Specific Antigens of *Lactobacillus acidophilus*

By HIROTAKA SHIMOHASHI AND MASAHICO MUTAI

*Yakult Institute for Microbiological Research, 1796 Yaho,
Kunitachi, Tokyo 186, Japan*

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Antigens specific for *Lactobacillus acidophilus* were investigated by double immunodiffusion in agar-gel. Antigenic materials were extracted from whole bacteria and some walls with cold trichloroacetic acid. Antisera were prepared by intravenous injection into rabbits of suspensions of whole organisms in solutions of bovine serum albumin, which had been heated and then washed. Four specific antigens were found as precipitinogens and denoted as antigens 11, 12, 13 and 14. Of 43 strains of *L. acidophilus* studied, 33 strains possessed antigen 11, six strains antigen 12, two strains antigen 13 and two strains antigen 14. Sugar compositions of wall preparations were analysed in an attempt to characterize the determinants of antigens 11 and 12. The walls contained glucose, galactose, hexosamine and sometimes glycerol, but no rhamnose was found. It was considered that α -glucopyranose was the major component of the determinant of antigen 11 since trehalose and maltose significantly inhibited the reaction between antibody 11 and its antigen; the determinant of antigen 12 was not clarified.

INTRODUCTION

Lactobacilli, particularly *L. acidophilus*, *L. fermentum* and *L. salivarius*, are normally found in the human intestine, mouth and saliva as well as in the faeces of animals. Physiological criteria have generally been used for determining the species even though most lactobacilli except *L. acidophilus* have been divided serologically into groups A to G (Sharpe, 1970). There is thus a need for the serological classification of *L. acidophilus* to be developed because of the wide distribution of this species.

Two main studies on the antigenic composition of *L. acidophilus* have been reported. Efthymiou & Hansen (1962) showed that *L. acidophilus* has many agglutinogens, some of which are also present in other lactobacilli, although they could not find a common antigen to this species. They recommended the use of live bacteria for the production of agglutinins rather than heated cells which have generally been employed for the preparation of precipitins. Sharpe (1970) reported antigens specific for *L. acidophilus* in the serological classification of lactobacilli by ring precipitin reactions, and assigned 17 of the 35 strains tested to two groups based on the specific antigens. Three antigens specific for *L. acidophilus* were shown, one of which consisted of a glycerol teichoic acid in the cell wall. However, neither study is completely satisfactory: the first because of the occurrence, in our experience, of spontaneous agglutination in many strains, and the other because only about one-half of the strains tested could be classified.

Subsequently, Sharpe *et al.* (1973) showed that an antiserum to *L. acidophilus* A-22 reacted with HCl extracts of strains of lactobacilli and some other Gram-positive cocci and that the antigenic material was a glycerol teichoic acid present in the cell membrane. Independently, we (Shimohashi, Kamiyama & Arai, 1973) also demonstrated a common antigen (designated

antigen 3) in the reaction of extracts of lactobacilli and staphylococci with an antiserum to *L. casei* C-5 (NIRD RO-94).

Preliminary experiments suggested that the preparation of suitable antisera for the serological classification of *L. acidophilus* was a difficult problem. Satisfactory antisera could not be obtained by methods using either live or heated bacteria. Fukazawa, Shinoda & Nakase (1973) obtained high titred antisera to the polysaccharides of *Cryptococcus neoformans* when rabbits were immunized with organisms conjugated with bovine γ -globulin by heating. Doyle *et al.* (1975) indicated the abilities of bacterial teichoic acids to bind bovine serum albumin (BSA), although the complexes were formed in 50 to 97% ethanol solution. We therefore mixed bacteria and BSA solution and heated the mixture in the hope of consolidating binding to the bacteria and increasing aggregation of BSA, which is known to increase the immunogenic activity (Draper & Hirata, 1968). Antisera obtained when treated and washed bacteria were used for immunization allowed the strains tested to be divided into groups. Each group possessed its own antigen, as determined in gel diffusion tests.

METHODS

Bacterial strains. Forty-three strains of *L. acidophilus* were used in the study. Of these, four strains (A-1, A-4, A-11 and A-28) were kindly supplied by Dr M. E. Sharpe, National Institute for Research in Dairying, Reading; six strains were obtained from the American Type Culture Collection; nine strains were isolated from human faeces; two strains were isolated from fermented milk; and two strains were of unknown origin (Table 1). The remaining 20 strains were isolated from rats (Table 2). The isolates were identified as *L. acidophilus* by physiological tests (Rogosa & Sharpe, 1959) and the biotype of each strain, based on the fermentation patterns of trehalose, melibiose and raffinose according to Mitsuoka (1969), was determined. In addition, some strains of other species of lactobacilli listed previously (Shimohashi, 1975) were used to examine cross-reactions. Organisms grown in MRS broth (de Man, Rogosa & Sharpe, 1960) at 37 °C for 18 to 24 h and then washed were used for antigen extraction, wall preparation or antiserum preparation.

