Physiological, Chemical and Ultrastructural Characteristics of *Corynebacterium rubrum*

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SUMMARY

A study of the ultrastructural characteristics, the physiological properties and an analysis of hydrolysates of phenol-treated cell walls of *Corynebacterium rubrum* were made. While the arabinogalactan-peptidoglycan complex found in the cell-wall hydrolysates was that common to the genera *Corynebacterium*, *Mycobacterium* and *Nocardia*, the ultrastructural patterns found, the physiological reactions observed, the sensitivity to nocardio-phages but not to corynebacteriophages or mycobacterial phages, and the (previously reported) presence of nocardomycolic acids, combined with the absence of corynemycolic acids and mycolic acids, indicate that this species belongs in the genus *Nocardia* rather than in the genus *Corynebacterium*.

It is proposed, therefore, that *Corynebacterium rubrum* be renamed *Nocardia corynebacterioides* nomen novum.

INTRODUCTION

Corynebacterium rubrum was described by Crowle (1962). Gordon (1966) proposed the reclassification of this species as a member of the 'rhodochrous' group. The ultrastructure of wild-types of the genera *Mycobacterium*, *Nocardia* and *Corynebacterium* has been investigated and revealed structural differences in their cell walls (Imaeda & Ogura, 1963; Kawate & Inoue, 1965; Farshtchi & McClung, 1967; Lickfeld, 1967; Montez & Black, 1967; Imaeda, Kanetsuna & Galindo, 1968; Beaman & Shankel, 1969; Hard, 1969).

The phenol-treated cell wall is composed of a mycolic acid-arabinogalactan-peptidoglycan complex, the common structural component of mycobacterial cell walls (Kanetsuna, 1968), suggesting that this complex may be of taxonomic significance.

The purpose of this study was to examine the physiological and ultrastructural characteristics of *Corynebacterium rubrum* in relation to its classification. Chemical and morphological analyses were made of cell wall components obtained by various chemical and enzymatic treatments.

METHODS

Corynebacterium rubrum ATCC14898 was maintained on tryptose-phosphate broth (TPB) Difco, supplemented with 5 % glycerol and 0.04 % Tween 80, for ultrastructural and chemical studies.

For physiological studies, nutrient broth, brain heart infusion (BH1) broth, glucose yeast extract medium (Waksman, 1950), Kirchener's medium (Crowle, 1962), Farshtchi's medium (Farshtchi & McClung, 1967), Middlebrook 7H9 glucose and Lowenstein-Jensen medium

(Difco) were used. Sugar tests were carried out according to the methods recommended by Barksdale, Li, Cummins & Harris (1957), Gordon & Mihm (1957, 1959); Edwards & Ewing, (1962) and Gordon (1966). Carbohydrates (1 %, w/v) were sterilized by filtration and added aseptically to the medium. Glucose was used at 1.5 % final concentration.

Organic acids as carbon source used at 0.2 % final concentration (Gordon & Mihm, 1957). Tests for indole production, methyl-red, Voges-Proskauer, H₂S production, growth in KCN, nitrate reduction and decarboxylation of amino acids were made by the methods recommended by Edwards & Ewing (1962).

Cultural condition for ultrastructural and chemical studies. Cultures in liquid media were aerated on an orbital shaker at 37° for morphological studies. For cell-wall preparations, organisms grown in liquid media for 1 week at 37° without shaking were killed with formaldehyde (0.5 % final concentration). After several hours the bacteria were sedimented at 3000g in a Sorvall KSB system and washed in distilled water. All procedures were performed at 23 to 25°.

Treatment of cell wall. The washed bacteria were freeze-dried in a 'Virtis' lyophilizer. The dried cells were extracted with neutral solvents at 23 to 25°: ethanol-ether 1:1 (v/v) for 1 week, chloroform for 24 h, acetone for 24 h, successively. Organisms without free lipids were washed with distilled water several times and centrifuged at 10000g for 15 min. They were disrupted in a mortar with dry ice, followed by sonication at 20 kHz for 1 h with a Branson sonifier in an ice bath.

The suspension was centrifuged at 1500g for 20 min to discard unbroken cells and the supernatant fluid was recentrifuged at 10000g for 30 min.

