The Mutation of Corynebacterium pyogenes to Corynebacterium haemolyticum

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SUMMARY: Corynebacterium pyogenes has been observed to give rise to mutants which are indistinguishable from Corynebacterium haemolyticum. C. pyogenes ferments lactose and xylose (Xyl+), elaborates a soluble haemolysin and/or a proteolytic enzyme (H+), and its cell walls contain glucose units in addition to certain other components. On horse blood agar C. pyogenes forms small colonies (s) surrounded by large zones of haemolysis. C. haemolyticum and the mutant derived from C. pyogenes ferment lactose but not xylose, produce no soluble haemolysin, and glucose cannot be detected in hydrolysates of their cell walls. The change in the basal structure of the cell wall is accompanied by a lack of immunochemical cross-reaction between the wild type and the mutant or C. haemolyticum. On horse blood agar both C. haemolyticum and the mutant produce relatively large colonies (L) surrounded by narrow bands of haemolysis. The possibility that a single mutation involving cell-wall structure may account for the apparent change from Xyl+H+ to Xyl−H− is discussed. It is suggested that neither C. pyogenes nor C. haemolyticum is a corynebacterium, and that taxonomically both organisms belong to the genus Streptococcus.

Corynebacterium pyogenes has long been known to be unique among the corynebacteria which cause disease in man in that it ferments lactose, does not form metachromatic granules and produces a soluble haemolysin (Brown & Orcutt, 1920; Lovell, 1937; Topley and Wilson’s Principles, 1955). Another corynebacterium, C. haemolyticum, which shares some of these properties, was described by MacLean, Liebow & Rosenberg (1946) who suggested that it resembled C. ovis and C. pyogenes. Evidence to be presented here indicates that C. haemolyticum is a mutant of C. pyogenes, and that as already suggested (Cummins & Harris, 1956) both of these organisms belong to the genus Streptococcus rather than the genus Corynebacterium.

METHODS

Sources of strains. Several strains of Corynebacterium pyogenes and a few strains of C. haemolyticum were isolated between 1945 and 1949 from the throats of patients suffering with acute pharyngitis, from urethral exudates and from cutaneous lesions. This part of the work was carried out at the
406th Medical General Laboratory, U.S. Army, Tokyo, Japan. Additional strains of *C. pyogenes* were kindly supplied by Professor R. Lovell, Dr I. A. Merchant, Dr F. Potoshka (Charles University, Prague), and Miss Dorothy Ballard (see Lovell, 1937, 1941; Merchant, 1935; Ballard, Upsher & Seely, 1947). A culture of strain NCTC 5224 maintained by the National Collection of Type Cultures (Colindale, London, N.W. 7, England) was obtained from Dr S. T. Cowan. The type strain of *C. haemolyticum*, 53-W-1, and the strain 53-W-2, were supplied by Dr A. A. Liebow, together with cultures of *C. ulcerans* (New York State Department of Health, Albany, N.Y., U.S.A.). Strains of *C. diphtheriae gravis, mitis, intermedius* and *C. xerosis* were provided by the United States Army Medical Department Research and Graduate School, Army Medical Centre, Washington, D.C. Several cultures of *C. ovis* were sent to us by Professor H. R. Carne (Carne, 1939). The biochemical patterns of these various strains are shown in Table 1. Information relative to the cell-wall components of representative corynebacteria and streptococci are given in Table 2.

**Culture media**

*Basal medium for biochemical reactions.* The proteolytic activity of *Corynebacterium pyogenes* excludes the use of Hiss’s serum water as a basal medium for carrying out the classical fermentation tests. Further, most peptones which are commercially available inhibit the growth of this organism in concentrations of 0.5–1% (w/v) unless they are first treated with charcoal. Proteose-peptone no. 3 (Difco) permits suitable growth from large inocula when used in the concentration indicated in the following formula: proteose-peptone no. 3 (Difco) 2.5 g.; \( \text{Na}_2\text{HPO}_4 \) 1.0 g.; NaCl 5.0 g.; distilled water 1 l. The pH value of this medium was adjusted to 7.4 and 8 ml. of a 0.2% (w/v) solution of phenol red was added. Volumes of 100 ml. were sterilized by autoclaving at 15 lb./sq.in. for 15 min.

