of *Bacillus licheniformis* (Button & Hemmings, 1976b), but it appears more probable that we did not succeed in isolating the intact lipid part of the lipoteichoic acid. This may be caused by acid degradation since the phosphogalactolipids, the presumable lipid portion, showed the same products at HF treatment (Veerkamp & van Schaik, 1974). Indirect evidence for the occurrence of a phosphogalactolipid as membrane anchor came also from some other experiments. The acid hydrolysate from lipoteichoic acid contained some galactose phosphate as stated by paper electrophoresis and phosphatase treatment. These results are comparable with those from acid hydrolysis

of glycerophosphorylgalactosyldiacylglycerol (Veerkamp & van Schaik, 1974). Analysis of the products after alkaline hydrolysis and after degradation of the lipoteichoic acid by periodate oxidation, hydrazinolysis and phosphodiesterase-phosphomonoesterase treatment revealed the presence of a small amount of galactosylglycerol identified by thin-layer chromatography on Silicagel 60 in system C. Permethylation analysis of native lipoteichoic acid revealed some 2,3,5-trimethylgalactitol originating from C-6 substituted galactofuranosyl groups. This compound was also found after permethylation of the phosphogalactolipids from *B.bifidum* subsp. *pennsylvanicum*. Therefore, glycerophosphoryl-galactosyldiacylglycerol or one of its acyl derivatives (Veerkamp & van Schaik, 1974) could form the lipid portion. The high contant of fatty acids and the recovery of galactosylglycerol after periodate oxidation as described above indicate that a diacyl derivative is the most probable lipid anchor of the lipoteichoic acid. Previously, such a component (lipid 6) was isolated from *B.bifidum* (Veerkamp & van Schaik, 1974).

3.4 Discussion

The lipoteichoic acids from *Bifidobacterium bifidum* subsp. *pennsylvanicum* were isolated from membranes or from desintegrated bacteria by extraction with phenol. Further purification was achieved by column chromatography on Sepharose 6B and DEAE-Sephacel (Figs. 3.1 and 3.2). The procedure described by Fischer *et al.* (1983) gives a 2.5 times higher yield than our procedure. In the latter procedure a part of the lipoteichoic acid may be lost during protoplast formation by lysozyme treatment (Coley *et al.*, 1975; Joseph & Shockman, 1975a). The purified lipoteichoic acid preparations contained a high amount of hexoses which were identified as galactose and glucose. No contamination with cell wall polysaccharide or teichoic acid (Hoelen, 1981; Veerkamp *et al.*, 1983) was detected.

The structure of the lipoteichoic acids from *B.bifidum* is summarized in Fig. 3.6. Four structural units were identified. NMR-analysis, together with the results from the chemical and enzymatic degradation experiments revealed the presence of a poly(1,2)glycerol phosphate chain, without substitution on C-3 of glycerol, as backbone of

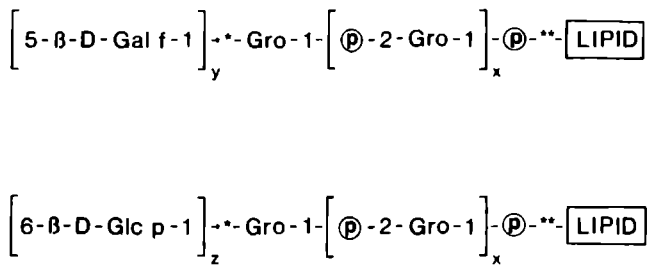


Fig. 3.6: Structure of the lipoteichoic acids from *B. bifidum* subsp. *pennsylvanicum*. Lipid = 3-O-(6'-(*sn*-glycero-1-phosphoryl)-diacyl-ß-D-galactofuranosyl)-*sn*-1,2-diacylglycerol.
Ⓟ indicates phosphate. *linkage either to C-2 or to C-3 of glycerol. **linkage to C-2 of glycerol phosphate of the lipid anchor.

the lipoteichoic acid molecules. Additional evidence for this structural unit came from preliminary biosynthesis experiments (see also chapter 4). Phosphatidylglycerol appeared to be the precursor for synthesis of the poly(glycerol phosphate) chains. Since phosphatidylglycerol functions as donor for *sn*-glycerol 1-phosphate units the backbone must consist of 1,2-phosphodiester linked units. HF hydrolysis, ussually applied to study the glycosidic substitution (Hether & Jackson, 1983; Wicken & Knox, 1975b), appeared to be not adequate for structural analysis because HF also split the galactofuranosyl linkages. By alkaline hydrolysis, permethylation analysis and NMR we identified two polysaccharide parts of the lipoteichoic acids, namely poly[(1→5)-ß-D-galactofuranose] and poly[(1→6)-ß-D-glucopyranose]. The structure of a polysaccharide containing both sugars alternately is not considered because HF split the galactofuranosyl linkages, but did not liberate glucose (Table 3.3, Fig. 3.3A). The polysaccharide part of the lipoteichoic acid will be linked to the terminal glycerol of the poly(glycerol phosphate) chain, since only a slight amount of glycerol was liberated during treatment with phosphodiesterase-phosphomonoesterase and the amount of glycerol strongly increased after degradation of the polysaccharide part by periodate oxidation and hydrazinolysis. This enzyme system needs a terminal glycerol for initiation (Fischer *et al.*, 1980; Schneider & Kennedy, 1978).

Due to the presence of terminal polysaccharides, the chain length

of the glycerol phosphate backbone could not be determined from periodate oxidation experiments as described by Hether & Jackson (1983). Assuming 4 fatty acids per molecule lipoteichoic acid we calculated an average chain length of 13 glycerol phosphate units linked to 32-35 hexose (galactose or glucose) molecules. There appeared to be some heterogeneity in the lipoteichoic acid preparations on account of chain length, as can be seen from DEAE-Sephacel elution of native lipoteichoic acid (Fig. 3.2), from the behaviour of deacylated lipoteichoic acid on Sephadex G-75 and from the large variation of the hexose to phosphate ratio.

The substitution of the terminal glycerol at C-2 or C-3 with one polysaccharide chain appears to be well in agreement with the apparent molecular weights observed at gelfiltration of the deacylation product and the degradation products after alkaline hydrolysis, although the presence of two saccharide chains of about 16 hexoses at C-2 and C-3 of the terminal glycerol could not be excluded completely. The polysaccharide chain will consist either of galactose or of glucose, since methylation analysis of native lipoteichoic acid revealed the presence of terminal galactofuranosyl- and glucopyranosyl groups, no heterooligosaccharide was found and a combination of two different chains of about 16 sugars does not appear to be probable on basis of biosynthetic consideration. The variation of the galactose/glucose ratio in the lipoteichoic acid preparations suggested differences in the proportion of the two lipoteichoic acids. We did not succeed in separating these different molecules. Within one purification procedure all column fractions tested showed the same galactose to glucose ratio.

The lipid moiety of lipoteichoic acids is usually a glycolipid or phosphoglycolipid normally present in the membrane (Button & Hemmings, 1976b; Wicken & Knox, 1975b). Recently, Fischer (1981) showed the relationship between the glycerophosphoglycolipids present in the membrane and lipid part of the lipoteichoic acid molecule within 33 different Gram-positive bacteria. A diacyl derivative of glycerophosphorylgalactosyldiacylglycerol, previously (Veerkamp & van Schaik, 1974) described as lipid 6 from *B. bifidum*, appeared to be most probably the lipid anchor of the lipoteichoic acids, on account of the high fatty acid content of the lipoteichoic acid preparations. The biosynthesis of this lipid by transfer of the *sn*-glycerol 1-phosphate of phosphatidylglycerol to diacylmonogalactosyldiacylglycerol (Veerkamp, 1976; Veerkamp

& van Schaik, 1974) indicates that the additional acyl groups are linked to the galactose molecule and that the free glycerol unit can be used for polymerization of glycerol phosphate units during lipoteichoic acid biosynthesis.

Thus far two types of glycerol lipoteichoic acids, in which repeating units are linked through phosphodiesters have been described. The first and main type consists of a linear 1,3-phosphodiester linked glycerol phosphate chain variously substituted with D-alanyl ester or glycosidic groups (see chapter 1). A second type shows the presence of one or more glycosides in the backbone of the molecule. The lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum* appear to be unique since a poly(1,2)glycerol phosphate backbone and terminal substitution of this backbone with polysaccharides have not been described before.

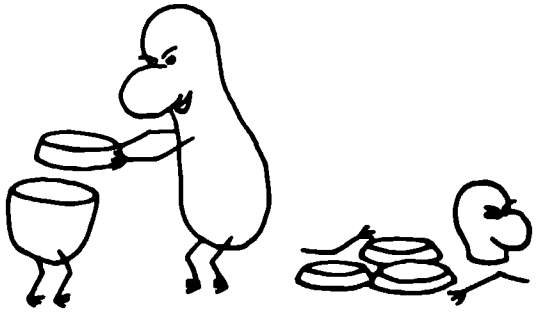
3.5 Summary

The lipoteichoic acids from *Bifidobacterium bifidum* subsp. *pennsylvanicum* were extracted from cytoplasmic membranes or from desintegrated bacteria with aqueous phenol and purified by gel chromatography. The lipoteichoic acid preparations contained phosphate, glycerol, galactose, glucose and fatty acids in a molar ratio of 1.0 : 1.0 : 1.3 : 1.2 : 0.3, respectively. Chemical analysis and NMR studies of the native preparations and of products from various acid and alkaline hydrolysis procedures gave evidence for the structure of two lipoteichoic acids. The lipid anchor appeared to be 3-O-(6'-(*sn*-glycero-1-phosphoryl)-diacyl-β-D-galactofuranosyl)-*sn*-1,2-diacylglycerol. The polar part showed two structural features not earlier described for lipoteichoic acids. A 1,2-(instead of the usual 1,3-)phosphodiester linked *sn*-glycerol phosphate chain is only substituted at the terminal glycerol unit with a linear polysaccharide, containing either β(1→5)-linked D-galactofuranosyl- or β(1→6)-linked D-glucopyranosyl groups.

Chapter 4

Phosphatidylglycerol as biosynthetic precursor for the poly(glycerol phosphate) backbone of bifidobacterial lipoteichoic acid

Adapted from Op den Camp *et al.* (1985c)



4

4.1 Introduction

The lipoteichoic acid molecules of *Bifidobacterium bifidum* subsp. *pennsylvanicum* consist of linear 1,2-phosphodiester linked glycerol phosphate backbones substituted at the terminal glycerol with a polysaccharide containing either galactose or glucose (chapter 3). The other end of the glycerol phosphate polymer is covalently linked to 3-O-(6'-(*sn*-glycero-1-phosphoryl)-diacyl-β-D-galactofuranosyl)-*sn*-1,2-diacylglycerol (chapter 3). Together with the experiments concerning some biological properties of this rather unique amphiphile (chapters 5, 6 and 7), we initiated to study its biosynthesis.

Fischer (1981) reviewed metabolic and structural evidence that glycerophosphoglycolipids of many Gram-positive bacteria are related to the lipoteichoic acid. Previously (Van Schaik & Veerkamp, 1976; Veerkamp 1976), the synthesis of lipid 6, the lipid anchor of the bifidobacterial lipoteichoic acid, by transfer of the *sn*-glycerol 1-phosphate from phosphatidylglycerol to the related galactolipid was reported. Studies concerning the biosynthesis of lipoteichoic acids (Emdur & Chiu, 1974, 1975; Ganfield & Pieringer, 1980; Glaser & Lindsay, 1974; Koga *et*

al., 1984) have given evidence that the glycerol phosphate moieties of these molecules also originate from phosphatidylglycerol. The unacylated *sn*-glycerol 1-phosphate of the lipid anchor represents the first unit of the lipoteichoic acid backbone (Fischer, 1981; Ganfield & Pieringer, 1980).

Since pulse labeling experiments *in vivo* with [^{14}C]glycerol were not possible with *B.bifidum* due to the absence of glycerol kinase, we studied lipoteichoic acid synthesis *in vitro* using a particulate enzyme preparation of this organism. Studies revealed that the *sn*-glycerol 1-phosphate unit from phosphatidylglycerol is incorporated in the bifidobacterial lipoteichoic acid. The role of the lipid anchor and of UDP-glucose and UDP-galactose in this process has also been investigated.

4.2 Methods

Growth of bacteria and preparation of bacterial fractions

Bifidobacterium bifidum subsp. *pennsylvanicum* was cultivated in 800 ml batches for 16 h under the conditions described in section 2.1. Lipoteichoic acid was isolated from desintegrated bacteria and purified by column-chromatography (section 2.2). A particulate membrane fraction was prepared by lysis of the bacteria with lysozyme, treatment with DNase and ultra-centrifugation (Veerkamp, 1974). The protein content of the particulate fraction was 54 ± 2% (mean ± S.D. of four preparations) as determined by the method of Lowry *et al.* (1951).

Preparation of labeled precursors

Phosphatidyl[^{14}C]glycerol (specific activity 0.05 µCi/µmol) was synthesized from *sn*-[1-^{14}C]glycerol 3-phosphate and CDP-dioleylglycerol and purified as described by Veerkamp (1976). CDP-[^{14}C]glycerol was prepared from *sn*-[1-^{14}C]glycerol 3-phosphate and CTP (Veerkamp, 1976). [^{14}C]oleic acid-labeled lipids were obtained by cultivation of *B.bifidum* subsp. *pennsylvanicum* in the presence of [^{14}C]oleic acid (250 µCi/l) and a Tween-80 concentration of only 0.1 g/l. The labeled lipids were fractionated on a silicagel column (Exterkate & Veerkamp, 1969) to separate glyco- and phosphoglycolipids.

Incorporation experiments

Incorporation of phosphatidyl[^{14}C]glycerol was performed using the assay mixture described by Ganfield and Pieringer (1980) with some modifications. Briefly, the indicated amount of phosphatidyl[^{14}C]-glycerol in chloroform/methanol (2:1, v/v) was transferred into the test tube. The solvent was removed by a stream of N_2. After addition of 75 µl of 50 mM Tris/HCl assay buffer (pH 7.0), containing 0.1% Triton X-100, 5 mM UDP-glucose and 5 mM UDP-galactose, the mixture was sonicated for 1 min in a ultrasonic waterbath (Bransonic B12). The reaction was started with the addition of about 750 µg particulate fraction (400 µg of protein) suspended in 50 µl of the same buffer. The mixture was usually incubated at 37°C for 4 h.

The incorporation was terminated with the addition of 2 ml chloroform/methanol (1:1, v/v). The protein was pelleted by centrifugation and washed once with 1 ml chloroform/methanol. The supernatants were combined and extracted with 1 ml of 0.2% NaCl (w/v). Saline-extractable radioactivity was determined by liquid scintillation counting in Opti-Fluor. The pellet was suspended in 0.5 ml of 0.1 M acetate buffer (pH 5.5) containing 20 mM $MgSO_4$ and extracted with 0.5 ml of 80% phenol (w/v) for 30 min at 60°C. The aqueous layer was collected by centrifugation (10,000 x g; 10 min) and the organic layer was washed with 0.5 ml of the acetate buffer. The aqueous layers were collected (lipoteichoic acid fraction) and the incorporated radioactivity was measured. We also determined the radioactivity remaining in the phenol residue.

In some experiments [^{14}C]oleic acid-labeled phosphoglycolipids, UDP[^{14}C]glucose or UDP[^{14}C]galactose replaced phosphatidylglycerol as the radioactive substrate. In these experiments 100 nmol of unlabeled phosphatidylglycerol was added to each test tube. The incorporation of UDP[^{14}C]glucose and UDP[^{14}C]galactose into the bifidobacterial lipoteichoic acid was also tested using the assay system described by Arakawa *et al.* (1981), with 7.5 µg lipoteichoic acid per test tube.

Identification of the radioactive products

The saline extract of the supernatant and the phenol extract of the pellet (lipoteichoic acid fraction) were washed twice with equal volumes of chloroform, concentrated and applied to a Sepharose 6B column (1.7 x

50 cm). The column was eluted with 0.2 M ammonium-acetate (pH 7.0), containing 0.02% NaN_3. Fractions of 5.5 ml were collected and assayed for radioactivity. The peaks containing radioactivity were further treated as described in section 4.3.

Strong alkaline hydrolysis was performed in 1 M NaOH for 1 h at 100°C. The hydrolysates were passed through a Dowex 50 H^+ column to remove Na^+. Products were identified by paper electrophoresis and thin-layer chromatography in system C (section 2.5).

4.3 Results

Characteristics of the biosynthetic reaction

The radioactivity from phosphatidyl[^{14}C]glycerol appeared to be incorporated in a product that coprecipitated with protein at chloroform/methanol addition and that could be extracted with aqueous phenol.