Sediments were treated with 0.5 % trypsin (Sigma) in 0.1 M-phosphate buffer pH 7.6 at 37° for 6 h to remove the remaining cytoplasmic components. Insoluble material was collected by centrifuging at 10000g for 20 min and washed in distilled water several times. The cell walls thus prepared were examined with a Baush & Lomb Spectronic 600 spectrophotometer at wavelengths ranging from 230 to 300 nm to check for the presence of nucleic acids as an indication of cytoplasmic contaminants. The preparation not showing any specific absorbance at 260 nm was used as the cell-wall fraction.

Treatment of the cell walls with 90 % (w/v) phenol was performed (Imaeda *et al.* 1968; Kanetsuna, 1968) to obtain the lipopolysaccharide peptidoglycan complex; 45 % (w/v) phenol was also used to eliminate free protein and lipopolysaccharide from the phenol-insoluble lipopolysaccharide-peptidoglycan complex. Cell walls were also treated with 0.1 % pancreatin in 0.1 M-phosphate buffer at pH 7.6 for 6 h, 5 % TCA at 4° for 72 h or 0.4 % sodium dodecyl sulphate (SDS) (Sigma).

Chemical analysis of phenol-treated cell-wall fractions. For the determination of sugars, lipids, amino sugars and amino acids, samples of cell walls were examined as previously described (Kanetsuna, 1968). Quantitative determination of amino acids was carried out with a JEOL amino acid analyser JLC-3BC.

Electron microscopy. Whole organisms and cell walls, either without treatment or after treatment with different neutral solvents, were positively or negatively stained with 2% sodium silicotungstate at pH 7.5.

To obtain ultrathin sections the organisms at various stages of growth were centrifuged, and fixed by the methods of Ryter & Kellenberger (1958). Fixation with 4 % formaldehyde in 0.15 M-tris-HCl buffer pH 7.4 containing 1 % NaCl and 0.2 % CaCl₂ by a modification of Stoeckenius method (Stoeckenius & Rowen, 1967; Imaeda *et al.* 1969) was also employed, followed by treatment with 1 % osmium tetroxide or 1 % potassium permanganate. After dehydration with acetone, cells were embedded in Araldite.

Fig. 1. Phase-contrast micrograph of cells of *Corynebacterium rubrum*. Note the cells in pairs, V forms, clumps and palisade arrangements.

Table 1. *Physiological characteristics of Corynebacterium rubrum*

Test	Results	Test	Results
Indole	—	Acid from lactose	—
Methyl-red	—	Acid from mannitol	—
Voges-Proskauer	—	Acid from mannose	+
Growth at 10°	+	Acid from melibiose	—
Growth at 40°	+	Acid from raffinose	—
Growth at 45°	—	Acid from rhamnose	—
Survival of 60°, 4 h	—	Acid from sorbitol	+
Sensitive to 0.01 % methyl violet	+	Acid from sorbose	—
Sensitive to 0.01 % pyronin B	+	Acid from sucrose	—
Sensitive to 0.01 % brilliant green	+	Acid from trehalose	—
Decomposition of casein	—	Acid from xylose	—
Decomposition of gelatin	—	Utilization of acetate	+
Decomposition of tyrosine	—	Utilization of citrate	+
Decomposition of xanthine	—	Utilization of malate	+
Hydrolysis of starch	—	Utilization of lactate	+
Hydrolysis of hippurate	—	Utilization of pyruvate	+
Catalase	+	Utilization of succinate	+
Urease	—	Growth in KCN medium	—
Nitrate reduction	—	Phenylalanine deaminase	—
Motility	—	Alanine	+
Acid from adonitol	—	Arginine	+
Acid from arabinose	—	Cysteine	+
Acid from cellobiose	—	Hydrogen sulphide production	—
Acid from dulcitol	—	Oxidation-fermentation test	—
Acid from fructose	+	medium (Hugh & Leifson, 1953)	—
Acid from galactose	—	Haemolysis	—
Acid from glucose	—		
Acid from inositol	—		
Acid from inulin	—		

* This activity is inhibited by warming the cells at 68° in water bath for 20 min.

Fig. 2. Electron micrograph showing negatively stained (silicotungstate) *Corynebacterium rubrum* after 20 min agitation in physiological saline. Note how the 'fibrillar-like' substance that covers the surface of the cell has been partially removed (arrows) by this treatment.

