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COMPOSITION OF CELL WALLS OF *CLOSTRIDIUM SORDELLII* AND *CLOSTRIDIUM BIFERMENTANS* AND ITS RELATION TO TAXONOMY

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CLOSTRIDIUM BIFERMENTANS (Tissier and Martelly, 1902) and *Cl. sordellii* (Sordelli, 1922; Hall and Scott, 1927) are in many respects similar organisms. Clark and Hall (1937) found that they are morphologically and culturally indistinguishable and serologically related—only the non-pathogenicity of the first and high pathogenicity of the second distinguish them. Stewart (1938), after investigation of some few strains, suggested that *Cl. sordellii* is a pathogenic variant of *Cl. bifermentans* and that they should be regarded as a single species: *Cl. bifermentans*. This suggestion has been accepted by some workers (Breed, Murray and Smith, 1957).

However, Huet and Aladame (1952) and Tataki and Huet (1953) were able to distinguish these species on the grounds of urease production and agglutination. Strains of *Cl. sordellii* are ureolytic; sera of rabbits immunised with ureolytic strains agglutinate ureolytic strains only. Some ureolytic strains are non-pathogenic. *Cl. bifermentans* is non-ureolytic and never pathogenic, but sera against *Cl. bifermentans* agglutinate both ureolytic (*Cl. sordellii*) and non-ureolytic (*Cl. bifermentans*) strains. Brooks and Epps (1959) investigated many pathogenic and non-pathogenic, ureolytic and non-ureolytic strains and found that in addition to ureolysis other features distinguish *Cl. sordellii* and *Cl. bifermentans*—colonial morphology, fermentation of carbohydrates, the results of the methyl red test, the production of indole and acetoin, gelatinolysis and the nature of the amino acids present in cultures. They concluded that separation of *Cl. sordellii* from *Cl. bifermentans* is justifiable.

The organisms are antigenically related (Clark and Hall; Stewart), but could be serologically distinguished (Tardieux and Nisman, 1952; Tataki and Huet). Also the spore antigens are distinctive (Meisel and Rymkiewicz, 1959), although antisera to spores of *Cl. bifermentans* reacted to low titres with spores of *Cl. sordellii* (Walker, 1963). By precipitation tests Ellner and Green (1963) were able to detect in *Cl. sordellii* and *Cl. bifermentans* species-specific antigens in addition to a common antigen shared by both species as well as some other clostridia studied. Recently Meisel and Świtalska (1967) studying 30 strains of

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Cl. sordellii and 35 of *Cl. bifermentans* found that neither "species" forms an antigenically homogeneous group; several distinct serological types could be detected, although more than 80 per cent. of strains could be classified by means of agglutination. These studies also seem to support the view that the species are distinct.

However, this taxonomic relationship is again a subject of controversy. It is relatively simple to obtain fully atoxic substrains from toxic strains of *Cl. sordellii*, but Huang (1959, cited by Huang, Tamai and Nishida, 1965) isolated a toxic strain from the heart of a rabbit infected with an atoxic strain; this toxic strain became atoxic on repeated subculture. This observation inspired the study of conditions that induce the changes of some properties of the strains. Nishida, Tamai and Yamagishi (1964), by heating a pathogenic and ureolytic strain of *Cl. sordellii* (strain 1734), selected a pathogenic substrain and also a non-pathogenic and non-ureolytic one. Tamai and Nishida (1964) observed that by progressive heating substrains could be obtained from *Cl. sordellii* strain 4708 that had lost not only ureolytic activity and toxicity but also some other properties that were regarded as distinctive by Brooks and Epps. These new substrains were biologically indistinguishable from *Cl. bifermentans*. Huang *et al.* continued this work and found that new substrains obtained by heat selection acquired the antigenicity of *Cl. bifermentans*.

In a preliminary report (Novotný, 1964*b*) I have demonstrated that the cell wall of *Cl. sordellii* strain P₃ contains glucose and that of *Cl. bifermentans* strain B₁ glucose, mannose and rhamnose; amino acid and amino sugar composition was, however, found to be quite similar in both these strains. In the present paper the study of the composition of cell walls of 16 strains each of *Cl. sordellii* and *Cl. bifermentans* is reported.

MATERIAL AND METHODS

Strains used

Clostridium sordellii, pathogenic (9 strains): P₁ (A. R. Prévot's strain no. 1); P₂ (A. R. Prévot's strain no. 82); P₃ (highly toxigenic strain selected in our laboratory from strain no. 82, and regarded as type culture in this paper); P₄ (A. R. Prévot's strain no. 1676); S₁ (strain 79 of A. Sordelli, obtained from Dr L. S. McClung as no. 127); S₂ (*Cl. oedematoides* "Bieber strain" of Dr Meleny, obtained from Dr L. S. McClung as no. 142); S₃ (NCTC 2919); S₄ and S₅ strains no. 8841 and 8854 of Dr L. D. S. Smith, obtained from Professor H. Meisel).

Cl. sordellii, non-pathogenic (7 strains): S₂₁, S₂₂, S₂₃, S₂₄, S₂₅ isolated in our laboratory from human faeces; S₂₆ isolated in our laboratory from intestines of mummies by Patočka and Šefrna (1944) (the spores of this strain had probably remained dormant for 150–280 yr); S₂₇ isolated from a case of clostridial myonecrosis.

Cl. bifermentans (16 strains): B₁ (Nancy Hayward's strain—NCTC 536—obtained from Dr L. S. McClung as no. 435, and regarded as a type culture in our laboratory); strains B₂, B₃, B₄, B₅, B₇, B₈, B₉, B₁₁, B₁₃, B₁₄, B₂₀, B₂₇, B₃₂, and B₄₀ isolated in our laboratory from human faeces; strain B₃₇ isolated in our laboratory from spoiled canned ham.

All strains are stored in Robertson's meat medium in the dark at laboratory temperature. The strains isolated in this laboratory were diagnosed by methods similar to those used by Brooks and Epps, with special attention to colonial morphology, pathogenicity in guinea-pigs, urease production and fermentation reactions.

Preparation of cells

The cells were grown in 750-ml volumes of medium containing (per litre): Tryptone Oxoid, 30 g; Yeast Extract Difco, 2 g; glucose, 1 g; NaCl, 5 g; pH before sterilisation 7.2–7.4. Several 1-litre flasks containing medium, immediately after autoclaving for 20 min. at 120°C, were cooled in running tap-water, 1 ml of actively growing culture in Vf-broth was

inoculated near the bottom level and the mixture was incubated at 37°C. The end of the exponential growth phase was reached in 5–6 hr. To harvest actively growing cells 4-hr cultures were rapidly cooled to 5°C and centrifuged in a Sharples centrifuge. Sedimented cells were washed while still in the rotor with half of the culture volume of ice-cold acid saline (0.85 per cent. NaCl solution containing 0.025M-KH₂PO₄, pH 5.0), removed from the rotor, washed twice with ice-cold acid saline at 3000*g* in the centrifuge and suspended in acid saline to appropriate density.

