Amino Acid Composition of Walls from Single and Filamentous Cells of *Clostridium acidurici*¹

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ABSTRACT

GAFFAR, ABDUL (Brigham Young University, Provo, Utah), DAVID R. TERRY, AND RICHARD D. SAGERS. Amino acid composition of walls from single and filamentous cells of *Clostridium acidurici*. J. Bacteriol. 91:1618-1624. 1966.—The walls from single and filamentous cells of *Clostridium acidurici* were shown to contain 11 amino acids: aspartic acid, serine, glutamic acid, proline, D-alanine, glycine, valine, methionine, valine, leucine, phenylalanine, and lysine. In the walls from cells grown at 37 C, D-alanine was the amino acid present in largest quantity, but in the walls from cells grown at 44 C there was a 50% reduction in the D-alanine content while the levels of the other amino acids were unchanged. Filamentous cells grown at 44 C, then brought to 37 C and transferred to fresh medium, fragmented into short cells within 30 min. Alanine racemase activity was the same in extracts from cells grown at both 37 and 44 C, suggesting that this enzyme was not the major controlling factor in the low content of D-alanine in filaments grown at 44 C. Spent medium from cultures grown at 44 C contained a significant amount of D-alanine, whereas there was no evidence of this amino acid in the spent medium from cultures grown at 37 C.

In another report (12), the formation of very long filaments of *Clostridium acidurici* was described, and some properties of the filaments were discussed. The filaments were incapable of forming cross walls at 44 C even when supplemented with fresh medium and at magnesium concentrations between 10^{-6} and 10^{-2} M, although the filaments were capable of forming longitudinal walls at this temperature. When the temperature was lowered to 37 C, the filaments rapidly formed cross walls and fragmented to short, single cells. These observations suggested the possibility that a specific repression of the enzyme system catalyzing the synthesis of cross walls occurred at 44 C, with the repression being relieved by lowering the temperature, and further suggested that there might be differences in the composition of walls from filaments grown at 44 C and single cells grown at 37 C. The present studies were undertaken to determine the amino acid composition of walls from single and fila-

mentous cells and to relate, if possible, any observed differences to specific enzyme systems.

MATERIALS AND METHODS

Growth of cells. Stock cultures of *C. acidurici* were maintained as described in another report (12). For growing large quantities of cells, cultures grown in screw-cap tubes (20 by 150 mm) were transferred into 1-liter bottles of liquid medium, and, after 12 to 16 hr of growth at 37 C, were transferred to 20-liter bottles of medium. The liquid medium in the 1-liter and 20-liter bottles was the same as was used for growing stock cultures, except that it did not contain agar. After 12 hr of growth, the cells were harvested by Sharples centrifugation, washed twice, and prepared for storage or further experiments. Approximately 8 to 10 g of cells (packed wet weight) was obtained from a 20-liter bottle of medium.

To assure adequate temperature control in the 20-liter bottles incubated at 44 C, a bottle of medium was placed in a 25-gal (94.6-liter) polyethylene container surrounded with 2 inches (5 cm) of foam rubber. The plastic container served as a constant-temperature bath. Water was pumped from a nearby heating bath by a high capacity, continuous-flow pump, which facilitated the circulation and stirring of the water. The temperature in the container was controlled within ± 0.1 C by a Yellow Springs Thermistemp

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Controller model 71, having a no. 403 regulating probe placed in the water within the large plastic container.

Preparation of cell walls. Cells grown at 37 or 44 C were disintegrated with a Braun model MSK homogenizer (Bronwill Scientific, Rochester, N.Y.) as follows. A 5-g amount of washed cells was suspended in 25 ml of distilled water and poured into a 75-ml Duran flask, followed by the addition of 40 g of 0.12-mm glass beads. The stoppered flask was shaken for 3 min at 4,000 oscillations per min, while being cooled with a stream of liquid CO₂. Essentially all of the bacteria were disrupted by this treatment as observed by microscopic examination.

