CELL WALL COMPOSITION IN THE CLASSIFICATION
OF GRAM POSITIVE ANAEROBES¹

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ABSTRACT. Four groups of strains of clostridia and anaerobic corynebacteria were examined by chemical and antigenic cell wall analysis. In each group it was possible to identify more than one pattern of cell wall sugar components, and these sugar patterns show a high degree of correlation with the subgroups revealed by cell wall agglutination tests. In the two groups tested, DNA/DNA homology tests showed that strains with the same cell wall sugar pattern had a high degree of homology (80-100%).

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In the last ten years a great deal of information has become available about the general structure of the cell wall of Gram-positive bacteria, and representatives of most of the commonly found genera have been examined. Less attention has perhaps been paid to gram-positive anaerobes, but wherever these have been examined (e.g. Salton and Ghuyssen, 1957; Salton and Pavlik 1960; Glendenning, 1958) their cell walls have shown no essential differences from those of other gram-positive organisms, either in electron microscope appearance or in broad chemical structure.

In this contribution, I wish to report the results of studies of cell wall composition in two groups of anaerobes. Firstly, various members of the genus Clostridium, and secondly, a group of "anaerobic diphtheroids" composed of strains of Propionibacterium acnes (Corynebacterium acnes) and strains which appeared to be more or less closely related to it. These studies have been made primarily for taxonomic purposes, and two aspects only of cell wall composition have been considered, broad chemical structure and serological specificity as determined by cell wall agglutination tests. The experimental results are given here in outline only and will be reported in detail elsewhere. In some cases, it has been possible to compare the results obtained by these methods with the degree of genetic homology between strains as determined by DNA competition experiments. The results of these DNA homology studies are reported by Dr. J. L. Johnson in the next paper.

METHODS

The bacteria were grown in a peptone-yeast-extract-glucose medium, containing also cysteine (0.06%) and bicarbonate (0.5%), under an atmosphere of CO₂. The washed organisms were shaken with ballotini

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beads in a Braun disintegrator, and after filtration to remove beads, the mixture was digested with pronase for 1 1/2 - 2 hrs. at 56°. Cell walls were deposited by centrifugation at 30,000 G, separated if necessary from unbroken cells by differential centrifugation, and washed X 3 in distilled water. The final cell wall fractions were lyophilized.

In the case of sporing organisms, precautions were taken to harvest the cells at a time when spore formation was minimal or absent. With Ci. limosum and Ci. botulinum, cultures were killed by the addition of formalin to a final concentration of 2 percent.

For chemical analysis, 5 mgm of cell walls were hydrolysed in 6 N HCl for 18 hrs. at 105°C for amino acids, and 10 mgm of cell walls hydrolysed in 2 N H₂SO₄ for 2 hrs. for sugars. After suitable neutralization, the hydrolysates were examined by paper chromatography and the amounts of different components present recorded roughly as +, ++, +++, or Tr, depending on the intensity of the spots. Cell wall agglutination tests were done as described in Cummins and Slade, 1962.

This brief outline of the methods used to prepare and analyze the cell wall fractions indicates that in chemical terms the analyses are crude, although well suited to the examination of large numbers of strains. No examination was made for the presence of teichoic acids, lipid or hexosamines, and it must be remembered that the mere examination of acid hydrolysates for sugars gives little indication of the type of polymer from which the substances are released. However, the results show that the determination of crude amino acid and sugar patterns in cell walls can be useful in taxonomy, provided that the limitations of the method are realized.

RESULTS

The following groups of strains have been examined:

1. Clostridium limosum, 17 strains
 2. Clostridium botulinum, 22 strains
 3. Clostridium butyricum, 29 strains
 4. Propionibacterium acnes and related organisms, 43 strains
1. Strains of Clostridium limosum.

This organism is a proteolytic anaerobic gram-positive sporing bacillus, with oval subterminal spores, which has been isolated from a variety of situations including soil, bovine liver infections and liver and kidney infections in alligators. It has proved pathogenic to mice in laboratory tests. A detailed description of Ci. limosum is being prepared for publication. (Cato, Cummins and Smith, 1970).

