Distribution of Cytochromes in Selected Species of Corynebacteria Pathogenic to Animals

By C. AdinAryana Ana Reddy* and M. Kao

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824, U.S.A.

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Evidence is presented for the first time for the presence of a b-type cytochrome in extracts of Corynebacterium renale, C. bovis, C. kutscheri and C. pseudotuberculosis, and b- and c-type cytochromes in extracts of Rhodococcus equi (syn. C. equi). Previous observations on the presence of a b-type cytochrome in C. pyogenes were extended.

INTRODUCTION

Previous studies have shown that certain strains of Corynebacterium diphtheriae contain cytochromes b, c and a, while other strains contain only cytochrome b (Pappenheimer et al., 1962; Scholes & King, 1965; Yaoi & Tamiya, 1928). In contrast, little conclusive evidence is available on the presence of cytochromes in coryneform bacteria of animal origin, except for a recent report demonstrating the presence of a b-type cytochrome in a strain of C. pyogenes (Reddy et al., 1977). Julak et al. (1978) reported the presence of a- and c-type cytochromes in a strain of C. ovis (syn. C. pseudotuberculosis) and a b-type cytochrome in a strain of C. pyogenes, but little supporting evidence was presented. The aim of this investigation was to determine the cytochrome content, a characteristic reported to be of some significance in bacterial taxonomy (Meyer & Jones, 1973), of selected species of corynebacteria pathogenic to animals.

METHODS

Bacterial strains. Corynebacterium bovis (BT435-76), Rhodococcus equi (syn. C. equi) (E222-70), C. kutscheri (MA76-74), C. pyogenes (SS7-74), C. pseudotuberculosis (MM120-71) and C. renale (CE565-73) were obtained from the culture collection of this department. Biochemical characteristics and other features of these strains have already been published (Reddy & Kao, 1978) and are typical for the respective species (Rogosa et al., 1974).

Maintenance and growth of cultures. Cultures were maintained as previously described (Reddy & Kao, 1978). All cultures, except C. pyogenes, were grown in 500 ml Bacto Brain Heart Infusion broth (BHI) supplemented with 0.2% (w/v) dextrose, contained in 1 litre foam-plugged Erlenmeyer flasks. The same medium, supplemented with 0.0002% (w/v) haemin was used for growing C. pyogenes. Inocula consisted of 3 ml BHI broth cultures incubated at 37°C for 48 h. Incubation was at 25°C on a rotary shaker at 200 rev. min⁻¹. Growth was monitored by following the increase in A₆₆₀ using a Bausch & Lomb Spectronic-20 spectrophotometer. Cells in the early stationary phase of growth were harvested by centrifugation at 15 000 g for 15 min at 4°C and were washed three times with 100 ml 0.02 M-phosphate buffer, pH 7.0. Cells were resuspended in the same buffer (approx. 1 g cells ml⁻¹), broken in a French pressure cell at 15 000 lbf in⁻² (104 MPa) and centrifuged as above to remove unbroken cells and large cell fragments. The supernatants, hereafter referred to as cell extracts, were collected and examined for the presence of cytochromes.

Difference spectra. The presence of cytochromes was determined by dithionite-reduced minus air-oxidized difference spectra as previously described (Reddy et al., 1977; White et al., 1962). All spectra were determined at room temperature with a Cary-15 split-beam scanning spectrophotometer, using 1.25 ml cell extract or pyridine haemochrome preparations in 1.5 ml Quarsil cuvettes with a 10 mm light path. The protein content of the cell extract was measured by the biuret method (Gornall et al., 1949) in the presence of 0.06% (w/v) sodium deoxycholate. Crystalline bovine serum albumin (Sigma) was used as the standard. Nomenclature for the cytochromes and pyridine haemochromes (see below) is that proposed by the Commission on Biochemical Nomenclature (1973).
Fig. 1. (a) Dithionite-reduced minus air-oxidized (solid line) and air-oxidized minus air-oxidized (broken line) difference spectra of R. equi cell extract (10.9 mg protein ml\(^{-1}\)). (b) Dithionite-reduced minus air-oxidized (solid line) and air-oxidized minus air-oxidized (broken line) difference spectra of pyridine haemochrome from acid/acetone extract of R. equi. Extract from 15.1 mg cell protein was present per ml of pyridine/KOH. (c) Dithionite-reduced minus air-oxidized (solid line) and air-oxidized minus air-oxidized (broken line) difference spectra of pyridine haemochrome from the residue left after acid/acetone extraction of R. equi cell extract as described for (b).

