A high-molecular-mass cell wall protein released from *Clostridium tyrobutyricum* by heat treatment

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Analysis of the cell wall of 4 strains of *Clostridium tyrobutyricum* reveals an unusually high protein content (35–40% dry weight). Brief heat treatment of whole cells of these strains causes release of two proteins, flagellin and a cell wall component of high molecular mass (110–125 kDa in the different strains). This component represents approx. 5% of the dry cell weight.

Clostridium tyrocutyricum *Cell wall* *Protein*

1. INTRODUCTION

Clostridium tyrobutyricum is the most important lactate fermenting *Clostridium* capable of multiplying in certain cheeses and causing subsequent spoilage mainly through gas and butyric acid formation [1,2]. Therefore, development of a serological test which would enable specific detection of *C.tryobutyricum* in milk, cheese or any other medium was begun. However, little is known about the structure and antigenic properties of the cell wall of this bacterium. Here, we present some preliminary results of a biochemical study of *C.tyrobutyricum* cell wall. They reveal some interesting features, notably the presence of a high-molecular-mass protein in large quantities in the cell wall preparations and the release of this protein by heating whole or deflagellated *Clostridia* cells.

2. MATERIALS AND METHODS

2.1. *Growth of* Clostridium tyrobutyricum

C.Tyrobutyricum strains 608, 500, 603 (ATCC 25755) and 596 obtained from the laboratory collection were grown at 37°C under anaerobic conditions at pH 7 in Reinforced Clostridial Medium without agar [3]. The cultures were inoculated to a final concentration of 10^3–10^5 cells/ml with exponentially growing cells prepared by growth from spores in the same medium. The cells were harvested by centrifugation at 4°C for 30 min at 10 000 × *g* and washed once with distilled water.

2.2. *Preparation of cell walls (adapted from [4], all operations at 0–4°C)*

Cells mixed with twice their weight of glass beads (diameter 0.15–0.2 μm) were subjected to mechanical disintegration of a Buhler vibrogen at maximum speed in the presence of protease inhibitors: iodoacetamide (3 mM), and phenylmethylsulfonyl fluoride (5 mM) until microscopic observations showed less than 1% intact cells. The homogenate was mixed with 5 vols distilled water, decanted and centrifuged successively at 200 × *g* (5 min) and 100 × *g* (20 min) to remove remaining glass beads and unbroken cells. Cell walls were then recovered by centrifugation at 10 000 × *g* (30 min) and washed by several centrifugations with distilled water (twice), phosphate buffer (10 mM KH_2PO_4–K_2HPO_4 pH 7, once), distilled water (twice), phosphate buffer (once) and finally with distilled water (twice). They were then lyophilised and stored at −20°C. The homogeneity of cell wall preparations was verified by electron microscopy.

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2.3. Preparation of deflaggelated cells [5]

Exponentially growing cells were resuspended (2×10^9 cells/ml) in 0.01 M Tris–HCl buffer (pH 6.8). The suspension was placed in a Sorvall omnimixer blender and treated at 4°C for 1 min at a speed control setting of 7.5. The suspension was then diluted 10-fold in Tris–HCl buffer and the cells were harvested by centrifugation for 30 min at $5000 \times g$ and washed once in Tris–HCl buffer.

2.4. Preparation of cell wall extracts

Cell walls were suspended (12.5 mg dry wt/ml) in 0.0625 M Tris–HCl buffer (pH 6.8) containing 2% SDS and 10% glycerol. After treatment at 100°C for 5 min and addition of 5% 2-mercaptoethanol, the extracted wall suspension was centrifuged at 4°C for 20 min at $12000 \times g$ and the insoluble wall material was discarded.

2.5. Preparation of heat extracts of whole or deflagellated cells

Cells were resuspended (10^9–10^{10} cells/ml) in phosphate-buffered saline [PBS; 0.137 M NaCl, 7 mM K_2HPO_4, 2.5 mM KH_2PO_4 (pH 7.3)]. The cell suspension was heated at 100°C for 5 min and centrifuged at 4°C for 20 min at $17000 \times g$. Cells were discarded and supernatant was stored at −20°C.

2.6. Analytical procedures

Protein determination: total cell wall potein was determined as in [6] and protein in heated cell extracts as in [7].

Neutral sugars were estimated according to [8] and uronic acids as in [9].

Hexosamine determination: the walls were hydrolysed (10 mg dry wt/ml) in 3 N HCl in sealed tubes at 95°C for 4 h. The hydrolysates were neutralised with 3 N NaOH and their hexosamine content was determined by the method of Elson Morgan as described in [10].

Total phosphorus was estimated as in [11] after acid hydrolysis of organic phosphates.

2.7. Gel electrophoresis

SDS-gel gradient electrophoresis was performed as in [12] on 12.5–20% SDS-polyacrylamide gel gradients using a 5% polyacrylamide stacking gel. After electrophoresis gels were washed for 60 min in 2% acetic acid, stained for 30 min in 0.1% Coomassie blue R250–50% ethanol–7% acetic acid and rinsed in 15% ethanol–7% acetic acid.

