Distribution of Cytochromes in Bacteria: Relationship to General Physiology

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A review of cytochrome occurrence in bacteria is presented which gives the taxonomic distribution of cytochromes and which relates this to general physiological characteristics. Data obtained from published research and recent experimental studies on a total of 169 species of bacteria suggested the existence of four major groupings: (i) the aerobic and facultatively anaerobic, heterotrophic gram positives (cytochrome pattern $aa_3.o.b.c$); (ii) the aerobic and facultatively anaerobic, heterotrophic gram negatives (cytochrome pattern either $a_1.d.o.b.c$, $a_1.o.b.c$ or $aa_3.o.b.c$); (iii) anaerobic and microaerophilic heterotrophs (cytochrome pattern b sometimes with $a_1/d/o$), and (iv) the chemo- and photo-autotrophs (cytochrome pattern c plus $a_1/aa_3/o/b$). The absence or minor presence of cytochrome c in facultatively anaerobic and anaerobic heterotrophs was confirmed and was also observed in plant and animal pathogens. Cytochrome d was confined in occurrence mainly to a small taxonomic group of organisms characterized by a high degree of adaptability to unstable habitats. This group was considered for further subdivision dependent upon the conditions causing the production of cytochrome d .

As part of an investigation into the occurrence of more than one spectral type of cytochrome oxidase in many bacteria, a survey of published data was carried out. In addition, a number of species were examined experimentally to extend existing data.

In contrast to previous reviews (14, 39, 56, 57, 89, 90), this study constitutes a general approach relating cytochrome composition to the physiology and taxonomy of a large number of bacterial species. Three general observations have been reported: firstly, that the cytochrome pattern $aa_3.o.b.c$ occurred predominantly in gram-positive bacteria, and the pattern $a_1.d.o.b.c$ occurred predominantly in gram-negative species (65); secondly, that cytochrome c was very often concluded to be absent from facultatively anaerobic species (42, 65); and, thirdly, that cytochromes a_1 and d always occurred together (86). This investigation evaluates and extends these suggestions.

MATERIALS AND METHODS

Analysis of published work. The following types of data from relevant scientific publications were assim-

ilated on organisms in the three major bacterial orders: (i) the taxonomic status of the species according to *Bergey's Manual of Determinative Bacteriology* (13) (i.e., the order, family, genus, species, strain, and response to Gram stain); (ii) the physiological type (e.g., heterotrophic, facultatively anaerobic); (iii) the culture conditions with respect to the terminal electron acceptor, the sources of carbon and nitrogen, and the phase of growth of the culture; and (iv) the qualitative cytochrome content. (Although the seventh edition of *Bergey's Manual* (1957) is considerably out of date, it was considered to be the most workable classification for the present study.) Where a publication failed to conclude whether an a type cytochrome was a_1 or aa_3 , the cytochrome was classified for the survey on the basis of the position of its α peak in the reduced minus oxidized spectrum, namely, a_1 , 586 to 596 nm, and aa_3 , 598 to 608 nm.

Experimental studies. Bacteria mainly from taxa not previously investigated were cultured at 30 °C in 600-ml batches of nutrient broth (Oxoid, England), 26 g plus 3 ml of Silicone MS Antifoam Emulsion RD per liter (Hopkins and Williams, England) in 21 tri-baffled flasks. Aeration was by gyratory shaking (180 rpm; oxygen dissolution rate, 144 mmol per liter per h as measured by a titrimetric procedure [26]).

Cells in either the logarithmic or stationary growth phase were harvested by centrifugation at 4 °C, homogenized in distilled water, recentrifuged, and resuspended by homogenization in 25 mM sodium phosphate buffer (pH 7.4) to an extinction at 680 nm of 50 to 80. Cell suspensions in 8- to 10-ml portions

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were disrupted by sonic treatment at 0 to 4 C by use of an MSE 100-W ultrasonic disintegrator at an amplitude of 7.8 μ m (peak to peak). In general, 3 min were required to disrupt gram-negative cells, 5 min for gram positives. Whole cells and cell-wall material were removed by centrifugation at 7,000 $\times g$ for 10 min, and the respiratory membranes were sedimented by further centrifugation at 157,000 $\times g$ for 1 to 2 h. The membranes were finally suspended in the same buffer to a protein concentration of 4 to 10 mg/ml as determined by a modified biuret method (41).

Cytochrome content was determined from dithionite-reduced minus oxidized, and dithionite-reduced plus CO minus dithionite-reduced difference spectra obtained at room temperature by use of a Shimadzu MPS 50-L split-beam recording spectrophotometer. The wavelength accuracy was often checked by use of a filter of known spectrum.