Extraction of soluble antigens. Walls were prepared as follows: mechanically disrupted bacteria were fractionated by centrifugation and treated with trypsin and ribonuclease at 37 °C for 4 h to degrade membrane attached to the walls (Shimohashi, 1975); the disruption of bacteria was examined microscopically. Soluble antigens were extracted from whole bacteria and some wall preparations by treatment with 10% (w/v) trichloroacetic acid (TCA) for 3 days. Cold ethanol (4 vols) was added to these extracts and the precipitates formed were dissolved in 0.15 M-phosphate buffered saline, dialysed against distilled water and lyophilized as previously described (Shimohashi, 1975). For comparison, antigens were also extracted with hot dilute HCl (Sharpe, 1955).

Preparation of antisera. Procedures for antiserum preparation were tested and compared, since satisfactory antisera to *L. acidophilus* strains A-1, A-4 and A-28 were not produced by the method previously used (Shimohashi *et al.*, 1973). Bacteria were treated as follows: (1) washed bacterial suspensions in saline (0.15 M-NaCl) with an E_{600} of 13 (E_{600} 1.3 at the 10-fold dilution) were heated at 60 °C for 1 h or not heated; (2) bacteria (1 g wet wt) suspended in 25 ml saline containing 1 g bovine serum albumin (Iwai Kagaku Co., Tokyo, Japan) were heated at 65 °C for 30 min, washed four times with saline and resuspended in formalinized saline to an E_{600} of 13; (3) bacterial suspensions (E_{600} 15) were mixed with equal volumes of Freund's complete or incomplete adjuvant. Rabbits were injected intravenously at 3 day intervals with 0.3, 0.5, 0.7, 0.8, 0.9, 1.0 and 1.3 ml of the suspensions prepared by method 1 or 2, or were given five injections of 1.0 ml intramuscularly at 7 day intervals using suspensions prepared by method 3. Rabbits were test-bled 6 days after the last injections. When the sera did not form clear precipitin lines in reactions of some antigen extracts in agar-gel, several further injections of 1.0 ml were given on consecutive days. Two rabbits were used for injection of each vaccine.

Serological methods. Antigenic compositions of the extracts were examined by double diffusion tests using agar-gel with wells of 3 mm diameter and 3 mm depth. In some experiments, antisera were absorbed with whole bacteria (5 ml antiserum/1.5 g packed organisms). The charge of antigenic materials in 0.05 M-veronal/HCl buffer pH 8.6 was examined by immunoelectrophoresis at 5 mA cm^{-1} for 60 min (Brathall, 1970). Specific components of antigens were investigated by inhibition tests of precipitin reactions as described previously (Shimohashi, Kodaira & Suegara, 1976). After determining the optimum ratio for antigen-antibody reaction by the addition of increasing amounts of soluble antigen to a series of tubes containing a constant volume of antiserum, increasing quantities of various sugars were added to the antiserum. The mixtures were allowed to stand at 37 °C for 1 h and then the appropriate quantity of antigen was added to

each tube. The tubes were incubated at 37 °C for 1 h and kept at 5 °C for a further 2 days. The resultant precipitates were washed twice with cold saline, the protein contents were estimated by the method of Lowry *et al.* (1951) and the percentage of inhibition was calculated. These inhibition tests were performed in duplicate on each dilution of a sugar.

Chemical analyses. Wall preparations or extracts from the walls (10 mg dry wt) were hydrolysed in 0.5 M-HCl at 100 °C for 15 h. The hydrolysates were taken to dryness under a stream of N₂. Neutral sugars of the hydrolysates were analysed by gas chromatography after methylation and trimethylsilylation and amino-sugars were analysed by the procedure of Elson and Morgan as previously described (Shimohashi, 1975). Protein was measured as before.