Table 4.1 *Effect of various factors on incorporation of phosphatidylglycerol in phenol-extractable polymer*

	Incorporation	
Addition	nmol PG/h.mg protein	%
None	0.71 ± 0.05	100
Triton X-100	1.76 ± 0.07	248
UDPglucose + UDPgalactose	1.37 ± 0.08	193
Triton X-100 + UDPglucose	1.73 ± 0.05	244
Triton X-100 + UDPgalactose	1.86 ± 0.04	262
Triton X-100 + UDPglucose + UDPgalactose	2.52 ± 0.20	355

An amount of 180 nmol phosphatidyl[^{14}C]glycerol was incubated with 750 µg membrane fraction in 50 mM Tris/HCl (pH 7.0) with the indicated additions in a total volume of 125 µl. Values are means ± S.D. of three experiments. Concentrations of factors were: Triton X-100, 0.1%; UDP-glucose and UDP-galactose, 5 mM. PG, phosphatidylglycerol.

The residual phenol layer contained only very small amounts of radioactivity. Extraction of the pellet with 0.2% NaCl (w/v) (Ganfield & Pieringer, 1980) appeared to be incomplete. The radioactivity incorporated into the saline extract of the supernatant represents mainly [^{14}C]glycerol which is liberated during synthesis of diphosphatidylglycerol from two phosphatidylglycerol molecules (Van Schaik & Veerkamp, 1976; Veerkamp, 1976). This was confirmed by thin-layer chromatography in System C. No products with a high molecular weight were found in the saline fraction. The incorporation into the phenol extract was stimulated by Triton X-100 (optimal concentration 0.1%) and by UDP-glucose and UDP-galactose, when both compounds were present (Table 4.1). Mg^{2+} did not affect the incorporation into the phenol extract but increased the release of [^{14}C]glycerol into the saline extract of the supernatant. CDP-glycerol did not inhibit the incorporation reaction up to 100 nmol. Radioactivity from CDP[^{14}C]glycerol (100 nmol/incubation), instead of phosphatidylglycerol, was not incorporated into the phenol-extractable lipoteichoic acid fraction.

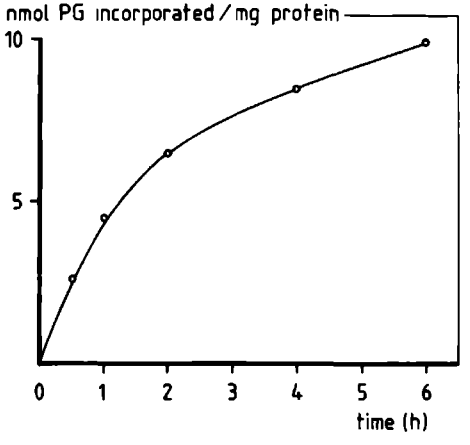


Fig. 4.1: Time course of incorporation of phosphatidyl[^{14}C]glycerol into the lipoteichoic acid containing fraction. The reaction mixture contained 180 nmol phosphatidyl[^{14}C]glycerol and 750 µg particulate fraction in a total volume of 125 µl assay buffer. At the times indicated the reaction was stopped by addition of 2 ml chloroform/methanol (1:1, v/v). Other details are described in section 4.2. Values are means of three experiments with a variation coefficient of less than 6%.

The incorporation appeared to be linear for at least 1 h (Fig. 4.1) and proportional with the amount of membrane fraction up to 800 µg of protein (Fig. 4.2). Maximal incorporation occurred within the pH range of 6-7 at 37°C.

Identification of the phenol-extractable product (lipoteichoic acid fraction)

In a large scale incorporation experiment we incubated phosphatidyl[^{14}C]glycerol (1 x 10^6 dpm) with 7.5 mg particulate fraction for 4 h at 37°C in a total volume of 1.25 ml assay buffer. The phenol extract of the pellet was applied to a Sepharose 6B column. The elution profile is shown in Fig. 4.3. 79% of the incorporated radioactivity eluted in a peak with an elution volume identical to purified lipoteichoic acid (chapter 3, Fig. 3.1). Some residual radioactivity eluted at the void volume and at the exclusion volume of the column. The main peak (fractions 9-14) was pooled, dialyzed against water and concentrated. 95% of the radioactivity appeared to be nondialyzable. A part of the isolated product was hydrolyzed with 1 M NaOH for 1 h at 100°C and

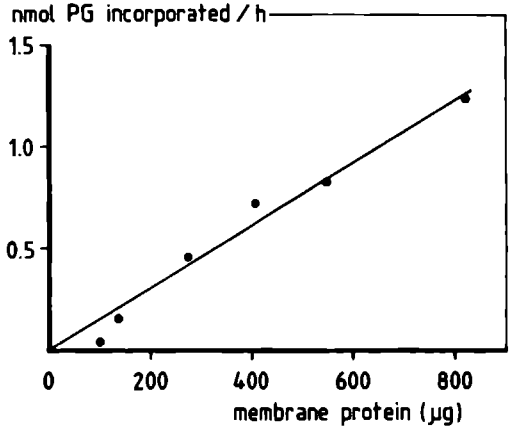


Fig. 4.2: Enzyme-dependence of the incorporation of phosphatidyl[^{14}C]-glycerol into the lipoteichoic acid containing fraction. The amounts of protein indicated were incubated with 180 nmol phosphatidyl[^{14}C]glycerol in a total volume of 125 µl assay buffer. Other details are described in section 4.2. Values are means of three experiments with a variation coefficient of less than 6%.

analyzed by paper electrophoresis. A spot identified as glycerol phosphate contained 60% of the radioactivity applied. 10% of the radioactivity was found in a slow-moving compound identical to that described in the alkaline hydrolysate of lipoteichoic acid (chapter 3) and 30% of the label remained at the origin and could be identified as glycerol by thin-layer chromatography in system C. From the results we may conclude that the radioactivity from phosphatidyl[^{14}C]glycerol coprecipitating with protein and extractable with aqueous phenol, is incorporated into lipoteichoic acid.

Determination of apparent Km and Vmax values

In order to determine the apparent Km and Vmax values for the incorporation of phosphatidyl[^{14}C]glycerol into the bifidobacterial lipoteichoic acid we measured the incorporation into the phenol extract using different amounts of phosphatidyl[^{14}C]glycerol per incubation (50–450 nmol). The results are shown in Fig. 4.4. From the Lineweaver-Burke plot (inset of Fig. 4.4), the apparent Km for phosphatidylglycerol was calculated to be 1.4 ± 0.1 mM and the Vmax 3.2 ± 0.1 nmol/h per mg protein (means ± S.D. of three experiments).

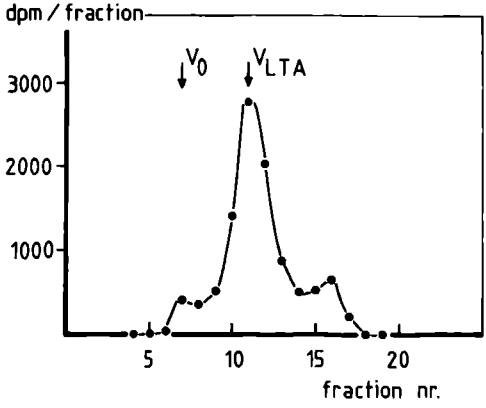


Fig. 4.3: Chromatography on Sepharose 6B of the phenol-extractable product that coprecipitated with protein. Elution was performed with 0.2 M ammonium-acetate (pH 7.0), containing 0.02% NaN_3. Fractions of 5.5 ml were collected and tested for radioactivity. V_o, 39 ml; V_{LTA}, 60 ml. LTA, purified lipoteichoic acid.

To test their acceptor function, [^{14}C]oleic acid-labeled phosphogalactolipids (100,000 dpm/incubation) replaced phosphatidylglycerol as the labeled precursor. The assay mixture was incubated for 24 h at 37°C. A small amount of radioactivity became saline-extractable during the incubation. Since it was not free oleic acid, the product was presumably formed by the addition of a number of glycerol phosphate units to a lipid acceptor. This indicates, that the phosphogalactolipids may serve as anchor for the polymerization of the poly(glycerol phosphate) backbone. The amount of saline-extractable radioactivity however was too low to be used for definitive characterization, probably due to the low specific activity of the product formed.

Lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum* contain high amounts of glucose or galactose present in a polysaccharide end chain (chapter 3). In order to determine the biosynthetic origin of this chain we measured the incorporation of UDP[^{14}C]glucose or UDP[^{14}C]galactose,

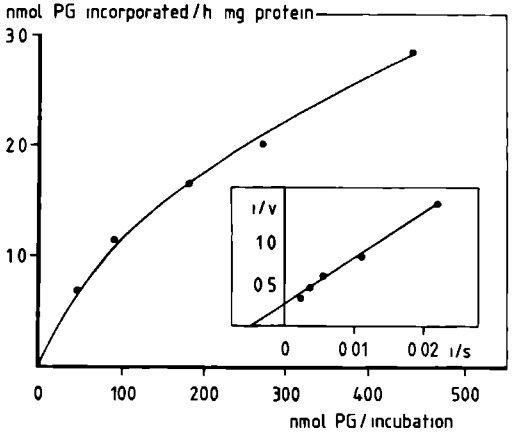


Fig. 4.4: Concentration-dependence of the incorporation of phosphatidyl-[^{14}C]glycerol into the lipoteichoic acid containing fraction. The indicated amounts of phosphatidyl[^{14}C]glycerol were incubated with 750 µg particulate fraction in a total volume of 125 µl assay buffer. Other details are described in section 4.2. Inset shows a Lineweaver-Burke plot. Values are means of three experiments with a variation coefficient of less than 7%.

in the presence of unlabeled UDP-galactose or UDP-glucose, into lipoteichoic acid using the method described for phosphatidylglycerol. Products were analyzed by Sepharose 6B column chromatography. We also used the glycosyl transferase assay described by Arakawa *et al.* (1981) to study the incorporation of the labeled UDP-glycosides. In this method purified lipoteichoic acid was used as acceptor molecule. Products were characterized by paper chromatography (Arakawa *et al.*, 1981) or Sepharose 6B column chromatography. In both assays we could not detect high molecular weight products, although we varied incubation time, pH, specific activity, Mg^{2+} concentration and buffer composition.

4.4 Discussion

The synthesis of the bifidobacterial lipoteichoic acid by transfer of *sn*-glycerol 1-phosphate units from phosphatidylglycerol is in agreement with its poly(1,2)glycerol phosphate structure (chapter 3). Lipid 6, the presumable lipid anchor (chapter 3) most probably serves as point of initiation although this could not definitely be proved. The results are comparable with those described for synthesis of the poly(1,3)glycerol phosphate backbone in *Staphylococcus aureus* (Glaser & Lindsay, 1974; Koch *et al.*, 1984), *Lactobacillus casei* (Taron *et al.*, 1983) and streptococci (Emdur & Chiu, 1974, 1975; Ganfield & Pieringer, 1980). CDP-glycerol, precursor of wall teichoic acid synthesis (Ward, 1981) could be excluded as precursor of the poly(glycerol phosphate) backbone since CDP-glycerol did not inhibit the incorporation of phosphatidylglycerol and CDP[^{14}C]glycerol was not incorporated into lipoteichoic acid. This is in agreement with our understanding of the structure of the lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum* since a poly(1,2)glycerol phosphate chain can not be synthesized from CDP-glycerol, transferring a *sn*-glycerol 3-phosphate group.

The absence of a Mg^{2+} effect on the incorporation was also reported by Ganfield and Pieringer (1980). In contrast with these findings Emdur and Chiu (1975) found a stimulating effect of Mg^{2+} ions. Previously (Veerkamp, 1976), we observed that the synthesis of diphosphatidylglycerol from phosphatidylglycerol was stimulated by Mg^{2+}. Therefore it may be expected that Mg^{2+} ions play an important role in the regulation of the phosphatidylglycerol turnover.

Phosphatidylglycerol has been described as a precursor for several membrane components in Gram-positive bacteria (Koch *et al.*, 1984; Koga *et al.*, 1984). For *B.bifidum* subsp. *pennsylvanicum* we have now established the role of this molecule in the synthesis of diphosphatidylglycerol, the phosphogalactolipids (Van Schaik & Veerkamp, 1975, 1976; Veerkamp, 1976) and lipoteichoic acid. Pulse chase experiments *in vivo* would give more information about the rate of the different reactions but these are obviated by the fact that *B.bifidum* subsp. *pennsylvanicum* can not incorporate glycerol due to the absence of glycerol kinase.

Normally, glycosylation of glycerol units of lipoteichoic acids occurs with UDP-glycosides as precursors (Arakawa *et al.*, 1981; Emdur & Chiu, 1975; Mancuso *et al.*, 1979). The lipoteichoic acids of *B.bifidum* subsp. *pennsylvanicum* consist however of an unsubstituted backbone with a glucose or galactose polymer linked to the terminal glycerol (chapter 3). UDP-glucose and UDP-galactose were not incorporated into the lipoteichoic acid by the particulate membrane fraction of *B.bifidum*. In this case the polysaccharide part may be synthesized on a lipid intermediate and then transferred to the lipoteichoic acid. The use of such a lipid for glycosylation was recently described in *Streptococcus sanguis* (Mancuso & Chiu, 1982). The biosynthetic reactions leading to the synthesis and transfer of the polysaccharide part remain to be established.

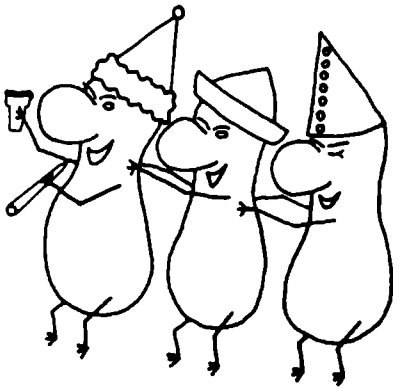
4.5 Summary

Phosphatidylglycerol functions as donor of the *sn*-glycerol 1-phosphate units in the *in vitro* synthesis of the 1,2-phosphodiester linked glycerol phosphate backbone of the lipoteichoic acids of *Bifidobacterium bifidum* subsp. *pennsylvanicum*. The incorporation was catalyzed by a membrane-bound enzyme system. At chloroform/methanol addition the product formed coprecipitated with protein. The material was phenol-extractable and co-eluted with purified lipoteichoic acid on Sepharose 6B. The reaction was stimulated by Triton X-100, UDP-glucose and UDP-galactose, but Mg^{2+} ions had no effect. The apparent values for Km and Vmax of the phosphatidylglycerol incorporation were 1.4 mM and 3.1 nmol/h per mg membrane protein, respectively. UDP-glucose and UDP-galactose were not directly used for the formation of the polysaccharide part of the lipoteichoic acids.

Chapter 5

Interaction of bifidobacterial lipoteichoic acid with human intestinal epithelial cells

Adapted from Op den Camp *et al.* (1985a)



5

5.1 Introduction

Lipoteichoic acids of Gram-positive bacteria have been shown to possess a high binding affinity to mammalian cell membranes (Beachey *et al.*, 1977, 1978, 1979; Chiang *et al.*, 1979; Courtney *et al.*, 1981; Knox & Wicken, 1973; Simpson *et al.*, 1980). Bihding occurs spontaneously via the lipid part of the lipoteichoic acid (Beachey *et al.*, 1978; Courtney *et al.*, 1981; Ofek *et al.*, 1975). Studies of the binding of lipoteichoic acid are of special interest, because lipoteichoic acid appears to play an important role in the adherence of several Gram-positive bacteria to epithelial and other mammalian cells (Beachey, 1981; Beachey & Ofek, 1976; Carruthers & Kabat, 1983; Courtney *et al.*, 1981). Lipoteichoic acids may also serve as a carrier for other antigens and bind them to target tissues, in which they may provoke immuno-cytotoxic reactions (Courtney *et al.*, 1981; Fiedel & Jackson, 1979).

The investigations described in this chapter, concerning the binding of the bifidobacterial lipoteichoic acids to human colonic epithelial cells (colonocytes), were performed in order to obtain more information about the biological activities and functions of these lipoteichoic acids

5.2 Methods

Growth of bacteria and preparation of lipoteichoic acid

B. bifidum subsp. *pennsylvanicum* was grown as described in section 2.1. Unlabeled lipoteichoic acid and [^{14}C]oleic acid-labeled lipoteichoic acid ([^{14}C]lipoteichoic acid) were extracted from cytoplasmic membranes with aqueous phenol and purified as described in section 2.2. The purified [^{14}C]lipoteichoic acid had a specific activity of about 190 dpm/µg.

Preparation of colonocytes

For preparation of free colonocytes, biopsies from colon resection surgery were incubated in appropriate buffers using the chelation-elution method described by Pinkus (1981). The suspension of isolated colonocytes was washed three times in 0.02 M phosphate-0.15 M NaCl (PBS) (pH 7.4), resuspended in PBS, and counted in a hemacytometer. Cell viability was estimated by trypan-blue exclusion. Although the viability of the isolated colonocytes varied between 40 and 80%, we could not detect any significant differences in their ability to bind lipoteichoic acid.