For the preparation of small quantities of cells for estimation of ureolytic activity or for preparation of formamide cell-extracts, a modification of the biphasic culture of Tyrrell, MacDonald and Gerhardt (1958) was found very useful. Two per cent. of Bacto Agar Difco was added to the above medium, and 7-ml volumes of the mixture were distributed in 16 × 120 mm tubes with aluminium caps, heated for 15 min. at 115°C and slanted. After solidification of the agar medium 5 ml of sterile fluid medium was added to each tube. The slanting was done so that the agar medium did not penetrate the surface of the fluid medium. Immediately after preparation, 0.1 ml of actively growing culture was inoculated into the bottom of the tubes and the cultures were incubated without any seal at 37°C. In these conditions also the end of exponential growth phase is reached in about 5–6 hr. On the average 2.8 mg dry weight of exponentially growing cells could be obtained from each tube with *Cl. sordellii* and 3.2 mg dry weight of cells of *Cl. bifermentans*.

Preparation of cell walls

A bacterial pellet (2–4 g wet weight) was suspended in 6–7 ml of cold acid-saline and disintegrated with ballotini glass beads for 3 min. in a rotary disintegrator (Novotný, 1964a), cooled with ice and salt. The beads were removed by filtering the slurry through a sintered glass filter (S2) with thorough washing of the material retained. The cell walls were isolated by the methods of Salton and Horne (1951), with the exceptions mentioned in *Results*. All operations were carried out at a temperature of <5°C. When the cell walls were not immediately analysed, they were freeze-dried.

Measurement of sensitivity of cells or isolated cell walls to enzymes

Log-phase cells washed twice with ice-cold acid physiological saline (pH 5) and re-suspended in that saline to a concentration of 1.7–2.0 mg dry weight of cells per ml were heated at various temperatures. One ml of this suspension was added at zero time to 4.0 ml of Tris-HCl-buffer (0.05M, pH 7.4), with or without enzyme, in calibrated test-tubes pre-heated to 37°C; the tubes were incubated at 37°C in a waterbath, and optical densities were read at 5-min. intervals in an LP photoelectrometer with a red OB2 filter. An optical density of 0.200 corresponded to 105 µg of dry weight of cells of *Cl. sordellii* strain P₃ per ml.

Freshly isolated cell walls prepared as above were suspended in acid physiological saline, heated in the same way as cell suspensions and diluted to appropriate density to a volume of 2.5 ml in Tris-HCl-buffer, with or without enzyme, in 1-cm stoppered cuvettes. Their optical density was measured at 600 nm in an SP 500 Unicam Spectrophotometer equipped with a constant temperature cell-housing at 37°C, and repeated at appropriate intervals for 1 hr. An optical density of 0.500 corresponded to about 600 µg dry weight of cell walls per ml.

When freeze-dried preparations of cell walls were used, the lyophilised product was soaked in distilled water and stored overnight at 2°–3°C before use.

Crystalline trypsin (Organopharma, Prague) was dissolved in Tris-HCl-buffer at the appropriate concentration shortly before use. Twice recrystallised isoelectric lysozyme prepared from egg white by the method of Alderton and Fevold (1946) was used.

Determination of ureolytic activity in cell suspension and cell-free extracts

Cells were harvested as above and resuspended in ice-cold acid physiological saline to a density of 7–8 mg dry weight per ml. One-half of the suspension was disintegrated in a

cooled rotary disintegrator. The slurry of disintegrated cells and glass beads was transferred to a centrifuge filter holder equipped with a sintered glass filter (S2) and centrifuged for 2 min. at 500*g*. The filtrate obtained was again centrifuged at 3000*g* for 10 min. to remove the intact cells and debris. The supernatant was considered to be a cell-free extract; it contained on the average 6.0 mg of dry weight per ml and living cells were not present.

One ml of the cell suspension or the cell-free extract under test was added to 1.0 ml of urea solution of Brooks and Epps in which the universal indicator was replaced by phenol red. The mixtures were incubated at laboratory temperature in the dark and observed for 24 hr. The same buffer solution with urea omitted was used for control.

Analytical methods

Determination of sugars. Preliminary experiments on the course of hydrolysis of cell walls of strains P₃ and B₁ showed that when samples were refluxed with 2*N*-HCl in a boiling waterbath, liberation of *N*-acetyl-amino sugars (determined by the method of Reissig, Strominger and Leloir, 1955) reached its maximum in 10 min. of hydrolysis and thereafter rapidly decreased; liberation of total hexosamines and sugars reached its maximum in 3 hr. Samples of cell walls were therefore hydrolysed in 2*N*-HCl in sealed ampoules for 3 hr in a boiling waterbath. After hydrolysis the volume of sample was diluted 20 times with water and transferred to a 1 × 2 cm column of Dowex 50, × 8, 100–200 mesh (H⁺ form). The effluent and wash were collected and evaporated in a vacuum rotator at 40°C. The residuum was dissolved in distilled water and total sugars were determined by the anthrone method (Ashwell, 1957) with glucose or a glucose-mannose-rhamnose mixture (molar ratio 2 : 2 : 1) as standard. For paper chromatography the evaporated residue was dissolved in pyridine (Malpress and Morrison, 1949).

Hexosamines retained in the Dowex 50 column were eluted with 10 ml *N*-HCl; the effluent was dried in a vacuum rotator at 40°C and the residue dissolved in water.

Paper chromatography of sugars. 10–30 µg of total sugars in pyridine were used for each spot on Whatman no. 3MM paper and developed in *n*-butanol-ethanol-water (52 : 32 : 16, by volume, Putman, 1957). The neutral red spot has an R_f value similar to that of rhamnose and was used as marker. The sugar spots were detected with anilin-oxalate reagent (Dzulyńska and Mikulaszek, 1954) or with silver nitrate reagent (Trevelyan, Procter and Harrison, 1950).

For the determination of the molar ratio of sugars on chromatograms, the method of Putman was used. Sheets of Whatman no. 3MM paper with serrated lower edges were washed for several days with 15 per cent. (v/v) acetic acid and dried before use. The sugars eluted from the paper were determined by the anthrone method by comparison with the standard curves for each sugar. Each sample was analysed in duplicate.

Hydrolysates that contained glucose and galactose were developed twice in the same solvent. In the first run the development was stopped when the neutral red spot had reached a point 15 cm above the lower edge. After drying, in the second run, the neutral red spot was allowed to reach the lower position.

Determination of amino sugars. Total amino sugars were determined by the method of Belcher, Nutton and Sambrook (1954), in which the chromogens of muramic acid as well as glucosamine are stable for at least 12 hr.

Qualitatively the hexosamines were determined by two-dimensional chromatography with the solvents of Redfield (1953). The spots were revealed with either the ninhydrin spray, the silver nitrate method of Trevelyan *et al.* or the Elson and Morgan spray of Partridge (1948). Commercial glucosamine recrystallised from dilute ethanol was used as a standard. Muramic acid standard was isolated from cell walls of *Micrococcus lysodeikticus* by a method similar to that of Strange and Kent (1959). The product recrystallised from ethanol was chromatographically pure and after ninhydrin deamination (Stoffyn and Jeanloz, 1954) a slowly migrating spot of the pentose derivative could be demonstrated.

In some hydrolysates the quantity of amino sugars was determined separately with the

cadmium-ninhydrin method as for amino acids. The cadmium-ninhydrin complex of muramic acid produced 53 ± 2 per cent. of the colour given by glucosamine.

