Plant & Cell Physiol. 17: 305-316 (1976)

Changes in cytochrome contents and respiratory activity of *Bacillus cereus* Strain T during germination, vegetative growth and sporulation

Takako Ankei-Yamada

Botanical Institute, Faculty of Science, Hiroshima University, Hiroshima 730, Japan

(Received November 10, 1975)

Vegetative cells of *Bacillus cereus* Strain T contain cytochrome *b*-562, a minor *b*-type component, in addition to known components, cytochrome $a+a_3$, cytochrome *b*-557 and cytochrome *c*-551. Also, the spores contain low but definite amounts of cytochromes *b*-562 and *c*-551, which were oxidized when the spores were shaken with air. Contents of cytochromes *a*, *b* and *c* per cell and per cell nitrogen, and the activity of glucose oxidation increased during spore germination and elongation. During the stage preceding first cell division, cytochrome contents per cell increased in parallel with the increase of cell nitrogen, while the activity of glucose oxidation decreased. During early exponential growth, the content of cytochrome *b* per cell nitrogen and respiratory activity with glucose again increased. When cells entered the sporulation stage, characterized by structural changes inside the cells, the activity of glucose oxidation began to decrease, while that of acetate or succinate oxidation started to increase. During the sporulation process, the contents of the three cytochrome components continued to increase and reached the highest level in cells containing completed spores, but the activity of respiration with endogenous or added substrates was negligible in these cells.

Formation or germination of spores in bacteria affords a suitable system for studying the correlation of structural or functional changes with metabolic alterations. Several investigators have compared the respiratory system between vegetative cells and spores of bacteria of the genus *Bacillus*, in particular, *B. subtilis* and *B. cereus*, since Keilin and Hartree in 1947 reported that the content of cytochrome *c* in spores of *B. subtilis* is about 6% of the content in vegetative cells (*11*). Tochikubo (*19*) demonstrated the presence of all of the cytochrome components, cytochromes *a*, a_3, *b*, *c*, c_1, and *o*, in spores of *B. subtilis*. He showed that concentrations of these cytochromes increased during germination and subsequent vegetative growth, but did not examine the change in the cytochrome system during the process of spore formation.

Vegetative cells of *B. cereus* have been reported to contain cytochromes *a*, a_3, *b*, and *c* (*5*, *7*, *17*). Contradictory results have been published concerning the cytochrome composition of spores of *B. cereus*. Some workers (*5*, *14*) reported that they could not detect cytochrome, while others (*1*, *6*) reported the presence of cytochromes in spores; even among the latter workers, there are disagreements about the position of absorption maxima of the cytochrome spectra.

Felix and Lundgren (*7*) compared the cytochrome contents of *B. cereus* between cells in exponential growth and cells containing forespores and found that the contents increased during sporulation, but they did not examine the changes in cytochrome contents during germination. Thus, although changes in cytochrome composition during germination were examined with *B. subtilis* and changes during sporulation with *B. cereus,* no report has been published dealing with the changes in cytochrome components during the whole life cycle of a particular spore-forming bacterium.

Several workers have compared the activity of oxygen uptake among intact cells from different developmental stages of aerobic spore formers. Many reports showed that respiratory activity is extremely low in dormant spores and that activity increases after the commencement of germination. However, studies on the time course of the development of respiratory activity from spores to the inception of cell division are scanty; moreover in these studies oxygen uptake was measured continuously with cells germinating in a flask containing either glucose (*10*) or nutrient broth (*13*), and the rate of oxygen uptake was not corrected for the increase of cell mass. Still fewer are studies on the change of respiratory activity of intact cells during the process of spore formation.

In the present study, changes in cytochrome components as well as in respiratory activity with several substrates of *B. cereus* strain T were examined with cells harvested from various stages of germination, vegetative growth and sporulation. Special care was taken to have a mass of cells germinating or sporulating synchronously, but it was not possible to establish a condition in which cells germinate, divide and sporulate synchronously in a culture starting from a single inoculation. Therefore, we examined the process of germination and that of sporulation in separate experiments.

With the methods used, more than 95% of the spores germinated, and the extent of synchrony was about 80% at the stage of the first cell division. In the series of experiments for sporulation, inoculated cells reached the stage of sporangia, the stage of appearance of a refractile spore in each cell, with 90 to 95% synchrony.