**Extraction of haem and preparation of pyridine haemochromes.** A modification of the method of Jacobs & Wolin (1963) was employed to prepare pyridine haemochromes. Cell extracts containing approximately 76 mg protein were lyophilized at \(-40^\circ\)C, mixed with 40 ml cold acetone and centrifuged at 15000 g for 15 min at 4 \(^\circ\)C. This acetone wash was repeated and protohaem in the washed pellet extracted three times with acid/acetone (40 ml cold acetone containing 0.4 ml 2.4 M HCl). The three acid/acetone extracts were pooled and dried under vacuum and the dry residue was resuspended in 3.5 ml pyridine plus 3.5 ml 0.2 M KOH to obtain the pyridine haemochrome.

Haem c, the prosthetic group of cytochrome c, which remains in the residue after acid/acetone extraction was resuspended in 3.5 ml pyridine and 3.5 ml 0.2 M KOH to obtain the pyridine haemochrome.

**RESULTS**

The dithionite-reduced minus air-oxidized difference spectrum of R. equi cell extracts (Fig. 1a) showed two \(\alpha\)-absorption maxima (562 and 555 nm), a broad \(\beta\)-absorption maximum (520 nm), and two \(\gamma\)-absorption maxima (a shoulder at 425 nm and a peak at 415 nm). These results suggest that both \(b\)- and \(c\)-type cytochromes are present in this organism. The broad \(\beta\)-absorption maximum apparently represents the combined \(\beta\)-peaks of \(b\)- and \(c\)-type cytochromes. Cytochromes of the \(b\)-type contain haem groups that are not covalently bound to the protein and are readily extractable with acid/acetone. The presence of a \(b\)-type cytochrome in R. equi was confirmed by the difference spectrum of the pyridine haemochrome derivative of the acid/acetone-extracted protohaem, which showed characteristic absorption maxima at 556, 525 and 420 nm for \(\alpha\)-, \(\beta\)-, and \(\gamma\)-peaks, respectively (Fig. 1b). The difference spectrum of the pyridine haemochrome derivative of the residue, left after acid/acetone extraction of cell extract, showed absorption maxima at 550, 520 and 420 nm, confirming the presence of haem c (Fig. 1c).

Dithionite-reduced minus air-oxidized difference spectra of cell extracts of C. renale (Fig. 2a), C. pyogenes (Fig. 2b), C. bovis (Fig. 3a), C. kutscheri (Fig. 3b) and C. pseudotuberculosis (Fig. 4) showed absorption maxima characteristic of \(b\)-type cytochrome. This was further confirmed by the difference spectra of pyridine haemochrome derivatives of the acid/acetone-extractable haem present in cell extracts of all the above species which also showed absorption maxima characteristic of a \(b\)-type cytochrome (Table 1). No \(c\)-type cytochrome or haem c was detectable in cell extracts of the above species.

**DISCUSSION**

It has been recognized for several years that the genus Corynebacterium (Rogosa et al., 1974) includes species which have little in common with each other. Corynebacterium species of human
Fig. 2. (a) Dithionite-reduced minus air-oxidized (solid line) and air-oxidized minus air-oxidized (broken line) difference spectra of *C. renale* cell extract (12.3 mg protein ml\(^{-1}\)). (b) Dithionite-reduced minus air-oxidized (solid line) and air-oxidized minus air-oxidized (broken line) difference spectra of *C. pyogenes* cell extract (10.4 mg protein ml\(^{-1}\)).

Fig. 3. (a) Dithionite-reduced minus air-oxidized (solid line) and air-oxidized minus air-oxidized (broken line) difference spectra of *C. bovis* cell extract (6.0 mg protein ml\(^{-1}\)). (b) Dithionite-reduced minus air-oxidized (solid line) and air-oxidized minus air-oxidized (broken line) difference spectra of *C. kutscheri* cell extract (3.8 mg protein ml\(^{-1}\)).