Binding experiments

Binding assays were performed in 1.5 ml Eppendorf tubes. Tubes containing 150 µl of PBS with various amounts of [^{14}C]lipoteichoic acid and colonocytes were incubated at 37°C with shaking. After a specified time, the assay was terminated by adding 1.5 ml of PBS to each tube. The cell-[^{14}C]lipotechoic acid complexes were collected by centrifugation, washed three times with 1.0 ml of PBS, resuspended in 0.5 ml of 0.1 M NaOH, and heated for 5 min at 96°C. Bound [^{14}C]lipoteichoic acid was determined by liquid scintillation counting in 10 ml of Opti-Fluor.

5.3 Results

Binding of lipoteichoic acid to colonocytes

The binding of lipoteichoic acid to colonocytes was cell concentration- (Fig. 5.1) and time-dependent (Fig. 5.2). Binding reached to a maximum within 60 min and was comparable with the binding of streptococcal lipoteichoic acid to buccal epithelial cells (Simpson *et al.*, 1980a). Binding of bifidobacterial lipoteichoic acid appeared to be reversible, since 45% of the bound [^{14}C]lipoteichoic acid was displaced by a 50-fold excess of unlabeled lipoteichoic acid within 10 min. [^{14}C]-lipoteichoic acid remained bound in PBS controls. Displacement of 80% of bound lipoteichoic acid has only been described for leukocytes (Courtney *et al.*, 1981). Other authors have found 40 to 55% displacement (Beachey *et al.*, 1977, 1978; Chiang *et al.*, 1979; Simpson *et al.*, 1980a). The occurrence of the added unlabeled lipoteichoic acid in micelles may be responsible for the inability to displace all of the bound lipoteichoic acid (Chiang *et al.*, 1979). Internalization into the

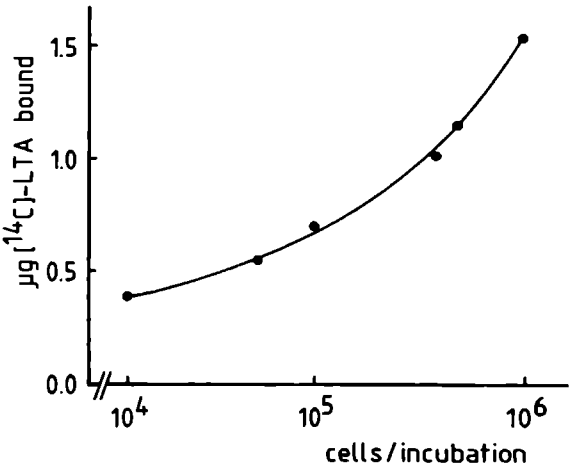


Fig. 5.1: Relationship between the number of colonocytes and binding of [^{14}C]lipoteichoic acid. The indicated numbers of cells were incubated with 50 µg of [^{14}C]lipoteichoic acid for 1 h at 37°C and bound [^{14}C]lipoteichoic acid was determined. Values are means of two separate experiments and corrected for non-specific binding. LTA, lipoteichoic acid.

colonocytes is not expected. Control incubations with a 50-fold excess of unlabeled lipoteichoic acid included in the binding assay revealed aspecific binding values which were always less than 10% of the experimental values.

To study the influence of deacylation of lipoteichoic acid on the binding, we labeled the polar part of the lipoteichoic acid with [U-^{14}C]glucose. Glycerol could not be used for labeling since it is not incorporated by *B. bifidum*. Extraction and purification was done as described in section 2.2. Experiments with [^{14}C]glucose-labeled lipoteichoic acid gave identical results as those with [^{14}C]oleic acid-labeled lipoteichoic acid, but the low specific activity of the former made it less useful for all experiments. Deacylation of the [^{14}C]-glucose-labeled lipoteichoic acid, by treatment with 0.1 M NaOH in ethanol for 30 min at 37°C, abolished the binding of the lipoteichoic acid. This is consistent with the requirement of ester-linked fatty acids for binding (Beachey, 1981; Beachey *et al.*, 1977; Chiang *et al.*, 1979).

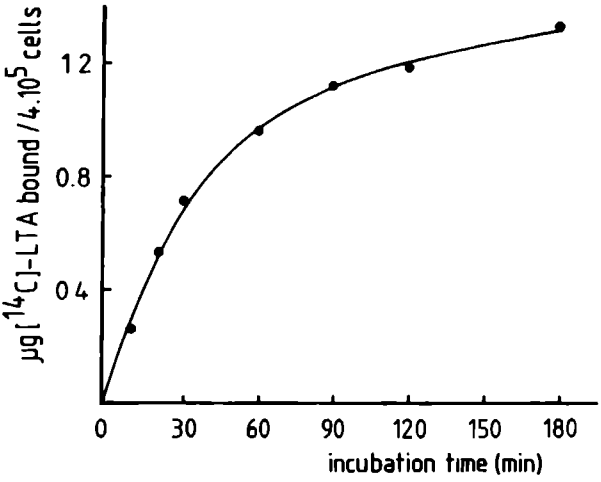


Fig. 5.2: Time-dependent binding of [^{14}C]lipoteichoic acid to human colonocytes. 50 µg of [^{14}C]lipoteichoic acid was incubated with 4 x 10^5 colonocytes at 37°C. At the indicated time intervals bound [^{14}C]lipoteichoic acid was determined.

Determination of the number of lipoteichoic acid binding sites and the dissociation constant

To determine the number of lipoteichoic acid binding sites per colonocyte and the affinity constant, we incubated 4 x 10^5 cells with various amounts of [^{14}C]lipoteichoic acid (50 to 1000 µg) for 1 h at 37°C. Bound [^{14}C]lipoteichoic acid was measured (Fig. 5.3) and the data were analyzed by the method of Scatchard (1949). The Scatchard-plot (Fig. 5.4) revealed a maximal binding (at saturation) of 5.49 µg lipoteichoic acid/4 x 10^5 colonocytes. Assuming of a molecular weight of 10,000 for the bifidobacterial lipoteichoic acid (chapter 3), we calculated that colonocytes possess a single population of about 8.3 x 10^8 binding sites per cell. The dissociation constant was 125 µM. Since bifidobacterial lipoteichoic acid also binds to erythrocytes, for comparison we determined the population of binding sites on these cells. Human erythrocytes appeared to possess a single population of about 2.1 x 10^6 binding sites per cell, with a dissociation constant of 44 µM.

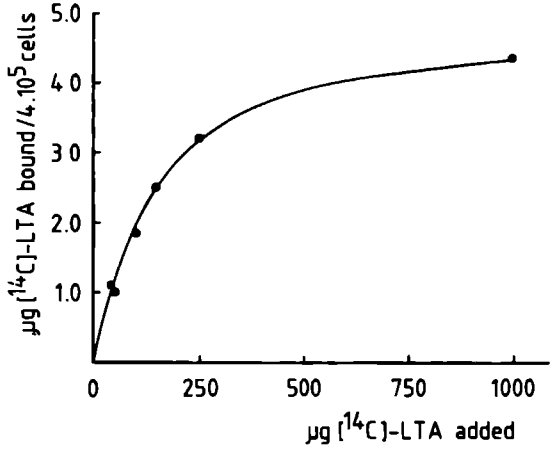


Fig. 5.3: Binding of [^{14}C]lipoteichoic acid as a function of lipoteichoic acid concentration. 4 x 10^5 colonocytes were incubated with different amounts of [^{14}C]lipoteichoic acid for 1 h at 37°C and bound [^{14}C]lipoteichoic acid was determined. Values are means of two separate experiments and corrected for non-specific binding.

The values for colonocytes as well as for erythrocytes are comparable with those described for other lipoteichoic acids (Chiang *et al.*, 1979; Simpson *et al.*, 1980a).

Inhibition experiments

We also tested the effect of some sugars and albumin on the binding of [^{14}C]lipoteichoic acid to colonocytes. None of the sugars tested (fucose, glucose, galactose, mannose, arabinose, and xylose) inhibited the binding at concentrations up to 25 mg/ml indicating that sugar moieties are not involved in the interaction between the epithelial cell membrane and the lipoteichoic acid. Albumin (5 mg/ml) markedly reduced the binding to 34 ± 3% of control (mean ± S.D. of three determinations), most probably by masking of the lipid part of the lipoteichoic acid. Simpson *et al.* (1980b) showed that streptococcal lipoteichoic acid binds readily to the fatty acid-binding sites of serum albumin.

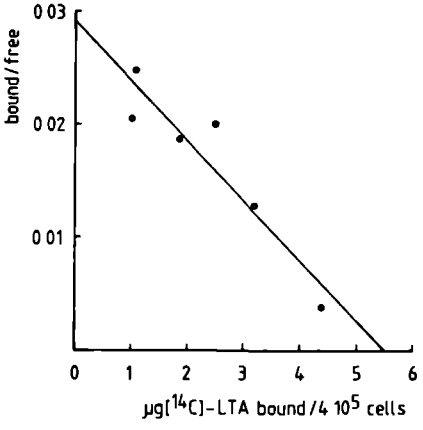


Fig. 5.4: Scatchard-plot of the binding of [^{14}C]lipoteichoic acid to human colonocytes. The ratio of bound to free [^{14}C]lipoteichoic acid is plotted as a function of bound [^{14}C]-lipoteichoic acid. The intercept of the abcissa (5.49 µg) corresponds to the maximal binding capacity of human colonocytes.

6.4 Discussion

The experiments decribed in this chapter revealed that bifidobacterial lipoteichoic acid binds in a specific and reversible way to colonocytes. Binding characteristics were comparable with those described for oral epithelial cells (Simpson *et al.*, 1980a). The lipoteichoic acid binds via the ester-linked fatty acids of the lipid part of the molecule, since deacylated lipoteichoic acid did not show binding activity and albumin effectively inhibited the binding. Because of the specificity of the binding and the importance of the lipid part, a protein or glycoprotein with fatty acid-binding sites appears to be the most probable receptor in the mambrane of the colonocytes. The binding of closely related fatty acids to this putative receptor will be about equal since the rather high percentage of oleic acid in the bifidobacterial lipoteichoic acid compared to streptococcal lipoteichoic acid (40 versus 24%)(Rudczynski & Jackson, 1978) did not change the binding characteristics. Glatz & Veerkamp (1983) reported that palmitic and oleic acid bound to the fatty acid-binding sites of albumin with the same affinity. Recently, evidence was obtained for the presence of fibronectin as a membrane receptor for streptococci on epithelial cells (Beachey & Simpson, 1982; Simpson & Beachey, 1983; Simpson *et al.*, 1982). Fibronectin is capable of binding lipoteichoic acid via its fatty acid-binding sites (Courtney *et al.*, 1983) as was described for albumin (Simpson *et al.*, 1980b). The importance of the lipid portion of the bifidobacterial lipoteichoic acid in its binding to colonocytes correlates well with the role of lipoteichoic acid as the main component responsible for surface hydrophobicity of whole bacteria (Miörner *et al.*, 1983; Ofek *et al.*, 1983). If the model depicted by Ofek *et al.* (1982)(see Fig. 1.5), concerning the interaction of lipoteichoic acid with cell wall proteins, can be applied to other bacteria, hydrophobic interactions mediated by lipoteichoic acid may play an important role in the adherence of bifidobacteria to intestinal epithelial cells.

5.5 Summary

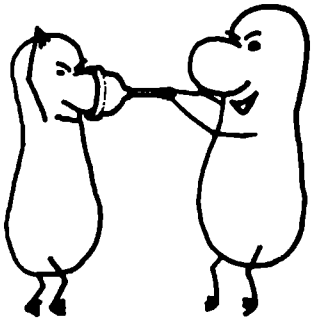
Lipoteichoic acids of Gram-positive bacteria are known to bind spontaneously to a variety of animal cell membranes. Binding of the

lipoteichoic acids of *Bifidobacterium bifidum* subsp. *pennsylvanicum* to human colonic epithelial cells appeared to be specific and cell concentration- and time-dependent. Binding was reversible. About 45% of bound [^{14}C]lipoteichoic acid was displaced by a 50-fold excess of unlabeled lipoteichoic acid. A single population of approximately 8.3 x 10^8 binding sites per cell was calculated from Scatchard-plot analysis, with a dissociation constant of 125 µM. Among the inhibitors tested only albumin markedly reduced lipoteichoic acid binding. Deacylation of lipoteichoic acid abolished the binding, indicating that ester-linked fatty acids are essential for binding.

Chapter 6

Immunochemical studies on the lipoteichoic acids of Bifidobacterium bifidum subsp. pennsylvanicum

Adapted from Op den Camp *et al.* (1985b)



6

6.1 Introduction

Bifidobacterium bifidum subsp. *pennsylvanicum* is a Gram-positive, anaerobic organism, which normal habitat is the human intestine (György *et al.*, 1954). The two unique membrane lipoteichoic acids of this bacterium, which could not be separated, consist of a linear 1,2-phosphodiester linked glycerol phosphate backbone, covalently linked to a membrane phosphogalactolipid and substituted at the terminal glycerol with a linear polysaccharide containing either galactose or glucose (chapter 3). These lipoteichoic acids possess affinity for mammalian cells (chapter 5). Since lipoteichoic acid may serve as a group-specific surface antigen (Knox & Wicken, 1973; Sharpe *et al.*, 1973) or as carrier for other bacterial antigens to target organs (Courtney *et al.*, 1981; Fiedel & Jackson, 1979), we have studied the serological characteristics of the lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum* to obtain more information about their biological functions.

Purified lipoteichoic acid preparations, containing a minor amount of contaminating protein, are only very weakly immunogenic (Knox & Wicken, 1973; Wicken & Knox, 1977). However, complexation with a carrier protein strongly enhanced the antibody response (Fiedel & Jackson,

1976). The antibodies formed against lipoteichoic acids appear to be specific for the poly(glycerol phosphate) backbone or for the glycosyl or D-alanyl-ester substituents (Knox & Wicken, 1973; Wicken & Knox, 1977).

This chapter reports the immunogenicity of the lipoteichoic acid of *B.bifidum* subsp. *pennsylvanicum* and the specificity of the induced antibodies. Furthermore we describe the cross-reactivity of these antibodies with phenol extracts from other strains of bifidobacteria and lactobacilli.

6.2 Methods

Bacterial strains

All strains of bifidobacteria except *Bifidobacterium bifidum* subsp. *pennsylvanicum* were obtained from Dr. V.Scardovi, Institute of Microbiology, University of Bologna, Italy and were cultivated in trypticase/phytone/glucose-medium as described by Scardovi *et al.* (1971). *B.bifidum* subsp. *pennsylvanicum* and the strains of lactobacilli were from our own collection and were grown as described (Exterkate *et al.*, 1971; section 2.1). All strains were incubated for 16 h at 37°C. Bacteria were harvested by centrifugation (8,000 x g; 10 min), washed twice with water and lyophilized.

Isolation of preparations from B.bifidum subsp. pennsylvanicum

Lipoteichoic acid was isolated from disintegrated bacteria by extraction with aqueous phenol and purified as described in section 2.2. Cell wall fractions, containing wall teichoic acid, were prepared according to Veerkamp *et al.* (1983). Cytoplasmic membranes were obtained by lysis of protoplasts followed by ultra-centrifugation (section 2.2).

Preparation of phenol extracts

Bacteria (40 mg dry weight) were suspended in 2 ml of 0.1 M acetate buffer (pH 4.7) and disrupted by sonication (Branson Sonifier 70W; 15 times for 45 s) under cooling. An equal volume of 80% phenol (w/v) was

added and extraction was performed at 65°C for 1 h, with shaking. After cooling the emulsion was centrifuged (10,000 x g; 30 min). The aqueous layer was collected and the phenol layer was washed with an equal volume of acetate buffer. The aqueous layers were combined, dialyzed against water, lyophilized and dissolved in 0.5 ml of water before use.

Preparation of antisera

New Zealand White rabbits of both sexes (weight 3-5 kg) were used for production of antisera. Antisera against formalin-killed *B. bifidum* subsp. *pennsylvanicum* cells were obtained by means of the immunization procedure of Emdur *et al.* (1974). Briefly, bacteria were treated with 67 mM formaldehyde for 4 h at 20°C, washed twice with saline and resuspended to a concentration of 10 mg/ml (dry weight). Rabbits were injected intramuscularly with 0.25 ml of this solution mixed with Freund's complete adjuvant. After five days a second injection was given intravenously, followed by subcutaneous injections every three days. Antibody titre was checked every week.