Materials and methods

Organism and culture medium. The organism used in this study was *Bacillus cereus* Strain T, which was provided by Y. Kobayashi of Hiroshima University. The medium was the G-medium, originally used by Stewart and Halvorson (*18*) for cultivation of *B. cereus* strain T. The composition of the medium was: glucose, 4 g; yeast extract (Difco), 2 g; $(NH_4)_2SO_4$, 5 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 1.6 g; $CaCl_2 \cdot 2H_2O$, 66 mg; $FeSO_4 \cdot 7H_2O$, 1.8 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 18 mg; $MnSO_4 \cdot H_2O$, 100 mg per 1000 ml (pH 7.2). In preparing this medium, (1) the mixture containing $(NH_4)_2SO_4$, K_2HPO_4, yeast extract, (2) glucose solution, (3) $CaCl_2$ solution, and (4) the mixture containing the other components, were prepared separately and aseptically mixed after autoclaving. Unless otherwise stated, cells were grown aerobically at 30°C by shaking culture on a reciprocating shaker at 120 strokes per min with an amplitude of 6 cm.

Germination. Spores were heat activated at 65°C for 20 min in distilled water, then mixed into the culture medium, which was kept at 30°C, by stirring

to make uniform suspension. The procedure was immediately followed by the start of shaking culture with either 100 ml of the culture medium in a 500-ml shaking flask or 1 liter of the medium in a 3-liter Erlenmeyer flask. The difference in culture size did not influence the outcome of germination and vegetative growth.

Sporulation. Three serial transfer inoculations were made to obtain reproducible exponential growth and almost synchronous sporulation in the main culture. The initial culture was inoculated from stock agar slant and incubated for about 16 hr. This culture was then inoculated into the second culture to make 1% inoculum. When cells in this second culture had grown to the late exponential phase (3.5 hr) and cell concentration had reached an optical density value of 0.30, 5 ml of the culture was transferred to 100 ml of fresh medium in the 500-ml shaking flask to start the main culture.

Resting suspensions of cells and spores. To prepare resting cell suspensions, culture fluids were chilled in an ice bath, and cells were collected and washed three or four times by centrifugation with 0.85% saline solution. In spectrophotometric measurements, cells were suspended in the saline solution and either used as such or stored in a freezer. When frozen samples were melted slowly, the suspensions showed cytochrome spectra identical with those of fresh suspensions. In manometric measurements, washed cells were suspended in 0.02 M phosphate buffer (pH 7.2).

Free spores were harvested 3 days after the start of the main culture for examination of the sporulation process. When it was necessary to collect a mass of spores, the main culture was performed by adding 70 ml of the preculture to 7 liter of the medium in a 10-liter bottle, and then passing air through the medium for 3 days. At the end of the culture, most of the cells had lysed and free spores had been liberated into the culture fluids. Spores were cleaned of the remaining intact cells and cell debris by differential centrifugation through distilled water; cells were first sedimented at $10,000 \times g$ for 5 min, then resuspended and sedimented at least 10 times by gradually decreasing centrifugal force that was $3,000 \times g$ for 5 min in the final centrifugation. Spores after these treatments were clean as judged by microscopic observations, and by measurement of their catalase activity which was reported to be about 5% in spores as in vegetative cells (*12*).

Spectrophotometric analysis. Absorption spectra of cytochromes in intact cells were measured with a Hitachi two-wavelength double beam spectrophotometer Model 356, using a 10-mm light path cuvette at room temperature or a 3-mm light path cuvette with a low-temperature attachment at liquid nitrogen temperature (77°K). Cytochromes were reduced with sodium dithionite and oxidized with air or ferricyanide. In precise measurements of reduced minus oxidized difference spectra, the absorption curves were corrected by the curve obtained when both sample and reference cuvettes contained the same oxidized cell suspensions.

The quantities of cytochromes *a*, *b*, *c* were determined from the height of α-peaks of reduced minus ferricyanide-oxidized difference spectra, measured at room temperature. The amount of cytochrome *a* ($+a_3$) was determined as the increment of absorbancy to the peak at 603 nm from a tangent line connecting points on the absorption curve at about 580 and 630 nm. The quantity of cytochrome *a* was calculated on the basis of $\Delta\varepsilon$ mM (reduced minus oxidized) at 603 nm to be 12.0 (*20*). Since absorption curves by cytochromes *b* and *c* overlap each other, the contents of each component were calculated from the following equations.

$$A_{557}=16\ X_b+4.0\ X_c$$

$$A_{551}=6.1\ X_b+24\ X_c$$

where X_b and X_c denote the millimolar concentrations of cytochromes *b* and *c*; A_{557} or A_{551} is the absorbancy increment to the peak at 557 or 551 nm from the tangent line connecting points on the absorption curve at 540 and 580 nm. The values of 16 and 6.1 are $\Delta\varepsilon$ mM (reduced minus oxidized) of cytochrome b_1 from *Escherichia coli* (*4*) at 557 and 551 nm, respectively. We used the value of extinction coefficients for cytochrome b_1, since the position of the α-peak of the main *b*-type cytochrome of *B. cereus,* 557–558 nm, suggests a similarity to cytochrome b_1. The values of 4.0 and 24 are $\Delta\varepsilon$ mM (reduced minus oxidized) of cytochrome *c* from horse heart, at 557 and 551 nm, respectively. To obtain the values of extinction coefficients of cytochrome *c*, we measured the reduced minus oxidized spectrum of a solution of crystalline horse heart cytochrome *c* (Boehringer Mannheim), the concentration of which was calculated from ε mM of the reduced form at 550 nm taken to be 27.5 (*2*).

