Identification of the Group G Antigen of Lactobacilli

By KENNETH W. KNOX,¹* LINDY K. CAMPBELL,¹
JUDY D. EVANS² AND ANTHONY J. WICKEN²

¹ Institute of Dental Research, United Dental Hospital, Sydney,
New South Wales 2010, Australia

² School of Microbiology, University of New South Wales, Kensington,
New South Wales 2033, Australia

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The group G antigen of lactobacilli was identified as a negatively-charged cell wall polysaccharide. The components of the preparation isolated from cell walls of *L. salivarius* subsp. *salivarius* by mild acid hydrolysis were glucose, galactose and lesser amounts of rhamnose, *N*-acetylglucosamine and phosphate. Quantitative serological studies on acid-released polysaccharide and enzymic lysates of cell walls showed that rhamnose was the immunodominant component. The antigen was also detected in *L. salivarius* subsp. *salicinus* but not in a recent isolate of *L. salivarius*, strain IV CL-37.

INTRODUCTION

The serological classification of lactobacilli developed by Sharpe (1955) enabled the majority of the examined strains to be classified into one of six serological groups designated A to F, and the respective grouping antigens have been identified (Sharpe, 1970; Knox & Wicken, 1976). Group G was defined as a result of later studies on strains of *Lactobacillus salivarius* in which it was shown that strains representative of the two subspecies *salivarius* and *salicinus* could be distinguished serologically from the previously defined groups (Rogosa & Sharpe, 1959; Sharpe, 1970). *Lactobacillus salivarius* has been shown to be prevalent in dental plaque and carious dentine (Bowen, 1967) and also in infected dental root canals (Sundqvist & Carlsson, 1974). Recently, a human isolate identified physiologically as *L. salivarius* (Jacques *et al.*, 1980) has been shown to cause dental caries in experimental animals (Fitzgerald *et al.*, 1980). The latter observation prompted an investigation aimed at identifying the group G antigen. These studies with type strains showed that the antigen is a cell wall polysaccharide but that the antigen is not detectable in the cariogenic strain.

METHODS

Organisms. Two subspecies of *L. salivarius* were identified in the studies by Rogosa and co-workers (Rogosa *et al.*, 1953; Sharpe, 1970). The respective type strains, *L. salivarius* subsp. *salivarius* (ATCC 11741) and *L. salivarius* subsp. *salicinus* (ATCC 11742), were kindly supplied by Dr M. E. Sharpe, National Institute for Research in Dairying, Reading, and Dr M. Rogosa, National Institute of Dental Research, Bethesda, Md, U.S.A., respectively.

The cariogenic strain of *L. salivarius* designated IV CL-37 was isolated and kindly supplied by Dr R. J. Fitzgerald, Veterans Administration Hospital, Miami, Fla, U.S.A.; the strain has the general physiological characteristics of this species but lacks the specific features of the two known subspecies (Jacques *et al.*, 1980).

Lactobacillus casei NIRD H831, which belongs to serological group B, was available from previous studies (Knox, 1963).

Medium. Organisms were grown for 17 h under anaerobic conditions (95 % N₂/5 % CO₂) without pH control at 37 °C in MRS medium (de Man *et al.*, 1960) containing 2 % (w/v) glucose. Organisms were harvested, washed twice with 0.85 % (w/v) NaCl and stored frozen.

Preparation of cell walls. Organisms were disrupted in a Braun model MSK cell homogenizer (Braun, Melsungen, West Germany) and the cell wall fraction was isolated as described previously (Campbell *et al.*, 1978).

Lysis of cell wall by an N-acetylmuramidase. Lysates of whole organisms and of cell walls were obtained by incubation with the M-1 enzyme preparation from *Streptomyces globisporus* 1829 under conditions similar to those used previously (Knox *et al.*, 1979). The preparation, which contains an N-acetylmuramidase, was kindly supplied by Dr K. Yokogawa, Dainippon Pharmaceutical Co. Ltd, Osaka, Japan (Yokogawa *et al.*, 1975). Incubation of 100 mg wall from *L. salivarius* subsp. *salivarius* with 2 mg enzyme at 37 °C and pH 5.4 for 40 h yielded 70 mg of soluble non-diffusible material.

Lysis of cell wall from *L. casei* NIRD DECP with a preparation of *Streptomyces albus* muralytic enzyme provided a source of group B antigen for serological tests. The fractionated lysate (fraction II) was available from previous studies where it was shown that rhamnose was the major and also the immunodominant component (Knox, 1963).