The results of cell wall analysis of 17 strains of Ci. limosum are shown in Table 1, and it can be seen that 13 of the 17 strains form a very homogeneous group both by sugar pattern and serological tests. The agreement between these two sets of results is perhaps not surprising since it is very likely that the antigenic determinants responsible for cell wall agglutination are those of the cell wall polysaccharide which is the source of the sugars obtained on cell wall hydrolysis. The results of cross absorption tests (not recorded in the table) have shown that the two strains used to prepare antisera to this group (1936-C and 2485) are serologically identical. Of the other 4 strains, two have a distinctive cell wall sugar pattern (Galactose +++, Glucose +, Rhamnose+) and seem to be serologically identical when tested against a serum to one of

TABLE I. COMPOSITION & SEROLOGICAL REACTIONS OF
CELL WALLS OF *CL. LIMOSUM*

Group	Number of Strains	CW Sugars				CW agglutinating titres with sera to strains:			
		Gal.	Glu.	Mann.	Rham.	1928	2755	1937-C	2485
1.	13 (includes strains 1936-C & 2485)	+	+	±	±	*-	-	160-640	160-640
2.	1	+	±	-	±	-	-	20	20
3.	2 (includes strain 1928)	+++	+	-	+	160-320	-	-	-
4.	1 (2755)	+	-	-	-	-	320	-	-

Major CW aminoacids in all strains; ala, glu, meso-DAP

*- = no reaction at dilution 1/20.

them (1928). One of the 2 remaining strains (Group 2) appear to be somewhat intermediate between the first two groups, while the last strain (2755) seems quite unrelated to the others. It is worth noting that if suspensions of formalin-killed intact cells are used in place of suspensions of purified cell wall fragments a totally different picture is obtained. The intact cell suspensions agglutinate with the homologous sera but there is little or no cross-reaction with other strains and no serological groups can be identified. Results for DNA homologies are not yet available for these strains, and in their absence, it is difficult to assess the possible relationships between these serological groups.

2. Strains of *C. botulinum*

Three well-defined cell wall sugar patterns were found in the 22 strains examined, as shown in Table II. The results of cell wall agglutination tests are also given in the Table and it can be seen that with sugar patterns II and III, the two sets of results agree very satisfactorily. Unfortunately, a satisfactory serum has not yet been prepared for strains showing sugar pattern I (proteolytic strains of toxigenic types A, B, and F). However, it is clear that these strains differ serologically from the other two groups.

The grouping of strains as shown by these tests agrees well with that found by other workers. For example, Walker and Batty (1964), using a fluorescent-antibody technique found that types A, B, and F (proteolytic strains) cross-reacted and that types C and D cross-reacted, but that there was no cross-reaction between the A, B, and F strains and the C and D strains: type E strains reacted only with Type E antiserum.

TABLE II. CELL WALL COMPOSITION INOF CLOSTRIDIUM BOTULINUM

(22 strains)

Mucopeptide Aminoacids.

Ala, Glu, meso-DAP, all strains

<u>Cell Wall Sugars.</u>		<u>Cell Wall Agglutination</u> <u>with sera to:</u>		
		Type C	Type D	Type E
I. Glucose.				
Type A	(2)	-	-	-
Type B, Prot.	(1)	-	-	-
Type F, Prot.	(2)	Tr. -	-	-
II. No sugar detected,				
(or trace Glucose only)				
Type C	(4)	+	+	-
Type D	(3)	+	+	-
III. Galactose and Glucose				
Type E	(8)	-	-	+
Type F, Non-Prot.	(2)	-	-	+

TABLE III. CELL WALL COMPOSITION IN CL. BUTYRICUM

Results in 29 strains

Mucopeptide amino acids. Ala, Glu, meso-DAP; all strainsCell wall sugars.

Group I. CW sugar; Glucose.

16 strains, received as:

<u>Cl. butyricum</u>	9
<u>Cl. multif fermentans</u>	5
<u>Cl. fallax</u>	2

Group II. CW sugars; Galactose, Glucose

13 strains, received as:

<u>Cl. butyricum</u>	9
<u>Cl. multif fermentans</u>	1
<u>Cl. amylolyticum</u>	1
<u>Cl. spp.</u>	2

3. Strains of C. butyricum.

Cell wall analyses were carried out on 29 strains which had been definitely or tentatively identified as C. butyricum by the results of routine tests carried out in Anaerobe Laboratory, Virginia Polytechnic Institute. The results are shown in Table III. As can be seen from the Table, not all of these strains were listed as Cl. butyricum when received; however, all except 5 were listed either as Cl. butyricum or Cl. multifementans. Two groups were identified on the basis of cell wall sugar patterns, (see Table III) and on re-examining the results of fermentation and other tests on these strains, some differences could be found between the 2 cell wall groups. These differences are not absolute, but in general strains of Group I (cell wall sugar, glucose) appear to grow more vigorously and produce a lower final pH in dextrin, glycogen and starch, than do strains in Group II (cell wall sugars, galactose and glucose).