3. RESULTS

3.1. Chemical composition of cell walls

The results of quantitative analyses of the wall preparations obtained from log phase cells of different strains of *C.tyrobutyricum* are summarized in table 1. Neutral sugars, hexosamines, phosphorus and uronic acids vary between strains. The protein contents are relatively high and do not vary significantly between strains.

3.2. Electrophoretic analysis of proteins released from SDS-treated cell wall preparations

The large amounts of protein associated with the *C.tyrobutyricum* cell walls led us to examine the electrophoretic pattern of the proteins solubilized by heating cell walls at 100°C for 5 min in Tris–HCl buffer containing 2% SDS. This treatment is a modification of that used in [13] to solubilize

Table 1

Chemical composition of the cell wall of different strains of *C.tyrobutyricum*[a]

Strain	Assayed component (% of cell wall dry wt)				
	Proteins	Sugars	Hexosamines	Phosphorus	Uronic acids
C.tyrobutyricum 608	36	8.5	9	1	1.5
C.tyrobutyricum 500	40	3	8	2.3	0.7
C.tyrobutyricum 603	39	7	10	3.4	1
C.tyrobutyricum 596	35	13	15	1.6	2

[a]Cells were harvested in exponential phase

E.coli cell walls in which 4 M urea was added. It has been shown in our laboratory that 4 M urea was unnecessary for the complete extraction of *C.tyrobutyricum* cell walls. The SDS–PAGE protein patterns of the cell wall extracts from different *C.tyrobutyricum* strains are shown in lanes 1–4 of fig.1. In the gel from which the photograph in fig.1 was taken approx. 50 protein bands with apparent molecular masses of 10–125 kDa and little analogy were visible in each lane but of these only about half can be seen in the photograph. All 4 strains contain a major protein component of high molecular mass: approx. 116 kDa for strain 608, 110 kDa for strains 500 and 596 and 125 kDa for strain 603.

Although electron microscopic examination of

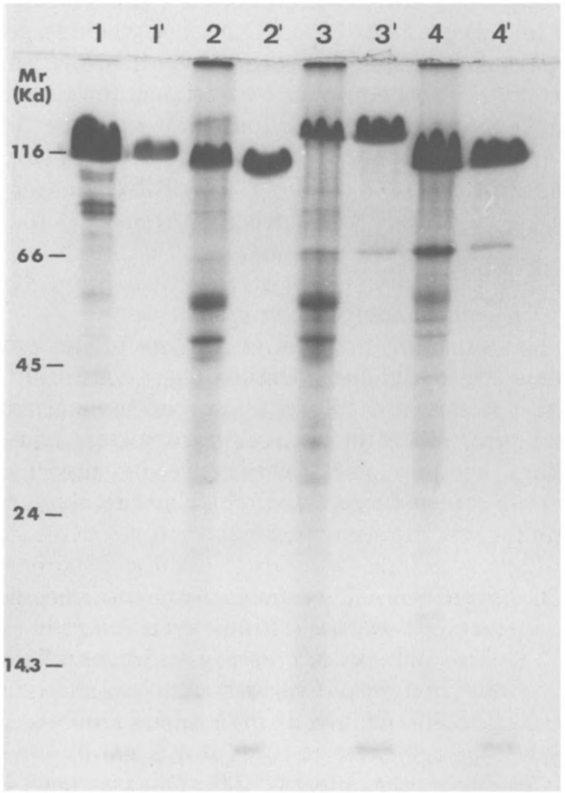

Fig.1. Electrophoretic patterns of protein components extracted from cell walls (1–4) and deflagellated intact cells (1′–4′) of different strains of *C.tyrobutyricum:* (1,1′) *C.tyrobutyricum* 608, (2,2′), *C.tyrobutyricum* 500, (3,3′) *C.tyrobutyricum* 603, (4,4′) *C.tyrobutyricum* 596. Proteins were extracted by heating suspensions of cell walls in 0.0625 M Tris–HCl, 2% SDS, 10% glycerol (pH 6.8), and of deflagellated cells in 0.137 M NaCl, 7 mM K_2HPO_4, 2.5 mM KH_2PO_4 (pH 7.3) at 100°C for 5 min (see section 2). The presence of traces of the major flagellar protein in lanes 3′ and 4′ (faint faster moving band) indicates incomplete deflagellation of the cells used to prepare these extracts. Molecular mass markers (Sigma): β-galactosidase, 116 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; trypsinogen, 45 kDa; lysozyme, 14.3 kDa.

