Low-Temperature Cytochrome Spectra of Anaerobic Actinomycetes

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A sensitive, low-temperature spectrophotometric technique was used to detect the presence and relative contents of cytochromes resembling hemoproteins in suspensions of intact cells of 15 strains of anaerobic actinomycetes. The cytochrome content of most cultures was increased by aerated cultivation. Anaerobically grown cells of *Actinomyces israelii* serotype 2 contained virtually no cytochromes whereas those of *A. israelii* serotype 1, *Actinomyces viscosus*, and *Actinomyces bovis*, in this order, show an increased concentration of only cytochrome *b* (hemoprotein 559). Under aerobic conditions, *A. viscosus* was able to synthesize cytochromes *c* and *a*. No correlation was found between spectrophotometric and benzidine test data. Cytochrome spectra may be useful in differentiating serotype 1 from serotype 2 of *A. israelii* and differentiating *A. bovis* from the other species of *Actinomyces* studied.

Despite the fact that the presence of cytochromes in anaerobic actinomycetes was first mentioned over 30 years ago (13), relatively little attention has been paid to the cytochromes in extensive studies on the physiology and biochemistry of these organisms (for a review of the latter, see reference 12). Our preliminary observations (15) suggested that application of a recently developed sensitive technique (9) for the study of cytochromes in these organisms might be instrumental in providing further information on the differences between aerobic and anaerobic members of the order *Actinomycetales* as well as on the utility of cytochrome spectra for identification purposes. This paper summarizes the results of experiments performed on 15 strains of anaerobic actinomycetes differing in their ability to grow at different levels of aeration.

MATERIALS AND METHODS

Bacterial strains. The list of strains used in this study is given in Table 1.

Methods. Cultures were grown in a liquid medium composed of a standard meat-peptone broth (MPB) supplemented with glucose (0.05% wt/vol) and, when indicated, with sodium thioglycollate (0.05% wt/vol), cysteine (0.1% wt/vol), or sodium ascorbate (0.1% wt/vol), as reducing agent. The autoclaved medium was dispensed into sterile flasks with cotton plugs, heated in a boiling water bath for 30 min to remove dissolved oxygen, and then poured into sterile 600-ml

flasks (up to their necks), rapidly cooled, and inoculated. The flasks were then stoppered with sterile rubber stoppers and incubated at 35 C for 7 to 10 days. In some of the experiments, organisms were grown under aerobic conditions either as stationary or as shaken cultures. Growth was stopped by cooling the cultures to 4 C, and cells were killed by adding Formalin to a final concentration of 0.5% (vol/vol). Cells were harvested by centrifugation and washed three times using cooled 1/15 M phosphate buffer (pH 7.0). The material was kept frozen until used.

Cytochromes were assayed using a low-temperature spectrophotometric procedure described previously (9). The concentration of hemoproteins was calculated from the absolute spectra in preparations reduced with sodium dithionite and was expressed in terms of optical density units per unit of dry weight of cells. The molar extinction coefficient of mammalian cytochrome *b* was used for calculation of the hemoprotein (as cytochrome *b*) content in micromoles per milligram of dry weight of cells as described elsewhere (3). For dry weight determinations, the samples were dried on membrane filters over anhydrous CaCl₂ for 24 h. Protoporphyrin was extracted with acid acetone (4). For qualitative detection of iron-porphyrins, a modified benzidine test (2) was employed.

Each set of experiments was performed at least three times.

RESULTS AND DISCUSSION

As one may see from the data presented in Table 1, all of the strains examined except those belonging to serotype 1 of *Actinomyces israelii* were characterized by notable absorp-

TABLE 1. *Relative content of hemoprotein (cytochrome b type) in cell suspensions of anaerobic actinomycetes^a*

Organisms ^b	α -Absorption maxima in liquid nitrogen		Hemoprotein content	
	b_{α_2}	b_{α_1}	OD units ($\Delta D \times 10^{-4}$ mg)	μmol (10^{-4} /mg)
<i>Actinomyces bovis</i> ATCC 13683	554	559	11.90	0.70
<i>A. viscosus</i> ATCC 15987	554	559	3.40	0.20
<i>A. viscosus</i> ATCC 19246 ^c	554	559	4.20	0.25
<i>A. israelii</i> serotype 1				
ATCC 12102	554 ^d	559	1.87	0.11
ATCC 10048	554 ^d	559	1.36	0.08
WVU 231	554 ^d	559	1.23	0.07
WVU 232	554 ^d	559	1.05	0.06
WVU 233	554 ^d	559	1.18	0.07
WVU 300	554 ^d	559	1.35	0.08
WVU 306	554 ^d	559	1.24	0.07
WVU 396	554 ^d	559	1.36	0.08
<i>A. israelii</i> serotype 2				
WVU 301	abs ^e	abs ^f	0-0.3	0
WVU 304	abs ^e	abs ^f	0-0.2	0
WVU 312	abs ^e	abs ^f	0-0.1	0
WVU 326	abs ^e	abs ^f	0-0.2	0

^a Cultures were grown in MPB with glucose and sodium thioglycollate.^b Cultures were provided by J. Slack of West Virginia University (WVU). Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; WVU, West Virginia University, Morgantown, W.Va.^c This strain was originally identified as *A. discofoliatus* (5).^d Usually not detected; very slight absorption "shoulder" noted occasionally.^e Absent.^f Practically absent in most cases; slight absorption noted occasionally.

tion maxima in the region of cytochrome *b*. This was especially evident with *A. bovis*. The absorption peak at 559 nm was asymmetrical, with an additional, smaller maximum at 554 nm (Fig. 1). Both maxima were sharply expressed without adding dithionite when thick suspensions of *A. bovis* and *A. israelii* cells were frozen in liquid nitrogen. This fact suggested that the principal hemoproteins were reduced by intracellular systems and hence may play some physiological role. However, the rate of response to dithionite or intracellular reductants varied with the two peaks. In response to dithionite, the absorption at 559 nm increased more sharply, possibly signifying the presence of a second absorbing compound.