Upon completion of the disruption process, the cell walls were separated and purified by a procedure essentially combining the methods of Cummins and Harris (3) and Allsop and Work (1). This procedure is outlined in Fig. 1. The glass beads were removed by sedimentation, and the cell homogenates were centrifuged at $1,000 \times g$ for 7 min to sediment any unbroken cells. The supernatant liquid was then centrifuged at $22,000 \times g$ for 30 min. The pellet was suspended in 0.1% trypsin and 0.1% ribonuclease (both obtained from Sigma Chemical Co., St. Louis, Mo.) and was incubated at 37 C (4 to 5 hr) until the ultraviolet absorbancy at 260 m μ of washed suspensions of the walls had fallen to a constant level. The low- and high-speed centrifugations were repeated, and the cell walls (sedimented at $22,000 \times g$) were suspended in 0.02 N HCl (200 ml). Pepsin (2 mg/ml) was added, and the mixture was incubated at 37 C for 18 hr. The low- and high-speed centrifugations were repeated, and the walls sedimented at $22,000 \times g$ were washed twice with 0.1 M potassium phosphate buffer (pH 7.1) and twice with distilled water. Finally, the purified walls were frozen and stored at -5 C.

Hydrolysis of cell walls. For amino acid analysis, 5 to 10 mg (dry weight) of cell walls was placed in sealed tubes with 2 ml of 6 N HCl and heated for 8 hr

at 105 C. The HCl was removed in vacuo over NaOH, by repeated addition and evaporation of water, and the sample was finally dissolved in 0.2 N sodium citrate buffer (pH 3.25).

Amino acid analysis. Amino acids and NH₃ were determined by ion-exchange chromatography by the method of Moore, Spackman, and Stein (6), with the use of Amberlite IR-120 (200-400 mesh) resin which had been selected for uniformity of size by repeated decantation and settling of the particles in a column of water (50 by 10 cm).

The resin was suspended in 0.2 N citrate buffer (pH 3.25), poured into a jacketed chromatographic column (1.4 by 150 cm), and packed in three successive portions under a pressure of 25 cm of mercury. Before use, or between applications of samples to the column, the resin was washed with 0.2 N NaOH and then equilibrated with 0.2 N citrate buffer at pH 3.25.

A column (0.9 by 15 cm) for analysis of basic amino acids was similarly prepared with resin that had been suspended in 0.35 N buffer at pH 5.28.

Operation of the columns. The columns were maintained at 50 C during operation and were used in conjunction with a Vanguard model 1000 fraction collector. To calibrate the columns, 1- μ mole samples of 19 known amino acids (see 6) were applied, and the position of each in the effluent was noted, together with the degree of recovery of each (which was essentially 100%). Identity of the amino acid in each peak emerging from the columns was confirmed by paper chromatography with known samples. During five determinations, good reproducibility in the peak positions was observed, with variations for any particular peak not exceeding ± 4 ml of the mean position of that peak on the effluent plot.

For determination of the neutral and acidic amino acids in cell walls, a 10-mg sample of hydrolyzed walls in 0.2 N citrate buffer (pH 3.25) was applied to the top of the column (1.4 by 150 cm) in such a way that it did not disturb the upper layer of the resin. The

FIG. 1. Outline of the procedure used for preparing cell walls of *Clostridium acidurici*.

sample was allowed to adsorb, and then 1 ml of buffer was applied to wash the sample into the resin. Citrate buffer (pH 3.25) was then applied, and 2-ml fractions totaling 280 to 290 ml were collected. The flow rate was maintained at 12 to 14 ml per hr. After elution of the alanine peak, the pH of the buffer was changed to 4.25, and an additional 280 ml of effluent was collected to complete the elution of all neutral and acidic amino acids.

For resolution and identification of basic amino acids, the 15-cm column described earlier was used. A sample of hydrolysate was applied to the column as before, and washed in with citrate buffer at pH 5.28. The elution was carried out at pH 5.28 and at a flow rate of 20 to 25 ml per hr.

Analysis of effluent. The effluent from the 150- and 15-cm columns was collected in 2-ml and 1-ml fractions, respectively, and 1-ml samples were analyzed photometrically by the modified ninhydrin method of Moore and Stein (7).

Desalting of the effluent for paper chromatography. To confirm the identity of each amino acid peak and to determine whether the material in each peak was homogeneous on a paper chromatogram, the effluent was desalted on Dowex 1 X-2 (bicarbonate form) by the method of Piez, Tooper, and Fosdick (9), and samples were examined by paper chromatography.

Paper chromatography. For the qualitative determination of amino acids in the desalted peaks, two-dimensional ascending paper chromatography was carried out on Whatman no. 1 paper, with methyl alcohol-water-pyridine (80:20:4) and tertiary butyl alcohol-methyl ethyl ketone-water-diethylamine (40:40:20:4) as solvents.