With the exception of corn starch, all carbohydrates used (see Table 1) were of A.R. grade and were employed as sterile filtrates in a final concentration of 1% (w/v). Corn starch was used as a 0.4% (w/v) suspension autoclaved at 10 lb./sq.in. for 10 min. The complete medium (sterile basal medium + sterile carbohydrate solution) in each case was autoclaved at 7 lb./sq.in. for 10 min., and aseptically tubed in 2 ml. amounts in tubes of small diameter. For fermentation tests under anaerobic conditions, 0.1% (w/v) sodium thioglycollate was incorporated in the basal medium and, in this case, the completed and tubed medium received an overlay of sterile mineral oil 4 mm. in depth. For observation of gelatin hydrolysis, 8% (w/v) gelatin was used. Skim milk 10% (w/v) was prepared from the Difco dried product.

*Medium for growth in bulk.* Suspensions of organisms for disintegration were prepared from cultures grown in screw-capped bottles containing 1 l. of medium. The basal medium was infusion broth to which was added before sterilization 0.1% (w/v) sodium phosphate (\( \text{Na}_2\text{HPO}_4 \), anhydrous). This medium was enriched before inoculation by the addition of sterile glucose + bicarbonate solution (10% (w/v) glucose + 10% (w/v) \( \text{NaHCO}_3 \) anhydrous) and
Mutation of C. pyogenes to C. haemolyticum

sterile horse serum, in the proportions of 2 ml. of each/100 ml. final medium. The bottles were inoculated with 4-5 ml. of overnight culture in the same medium, and incubated for a further 48 hr. Formaldehyde solution was then added to a final concentration of 0.5% HCHO and the culture allowed to stand at room temperature for 24 hr.

**Cell-wall analysis.** The preparation of cell-wall fractions, and the chromatographic examination of the products of hydrolysis, were done as previously described (Cummins & Harris, 1956). This involved disintegration of the organisms in a Mickle shaker, and treatment of the cell-wall fraction first with trypsin and ribonuclease and then with pepsin. The purified cell-wall material was hydrolysed, and the hydrolysates examined by two-dimensional paper chromatography. The amounts of the various components present were scored as +++, ++, +, ±, tr. or – depending on the size and intensity of the spots.

**RESULTS**

**Cultural characteristics of Corynebacterium pyogenes and C. haemolyticum**

One of the principal differences between *Corynebacterium pyogenes* and the variants of it described below is that of colony size, and this is indicated for the various strains under discussion by the letters s (=small colony) or L (=Large colony) in brackets after the strain number, e.g. 637 (s) 13081 (L), etc.