Manometry. Manometric flasks contained 1.0 ml of cell suspension, 1.0 ml of 0.1 M phosphate buffer (pH 7.2) and 0.5 ml of water in the main compartment, 0.5 ml of 0.1 M substrate in the side arm, and 0.2 ml of 20% KOH in the center well. Oxygen uptake was measured every 5 to 10 min at 30°C. Respiratory activity, which was expressed as $QO_2(N)$, was calculated from the data of the first 40 or 50 min, since prolonged estimation sometimes resulted in a change of the rate of oxygen uptake. The value of the endogenous activity was subtracted.

All the values of cytochrome contents and respiratory activity of cells and spores in this report are the means of three to seven independent measurements.

Cell concentration. The total number of cells and spores was counted directly under a microscope with a Thoma counting chamber. To make the septa of dividing cells easily visible, cells were suspended in cold 5% trichloroacetic acid before counting (*16*). Growth was followed by the changes in turbidity of culture solution in an 18-mm test tube with a Hitachi EPO-B photometer using a 660-nm filter and expressed as absorbancy. Dense culture solutions were diluted to a range in which turbidity readings were proportional to cell concentrations. Nitrogen contents of cells and spores were determined by a micro-Kjeldahl method.

Results

Cytochrome components in vegetative cells and spores

Fig. 1 shows the reduced cytochrome spectra of vegetative cell measured at room temperature and 77°K. Fig. 5 and 7 show the reduced minus oxidized difference spectra, measured at room temperature, of vegetative cells from various developmental stages. These spectra reveal the feature of cytochrome components in vegetative cells of *B. cereus* Strain T. The peaks or shoulders at 600, 445 nm (room temperature) and at 599, 440 nm (77°K) in reduced spectra, and at 600 to 603 nm in reduced minus oxidized difference spectra, show the presence of the cytochrome complex, $a+a_3$. The peaks or shoulders at 557, 528, 423 nm (room temperature) and at 555, 520, 425 nm (77°K) in reduced spectra, and at 557–558,

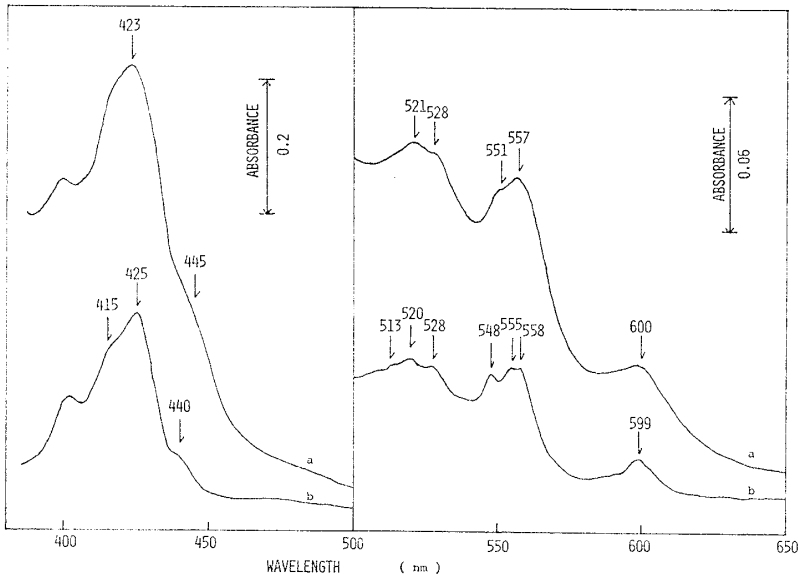

Fig. 1. *Reduced cytochrome spectra of vegetative cells at room temperature* (a) *and at liquid nitrogen temperature* (b). Dithionite was added to the suspensions of cells from the sporangial stage.

530 nm in the difference spectra suggest the presence of a *b*-type cytochrome resembling cytochrome b_1. The peaks in reduced, 77°K spectrum at 558 and 528 nm indicate the presence of another *b*-type cytochrome, the reduced form of which probably has maximum absorption at about 562 nm at room temperature. The peaks and shoulders at 551, 521 nm (room temperature) and 548, 513, 415 nm (77°K) in reduced spectra, and at 551, 522 nm in the difference spectra show the presence of a cytochrome *c*.