Amino acid determination. The sample of cell walls was hydrolysed in 6N-HCl in sealed ampoules at 105°C for 18 hr. The HCl was removed by repeated evaporation in a vacuum rotator at 50°C and the residue dissolved in 10 per cent. (v/v) propanol.

Qualitatively the amino acids were identified by the two-dimensional chromatography method of Redfield using the ninhydrin spray of Cowgill and Pardee (1957).

Quantitatively the amino acids were determined in one-dimensional paper chromatography. The chromatograms on 3MM Whatman paper were developed in *tert*-butanol, methanol, water and concentrated formic acid (40 : 25 : 9.5 : 0.5 by volume) for about 50 hr. The spots were quantitatively measured by use of the cadmium-ninhydrin reagent of Heilmann, Barrolier and Watzke (1957, as modified by Altfield and Morris, 1961). On each paper, together with the sample to be analysed, three concentrations of reference amino acids were developed and a calibration extinction graph was made for each chromatogram separately. Each sample was analysed in duplicate. Meso-diaminopimelic acid was determined by the method of Hoare and Work (1955).

Total phosphorus in isolated cell walls was determined by the method of Allen (1940).

Agglutination tests

Antisera were prepared by intravenous immunisation of rabbits with formolised antigens, 2 ml twice weekly for 2 mth. The rabbits were bled 6 days after the last injection. Sera so prepared agglutinated homologous suspensions to a titre of 2560 or more. The antisera were diluted before use so that the titre with the homologous antigen was 640. Antisera were prepared against strains P₂, P₃, P₄, S₂₇, B₁, B₅, B₂₀, B₃₂ and B₃₇.

Formolised antigen was prepared by suspending exponentially growing cells in physiological saline containing 0.4 per cent. formaldehyde and 1 in 10,000 Merthiolate. After 2 hours' standing the suspension was twice washed at 10,000*g* and finally suspended to contain *c.* 1 mg dry weight of cells per ml in formol-saline. For immunisation with *Cl. sordellii*, strains exposed to formaldehyde for at least 5 days had to be used to avoid killing the rabbits.

For agglutination, the suspensions were diluted to contain 250–300 µg dry weight of cells per ml. Dilutions of sera mixed with the same volume of antigen were incubated at 37°C for 4 hr and read after they had stood at room temperature overnight.

RESULTS

Properties of cell walls of Cl. sordellii and Cl. bifermentans

The cells of *Cl. sordellii*—especially those of strain P₃—harvested in the log phase are extremely prone to lysis when they are suspended in a buffer of pH 6–8; in about 1 hr the cells are almost completely autolysed. Much of the autolysis can be prevented by suspending the cells at pH 5 or by heating them for 60 min. at 60°C (Novotný and Čáslavská, 1967). Log-phase cells of *Cl. bifermentans* strain B₁ are also very unstable in nearly neutral buffer, although the optical density decrease was in every case less pronounced than with *Cl. sordellii*; in 1 hr the optical density at pH 7.4 (Tris buffer, 0.05M) and 37°C decreases to 67 per cent. of the original level. With *Cl. bifermentans* also the autolysis of cells could be prevented by heating them for 60 min. at 60°C, or for 20 min. at 100°C, or by autoclaving them for 20 min. at 120°C. Such heated cell suspensions of both organisms are extremely sensitive to the presence of trypsin (fig. 1), although against unheated cell suspensions trypsin has little activity (Novotný and Čáslavská). Isolated cell walls of both organisms

behaved similarly; unheated cell walls, as well as those heated to the same temperatures as the cell suspensions, rapidly lysed in the presence of trypsin, as could be seen by following the optical densities (fig. 2). The isolated cell walls of both organisms are not sensitive to lysozyme, whether they are heated or not. However, trypsin sensitivity of cell walls of both organisms diminished when the cell walls were freeze-dried and again resuspended in a buffer. This

FIG. 1.

FIG. 2.

FIG. 1.—Effect of 50 µg trypsin per ml at 37°C on cells of *Clostridium sordellii* and *Cl. bifermentans* heated for 20 min. at 100°C. ●—● *Cl. sordellii*, P₃ cells with trypsin. ○—○ Control test without added trypsin. ■—■ *Cl. bifermentans*, B₁ cells with trypsin. □—□ Control without added trypsin.

FIG. 2.—Effect of 50 µg trypsin per ml at 37°C on isolated cell walls of *Cl. sordellii* and *Cl. bifermentans* heated for 20 min. at 100°C. ●—● *Cl. sordellii*, P₃ cell walls with trypsin. ○—○ Control without added trypsin. ■—■ *Cl. bifermentans*, B₁ cell walls with trypsin. Control without trypsin in figure omitted for simplicity, because the values were very similar to those for *Cl. sordellii* without added trypsin.

unexpected behaviour of cells as well as of cell walls of these clostridia rendered impossible the use of common methods of cell wall preparation (Cummins and Harris, 1956). Trypsin digestion and heating of cell walls were therefore omitted.

Much of the autolysis could be prevented by rapidly chilling the cultures during harvesting and washing the suspensions with ice-cold acid-saline (pH 5). After disintegration the cell walls were isolated by differential centrifugation

and subsequent washing by centrifugation only. All manipulations up to the freeze-drying of the end product were done as rapidly as possible. After three washings with acid-saline and 3–5 washings with distilled water, the suspension of cell walls was no longer contaminated by optically dense particles. If optically dense debris was present, a hump was observed on the curve of optical density at wavelengths in the region of 260 nm, and after hydrolysis, ribose was detected in some preparations. In suspensions of cell walls used for analysis a smooth curve of absorption spectra was observed without any evidence of peaks at wavelengths in the ultraviolet. The yield of cell walls by these methods was 12–18 per cent. of dry weight of cells. However, these figures are probably rather low, as it is likely that there were losses during preparation.

Chemical composition of cell walls

The results of the analysis of two cell-wall preparations each of *Cl. sordellii* strain P₃ and *Cl. bifermentans* strain B₁ are given in table I. The amino acid constitution of cell walls of both strains was similar qualitatively and quantitatively: no clear-cut difference could be detected. About one-third of the dry weight of the cell walls is represented by amino acids. However, it is interesting that beside meso-diaminopimelic acid lysine was constantly found. Originally, it had been supposed that lysine was an impurity, but 6 other preparations of cell walls of *Cl. sordellii* strain P₃ and 3 preparations of cell walls of *Cl. bifermentans* strain B₃, were all found to contain diaminopimelic acid and lysine, as did hydrolysates of isolated cell walls of strains S₂, S₃, S₂₁, B₄, B₈, B₉, B₁₁, B₁₃, B₃₇ and B₄₀, which also contained the same amino acids. In some hydrolysates faintly visible spots of some other amino acids were also observed, but these are probably from impurities and are not constantly present. Only one exception was observed: in strain S₃ the cell-wall hydrolysate showed no diaminopimelic acid—only lysine and a spot due to an unidentified amino acid. The molar ratio of amino acids in cell walls of this strain was 1.0 : 1.6 : 2.6 : 2.0 : 1.6 for Glu, Ala, Gly, Lys, Asp (about 32 per cent. of the dry weight of cell walls was amino acids). It appears, therefore, that some exceptions in the pattern of amino acids may exist, but in most preparations so far studied the constitution of amino acids was very similar.

