Chemical Composition of the Cell Wall of Lactic Acid Bacteria and Related Species

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In order to examine the relationship between biological activities and the cell wall content, the murein type and the teichoic acid of the cell wall from five strains of bacteria were studied. Two of these *Lactobacillus casei* CRL 431 and *L. acidophilus* CRL 730, are used in a commercial fermented milk (BIO MILK), which is believed to be beneficial for health. The other strains, *Lactococcus lactis* CRL 526, *Pediococcus pentosaceus* CRL 923 and *Propionibacterium acidipropionici* CRL 1198 were included in order to compare the cell wall structures of active and inactive strains.

A method was designed to confirm the amino acids of the peptidoglycan in impure substrates.

Four of the studied strains, *L. casei*, *L. acidophilus*, *L. lactis* and *P. acidipropionici*, contained glycerol teichoic acids. *L. casei*, *L. acidophilus*, *P. pentosaceus* and *L. lactis* contained A4α type murein, while *P. acidipropionici* contained A3γ type.

The capacity of orally administered peptidoglycans of the studied strains to stimulate phagocytosis by mouse peritoneal macrophages was analyzed. Only the PG of *L. casei* showed this activity. No differences were observed between active and inactive strains with respect to the chemical composition of the peptidoglycan. Therefore the biological activity is unlikely to be due to the peptidoglycan structure.

Key words murein; teichoic acid; lactic acid bacteria; propionibacteria

Bacterial cell wall fragments and peptidoglycans exhibit various activities, including: a) induction of humoral and cellular triggering, b) activation of lymphocytes, c) activation of macrophages and d) induction of chronic inflammatory response.[1)]

In our laboratory two strains of lactobacilli being *Lactobacillus acidophilus* CRL 730 and *Lactobacillus casei* CRL 431 have been isolated from the feces of healthy children. These microorganisms were used to obtain a fermented milk (Bio Milk) with inhibitory activity against various pathogens.[2,3)] Immunological studies confirmed the beneficial effect of these strains when administered orally.[4-6)] Given these observations we focused our attention on the possible relationship between the biological effect of these lactic acid bacteria and the chemical composition of their cell wall. In the present work, we studied the chemical components of the cell walls of these two bacteria, in comparison with those of several other bacteria. The latter included *Propionibacterium acidipropionici* CRL 1198, the cell walls of which stimulate the immune system,[7)] and *L. lactis* and *P. pentosaceus*, which do not show immune-stimulating properties when orally administered (data not shown). Furthermore, the capacity of orally administered peptidoglycans isolated from the different strains to stimulate phagocytosis by mouse peritoneal macrophages was examined.

The cell wall of gram-positive bacteria consists of peptidoglycan (PG) and an additional matrix containing polysaccharides, teichoic acids and proteins. In a previous paper the cell walls of the same bacterial strains were analyzed for hexosamines and neutral sugars.[8)] The present work deals with the amino acid composition of the cell wall and its PG. To define the PG-type in one of the studied strains, *L. casei* CRL 431, it was necessary to develop a new assay, which is described in materials and methods. We also examined the teichoic acids of the cell walls, since they play a role in the adhesion of microorganisms to the epithelial tissue.[9)]

Materials and Methods

Microorganisms and Growth Conditions *Lactobacillus casei* CRL 431 and *Lactobacillus acidophilus* CRL 730 were isolated from the feces of healthy children. *Propionibacterium acidipropionici* CRL 1198 was isolated from Swiss cheese, *Lactococcus lactis* CRL 526 from Tafi cheese (a local handmade cheese from the Tucumán region, Argentina) and *Pediococcus pentosaceus* CRL 923 from cabbage. The strains were grown in Laptg medium[10)] at 37 °C on a gyratory stirring machine, except for *L. lactis* CRL 526, which was incubated at 30 °C, and *P. pentosaceus* CRL 923, which was grown in MRS medium[11)] at 37 °C. After incubation, each culture was immediately cooled to 4 °C to prevent autolysis.