TABLE IV. CELL WALL COMPOSITION IN ANAEROBIC DIPHTHEROIDS

	<u>Mucopeptide</u>	<u>Sugars</u>	<u>Serological Reactions*</u>		<u>Number of Strains</u>
			<u>Serum R15</u>	<u>Serum 0162</u>	
Group I	Ala, Glu, Gly LL-Dap	Galactose + Glucose + Mannose ±	320-1280	<20	35
Group II	Ala, Glu, Gly LL-DAP	Glucose + Mannose ±	<20-80	40-80	6

*Serum R15 was prepared against a Group I strain, and serum 0162 against a Group II strain.

The results of DNA homology studies on these strains, reported in detail by Dr. Johnson in the next paper, seem to confirm the existence of the 2 groups. The degree of homology between strains within each group ranged between 75% and 95%, while if strains of one group are tested for homology against a reference DNA prepared from a member of the other group, the degree of homology was considerably lower, varying between 20% and 40%.

It has not been possible to produce satisfactory antisera for these strains and we do not yet know whether each group is characterized by a distinctive cell wall antigen. However, there is some preliminary evidence from immunodiffusion tests that strains in Group II do have such an antigen in common.

"Anaerobic Diphtheroids"

In this collection of 43 strains, two clear-cut groups could be distinguished on the basis of cell wall sugar patterns and cell wall agglutination tests (Table IV). It appears from the serological results that both groups share a common antigenic determinant in their cell walls, since strains in Group II cross-react at rather lower titres with a serum prepared against a strain belonging to group I. However, absorption of this serum with cell walls of a group II strain abolished the cross-reaction while leaving the titre against group I strains

undiminished. The results suggest that the major antigenic determinant of the group I strains is connected with the presence of galactose in the cell wall polysaccharide, since this sugar is not present in strains of Group II.

The DNA homology results appear to correlate with the cell wall sugar groupings. DNA homology values of group I strains are from 95-100 percent with a reference organism from group I, whereas DNA's from group II strains showed in general a slightly lower degree of homology (89-92 percent) to the same reference DNA.

As indicated in Table IV, the characteristic mucopeptide amino acid pattern in these strains is alanine, glutamic acid, glycine and LL-DAP. However, in at least one similar strain only meso-DAP was present, and the amount of glycine was minimal. By cell wall sugar pattern, this strain belonged to Group II, and it behaved serologically in the same way as other group II strains. It is of considerable interest that this strain showed the same relatively high degree of homology to the group I reference DNA as do other group II strains, despite the fact that its mucopeptide contains a different isomer of DAP.

DISCUSSION

It is very likely, as pointed out above, that both cell wall sugar patterns and cell wall serological reactions are functions of the same polymer, namely the cell wall polysaccharide. Sugar patterns made up of one or two of the common hexoses (e.g. glucose alone, galactose alone, or glucose and galactose) are so common in the Gram-positive bacteria that they can be of little value in assigning organisms to the broader taxonomic groups (genus, family or above). Even in the case of species in the same genus, it may be noted that in the present results both Clostridium butyricum and Clostridium botulinum have subgroups within each which are distinguished by having glucose alone or glucose and galactose as their cell wall sugars, but this does not necessarily indicate that strains of Cl. botulinum which have glucose as cell wall sugar are more closely related than their fellows to those strains of Cl. butyricum whose walls contain glucose. Immunological techniques are valuable in this situation, because the specificity of antigen-antibody reactions is such that they will distinguish clearly between cell wall polysaccharides which appear to be similar in terms of crude sugar patterns.

At the moment the main value of the type of analysis proposed here is in the further investigation of a group of strains which have already been grouped on morphological and/or physiological grounds. In such a collection of strains, the analysis of cell wall sugar patterns may be a useful indication of the homogeneity or otherwise of the group. This is well illustrated by the results quoted here for Cl. butyricum, where the finding of 2 distinct cell wall sugar patterns among strains tentatively assigned to this species prompted a further study of the strains.

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