Antisera against purified lipoteichoic acid preparations of *B. bifidum* subsp. *pennsylvanicum* were prepared using a methylated bovine serum albumin (mBSA)/lipoteichoic acid complex (Fiedel & Jackson, 1976). For each injection, 2 mg complex was suspended in 1 ml water, and 1 ml Freund's incomplete adjuvant was added. After the mixture was emulsified, 2 ml of 2% Tween-80 (w/v) in phosphate-buffered saline (pH 7.2; PBS) was added. The mixture was vigorously vortexed and injected intravenously. Injections were repeated weekly (booster) and antibody production was examined one day before the next booster. Pre-immune blood was collected prior to the first injection.

Serological methods

The passive haemagglutination assay (PHA) and the haemagglutination inhibition assay were performed according to Antonissen *et al.* (1981). The optimal sensitizing activities for lipoteichoic acid and mBSA were 50 and 5 µg/ml PBS, respectively. The sensitizing ability of the phenol extracts from different strains of bacteria was tested by adding 100 µl phenol extract (equivalent to 8 mg bacteria, dry weight) to 5 ml of a suspension of rabbit erythrocytes (1%, v/v, in PBS). After incubation

for 30 min at 37°C, the erythrocytes were washed twice with PBS, resuspended to the original concentration and tested with positive serum of known reactivity.

The quantitative precipitin reaction was done as described by Knox *et al.* (1970). Briefly, 50 µl of antiserum was incubated with different amounts of lipoteichoic acid (5 - 75 µg) or cell wall fractions (25 - 75 µg) in 0.85% NaCl (w/v) to a total volume of 0.6 ml for 1 h at 37°C, followed by 2 d at 4°C. The precipitate was washed twice with cold saline, dissolved in 0.1 M NaOH and analyzed for protein using the method of Lowry *et al.* (1951) with bovine serum albumin as standard. In order to determine cross-reactivity, phenol extracts (10 µl) of different strains were tested for their ability to precipitate antibodies. For precipitin inhibition tests we first determined the optimum ratio for antigen-antibody formation. The inhibitor tested was preincubated with antiserum for 30 min at 37°C and then the appropriate amount of antigen was added. Inhibition tests were performed in triplicate.

Enzyme-linked immunosorbent assay (ELISA) was performed as described by Kessler & Thivierge (1983) with the modification that we used swine anti-rabbit immunoglobulin conjugated peroxidase with o-phenylenediamine as substrate for the detection of bound antibody. Optimal sensitation was achieved with 2.5 µg lipoteichoic acid/ml PBS (150 µl/well).

Counter immunoelectrophoresis was performed on slides coated with 1.3% agar-agar (w/v) in 0.4 M Veronal buffer (pH 8.0). Troughs were filled with 8 µl antigen solution (1 mg/ml PBS) and 8 µl antiserum. Electrophoresis was performed for 1 h at 10 V/cm with a constant current of 20 mA.

Separation of IgG from other immunoglobulins was performed as described by Goudswaard *et al.* (1978) using Protein-A-Sepharose affinity chromatography.

Absorption of antiserum with whole bacteria

Positive serum (diluted 1:16) was incubated for 30 min at 37°C with the amounts of bacteria indicated under Results. After sedimentation of the bacteria by centrifugation (10,000 x g; 10 min) sera were tested by passive haemagglutination and compared to serum incubated without bacteria.

6.3 Results

Immunogenicity of whole bacteria, cell walls and lipoteichoic acid

The injection of formalin-killed cells or cell walls of *B. bifidum* subsp. *pennsylvanicum* into rabbits according to the procedure of Emdur *et al.* (1974) resulted in only a very weak immune response to lipoteichoic acid as detected by PHA (titre 64) and counter immunoelectrophoresis. However, when rabbits were given intravenous injections of lipoteichoic acid/mBSA complexes (phosphate/protein ratio 1:13) emulsified in Freund's incomplete adjuvant a strong antibody response to detectable by counter immunoelectrophoresis, PHA and ELISA. Antibody production to mBSA was either undetectable or negligible (PHA-titers 0 - 16). Fig. 6.1 shows the PHA-titers of the rabbit sera after serial injections (boosters) with the lipoteichoic acid/mBSA complex. Although there was some variation in the titers, each animal gave a strong immune response. The anti-lipoteichoic acid titre rose to a maximum level within 20 days. After the boosters were stopped, the titre rapidly decreased. For further experiments we used antiserum obtained from the animals when the anti-lipoteichoic acid titre was at maximum (30 - 40

Fig. 6.1: Anti-lipoteichoic acid titre (PHA) after serial injections (weekly) with lipoteichoic acid/mBSA complexes. The arrow indicates when immunization wwas stopped. Values are means for three animals.

days after starting injections). The injected animals did not show any anaphylactoid reaction as was described for the lipoteichoic acid from *Streptococcus pyogenes* (Fiedel & Jackson, 1976, 1979). Identical results as with PHA were obtained using ELISA for detection.

Specificity of the haemagglutination reaction

The haemagglutination reaction between antiserum and lipoteichoic acid-coated erythrocytes was strongly inhibited by lipoteichoic acid; the minimal inhibitory concentration (m.i.c.) was 1 ng/well, at a serum dilution of 1:250. Deacylated lipoteichoic acid and cytoplasmic membrane preparations were less potent inhibitors (m.i.c. 2.0 and 1.0 µg/well, respectively). Preparations of cell wall mannitol teichoic acid together with the glycosylated rhamnose polymer (Veerkamp *et al.*, 1983) did not show inhibitory activity. Further we observed that, in contrast with the results of Antonissen *et al.* (1981), the haemagglutination reaction was not affected by simple sugars, glycerol or glycerol phosphate.

The haemagglutination inhibition assay (Antonissen *et al.*, 1981) appeared to be an excellent tool for the determination of small amounts of lipoteichoic acid during the purification procedure. For instance, we could demonstrate the solubilization of a part of the lipoteichoic acid from whole bacteria during protoplast formation as was suggested in chapter 3. We also used the assay to screen fractions from column chromatography.

Detection of IgM and IgG antibodies

Treatment of the anti-lipoteichoic acid serum with 0.1 M 2-mercaptoethanol resulted in a decrease of the PHA-titre from 4096 to 1024, indicating the presence of IgM antibodies. We also separated the IgG antibodies from the other immunoglobulins on Protein-A-Sepharose (Goudswaard *et al.*, 1978). The isolated IgG-fraction contained no IgM or IgA as was proved by immunoelectrophoresis against anti-rabbit IgM and anti-rabbit IgA. The PHA-titers of the purified IgG-fraction and the residual serum fraction were both 2048. The results indicate that both the IgM- and IgG-antibodies contribute to the reactivity of the anti-lipoteichoic acid serum.

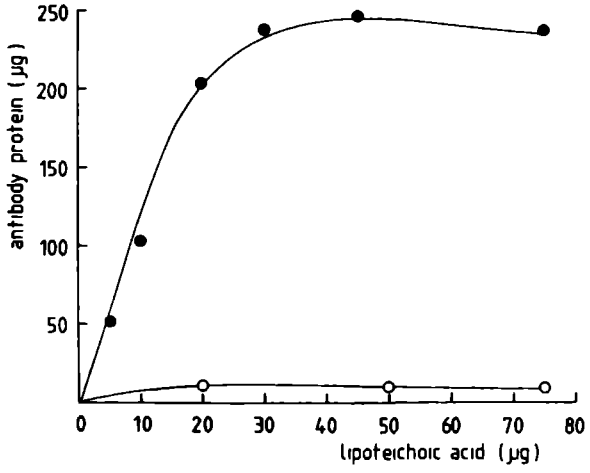


Fig. 6.2: Quantitative precipitin curve for lipoteichoic acid from *B. bifidum* subsp. *pennsylvanicum*. Lipoteichoic acid (●), deacylated lipoteichoic acid (○).

Quantitative precipitin reaction

In order to obtain information about the immunodominant groups of the lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum* we studied the precipitation of antibody by lipoteichoic acid and the inhibition of this reaction by several chemically defined substances. The quantitative precipitin curve for native and deacylated lipoteichoic acid is shown in Fig. 6.2. The maximum precipitation of antibody occurred with about 30 µg lipoteichoic acid and the maximum amount of reacting antibody (serum collected after 30 - 40 days) was 4.6 mg/ml serum. Deacylated lipoteichoic acid could only precipitate a small amount of antibody (less than 5% of the native product). This is probably due to the decrease in apparent molecular weight caused by the disturbance of the micellar structure of the native material. The mannitol teichoic acid of the cell wall did not react in the quantitative precipitin reaction, as expected, since there is no structural similarity between the wall teichoic acid and the membrane lipoteichoic acid of *B. bifidum* subsp. *pennsylvanicum*.

The inhibition of the precipitin reaction by several chemically

Table 6.1 *Inhibition of precipitin reaction between lipoteichoic acid of B.bifidum subsp. pennsylvanicum and its antibody*

Component	Amount (µmol)	Inhibition (%)
Glycerol	100	36 ± 4
Glycerol phosphate*	100	66 ± 2
	25	44 ± 4
G_3P_2**	0.5	30 ± 3
G_2P **	0.5	31 ± 2
Galactose	100	18 ± 1
Glucose	100	15 ± 1
Methyl β-D-glucoside	100	7 ± 2
Methyl β-D-galactoside	100	6 ± 1
Melibiose	100	10 ± 2
Cellobiose	100	10 ± 3
Gentiobiose	100	4 ± 2
Mannitol	100	0 ± 1
Rhamnose	100	0 ± 1

Results are expressed as percentage inhibition of the precipitin reaction between 20 µg of lipoteichoic acid and 50 µl of serum in a total volume of 600 µl and are means ± S.D. of three experiments.
* α- and β-glycerol phosphate had the same effect.
**Glycerol-phosphoryl-glycerol-phosphoryl-glycerol (G_3P_2) and glycerol-phosphoryl-glycerol (G_2P) were prepared by mild alkaline deacylation from diphosphatidylglycerol and phosphatidylglycerol, respectively.

defined components is compiled in Table 6.1. The amount of lipoteichoic acid used in the inhibition assay was 20 µg, resulting in the precipitation of about 200 µg antibody protein in the absence of inhibitors. Glycerol, glycerol phosphate, glycerol-phosphoryl-glycerol-phosphoryl-glycerol (G_3P_2) and glycerol-phosphoryl-glycerol (G_2P) were very effective inhibitors; galactose, glucose, their methyl β-glycosides, melibiose cellobiose and gentiobiose were less effective. The cell wall components mannitol and rhamnose had no effect. From these results we may conclude that the glycerol phosphate backbone (1,2-phosphodiester

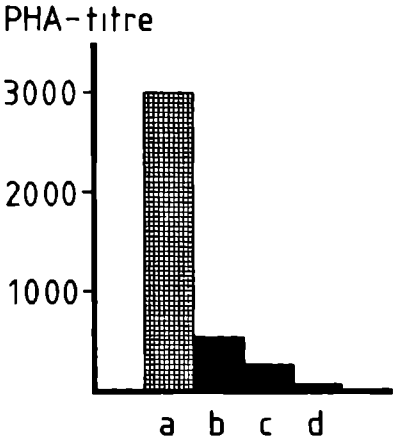


Fig. 6.3: Effect of treatment of antiserum with *B.bifidum* subsp. *pennsylvanicum* on the anti-lipoteichoic acid titre (PHA); a, titre before absorption; b, serum absorbed with 2.75 mg/ml bacteria/ml (dry weight); c, 5.5 mg/ml; d, 11.0 mg/ml; e, 22.0 mg/ml.

linked) is the major immune determinant of the lipoteichoic acids of *B.bifidum* subsp. *pennsylvanicum*. The polysaccharide parts containing galactose or glucose appear to be less important for the immune response.

Inhibition of haemagglutination by whole cells of B.bifidum subsp. pennsylvanicum

After treatment of antiserum (diluted 1:16) with 2.75 - 22.0 mg bacteria/ml (dry weight), the PHA-titre decreased strongly (Fig. 6.3). Total absorption required about 11 mg bacteria/ml. The absorption of antibodies directed against lipoteichoic acid by whole bacteria indicates that the lipoteichoic acids must be present at or near the surface of this bacterium.

In order to determine the proportion of total lipoteichoic acid reactive at the bacterial surface we also measured the total lipoteichoic acid content. We prepared a phenol extract from disintegrated bacteria. Extraction of lipoteichoic acid by this method appears to be complete, as was shown by Fischer *et al.*, (1983). A volume of 10 µl of this phenol extract, equivalent to 0.8 mg bacteria (dry weight), precipitated an amount of antibody equal to that precipitated by 20 µg lipo-

Table 6.2 *Effect of absorption of antiserum with bacteria on the haemagglutination titre*

Serological group *	Species	Strain	Titre
A	*B. angulatum*	ATCC 27535	2048
B	*B. longum*	ATCC 15707	512
B	*B. bifidum*	ATCC 15696	64
B	*B. bifidum*	*pennsylvanicum*	64
C	*B. magnum*	ATCC 27540	256
D	*B. boum*	ATCC 27917	256
E	*B. pseudolongum*	ATCC 25526	512
F	*B. subtile*	ATCC 27537	256
G	*B. suis*	ATCC 27533	512
H	*B. asteroides*	ATCC 25909	128
	L. fermentum	9H	2048
	L. bulgaricus	9LB	2048
	L. helveticus	9BB	2048
	L. arabinosus	9K	2048
	L. plantarum	9P	2048

11.0 mg bacteria (dry weight) were shaken with 1 ml of diluted (1:16) antiserum. The titre before absorption was 2048 and the table gives the values after absorption. The results shown are from one representative experiment out of three.
* Classification according to Sgorbati & London (1982).

teichoic acid. The total content of lipoteichoic acid therefore represents about 2.5% of the dry weight of *B. bifidum* subsp. *pennsylvanicum*. As we showed above, 11 mg bacteria (dry weight), containing 275 µg lipoteichoic acid, are needed for total absorption of 1 ml of diluted (1:16) antiserum. Since this antiserum contained 288 µg of antibody protein per ml, sufficient to precipitate 37.5 µg lipoteichoic acid, we concluded that only 14% of the total lipoteichoic acid content was reactive at the surface of intact bacteria.

Table 6.3 *Cross-reactivity of antiserum to the lipoteichoic acids of B.bifidum subsp. pennsylvanicum with phenol extracts from other bacteria*

Species	PHA-titre	Quantitative precipitin reaction	
		Antibody protein (µg)	Cross-reactivity (%)
B.angulatum	40	47	29
B.longum	320	135	82
B.bifidum	320	178	108
B.bifidum (pennsylv.)	320	165	100
B.magnum	160	44	27
B.boum	320	223	135
B.pseudolongum	80	41	25
B.subtile	80	38	23
B.suis	320	70	42
B.asteroides	320	87	53
L.fermentum	10	11	6
L.bulgaricus	20	9	6
L.helveticus	40	17	10
L.arabinosus	20	14	8
L.plantarum	20	15	9

The PHA-titre of positive serum was determined using rabbit erythrocytes sensitized with 100 µl phenol extract. The quantitative precipitin reaction of 10 µl phenol extract with 50 µl of serum in a total volume of 600 µl saline was tested and expressed as µg protein precipitated. The cross-reaction was determined from the former values and expressed as percentage of the homologous reaction. Values are means of four experiments using antisera from two different animals.

Cross-reactivity with other strains of bifidobacteria and lactobacilli

To test the ability of other strains of bacteria to absorb antibodies to the lipoteichoic acids of *B.bifidum* subsp. *pennsylvanicum* we,

applied the procedure described above using 11 mg bacteria (dry weight) per ml of diluted (1:16) antiserum. Representative strains of bifidobacteria of the serological groups indicated by Sgorbati & London (1982) were examined together with five strains of lactobacilli. The results are shown in Table 6.2. A second strain of *B.bifidum*, ATCC 15696, showed results similar to those of *B.bifidum* subsp. *pennsylvanicum*. Of the other bifidobacteria, *B.asteroides* had a marked effect. All other strains of bifidobacteria only partially absorbed antibodies to the lipoteichoic acid of *B.bifidum* subsp. *pennsylvanicum*, except for *B.angulatum* which did not absorb any antibodies. The strains of lactobacilli tested had no effect on the PHA-titre.

Cross-reactivity was also tested using phenol extracts of the different strains. Phenol extracts of Gram-positive bacteria are generally accepted to be crude teichoic acid preparations. Both membrane and wall teichoic acids will be extracted but the latter will not react in the passive haemagglutination assay. The extracts were tested for their ability to sensitize erythrocytes in the passive haemagglutination assay and to precipitate antibody protein in the quantitative precipitin reaction, compared to the homologous reactions. The results are compiled in Table 6.3. The two *B.bifidum* strains gave identical results. Strong cross-reactivity was observed with *B.longum* and *B.boum*, and moderate reactivity with *B.suis* and *B.asteroides*, while the other strains of bifidobacteria tested showed low cross-reactivity. The cross-reactivity of the lactobacilli did not exceed 10%, determined with the precipitin reaction. All positive haemagglutination reactions were fully inhibited by preincubation of the antiserum with lipoteichoic acid of *B.bifidum* subsp. *pennsylvanicum*.