Cell Wall Isolation The harvested cells were washed four times with saline solution (NaCl, 0.9%) and resuspended in cold distilled water (30% w/v). The washed cells were ruptured by two passages through a French-X press at 2000 $g \cdot s \cdot cm^{-2}$ (28000 psi). The obtained suspension was heated at 75 °C for 15 min in order to remove autolytic enzymes. The cell walls were purified by differential centrifugation at 4 °C as follows[12)]: centrifugation at $1000 \times g$ for 40 min afforded a supernatant, which was centrifuged at $25000 \times g$ for 20 min. The resultant pellet was resuspended in distilled water and then centrifuged at $1000 \times g$ for 10 min to give a supernatant, which was centrifuged at $20000 \times g$ for 15—20 min to afford a pellet that contained purified cell wall material.

This procedure was carried out 3 times successively: first with water, then with 1 M NaCl and again with water. The cell wall material thus obtained was washed four times with distilled water to remove traces of cytoplasmic material. The purity of the preparations was confirmed by electron microscopy. The cell wall material was lyophilized after purification.[13)]

Preparation of Bacterial Peptidoglycan Proteins and nucleic acids were removed from the cell wall preparation by trypsin, RNase and DNase digestion. The cell walls thus obtained were resuspended in phosphate buffer (KH_2PO_4–Na_2HPO_4, 0.05 M, pH 7.0), containing 2% sodium dodecyl sulfate (SDS), and subjected to boiling for 30 min in order to remove the proteins. Then they were resuspended in the same buffer without SDS for washing, which was carried out four times in order to obtain the maximum possible removal of soluble substances.

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The teichoic acids were then removed from the resulting material by resuspending it in a 10% (w/v) trichloroacetic acid (TCA) solution (1.5 ml per 50 mg of cell wall), and stirring the mixture at 4 °C for 16 h. Then the material was centrifuged at 17000 × *g*. The supernatant contained the teichoic acids.The pellet was afterwards washed 3 times with distilled water.[14] The removal of the teichoic acids was followed by assay of organic phosphorus.

Teichoic Acid Preparation The supernatant obtained by the treatment of the cell walls with TCA was used for this experiment. Cold ethanol (2 vol.) was added to the combined extracts which were then kept at 4 °C for 24 h. Precipitated teichoic acid was removed by centrifugation and reprecipitated from 10% (w/v) TCA solution by addition of cold ethanol (1 vol.). The precipitate was kept at 4 °C for 24 h, then collected in a centrifuge, washed with cold acetone, ethanol and ether, and dried in a desiccator (vacuum).

Analytical Methods Proteins were analyzed by the method of Lowry *et al.*[15]

Ribitol and glycerol were analyzed by HPLC (Gilson), after the samples had been hydrolyzed for 2 h with 2 N H_2SO_4 at 100 °C. Samples (20 μl, loop injected) were run on an RHM-Monosaccharides column (Phenomenex, 300 × 7.8 mm) at a flow rate of 0.6 ml/min and a temperature of 55 °C. Sample components were identified and quantified by refractive index measurements (differential refractometer, Model 2142, LKB).

Amino acids were analyzed after hydrolysis of 2 mg of cell wall material in 4 mol·l^{-1} HCl at 100 °C for 4 h, using a Beckman Automatic Analyzer CL 119.

The configurations of the amino acids were determined by measuring the amino acids contents of the hydrolysates before and after incubation with D-alanine oxidase (alanine), L-lysine decarboxylase (lysine), or L-glutamic acid decarboxylase (glutamic acid), as described by Kandler and König.[16] The difference between the concentrations before and after was determined as Δamino acids (ΔAA).

The N-terminal amino acid of the interpeptide bridge was determined by dinitrophenylation of undegraded PG.[17]

The peptidoglycan structure was determined by the method of Schleifer and Kandler.[17] Partial acid hydrolysates were examined by two-dimensional TLC, and peptides were identified on the basis of their chromatographic mobilities and staining characteristics.[17]

Design of an Analytical Method to Define the Amino Acids of the Peptidoglycan Determining the amino acids in the partially purified PG of *L. casei*, we obtained very similar values for both lysine (Lys) and diaminopimelic acid (DAP). To define which was the di-aminated amino acid of the PG we designed the following assay. Samples of the PG were hydrolysed with trypsin and α-chymotrypsin, both of which endoproteinases act only on amino acids of L-configuration and not on the PG, which contains L- and D-amino acids. Therefore, this treatment will eliminate amino acids present as impurities in PG.