RESULTS

Preparation of antisera against L. acidophilus

Lactobacillus acidophilus strains A-28, A-I, A-4 and Ra-69 of different biotypes were used for preparation of antisera. Bacteria of each strain were treated by the various methods described and injected into rabbits. Satisfactory antisera were only produced by intravenous injection of organisms treated with BSA, when clear precipitin lines against certain soluble antigens were formed in agar-gels. In a ring test, the precipitin titres of antisera were between 1:16 and 1:32 in the homologous reactions. The antisera did not react with BSA solution and MRS broth in agar-gel or in a ring test.

Antigenic composition of L. acidophilus

The reactions of TCA extracts (0.1 to 3 mg ml⁻¹ in saline) from whole organisms with undiluted anti-A-28, anti-A-I, anti-A-4 and anti-Ra-69 sera were examined by two-dimensional immunodiffusion in agar. Extracts of strains A-28, ATCC4963, LA-2 and ATCC9857 formed an identical band to anti-A-28 serum and did not react with the other three antisera; the causal antigen was designated antigen 11. In a reaction with anti-A-I serum, extracts of strains A-I, A-II and ATCC4356 had the same antigen, and they did not react with the other antisera; the antigen was designated antigen 12. Anti-A-4 serum reacted with an identical line to the extracts of strains A-4 and 790, and anti-Ra-69 serum reacted specifically with strains Ra-69 and Ra-74; these antigens were designated antigens 13 and 14, respectively. Differences in such antisera were clarified further by absorption with whole bacteria. Anti-A-28 serum absorbed with each of the homologous strains did not react with any extracts. Anti-A-I, anti-A-4 and anti-Ra-69 sera similarly lost their reactivity after absorption with each of their homologous strains. Conversely, the four antisera retained their reactivity when they were absorbed with each of the heterologous strains. In addition, all extracts possessed antigen 3, since they reacted with antiserum against *L. casei* strain C-5 (NIRD RO-94) showing the precipitation pattern specific for this antigen (Shimohashi *et al.*, 1973). No other precipitin lines were found between these extracts and the antisera.

The antigenic formulae of all strains were determined by the same methods and are shown together with their biotypes and sources in Tables 1 and 2. Antigen 11 or 12 was present in the majority of strains tested, while antigen 13 or 14 was present in only a few strains. Each of the four antigens was specific for *L. acidophilus* since extracts of other species of lactobacilli did not react with these antisera, whereas antigen 3 was present in all strains examined.

Subsequently, the cellular location of such antigens was investigated by examining whether or not they were extracted from wall preparations with TCA. Walls were obtained from strains A-28, LA-2 and ATCC4963 (antigen 11); strains A-I, A-II and ATCC4356 (antigen 12); strain A-4 (antigen 13); and strain Ra-69 (antigen 14). The wall extracts (0.1 to 2 mg ml⁻¹) reacted with the same bands as those formed by the whole organism extracts with the four antisera to *L. acidophilus*. Therefore, it appears that antigens 11, 12, 13 and 14 occur in the wall. Antigen 3 was not found in the wall preparations.

Antigens were then prepared from whole organisms or wall preparations by extraction

Table 1. *Antigenic composition of Lactobacillus acidophilus strains derived from humans and other sources*

Antigen	Biotype*	Strain	Source
11	I	ATCC9857	Vaginal tract
		ATCC19992	Faeces
		LA-1, LA-2, LA-3, LA-4, LA-5, LA-6, LA-7, LA-8, YITO163	Human faeces
		YITO164	Fermented milk
		A-28, ATCC4963, ATCC11506	Unknown
12	II	ATCC4356	Human
		YITO165	Fermented milk
		A-1†, A-II, ATCC4357, YITO097	Unknown
13	III	A-4, 790	Unknown

* Determined according to Mitsuoka (1969). † Biotype II or III.

Table 2. *Antigenic composition of Lactobacillus acidophilus strains isolated from the intestinal tract of rats*

Antigen	Biotype*	Strain
II	I	Ra-19, Ra-22, Ra-27, Ra-28, Ra-29, Ra-35, Ra-57
II	II	Ra-1, Ra-3, Ra-4, Ra-7, Ra-15, Ra-21, Ra-30, Ra-42
II	III	Ra-11, Ra-16, Ra-38
14	IV	Ra-69, Ra-74

* Determined according to Mitsuoka (1969).

with hot dilute HCl instead of cold TCA. Antigens present in HCl extracts were essentially similar to those in the TCA extracts. However, an additional antigen reacting with anti-Ra-69 serum was present in HCl extracts of whole organisms of most strains of *L. acidophilus* isolated from rats (with the exception of strains Ra-7 and Ra-57) and also in strains A-4 and 790, although it was not found in extracts (HCl or TCA) of walls or in TCA extracts of the whole organisms. This antigen is not named here because the relationships between it and others present in membranes of other lactobacilli have not been investigated in detail, but it was clearly different from antigen 3. Antigens having such characteristics have been found in some other species of lactobacilli (Shimohashi, 1975; Shimohashi *et al.*, 1976). This antigen was also found in all strains of *L. casei*, *L. bulgaricus*, *L. fermentum* and *L. salivarius* examined, and in some strains of *L. helveticus* and *L. plantarum*.