Fig. 4. Dithionite-reduced minus air-oxidized (solid line) and air-oxidized minus air-oxidized (broken line) difference spectra of *C. pseudotuberculosis* cell extract (7.7 mg protein ml\(^{-1}\)).
Table 1. Absorption maxima of the pyridine haemochrome derivatives of protohaem extracts from different corynebacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Cytochrome</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
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</thead>
<tbody>
<tr>
<td>C. renale</td>
<td>b</td>
<td>556</td>
<td>523</td>
<td>420</td>
</tr>
<tr>
<td>C. pyogenes</td>
<td>b</td>
<td>558</td>
<td>525</td>
<td>423</td>
</tr>
<tr>
<td>C. bovis</td>
<td>b</td>
<td>556</td>
<td>526</td>
<td>424</td>
</tr>
<tr>
<td>C. kutscheri</td>
<td>b</td>
<td>556</td>
<td>526</td>
<td>424</td>
</tr>
<tr>
<td>C. pseudotuberculosis</td>
<td>b</td>
<td>556</td>
<td>523</td>
<td>418</td>
</tr>
</tbody>
</table>

and animal origin have been shown to differ considerably in the composition of their cell walls (Cummins & Harris, 1956; Cummins, 1962), phospholipids (Komura et al., 1975), mycolic acids (Goodfellow et al., 1976; Keddie & Cure, 1977), menaquinones (Collins et al., 1977), metabolic products (Reddy & Kao, 1978), and a number of other biochemical and serological features (Rogosa et al., 1974). Thus, it is surprising that, with the exception of R. equi, all the species examined here are very similar in their cytochrome content. It should be added, however, that only a limited number of strains have been examined in this study and it is possible that examination of a broader spectrum of strains will show greater variability in cytochrome content among the corynebacteria.

The results show that R. equi is different from the other species tested here in containing both b- and c-type cytochromes. This organism also differs from these species (C. diphtheriae, C. pseudotuberculosis, C. kutscheri, C. bovis, C. renale and C. pyogenes) in the following: mycolic acids (Goodfellow et al., 1976; Keddie & Cure, 1977), biochemical reactions (Reddy & Kao, 1978), production of large salmon- to white-coloured mucoid colonies on blood agar and non-production of acid from sugars (Reddy & Kao, 1978). Numerical taxonomy has also revealed differences (Harrington, 1966; Jones, 1975), and hence the transfer of C. equi to the new genus Rhodococcus (as R. equi) (Goodfellow & Alderson, 1977) appears to be justified.

It is generally agreed that C. pseudotuberculosis, C. kutscheri and to some extent C. bovis and C. renale are closely related organisms and should remain in the genus Corynebacterium (Harrington, 1966; Jones, 1975; Rogosa et al., 1974). In agreement with this, all of these species contained a b-type cytochrome. It should be pointed out, however, that C. bovis and C. renale are very different from the remaining three species with regard to acid metabolic products (Reddy & Kao, 1978). Furthermore, C. bovis has been shown to be different from the other four species in containing dihydromenaquinone with nine, as opposed to eight isoprene units (Collins et al., 1977). Therefore, based on this preliminary study employing limited numbers of bacterial strains, the presence of a b-type cytochrome appears to be a common property of the coryneform bacteria but is apparently not indicative of the interspecies relationships amongst them. However, a definitive conclusion must await the results of in-depth study employing larger numbers of strains grown under several different growth conditions.

Corynebacterium pyogenes is different from all the other corynebacteria studied here in that it is generally catalase-negative, liquefies gelatin, and causes acid coagulation and peptonization of litmus milk (Reddy & Kao, 1978; Roberts, 1968; Rogosa et al., 1974). It also differs from C. diphtheriae, and related corynebacterial species of animal origin, in cell wall composition (Cummins & Harris, 1956; Cummins, 1962), serology (Rogosa et al., 1974) and in the absence of mycolic acids (Goodfellow et al., 1976). Indeed, numerical taxonomic studies have confirmed the difference between the two organisms (Harrington, 1966; Jones, 1975). However, as with other corynebacterial species examined, C. pyogenes strain SS 7-74 contained a b-type cytochrome. This extends previous findings on strain 5 (ATCC 33157) of C. pyogenes (Reddy et al., 1977).

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REFERENCES

Cytochromes in corynebacteria