**Strains 14-1-s (Lovell), 637-s and 13081-s of Corynebacterium pyogenes.** Following 24 hr. of incubation on blood agar *Corynebacterium pyogenes* (s) strains formed tiny colonies (in contrast to large (L) colony forms to be discussed later) surrounded by large zones of haemolysis (2 to 3 times the diameter of the colony). For some strains growth was much enhanced when the CO₂ tension in the atmosphere was increased. The individual organisms were wedge-shaped, from 0.5 to 2 μ in length, and were Gram-positive. When stained with methylene blue or toluidine blue metachromasia was not evident. Colonies on inspissated serum (Loeffler's slopes) produced marked pitting as they digested the coagulated protein. Skim milk was readily clotted and in a few days the clot was completely digested. All strains fermented lactose and xylose (see Table 1); all failed to show any catalase activity, even when the organisms were first disintegrated in the Mickle shaker. With sodium caseinate it could be shown that coagulation of casein and digestion of the coagulum were both dependent upon the presence of calcium ions (see also Brown & Orcutt, 1920). Cell-free preparations preserved with either 0.01% (w/v) thiomersalate or 0.5% (w/v) phenol caused the precipitation of casein and the subsequent digestion of the precipitate. The same preparations were haemolytic for human, guinea-pig, horse and rabbit erythrocytes. The haemolysin was stable to oxygen; there was no enhancement of its activity by reducing agents. Lovell (1941, 1944) demonstrated that antitoxin prepared against *C. pyogenes* toxin neutralized the toxin in vivo and the haemolysin in vitro. With antitoxin, provided by Professor Lovell, we were able to neutralize both the toxic action
for mice of our crude cell-free extracts as well as their haemolytic activity. Sera prepared against strains 687-s and 14-1-s showed cross-agglutination to within 50–100% of their homologous titres; strain 13081-s antiserum was not prepared.

**Strains 53-W-1 and 53-W-2 of Corynebacterium haemolyticum.** On blood agar, after incubation for 24 hr., *Corynebacterium haemolyticum* grew as easily

Table 1. **Fermentation reactions given by various corynebacteria with the indicated sugars and alcohols**

All organisms gave negative reactions with mannitol, sorbitol, dulcitol, arabinose, rhamnose and raffinose.

<table>
<thead>
<tr>
<th>C. diptheriae</th>
<th>Glucose, fructose</th>
<th>Maltose, dextrose</th>
<th>Galactose</th>
<th>Sucrose</th>
<th>Starch</th>
<th>Lactose</th>
<th>Trehalose</th>
<th>Xylose</th>
<th>Inositol</th>
<th>Glycerol</th>
<th>Gelatin liquefaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>gravis (53-A-7)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>mitis (53-A-4)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>intermedius (53-A-9)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Corynebacterium ulcerans</em> (39164)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Corynebacterium ovis</em> (CS1R-1, -2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Corynebacterium xerosis</em> (53-K-1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

**Streptococcus pyogenes**

*Corynebacterium pyogenes*

Small colony types

- Lovell (14-1-s)
- (14-8-s)
- Merchant (P-14-s)
- The authors' (637s)
- (13081s)

Large colony types

- variants from (s) strains
- (14-1-L)
- (P-14-L)
- (637L)
- (13081L)

received as (L) strains

- Potoshka I, II, III L
- Potoshka IV L
- The authors' six strains L
- The authors' two strains L

**Corynebacterium haemolyticum**

- Liebow 53/W/1
- Liebow 53/W/2


visible colonies about twice the diameter of those of *C. pyogenes*, and surrounded by a narrow band of haemolysis just exceeding the diameter of the colony. The individual organisms were from 2 to 10 μ. in length, were always tapered and usually occurred in mats of several cells. They were Gram-positive
and when stained with metachromatic dyes did not show metachromasia. All strains fermented lactose and failed to ferment xylose. They showed feeble catalase activity in that the evolution of gas from a mixture of \( \text{H}_2\text{O}_2 \) and organisms was hardly discernible; fresh extracts obtained by breaking organisms up in a Mickle disintegrator, however, exhibited definite catalase activity. All strains coagulated milk but did not lyse the coagulum. Cell-free extracts showed neither haemolytic nor proteolytic activity. The organisms were capable of growing from small inocula on such ordinary laboratory media as Neopeptone broth. The growth of the organisms in matted clumps made them entirely unsuitable for the preparation of agglutinable suspensions.

Other ways in which various strains of \textit{Corynebacterium haemolyticum}, \textit{C. pyogenes} and other corynebacteria differ from one another are shown in Table 1.