After this treatment we divided the material into two aliquots. One of these was dialyzed in benzoylated dialysis tubing, while the other was not. Then the amino acids of both aliquots were determined. The decrease of a certain amino acid in the dialyzed aliquot when compared with the non-dialysed aliquot indicated that this amino acid was an impurity and not a component of the PG.

Animals Swiss albino mice, each weighing 25 to 30 g, were obtained from the random-bred colony kept at our department. The animals were housed in plastic cages and kept at room temperature. Each experimental group consisted of 16 to 20 mice.

Feeding and Inoculation Procedure Animals assigned to oral administration of peptidoglycans were fed daily for 2, 5 and 7 consecutive days with 50 μg of the compound, suspended in 0.05 ml of sterile distilled water.

Macrophage Collection The mice were killed by cervical dislocation, and the peritoneal fluid was collected in 5 ml of modified Hanks medium (containing 100 U of penicillin and streptomycin per ml and 0.1% bovine serum albumin, but without glucose and stain) after gentle massage of the abdomens of the animals. Portions of the peritoneal cell suspension containing 10^6 cells per ml were used for *in vitro* phagocytosis assays.

Phagocytosis Assays *in Vitro* To measure the phagocytic activity, aliquots of peritoneal macrophages (10^6 cells per ml) were incubated for 15 min at 37 °C with the same volume of a yeast suspension (*Saccharomyces cerevisiae*; 10^7 cells per ml). The incubation was stopped in an ice-cold bath, the mixture centrifuged for 5 min at 1500 × *g*, and the sediment observed with a Zeiss microscope immersion method. We estimated the percentage of macrophage that had ingested yeast by counting 200 cells. Opsonization of yeast was carried out by incubation of a bacterial suspension (10^7 cells per ml) with fresh mouse serum for 15 min at 37 °C.

Statistical Techniques Analysis of variance for four levels, including the control, as well as a test which compares the different media, was done with the Minitab Statistic Program, release 8.21.

Results

Determination of the Teichoic Acids of the Cell Wall In a previous paper,[8] in which we studied these five strains, all strains contained teichoic acids except for *P. pentosaceus*. In the present study we have defined the teichoic acid type of the cell wall of the four strains, examining the presence of glycerol and ribitol. In all cases the polymers turned out to be glycerol-phosphate (Table 1).

Determination of the PG in the Bacterial Cell Walls The first step was qualitative and quantitative analysis of the amino acid composition of the cell wall (Table 2) and of the partially purified PG (Table 3). The highest values were used to calculate the molar ratio of each amino acid for the studied strains, which are shown in Table 4. In *L. acidophilus*, *L. lactis* and *P. pentosaceus*, the observed proportions suggest the presence of L-Lys–Asp PG type, corresponding to the A4α variation. This was as expected, according to the results of Schleifer and Kandler.[17] As the proportion in *L. casei* CRL 431 was not very clear, it was necessary to design a method to define which were the amino acids of the PG. The results are shown in Table 5. DAP can be discarded as a PG amino acid, due to its marked decrease after the treatment, and thus *L. casei* also has an L-Lys–Asp structure. *P. acidipropionici* showed a different molar ratio and PG type (DAP-Gly) (Table 4), in accordance with the results found by Schleifer and Kandler.[17]

To obtain more information about the amino acid composition of the PG we analyzed the configuration of some of the components (Table 6); D-Ala was present in all the studied PGs. Lysine (Lys) was found in four of the five strains, and only in the L-configuration. L-Glutamic acid (L-Glu) was not found in any of the studied strains, which suggests that this amino acid would be present in the D-form.

By determining the N-terminal residue of the PG we could confirm the di-aminated amino acid of these peptides. As we expected, it was DAP in *P. acidipropionici* and Lys in the other strains (Table 7).