Our observation revealed the presence of cytochromes in spores that had been

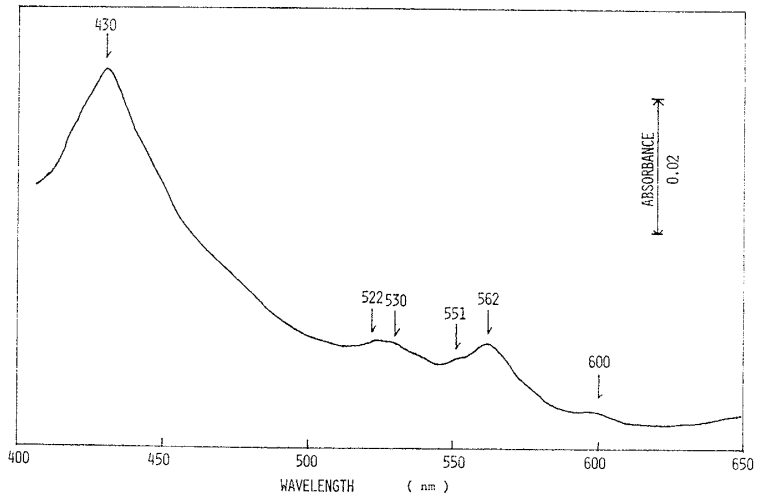

Fig. 2. *Reduced minus oxidized difference spectrum of dormant spores.* Spores were purified by repeated differential centrifugations and washing with HCl. Cytochromes were reduced with dithionite and oxidized with air, and the difference spectrum was measured at room temperature. The concentration of the spore suspension was 0.92 mg cell nitrogen per ml.

cleaned by repeated differential centrifugations. Since this observation contradicts that by some previous workers who reported the absence of cytochromes in spores of *B. cereus* (*5*, *14*), we attempted further purification of spores to eliminate possible contamination by cell fragments, by suspending spores in cold 0.01 N HCl for a few minutes and subsequently washing them 4 times with distilled water (*3*). Spores after such treatment germinated normally when heat activated at 65°C for 20 min. Dithionite-reduced minus air-oxidized difference spectrum of the suspension of a clean spore sample is shown in Fig. 2. The spectrum has peaks at 560 to 562, 551, 530, 522 and 430 nm. These peaks show the presence of cytochromes *b* and *c* in spores. The dithionite-reduced cytochromes could be reoxidized when spore suspensions were shaken with air. Further change in the spectrum did not occur when ferricyanide was added to the air-oxidized sample. Although absorption by cytochrome *a* at 600 nm is slight in the spectrum shown in Fig. 2, a preliminary measurement with a low-temperature spectrum also suggested the presence of cytochrome *a* in spores.

Changes in respiratory activity and cytochrome contents during germination and vegetative growth

Curve a in Fig. 3 shows the change in turbidity of cell suspensions during the process of germination. When dormant spores were heat activated at 65°C for 20 min and transferred to the G-medium at 30°C, spores started germination, and in about 10 min turbidity of spore suspensions decreased due to the disappearance of refractility of spores. The turbidity of the suspension remained unchanged during

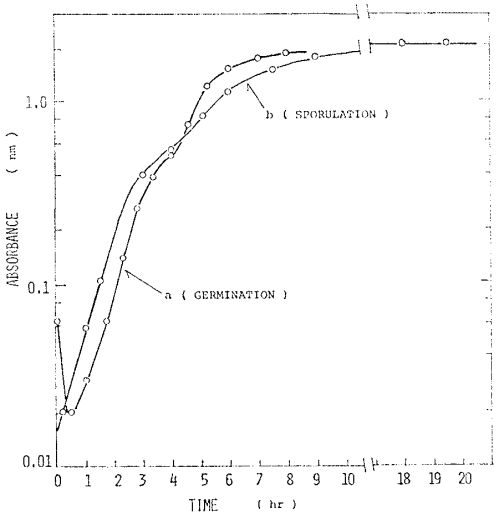

Fig. 3.

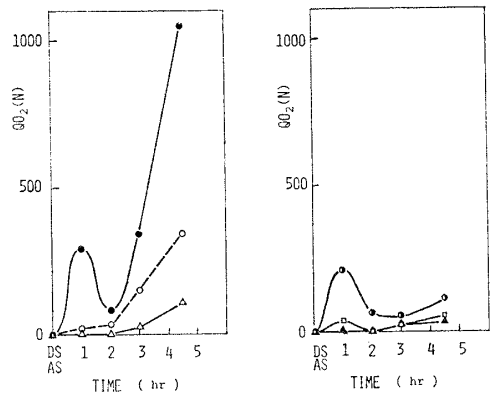

Fig. 4.