The mutation of \textit{Corynebacterium pyogenes} to \textit{C. haemolyticum}

When \textit{Corynebacterium pyogenes} strains 14-1-s, 687-s and 13081-s were repeatedly subcultured on blood agar there occasionally appeared a large colony variant, \( L \), which possessed all of the cultural and biochemical properties of \textit{C. haemolyticum}. Three such mutants were designated 14-1-L, 647-L and 13081-L. Over a period of several years no reverse mutation among them from \( L \) to \( s \) has been observed.

All the strains obtained from Dr Potoshka labelled \textit{Corynebacterium pyogenes} were large colony strains, as were at least eight distinct isolates sent to us as reference cultures from various hospitals in Japan. All strains received from Professor Lovell and from Dr Merchant were small colony strains, or what we assume to be the wild type \textit{C. pyogenes}. In all, four wild types, two from animal sources and two from human cases, were examined for evidence of the mutation from \( s \) to \( L \). Four \( L \) variants, one from each of the wild types, were obtained. Selection was carried out on the basis of colony morphology alone. All four mutants gave identical fermentation and proteolytic reactions which were indistinguishable from those of \textit{C. haemolyticum}. Since the \( L \) mutant grows fairly well on ordinary laboratory media, whereas the \( s \) does not, selection on such media may account for the preponderance of \( L \) variants in reference cultures from hospital laboratories.

Cell-wall composition of \textit{Corynebacterium pyogenes} \( s \) and \( L \)

Determinations of the major components of the cell walls of eight strains of \textit{Corynebacterium pyogenes} \( s \) and \( L \) were made. In all cases the main amino acid components were alanine, glutamic acid and lysine, and the characteristic sugar was rhamnose. This pattern of cell-wall composition closely resembles that previously reported for streptococci of different Lancefield groups, but it is decidedly unlike that of various corynebacteria (Cummins & Harris, 1956) where the characteristic amino acids of the cell wall are alanine, glutamic acid and diaminopimelic acid, and the characteristic sugars are arabinose and galactose (see Table 2).

The cell-wall composition of the wild type \textit{Corynebacterium pyogenes}
differed from that of the L mutant. Glucose was regularly present in the wild type and absent from *C. haemolyticum* and the other L variants. Mannose was sometimes detected in the wild type but not in the mutant strains (Table 3).

Table 2. **Principal products of hydrolysis of cell-wall preparations from various corynebacteria, streptococci, Corynebacterium pyogenes and C. haemolyticum**

Data from Cummins & Harris, 1956.

<table>
<thead>
<tr>
<th></th>
<th>Arabinose</th>
<th><em>Rhamnose</em></th>
<th>Galactose</th>
<th>Glucose</th>
<th>Manose</th>
<th>Glucosamine</th>
<th>Galactosamine</th>
<th>Unknown hexosamine</th>
<th>Alanine</th>
<th>Glutamic acid</th>
<th>Lysine</th>
<th>Diaminopimelic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corynebacteria (C. diphtheriae, C. hofmannii, C. xerosis, C. renale, C. ovis, C. ulcerans, C. equi, C. murium)</td>
<td>++</td>
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<tr>
<td>Streptococci (representatives of groups A, B, C, D, E, F, G)</td>
<td></td>
<td>--</td>
<td>++</td>
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<td>++</td>
<td>+</td>
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<tr>
<td><em>C. pyogenes</em> (6 strains)</td>
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<tr>
<td><em>C. haemolyticum</em>, strain 53/W/1</td>
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<tr>
<td><em>C. murium</em> cell walls contained a small amount of rhamnose</td>
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</tbody>
</table>

Table 3. **Cell-wall composition in Corynebacterium pyogenes, C. haemolyticum and the large (L) colony mutants**