After development, but before treating with ninhydrin, the paper was sprayed with 0.1 M borate buffer, pH 9.3, and dried in hot air to eliminate the background color otherwise caused by the diethylamine. The amino acids were located by spraying the paper with a mixture of ninhydrin, collidine, and glacial acetic acid (50 ml of 0.1% ninhydrin in ethyl alcohol plus 2 ml of collidine and 15 ml of glacial acetic acid). The spots were developed in a hot-air oven at 105 C in 5 to 10 min.

Determination of D-alanine (and total D-amino acids). Identification of the optical isomer of alanine present in the peaks eluted from the column, or in other samples, was accomplished by a method suggested by Strominger and Threnn (11). D-Amino acid oxidase converts certain D-amino acids to their corresponding keto acids which can be measured colorimetrically. A sample from the chromatographic column, or a sample of hydrolyzed cell wall material in sodium pyrophosphate buffer (pH 8.3) was mixed with 1 μ liter (900 μ g/ml) of flavin adenine dinucleotide (FAD), 1 μ liter of catalase (500 μ g/ml; Sigma Chemical Co.) and 0.5 ml (2 mg/ml) of D-amino acid oxidase (Sigma Chemical Co.). The final volume was made up to 1 ml with oxygenated sodium pyrophosphate buffer, and the mixture was incubated for 2 hr at 37 C. Proteins were removed by treatment with 15% (w/v) trichloroacetic acid and centrifugation, after which pyruvate in the mixture was determined by the method of Friedemann and Haugen (4).

Determination of protein. The protein concentration in crude extracts was determined turbidimetrically at 600 m μ after precipitation with 5% trichloroacetic acid by the method of Stadtman, Novelli, and Lipmann (10). Crystalline bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) was used as a standard.

Assay for alanine racemase. Alanine racemase activity in extracts was assayed by a method suggested by Wood and Gunsalus (13) in the following manner. To 0.3 ml of 1.0 M potassium phosphate buffer (pH 8.1) were added 0.1 ml (100 μ g/ml) of pyridoxal phosphate, 0.2 ml of 0.01 M glutathione, 0.1 to 0.4 ml of cell extracts, 0.2 ml of 0.2 M L-alanine, 0.02 ml (900 μ g/ml) of FAD, 0.02 ml (500 μ g/ml) of catalase, and 0.5 ml (4 mg/ml) of D-amino acid oxidase. The volume was adjusted to 2 ml with distilled water, and the mixture was incubated at 37 C for 1 hr. Alanine racemase present in the cell extracts converted L-alanine to D-alanine, which in turn was converted to pyruvic acid by D-amino acid oxidase. The pyruvic acid liberated from the D-alanine was determined as described above. Control samples of boiled *C. acidurici* extract were employed to determine the level of free D-alanine present in the extract and converted to pyruvate by D-amino acid oxidase.

RESULTS

Amino acid analysis of cell walls. The site where the cross wall or septum is formed within the cell upon division may be a very active site for the synthesis of cell wall material. Since there was a suppression of cross wall formation in cells of *C. acidurici* grown at 44 C (12), it seemed logical to examine the walls of both single and filamentous cells to determine whether differences existed in their chemical composition. The walls from both types of cells were prepared by the procedure outlined in Fig. 1. The yield of walls amounted to 16 to 20% of the total dry weight of the cells. When examined electron microscopically, the walls appeared to be homogeneous and free from cellular debris. Ion-exchange chromatography of hydrolyzed cell walls from the cells grown at both 37 and 44 C showed the presence of 11 amino acids. They were aspartic acid, glutamic acid, proline, glycine, alanine, valine, methionine, leucine, phenylalanine, and lysine (Fig. 2 and 3). In the walls from cells grown at 37 C, the alanine peak was the largest, but in the walls from cells grown at 44 C there was a 50% reduction in the alanine concentration, while the concentration of the other amino acids remained unchanged. It was observed in other experiments that, when the filamentous cells grown at 44 C were placed at 37 C and supplemented with fresh medium, the inhibition of cross wall formation was relieved, and the filaments fragmented into many single cells within 30 min.

FIG. 2. Chromatographic analysis of neutral and acidic amino acids from hydrolyzed cell walls of organisms grown at: ●, 37 C; and ○, 44 C; 10 mg of hydrolyzed walls was applied to the 150-cm column. The number above each peak represents the number of micromoles of that amino acid per 10 mg of cell walls from organisms grown at 37 C.