Cell wall polysaccharide. Cell wall from *L. salivarius* subsp. *salivarius* (200 mg) was heated at 60 °C in 0.05 M-H₂SO₄ (20 ml) for 20 min, the soluble fraction was recovered by centrifugation and the residue was heated for a further 20 min in 20 ml 0.05 M-H₂SO₄; the procedure was repeated until the total period of heating equalled 2 h. The soluble extracts were pooled, dialysed and freeze-dried to yield 50 mg polysaccharide. This procedure, which was similar to that used previously with other lactobacilli (Hall & Knox, 1965), was chosen as a result of preliminary experiments where it was shown that the percentage of the total cell wall carbohydrate present in the soluble fraction after heating in 0.05 M-H₂SO₄ at 60 °C for 1, 2 and 3 h was 25, 30 and 35 %, respectively. The product was tentatively designated as a polysaccharide on the basis of a carbohydrate content of 52 % as determined by the primary cysteine-sulphuric acid reaction with glucose as standard (Dische, 1955).

Serology. Antisera were prepared in male New Zealand white rabbits against *L. salivarius* subsp. *salivarius*, *L. salivarius* IV CL-37 and *L. casei* NIRD H831 by the intravenous injection of heat-killed organisms in 0.85 % NaCl at 2 to 3 d intervals according to the following schedule: 0.2 ml, 0.5 ml, 1.0 ml and 1.5 ml of suspension with an absorbance of 1.0 at 600 nm in a 1 cm cell, followed by 1.0 ml and four injections of 1.25 ml of suspension with an *A*₆₀₀ of 2.0. Rabbits were bled 7 d after the last injection.

A qualitative examination of the reactivity of antisera was made by examining extracts in the ring precipitin test; the extracts were obtained by the action of M-1 enzyme on organisms and cell wall preparations, and by extracting organisms by the Lancefield procedure in which approximately 60 mg cells were heated in 2 ml 0.1 M-HCl at 100 °C for 15 min.

Antisera were further examined by the quantitative precipitin method (Knox *et al.*, 1970) and by gel electrophoresis (Campbell *et al.*, 1978); the electrophoretic studies were kindly performed by Lyn Hardy.

Chemical procedures. Hydrolysis of polysaccharide and cell wall preparations was carried out in 1, 2 and 4 M-HCl for various periods at 100 °C in sealed tubes. Samples for chromatographic analysis were dried *in vacuo* over P₂O₅ and NaOH while samples for quantitative analysis were neutralized immediately with NaOH of equivalent molarity. Paper chromatography of acid hydrolysates and appropriate standards was carried out in the following solvent systems: (I) propan-1-ol/aqueous ammonia (sp.gr. 0.88)/water (6:3:1, by vol.; Hanes & Isherwood, 1949), Whatman paper no. 4, ascending; (II) butan-1-ol/pyridine/water (6:4:3, by vol.; Jeanes *et al.*, 1951), Whatman paper no. 1, descending; (III) ethyl acetate/pyridine/water (5:2:5, by vol., upper layer; Sastry & Kates, 1964), Whatman paper no. 1, descending. Spray reagents were those described previously (Wicken & Knox, 1970). Glucose was determined by the glucose oxidase method of Huggett & Nixon (1957), galactose by galactose dehydrogenase (Wallenfels & Kurz, 1962), rhamnose by the procedure of Dische & Shettles (1948), glucosamine by the procedure of Strominger *et al.* (1959) and phosphate by the procedure of Ames (1966). Extraction of cell walls with trichloroacetic acid and hot aqueous phenol was performed as described previously (Wicken & Knox, 1970; Wicken *et al.*, 1973).

RESULTS AND DISCUSSION

Qualitative serological examination of L. salivarius strains

Antisera to *L. salivarius* subsp. *salivarius* gave a strong, positive ring precipitin test with M-1 enzyme digests of homologous organisms and cell walls, and with Lancefield acid extracts of organisms. The antisera also reacted strongly with Lancefield acid extracts of *L. salivarius* subsp. *salicinus*. However, enzymic and acid extracts of *L. salivarius* IV CL-37 failed to give a detectable serological reaction and antisera to this strain failed to give a reaction with extracts of homologous organisms or with extracts of the type strains. These results confirm that the two type strains of *L. salivarius* subsp. *salivarius* and subsp. *salicinus*

Fig. 1. Immunoelectrophoresis of soluble extracts from *L. salivarius* subsp. *salivarius* against homologous antiserum (rabbit 546). The respective contents of wells 1, 2 and 3 were cell wall polysaccharide (2.5 mg ml^{-1}), M-1 lysate of cell wall (10 mg ml^{-1}) and M-1 lysate of whole cells (12.5 mg ml^{-1}). Electrophoresis was run at 7.5 V cm^{-1} for 60 min at 10°C with the anode (+) being on the right of the plate.

belong to the same serological group (Rogosa & Sharpe, 1959) but indicate that the cariogenic strain IV CL-37 lacks the grouping antigen. The cell wall of IV CL-37 lacks detectable amounts of rhamnose and this probably accounts for the negative reaction as the results described below show that rhamnose is the immunodominant component of the group antigen.

