Cytochrome Difference Spectra of Acetic Acid Bacteria

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The cytochrome difference spectra of 15 strains of the genus *Acetobacter* were found to differ from those of 7 strains of the genus *Gluconobacter*. The *Acetobacter* strains contained cytochrome a_1 , which was lacking in the *Gluconobacter* strains. Within the genus *Acetobacter*, the Peroxydans group of Frateur could be separated through its cytochrome d content from the Oxydans and Mesoxydans groups.

The generic name *Acetobacter* was suggested by Beijerinck in 1900 for the acetic acid bacteria (5). Asai (1, 2) divided the genus *Acetobacter* into the genera *Acetobacter* and *Gluconobacter* based on their different oxidative capacities for ethanol and glucose. Frateur (11) differentiated the acetic acid bacteria into four groups mainly on the basis of their oxidative metabolism. His Suboxydans group corresponds to the genus *Gluconobacter*, whereas the Peroxydans, Mesoxydans, and Oxydans groups correspond to the genus *Acetobacter*. Leifson (14), without knowledge of Asai's (1, 2) work, divided the genus *Acetobacter* into the non-acetate-oxidizing genus *Acetomonas*, whose motile strains are polarly flagellated, and the acetate-oxidizing genus *Acetobacter*, whose motile strains have peritrichous cells. The name *Acetomonas* is now regarded as being synonymous with *Gluconobacter*. Other attempts to group the acetic acid bacteria by biochemical and physiological means have been reviewed by Asai (3).

The cytochromes of the acetic acid bacteria have been studied mainly with strains of *Acetobacter peroxydans*, *Acetobacter pasteurianus*, and *Gluconobacter suboxydans*, and the following cytochromes were found: in *A. peroxydans*, a_1 , a_2 (d), a_4 , b , c , and c_1 ; in *A. pasteurianus*, a_1 , b , c , and c_1 ; and in *G. suboxydans*, b , c , c_1 , and o (18). Additional data on cytochromes of acetic acid bacteria have been reviewed and tabulated by Nakayama and De Ley (16).

In this study, the cytochrome contents of 22 representative strains of acetic acid bacteria were determined and correlated with the existing classification of the acetic acid bacteria.

MATERIALS AND METHODS

Bacterial strains. The strains used, their origins, and

their biochemical properties as determined by the methods of Carr (6) are listed in Table 1.

Growth conditions. *A. peroxydans* and *A. ascendens* were grown in a complex medium containing per liter of phthalate buffer (0.05 M, pH 5.5): malt extract (Wander), 15 g; yeast extract (Oxoid), 5 g; $\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$, 0.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; and ethanol, 5 g. *A. aceti* was grown in the same medium without the inclusion of malt extract. All other strains were grown in this medium without malt extract and with glucose (5 g/liter) instead of ethanol. The cells were grown aerobically in 1-liter Erlenmeyer flasks containing 200 ml of medium on a gyratory shaker at 30 C.

Cell-free extract. All procedures were carried out at 4 C. The cells were collected at the end of the exponential growth phase, washed twice with $\text{NaOH-KH}_2\text{PO}_4$ buffer (0.06 M, pH 6.5), and resuspended at a concentration of 1 g of cells (wet weight) per 10 ml of the same buffer. The cells were disrupted by a Branson sonifier-S75 at the highest intensity for 2 min with intermittent cooling. The sonically treated cells were centrifuged at $40,000 \times g$ for 10 min, and the resultant supernatant, designated as "cell-free extract," was employed for the experiments. Protein was determined by the biuret method of Layne (13).

Cytochrome difference spectra. The reduced-minus-oxidized cytochrome difference spectra were measured at room temperature with a Beckman-DB recording spectrophotometer between 400 and 700 nm. The light path was 1 cm; the scanning rate was 80 nm/min. The cuvette with the oxidized cell-free extract contained per milliliter: $\text{NaOH-KH}_2\text{PO}_4$ buffer (pH 6.5), 0.05 mmol; H_2O_2 , 8.8 μmol ; and protein, 2 to 18 mg. The cuvette with the reduced extract contained per milliliter: $\text{NaOH-KH}_2\text{PO}_4$ buffer (pH 6.5), 0.05 mmol; D-glucose, 0.35 mmol; and protein, 2 to 18 mg.

RESULTS

The positions of the absorption bands of the cytochrome difference spectra of the individual strains are listed in Table 2.