The ultrathin sections were cut with a MT-2 Porter-Blum ultramicrotome and double stained with uranyl acetate and lead citrate. The electron microscopes used were a Hitachi HU-11B, HS-6 and JEM 7-A.

Fig. 3. Electron micrograph of a negatively stained (silicotungstate) *Corynebacterium rubrum* cell. Treated with acetone. The fibrillar-like substance shown in Fig. 2 appears now as a crystal-like structure (arrows).

RESULTS

Physiology and gross morphology of Corynebacterium rubrum

Corynebacterium rubrum is aerobic. It grows in tryptose phosphate, nutrient broth, brain–heart infusion broth (Difco), glucose–yeast extract medium and Lowenstein–Jensen media, at 37°, in chemically defined media such as Kirchener's medium and Middlebrook 7H9 glucose (Difco). In stationary liquid media growth starts with the production of a fine

Fig. 4. (a) Electron micrographs of negatively stained (silicotungstate) *Corynebacterium rubrum* cells. (a) Treatment with 0.1 % pancreatin (6 h at 37°) partially removed the fibrillar-like substance from the surface, disclosing an underlying network of fibrils 8 to 10 nm in diam. (arrows). (b) After phenol extraction, both the outermost fibrillar-like substance and the underlying network of fibrils observed in (a) were removed. An innermost network of fibrils (arrows) with a mean diam. of about 4 nm is now detected.

dark-orange sediment which after about 35 h at 37° forms an orange-red film on the surface of the media. Colour becomes more intense in the glucose yeast extract media. In chemically defined media the orange-red film appears more rapidly and has a dry appearance. On solid media orange-red pigmented colonies with a granular central region are surrounded by a smooth and shiny peripheral zone.

By phase-contrast bacterial cells were seen in pairs, V forms, clumps and palisade arrangements (Fig. 1). It is Gram-positive, not acid-fast, and has metachromatic granules and lipophilic granules in its cytoplasm when stained by Albert's method or with oil red O. Results of the physiological tests applied to the *Corynebacterium rubrum* are presented in Table 1.

Corynebacterium rubrum is sensitive to 10 i.u. bacitracin/ml, and 2 i.u. penicillin/ml in quantitative antibiotic sensitivity test and sensitive to 2 mg lincomycin, 10 µg oxytetracycline, 10 µg chloramphenicol, 5 mg neomycin, 2 mg erythromycin, 1 µg sulphathiazole, 25 µg ampicillin, 2 µg dehydrostreptomycin, 30 µg novobiocin and 25 µg cephalotin in qualitative sensitivity test with antibiotic discs (Difco).

Fig. 5. Electron micrograph of a longitudinal section of a *Corynebacterium rubrum* cell after double fixation with formaldehyde and OsO_4 . The fibril-like structure (arrows) is observed adherent to the outermost surface of the cell. Note the vesicular structure of the mesosome (*m*) in relation to the septum (*s*). Remains of mesosome membranes are also detected (*mm*).

Ultrastructure

Negatively stained cells of *Corynebacterium rubrum* are covered with a fibril-like substance (Fig. 2) which can be partially removed by sonication and neutral solvents. Treatment with neutral solvents transforms fibrils into a crystal-like substance *in situ* or after they are

Fig. 6. A positively stained (silicotungstate) *Corynebacterium rubrum* cell, during active division. The formation of the septum is completed and the mesosomes with a tubular appearance are seen at both sides of the septum. Structures similar to those described as polyphosphate granules are also detected within the cells.

removed from the cell surface (Fig. 3). The shape of the crystal-like material is not altered by treatment with 0.5 % trypsin, pancreatin or pepsin. This outermost fibrillar layer, which may correspond to the outermost diffuse dense layer seen in ultrathin sections (Fig. 5) is a unique surface substance of this species and has never been observed by us on the surface of any mycobacterium, nocardia or corynebacterium.

When the organisms were agitated for 20 min with physiological saline, the outermost fibrillar layer was partially removed disclosing an underlying layer. Treatment with 0.1 % pancreatin at 37°, or 5 % TCA at 4° also removed the outermost layer. The underlying layer was composed of a network of fibrils of 8 to 10 nm diam. (Fig. 4*a*). SDS (0.4 %) and phenol removed completely the innermost fibrillar network layer containing fibrils of about 4 nm diam. (Fig. 4*b*).