<table>
<thead>
<tr>
<th></th>
<th>Arabinose</th>
<th>Rhamnose</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Manose</th>
<th>Glucosamine</th>
<th>Galactosamine</th>
<th>Unknown hexosamine</th>
<th>Alanine</th>
<th>Glutamic acid</th>
<th>Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. pyogenes</em>, NCTC 5224</td>
<td></td>
<td>++</td>
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<tr>
<td><em>C. haemolyticum</em>, strain 53-W-1</td>
<td></td>
<td>++</td>
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<tr>
<td><em>C. pyogenes</em>, strain 637-s</td>
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<td>++</td>
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<tr>
<td><em>C. pyogenes</em>, strain 637-L</td>
<td></td>
<td>++</td>
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<tr>
<td><em>C. pyogenes</em>, strain 14-1-s</td>
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<td>++</td>
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<tr>
<td><em>C. pyogenes</em>, strain 14-1-L</td>
<td></td>
<td>++</td>
<td></td>
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<tr>
<td><em>C. pyogenes</em>, strain 13081-s</td>
<td></td>
<td>++</td>
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<tr>
<td><em>C. pyogenes</em>, strain 13081-L</td>
<td></td>
<td>++</td>
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</tbody>
</table>

No differences were found in the amino acid or hexosamine distributions in any of the strains except 14-1-L, in which galactosamine was not detected. The substance referred to as ‘unknown hexosamine’ in Table 3 (originally described by Strange & Powell, 1954) has recently been more fully characterized; its probable structure is 3-α-carboxyethyl-hexosamine (Strange, 1956; Strange & Dark, 1956).
Precipitation tests with antisera prepared against cell-wall fragments

Crude cell-wall fractions prepared from Corynebacterium pyogenes NCTC 5224 were washed but not treated with trypsin or pepsin and were used as antigen for rabbit immunization. The antiserum obtained gave strong precipitation reactions with formamide extracts (Fuller, 1938) of whole organisms of the homologous strain. Similar extracts of strains 637-s, 13081-s, 14-1-s and six other strains of C. pyogenes s gave equally strong reactions with this serum. Extracts of strains 637-L, 13081-L and 14-1-L did not react, nor did an extract of 58-W-1, the type strain of C. haemolyticum.

Table 4. Serological reactions of formamide extracts in relation to the sugars and hexosamines present in cell walls

<table>
<thead>
<tr>
<th>Content of sugars and hexosamines</th>
<th>Reaction of formamide extracts.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sera</td>
</tr>
<tr>
<td></td>
<td>C. pyogenes, NCTC 5224</td>
</tr>
<tr>
<td></td>
<td>C. pyogenes, 637-L</td>
</tr>
<tr>
<td></td>
<td>Streptococcus sp., group A</td>
</tr>
<tr>
<td></td>
<td>Streptococcus sp., group G</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>++</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>+</td>
</tr>
<tr>
<td>'Unknown' hexosamine</td>
<td>+</td>
</tr>
</tbody>
</table>

Antiserum obtained following the immunization of rabbits with similar cell-wall fractions from strain 637-L did not give precipitin reactions with strains 637-s, 13081-s, 14-1-s, or any of the other s strains of Corynebacterium pyogenes. This 637-L antiserum gave clear-cut reactions with antigens prepared from strains 13081-L, 14-1-L, and C. haemolyticum 53-W-1. Extracts from both s and L strains uniformly failed to react with group A streptococcal antiserum, and this lack of reaction is of interest in the case of the L variants, in view of the rather close resemblance between their cell-wall compositions and that of Streptococcus pyogenes (see Table 4).

Dr R. E. O. Williams (Streptococcal Reference Laboratory, Colindale, London) kindly examined the reactions of these extracts with antisera to streptococci of other Lancefield groups, and found that extracts from s strains cross-reacted with several different group G sera, but not with other group sera. Extracts of L strains did not react with any grouping sera. The cross-reaction with group G sera was also given by extracts of a purified cell-wall
fraction from *Corynebacterium pyogenes* NCTC 5224. Since this fraction had been treated with trypsin and pepsin during its preparation, it seems unlikely that the reactions with group G antiserum were due to a minor antigen.