Fig. 3. *Changes in the turbidity of cell suspensions.* Curve a. Turbidity change during germination, elongation and vegetative growth. Spores were heat activated at 65°C for 20 min in distilled water, then mixed in the culture medium at 0 hr. Curve b. Turbidity change during vegetative growth and sporulation. Details of the growth conditions are described in **Materials and methods.**

Fig. 4. *Changes in respiratory activity during germination, elongation and vegetative growth.* Dormant spores (DS), heat activated spores (AS), and cells harvested at various intervals after the start of germination, were assayed for their respiratory activity with the following substrates. ●, glucose; △, acetate; ◑, pyruvate; □, lactate; ▲, succinate; and ○, endogenous. For the growth curve, consult Fig. 3 (curve a).

the subsequent 30 min, and thereafter increased due to cell elongation and cell proliferation. At 1 hr after the commencement of germination, the suspension contained cells that had grown longer breaking their spore coats. First cell division took place at 2 hr with about 80% synchrony, and second cell division at 2.5 hr. Cells continued division, and the pH of the culture media decreased during growth reaching a minimum of 4.8 at about 4 hr, then gradually increased. According to previous workers (*15*), this stage of minimum pH of the media corresponds to the commencement of sporulation.

The activities of oxygen uptake with five substrates were compared among dormant spores, heat-activated spores, and cells harvested at 1, 2, 3 and 4.5 hr after the commencement of germination. The result is shown in Fig. 4. The oxygen uptake of dormant spores and heat-activated spores was negligible, even when manometry was carried out with as much as 10 mg dry weight of spores per flask. The activity of glucose oxidation was apparent at 1 hr after the beginning of germination, decreased at 2 hr when cells underwent the first division and then rapidly increased during the vegetative growth. The activity of pyruvate oxidation was also high at 1 hr and low at 2 hr, and increased to some extent during vegetative growth. Lactate oxidation, although the level was lower, followed almost the same pattern as pyruvate oxidation. The oxidation of acetate and succinate was not apparent until the start of cell multiplication.

The pattern of cytochromes in cells changed during spore germination and vegetative growth. Fig. 5 shows dithionite-reduced minus ferricyanide-oxidized difference spectra of cells, measured with the same cell concentrations (on the bases of cell nitrogen per ml), at 1.0 hr and 3.0 hr after the start of germination. The quantitative changes of the contents of each cytochrome component, expressed on the basis of per cell nitrogen or per cell, in the process of spore germination and vegetative growth are presented in Table 1. During germination of heat-activated spores (0 hr to 1.5 hr), three cytochrome components were rapidly synthesized in the cells; particularly, the contents of cytochromes *a* and *c* per cell nitrogen showed a distinct increase during 0 to 1.0 hr, suggesting preferential synthesis of these cytochromes over gross proteins. From 1.0 to 2.0 hr during germination, the amount of the three cytochromes per cell nitrogen was almost constant, but their amount per cell dropped sharply at 2.0 hr due to cell divisions. During vegetative growth until the commencement of sporulation, i.e., from 2.0 to 4.5 hr, the content per cell

Table 1 *Amounts of cytochromes in spores, germinated cells and vegetative cells*

Time after start of germination (hr)	Cytochrome			Cytochrome		
	a	*b*	*c*	*a*	*b*	*c*
	(nmole/mg cell-N)			(nmole/10^{10} cells)		
0.0 [a]	0.00	0.24	0.028	0.00	0.065	0.008
1.0	0.38	0.27	0.11	0.57	0.41	0.17
1.5	0.40	0.27	0.088	2.25	1.52	0.51
2.0 [b]	0.48	0.29	0.090	1.69	1.02	0.32
3.0	0.50	0.57	0.10	1.22	1.39	0.24
4.5	0.39	0.65	0.14	—	—	—

[a] 0.0 hr: dormant spore.

[b] 2.0 hr: end of first cell division.

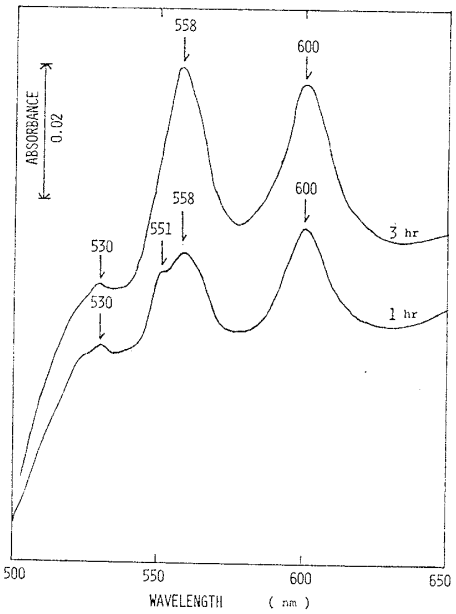

Fig. 5.