Rhamnose is also the immunodominant component of group B lactobacilli (Knox, 1963) and for this reason the possibility of a cross-reaction was examined. However, in the qualitative precipitin test there was no detectable reaction of group B antiserum with *L. salivarius* subsp. *salivarius* extract or of the *L. salivarius* subsp. *salivarius* antiserum with group B antigen.

The polysaccharide fraction obtained from the cell wall of *L. salivarius* subsp. *salivarius* by dilute acid hydrolysis was examined by immunoelectrophoresis against homologous antiserum. The results (Fig. 1) showed that there was a major negatively charged component and a minor faster-moving component. The faster-moving component was more apparent in enzymic digests of cell wall and whole cells (Fig. 1). The major component is presumed to be the group G antigen and the charge to be due to the phosphate subsequently shown to be present.

Chemical analysis of cell walls of L. salivarius subsp. salivarius and the isolated polysaccharide fraction

To identify the group G antigen, cell wall fractions from the type strain *L. salivarius* subsp. *salivarius* ATCC 11741 were analysed. Sharpe and co-workers (1964) recorded that the cell walls of unspecified group G strains lacked a teichoic acid though the results of the analyses were not given.

The negative charge of the antigen (Fig. 1) suggested the presence of a phosphate component and quantitative analysis of fractions from *L. salivarius* subsp. *salivarius* showed the presence of $0.38 \mu\text{mol}$ phosphorus per mg dry wt cell wall and $0.49 \mu\text{mol}$ phosphorus per mg dry wt polysaccharide extracted with hot dilute acid. However, extraction of cell walls with cold 10% (w/v) trichloroacetic acid and hot 45% (w/v) aqueous phenol (methods which have been used to extract wall and membrane teichoic acids, respectively) failed to solubilize any phosphate-containing material. In further confirmation of the absence of teichoic acids, paper chromatography in solvent I of acid and alkali hydrolysates of cell wall and the polysaccharide fraction did not show the presence of polyols or polyol-phosphates.

The products released on hydrolysis of the polysaccharide preparation for 3 h in 2 M-HCl at 100°C were examined by paper chromatography in solvents II and III. The carbohydrate

Table 1. *Estimation of carbohydrate components of L. salivarius subsp. salivarius polysaccharide following hydrolysis in HCl at 100 °C for different periods*

HCl concn (M)	Time (h)	Carbohydrate component [μg (mg polysaccharide) $^{-1}$]			
		Glucose	Galactose	Rhamnose	Glucosamine
2	2	253*	413*	85.3*	14.7
2	3	259	416	76.0	16.0
2	4	245	405	69.3	18.7
4	4	53	188	10.7	25.3*

* Data used in calculation of molar ratios of component sugars (see text).

components detected were glucose, galactose, rhamnose and a hexosamine with the chromatographic properties of glucosamine; confirmation of glucosamine was obtained by ninhydrin oxidation to the corresponding pentose, arabinose.

Tue M-1 digest of cell wall showed the same monosaccharide constituents together with the expected peptidoglycan components. The molar ratio of glucose to phosphorus in the M-1 digest was 2.36:1.00 compared with 2.86:1.00 for the polysaccharide fraction (see below).

Acid hydrolysis (2 M-HCl, 3 h) of the polysaccharide also released phosphorus as inorganic phosphate. Alkaline hydrolysis (1 M-NaOH, 3 h, 100 °C) followed by chromatography in solvent I indicated the presence of a presumptive sugar phosphate, which was distinguished from polyol-phosphate by its mobility ($R_{\alpha\text{-glycerophosphate}} = 0.86$). Treatment of the polysaccharide fraction with alkaline phosphomonoesterase failed to release inorganic phosphate.

Incomplete hydrolysis of the polysaccharide fraction on heating at 100 °C for 3 h in 2 M-HCl was indicated by the presence of presumptive oligosaccharides near to the origin of chromatograms; different conditions of hydrolysis were therefore examined. The results (Table 1) showed that the maximum amounts of glucose, galactose and rhamnose were detectable after 2 to 3 h hydrolysis in 2 M-HCl whereas maximum hexosamine was detectable on longer hydrolysis in 4 M-HCl. A combination of these results gave a molar ratio of D-glucose:D-galactose:rhamnose:glucosamine:phosphorus of 2.86:4.67:1.05:0.24:1.0 and represents a recovery of 82.5 % of the weight of the polymer (without allowing for water of hydrolysis). On the basis of the total carbohydrate content of the cell wall and the isolated polysaccharide as determined by the primary cysteine-sulphuric acid reaction, the polysaccharide represents approximately 40 % of the cell wall, a value which is similar to that for grouping antigens in other lactobacilli.