FIG. 3. Chromatographic analysis of basic amino acids from hydrolyzed cell walls of organisms grown at: ●, 37 C; and ○, 44 C; 5 mg of hydrolyzed walls was applied to the 75-cm column. The number representing the lysine concentration was calculated per 10 mg of cell walls, as in Fig. 2.

Determination of optical isomers. The optical specificity of each of the amino acids collected from the ion-exchange column was determined by the use of D-amino acid oxidase. The amino acid-containing fractions were desalted by the procedure described in Materials and Methods, and were subjected to analysis. As shown in Table 1, no fraction other than the one containing alanine reacted with the enzyme, showing that alanine was present as the D isomer. The optical configuration of glutamic acid could not be determined by this method, since D-glutamate does not react with D-amino acid oxidase.

TABLE 1. Action of D-amino acid oxidase on each of the amino acid peaks from the ion-exchange column*

Peak no.	Amino acid	Keto acid formed (μmoles/10 mg of walls)	
		From 37 C cell walls	From 44 C cell walls
1	Aspartic acid	<0.01	<0.01
2	Serine	<0.01	<0.01
3	Glutamic acid	<0.01 †	<0.01*
4	Proline	<0.01	<0.01
5	Glycine	0.045	0.03
6	Alanine	2.8	1.5
7	Valine	<0.01	0.01
8	Methionine	<0.01	<0.01
9	Leucine	<0.01	<0.01
10	Phenylalanine	<0.01	<0.01
11	Lysine	<0.01	<0.01

* The sample on the column consisted of 10 mg of hydrolyzed cell walls from cells grown at 37 and 44 C. The effluent containing each peak was desalted, concentrated to 2 ml, and assayed as described in Materials and Methods for the determination of D-alanine.

† The configuration of the glutamic acid could not be determined by this method, since D-glutamic acid does not react with D-amino oxidase.

All of the amino acid peaks were desalted and subjected to paper chromatography together with known amino acids to confirm the identity and homogeneity of each peak. A very slight overlapping of the alanine and glycine peaks

occurred, which could be inferred from the curves in Fig. 2 and also from the data in Table 1 wherein a very low activity of D-amino acid oxidase on the material from the glycine peak was apparent. All of the other peaks were homogeneous and corresponded to the known single amino acids applied to the paper chromatogram.

Comparison of the total D-amino acid content (as indicated by reactivity with D-amino acid oxidase) in hydrolysates of walls from cells grown at 37 and 44 C also showed a reduction by 50% in the D-amino acid content of the walls from the filaments (Table 2). This information correlated well with the 50% reduction in D-alanine from the 44 C filaments (Fig. 2). It is also shown in Table 2 that approximately 85% of the total alanine was present as the D-isomer.

Alanine racemase activity in extracts. Since alanine racemase converts L-alanine to D-alanine, it seemed possible that this enzyme could be the controlling factor in the reduced concentration of D-alanine in cells grown at 44 C if this enzyme were repressed at the higher temperature. Accordingly, alanine racemase activity was measured in extracts from cells grown at 37 C and filaments grown at 44 C to establish the presence of the enzyme, and to determine whether there was any difference in the activities from cells grown at the different temperatures. Table 3 shows that when assayed at 37 C there was not a significant difference in the racemase activity of such extracts. This led to three possibilities: (i) that this enzyme was not the controlling factor in the low content of D-alanine in filaments grown at 44 C; (ii) that, in the procedure used to measure alanine racemase activity, the D-alanine formed from the L-isomer was selectively converted to pyruvate by D-amino acid oxidase, but some of the pyruvate so formed was disappearing

TABLE 3. Activity of alanine racemase in extracts from cells grown at 37 and 44 C*

Growth temp	D-Alanine formed (μ moles per 10 min per mg of protein)
C	
37	0.012
44	0.013

* The reaction mixture was as described in Materials and Methods for alanine racemase.

by way of other reactions, thus giving an apparent racemase activity lower than actually present; or (iii) that the temperature-response curves for the enzyme derived from the respective types of cells might appear differently over a temperature gradient between 37 and 44 C. The second possibility was ruled out since control assays containing known concentrations of pyruvate together with extracts from the respective cells showed no significant disappearance of pyruvate. In considering the third possibility, it was of interest to determine whether the alanine racemase produced in filaments at 44 C responded differently to a temperature gradient than the enzyme produced in cells at 37 C. Figure 4 shows the in vitro temperature response of the racemase from extracts of both types of cells over a temperature range between 30 and 50 C. It is noted that the activity of the enzyme from both cell sources is strictly parallel over the entire range, with greatest activity occurring at 40 C. The rate at 44 C was slightly lower than the rate at 37 C, and approximately half the rate at 40 C.