Chemical analyses of wall preparations and extracts from the walls

Neutral sugars, amino-sugars and the protein content of wall preparations and of TCA extracts of isolated walls were analysed (Table 3). The walls and extracts of strains A-28 and LA-2 contained large quantities of glucose, and trace amounts of glycerol. In both preparations (wall and extract), the glucose content of strain A-4 was higher than that of strain A-1, but considerably lower than that of strain A-28; strains A-1 and A-4 also contained a small amount of glycerol. The amounts of material extracted from walls of strains A-1 and A-4 were small compared with those extracted from the other strains (about 3 and 10% respectively of the amount extracted from strain A-28). Both preparations of strain Ra-69 contained larger amounts of galactose than did the other strains examined.

Characteristics of antigens 11 and 12

The electrophoretic mobilities of antigens 11 and 12 in wall extracts prepared by extraction with TCA were compared by immunoelectrophoresis using 0.05 M-veronal/HCl buffer pH 8.6 (Fig. 1). Extracts of strains A-28 and LA-2 formed one precipitin arc in the reaction

Table 3. Sugar and protein composition of wall preparations of *Lactobacillus acidophilus* strains and their extracts

Extracts were prepared from wall preparations with TCA. Analysis results are expressed as percentages of total dry weight.

Preparation	Antigen present	Glycerol	Glucose	Galactose	Amino-sugar	Protein
A-28 Wall	11	tr	69.4	0.3	5.6	15.0
Extract		tr	100	0	0.2	0
LA-2 Wall	11	tr	80.0	10.3	4.4	9.0
Extract		tr	85.6	15.2	0.2	0
A-I Wall	12	5.4	4.8	16.8	6.1	30.4
Extract*		3.4	10.4	18.0	4.6	14.2
A-4 Wall	13	4.7	12.7	10.0	5.9	32.3
Extract*		4.0	40.4	21.8	2.8	6.2
Ra-69 Wall	14	0.1	3.4	54.4	6.0	19.4
Extract		0.1	3.3	95.3	0.8	0

tr, Trace.

* The lyophilized extract contained material insoluble in 0.05 M-HCl (or saline) before hydrolysis and unknown sugar(s).

Fig. 1. Immunoelectrophoretic patterns of antigens 11 and 12 in wall extracts of *Lactobacillus acidophilus* strains. Immunoelectrophoresis was performed using 0.05 M-veronal/HCl buffer pH 8.6 at 5 mA cm⁻¹ for 60 min. Extracts of: 1, strain A-28; 2, strain LA-2; 3, strain A-I; 4, strain A-II. a, Anti-A-28 serum; b, anti-A-I serum.

with anti-A-28 serum, while a second arc was present in the reaction of anti-A-I serum with extracts of strains A-I and A-II.

Immunologically dominant components of antigens 11 and 12 were examined by quantitative precipitin reactions. When sugars which inhibit antibody 11 were compared, trehalose [(1 → 1)-α] was the most effective inhibitor of the reaction system (Table 4). The results demonstrate that α-glucosidic linkages inhibit the reaction whereas β-glucosidic linkages do not. In contrast, when attempts were made to inhibit the reaction of antigen 12 and its antibody using the extract of strain A-I or A-II and anti-A-I serum, no effective inhibitor was found although glucosamine (100 μmol) caused slight inhibition.

Anti-A-28 serum was absorbed with whole organisms of antigenically heterologous strains A-I and A-4 to remove antibodies produced in low levels against common antigens present in membranes. The absorbed anti-A-28 serum gave a cross-reaction with dextrans (Sigma) from *Leuconostoc mesenteroides*, but not with soluble starch or oyster glycogen

Table 4. *Inhibitory effects of sugars on the precipitin reaction between antigen 11 and its antibody*

The inhibition test was carried out using the reaction between the wall extract (30 µg) of strain A-28 and anti-A-28 serum. (A similar result was obtained for the reaction between the wall extract of strain LA-2 and anti-A-28 serum.)