In ultrathin sections the cell wall is composed of an outermost diffuse layer and a middle moderately dense layer 10 nm thick which adheres to the plasma membrane. The total thickness of the cell wall is about 18 to 20 nm. The plasma membrane is often seen to be continuous with the mesosome.

In the cytoplasm, granules possibly representing polyphosphate are observed along with other cytoplasmic structures such as ribosomes and a nuclear region (Fig. 5, 6). The mesosome in positively stained cells is revealed as a tubular structure which appears to be lamellar or vesicular in ultrathin sections (Fig. 6); occasionally the mesosome shows contact with the nuclear area.

Table 2. Chemical composition of the phenol-treated cell walls of *Corynebacterium rubrum*

Values are expressed in % (w/w)				
Strain	Pentose as arabinose	Hexose as galactose	Amino sugar as glucosamine	Amino acid as alanine
<i>C. rubrum</i>	13.7	24.9	6.2	22.6

Table 2 shows the chemical composition of the phenol-treated cell walls of *Corynebacterium rubrum*. This fraction has α , ϵ -diaminopimelic acid, glutamic acid and alanine as the principal amino acids and glucosamine and muramic acid as the principal amino sugars.

The molar ratio of diaminopimelic acid, glutamic acid, alanine, muramic acid and glucosamine is 1:1:1.9:0.9:0.9. The sugars contained in this fraction are arabinose and galactose. These components may be considered to form an arabinogalactan-peptidoglycan complex, as reported in *Mycobacterium* and *Nocardia* (Cummins & Harris, 1958; Kanetsuna, 1968; Cunto, Kanetsuna & Imaeda, 1969). There are not mycolic acids in this fraction.

DISCUSSION

On physiological characteristics, *Corynebacterium rubrum* has been included within the 'rhodochrous' group. The physiological assays that were carried out during this investigation lead us also to consider that this species does not belong to the genus *Corynebacterium* but rather to be more closely related to *Mycobacterium* or *Nocardia*. However, the ultrastructure of *C. rubrum* is different from that observed in wild-type of strains of *Corynebacterium*.

For example, the presence of an outermost dense cell-wall layer in *Corynebacterium minutissimum*, *Corynebacterium diphtheriae* and *Corynebacterium ovis* (Lickfeld, 1967; Montez & Black, 1967; Hard, 1969) and the multiseptum formation sometimes observed in *C. diphtheriae* (Lickfeld, 1967) make these species very different from *Corynebacterium rubrum*. On the other hand, several mutants of mycobacteria which contain red pigment show very similar ultrastructural characteristics to those of *C. rubrum* (T. Imaeda, personal communication).

Many human and animal pathogenic corynebacteria and many mycobacteria and nocardia cell walls are characterized by arabinose, galactose and α , ϵ -diaminopimelic acid (Cummins & Harris, 1958). The presence of this polymer in the *Corynebacterium rubrum* cell wall is not conclusive evidence that this species belongs to the genera *Corynebacterium*, *Mycobacterium* or *Nocardia*, but the isolation of nocardomycolic components from cell-wall analyses of *C. rubrum* (M. A. Lanelle, B. L. Beaman and W. L. Barksdale, personal communication) showed it to have low-molecular-weight nocardomycolic acids, suggesting that its taxonomic position was closer to *Nocardia rubra* than to *Nocardia asteroides* or *Nocardia brasiliensis*. Lechevalier, Horan & Lechevalier (1971) also reported the presence of nocardomycolic acid in *C. rubrum* and suggested that *C. rubrum* was lysed only by nocardiphages, but not by mycobacterial phages or by corynebacteriophages (Arden, 1970).

Based on the comparisons of ultrastructure, physiological characteristics, cell-wall composition and phage sensitivity it is concluded that *Corynebacterium rubrum* should be included in the genus *Nocardia*. It is proposed that this species be named *Nocardia*

corynebacteroides. The change in specific epithet is needed because the combination *Nocardia rubra* is pre-empted by *Nocardia rubra* (Kruse) Chalmers & Christopherson, which combination, according to Buchanan, Holt & Lessel (1966), is validly published and legitimate.

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