Studies on spent medium. Since the cell walls from filaments grown at 44 C showed a 50% reduction in D-alanine, and since alanine racemase activity was similar in extracts from cells grown at 37 and 44 C, it seemed possible that the amino acid was being synthesized at 44 C, but, instead of being incorporated into cell wall material, it was being excreted into the medium. The spent media after growth at 37 and 44 C were, therefore, concentrated 500-fold and assayed for D-alanine content by paper chromatography and by the action of D-amino acid oxidase (Table 4). The spent medium from the 37 C cultures contained no D-alanine, but the medium from the 44 C cultures contained this amino acid at a level slightly higher than that found in the cells from an equivalent volume of culture grown at 37 C.

DISCUSSION

Studies on cell wall replication by means of electron microscopy of ultrathin sections (8), fluorescent-antibody techniques (2), and radio-

TABLE 2. Comparison of the total alanine and the D-amino acid content in walls of cells grown at 37 and 44 C^a

Growth temp	Total alanine ^b	Total D-amino acids ^c	D-Alanine ^d
C			
37	3.2	2.9	2.8
44	1.6	1.6	1.5

^a The reaction mixture for determination of D-amino acids was as described in Materials and Methods. Amounts are given in micromoles per 10 mg of walls.

^b Determined by reaction with ninhydrin as peak 6, Fig. 1.

^c The neutralized hydrolysate of 10 mg of cell walls was assayed directly.

^d From Table 1.

FIG. 4. Activity of alanine racemase in extracts of: ○, cells grown at 37 C; and △, filaments grown at 44 C.

TABLE 4. Relationship of the D-alanine content of cells and spent medium to the temperature of growth*

Growth temp	D-Alanine content	
	Medium containing cells	Spent medium minus cells
C	μmoles/liter	μmoles/liter
37	5.7	0
44	2.7	6.9

* Amounts of 88 and 83 mg (dry weight) of cells were obtained per liter of culture at 37 and 44 C, respectively. Samples (10 mg) of each were hydrolyzed and assayed for D-alanine as outlined in Materials and Methods. The spent medium (1 liter), freed from cells by centrifugation, was concentrated 500-fold in a Buchler PTFE-1 flash evaporator and assayed for D-alanine as above. The yeast extract used in medium contained no D-alanine.

active tracer techniques (5) have shown that, prior to division, cells (bacilli and cocci) form a septum which can be seen as a double layer of wall material. Upon division, the daughter cells separate between these layers, yielding cells having a portion of older, thicker wall which was originally present in the organism, and a region of newly formed, thinner wall derived from the septum. This region of newly formed material undoubtedly is an area of intense metabolic activity, particularly of cell wall synthesis. In view of such information, it was considered significant to examine the cell walls of normal and filamentous cells of *C. acidiurici* to determine whether chemical differences in composition of the walls existed, since the filaments are incapable of forming cross walls (12). The data shown in Fig. 2 and 3 indicate a 50% reduction in the D-alanine content of walls from cells grown at 44 C, yet there was no significant change in the levels of the other amino acids.

The presence of alanine racemase at the same level in organisms grown at both 44 and 37 C (Table 3) indicates that this enzyme is available for supplying D-alanine to the filaments, but the fact that the activity of the racemase is slightly less at 44 C than at 37 C (Fig. 4) may be a factor in the reduced D-alanine content of the filaments. However, the accumulation of D-alanine in the medium at 44 C would indicate that the amino acid is synthesized but may not be efficiently incorporated into cross wall material at the elevated temperature. This idea is further supported by the observation that, when the temperature of the filamentous cells is lowered to 37 C and the filaments are supplied with additional substrate, they form cross walls and fragment to single cells within 30 min.

It is premature to speculate on the precise site of the cellular control mechanism resulting in the repression of cross wall formation in *C. acidiurici*, since studies have not yet been made on the muramic acid and teichoic acid linkages in this organism, but such studies are to be made. It is apparent, however, that the cellular processes related to the incorporation of D-alanine into cross wall material may play a prominent role in directing the cells either to form cross walls and divide at temperatures below 42 C, or to form filaments devoid of cross walls at temperatures above 42 C.

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