6.4 Discussion

The weak antibody response to lipoteichoic acid of *B.bifidum* subsp. *pennsylvanicum* induced by formalin-killed bacteria is most probable due to the location of this antigen (Knox & Wicken, 1973). The purified lipoteichoic acid preparation contained only very small amounts of nucleic acid and protein (chapter 3). We applied the method described by Fiedel & Jackson (1976) to obtain antibodies directed towards lipoteichoic acid since purified lipoteichoic acid is only very weak immuno-

genic, unless complexed with protein (Fiedel & Jackson, 1976; Knox & Wicken, 1973; Wicken & Knox, 1977). Complexation with mBSA made the lipoteichoic acid preparation strongly immunogenic (Fig. 6.1), while there was hardly response to mBSA.

The antibodies raised appeared to be specific for the glycerol phosphate backbone of the lipoteichoic acid molecule and to a minor extent for the polysaccharide part. The inhibition of the quantitative precipitin reaction by G_3P_2 (derived from diphosphatidylglycerol), containing 1,3-phosphodiester linked glycerol phosphates (Table 6.1), seems in contrast with the presence of unsubstituted 1,2-phosphodiester linked glycerol phosphate chains as structural units of the lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum* (chapter 3). Wicken & Knox (1971) showed that the glycosylated 2,3-phosphodiester linked glycerol phosphate teichoic acid of *Bacillus stearothermophilus* did not cross-react with antibodies specific for a 1,3-phosphodiester linked glycerol phosphate backbone. However, in that case the high degree of glycosylation of the teichoic acid may strongly affect reactivity as was observed with streptococcal lipoteichoic acid (Kessler & Thivierge, 1983). Therefore, we may conclude that the unsubstituted 1,2-phosphodiester linked backbone is the major immunodominant group and small molecules with 1,3-phosphodiester linkages (G_3P_2) may be cross-reactive.

By means of a combination of phenol extraction and the quantitative precipitin reaction, we estimated the total amount of lipoteichoic acid as 2.5% of bacterial mass. This value is comparable with those described for other Gram-positive bacteria ranging from 1.0 to 2.5% (Hewett *et al.*, 1970; Huff, 1982; Knox & Wicken, 1973). Lipoteichoic acid appeared to be present as a surface component since whole bacteria absorbed antibodies directed against these molecules (Fig. 6.3). Identical results were obtained for *Lactobacillus fermenti* (Hewett *et al.*, 1970) and streptococci (Mïörner *et al.*, 1983). Previous studies concerning the location of lipoteichoic acids (Van Driel *et al.*, 1973; Joseph & Shockman, 1975a) led to the hypothesis that lipoteichoic acid molecules, held in the membrane by hydrophobic bonds involving their lipid parts, may extend through the network of the cell wall to the outer surface to act as surface antigen. Alternatively, lipoteichoic acid molecules, conjugated with protein, may be present at the bacterial surface. The role of lipoteichoic acid as surface component is well established since these molecules are responsible for surface hydro-

phobicity (Miörner *et al.*, 1983; Ofek *et al.*, 1983) and play a role in the bacteria-eukaryotic cell interaction (Beachey, 1981). The quantitative study revealed that only 14% of the total lipoteichoic acid content was reactive at the bacterial surface. Whole cells of *B.bifidum* subsp. *pennsylvanicum* appear to be strongly hydrophobic (chapter 7).

Several strains of bifidobacteria, representatives of the eight serological groups described by Sgorbati & London (1981), were tested for their cross-reactivity with antiserum specific for the lipoteichoic acid of *B.bifidum* subsp. *pennsylvanicum* in order to obtain information about the presence and structural conformity of lipoteichoic acids among this genus. Absorption of antibodies by whole bacteria may be influenced by the location of lipoteichoic acid (Van Driel *et al.*, 1973; Knox & Wicken, 1973). Therefore we also studied the cross-reactivity of phenol extracts of the different strains. From the results (Tables 6.2 and 6.3) we conclude that all of the strains tested, except *B.angulatum*, contain teichoic or lipoteichoic acid molecules more or less structurally and serologically related to the lipoteichoic acids of *B.bifidum* subsp. *pennsylvanicum*. The low cross-reactivity of the lactobacilli tested is in agreement with results obtained by Sharpe *et al.*, 1973), concerning the reactivity of acid extracts of bifidobacteria with antiserum to *L.acidophilus*. Wicken & Knox (1977) reported, on the basis of passive haemagglutination experiments, the presence of lipoteichoic acids in phenol extracts of ten out of fourteen species of bifidobacteria, but no information about the sera tested, the species used and the sensitizing activity of the extracts was given. Further conclusions about relationships of the teichoic and lipoteichoic acids within the genus *Bifidobacterium* will need more detailed studies of structural and serological properties of these bacterial antigens. Serological grouping, with lipoteichoic acid as a group specific antigen, as was described for other Gram-positive bacteria (Knox & Wicken, 1973) may then become possible.

6.5 Summary

Antisera to lipoteichoic acid of *Bifidobacterium bifidum* subsp. *pennsylvanicum* were obtained by injecting lipoteichoic acid/mBSA complexes into rabbits. The glycerol phosphate backbone is primarily responsible for the serological specificity while the polysaccharide

part of the molecule plays a minor role. Whole cells of *B. bifidum* subsp. *pennsylvanicum* were capable of absorbing antibodies indicating the presence of lipoteichoic acid (14% of the total content) at or near the bacterial surface. Cross-reactivity with strains of the genera *Bifidobacterium* and *Lactobacillus* was tested using absorption of antiserum by whole bacteria and reactivity of phenol extracts. The results indicated that lipoteichoic acid is a common antigen within the genus *Bifidobacterium*. The cross-reactivity with the lactobacilli tested was very low.

Chapter 7

Some aspects of cell surface hydrophobicity of Bifidobacterium bifidum subsp. pennsylvanicum

Adapted from Op den Camp *et al.* (1985d)

7

7.1 Introduction

The interaction of bacteria with eukaryotic cells is a complex event in which several attractive and repulsive forces operate between the surfaces of the bacteria and the eukaryotic cells (Beachey, 1983; Rogers, 1979; Van Houte, 1983). These forces include specific interactions between bacterial adhesins and receptors on the host cell membrane, net surface charge and relative hydrophobicity of the cell surfaces.

The contribution of adhesin-receptor interactions to the binding of bacteria to host cells and the possible role of lipoteichoic acid as bacterial adhesin is discussed in chapter 5. The hydrophobic character of bacteria plays a central role in their interaction with mammalian cells and inert surfaces (for review see Rosenberg, 1984). The nature of the molecules conferring hydrophobicity on the bacterial surface has not yet been identified, but recently it was suggested that lipoteichoic acid is the main component responsible for surface hydrophobicity in group A streptococci (Miörner *et al.*, 1983; Ofek *et al.*, 1983). This idea is compatible with the proposed model for the orientation of lipoteichoic acids and proteins on the surface of this group of bacteria

(Ofek *et al.*, 1982; Fig. 1.5). In this model the lipid part of the lipoteichoic acid molecule is available to react with hydrophobic receptors on the epithelial cells.

This chapter reports some experiments concerning the surface hydrophobicity of *Bifidobacterium bifidum* subsp. *pennsylvanicum*. The role of the lipoteichoic acids is investigated. Furthermore, the cell surface hydrophobicity of some representative members of the genus *Bifidobacterium* is compared.

7.2 Methods

Bifidobacterium bifidum subsp. *pennsylvanicum* and the other strains of bifidobacteria were cultivated as described in section 2.1 and section 6.2, respectively. Unlabeled lipoteichoic acid and [^{14}C]oleic acid-labeled lipoteichoic acid were prepared as outlined in section 2.2. Fatty acid-free serum albumin was prepared by the method of Glatz & Veerkamp (1983b).

Assay procedure for testing surface hydrophobicity

Bacteria were harvested by centrifugation, washed twice and resuspended in PUM-buffer (22.2 g $K_2HPO_4.3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea and 0.2 g $MgSO_4.7H_2O$ per 1 distilled water; pH 7.1). Hydrophobicity was measured using the rapid and simple assay described by Rosenberg *et al.* (1980). Briefly, to 1.2 ml of a turbid bacterial suspension (absorbance at 550 nm 1.0 to 1.5), 0.2 ml of octane or xylene was added. Tubes were preincubated for 30 min at 30°C and mixed uniformly for 120 s. After the phases were allowed to separate, the aqueous phase was carefully removed and its absorbance at 550 nm was measured. Decrease of absorbance of the aqueous phase was used as a measure of cell surface hydrophobicity.

Treatments of B.bifidum subsp. pennsylvanicum

These experiments were performed using a standard suspension of bacteria with an absorbance at 550 nm of 1.00. After treatment the bacteria were washed twice with PUM-buffer, resuspended to the original volume and the surface hydrophobicity was assayed as described above.

Bacteria were treated with trypsin (EC 3.4.4.4) by adding 10 mg trypsin to 10 ml of a standard suspension in 0.15 M NaCl-0.02 M phosphate (pH 7.4, PBS). The mixture was incubated for 30 min at 37°C. Digestion with pepsin (EC 3.4.4.1) was performed by adding 2 mg pepsin to 10 ml of a standard suspension in 0.1 M acetate buffer (pH 4.5 or 5.8). After incubation for 30 min at 37°C the reaction was terminated by raising the pH to 7.5 with a few drops of 7.5% $NaHCO_3$ (w/v).For treatment with hot acid a standard suspension in 0.2 M HCl was prepared. The suspension was incubated for 30 min at 95°C, cooled and neutralized with 2 M NaOH. Heat treatment was performed by incubating a standard suspension in PBS for 10 min at 95°C.

Penicillin treatment and assay of excreted lipoteichoic acid

An exponentially growing culture of *B. bifidum* subsp. *pennsylvanicum* (absorbance at 550 nm 1.30) was divided into two portions. One part served as control and to the other part penicillin was added (0.6 µg/ml). At the different time intervals samples were taken and absorbance was measured at 550 nm. Bacteria were removed by centrifugation (8,000 x g for 10 min) and the supernatants (0.5 ml) were extracted with an equal volume of 80% phenol (w/v) for 1 h at 56°C. The mixture was centrifuged (10,000 x g for 15 min) and the phenol layer was washed once with an equal volume of water. The aqueous layers were combined, dialyzed against water, dried under reduced pressure and dissolved in 0.5 ml of PBS.

Excreted lipoteichoic acid was assayed by testing the sensitizing activity of the phenol extracts in the enzyme-linked immunosorbent assay performed according to Kessler & Thivierge (1983) with some modifications. Briefly, sensitization was carried out with 150 µl phenol extract per well for 16 h at 20°C. The plates were washed five times with PBS containing 0.05% Tween-20 (v/v) and 0.1% BSA (w/v). 100 µl of anti-lipoteichoic acid serum (chapter 6, diluted 1:1000) was added per well and the plates were incubated for 1 h at 20°C. After the plates were washed as before, 100 µl of swine anti-rabbit immunoglobulin conjugated to peroxidase (diluted 1:1000) was added per well. After 1 h at 20°C the plates were washed again and 100 µl substrate solution (24.3 mM citric acid, 51.4 mM Na_2HPO_4, 0.04% H_2O_2 (30%, v/v) and 0.04% o-phenylenediamine (w/v)) was added per well. The enzyme reaction was

stopped after 30 min at 20°C by addition of 100 µl 2 M H_2SO_4. Plates were scanned at 492 nm with a Titertek Multiscan. The ability of the phenol extracts to sensitize the titertek plates was expressed as absorbance at 492 nm.

7.3 Results

Surface hydrophobicity of B.bifidum subsp. pennsylvanicum

The affinity of *B.bifidum* subsp. *pennsylvanicum* towards hydrocarbons was used to measure the cell surface hydrophobicity of the bacteria (Rosenberg *et al.*, 1980). The bacteria appeared to absorb readily to octane as well as to xylene. The decrease of absorbance as a function of hydrocarbon volume added is shown in Fig. 7.1. Addition of 0.2 ml of the hydrocarbons to the assay tubes removed up to 99% of the bacteria from the aqueous phase. Hydrophobicity did not differ more than 5% when using exponentially growing cells instead of stationary phase cells. This is in contrast with group A streptococci (Ofek *et al.*, 1983) which appeared

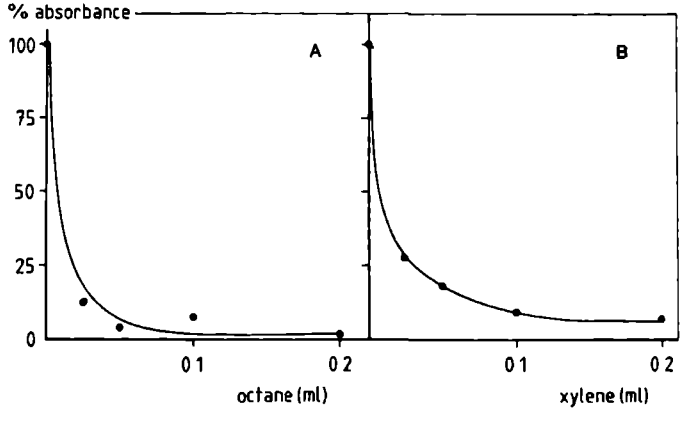


Fig. 7.1: Adherence of *B.bifidum* subsp. *pennsylvanicum* to octane (A) and xylene (B). Washed stationary phase cells were suspended in PUM-buffer to an absorbance of 1.00 at 550 nm and assayed for hydrophobicity. Results are expressed as percentage of the initial absorbance at 550 nm of the aqueous phase as a function of hydrocarbon volume added and are from one representative experiment out of three.

to be more hydrophilic when growing exponentially due to the presence of a capsule. From the results we conclude that *B.bifidum* subsp. *pennsylvanicum* has a strongly hydrophobic cell surface.

Changes in hydrophobicity by treatment of bacteria

In order to obtain information about the nature of the hydrophobic factor(s) on the bacterial surface we studied the effect of several treatments of *B.bifidum* subsp. *pennsylvanicum* on hydrophobicity. The results are compiled in Table 7.1. Trypsin digestion had a marked effect on the adherence of bacteria to octane. Pepsin digestion (at pH 4.5) and treatment with penicillin or hot acid showed moderate effects. Heating the bacterial suspension and pepsin treatment at suboptimal pH (pH 5.8) did not influence hydrophobicity.

Table 7.1 *Effects of treatments of B.bifidum subsp. pennsylvanicum on adherence to octane droplets*

Treatment	Adherence (%)
None	97 ± 3
Trypsin (1 mg/ml)	57 ± 7
Pepsin (200 µg/ml) pH 4.5	78 ± 5
pH 5.8	91 ± 4
Heating, 10 min at 95°C	94 ± 5
0.2 M HCl, 30 min at 95°C	83 ± 3
Penicillin *	86 ± 2

Suspensions of stationary phase cells of *B.bifidum* subsp. *pennsylvanicum* were treated as described in section 7.2. After treatment the bacteria were washed twice with PUM-buffer, resuspended to an absorbance at 550 nm of 1.00 and tested for hydrophobicity using 0.2 ml octane. Results are expressed as % adherence to octane after mixing and are means ± S.D. of three experiments.
* Penicillin treated-cells were obtained by incubating exponentially growing bacteria with penicillin (0.6 µg/ml) for 5 h.

We also studied the influence of albumin. The partition of bacteria into the octane layer was completely inhibited by adding 100 µg fatty acid-free serum albumin to the test tubes. When the pretreatment of a standard suspension of bacteria in PBS with up to 1 mg albumin was followed by washing twice with PUM-buffer no effect on hydrophobicity was observed. Therefore, albumin decreases the surface hydrophobicity either by binding loosely to the hydrophobic sites on the bifidobacterial surface or by coating the octane droplets.

Excretion of lipoteichoic acid

The excretion of lipoteichoic acid into the surrounding medium during penicillin treatment (0.6 µg/ml) was studied using an exponentially growing culture of *B. bifidum* subsp. *pennsylvanicum*. Controls without additions were used to examine excretion during normal growth.

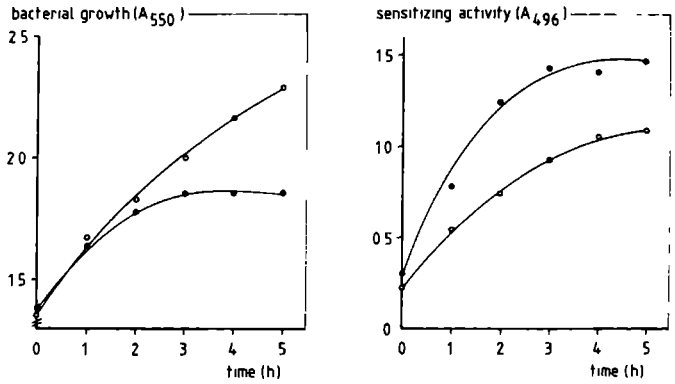


Fig. 7.2: Excretion of lipoteichoic acid into the supernatant during exponential growth in the absence (O) or in the presence (●) of penicillin (0.6 µg/ml). Bacterial growth was monitored turbidometrically at 550 nm (A). To measure the excretion of lipoteichoic acid, phenol extracts of the supernatants were tested for their ability to sensitize micro-titertek plates in ELISA as described in section 7.2 (B). Values are means of two experiments.