Analogous but less intensive absorption was evident in dithionite-reduced suspensions of other anaerobic actinomycetes (Table 1), including *A. viscosus*, which is considered to be

the most "aerobic" member of the group (14). The spectral patterns were similar in all cases, but maxima were less pronounced with organisms having low hemoprotein content.

When potassium ferricyanide (as oxidizing agent) was added to cell suspensions, the absorption maxima in the cytochrome *b* region disappeared completely. Extraction of thick cell suspensions with acid acetone also eliminated absorption. The treatment is thought to split noncovalently bound protohemes from the proteins of cytochrome *b*, catalase, and some peroxidases (4). These results provide further evidence for the presence of heme-containing proteins of cytochrome *b* type.

It should be noted that an asymmetrical absorption peak for cytochrome *b* has been previously recorded, and it was especially evident with purified cytochrome *b* from *Sclerotinia libertiana* (16).

FIG. 1. Low-temperature spectra of cell suspensions of *A. bovis* and *A. israelii*. a, Thick suspension (12 mg dry wt/ml) of *A. bovis* ATCC 13683. Dithionite added (1); thick suspension of cells of *A. bovis* ATCC 13683 without dithionite (2). b, *A. israelii* ATCC 10048 (serotype 1), 10 mg dry wt/ml, dithionite added (1); *A. israelii* WVU 304 (serotype 2), 10 mg dry wt/ml, dithionite added (2).

TABLE 2. Comparison of hemoprotein-559 content in *A. israelii* cells grown in broth with and without thioglycollate

Organisms ^a	Serotype	Hemoprotein content (OD units [$\Delta D \times 10^{-4}$]/mg dry biomass) in cells grown in media:	
		+ Thioglycollate	- Thioglycollate
<i>Actinomyces israelii</i> ATCC 10048	1	1.0-1.3	2.5-3.4
<i>A. israelii</i> WVU 304	2	0	0-1.45

^a Abbreviations as in footnote to Table 1.

The hemoprotein (cytochrome *b*) content per dry weight unit of cells of *A. bovis* was comparable to that in cells of aerobic actinomycetes when dithionite was employed as a reducing agent. However, in their cytochrome *b* spectra pattern, the anaerobic cultures differed from the aerobic actinomycetes (15).

The hemoprotein content of cells of *A. israelii* serotype 1 differed significantly from that of cells of serotype 2. The absorption maximum at 559 nm was practically absent (Fig. 1b) in serotype 2 cells grown in medium supplemented with sodium thioglycollate or other reductant. The same cultures grown in medium without reductants increased their hemoprotein content to equal that of serotype 1 cells grown in medium with reductant (Table 2). It then follows that the hemoprotein content of *A. israelii* cells depends upon the redox condition of cultivation. Cytochrome *b* was observed in serotype 1 and 2 cells when grown under different conditions.

FIG. 2. Low-temperature spectrum of *A. viscosus* cell suspension. Dithionite added. Cells grown under aerobic conditions.

When the more "aerobic" *A. viscosus* strain ATCC 19246 was grown in stationary or shaken culture with free access of air, the hemoprotein content per biomass unit increased two- to threefold. Absorption at 549 nm became evident as a small "shoulder." In cell suspensions grown in shaken culture, a notable absorption maximum was seen at 595 nm and a

broad peak without a sharply defined maximum was seen at 630 to 640 nm (Fig. 2). Both maxima disappeared after cell treatment with potassium ferricyanide. There is thus a possibility that under aerobic conditions these cells were able to synthesize other components of the cytochrome chain, notably cytochromes of type *c* and *a*.

Our attempts to use simple methods for characterizing cytochromes in anaerobic actinomycetes so far have failed. Specifically, we were unable to correlate low-temperature spectrophotometric data with the intensity of color developed with the benzidine test. On the one hand the benzidine test gave negative results, whereas a low-temperature absorption peak in dithionite-reduced suspensions was quite evident. Possible cases of such a discrepancy were discussed earlier (7). On the other hand, there were some cases where the benzidine test gave positive results but low-temperature spectrophotometry failed to reveal noticeable absorption in dithionite-reduced samples. Such data seem to suggest the presence in cells of anaerobic actinomycetes of unidentified porphyrin compounds, since catalase activity is lacking in a majority of these organisms.

According to published spectrophotometric data, cytochromes of types *b*, *c*, and *a* are present in aerobic actinomycetes. The *b* type cytochrome has been partially purified (1, 6). However, a detailed comparison of the cytochrome spectra of anaerobic actinomycetes with those reported for aerobes (10, 11) is difficult at present because of the lack of information on low-temperature spectra in aerobes generally and on purified cytochrome *b* in particular. The data presently available indicate that the anaerobic actinomycetes lack cytochrome *a* and possibly also *c*. In the more aerobic actinomycetes such as *A. viscosus*, cytochromes *a* and *c* might be synthesized in response to increased aeration. The total cytochrome *b* content in the anaerobic actinomycetes grown under restricted aeration (with the exception of *A. bovis*) is much lower than that in the aerobes.

The precise physiological role of the hemoprotein resembling cytochrome *b* revealed in the present study in cells of most anaerobic actinomycetes is difficult to evaluate. However, the differences in relative content of this component in cells grown under standard conditions might be of value in distinguishing between serotype 1 and serotype 2 of *A. israelii*. Should our data on *A. bovis* be confirmed with other strains of this species, the differentiation of this species from other anaerobic actinomycetes may be facilitated.

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