There is about 20 per cent. of total hexosamines, as judged from the Elson and Morgan test, in cell walls of *Cl. sordellii* strain P₃, in *Cl. bifermentans* strain B₁ about 10 per cent. only; the results are not corrected for loss during hydrolysis. Qualitatively, glucosamine and muramic acid are present in cell walls of both organisms. When glucosamine and muramic acid are determined separately by the cadmium-ninhydrin method, the total amount of hexosamines was even higher in 6N-HCl hydrolysates of some samples investigated (table I). In 2N-HCl hydrolysates about 25 per cent. of total hexosamines was determined by this method in *Cl. sordellii* cell walls and c. 15 per cent. in cell walls of *Cl. bifermentans* strain B₁. The results obtained by the Elson and Morgan method for total hexosamines are therefore probably underestimates.

TABLE I
Quantitative analysis of cell walls of *Cl. sordellii* strain P₃ and *Cl. bifermentans* strain B₁

Hydrolysis	Substance	Amount of substance in							
		<i>Cl. sordellii</i> strain P ₃				<i>Cl. bifermentans</i> strain B ₁			
		1st preparation		2nd preparation		1st preparation		2nd preparation	
		per cent.	molar ratio *	per cent.	molar ratio *	per cent.	molar ratio *	per cent.	molar ratio *
6N-HCl 18 hr at 105°C	Glutamic acid	7.25	1.00	11.53	1.00	7.33	1.00	13.20	1.00
	Alanine	9.32	2.13	9.88	1.41	6.22	1.40	7.92	0.99
	meso-Diaminopimelic acid	7.54	0.80	7.57	0.53	6.27	0.66	13.30	0.78
	Glycine	1.96	0.53	7.78	1.32	4.42	1.18	1.97	0.29
	Lysine	1.42	0.20	3.44	0.30	1.90	0.26	3.25	0.25
	Aspartic acid	1.28	0.20	4.33	0.42	3.82	0.58	2.56	0.21
	Glucosamine	...	...	...	...	4.65	0.52	...	...
	Muramic acid	...	...	...	...	6.60	0.53	...	...
2N-HCl 3 hr at 100°C Retained by Dowex 50 Not retained by Dowex 50	Total hexosamines as glucosamine	21.0	...	19.2	...	9.4	...	8.9	...
	Total sugars as glucose	14.0	...	11.0	...	23.8	...	21.8	...
	as artificial mixture of glucose, mannose and rhamnose	...	...	...	...	33.3	...	29.4	...

* Glutamic acid equals 1.0.

Monosaccharides that could be detected in cell-wall hydrolysates distinguished strains P_3 and B_1 strikingly. In hydrolysates of cell walls of *Cl. sordellii* strain P_3 , only glucose was found, whereas cell walls of *Cl. bifermentans* strain B_1 contained glucose, mannose and rhamnose (table IV). The total sugar content in cell walls of *Cl. bifermentans* strain B_1 is about 30 per cent.—twice the amount found in cell walls of *Cl. sordellii* strain P_3 .

Isolated cell walls of both strains were extracted with formamide (Krause and McCarty, 1961): 10–30 mg of lyophilised preparations were suspended in 1.0 ml formamide and after heating for 20 min. at 160–170°C in an oil-bath and cooling, 2.5 ml of acid ethanol (0.1N-HCl in 90 per cent. ethanol) was added and the mixture centrifuged for 20 min. at 3000*g*. To the supernatant 5 ml acetone was added. The precipitate formed at 3°C was collected by centrifugation next day, dialysed against water, lyophilised and weighed. 18–19 per cent. of dried extract was obtained from cell walls of *Cl. sordellii* strain P_3 , and 21–22 per cent. from those of *Cl. bifermentans* strain B_1 . Sedimented extracted cell walls were washed with acid ethanol-water (or dialysed against water) and freeze-dried. The formamide-ethanol-acetone mixture remaining after precipitation of extract was reduced in a vacuum rotator, HCl was added to 6*N* and the mixture hydrolysed. The first two fractions of cell walls obtained by formamide treatment were hydrolysed in 2*N*-HCl as well as in 6*N*-HCl.

In the formamide extract neither diaminopimelic acid nor muramic acid could be detected, and in extracted cell walls scarcely perceptible amounts of lysine and aspartic acid were present. This holds for cell walls of both organisms examined. The molar ratios of amino acids in these three fractions are given in table II. In the formamide extract of both organisms glucosamine was detected and in the extract of *Cl. sordellii* strain P_3 glucose also, but the extract of *Cl. bifermentans* strain B_1 contained all the three sugars detected in the whole cell walls: glucose, mannose and rhamnose, probably in the same molar ratio. However, the majority of sugars remained bound to extracted cell walls. The material remaining in formamide-ethanol-acetone mixture differs from formamide extract as well as from extracted cell walls in that it contains the highest ratio of lysine to glutamic acid. Therefore it probably contains some fragments other than traces of extracted cell walls or formamide extract or both.

Formamide extract is soluble in water at pH 7.8 and is serologically active. In double diffusion precipitation in agar gels (Oakley and Fulthorpe, 1953), extract of *Cl. sordellii* strain P_3 reacted to give a broad band with homologous agglutinating sera and a narrow band with *Cl. bifermentans* sera. Similarly, *Cl. bifermentans* strain B_1 extract reacted with a broader band with homologous than with heterologous (P_3) antisera.

The cell walls remaining after formamide extraction were dialysed against water to remove the rest of the solvents (Allsop and Work, 1963). Although the density of the cell wall suspension before formamide treatment decreased rapidly in the presence of trypsin, the extracted cell walls were fully resistant to trypsin (25–100 μ g per ml of suspension) as well as to lysozyme (25 μ g per ml); there was no decrease in optical density in 24 hr at 37°C. Higher concentrations of lysozyme agglutinate the cell walls. Electron-microscopically,

the extracted cell walls still have the appearance of cell walls although they are much more delicate than before extraction.

Because the phosphorus content is much less than 0.5 per cent. of dry weight of cell walls, polyolophosphates were probably not present in the cell walls of either strain. However, about 30 per cent. of material in cell walls of *Cl. sordellii* strain P₃, and about 20 per cent. of that of *Cl. bifermentans* strain B₁, was not accounted for by the methods employed.

TABLE II

Molar ratios of amino acids in fractions of cell walls obtained by formamide extraction

Fraction	Strain	Molar ratio * in fractions of						Per cent. of amino acids
		Glu †	Ala	DAP	Gly	Lys	Asp	
Formamide extract	<i>Cl. sordellii</i> strain P ₃	1.0	0.94	0	2.30	0.43	2.10	13.8
	<i>Cl. bifermentans</i> strain B ₁	1.0	0.59	0	1.00	0.52	0.67	22.0
Extracted cell walls	<i>Cl. sordellii</i> strain P ₃	1.0	2.20	1.10	0	traces	traces	18.6
	<i>Cl. bifermentans</i> strain B ₁	1.0	1.75	0.60	0.20	traces	traces	21.0
Formamide-ethanol-acetone mixture	<i>Cl. sordellii</i> strain P ₃	1.0	3.00	0.73	1.32	0.67	0.93	...
	<i>Cl. bifermentans</i> strain B ₁	1.0	1.46	0.26	1.10	0.70	1.10	...