Distilled water was used to make up the growth medium, and glass-distilled, deionized water was used for the preparation and examination of respiratory membranes. Reagents were of analytical grade.

RESULTS

The data obtained in the present work, plus those gathered from the literature, are summarized in Tables 1 to 4, which include the sources of data.

General observations. Few of the publications examined contained complete information; the presence or absence of cytochrome *o* was uncertain in nearly two-thirds of the species due to the omission of a reduced plus CO minus reduced spectrum. Where suitable spectra were obtained, cytochrome *o* was found to be present in 75% of the species.

There was, in the majority of cases, a close correlation between the cytochrome content and the taxonomic family.

The absence of a distinct absorption peak(s) or shoulder corresponding to cytochrome *c*

occurred not only in heterotrophic, facultative anaerobes but also in species pathogenic for plants and animals, and in several other heterotrophic species.

Although the quantitative and, to a lesser extent, the qualitative cytochrome content varied with growth conditions in many cases, four groups of physiologically and/or taxonomically related species could be distinguished on the basis of their total cytochrome content, as follows.

Group A. The aerobic and facultatively anaerobic, heterotrophic, gram-positive bacteria of the orders *Eubacteriales* and *Actinomycetales* indeed contained predominantly the *aa₃.o.b.c* pattern (see Table 1). In some cases either cytochrome *aa₃* or *o* was absent, and in a few cases (*Bacillus megaterium* [KM] [14], *Micrococcus denitrificans* [81], and *Bacillus subtilis* [D473]—see Table 5) cytochrome *aa₃* predominated during logarithmic growth and cytochrome *o* was present particularly in the stationary phase of growth.

Certain exceptions to the general pattern occurred: in the family *Bacillaceae*, cytochrome *a₁* and *d* were observed "in some cultures" of the unusual insect pathogen *B. popilliae* (71), and cytochrome *a₁* was concluded to be present in *B. licheniformis* S294 (82), although in another strain of this species the expected cytochrome *aa₃* was present in place of *a₁* (see Table 5). In the *Micrococcaceae*, *M. denitrificans* was unusual in its ability to produce a cytochrome *cd* (nitrite reductase) complex when grown anaerobically with nitrate as terminal acceptor (62). This organism was subsequently transferred to a new genus, *Paracoccus* Davis (28).

Arthrobacter globiformis NCIB 8602 (family *Corynebacteriaceae*) appeared to be an excep-

TABLE 1. Cytochrome pattern of aerobic and facultatively anaerobic, gram-positive heterotrophs

Cytochrome pattern	Order	Family	No. of species in agreement	Exceptions	Sources of information
<i>aa₃.o.b.c.</i> (<i>aa₃</i> or <i>o</i> are sometimes absent; <i>c</i> is often absent)	<i>Actinomycetales</i>	<i>Actinoplanaceae</i>	10	0	(31, 52, — ^a)
		<i>Mycobacteriaceae</i>	6	0	(8, 34, 40, 57, 62, 76)
		<i>Streptomycetaceae</i>	11	0	(43, 66)
	<i>Eubacteriales</i>	<i>Bacillaceae</i>	14	2	(14, 17, 18, 19, 29, 32, 61, 63, 65, 71, 82, 83, 85, 93, 98)
		<i>Brevibacteriaceae</i>	2	0	(— ^a)
		<i>Corynebacteriaceae</i>	5	0	(42, 69, 80)
		<i>Micrococcaceae</i>	6	1	(17, 35, 48, 49, 56, 79, 81, 85, 100, 105)

^a —, See Table 5.

TABLE 2. Cytochrome patterns of aerobic and facultatively anaerobic, gram-negative heterotrophs (group B)

Cytochrome pattern	Order	Family	No. of species in agreement	Exceptions	Sources of information
Group B-1: $a_1.d.o.b.c$; c is often absent, a_1 and d are often present only after log phase	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	20	1	(7, 9, 10, 46, 59, 60, 77, 85, 89, 97)
	<i>Eubacteriales</i>	<i>Achromobacteraceae</i>	1	0	(6)
		<i>Azotobacteraceae</i>	2	0	(17, 27, 51, 85)
		<i>Brucellaceae</i>	4	0	(36, 106, 103, $-^a$)
		<i>Enterobacteriaceae</i>	11	0	(17, 33, 37, 85)
		<i>Rhizobiaceae</i>	0	1	($-^a$)
Group B-2: $a_1.o.b.c$.	<i>Pseudomonadales</i>	<i>Methanomonadaceae</i>	2	0	(75, $-^a$)
		<i>Pseudomonadaceae</i>	7	2	(17, 21, 27, 72, 85)
		<i>Spirillaceae</i>	3	0	(24, 25)
Group B-3: $aa_3.o.b.c$.	<i>Pseudomonadales</i>	<i>Caulobacteraceae</i>	1	0	($-^a$)
	<i>Eubacteriales</i>	<i>Pseudomonadaceae</i>	4	2	(20, 21, 22, 47, 89)
		<i>Rhizobiaceae</i>	2	0	(4, 5, 44)

^a —, See Table 5.