It has been shown conclusively by McCarty (1952) that the group antigen of *Streptococcus pyogenes* is the polysaccharide moiety of the cell walls, and this is presumably true also of streptococci of other groups, and of *Corynebacterium pyogenes* and *C. haemolyticum*. The sugar components found in the cell walls of two group G strains were rhamnose and galactose, together with three hexosamines (Cummins & Harris, 1956; and unpublished observations). The cross-reaction between these strains and *C. pyogenes*, and the lack of it in the case of strain 14-1-L and *S. pyogenes*, shows that there is little correspondence between qualitative chemical composition and immunological specificity. The latter property is presumably due more to the special arrangement of specific groups at the surface of the polysaccharide molecule than to the actual monosaccharide units themselves. This point is also illustrated in the work of McCarty (1956) on the serological specificity of cell-wall polysaccharides in a group A streptococcus and a variant of it whose polysaccharide did not react with group A serum. Both contained rhamnose and glucosamine as the principal components of the polysaccharide, but in the variant strain specificity seemed to depend on a rhamnose-rhamnose linkage, while in the parent this linkage was masked by side chains of N-acetylglucosamine which determined the original group A specificity.

**DISCUSSION**

It seems evident from the observations presented here that the bacterium described as *Corynebacterium haemolyticum* is actually a mutant form of *C. pyogenes*. The wild type, *C. pyogenes*, ferments lactose and xylose, produces a soluble haemolysin and/or proteinase and contains glucose in its cell wall, while the mutant *C. haemolyticum* ferments lactose, but not xylose, does not produce a soluble haemolysin, and glucose is not detectable in its cell-wall hydrolysates. Whether or not a single mutation could account for such a seemingly multiple change as that manifest in the s to L variation, is certainly open to question. There is a possibility that the colonial and haemolytic characters expressed in the L mutant may represent the culmination of a chain of mutational steps. On the other hand, perhaps the change in the cell-wall character CWgluc$^+\rightarrow$CWgluc$^-$ is the only change which takes place during the change from small to large colony form, and that the apparent alteration in fermentation pattern obtains because xylose is barred from reaching the interior of the cell as a result of the altered nature of the cell wall. On Mueller & Miller's semi-defined casein hydrolysate medium (1941) strain 687-s grows with xylose or glucose as a carbon source; strain 687-L does not grow with xylose as a carbon source but grows when glucose is present. It has been mentioned that one manner in which s and L colonies differ lies in the size of the haemolytic zone that develops around colonies on a blood plate. Both colonies are obviously haemolytic, but in the case of L the haemolysin never
Mutation of C. pyogenes to C. haemolyticum

reaches a detectable concentration in liquid medium. The ability to produce haemolysin may be the same in both the wild type and the mutant, but its liberation in detectable amounts may be possible only in the case of the wild type. The same phenomenon may account for the lack of proteolytic ability found in L cultures. We have already indicated the possibility that the haemolysin and the proteolytic enzyme are identical. That the change in cell-wall composition is a significant one is suggested by the lack of immunological cross-reaction between s and L strains.

There are other minor differences to be found between certain s and L pairs, some of which are recorded in Table 1. It is evident from Table 1 that all strains of Corynebacterium pyogenes and C. haemolyticum ferment lactose. We feel strongly that these organisms are 'lactic acid bacteria', probably streptococci; certainly they have little in common with other corynebacteria. The metabolism of C. pyogenes is that associated with facultative anaerobes and the pattern of its cell-wall components is quite different from that found in such corynebacteria as C. diphtheriae and C. ovis. The fact that C. pyogenes commonly assumes the morphology of a bacillus has no doubt been in large part responsible for its being placed initially among the corynebacteria. This morphological characteristic is an inconstant one, however, for under appropriate conditions these organisms grow as cocci in short chains (Brown & Orcutt, 1920).

REFERENCES


W. L. Barksdale and others


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