* Glutamic acid equals 1.0.

† Glu = Glutamic acid; Ala = alanine; DAP = diaminopimelic acid; Gly = glycine; Lys = lysine; Asp = aspartic acid.

Monosaccharides in cell wall hydrolysates of other strains of Cl. sordellii and Cl. bifermentans

An essential difference between strains P₃ and B₁ was the nature of the monosaccharides present in cell-wall hydrolysates. Therefore qualitative and in some cases quantitative analysis of sugars in cell-wall hydrolysates of further strains was undertaken.

As the isolation of cell walls is a time-consuming process for routine diagnostic purposes, cell suspensions of the strains under test were treated as follows.

4–5-hr cultures in 5–10 “biphasic slant tubes” were harvested and the cells washed twice with acid-saline in the centrifuge at 3000*g*. The supernatant from the last washing was discarded and, to avoid frothing during subsequent heating with formamide, all droplets of physiological saline above the pellet of bacteria were carefully removed with filter-paper strips. Sedimented cells were extracted with formamide by the same methods as in the case of isolated cell walls. Precipitated polysaccharide collected by centrifugation was dissolved in 0.3*M* ammonium acetate, hydrochloric acid was added to a final concentration of 2*N*, and the mixture was sealed in an ampoule and hydrolysed in a boiling waterbath for 2–3 hr. The hydrolysate was cooled, diluted 10–20 times with water and passed through a 1 × 2 column of Dowex 50, × 8, 100–200 mesh (H⁺ form). The effluent and water wash of this column were collected and evaporated to dryness in a vacuum rotator at 40°C. The sugars

dissolved in pyridine are spotted for chromatography. An equivalent of 30–50 mg wet weight of cells is sufficient for transfer to the spot on the starting line. The chromatogram is developed in an *n*-butanol, ethanol, water solvent (52 : 32 : 16, by volume).

The results of sugar determinations in cell-wall hydrolysates or formamide extract hydrolysates are in table III. All strains corresponding to *Cl. sordellii*

TABLE III

Monosaccharides present in hydrolysates of cell walls or formamide extracts of cells of strains of Cl. sordellii and Cl. bifermentans

Strain			Presence (+) or absence (–) in hydrolysates, of				Sugars detected in hydrolysates of	
No.	toxin	ureolysis	glucose	mannose	galactose	rhamnose	isolated cell walls	formamide extract of cells
P ₁	+	+	+	–	– *	–	...	+
P ₂	+	+	+	–	– *	–	+	+
P ₃	+	+	+	–	– *	–	+	+
P ₄	+	+	+	–	–	–	+	...
S ₁	+	+	+	–	– *	–	+	+
S ₂	+	+	+	–	– *	–	+	+
S ₃	+	–	+	–	–	–	+	...
S ₄	+	+	+	–	– *	–	...	+
S ₅	+	+	+	–	–	–	...	+
S ₂₁	–	–	+	–	– *	–	+	+
S ₂₂	–	+	+	–	– *	–	...	+
S ₂₃	–	+	+	–	– *	–	...	+
S ₂₄	–	+	+	–	– *	–	+	+
S ₂₅	–	+	+	–	– *	–	...	+
S ₂₆	–	+	+	–	– *	–	+	+
S ₂₇	–	+	+	–	– *	–	...	+
B ₁	–	–	+	+	–	+	+	+
B ₂	–	–	+	+	–	+	+	+
B ₃	–	–	+	+	–	+	...	+
B ₄	–	–	+	+	–	+	+	...
B ₅	–	–	+	+	–	+	...	+
B ₇	–	–	+	+	–	+	...	+
B ₈	–	–	+	+	–	+	+	...
B ₉	–	–	+	+	–	+	+	...
B ₁₁	–	–	+	–	+	+	+	+
B ₁₃	–	–	+	–	+	+	+	+
B ₁₄	–	–	+	+	–	+	...	+
B ₂₀	–	–	+	+	–	+	...	+
B ₂₇	–	–	+	+	–	+	...	+
B ₃₂	–	–	+	+	–	+	...	+
B ₃₇	–	–	+	–	+	+	+	+
B ₄₀	–	–	+	+	–	+	+	...

* Present in traces in formamide extract of cells only.

(ureolytic, pathogenic or non-pathogenic) possess glucose in their cell walls. On the other hand, 16 other strains in our collection that are designated *Cl. bifermentans* contained 3 sugars; 13 of them glucose, mannose and rhamnose—the remaining 3 strains, surprisingly, glucose, galactose and rhamnose. In two strains (B₂, B₄) the molar ratios of glucose, mannose and rhamnose were similar

to that of type strain B₁, in another two (B₈, B₉) the ratios were different (table IV). But in the three strains yielding glucose, galactose and rhamnose in hydrolysates, there were about 3 moles of galactose and 3 of rhamnose to one mole of glucose.

TABLE IV

*Molar ratios * of monosaccharides in cell-wall hydrolysates of some strains of Cl. bifermentans*

A. "Typical" strains

Strain	Molar ratio in cell-wall hydrolysates, of		
	glucose	mannose	rhamnose
B ₁	1st expt	1.00	0.85
	2nd expt	1.00	0.56
B ₂		1.00	0.86
B ₄	1st expt	1.00	0.75
	2nd expt	1.00	0.71
B ₈	1st expt	1.00	1.14
	2nd expt	1.00	1.00
B ₉		1.00	0.48

B. "Atypical" strains

Strain	Molar ratio in cell-wall hydrolysates, of		
	glucose	galactose	rhamnose
B ₁₁	1.00	2.10	2.58
B ₁₃	1st expt	1.00	3.61
	2nd expt	1.00	3.86
B ₃₇	1st expt	1.00	2.86
	2nd expt	1.00	2.70

* Glucose equals 1.0.

It is clear from table III that formamide extracts of intact cells are sufficient for diagnostic purposes. But there is a difference between these and isolated cell-wall hydrolysates. In hydrolysates of formamide extract of whole cells of all strains ribose was present, which is extracted from the cytoplasm. However, in all hydrolysates of formamide extract of whole cells of *Cl. sordellii* (pathogenic or non-pathogenic) a faint spot of galactose could be detected too. This sugar was never detected in isolated cell walls and its source was therefore investigated.

The cells of *Cl. sordellii* strain P₃ were autolysed in the presence of 0.33M sucrose and protoplasts were prepared (Novotný and Čáslavská). After repeated washings with 0.33M sucrose, the protoplasts were osmotically shocked and ghosts collected after centrifugation at 20,000*g* for 20 min. The sedimented plasma membranes were washed with 0.05M Tris buffer (pH 7.4), dialysed against water and hydrolysed in 2N- and in 6N-HCl. In the hydrolysate of these plasma membranes muramic acid and diaminopimelic acid as well as glucose were absent, which shows that the plasma membranes were free from cell wall, but a spot of galactose could be detected. It seems therefore that the source of galactose in formamide extract of whole cells of *Cl. sordellii* strains is the plasma membrane.