The oligopeptides obtained by partial hydrolysis (Table 8) confirm the suggested structure and show that the amino acid sequence of the PG peptide is as illustrated in Fig. 1.

Study of the Stimulation of Peritoneal Macrophages by Peptidoglycans from the Studied Strains Figure 2 shows

Table 1. Glycerol and Ribitol Contents of the Teichoic Acids

Microorganism	Glycerol (nmol/mg of cell wall)	Ribitol (nmol/mg of cell wall)
L. acidophilus	80	—
L. casei	98.3	—
P. acidipropionici	29.8	—
L. lactis	52.1	—

Table 2. Amino Acid Composition of the Cell Walls of the Studied Microorganisms

	L. casei	*L. acidophilus*	*P. acidipropionici* (nmol/mg of cell wall)	*L. lactis*	*P. pentosaceus*
Neutral polar amino acids					
Gly	151	569	399	727	201
Tyr	40	127	26	59	60
DAP	6	20	250	2	2
Acidic hydrophilic amino acids					
Asp	115	430	56	124	388
Glu	203	740	450	772	428
Basic hydrophilic amino acids					
Lys	89	325	81	243	317
Arg	80	233	79	105	91
His	23	103	24	38	36
Neutral hydrophobic amino acids					
Ala	159	771	565	776	538
Val	144	540	138	240	188
Pro	65	183	60	176	64
Ile	89	357	70	172	148
Leu	137	406	118	214	183
Phe	63	221	47	110	88
Met	35	18	23	55	46

Table 3. Amino Acid Composition of Peptidoglycan of the Studied Microorganisms

	L. casei	*L. acidophilus*	*P. acidipropionici* (nmol/mg of cell wall)	*L. lactis*	*P. pentosaceus*
Neutral polar amino acids					
Gly	15	139	457	29	9
Tyr	3	38	11	3	2
DAP	8	16	223	9	2
Acidic hydrophilic amino acids					
Asp	24	424	50	100	390
Glu	33	515	589	125	450
Basic hydrophilic amino acids					
Lys	12	350	27	96	320
Arg	8	64	25	5	4
His	2	26	10	4	4
Neutral hydrophobic amino acids					
Ala	37	725	762	190	600
Val	12	126	47	8	7
Pro	3	50	17	4	2
Ile	6	93	21	12	10
Leu	11	122	40	13	5
Phe	3	55	19	5	9
Met	2	37	9	47	18

Table 4. Characteristics of the Peptidoglycans of the Studied Microorganisms

Microorganism	Molar ratio						Type	Variation
	Ala	Glu	Lys	Asp	DAP	Gly		
L. casei	1.12	1	0.97	0.84			L-Lys-Asp	A4α
L. acidophilus	1.41	1	0.68	0.82			L-Lys-Asp	A4α
L. lactis	1.52	1	0.77	0.8			L-Lys-Asp	A4α
P. pentosaceus	1.33	1	0.71	0.87			L-Lys-Asp	A4α
P. acidipropionici	1.30	1			0.38	0.78	DAP-Gly	A3γ

the results of determination of the phagocytic activity of peritoneal macrophages from mice. It appears that only peptidoglycans obtained from the cell wall of *L. casei* CRL 431 had a significant effect on the immune system.

Discussion

In the present study the chemical composition of the cell walls of four strains of lactic acid bacteria and one strain of a very closely related genus, *Propionibacterium*, has been analyzed. Further, it was found that orally administered peptidoglycans stimulated phagocytosis by

peritoneal macrophages in the case of *L. casei* CRL 431, but not in any of the other strains (Fig. 2).

The immunological activities of the teichoic acids of both the cell wall and the membrane are well known,[18] as is the role of these acids in the adhesion of microorganisms to epithelial tissue.[9,19] Among the strains studied here, no relationship was apparent between the teichoic acid composition and the different biological activities of these bacteria. The teichoic acids are present in both active (*L. casei*) and inactive strains (*L. lactis*, *L. acidophilus*, *P. acidipropionici*), and all were of the glycerol-phosphate type.