Three different types of cytochrome difference spectra of acetic acid bacteria could be

TABLE 1. Physiological and biochemical properties of the strains studied^a

Strain	LBG no.	Origin	Overoxi- dation of ethanol	Cata- lase	Growth in Hoyer's medium	Acid from glucose	Dihydroxy- acetone from glycerol	Produc- tion of cellulose	Brown pigment
Peroxydants group ^b									
<i>Acetobacter peroxydans</i>	4110	ATCC 838	+ ^c	—	+	—	—	—	—
<i>A. peroxydans</i>	4392	ATCC 12874	+	—	+	—	—	—	—
Oxydants group ^b									
<i>A. rancens</i>	4031	LBG	+	+	—	+	—	—	—
<i>A. rancens</i>	4032	ELV	+	+	—	+	—	—	—
<i>A. rancens</i>	4098	NCIB 8619	+	+	—	+	—	—	—
<i>A. rancens</i>	4112	ATCC 9325	+	+	—	+	—	—	—
<i>A. rancens</i>	4313	NCTC 6428	+	+	—	+	—	—	—
<i>A. ascendens</i>	4194	Lab. for Microbiol., Univ. of Delft, no. 5	+	+	—	—	—	—	—
Mesoxydants group ^b									
<i>A. acetii</i>	4106	LBG	+	+	+	+	—	—	—
<i>A. acetii</i>	4114	NCIB 8554	+	+	+	+	+	—	—
<i>A. xylinum</i>	4095	NCIB 8747 (celluloseless mutant)	+	+	—	+	+	—	—
<i>A. xylinum</i>	4113	ATCC 10245	+	+	—	+	—	+	—
<i>A. xylinum</i>	4168	NCIB 7029	+	+	—	+	+	+	—
<i>A. liquefaciens</i>	4101	Inst. of Appl. Microbiol., Univ. of Tokyo	+	+	—	+	+	—	—
<i>A. mesoxydans</i>	4096	NCIB 8622	+	+	—	+	+	—	—
Suboxydants group ^b									
<i>Gluconobacter melanogenus</i>	4093	NCIB 8086	—	+	—	+	+	—	+
<i>G. oxydans</i>	4099	CN 1221	—	+	—	+	+	—	—
<i>G. oxydans</i>	4100	CN 2605	—	+	—	+	+	—	—
<i>G. oxydans</i>	4102	IFO 3289	—	+	—	+	+	—	—
<i>G. oxydans</i>	4104	IFO 3291	—	+	—	+	+	—	—
<i>G. oxydans</i>	4111	ATCC 621	—	+	—	+	+	—	—
<i>G. oxydans</i>	4315	NCTC 3734	—	+	—	+	—	—	—

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CN, National Collection of Type Cultures, London, England; ELV, Eidgenössische Forschungsanstalt Wädenswil; IFO, Institute for Fermentation, Osaka, Japan; LBG, Mikrobiologisches Institut der ETH, Zürich, Switzerland; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, London, England.

^b Frateur's groups (11).

^c +, Positive reaction; —, negative reaction.

TABLE 2. Positions of the maxima (nm) of the cytochrome difference spectra of acetic acid bacteria

Strain no.	Soret region γ -bands		β -bands		α -bands			
Peroxydans group								
4110	420	440s ^a	520	528s	548s	552	590	630
4392	424	440s	522	528s	548s	553	590	630
Oxydans group								
4031	425	440s	521	528s	548s	554	590	
4032	428	440s	524		548s	555	590	
4098	424	440s	520	528s	548s	554	590	
4112	421	440s	520	528s	548s	550	590	
4313	423	440s	520	526s	548s	554	590	
4194	422	440s	520	526s	548s	550	(590) ^b	
Mesoxydans group								
4106	423	440s	522	528s	548s	552	590	
4114	426	440s	520	528s	548s	552	590	
4095	424	440s	520	528s	548s	552	590	
4113	424	440s	521	528s	548s	552	590	
4168	422	440s	522	528s	548s	552	(590)	
4101	421	440s	522		548s	552	(590)	
4096	423	440s	520	528s	548s	552	(590)	
Suboxydans group								
4093	420		522		548s	553		
4099	420		520		548s	551		
4100	422		522		548s	551		
4102	422		520		548s	550		
4104	424		523		548s	552		
4111	420		522		548s	551		
4315	421		521		548s	552		

^a s, Shoulder.^b (590), Very weak band.

distinguished corresponding to the Peroxydans, Oxydans-Mesoxydans, and Suboxydans groups of Frateur (11). The first group was formed by both strains of *A. peroxydans* (Fig. 1); the γ -band of cytochrome *c* and *b* at 420 to 424 nm has a shoulder at 440 nm that corresponds to cytochrome *a*₁. The β -band of the cytochromes *c* and *b* appears at 520 to 522 nm with a shoulder at 528 nm. Three α -bands were found: one at 552 to 553 nm with a shoulder at 548 nm corresponding to the cytochromes *c* and *c*₁ and a very faint shoulder at 560 nm corresponding to cytochrome *b*; another faint band at 590 nm due to cytochrome *a*₁; and the third band, the α -band of cytochrome *d*, at 530 nm.