*Ureolytic activity of cell suspensions and cell-free extracts
of Cl. sordellii and Cl. bifermentans*

The strain S₂₁ was in the laboratory collection identified originally as *Cl. bifermentans*; this strain is non-pathogenic and, when tested by the usual methods, it has no ureolytic activity. The colonial morphology of this strain was, however, similar to that of *Cl. sordellii*, but it was assumed that it was an R-type colony form of *Cl. bifermentans*. However, after it was shown that the cell walls contained glucose—a cell-wall sugar typical of *Cl. sordellii* strains—the organism was reclassified as *Cl. sordellii* without ureolytic activity. Because it was found that in *Cl. sordellii* strain P₃ the ureolytic activity was approximately 1.6 times greater after disintegration than that of intact cell suspensions (Novotný, 1964*a*), it was assumed that urease as an intracellular enzyme is not able to hydrolyse urea outside the cells unless some kind of transport mechanism is functioning. The cells of strain S₂₁ were therefore disintegrated and thereafter it was found that this cell-free extract hydrolysed urea almost immediately—as do cell suspensions or cell-free extracts of most active strains. Other strains of *Cl. sordellii* and *Cl. bifermentans* were also examined in this way (table V). Of strains containing glucose in their cell walls, S₃ and S₂₁ appeared as non-ureolytic when cell suspensions only were examined. The other strains were significantly ureolytic. All these strains were significantly ureolytic after disintegration. On the other hand, in cell suspensions of strains containing glucose, mannose and rhamnose or glucose, galactose and rhamnose in their cell walls there was never any urease activity. However, after disintegration the cell-free extracts of these strains did increase the pH of urea solution—and in some of them (e.g., B₅, B₈ and B₉) the reaction occurred immediately, as it did in most active *Cl. sordellii* strains. The increase in pH was associated with liberation of ammonia, and as in control tests with buffer solution with urea omitted no ammonia was liberated, it appears that strains of *Cl. bifermentans* also contain urease intracellularly.

Agglutination of strains investigated

It has been accepted that *Cl. sordellii* and *Cl. bifermentans* are serologically related species, though neither forms a homogeneous group (Meisel and

Świtalska). We were able to see some differences in cell-wall composition and we wondered whether these differences had anything to do with the serological heterogeneity of the strains, particularly whether strains containing glucose, galactose and rhamnose in their cell walls would be serologically different from

TABLE V

Ureolytic activity of cell suspensions and cell-free extracts of Cl. sordellii and Cl. bifermentans

Sugar in cell walls	Strain	Ureolytic activity of	
		cell suspension	cell-free extract
Glucose	P ₁	+++	++
	P ₂	++	++
	P ₃	+++	+++
	P ₄	+	++
	S ₁	+++	+++
	S ₂	+	++
	S ₃	—	+
	S ₂₁	—	+++
	S ₂₂	++	++
	S ₂₃	++	++
	S ₂₄	+	++
	S ₂₅	+	++
	S ₂₆	++	++
	S ₂₇	++	++
Glucose, mannose and rhamnose	B ₁	—	++
	B ₂	—	+
	B ₃	—	+
	B ₄	—	++
	B ₅	—	+++
	B ₇	—	++
	B ₈	—	+++
	B ₉	—	+++
	B ₁₄	—	+
	B ₄₀	—	++
Glucose, galactose and rhamnose	B ₁₁	—	+
	B ₁₃	—	+
	B ₃₇	—	++

+++ = Reaction positive immediately after urea solution added; ++ = reaction positive within 4–12 hr; + = reaction positive within 12–24 hr; — = reaction negative in 24 hr.

the strains with glucose, mannose and rhamnose. However, clear-cut differences of this kind could not be found (table VI). Two strains with glucose, galactose and rhamnose (B₁₁ and B₃₇) are agglutinated by three (out of four) sera prepared against strains with glucose, mannose and rhamnose in their cell walls (B₅, B₂₀ and B₃₂ respectively), and a serum against strain B₃₇ behaved like *Cl. bifermentans* agglutinating sera—it agglutinated not only 5 strains of *Cl. bifermentans* (B₄, B₅, B₈, B₂₇, B₃₂) but also 11 strains of *Cl. sordellii*.

Strain B₁₃ is not agglutinated by "glucose, mannose, rhamnose" sera, but this is probably not due to the simple presence of glucose, galactose and rhamnose as compared with strains B₁₁ and B₃₇. On the other hand, strain B₁, which was selected as a type species for *Cl. bifermentans* in this study, was unfortu-

TABLE VI
Agglutination of strains of Cl. sordellii and Cl. bifermentans

Sugar in cell wall	Strain	Titre against strain of rabbit sera immunised with antigen:								
		P ₂	P ₃	P ₄ *	S ₂₇	B ₁	B ₅	B ₂₀	B ₃₂	B ₃₇
Glucose	P ₁	640	640	320	320	40	640	80	640	80
	P ₂	640	640	320	320	640	0	0	20	40
	P ₃	320	640	160	160	0	0	0	0	0
	P ₄	40	0	320	0	0	80	80	640	20
	S ₁	640	640	160	640	0	0	0	0	640
	S ₂	640	640	160	640	0	0	0	0	640
	S ₃	40	160	0	0	640	80	80	0	40
	S ₄	640	0	0	640	0	0	0	0	0
	S ₅	0	0	0	0	0	0	0	0	0
	S ₂₁	0	40	0	0	640	0	20	320	0
	S ₂₂	640	640	320	640	0	0	0	0	640
	S ₂₃	640	640	160	160	0	0	0	0	80
	S ₂₄	640	640	640	640	160	320	0	80	640
	S ₂₅	640	640	640	40	320	160	0	0	640
	S ₂₆	20	320	640	0	640	20	0	20	40
	S ₂₇	640	40	160	640	0	0	0	0	0
Glucose, mannose and rhamnose	B ₁	0	0	0	0	640	0	0	0	0
	B ₂	20	0	0	0	0	0	160	640	20
	B ₃	0	0	0	0	0	0	0	0	0
	B ₄	40	40	20	40	40	640	640	640	640
	B ₅	20	0	20	20	0	640	640	640	640
	B ₇	0	0	0	0	320	0	0	0	0
	B ₈	20	0	0	20	320	640	640	320	40
	B ₉	0	0	0	0	0	0	640	640	0
	B ₁₄	0	0	0	0	0	0	640	640	0
	B ₂₀	40	0	0	0	0	80	640	160	0
	B ₂₇	20	0	0	0	0	640	80	320	640
	B ₃₂	0	0	0	0	0	80	80	640	640
	B ₄₀	0	0	0	0	160	640	640	640	20
Glucose, galactose and rhamnose	B ₁₁	40	0	0	0	0	640	640	640	640
	B ₁₃	0	0	0	0	0	0	0	0	640
	B ₃₇	0	0	0	0	0	640	40	640	640

Titres lower than 20 represented by 0.

* A weak serum used undiluted, with a titre of 320 against the homologous strain; the others were diluted so that they gave a titre of 640 with the homologous strain (see *Methods*, p. 85). Homologous titres in italics.

nately not agglutinated by any heterologous serum used. It seems, therefore, that simple estimation of sugars in cell walls of these strains cannot explain the observed heterogeneity in agglutination tests.