The excreted lipoteichoic acid was isolated from the supernatant by phenol extraction. The results are shown in Fig. 7.2. No lysis was observed. Lipoteichoic acid was excreted during normal growth but on addition of penicillin a marked increase of release was observed. The decrease of surface hydrophobicity observed after penicillin treatment may relate to this phenomenon. Previously, the presence of extracellular lipoteichoic acid during normal growth was reported for streptococci and lactobacilli (Markham *et al.*, 1975).

Adherence of lipoteichoic acid to hydrocarbons

To test the ability of purified lipoteichoic acid to adsorb to hydrocarbons we used 30 µg [^{14}C]oleic acid-labeled lipoteichoic acid in 1.2 ml of PUM-buffer instead of the standard bacterial suspension in the hydrophobicity assay procedure. After preincubation for 30 min at 30°C and mixing, 17 and 11% of the labeled lipoteichoic acid appeared to

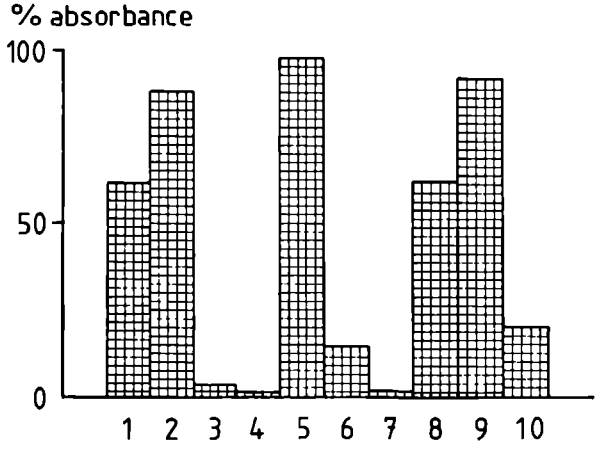


Fig. 7.3: Cell surface hydrophobicity among the genus *Bifidobacterium*. Hydrophobicity was measured by mixing 1.2 ml of a suspension of bacteria with 0.2 ml of octane. Decrease in absorbance at 550 nm relative to the initial suspension was used as a measure of hydrophobicity. Initial absorbance at 550 nm varied between 1.30 and 1.45. Values are means of two experiments. Bacterial strains: (1) *B.angulatum*; (2) *B.longum*; (3) *B.bifidum*; (4) *B.bifidum* subsp. *pennsylvanicum*; (5) *B.magnum*; (6) *B.boum*; (7) *B.pseudolongum*; (8) *B.subtile*; (9) *B.suis*; (10) *B.asteroides*.

adhere to 0.2 ml of octane and xylene, respectively. The presence of the lipoteichoic acid in micelles may influence this partition, since hydrophobic sites may be masked in this configuration.

Hydrophobicity among the genus Bifidobacterium

Ten representative strains of the genus *Bifidobacterium* were tested for cell surface hydrophobicity. The results are presented in Fig. 7.3. A great divergence in cell surface properties was observed. The two strains of *B. bifidum*, *B. boum* and *B. pseudolongum* appeared to possess strongly hydrophobic cell surfaces. Moderate hydrophobicity was observed with *B. angulatum*, *B. subtile* and *B. asteroides*. *B. longum*, *B. magnum* and *B. suis* attached poorly to octane indicating that their cell surfaces possess hydrophilic properties.

7.4 Discussion

The adherence of bacteria to hydrocarbons provides a simple method for measuring cell surface hydrophobicity (Rosenberg *et al.*, 1980). This method was used in several recent investigations concerning bacterial surface properties (Gibbons & Etherden, 1983; Nesbitt *et al.*, 1982; Rosenberg, 1984; Rosenberg *et al.*, 1981). *Bifidobacterium bifidum* subsp. *pennsylvanicum* appeared to possess a strongly hydrophobic cell surface (Fig. 7.1).

Studies concerning the nature of the molecule(s) conferring hydrophobicity on the bacterial surface have revealed that various surface components may be implicated. Some authors reported the association of pili or fimbriae on the bacterial surface with hydrophobicity (Boedeker & Cheney, 1983; Gibbons *et al.*, 1983). McBride *et al.* (1984) found high-molecular weight proteins in cell extracts of hydrophobic strains of *Streptococcus mutans* which were absent in extracts of strains with reduced hydrophobicity. In contrast lipoteichoic acid was reported as the main component responsible for surface hydrophobicity in group A streptococci (Mörner *et al.*, 1983, 1984; Ofek *et al.*, 1983). We observed that the hydrophobicity of *B. bifidum* subsp. *pennsylvanicum* could be reduced by treatment of the bacteria with trypsin, pepsin (at pH 4.5), 0.2 M HCl and penicillin (Table 7.1) or by the presence of albumin in

the assay buffer. Similar effects were observed studying hydrophobic properties of streptococci (Miörner *et al.*, 1983, 1984; Ofek *et al.*, 1983; Wadström *et al.*, 1984) and *Staphylococcus aureus* (Jonnson & Wadstrom, 1984). The penicillin treatment resulted in an increase of lipoteichoic acid excretion (Fig. 7.2) as was observed with other bacteria (Alkan & Beachey, 1978; Brisette *et al.*, 1982; Horne & Thomasz, 1979; Utsei *et al.*, 1983). This increase may relate to the observed decrease of hydrophobicity after penicillin treatment. Purified lipoteichoic acid adsorbed to octane and xylene. The results of the enzyme treatments suggest that cell wall proteins also mediate hydrophobicity but loss of lipoteichoic acid by these procedures can not be excluded (Ofek *et al.*, 1983). Therefore, the most reasonable explanation will be that both proteins and lipoteichoic acids, independently or in dynamic complexes, are responsible for the hydrophobic character of the bifidobacterial cell surface.

Among the strains of the genus *Bifidobacterium* tested a great divergence in cell surface hydrophobicity was observed (Fig. 7.3). The same divergence was found in a comparison of strains of the genera *Streptococcus* (Gibbons & Etherden, 1983; Wadström *et al.*, 1984), *Staphylococcus* (Hogt *et al.*, 1982; Rosenberg *et al.*, 1980) and *Bacteroides* (Gibbons & Etherden, 1983). Surface hydrophobicity of the different strains did not correlate with the presence of lipoteichoic acid as surface component detected with antiserum to the lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum* (section 6.3, Table 6.2). This is not unexpected, since the presence of polysaccharide capsules may strongly affect hydrophobic surface properties (Ofek *et al.*, 1983; Rosenberg *et al.*, 1983; Wädstrom *et al.*, 1984).

7.5 Summary

The cell surface properties of *Bifidobacterium bifidum* subsp. *pennsylvanicum* were studied by mixing standard suspensions of bacteria with octane or xylene. *B. bifidum* subsp. *pennsylvanicum* was shown to possess a strongly hydrophobic cell surface. Hydrophobicity of the bacteria could be reduced by treatment with trypsin, pepsin (at pH 4.5), HCl and penicillin. The latter treatment resulted in an increased excretion of lipoteichoic acid. Albumin was capable of inhibiting the adherence to

octane when it was present in the assay buffer. The data suggest that both protein and lipoteichoic acid may be responsible for cell surface hydrophobicity. A great divergence in cell surface properties was observed among the genus *Bifidobacterium*.

Chapter 8

Survey and Summary

8

8.1 Introduction

Bifidobacterium bifidum subsp. *pennsylvanicum* is a Gram-positive, rod shaped, anaerobic organism normally present in the human intestine. The intestinal microflora of breast fed infants consists largely of these bacteria. In the interactions between bacteria and eukaryotic cells the bacterial cell envelope plays a central role. Previously, several components of the bifidobacterial cell membrane and cell wall were extensively studied (section 1.1). The investigations described in this thesis deal with the lipoteichoic acids of *B.bifidum* subsp. *pennsylvanicum*. By their amphiphilic character these molecules are part of both cell wall and cell membrane. The aspects of these molecules studied are their structure, biosynthesis and biological activities.

8.2 Structure and biosynthesis of the lipoteichoic acids

Chemical analysis of the purified lipoteichoic acid preparation shows the presence of glycerol, phosphate and fatty acids together with large amounts of galactose and glucose (chapter 3). NMR study and

chemical analyses of native and acid or alkaline degraded lipoteichoic acid have given evidence for the structure of two lipoteichoic acids (Fig. 3.6). The lipid anchor of the molecules is identical to lipid 6, previously described by Veerkamp & Van Schaik (1974). A poly(1,2)-glycerol phosphate chain is covalently linked to this lipid. This backbone is substituted at the terminal glycerol unit with a linear polysaccharide, containing either β(1→5)-linked D-galactofuranosyl groups or β(1→6)-linked D-glucopyranosyl groups. The lipoteichoic acids of *B. bifidum* subsp. *pennsylvanicum* are unique since a poly(1,2)glycerol phosphate backbone, instead of the usual poly(1,3)-linked backbone and a linear polysaccharide chain have not been described before (Wicken & Knox, 1980; section 1.2).

Lipid 6, the lipid anchor of the bifidobacterial lipoteichoic acid, is synthesized by transfer of the *sn*-glycerol 1-phosphate from phosphatidylglycerol to the related galactolipid (Van Schaik & Veerkamp, 1976; Veerkamp, 1976). From the experiments described in chapter 4 it is obvious that lipid 6 may serve as point of initiation for the synthesis of the poly(1,2)glycerol phosphate backbone of the lipoteichoic acid from phosphatidylglycerol. Phosphatidylglycerol functions as donor of *sn*-glycerol 1-phosphate units and the incorporation is catalyzed by a membrane-bound enzyme system. The reaction is stimulated by Triton X-100, UDP-glucose and UDP-galactose but Mg^{2+} had no effect (chapter 4). The pathways leading to the synthesis of the poly(1,2)glycerol phosphate backbone are identical to those described for the poly(1,3)glycerol phosphate backbone of other bacteria (Ganfield & Pieringer, 1980; Koch *et al.*, 1984; Fig. 1.3). The biosynthetic reactions leading to the synthesis and transfer of the linear polysaccharide chains remain to be established. UDP-glucose and UDP-galactose were not directly used for the formation of these structural units.

8.3 Biological activities of the lipoteichoic acids

Interest in amphiphilic molecules stems from their many different biological properties. Lipoteichoic acids share several properties with the best known amphiphiles, the lipopolysaccharides of Gram-negative bacteria (Wicken & Knox, 1980). The most outstanding characteristics are binding to a variety of eukaryotic cell membranes and immunogenicity.

Lipoteichoic acids of *B.bifidum* subsp. *pennsylvanicum* bind spontaneously to human intestinal epithelial cells (colonocytes) and erythrocytes (chapter 5). Binding is specific and cell concentration and time dependent. The binding process is reversible, 45% of bound labeled lipoteichoic acid was displaced by a 50-fold excess of unlabeled lipoteichoic acid. Colonocytes possess a single population of 8.3×10^8 binding sites per cell for the bifidobacterial lipoteichoic acid, with an apparent dissociation constant of 125 μM. Ester-linked fatty acids are essential for the binding. This is in agreement with a common and important property of all amphiphiles, e.g. the hydrophobic lipid portion of these molecules is essential for biological activities.

The immunogenicity of the lipoteichoic acids of *B.bifidum* subsp. *pennsylvanicum* is described in chapter 6. Immunization of rabbits with complexes of lipoteichoic acid and methylated albumin results in a strong antibody response to lipoteichoic acid. The induced antibodies are mainly directed against the poly(1,2)glycerol phosphate backbone. Small molecules with 1,3-phosphodiester linkages are cross-reactive. The polysaccharide parts play a minor role. Whole bacteria are capable to absorb the induced antibodies, indicating that lipoteichoic acid is present at or near the bacterial surface. This is in agreement with the proposed role of lipoteichoic acid in the bacteria-eukaryotic cell interaction and in mediating surface hydrophobicity. Cross-reactivity with the antiserum to the lipoteichoic acids of *B.bifidum* subsp. *pennsylvanicum* is observed with cells and phenol extracts of other strains of the genus *Bifidobacterium* (section 6.3). From these results we conclude that lipoteichoic acid is a common antigen within this genus. Cross-reactivity with strains of the genus *Lactobacillus* was very low.

Some experiments concerning bacterial surface hydrophobicity are described in chapter 7. *B.bifidum* subsp. *pennsylvanicum* exhibits a strongly hydrophobic cell surface, since cells from this organism readily adhere to octane and xylene. The hydrophobicity of the bacteria could be reduced by treatment with trypsin, pepsin, acid and penicillin. The decrease of hydrophobicity after the latter treatment may relate to the observed increase of lipoteichoic acid excretion during growth in the presence of penicillin. The results suggest that both protein and lipoteichoic acid may be responsible for cell surface hydrophobicity. The role of lipoteichoic acid is in agreement with the presence of these molecules at the bacterial surface (section 6.3). Within the genus

Bifidobacterium a great divergence in surface properties was observed varying from hydrophilic to strongly hydrophobic.

8.4 Concluding remarks

The investigations described in this thesis have extended the knowledge of the cell envelope of *B. bifidum* subsp. *pennsylvanicum*. After structural studies concerning the fatty acids, the glycolipids and the phosphoglycolipids of the cytoplasmic membrane and the peptidoglycan, a glucosylated rhamnose polymer and a mannitol teichoic acid of the cell wall (section 1.1), the structures of the lipoteichoic acids have now been elucidated. Although these lipoteichoic acids appear to have an unique structure, their biosynthesis and biological properties were comparable to those of the usual (glycosylated) poly(1,3)glycerol phosphate lipoteichoic acids. It seems worthwile to investigate whether the unique poly(1,2)glycerol phosphate lipoteichoic acids are common within the genus *Bifidobacterium*. Another line of research might be the study of the proteins of the cell envelope of *B. bifidum* subsp. *pennsylvanicum*, and the role of the cell envelope components in the interaction of this bacterium with its host.

Samenvatting

Bifidobacterium bifidum subsp. *pennsylvanicum* is een Gram-positieve, staafvormige, anaerobe bacterie die behoort tot de normale flora van de menselijke darm. De bacteriële celenvelop, opgebouwd uit celmembraan en celwand, speelt een grote rol bij de interactie tussen bacteriën en de gastheer. Voor een beter inzicht in deze processen is het noodzakelijk om de opbouw van de bacteriële celenvelop te bestuderen. Hoofdstuk 1 geeft een overzicht van de historie van *B. bifidum* subsp. *pennsylvanicum* en van de verschillende componenten die deel uit kunnen maken van de celenvelop. Tevens wordt aangegeven wat reeds bekend is over de celenvelop van *B. bifidum*. De rest van de algemene inleiding is gewijd aan de component van de celenvelop waar deze dissertatie zich mee bezighoudt: het lipoteichonzuur. Lipoteichonzuren zijn amphiphiele moleculen, d.w.z. moleculen die opgebouwd zijn uit een polair en een apolair gedeelte. Ze maken deel uit van zowel de celmembraan als de celwand en komen voor in de meeste Gram-positieve bacteriën. Het lipoteichonzuur is vaak een van de oppervlakte antigenen. Lipoteichonzuren binden spontaan aan eukaryotische cellen en er bestaan aanwijzingen dat ze een rol spelen bij de hechting van bacteriën aan deze cellen.

In hoofdstuk 2 worden de materialen en methoden beschreven die bij het verdere onderzoek veelvuldig werden gebruikt. Tot deze methoden behoren: de kweek van *B. bifidum* subsp. *pennsylvanicum*, algemene analyse technieken, electroforese en dunnelaag chromatografie en de isolatie en zuivering van het bifidobacteriële lipoteichonzuur.

Het structuur-analytisch werk wordt beschreven in hoofdstuk 3. Uit chemische analyse bleek dat het gezuiverde lipoteichonzuur preparaat glycerol, fosfaat, galactose, glucose en vetzuren bevat in een molaire verhouding van 1.0 : 1.0 : 1.3 : 1.2 : 0.3. De exacte structuur van de lipoteichonzuren kon verder worden vastgesteld met behulp van NMR en chemische analyses van het intacte lipoteichonzuur en van de produkten na verschillende zure en alkalische hydrolyse procedures. Het apolaire gedeelte (lipide anker) van het lipoteichonzuur heeft de volgende structuur: 3-O-(6'-*sn*-glycero-1-phosphoryl)-diacyl-β-D-galactofuranosyl) -*sn*-1,2-diacylglycerol. Het polaire gedeelte, covalent gebonden aan het

lipide anker bestaat uit een glycerolfosfaat keten waarin de units via 1,2-fosfodiesters verbonden zijn. Deze keten is eindstandig gesubstitueerd met een lineair polysaccharide, samengesteld uit β(1→5)-gebonden D-galactofuranosyl groepen of β(1→6)-gebonden D-glucopyranosyl groepen. De lipoteichonzuren van *B.bifidum* subsp. *pennsylvanicum* zijn uniek en wijken af van alle tot nu toe beschreven structuren van lipoteichonzuren.