Sugar	Inhibition (%)		
	100 µmol	50 µmol	10 µmol
D-Galactose	0	0	0
D-Glucose	26.3	15.0	7.3
Methyl- α -D-glucoside	43.9	34.0	16.3
Methyl- β -D-glucoside	15.2	8.9	0
Trehalose [(1 $\rightarrow$ 1)- α]	82.0	61.5	50.9
Maltose [(1 $\rightarrow$ 4)- α]	47.3	39.2	25.2
Cellobiose [(1 $\rightarrow$ 4)- β]	14.9	8.0	0
Gentiobiose [1 $\rightarrow$ 6)- β]	13.9	8.8	0

Table 5. *Cross-reaction of dextrans with antiserum to Lactobacillus acidophilus strain A-28*

Anti-A-28 serum was absorbed with whole organisms of strains A-1 and A-4.

Dextran	Molecular weight	Cross-reaction* (%)	Reaction in capillary tube with dextran at (mg ml ⁻¹):			
			1	0.1	0.01	0.001
D-3759	72000	16	+	+	±	—
D-4876	170000	28	+	+	+	—
D-5376	2000000	45	+	+	+	—
Wall extract of strain A-28		100	+	+	+	±

* Values determined by quantitative precipitin reactions.

(Sigma) (Table 5). Table 5 also shows the maximum amount of antibody in the antiserum precipitated by the dextrans, expressed as a percentage of the maximum amount precipitated by the wall extract of strain A-28 in the quantitative precipitin method. In agar-gel, the extract of strain A-28 and dextran D-5376 reacted with the anti-A-28 serum giving a spur, thus indicating a partial identity of both antigens. The antigenic material extracted from the walls of strain A-28, containing antigen 11, was a carbohydrate, hydrolysates of which contained only glucose. Moreover, α -glucosylglucose effectively inhibited the reactivity of antibody 11, and anti-A-28 serum cross-reacted with dextrans. On the basis of these observations, α -glucosylglucose is considered to be the immunodominant component of antigen 11.

DISCUSSION

Some antigens specific for *Lactobacillus acidophilus* were found as precipitinogens in agar-gel diffusion and were named in sequence from the antigens described previously (Shimohashi *et al.*, 1976). Antigen 11 was present in all the strains of biotype I (these included all strains of *L. acidophilus* isolated from human faeces), and in the strains of biotypes II and III isolated from rats. The strains having antigen 12 comprised a group belonging to biotype II, as did the two strains having antigen 13. Antigen 14 was present in two strains of biotype IV from rats; further examination of the many strains of this biotype is necessary to determine whether antigenicity and biotype are related.

Mitsuoka (1969) divided *L. acidophilus* isolated from faeces of various animals into several biotypes to show differences in the resident strains and he found that most isolates from

humans belonged to biotype I. Gilliland, Speck & Morgan (1975) have also described differences in the biochemical properties and GC contents of *L. acidophilus* isolated from faeces of humans, pigs and chickens. Earlier, Sharpe (1970) reported that the serological characteristics of *L. acidophilus* coincided with the patterns of their lactic dehydrogenases revealed by Gasser (1970). However, comparison of four strains used both in his biochemical study and in our present study did not show such good agreement between serological characteristics and enzyme patterns. For example, strains A-II and ATCC4356 both had antigen 12 and strain A-4 had antigen 13, although strains A-4 and A-II belonged to Gasser's group II and strain ATCC4356 to group III.

TCA proved to be a better agent than HCl for the extraction of the specific antigens of *L. acidophilus*. Although the antigens were extracted equally well by each method, a cross-reactive antigen, different from antigen 3, was found in HCl extracts from whole organisms of most *L. acidophilus* strains from rats and several other lactobacilli species. This was not present in the TCA extracts. Analysis of the extracts prepared from walls suggests that the antigens are carbohydrate in nature. Coyette & Ghuysen (1970) found glucose, galactose, rhamnose and glycerol in the wall of one strain of *L. acidophilus* whereas we have been unable to detect rhamnose in measurable amounts.

The determinant of antigen 11 is considered to be α -glucoside for the following reasons. Hydrolysates of wall extract of strain A-28 consisted almost entirely of glucose; anti-A-28 serum reacted with dextrans as polymers of α -linked glucose units (Larm, Lindberg & Svensson, 1971); and α -glucosylglucose effectively inhibited antibody 11. The characteristics of antigen 12 were not determined although the material was shown by immunoelectrophoresis to bear no anionic charge at pH 8.6. It therefore seems unlikely that it is a teichoic acid.

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