Table 5. Amino Acid Composition of Peptidoglycan of *L. casei* Determined by Using a Specially Designed Method

Amino acids	Non-dialyzed nmol/mg (%)	Dialyzed nmol/mg (%)
Neutral polar amino acids		
Gly	11.2 (10.7)	28.8 (10.0)
Tyr	1.7 (1.6)	5.5 (2.0)
DAP	4.8 (4.6)	1.1 (0.4)
Acidic hydrophilic amino acids		
Asp	12.7 (12.2)	31.5 (11.2)
Glu	11.1 (10.7)	31.6 (11.2)
Basic hydrophilic amino acids		
Lys	5.5 (5.3)	14.8 (5.3)
Arg	2.1 (2.0)	3.3 (1.2)
His	0.4 (0.4)	2.2 (0.8)
Neutral hydrophobic amino acids		
Ala	10.6 (10.2)	26.8 (9.6)
Val	10.6 (10.1)	33.5 (11.9)
Pro	4.8 (4.6)	13.1 (4.7)
Ile	4.1 (5.0)	11.3 (4.0)
Leu	8.4 (8.1)	23.4 (8.3)
Phe	2.9 (2.8)	8.9 (3.2)
Met	0.4 (0.4)	2.5 (0.9)

Table 6. Configuration of the Amino Acids of the Peptidoglycan

Microorganism	D-Ala	L-Lys	L-Glu
	ΔAA (nmol/mg PG)		
L. casei	+	+	−
	(10)	(11)	(0)
L. acidophilus	+	+	−
	(250)	(280)	(0)
L. lactis	+	+	−
	(60)	(78)	(0)
P. acidipropionici	+	−	−
	(120)	(0)	(0)
P. pentosaceus	+	+	−
	(140)	(190)	(0)

With respect to the PG structure of the cell wall, the bacteria used in the fermented milk, which has a proven positive effect on the immune system, did not show any difference in the PG peptides from those of the other strains of the same species. Thus, the biological activity is not related to a different murein type. Moreover, the A4α variation of the PG is not only present in an active immune-stimulating strain (*L. casei*), but also in inactive strains (*L. lactis*, *L. acidophilus* and *P. pentosaceus*). *P. acidipropionici*, another active strain, has a different PG structure: A3γ. From these results we may conclude that neither the teichoic acid type nor the chemical structure of the PG determines the immune-stimulating ability observed upon oral administration of the studied bacteria. Nevertheless, cell wall components of both active and inactive bacteria do have an immune stimulating capacity when inoculated intravenously or subcutaneously, as has been widely demonstrated for other bacteria.[20]

Further studies are necessary to characterize the stimulating activity of the isolated cell walls and their components on the immune system, and to elucidate the mechanism involved. The method described here to define

Table 7. Amino Acids of the *N*-terminal Residue of the PG Peptides

Microorganism	Di-aminated amino acid
L. casei	Lys
L. acidophilus	Lys
L. lactis	Lys
P. acidipropionici	DAP
P. pentosaceus	Lys

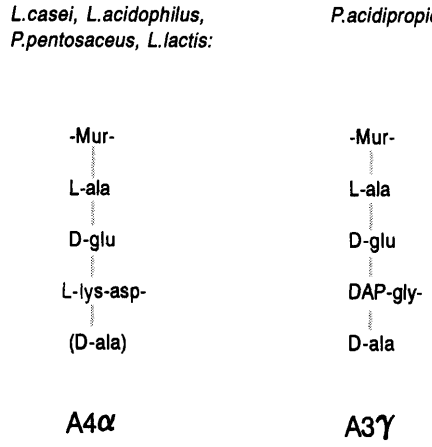

Fig. 1. Peptide Structure of the Peptidoglycan

Table 8. PG Oligopeptides Obtained by Partial Hydrolysis

Microorganism	Possible oligopeptide sequence according to Rala[a]				
	L-Lys–D-Ala	Mur–L-Ala	D-Glu–DAP–D-Ala	D/L-Glu–L-Lys	DAP–D-Ala
L. casei	+	+	−	+	−
L. acidophilus	+	+	−	+	−
L. lactis	+	+	−	+	−
P. acidipropionici	−	+	+	−	+
P. pentosaceus	+	+	−	+	−

a) Rala: Alanine relative.