Of the pathogenic strains of *Cl. sordellii*, strain S₅ behaves extraordinarily; it is not significantly agglutinated by any sera prepared in this or in Professor

Meisel's Laboratory (Meisel and Świtalska). In chromatograms of hydrolysates of formamide extract of cells of this strain a slowly migrating reducing spot reacting as hexose with aniline oxalate was found. This spot has not yet been identified (possibly it is a uronic acid), and it was not detected in any other strain so far investigated. Although it is not yet known whether this unidentified compound originates from material responsible for non-agglutinability with *Cl. sordellii* and *Cl. bifermentans* antisera, it could not be an over-all explanation for heterogeneity of the *Cl. sordellii* group, because strains P₄, S₃ and S₄ also agglutinate very weakly with some sera, even though this compound is not present in their cell-wall hydrolysates.

DISCUSSION

The experiments on a large number of strains reported in this paper have confirmed previous work (Novotný, 1964*b*) showing that the cell walls of *Cl. sordellii* strain P₃ and *Cl. bifermentans* strain B₁ have similar amino acid patterns but different sugar patterns. They show that cell walls of all urease-positive, pathogenic or non-pathogenic strains designated as *Cl. sordellii* contained glucose, whilst those of urease-negative strains classified as *Cl. bifermentans* contained 3 sugars: either glucose, mannose and rhamnose or glucose, galactose and rhamnose. If we accept the opinion of Cummins and Harris (1956) that the amino acids present in cell walls are characteristic of a genus and sugars seem to characterise the species within the genus, then we are dealing with one genus, but three species: *Cl. sordellii* (glucose polymer), *Cl. bifermentans* (glucose, mannose, rhamnose) and 3 strains similar in many respects to *Cl. bifermentans*, but containing in their cell walls glucose, galactose and rhamnose. It appears, therefore, that the possibilities of "chemical anatomy" as suggested by Cummins and Harris could also be useful in solving the taxonomic questions of clostridia. In the *Cl. sordellii*-*Cl. bifermentans* group the results in cell-wall composition seem to be consistent; however, some exceptions were observed. In strain S₃ no diaminopimelic acid was detected; it is present in other strains investigated. In strain S₅ an unidentified sugar component is present that was not detected in other strains. Moreover, when glucose was present in cell walls the organism produced urease, with two notable exceptions: strain S₃ (pathogenic) and S₂₁ (non-pathogenic) contained glucose in their cell walls, but were not ureolytic. Bacteria known to be urease-negative may, however, contain intracellularly active ureolytic enzyme, e.g., *Klebsiella pneumoniae*, *Bacterium anitratum* (Jeffries, 1964). In some ureolytic bacteria the ureolytic activity increases after disintegration (e.g., in our strains S₂, P₃, P₄, S₂₄, S₂₅), but not, however, as a rule; e.g., in *Micrococcus lysodeikticus* the ureolytic activity after disintegration decreases (Novotný, 1964*a*). It seems, therefore, that intracellular production of urease may not necessarily mean that extracellular urea would be hydrolysed; a urea transport system may be needed as well. This seems very probable; strain S₂₁, originally identified as *Cl. bifermentans* because it was non-pathogenic and had no urease activity, was reclassified as *Cl. sordellii* because its cell walls contained glucose

only; cell-free extracts of this strain are very actively ureolytic. Similarly, cell suspensions of the pathogenic strain S₃ were also urease-negative in our test, although cell-free extracts have a high degree of activity. These strains are probably mutants that have lost the supposed transport system for urea. Unfortunately, in *Cl. bifermentans*, although no cell suspension could hydrolyse urea, cell-free extracts did contain a little urease; so the distinction is not absolute. Possibly the real difference between *Cl. bifermentans* and *Cl. sordellii* in this respect is that the former never possesses the supposed urea transport system.

It may be concluded that both *Cl. sordellii* and *Cl. bifermentans* are valid species; they are, however, related in many respects. The type strains as well as some other strains of each species contained in their cell walls diaminopimelic acid and lysine. A suggestion has been made that one of these amino acids, but not both, is always present in cell walls of Gram-positive bacteria (Work, 1957). The presence of both these amino acids in cell walls of *Cl. sordellii* and *Cl. bifermentans* does not necessarily refute this suggestion, since the evidence suggests that the diaminopimelic acid is derived from mucopeptide, whereas the traces of lysine detectable in cell-wall remnants after extraction are derived from another peptide removable together with a part of the cell wall by formamide—a “microcapsule”—a situation similar to that in *Bacillus cereus*, whose cell walls contain diaminopimelic acid and lysine, but in different peptides (Kronish, Mohan and Schwartz, 1964).

In both *Cl. sordellii* and *Cl. bifermentans* the majority of the carbohydrate still remains bound to the cell walls after formamide extraction. Moreover, in both cell-wall types of *Cl. bifermentans* the composition of the carbohydrate complex seems to be similar throughout the whole cell wall. It seems therefore probable that smaller units of this carbohydrate are bound to the mucopeptide as well as to the “microcapsular” compound extractable by formamide.

Although in both species the cells and isolated cell walls are lysis-prone, the autolytic properties of *Cl. sordellii* strains are much more marked than those of *Cl. bifermentans*. In both species the autolysis of cells may be prevented by heating. Heated cells and heated or unheated isolated cell walls are extraordinarily sensitive to trypsin; the optical densities of suspensions of cells or isolated cell walls of *Cl. sordellii* treated with trypsin decreased to lower levels than those of *Cl. bifermentans*, suggesting that the fragments produced are different.

In strain P₃ of *Cl. sordellii* after autolysis or trypsin treatment the cell wall disintegrates into fibres or scales (Novotný and Čáslavská, 1967). The lysyl bond would therefore be expected to play a significant part in the stability of the cell walls of this organism. However, little or no lysine is present in the cell walls extracted with hot formamide, and it is rather surprising that extracted cell walls should, in the absence of significant amounts of lysine, still retain the appearance of cell walls and should not disintegrate into fibres. During further work it was shown (unpublished observation) that many proteolytic enzymes have an action on cell walls similar to that of trypsin; it follows, therefore, that lysyl itself is not the bond that holds the morphological units of cell walls

together. In this connexion it should be stressed that a mild treatment such as freeze-drying abolishes the sensitivity of cell walls to trypsin, and it may be expected also that hot formamide treatment may considerably modify the cell-wall structure.

Without doubt, mutants of the two species may be isolated by heating the cultures or by animal inoculation (Nishida *et al.*, 1964; Tamai and Nishida, 1964; Huang *et al.*, 1965). Such mutation may occur by modification of a single gene; but it can hardly be assumed that such a limited mutation could account for the change from *Cl. sordellii* to *Cl. bifermentans*, especially if we include the great difference in the carbohydrate composition of the cell walls. Our results indicate that the sugar composition of cell walls in strains we have investigated is rather stable: some of our strains (S₂₁, S₂₄, S₂₆, B₂, B₄, B₅, B₁₃, B₂₇) were primarily isolated from unheated as well as heated inocula (20 min. at 80°C and 5–20 min. at 100°C), and substrains so obtained show no change in the monosaccharide composition of their cell walls. It is unknown whether substrains obtained from *Cl. sordellii* by Japanese workers are mutants that have lost their toxicity and supposed transport mechanism for urea, but still contain glucose in their cell walls, or whether these substrains have also mutated in respect of this carbohydrate composition. Because antigenic heterogeneity, as shown, seems to be unrelated to the results of simple sugar analysis, such mutations could not be discovered by agglutination reactions. It would be well worth while to find out the answer to this question.