The difficulty in achieving complete hydrolysis of the polysaccharide, as indicated by paper chromatography and the results of quantitative analysis for glucosamine, may be related to the presence of the sugar phosphate detected in alkaline hydrolysates. A recent study (Yadomae *et al.*, 1979) on the structure of the pneumococcus type XIX polysaccharide showed the presence of phosphoric acid esters that were essentially resistant to hydrolysis in 4 M-HCl at 100 °C for 3 h. The components of this polysaccharide were D-glucose, L-rhamnose and N-acetylmannosamine, which was phosphorylated, and thus it resembles the *L. salivarius* polysaccharide.

Quantitative precipitin studies on the group G antigen

Antisera prepared by injecting rabbits 545 and 546 with *L. salivarius* subsp. *salivarius* were examined by the quantitative precipitin method for reactivity with the M-1 digest of cell wall; the amounts of antibody precipitated were 0.53 and 0.29 mg ml $^{-1}$, respectively. The reactivity with the isolated polysaccharide component was even less, as shown in Fig. 2, which compares the precipitin curves obtained with 0.3 ml antiserum 545. The greater reactivity of the

Fig. 2. Reaction between soluble cell wall fractions from *L. salivarius* subsp. *salivarius* and 0.3 ml homologous antiserum (rabbit 545) as determined by the quantitative precipitin reaction: ●, soluble fraction obtained with M-1 enzyme; ■, polysaccharide fraction released by dilute acid.

Table 2. Inhibition of precipitin reaction between group G antiserum and preparations of homologous antigen by component carbohydrates

The reactions of two antigen preparations from *L. salivarius* subsp. *salivarius* were examined, namely the M-1 lysate of cell wall (15 μg) with 0.2 ml antiserum 545 and the isolated polysaccharide (7.5 μg) with 0.3 ml antiserum 545. Results are expressed as the percentage inhibition of reaction as determined by the quantitative precipitin method.

Antigen	Inhibitor	Percentage inhibition		
		Amount of inhibitor (μmol):		
		25	50	100
M-1 lysate	Rhamnose	34	47	57
	Glucose	—	18	26
	Galactose	—	20	38
	N-Acetylglucosamine	—	6	16
Polysaccharide	Rhamnose	43	53	68
	Glucose	—	22	36
	Galactose	—	31	40
	N-Acetylglucosamine	—	13	20

—, Not tested.

wall lysate is probably due to the higher molecular weight of this fraction, as this is known to be an important factor in determining the precipitability of antibody.

The specificity of group G antiserum was examined by determining the ability of the carbohydrate components to inhibit the precipitin reaction between antiserum to *L. salivarius* subsp. *salivarius* and two preparations of antigen, namely the M-1 lysate of cell wall and the polysaccharide isolated following dilute acid hydrolysis. The results (Table 2) show that in each case rhamnose is the most effective inhibitor and therefore is the immunodominant component. The similarity between the results obtained with the two preparations also indicates that they are similar in their antigenic specificities. From this it may be concluded that the additional minor antigenic component, which is present in greater amounts in wall lysates, does not have a significant effect on the specificity of the reaction and that the relatively small amount of polysaccharide antigen released by dilute acid is representative of the total cell wall polysaccharide. Further, the previously noted low molar proportion of rhamnose in relation to glucose and galactose together with the results of the serological studies would be consistent with a polysaccharide structure in which rhamnose units are predominantly present as terminal units in side-chains attached to a 'core' composed primarily of glucose and galactose.

The identification of the group G antigen as a phosphorylated polysaccharide distin-

guishes this antigen from those present in the other six groups. In groups B and C the antigens are polysaccharides (Knox, 1963) and in the remaining groups they are teichoic acids – wall glycerol teichoic acids in groups A and E, a ribitol teichoic acid in group D and a lipoteichoic acid in group F (Sharpe, 1970; Knox & Wicken, 1976).

An alternative serological nomenclature has recently been proposed by Shimohashi and co-workers (1976, 1977). A comparison of the two systems is difficult as the Shimohashi system presents antigenic formulae based on a complex array of reactive components that are given numerical sequence but have not been identified. This is in sharp contrast to the system that identifies the seven serological groups A to G on the basis of their respective major and characterized antigens, and for this reason, the latter system would still appear to be preferable.

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