Frateur's Oxydans and Mesoxydans group could not be differentiated by their cytochrome difference spectra; the position of the main peak in the Soret region varies between 421 and 428 nm with the shoulder of cytochrome *a*₁ at 440 nm, the β -band absorbs at 520 to 524 nm with a shoulder at 528 nm, and the two α -bands lie at 550 to 555 nm with

shoulders at 548 and 590 nm (Fig. 2). Cytochrome *d* was not present. The α -band of cytochrome *a*₁ at 590 nm was sometimes very difficult to recognize, but the presence of cytochrome *a*₁ could always be seen by its γ -band at 440 nm.

Strains of the genus *Gluconobacter* were clearly differentiated from those of the genus *Acetobacter* by the absence of type-*a* cytochromes (Fig. 3). There was no cytochrome *a*₁-shoulder on the Soret peak. The peak of the β -band lies between 520 and 523 nm, and only one α -band at 550 to 553 nm with a shoulder at 548 nm is visible. The α -band of cytochrome *b* can be seen in the strains of *Acetobacter* and *Gluconobacter* as a weak shoulder on the α -band of the *c*-type cytochromes in the region of 560 nm.

DISCUSSION

The acetic acid bacteria studied can be divided into two main groups and one subgroup

A. peroxydans 4392

FIG. 1. Reduced-minus-oxidized difference spectrum of a cell-free extract of *A. peroxydans* 4392. The protein concentration of the assay mixture was 18.6 mg/ml. The peaks at 424, 522 to 528, and 548 to 553 nm with a very faint shoulder at 560 nm correspond to the γ , β , and α bands of the *c*- and *b*-type cytochromes. The shoulder at 440 nm and small peak at 590 nm correspond to the γ - and α -bands, respectively, of cytochrome a_1 . The absorption band with a maximum at 630 nm corresponds to the α -peak of cytochrome *d*.

A. aceti 4114

FIG. 2. Reduced-minus-oxidized difference spectrum of a cell-free extract of *A. aceti* 4114. The protein concentration of the assay mixture was 6.1 mg/ml. Legend is the same as in Fig. 1 except for the absence of the peak at 630 nm.

according to their cytochrome content. One main group is formed by all overoxidizing acetic acid bacteria belonging to the genus *Acetobacter*. They contain cytochrome a_1 . The other main group is formed by the strains that do not oxidize acetate, and they show no a -type cytochromes. The Peroxydans group of Frateur (11) forms a subgroup within the genus *Acetobacter* that can be distinguished from the Oxydans and Mesoxydans groups by its cytochrome *d* content. The Oxydans and Mesoxydans groups, which are separated only by their relative ability to form ketoses from polyalcohols (11), show no differences in their cytochrome difference spectra. In Table 3, the bands of the three types of cytochrome difference spectra obtained are compared with the spectra of acetic acid bacteria strains reported in the literature.

The α -band of *A. peroxydans* at 552 to 553 nm with a shoulder at 548 nm corresponds to the peaks at 554 and 550 nm described by Chin (Abstr. Commun. Int. Congr. Biochem. Biophys., Paris, p. 277, 1952), to the peak of 553 $\pm$ 5 nm of a reduced cell suspension (20), to the cytochrome band of a thick cell suspension with a maximum at 555 nm (8), and to the band of a cell paste extending from 546 to 563 nm observed by De Ley and Vervloet (9). The α -band of cytochrome a_1 at 590 nm is probably

G. oxydans 4104

FIG. 3. Reduced-minus-oxidized difference spectrum of a cell-free extract of *G. suboxydans* 4104. The protein concentration of the assay mixture was 15.1 mg/ml. Legend is the same as in Fig. 1 except for the absence of the peaks at 630 and 590 nm and the shoulder at 440 and 528 nm.