In hoofdstuk 4 worden de experimenten beschreven die betrekking hebben op de biosynthese van de bifidobacteriële lipoteichonzuren. Fosfatidylglycerol blijkt te functioneren als precursor bij de *in vitro* synthese van de 1,2-fosfodiester gebonden glycerolfosfaat ketens van de lipoteichonzuren door een membraangebonden enzym systeem. Het lipide anker fungeert vermoedelijk als acceptor voor de eerste glycerol fosfaat unit. De incorporatie wordt gestimuleerd door Triton X-100, UDP-galactose en UDP-glucose. De synthese van de polysaccharide ketens is niet opgehelderd. UDP-galactose en UDP-glucose zijn niet de directe precursors voor deze structurele eenheden.

De volgende hoofdstukken beschrijven het onderzoek naar enkele biologische functies van de lipoteichonzuren. Lipoteichonzuren binden spontaan aan een aantal verschillende dierlijke celmembranen. De binding van de lipoteichonzuren van *B.bifidum* subsp. *pennsylvanicum* aan humane darmepitheelcellen (colonocyten) wordt beschreven in hoofdstuk 5. Voor de experimenten werd gebruik gemaakt van [^{14}C]-oliezuur gelabeld lipoteichonzuur. De binding is tijd- en celconcentratie-afhankelijk en reversibel; 45% van de gebonden hoeveelheid gelabeld lipoteichonzuur kon vervangen worden door een 50x zo grote hoeveelheid ongelabeld lipoteichonzuur. Colonocyten bevatten ongeveer 8.3 x 10^6 bindingsplaatsen per cel voor het lipoteichonzuur, met een dissociatie constante van 125 μM.

In hoofdstuk 6 worden de immunologische eigenschappen van de lipoteichonzuren besproken. Antilichamen tegen bifidobacterieel lipoteichonzuur kunnen opgewekt worden door injectie bij konijnen van complexen van lipoteichonzuur en gemethyleerd albumine. Het gemethyleerde albumine dient als carrier en versterkt de immunogene werking van het lipoteichonzuur. De glycerolfosfaat keten is de voornaamste immuundeterminant, terwijl het polysaccharide gedeelte van het molecuul hierbij een kleinere rol speelt in dit proces. Bij absorptie van antiserum met intacte bacteriën daalt de anti-lipoteichonzuur titer aanzienlijk. Dit

suggereert dat lipoteichonzuur aan het celoppervlak voorkomt. Dit is in overeenstemming met de rol die men lipoteichonzuur toedenkt in de bacterie-gastheer interactie. Het antiserum tegen de lipoteichonzuren van *B. bifidum* subsp. *pennsylvanicum* vertoont kruisreactiviteit met intacte bacteriën en fenol extracten van andere stammen van het genus *Bifidobacterium*. Deze resultaten geven aan dat lipoteichonzuren algemeen voorkomen binnen dit genus. De kruis-reactiviteit met het genus *Lactobacillus* was laag.

Het onderzoek naar het karakter van het celoppervlak van *B. bifidum* subsp. *pennsylvanicum* in relatie tot de lipoteichonzuren wordt beschreven in hoofdstuk 7. De bacteriën bezitten een sterk hydrofoob celoppervlak aangezien ze een grote affiniteit voor octaan en xyleen vertonen. Het hydrofobe karakter van het celoppervlak kan worden verminderd door de bacterien te behandelen met trypsine, pepsine, zuur en penicilline. Deze laaste behandeling heeft ook een toename van de lipoteichonzuur excretie tot gevolg. De resultaten leiden tot de conclusie dat zowel lipoteichonzuur als eiwitcomponenten verantwoordelijk kunnen zijn voor de hydrofobe eigenschappen van het celoppervlak. Binnen het genus *Bifidobacterium* varieerde het karakter van het celoppervlak van hydrofiel tot sterk hydrofoob.

De resultaten beschreven in deze dissertatie hebben geleid tot een beter begrip van de structuur en eigenschappen van de celenvelop van *B. bifidum* subsp. *pensylvanicum*. Nader onderzoek verdient de samenstelling en de rol van de eiwitcomponenten van deze celenvelop en de rol van de verschillende bestandelen in de interactie tussen bacterie en gastheer.

References

Alkan, M.L. and Beachey, E.H. (1978) *J. Clin. Invest.* 61: 671-677.

Antonissen, A.C.J.M., van Kessel, K.P.M., van Dijk, H. and Willers, J.M.N. (1981) *J. Immunol. Meth.* 44: 351-357.

Arakawa, H., Shimada, A., Ishimoto, N. and Ito, E. (1981) *J. Biochem.* (Tokyo) 89: 1555-1563.

Archibald, A.R. and Baddiley, J. (1966) *Adv. Carbohydr. Chem.* 21: 323-375.

Baer, E. and Kates, M. (1950) *J. Biol. Chem.* 185: 615-623.

Bartlett, G.R. (1959) *J. Biol. Chem.* 234: 466-468.

Batley, M., Packer, N. and Redmond, J. (1981) in *Chemistry and Biological Activities of Bacterial Surface Amphiphiles* (Shockman, G.D. and Wicken, A.J., eds) Acad. Press, New York pp. 125-136.

Beachey, E.H. (1981) *J. Infect. Dis.* 143: 325-345.

Beachey, E.H. and Ofek, I. (1976) *J. Exp. Med.* 143: 759-771.

Beachey, E.H. and Simpson, W.A. (1982) *Infection* 10: 107-111.

Beachey, E.H., Chiang, T.M., Ofek, I. and Kang, A.H. (1977) *Infect. Immun.* 16: 649-654.

Beachey, E.H., Dale, J.B., Simpson, W.A., Evans, J.D., Knox, K.W., Ofek, I. and Wicken, A.J. (1978) *Infect. Immun.* 23: 618-625.

Beachey, E.H., Dale, J.B., Grebe, S., Ahmed, A, Simpson, W.A. and Ofek, I. (1979) *J. Immunol.* 122: 189-195.

Beachey, E.H., Simpson, W.A., Ofek, I., Hasty, D.L., Dale, J.B. and Whitnack, E. (1983) *Rev. Infect. Dis.* 5: S670-S677.

Beerens, H., Romond, C. and Neut, C. (1980) *Amer. J. Clin. Nutr.* 33: 2434-2439.

Bezkorovainy, A., Grohlich, D. and Nichols, J.H. (1979) *Amer. J. Clin. Nutr.* 32: 1428-1432.

Bock, K. and Pedersen, C. (1983) *Adv. Carbohydr. Chem. Biochem.* 41: 27-66.

Boedeker. E.C. and Cheney, C.P. (1983) in *Affinity Chromatography and Biological Recognition* (Chaiken, J.M. et al., eds.) Acad. Press Orlando pp. 501-503.

Bolton, R.W. (1981) *J. Dent. Res.* 60: 878-882.

Brautigan, V.M., Childs III, W.C. and Neuhaus, F.C. (1981) *J. Bacteriol.* 146: 239-250.

Brisette, J.L., Shockman, G.D. and Pieringer, R.A. (1982) *J. Bacteriol.* 151: 838-844.

Bullen, C.L. and Tearle, P.V. (1976) *J. Med. Microbiol.* 9: 335-344.

Bullen, C.L., Tearle, P.V. and Willis, A.T. (1976) *J. Med. Microbiol.* 9: 325-333.

Button, D. and Hemmings, N.L. (1976a) *Biochemistry* 15: 989-995.

Button, D. and Hemmings, N.L. (1976b) *J. Bacteriol.* 128: 149-156.

Cabacungan, E., and Pieringer, R.A. (1981) *J. Bacteriol.* 147: 75-79.

Carruthers, M.M. and Kabat, W.J. (1983) *Infect. Immun.* 40: 444-446.

Chiang, T.M., Alkan, M.L. and Beachey, E.H. (1979) *Infect. Immun.* 26: 316-321.

Childs III, W.C. and Neuhaus, F.C. (1980) *J. Bacteriol.* 143: 293-301.

Cleveland, R.F., Daneo-Moore, L., Wicken, A.J. and Shockman, G.D. (1976a) *J. Bacteriol.* 127: 1582-1584.

Cleveland, R.F., Holtje, J.-V., Wicken A.J., Thomasz, A., Daneo-Moore, L. and Shockman, G.D. (1975) *Biochem. Biophys. Res. Comm.* 67: 1128-1135.

Cleveland, R.F., Wicken, A.J., Daneo-Moore, L. and Shockman, G.D. (1976b) *J. Bacteriol.* 126: 192-197.

Coley, J., Duckworth, M. and Baddiley, J. (1975) *Carbohydr. Res.* 40: 41-52.

Courtney, H., Ofek, I., Simpson, W.A. and Beachey, E.H. (1981) *Infect. Immun.* 32: 625-631.

Courtney, H.S., Simpson, W.A. and Beachey, E.H. (1983) *J. Bacteriol.* 153: 763-770.

De Boer, W.R., Kruyssen, F.J., Wouters, J.T.M. and Kruk, C. (1976) *Eur. J. Biochem.* 62: 1-6

De Boer, W.R., Wouters, J.T.M., Anderson, A.J. and Archibald, A.R. (1978) *Eur. J. Biochem.* 85: 433-436.

Doyle, R.J., Chaterjee, A.N., Streips, U.N. and Young, F.E. (1975) *J. Bacteriol.* 124: 341-347.

Emdur, L.J. and Chiu, T.H. (1974) *Biochem. Biophys. Res. Comm.* 59: 1137-1144.

Emdur, L.J. and Chiu, T.H. (1975) *FEBS Lett.* 55: 216-219.

Emdur, L.J., Saralkar, C., McHugh, J.G. and Chiu, T.H. (1974) *J. Bacteriology* 120: 724-732.

Exterkate, F.A. (1970) Thesis, University of Nijmegen.

Exterkate, F.A. and Veerkamp, J.H. (1969) *Biochim. Biophys. Acta* 176: 65-77.

Exterkate, F.A. and Veerkamp, J.H. (1970) *Biochim. Biophys. Acta* 231: 545-549.

Exterkate, F.A., Vrenzen, G.J. and Veerkamp, J.H. (1970) *Biochim. Biophys. Acta* 219: 141-154.

Exterkate, F.A., Otten, B.J., Wassenberg, H.W. and Veerkamp, J,H, (1971) *J. Bacteriol.* 106: 824-829.

Fiedel, B.A. and Jackson, R.W. (1976) *Infect. Immun.* 13: 1585-1590.

Fiedel, B.A. and Jackson, R.W. (1979) *Med. Microbiol. Immun.* 167: 251-260.

Fiedler, F. (1981) in *Chemistry and Biological Activities of Bacterial Surface Amphiphiles* (Shockman, G.D. & Wicken, A.J. Eds.) Acad. Press. pp. 195-208.

Fiedler, F. and Glaser, L. (1974a) *J. Biol. Chem.* 249: 2684-2689.

Fiedler, F. and Glaser, L. (1974b) *J. Biol. Chem.* 249: 2690-2695.

Fischer, W. (1981) in *Chemistry and Biological Activities of Bacterial Surface Amphiphiles* (Shockman, G.D. & Wicken, A.J. eds.) Acad. Press pp. 209-228.

Fischer, W. and Koch, H.U. (1983) in *The Target of Penicillin*, Walter de Gruyter & Co., Berlin- New York, pp. 179-184.

Fischer, W. and Landgraf, H.R. (1974) *Biochim. Biophys. Acta* 380: 227-244.

Fischer, W., Koch, H.U., Rösel, P. and Fiedler, F. (1980a) *J. Biol. Chem.* 255: 4557-4562.

Fischer, W., Koch, H.U., Rösel, P., Fiedler, F. and Schmuck, L. (1980b) *J. Biol. Chem.* 255: 4550-4556.

Fischer, W., Laine, R.A. and Nakano, M. (1978) *Biochim. Biophys. Acta* 528: 298-308.

Fischer, W., Nakano, M., Laine, R.A. and Bohrer, W. (1978) *Biochim. Biophys. Acta* 528: 288-297.

Fischer, W., Schmidt, A., Jann, B. and Jann, K. (1982) *Biochemistry* 21: 1279-1284.

Fischer, W., Koch, H.U. and Haas, R. (1983) *Eur. J. Biochem.* 133: 523-530.

Ganfield, M.-C.W. and Pieringer, R.A. (1975) *J. Biol. Chem.* 250: 702-709.

Ganfield, M.-C.W. and Pieringer, R.A. (1980) *J. Biol. Chem.* 255: 5164-5169.

Gibbons, R.J. and Etherden, J. (1983) *Infect. Immun.* 41: 1190-1196.

Gibbons, R.J., Etherden, I. and Skobe, Z. (1983) *Infect. Immun.* 41: 414-417.

Glaser, L. and Lindsay, B. (1974) *Biochem. Biophys. Res. Comm.* 59: 1131-1136.

Glatz, J.F.C. and Veerkamp, J.H. (1983a) *Anal. Biochem.* 132:89-95.

Glatz, J.F.C. and Veerkamp, J.H. (1983b) *J. Biochem. Biophys. Meth.* 8: 57-61.

Glick, M.C., Sall, T., Zilliken, F. and Mudd, S. (1960) *Biochim. Biophys. Acta* 37: 361-363.

Gorin, P.A.J. and Mazurek, M. (1975) *Can. J. Biochem.* 53: 1212-1223.

Goudswaard, J., van der Donk, J.A., Noordzij, A. van Dam, R.H. and Vaerman, J.-P. (1978) *Scand. J. Immunol.* 8: 21-28.

György, P. and Rose, C.S. (1955) *Proc. Soc. Exp. Biol. Med.* 90: 219-223.

György, P., Norris, R.F. and Rose, C.S. (1954) *Arch. Biochem. Biophys.* 48: 193-201.

György, P., Jeanloz, R.W., von Nicolai, H. and Zilliken, F. (1974) *Eur. J. Biochem.* 43: 29-33.

Hether, N.W. and Jackson, L.L. (1983) *J. Bacteriol.* 156: 809-817.

Hewett, M.J., Knox, K.W. and Wicken, A.J. (1970) *J. Gen. Microbiol.* 60: 315-322.

Hewett, M.J., Wicken, A.J., Knox, K.W. and Sharpe, M.E. (1976) *J. Gen. Microbiol.* 94: 126-130.

Hoelen, G.E.J.M. (1981) Thesis, University of Nijmegen.

Hogt, A.H., Dankert, J., Feijen, J. and de Vries, J.A. (1982) *Antonie van Leeuwenhoek* 48: 496-498.

Horne, D. and Thomasz, A. (1979) *J. Bacteriol.* 137: 1180-1184.

Huff, E. (1982) *J. Bacteriol.* 149: 399-400.

Hughes, A.H., Hancock, I.C. and Baddiley, J. (1973) *Biochem. J.* 132: 83-93.

Jansson, P.E., Kenne, L., Liendgren, H., Lindberg, B. and Lönngren, J. (1976) *Chem. Commun. Univ. Stockholm* 8.

Jonnson, P. and Wadström, T. (1984) *Current Microbiol.* 10: 203-210.

Joseph, R. and Shockman, G.D. (1975a) *J. Bacteriol.* 122: 1375-1386.

Joseph, R. and Shockman, G.D. (1975b) *Infect. Immun.* 12: 333-338.

Kessler, R.E. and Thivierge, B.H. (1983) *Infect. Immun.* 41: 549-555.

Knox, K.W. and Wicken, A.J. (1970) *J. Gen. Microbiol.* 67: 251-254.

Knox, K.W. and Wicken, A.J. (1973) *Bacteriol. Rev.* 37: 217-257.

Knox, K.W., Hewett, M.J. and Wicken, A.J. (1970) *J. Gen. Microbiol.* 60: 303-314.

Knox, K.W., Markham, J.L. and Wicken, A.J. (1976) *Infect. Immun.* 13: 647-652.

Koch, H.U. and Fischer, W. (1978) *Biochemistry* 17: 5275-5281.

Koch, H.U., Haas, R. and Fischer, W. (1984) *Eur. J. Biochem.* 138: 357-383.

Koga, Y., Nishihara, M. and Morii, H. (1984) *Biochim. Biophys. Acta* 793: 86-94.