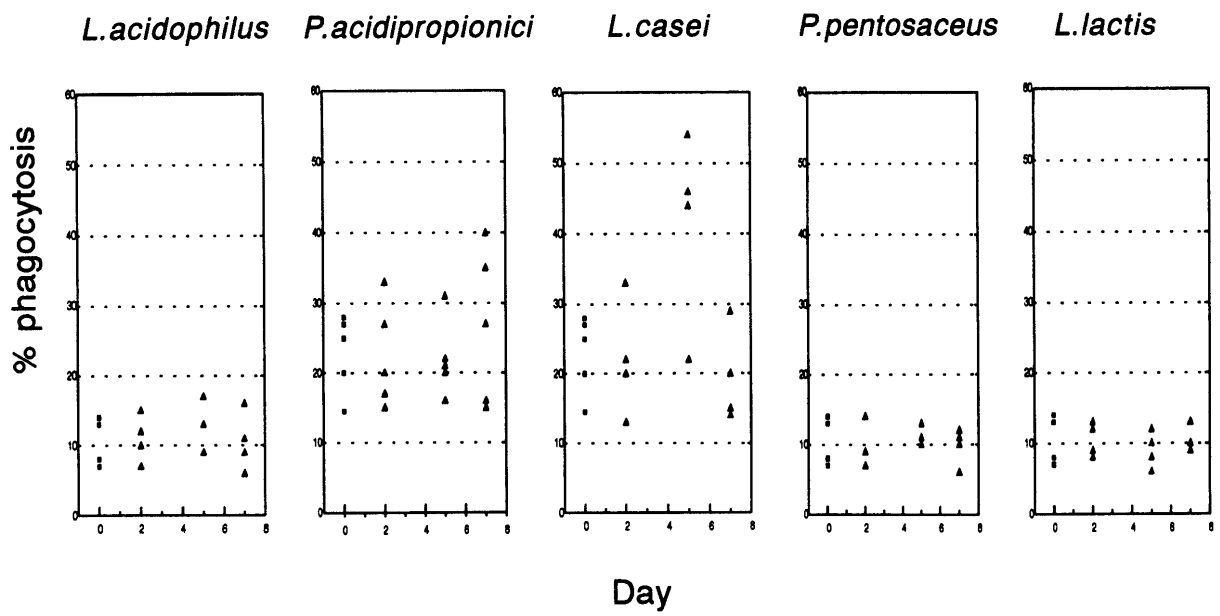

Fig. 2. Effect of Peptidoglycan on the Phagocytic Capability of Peritoneal Macrophages in Mice

■, normal mice; ▲, peptidoglycan suspension (50 μg/d). *L. acidophilus* CRL 730, $p_{2\,days} > 0.01$, $p_{5\,days} > 0.01$, $p_{7\,days} > 0.01$. *L. casei* CRL 431, $p_{2\,days} > 0.01$, $p_{5\,days} < 0.01$, $p_{7\,days} > 0.01$. *L. lactis* CRL 526, $p_{2\,days} > 0.01$, $p_{5\,days} > 0.01$, $p_{7\,days} > 0.01$. *P. acidipropionici* CRL 1198, $p_{2\,days} > 0.01$, $p_{5\,days} > 0.01$, $p_{7\,days} > 0.01$. *P. pentosaceus* CRL 923, $p_{2\,days} > 0.01$, $p_{5\,days} > 0.01$, $p_{7\,days} > 0.01$.

the amino acids comprising the peptide structure of the PG is simple, rapid and does not require a highly purified PG, so it is likely to be readily applicable to other cell-wall studies.

Acknowledgements This work was financially supported by BID-CONICET No. 314 and program of CIUNT No. 26DO65.

The authors wish to thank Lic. Elena Bru de Lavanda for statistical advice.

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