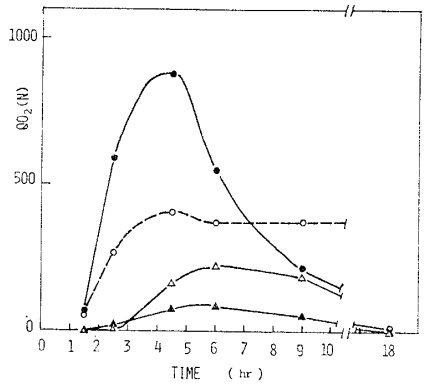

Fig. 6.

Fig. 5. *Reduced minus oxidized difference spectra of germinated, elongated cells (1.0 hr), and of exponentially growing cells (3.0 hr).* Cytochromes were reduced with dithionite and oxidized with ferricyanide, and the difference spectra were measured at room temperature. Cell concentrations in optical cuvettes were adjusted to 3.0 mg cell nitrogen per ml.

Fig. 6. *Changes in respiratory activity during vegetative growth and sporulation.* Cells harvested at various intervals after the start of exponential growth were assayed for their respiratory activity with the following substrates. ●, glucose; △, acetate; ▲, succinate; and ○, endogenous. For the growth curve, consult Fig. 3 (curve b).

nitrogen of cytochrome *b* increased, but cytochrome *c* remained at an almost constant level and cytochrome *a* showed a tendency to decrease.

Changes in respiratory activity and cytochrome contents during vegetative growth and sporulation

When cells, precultured until the late exponential phase as described in **Materials and methods,** were inoculated into fresh G-medium, they grew without an appreciable lag period; the growth rate was 1.9 to 2.0 doublings per hr (curve b in Fig. 3). The pH of the culture media decreased during the exponential growth and reached a minimum of 4.8 at about 4.0 hr. As described previously, this stage of minimum pH corresponds to the commencement of sporulation. Thereafter the pH of the media gradually increased and morphological changes occurred inside the cells. At 6 hr, distinct granular structures could be seen in the cells. At about 18 hr, refractile spores appeared with 90 to 95% synchrony in the cells, which served as sporangia. At about 30 hr, cell lysis occurred and spores were liberated.

The respiratory activities of cells from various developmental stages were measured with glucose, acetate and succinate. The result is shown in Fig. 6. The activity was low in cells from the early exponential phase. The activity of glucose oxidation increased during mid-exponential growth, reached maximum intensity at 4.5 hr, and decreased during the phase of sporulation. The activity of acetate or succinate oxidation was low during exponential growth, while it increased in the early sporulation stage and decreased slightly during subsequent sporulation.

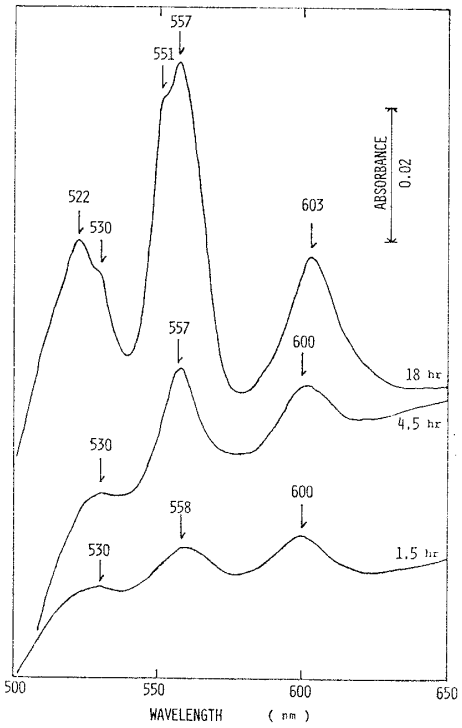

Fig. 7. *Reduced minus oxidized difference spectra of cells at the exponential phase (1.5 hr), the stage of commencement of sporulation (4.5 hr), and the sporangial stage (18 hr).* Cytochromes were reduced with dithionite and oxidized with ferricyanide, and the difference spectra were measured at room temperature. Cell concentrations in optical cuvettes were adjusted to 1.5 mg cell nitrogen per ml.

Remarkably, respiration with added substrates, or with endogenous substrate, was negligible in sporangial cells.