Kuramitsu, H.K., Wondrack, L. and McGuinness, M. (1980) *Infect. Immun.* 29: 376-382.

Lambert, P.A., Hancock, I.C. and Baddiley, J. (1973) *Biochem. J.* 149: 519-524.

Lambert, P.A., Hancock, I.C. and Baddiley, J. (1977) *Biochim. Biophys. Acta* 472: 1-12.

Lambert, R., Saito, Y. and Veerkamp, J.H. (1965) *Arch. Biochem. Biophys.* 110: 341-344.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193: 265-275.

Mancuso, D.J., Chiu, T.H. (1982) *J. Bacteriol.* 152: 616-625.

Mancuso, D.J., Junker, D.D., Hsu, S. and Chiu, T.H. (1979) *J. Bacteriol.* 140: 547-554.

Markham, J.L., Knox, K.W., Wicken, A.J. and Hewett, M.J. (1975) *Infect. Immun.* 12: 378:386.

Mata, L.J. and Urrutia, J.J. (1971) *Ann. N.Y. Acad. Sci.* 176: 93-108.

Mata, L.J., Urrutia, J.J., Garria, B., Fernadez, R. and Behar, M. (1969) *Amer. J. Dis. Child.* 117: 142-146.

Matsunaga, T., Okubo, A., Fukami, M., Yamozaki, S. and Toda, S. (1981) *Biochem. Biophys. Res. Comm.* 102: 524-530.

Mauck, J. and Glaser, L. (1972) *Proc. Nat. Acad. Sci.* 69: 2386-2390.

Miörner, H., Johansson, G. and Kronvall, G. (1983) *Infect. Immun.* 39: 336-343.

Miörner, H., Havlicek, J. and Kronvall, G. (1984) *Acta Pathol. Microbiol. Immun. Scand.* 92B: 23-30.

Mitsuoka, T. and Kaneuchi, C. (1977) *Amer. J. Clin. Nutr.* 30: 1799-1810.

Molenkamp, G.C. (1975) Thesis, University of Nijmegen.

Nakano, M. and Fischer, W. (1978) *H.-P. Z. Physiol. Chem.* 359: 1-11.

Nash, T. (1953) *Biochem. J.* 55: 416-421.

Nealon, T.J. and Mattingly, S.J. (1984) *Infect. Immun.* 43: 523-530.

Nesbitt, W.E., Doyle, R.J. and Taylor, K.G. (1982) *Infect. Immun.* 38: 637-644.

Neut, C.C. and Beerens, R.H. (1980) *Reprod. Nutr. Develop.* 20: 1679-1684.

Nichols, J.H., Bezkorovainy, A. and Londau, W. (1974) *Life Sciences* 14: 967-976.

Norris, R.F., Flanders, T., Tomarelli, R.M. and György, P. (1950) *J. Bacteriol.* 60: 681-696.

O'Brien, P.J., Glick, M.L. and Zilliken, F. (1960) *Biochim. Biophys. Acta* 37: 357-360.

Ofek, I. and Beachey, E.H. (1980) in *Bacterial Adherence, Receptors and Recognition*, Serie B, vol. 6 (Beachey, E.H., ed.) Chapmann and Hall, London pp. 1-27.

Ofek, I., Beachey, E.H., Jefferson, W. and Campbell, G.L. (1975) *J. Exp. Med.* 141: 990-1003.

Ofek, I., Simpson, W.A. and Beachey, E.H. (1982) *J. Bacteriol.* 149: 426-433.

Ofek, I. Whitnack, E. and Beachey, E.H. (1983) *J. Bacteriol.* 154: 139-145.

Op den Camp, H.J.M., Veerkamp, J.H., Oosterhof, A. and van Halbeek, H. (1984) *Biochim. Biophys. Acta* 795: 301-313.

Op den Camp, H.J.M., Oosterhof, A. and Veerkamp, J.H. (1985a) *Infect. Immun.* 47: in Press.

Op den Camp, H.J.M., Peeters, P.A.M., Oosterhof, A. and Veerkamp, J.H. (1985b) *J. Gen. Microbiol.* in Press.

Op den Camp, H.J.M., Oosterhof, A. and Veerkamp, J.H. (1985c) *Biochem.J.* submitted for publication.

Op den Camp, H.J.M., Oosterhof, A. and Veerkamp, J.H. (1985d) *Antonie van Leeuwenhoek* submitted for publication.

Orla-Jensen, S. (1924) *Lait* 4: 468-474.

Oshima, M. and Ariga, T. (1976) *FEBS Lett.* 64: 440-442.

Pazur, J.H., Dropkin, D.J. and Forsberg, L.S. (1978) *Carbohydr. Res.* 66: 155-160.

Pieringer, R.A., Ganfield, M.-C.W., Gustow, E. and Cabacungan, E. (1981) in *Chemistry and Biological Activities of Bacterial Surface Amphiphiles* (Shockman, G.D. & Wicken, A.J., eds.) Acad. Press pp.167-179.

Pinkus, L.M. (1981) *Meth. Enzymol.* 77: 154-162.

Powell, D.A., Duckworth, M. and Baddiley, J. (1974) *FEBS Lett.* 41: 259-263.

Râsić, J.L. and Kurmann, J.A. (1983) *Bifidobacteria and their role, Experientia Supplementum* vol. 39 Birckhäuser Verlag, Basel-Boston-Stutgart.

Rogers, H.J. (1979) in *Adhesion of Microorganism to Surfaces* (Elwood, D.C., Melling, J. and Rutter, P., eds.) Acad. Press, New York pp. 29-55.

Rogosa, M. (1974) in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (Buchanan, R.E. & Gibbons, N.E., eds.) Williams & Wilkins, Baltimore pp. 669-676.

Rosenberg, E., Kaplan, N., Pines, O., Rosenberg, M. and Gutnick, D. (1983) *FEMS Microbiol. Lett.* 17: 157-160.

Rosenberg, M. (1984) *FEMS Microbiol. Lett.* 22: 289-295.

Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) *FEMS Microbiol. Lett.* 9: 29-33.

Rosenberg, M., Perry, A., Bayer, E.A., Gutnick, D.L., Rosenberg, E. and Ofek, I. (1981) *Infect. Immun.* 33: 29-33.

Rudczynski, A.B. and Jackson, R.W. (1978) *Immunochem.* 15: 83-91.

Scardovi, V. (1981) in *The Prokaryotes* (Starr, P. *et al.*, eds.) Springer Berlin-Heidelberg-New York pp. 1951-1961.

Scardovi, V., Trovatelli, L.D., Zani, G., Crociani, F. and Matteuzzi, D. (1971) *Int. J. Syst. Bacteriol.* 21: 276-294.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* 51: 660-672.

Schneider, J.E. and Kennedy, E.P. (1978) *J. Biol. Chem.* 253: 7738-7743.

Sgorbati, B. and London, J. (1982) *Int. J. Syst. Bacteriol.* 32: 37-42.

Sharpe, M.E., Brock, J.H., Knox, K.W. and Wicken, A.J. (1973) *J. Gen. Microbiol.* 74: 119-126.

Silvestri, L.J., Craig, R.A., Ingram, L.O., Hoffman, E.M. and Bleiweis, A.S. (1978) *Infect. Immun.* 22: 107-118.

Simpson, W.A. and Beachey, E.H. (1983) *Infect. Immun.* 39: 275-279.

Simpson, W.A., Ofek, I., Sarasohn, C., Morrison, J.C. and Beachey, E.H. (1980a) *J. Infect. Dis.* 141: 457-462.

Simpson, W.A., Ofek, I. and Beachey, E.H. (1980b) *J. Biol. Chem.* 255: 6092-6097.

Simpson, W.A., Ofek, I., and Beachey, E.H. (1980c) *Infect. Immun.* 29: 119-122.

Simpson, W.A., Hasty, D.L., Mason, J.M. and Beachey, E.H. (1982) *Infect. Immun.* 37: 805-810.

Spiro, R.G. (1972) *Meth. Enzymol.* 28B: 3-43.

Suginaka, H., Shimatani, M., Ogawa, M. and Kotani, S. (1979a) *J. Antibiotics* 32: 73-77.

Suginaka, H., Shimatani, M., Ohno, Y. and Yano, J. (1979b) *FEMS Microbiol. Lett.* 5: 353-355.

Taron, D.J., Childs III, W.C. and Neuhaus, F.C. (1983) *J. Bacteriol.* 154: 1110-1116.

Theodore, T.S. and Calandra, G.B. (1981) *Infect. Immun.* 33: 326-328.

Toon, P., Brown, P.E. and Baddiley, J. (1972) *Biochem. J.* 127: 399-409.

Utsei, Y., Ohyo, S., Takenouchi, Y., Tajima, M. and Sugawara, S. (1983) *J. Antibiotics* 36: 1380-1386

Van Driel, D., Wicken, A.J., Dickson, M.R. and Knox, K.W. (1973) *J. Ultrastruct. Res.* 43: 493-497.

Van Houte, J. (1983) *Rev. Infect. Dis.* 5: S659-S669.

Van Schaik, F.W. (1974) Thesis, University of Nijmegen.

Van Schaik, F.W. and Veerkamp, J.H. (1975) *Biochim. Biophys. Acta* 388: 213-225.

Van Schaik, F.W. and Veerkamp, J.H. (1976) *FEBS Lett.* 67: 13-16.

Veerkamp, J.H. (1971) *Arch. Biochem. Biophys.* 143: 204-211.

Veerkamp, J.H. (1972) *Biochim. Biophys. Acta* 273: 359-367.

Veerkamp, J.H. (1976) *Biochim. Biophys. Acta* 441: 403-411.

Veerkamp, J.H. and van Schaik, F.W. (1974) *Biochim. Biophys. Acta* 348: 370-387.

Veerkamp, J.H., Lambert, R. and Saito, Y. (1965) *Arch. Biochem. Biophys.* 112: 120-125.

Veerkamp, J.H., Hoelen, G.E.J.M. and Op den Camp, H.J.M. (1983) *Biochim. Biophys. Acta* 755: 439-451.

Wadström, T., Schmidt, K.H., Kühnemund, O., Havlicek, J. and Köhler, W. (1984) *J. Gen. Microbiol.* 130: 657-664.

Wallenfels, K. and Kurtz, G. (1966) *Meth. Enzymol.* 9: 112-116.

Ward, J.B. (1981) *Microbiol. Rev.* 45: 211-243.

Wicken, A.J. (1980) in *Bacterial Adherence, Receptors and Recognition* (Beachey, E.H. ed.) Series B, vol. 17, pp.139-158 Chapmann and Hall, London.

Wicken, A.J. and Knox, K.W. (1970) *J. Gen. Microbiol.* 60: 293-301.

Wicken, A.J. and Knox, K.W. (1971) *J. Gen. Microbiol.* 67: 251-254.

Wicken, A.J. and Knox, K.W. (1975a) *Science* 187: 1161-1167.

Wicken, A.J. and Knox, K.W. (1975b) *Infect. Immun.* 11: 973-981.

Wicken, A.J. and Knox, K.W. (1977) *Progress in Immunology* 3: 135-143.

Wicken, A.J. and Knox, K.W. (1980) *Biochim. Biophys. Acta* 604: 1-26.

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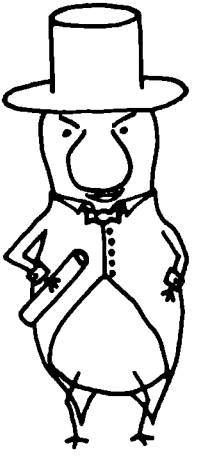
Curriculum vitae

Huub J.M. Op den Camp werd op 4 februari 1956 geboren te Roermond. In 1974 behaalde hij het diploma Atheneum-B aan het Bisschoppelijk College te Roermond waarna hij in datzelfde jaar de studie biologie aan de Katholieke Universiteit te Nijmegen begon. Het kandidaatsexamen (Blg) werd behaald op 7 juni 1977. De doctoraalstudie, met hoofdvak Microbiologie (Drs. D.B. Janssen, Dr. C. van der Drift en Prof. Dr. Ir. G.D. Vogels) en bijvakken Exobiologie II (Dr. W. Heinen) en Geschiedenis van de Biologie (Prof. Dr. P. Smit), werd afgesloten op 3 juni 1980. Tijdens de doctoraalsfase werkte hij als student-assistent bij diverse praktika voor kandidaatstudenten biologie.

Van 15 augustus 1980 tot 1 september 1983 was hij als wetenschappelijk ambtenaar in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO) via de Stichting Biologisch Onderzoek Nederland (BION). In deze periode verrichtte hij onder leiding van Prof. Dr. J.H. Veerkamp het in dit proefschrift geschreven onderzoek. Tevens werd gedurende deze tijd een bijdrage geleverd in het onderwijs aan studenten medicijnen.



Stellingen

I

De tegenstrijdige resultaten verkregen bij de omzetting van acetaat naar methaan door *Methanosarcina barkeri* stam MS en stam 227 geven aanleiding om te veronderstellen dat deze stammen niet als één soort beschouwd mogen worden.

Krzycki, J.A. & Zeikus, J.G. (1984) *FEMS Microbiol. Lett.* 25: 27-32.
Baresi, L. (1984) *J. Bacteriol.* 160: 365-370.

II

Het verdient aanbeveling om bij immuunchemische bepaling van kruisreactiviteit, met betrekking tot oppervlakte antigenen, naast intacte cellen ook extracten van de bacteriële celenvelop te bestuderen.

Dit proefschrift, hoofdstuk 6.
Knox, K.W. & Wicken, A.J. (1973) *Bact. Rev.* 37: 217-257.

III

Aangezien extra toediening van leucine als enig vertakt aminozuur resulteert in verminderde groei is het onbegrijpelijk dat Mendell *et al.* leucine getest hebben als potentieel geneesmiddel voor Duchenne spierdystrofie.

Mendell, J.R., Griggs, R.C., Moxley, R.T., Fenichel, G.M., Brook, M.H., Miller, P., Province, M.A., Dodson, W.E. & the CIDD-group (1984) *Muscle & Nerve* 7: 535-541.
Harper, A.E., Miller, R.H. & Block, K.P. (1984) *Ann Rev. Nutr.* 4: 409-454.
Block, K.P. & Harper, A.E. (1984) *Metabolism* 33: 559-566.

IV

De veronderstelling van Inouye *et al.* dat de sequentie van de promoter regio van de TOL genen in *Pseudomonas putida* afwijkt van de consensus sequentie van *Escherichia coli* promoters wordt onvoldoende ondersteund door de beschreven experimenten.

Inouye, S., Ebina, Y., Nakazawa, A. & Nakazawa, T. (1984) *Proc. Natl. Acad. Sci. USA* 81: 1688-1691.

V

Het is onwaarschijnlijk dat adsorptie van radioactieve metalen door microorganismen een belangrijke rol zal spelen bij het oplossen van het radioactief afval probleem.

Strandberg, G.W. (1982) in Impact of applied genetics in pollution control (Kulpa jr., C.F., Irvine, R.L. & Sojka, S.A., eds.) pp. 126-142.

VI

Bij een combinatietherapie van doxorubine en 5-fluorouracil moet de arts rekening houden met de mogelijkheid van een cumulatieve cardiotoxiteit.

Beretta, G., Labianca, R., Montinari, F. & Luporini, G. (1982) *J. Cancer Res. Clin. Oncol.* 104: 321-322.
Azuma, J., Sperelakis, N., Hasegawa, H., Tanimoto, T., Vogel, S., Agura, K., Awata, N., Sawanura, A., Harada, H., Ishiyama, T., Morita, Y. & Yamamura, Y. (1981) *J. Molec. Cell. Cardiol.* 13: 381-397.

VII

Researchgroepen die onderzoek doen aan "identieke" lipide bindende eiwitten dienen ook dezelfde nomenclatuur te gebruiken.

Dempsey, M.E. (1984) *Curr. Topics Cell. Regul.* 24: 63-86.

Het feit dat *Lactobacillus bifidus* reeds in Bergey's Manual van 1974 als *Bifidobacterium bifidum* vermeld wordt is, gezien de etikettering, nog steeds niet doorgedrongen tot een aantal fabrikanten van melkprodukten.

Rogosa, M. (1974) in Bergey's Manual of Determinative Bacteriology, 8th ed. (Buchanan, R.E. & Gibbons, N.E., eds.) Williams and Wilkins, Baltimore pp. 669-676.

IX

De term openbaar (=voor iedereen toegankelijk) vervoer heeft door de prijsstelling zijn oorspronkelijke inhoud verloren.

X

Een goede en open vertrouwensrelatie tussen de Universiteit en het bedrijfsleven is voor beide partijen van groot, zoniet vitaal belang.

XI

Tegenvallende bowlingscores zijn meestal niet te wijten aan slechte baancondities maar aan de bowler zelf.

Nijmegen, 12 april 1985 H.J.M. Op den Camp