SUMMARY

The cell walls of *Clostridium sordellii* and *Cl. bifermentans* agreed in their amino acid and amino sugar pattern, but differed strikingly in their monosaccharide patterns. Sixteen *Cl. sordellii* strains contained only glucose as monosaccharide in their cell walls, whilst in *Cl. bifermentans* three sugars were found: in 13 strains glucose, mannose and rhamnose and in the 3 others glucose, galactose and rhamnose. The sugar pattern in formamide extracts of cells is sufficient for diagnostic purposes.

Out of 16 *Cl. sordellii* strains 14 are ureolytic—the remaining two (one of which is pathogenic) are not. These strains are probably mutants that have lost a postulated transport mechanism for urea. Cell-free extracts of both *Cl. sordellii* and *Cl. bifermentans* possessed urease activity. Possibly *Cl. bifermentans* never possessed the postulated urease transport mechanism and is therefore not ureolytic.

Antigenic heterogeneity of *Cl. sordellii* and *Cl. bifermentans* strains, as determined by agglutination tests, could not be explained by the results of simple cell-wall analysis.

The physiology of *Cl. sordellii* and *Cl. bifermentans* cell walls is similar in many respects. The autolytic properties are particularly striking; autolysis could be prevented by heating. Heated cells (60 min. at 60°C, 20 min. at 100° or 120°C) are extremely sensitive to trypsin. The optical densities of suspensions of isolated cell walls of both organisms, whether heated or not, decreased rapidly in the presence of trypsin.

Although there are many similarities between *Cl. sordellii* and *Cl. bifermentans*, they are better regarded as separate species; the monosaccharide pattern in their cell walls seems sufficient to distinguish them.

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REFERENCES

- ALDERTON, G., AND FEVOLD, H. L. . . . 1946. *J. Biol. Chem.*, **164**, 1.
 ALLEN, R. J. L. 1940. *Biochem. J.*, **34**, 858.
 ALLSOP, JENNIFER, AND WORK, ELIZABETH 1963. *Ibid.*, **87**, 512.
 ALTFIELD, GLADYS N., AND MORRIS, 1961. *Ibid.*, **81**, 606.
 C. J. O. R.
 ASHWELL, G. 1957. *Meth. Enzym.*, **3**, 84.
 BELCHER, R., NUTTEN, A. J., AND 1954. *Analyst*, **79**, 201.
 SAMBROOK, C. M.
 BREED, R. S., MURRAY, E. G. D., AND 1957. *Bergey's Manual of determinative bac-*
 SMITH, N. R. *teriology*, 7th ed., London, p. 665.
 BROOKS, M. ELIZABETH, AND EPPS, 1959. *J. Gen. Microbiol.*, **21**, 144.
 H. B. G.
 CLARK, F. E., AND HALL, I. C. . . . 1937. *J. Bact.*, **33**, 23.
 COWGILL, R. W., AND PARDEE, A. B. . 1957. *Experiments in biochemical research*
 techniques, New York, p. 52.
 CUMMINS, C. S., AND HARRIS, H. . . . 1956. *J. Gen. Microbiol.*, **14**, 583.
 DŽULYŃSKA, JANINA, AND MIKULASZEK, E. 1954. *Acta biochim. Pol.*, **1**, 191.
 ELLNER, P. D., AND GREEN, S. S. . . . 1963. *J. Bact.*, **86**, 605.
 HALL, I. C., AND SCOTT, J. P. 1927. *J. Infect. Dis.*, **41**, 329.
 HEILMANN, JOHANNA, BARROLIER, J., AND 1957. *Hoppe-Seyler's Z. physiol. Chem.*, **809**,
 WATZKE, E. 219.
 HOARE, D. S., AND WORK, ELIZABETH . 1955. *Biochem. J.*, **61**, 562.
 HUANG, C. T., TAMAI, K., AND NISHIDA, S. 1965. *J. Bact.*, **90**, 391.
 HUET, M., AND ALADAME, N. 1952. *Annls Inst. Pasteur, Paris*, **82**, 766.
 JEFFRIES, C. D. 1964. *Archs Path.*, **77**, 544.
 KRAUSE, R. M., AND MCCARTY, M. . . . 1961. *J. Exp. Med.*, **114**, 127.
 KRONISH, D. P., MOHAN, R. R., AND 1964. *J. Bact.*, **87**, 581.
 SCHWARTZ, B. S.
 MALPRESS, F. H., AND MORRISON, A. B. . 1949. *Nature, Lond.*, **164**, 963.
 MEISEL, H., AND RYMKIEWICZ, DANUTA . 1959. *Medycyna dośw. Mikrobiol.*, **11**, 1.
 MEISEL, H., AND ŚWITALSKA, ALICJA . 1967. *Ibid.*, **19**, 49.
 NISHIDA, S., TAMAI, K., AND YAMAGISHI, 1964. *J. Bact.*, **88**, 1641.
 T.
 NOVOTNÝ, P. 1964a. *Nature, Lond.*, **202**, 364.
 " 1964b. *Folia microbiol.*, **9**, 179.
 NOVOTNÝ, P., AND ČÁSLAVSKÁ, JANA . 1967. *Ibid.*, **12**, 274.
 OAKLEY, C. L., AND FULTHORPE, A. J. . 1953. *J. Path. Bact.*, **65**, 49.
 PARTRIDGE, S. M. 1948. *Biochem. J.*, **42**, 238.
 PATOČKA, F., AND ŠEFRNA, K. 1944. *Sb. lék.*, **46**, 84.
 PUTMAN, E. W. 1957. *Meth. Enzym.*, **3**, 62.
 REDFIELD, R. R. 1953. *Biochim. biophys. Acta*, **10**, 344.

- REISSIG, J. L., STROMINGER, J. L., AND 1955. *J. Biol. Chem.*, **217**, 959.
LELOIR, L. F.
- SALTON, M. R. J., AND HORNE, R. W. . 1951. *Biochim. biophys. Acta*, **7**, 177.
- SORDELLI, A. 1922. *C. r. Séanc. Soc. Biol.*, **87**, 838.
- STEWART, SARAH E. 1938. *J. Bact.*, **35**, 13.
- STOFFYN, P. J., AND JEANLOZ, R. W. . 1954. *Archs Biochem. Biophys.*, **52**, 373.
- STRANGE, R. E., AND KENT, L. H. . . 1959. *Biochem. J.*, **71**, 333.
- TAMAI, K., AND NISHIDA, S. 1964. *J. Bact.*, **88**, 1647,
- TARDIEUX, P., AND NISMAN, B. . . . 1952. *Annls Inst. Pasteur, Paris*, **82**, 458.
- TATAKI, H., AND HUET, M. 1953. *Ibid.*, **84**, 890.
- TISSIER, H., AND MARTELLY, 1902. *Ibid.*, **16**, 865.
- TREVELYAN, W. E., PROCTER, D. P., AND 1950. *Nature, Lond.*, **166**, 444.
HARRISON, J. S.
- TYRRELL, ELIZABETH A., MACDONALD, 1958. *J. Bact.*, **75**, 1.
R. E., AND GERHARDT, P.
- WALKER, P. D. 1963. *J. Path. Bact.*, **85**, 41.
- WORK, ELIZABETH 1957. *Nature, Lond.*, **179**, 841.