Reduced minus oxidized difference spectra of the same concentrations of cell suspensions from three different stages are shown in Fig. 7. Distinct quantitative change in the contents of cytochromes is apparent among the three suspensions. The figure also reveals the shift of absorption maxima of cytochrome *a* ($+a_3$) from 600 nm in exponential cells to 603 nm in sporangial cells. The quantitative changes of three cytochrome components, expressed per cell nitrogen or per cell, during the process of exponential growth and sporulation are shown in Table 2. The contents of the three cytochromes were low in cells from the mid-exponential stage (1.5 hr). The contents increased during subsequent growth (1.5 to 2.5 hr). During the transition from vegetative growth to sporulation (2.5 to 4.5 hr), the contents of cytochromes *b* and *c* remained almost constant, while that of cytochrome *a* decreased slightly. The contents of the three cytochromes in cells of the sporangia stage were the highest of all the developmental stages.

Table 2 *Amount of cytochromes in vegetative cells, sporulating cells and sporangial cells*

Time after inoculation in fresh medium (hr)	Cytochrome (nmole/mg cell-N)			Cytochrome (nmole/10^{10} cells)		
	a	*b*	*c*	*a*	*b*	*c*
1.5	0.24	0.14	0.033	0.68	0.40	0.09
2.5	0.51	0.52	0.12	1.24	1.26	0.29
4.5 [a]	0.39	0.66	0.13	0.76	1.29	0.25
18.0 [b]	0.93	1.47	0.57	1.93	3.06	1.19

[a] 4.5 hr: start of sporulation.

[b] 18 hr: completion of sporangia formation.

Discussion

Cytochrome spectra of *B. cereus* have been measured by Schaeffer with strain Caron (*17*), by Doi and Halvorson with Strain T (*5*), and Felix and Lundgren with Strain ATCC 4342 (*7*). These workers recognized the presence of cytochromes $a+a_3$, *b* and *c* with characteristic absorption peaks of α-bands at 600–603, 556–557, 550–552 nm in room temperature reduced or reduced minus oxidized spectra. Our observation agreed with these results, and further revealed the presence of another cytochrome *b* by the position of the α-peak at 562 nm in room-temperature reduced minus oxidized spectrum of spores, and by the position of α- and β-peaks at 558 and 527 nm in 77°K reduced spectrum of vegetative cells.

Reports about the presence or absence of cytochromes in spores of *B. cereus* have been contradictory; Nakada et al. (*14*) and Doi and Halvorson (*5*) could not obtain conclusive evidence for the presence of cytochromes in spores, while Felix and Lundgren (*6*) reported that Triton X-100 soluble extracts of membrane fractions from spores of Strain ATCC 4342 showed cytochrome spectra similar to those from vegetative cells. Recently Bahnweg and Douthit (*1*) measured the absorption curve of intact spores of *B. cereus* Strain T suspended in glycerol, and obtained a spectrum which showed peaks at 611, 567 and 531 nm. The authors inferred the presence of cytochromes *a* and *b* in the spores, but the assignment appears to need reexamination, since the positions of the peaks are unusual for those of cytochromes and the pigments were found to be resistant to oxidation. The present result showed the presence of cytochromes *b* and *c* in spores, with absorption peaks and shoulders in reduced minus oxidized spectrum at about 562, 551, 530, 522 and 430 nm. Although the position of the α-peak of *b*-type cytochrome, about 562 nm, differs from that of b_1-type cytochrome (at 557 nm) predominant in vegetative cells, the presence of the former *b*-type cytochrome as a minor component in vegetative cells was disclosed by 77°K spectrum. The reduced forms of cytochromes *b* and *c* in spores were oxidized by air. A preliminary measurement of 77°K spectrum of spores suggested the presence of cytochrome *a* in spores. Unpublished results by Ankei and Hino showed the definite presence of cytochromes *a*, *b*-562, *b*-557 and *c* in spores of *B. cereus* Strain No. 2. Thus, spores of *B. cereus* appear to have cytochrome components similar to those of vegetative cells. The possibility of contamination by vegetative cell fragments in the spore sample may be excluded by the fact that the same spectra were obtained with spore suspensions before and after HCl washing of the purified spores.

The present results showed that there were parallel changes between cytochrome contents and respiratory activity in some developmental stages of *B. cereus*. When heat-activated spores germinated, the concentration of cytochromes *a* and *c* increased rapidly with concomitant activation of the ability of glucose oxidation. During the stage from early exponential growth to the commencement of sporulation, the concentration of cytochrome *b* increased in parallel with the increase of the activity of glucose and endogenous respiration. On the other hand, there was no correlation between the concentration of cytochromes and respiratory activity during the stage from the commencement of sporulation to the completion of sporangia. Remarkably, sporangial cells, with the highest concentrations of cytochromes of all the developmental stages, showed negligible respiratory activity with

endogenous or added substrates. Apparently, cytochromes at this stage are not functional for production of energy. During the later stage, most of the cytochromes in the sporangial cells must have been discarded into the culture media, since free spores contained only a small amount of cytochromes.