TABLE 3. Cytochrome pattern of anaerobic and microaerophilic heterotrophs (group C)

Cytochrome pattern	Order	Family	No. of species in agreement	Exceptions	Sources of information
Either no cytochromes, or cytochrome b sometimes also with $a_1/d/o$. (No c observed.)	<i>Eubacteriales</i>	<i>Bacillaceae</i> (Clostridia)	3	0	(30)
		<i>Bacteroidaceae</i>	3	0	(104)
		<i>Lactobacillaceae</i>	13	0	(11, 15, 30, 107)
		<i>Propionibacteriaceae</i>	5	0	(88, 101)

TABLE 4. Cytochrome pattern of autotrophs (group D)

Cytochrome pattern	Order	Family	No. of species in agreement	Exceptions	Sources of information
c , together with $a_1/aa_3/o/b$. (No d .)	<i>Pseudomonadales</i>	<i>Athiorhodaceae</i>	4	0	(56, 58, 78, 91, 102)
		<i>Chlorobacteriaceae</i>	2	0	(39, 56)
		<i>Nitobacteriaceae</i>	3	0	(2, 3, 45, 74, 90, 95, 96)
		<i>Spirillaceae</i>	3	0	(23, 50, 73)
		<i>Thiobacteriaceae</i>	5	0	(1, 70, 94, 99)
		<i>Thiorhodaceae</i>	1	0	(68)

tion that proved the rule: the cytochrome pattern of this gram-variable organism was, in the logarithmic phase, that of a typical gram-positive, heterotrophic aerobe ($aa_3.o.b.c$), but after the logarithmic phase, cytochromes aa_3 and c disappeared and were replaced by cytochromes a_1 and d to yield the pattern of a typical gram-negative, heterotrophic, facultative anaerobe of subgroup B-2, i.e., $a_1.d.o.b.c$ (see

below and Table 5). Concomitant with this change was the appearance, in mixed chains, of gram-negative as well as the original gram-positive cells. This organism is of further interest since it is the only organism observed to produce four spectrally distinct classes of CO-binding cytochromes.

Group B. The aerobic and facultatively anaerobic, heterotrophic, gram-negative bac-

teria of the orders *Eubacteriales* and *Pseudomonadales* are a group which have been distinguished from bacteria in group A on the basis of the regulation of their citrate synthase enzyme and, hence, their general intermediary metabolism (53). This group could be split into three subgroups on the basis of cytochrome pattern dependent upon the *a* type cytochrome(s) present (see Table 2). If no *a* type cytochromes were detected, species were classified according to the usual taxonomic considerations. (It was assumed here that *a* type cytochromes may well have been produced by these species under suitable conditions of culture.)

As a result of these subdivisions, representatives of only two families appeared in more than one group: the *Pseudomonadaceae*, the largest family in terms of number of species and one whose taxonomy remains the subject of intensive research, and the *Rhizobiaceae*, which contains three genera, *Agrobacterium*, *Chromobacterium*, and *Rhizobium*, which are related mainly by the relatively crude criterion of morphological similarity.

Exceptions to these groupings were generally of a minor nature, e.g., the absence of cytochrome *b* in *Pseudomonas testosteroni* and *P. acidovorans* (89); the notable exceptions were *P. riboflavina*, which contained cytochromes

aa₃ and *d* (7), and *Chromobacterium violaceum*, which contained cytochromes *d* and *o* but no *a₁* (see Table 5). The latter feature may be attributable to the physiological production of hydrogen cyanide by this species (16).

The organisms producing cytochrome *d* merit some additional consideration. They appeared to be divided into the following: facultatively anaerobic species, which produced fairly large amounts of cytochromes *a₁* and *d* in the late logarithmic or stationary phases of growth (6, 16); aerobic species, which produced only small quantities of cytochromes *a₁* and *d* in the stationary phase unless cultured under severe oxygen limitation (see Table 5); and species which produced significant amounts of cytochromes *d* and/or *a₁* mainly when grown anaerobically with nitrate as the terminal electron acceptor (67, 103, 108). It must be stressed, therefore, that for taxonomic use the cytochrome pattern of these species should be examined under a variety of growth conditions.