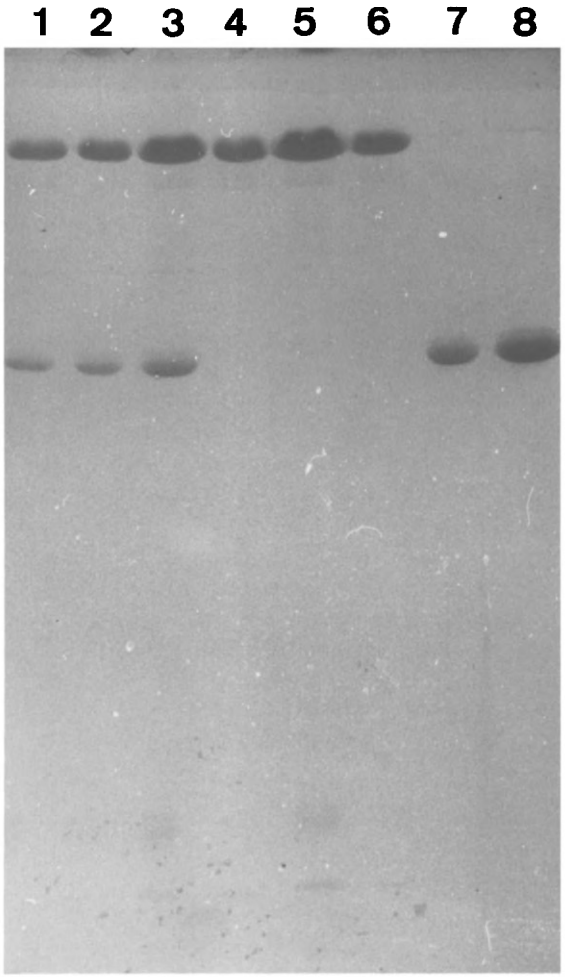

Fig.2. Electrophoretic patterns of protein components extracted by heating suspensions of whole or deflagellated cells of *C.tyrobutyricum* 608 in 0.137 M NaCl, 7 mM K_2HPO_4, 2.5 mM KH_2PO_4 (pH 7.3) for 5 min at 100°C (see section 2). (1,2,3) Extract of whole cells, (2, 4, 6 μg protein); (4,4,6) extract of deflagellated cells (2, 6, 4 μg protein); (7,8) flagellin prepared from *C.tyrobutyricum* 608 according to [5] (2, 4 μg protein).

cell wall preparations revealed the presence of plasma membrane contamination the amounts observed were too small to account for the large quantities of low molecular mass proteins released from the preparations by extraction with Tris–HCl–SDS at 100°C. Moreover, treatment of the cell wall preparations with the non-ionic detergent Triton X-100 under molder conditions [1% Triton X-100 in 0.0625 M Tris–HCl (pH 6.8) 5°C, 120 min] was found to release as many proteins, including the high-molecular mass species, as extraction with Tris–HCl–SDS, although in lower yield. We conclude that the low-molecular-mass species seen in fig.1 are probably not derived from plasma membrane contamination.

3.3. *Effect of heat on whole and deflagellated* C.tyrobutyricum *cells*

In the course of preparing anisera against *C.tyrobutyricum,* whole cells of strain 608 were heated for 5 min at 100°C in PBS, and the supernatant was examined by SDS–PAGE as in the case of cell wall extracts. A very simple protein pattern was observed (fig.2) mainly composed of two proteins. One of the two migrated at the same position, as the major protein (116 kDa) demonstrated in the cell wall preparations. When deflagellated cells were heated under identical conditions, only this protein was detected. The second protein solubilized by heating whole cells had a lower molecular mass and was shown to be the major component of flagella by electrophoretic comparison (fig.2) with flagellin prepared from *C.tyrobutyricum* by standard methods [5]. This treatment applied to deflagellated cells of strains 500, 603 and 596 of *C.tyrobutyricum* produced an equally simple SDS–PAGE protein pattern (fig.1).

The amounts of protein released by heating deflagellated cells were determined (table 2) and the results show that this high-molecular-mass protein represents 4.6–5.6% of the dry weight of deflagellated cells.

Table 2

Protein released by heating deflagellated cells of different strains of *C.tyrobutyricum* in PBS

Strain	% dry wt cells (Bradford)
C.tyrobutyricum 608	5.6
C.tyrobutyricum 500	4.6
C.tyrobutyricum 603	4.6
C.tyrobutyricum 596	4.9

4. DISCUSSION

This study demonstrates that the cell wall of 4 *C.tyrobutyricum* strains contains large quantities of protein consisting mainly of one high-molecular-mass component. This protein is readily released on heating whole cells in PBS, and can be prepared in a relatively pure state and large amounts by heating deflagellated cells in the same way. The high protein content of the cell wall of *C.tyrobutyricum* is unusual since the cell walls of Gram-positive bacteria are generally considered to be made up mainly of peptidoglycan, teichoic or teichuronic acids and/or other polysaccharides but little protein (10% or less [14]).

The localization of the high-molecular-mass component of the cell wall of *C.tyrobutyricum* is not yet known. However, many bacteria including *Clostridium* species are known to possess regular arrays composed of hexagonally or tetragonally arranged protein subunits of high molecular mass on the outer surface of their cell walls [15]. Authors in [16] observed an ordered layer of glycoprotein of 140 kDa in *C.thermosaccharolyticum* and *thermohydrosulfuricum* and those in [17] also observed an ordered layer of protein of 195 kDa on *C.botulinum* type A. In the case of *C.tyrobutyricum,* such regular arrays have not yet been demonstrated but the high-molecular-mass component found in its cell wall may perhaps belong to a structure of this type.

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