TABLE 3. Absorption bands of acetic acid bacteria cytochromes (nm)

Organisms and references	γ -bands		β -bands	α -bands			
Peroxydans group	420-424	440s ^a	520-522 528s	548s	552-553	590	630
<i>Acetobacter peroxydans</i> (Chin, Abstr., 1952) . . .				550	554		612 635
<i>A. peroxydans</i> (20) . . .					553 $\pm$ 5		
<i>A. peroxydans</i> (8) . . .			518-525-536	546	555	565	587-599-613
<i>A. peroxydans</i> (9) . . .			518-536	546	563	595	613
Oxydans and Mesoxydans group	421-428	440s	520-524 528s	548s	550-555	590	
<i>A. pasteurianus</i> (17) . . .	428	445	523		554	588	
<i>A. pasteurianus</i> , solubilized particles (12).	430	440-445	528-532		558-560	590-595	
<i>Acetobacter</i> sp., alcohol-cytochrome-553 reductase (15)	417		522		553		
<i>Gluconobacter liquefaciens</i> (19)	418		523		553		
<i>Acetobacter</i> different strains (16)			498 523-531	548	553-556-566	586-591	
Suboxydans group	420-424		520-523	548s	550-553		
<i>A. suboxydans</i> (17) . . .	422		525		554		
<i>A. suboxydans</i> , solubilized particles (12)	426-428		528-532		558-560		
<i>A. suboxydans</i> (10) . . .			524		552		
<i>G. cerinus</i> , cell-free extract (21)	427		523 530s		554 564s		
<i>A. suboxydans</i> , respiratory particles (7)	426		523 528	549s	553 558s 564		
<i>G. suboxydans</i> (16) . . .			523		554 565	590	
<i>G. melanogenus</i> (16) . .			530	548	568		

^a s, Shoulder.

identical to the band at 587 to 613 nm with its maximum at 599 nm, as reported by De Ley and Schell (8), and the band extending from 595 to 613 nm (9). The α -band of cytochrome a_4 at 612 nm described by Chin (Abstr., 1952) and reported again as a faint band by Atkinson (4) was not found in the two strains investigated. The α -band of cytochrome d at 630 nm corresponds to the band at 635 nm found by Chin (Abstr., 1952) in *A. peroxydans*. Differences in the position of the absorption bands reported by other authors may be due to their use of intact cells.

The maxima of the cytochrome difference spectra of cell-free extracts from strains of the Oxydans and Mesoxydans groups were observed at 421 to 428, 520 to 524, 550 to 555, and at 590 nm, with shoulders at 440, 428, and 548 nm. Smith (17) reported the maxima at 428, 445, 523, 554, and 588 nm from her studies with intact cells of *A. pasteurianus*, and King and Cheldelin (12) observed maxima at 430, 440 to 445, 528 to 532, 558 to 560, and 590

to 595 nm with solubilized particles from *A. pasteurianus*. Here differences of 2 to 6 nm were also observed between the data of solubilized particles and intact cells. Nakayama and De Ley (16) reported bands of cytochrome spectra of intact cells of various acetic acid bacteria at 523 to 531, 548 to 566, and at 586 to 591 nm.

Strains of the Suboxydans group showed three maxima at 420 to 424, 520 to 523, and at 550 to 553 nm, with a shoulder at 548 nm. Smith (17) observed maxima at 422, 525, and 554 nm when using intact cells of *G. suboxydans*, and Fewster (10), using fully active pink organisms grown on glucose in the presence of chalk, reported maxima at 524 and 552 nm, which disappeared after incubation of the cells for 72 h in a chalk-free glucose medium and were replaced by bands at 559 and 530 nm. The peaks of solubilized particles of *G. suboxydans* had maxima at 426 to 428, 528 to 532, and 558 to 560 nm (12). The difference spectrum of a cell-free extract of *Glucobacter*

cerinus reduced by fructose showed three peaks at 427, 523, and 554 nm, and faint shoulders at 530 and 564 nm (21). A low-temperature difference spectrum of respiratory particles from *G. suboxydans* showed a γ -band with a peak at 426 nm, a β -band with peaks at 523 and 528 nm, and an α -band with four components absorbing at 549, 553, 558, and 564 nm (7). In *G. suboxydans* (Fig. 3), a slight shoulder at 560 to 565 is visible on the α -peak, which might correspond to the shoulder at 564 nm observed by Yamada et al. (21) and the maxima at 564 nm reported by Daniel (7).

The foregoing survey together with our results would therefore support a division of the acetic acid bacteria into the two genera *Acetobacter* and *Gluconobacter*.

The *G. suboxydans* strain reported by Nakayama and De Ley (16) showing a cytochrome a_1 band at 590 nm forms the only exception to this cytochrome classification of the acetic acid bacteria. It would be of interest to know if this particular strain does really not overoxidize ethanol, and if it also shows the γ band of cytochrome a_1 at 440 nm.

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REPRINT REQUESTS

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