Group C. The anaerobic and microaerophilic heterotrophs of the order *Eubacteriales* (see Table 3) were for a long time thought not to contain cytochromes, but in the last decade cytochromes (always including cytochrome *b*) were found to be present in several species (although usually associated with the inclusion of heme in the growth medium). Cytochrome *b*

TABLE 5. *Qualitative cytochrome content of bacteria examined experimentally*

Species	Cytochrome content of cells in logarithmic (log) or stationary (stat) phase of growth
<i>Acinetobacter lwoffii</i> [4B-P. D. J. Weitzman]	<i>o b c^a</i> (log) <i>a₁^a d^a o b c^a</i> (stat)
<i>Agromyces ramosus</i> ^b	<i>aa₃ o b c</i> (log and stat)
<i>Arthrobacter globiformis</i> [NCIB 8602]	<i>aa₃ o b c</i> (log) <i>a₁ d o b</i> (stat)
<i>Bacillus licheniformis</i> ^c	<i>aa₃ o b c</i> (log and stat)
<i>Bacillus subtilis</i> [D-473] ^b	<i>aa₃ b c</i> (log) <i>aa₃ o^a b c</i> (late stat)
<i>Brevibacterium linens</i> [ATCC 9174]	<i>aa₃ o^a b c</i> (log and stat)
<i>Caulobacter vibrioides</i> [ATCC 11763]	<i>aa₃ o b c</i> (log and stat)
<i>Chromobacterium violaceum</i> [D-301] ^b	<i>d o b c^a</i> (stat)
<i>Cellulomonas biazotea</i> [NCIB 8077]	<i>aa₃ o b c</i> (log and stat)
<i>Hydrogenomonas facilis</i> ^d	<i>a₁ o b c</i> (stat)
<i>Kurthia zopfii</i> [C-5] ^b	<i>o b</i> (log and stat)
<i>Listeria monocytogenes</i> ^b	<i>b</i> (stat)
<i>Xanthomonas hyacinthi</i> [NCPBP 519]	<i>o b</i> (log) <i>a₁ d o b</i> (stat)

^a Trace amount.

^b The culture collection of the Medical Research Council's Microbial Systematics Research Unit, University of Leicester, England.

^c Strain supplied by T. Coulgate.

^d Strain provided by J. Heptinstall.

FIG. 1. Distribution of cytochromes in bacteria.

was also found to mediate the anaerobic reduction of fumarate (88, 104).

The families *Lactobacillaceae* and *Propionibacteriaceae* prompted special interest since the a type cytochromes in these families were a_1 and d rather than aa_3 as in other gram positives. This observation suggested a relationship between these species and the gram-negative group B-1 (see above). This relationship could be a direct evolutionary relationship, or it may be connected to their high degree of adaptability (see Discussion). This feature also strongly excludes from the *Lactobacillaceae* the atypical *Microbacterium thermosphactum* (at present reservedly classified with the *Corynebacteriaceae*), which produces cytochromes aa_3 and b (42).

The only group of bacteria in which the presence of cytochromes has not been detected is the genus *Clostridium*. Perhaps these organisms have been anaerobes throughout their evolution.

Group D. The chemo- and photo-autotrophs of the order *Pseudomonadales* (see Table 4) are a complex and diverse group characterized in particular by the omnipresence of cytochrome(s) c . The properties of these cytochromes c appear to be quite variable (for review, see references 53, 54), and it was speculated that these observations could be explained on the basis of cytochrome c having a greater potential for diversification and for evolution as a component in a wide variety of

electron transport systems, such as occur in the autotrophic bacteria.

The data presented in Tables 1 to 4 were summarized diagrammatically in order to emphasize the taxonomic distribution of each class of cytochrome (see Fig. 1). Note the relatively small taxonomic region in which cytochrome d occurs.

DISCUSSION

The results obtained with the recent mathematical analysis of Shipp (84) suggest strongly that two or more cytochromes may exhibit only a single absorption peak at room temperature. These findings cast serious doubts on a conclusion that heterotrophic, facultative anaerobes and pathogens lack cytochrome c . However, it would seem likely that, although cytochrome c may be present, it is a minor component in these bacteria and must represent a considerable difference in the aerobic oxidation pathway.

The above technique may serve as a refinement to the use of cytochrome composition as a taxonomic characteristic.

It is interesting to speculate that the bacteria (mainly group B-1), which commonly contain three CO-binding cytochromes, are species which are particularly adaptable in unstable environmental conditions, with respect not only to the terminal electron acceptor but also

the source of carbon; that their natural habitats are highly competitive, and that these features are intimately related to their possession of such complex electron transport systems.

This study should assist workers in the field of bacterial electron transport to take a broader, more biological view of the subject; and it is hoped that this would lead to a greater understanding of the relationships between individual electron transfer systems and the general physiology of the bacteria in their natural habitat.

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REPRINT REQUESTS

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