Our observation is consistent with reports by previous workers that respiratory activity increases during spore germination. However, we noticed that the activity of succinate and acetate oxidation was negligible even when germinated cells showed considerable activity of glucose oxidation. This observation agrees with findings by Hanson et al. (*9*) and Goldman and Blumenthal (*8*) that some enzymes in the tricarboxylic acid cycle are missing from germinated cells and young vegetative cells. Goldman and Blumenthal (*8*) also showed that glucose is catabolized mainly by the Embden-Meyerhof pathway during most stages in the life cycle of *B. cereus* T. They further revealed that the pentose-monophosphate pathway occupies a significant role in glucose catabolism during spore germination and subsequent elongation, but the contribution of this pathway is negligible at the stage of first cell division. Their observation partly explains our result that glucose oxidation was active at the stage of elongation but negligible at the stage of first cell division. However, the variation of the contribution of the pentose-monophosphate pathway cannot explain the variation of the activity of pyruvate oxidation, which showed a pattern similar to that of glucose oxidation.

The increase of the activity of succinate and acetate oxidation during the early stage of germination and the late-exponential phase probably reflects the increasing operation of the tricarboxylic acid cycle, demonstrated by previous workers (*8*, *9*). The cause of active glucose oxidation in the late-exponential phase (at 3 hr in Fig. 4 and at 2.5 hr in Fig. 6) must be elucidated, since the result of previous workers showed that both the pentose-monophosphate pathway and the tricarboxylic acid cycle are almost inactive at this stage. Also unknown is the cause of almost complete loss of endogenous and substrate oxidation in sporangial cells. The loss is probably caused by the breakdown of some of the proteins of respiratory enzymes, concomitant with the breakdown of many proteins in mother cells to supply building blocks for spore constituents.

The author is grateful to Dr. Y. Kobayashi of Hiroshima University for his valuable advice, and wishes to thank Prof. S. Hino of Hiroshima University for his guidance and discussion throughout the study.

References

(*1*) Bahnweg, G. and H. A. Douthit: Cytochrome pigments in spores of *Bacillus cereus* T. *J. Bacteriol.* 121: 737–739 (1975).

(*2*) Butt, W. D. and D. Keilin: Absorption spectra and some other properties of cytochrome *c* and of its compounds with ligands. *Proc. Roy. Soc. London* B 156: 429–458 (1962).

(*3*) Church, B. D., H. Halvorson and H. O. Halvorson: Studies on spore germination: Its independence from alanine racemase activity. *J. Bacteriol.* 68: 393–399 (1954).

(*4*) Deeb, S. S. and L. P. Hager: Crystalline cytochrome b_1 from *Escherichia coli*. *J. Biol. Chem.* 239: 1024–1031 (1964).

(*5*) Doi, R. H. and H. Halvorson: Comparison of electron transport systems in vegetative cells and spores of *Bacillus cereus*. *J. Bacteriol.* 81: 51–58 (1961).

(*6*) Felix, J. A. and D. G. Lundgren: Some membrane characteristics of *Bacillus cereus* during growth and sporulation. In *Spores* V. Edited by H. O. Halvorson, R. Hanson and L. L. Campbell. p. 35–44. American Soc. Microbiol., Washington, D. C., 1972.

(*7*) Felix, J. A. and D. G. Lundgren: Electron transport system associated with membranes of *Bacillus cereus* during vegetative growth and sporulation. *J. Bacteriol.* 115: 552–559 (1973).

(*8*) Goldman, M. and H. J. Blumenthal: Changes in terminal respiratory pathways of intact cells of *Bacillus cereus* at various stages of development. ibid. 87: 387–390 (1964).

(*9*) Hanson, R. S., V. R. Srinivasan and H. O. Halvorson: Biochemistry of sporulation II. Enzymatic changes during sporulation of *Bacillus cereus*. ibid. 86: 45–50 (1963).

(*10*) Hyatt, M. T. and H. S. Levinson: Utilization of phosphates in the postgerminative development of spores of *Bacillus megaterium*. ibid. 77: 487–496 (1959).

(*11*) Keilin, D. and E. F. Hartree: Comparative study of spores and vegetative forms of *Bacillus subtilis*. *Antonie van Leeuwenhoek J. Microbiol.* 12: 115–128 (1947).

(*12*) Lawrence, N. L. and H. O. Halvorson: Studies on the spores of aerobic bacteria IV. A heat resistant catalase from spores of *Bacillus terminalis*. *J. Bacteriol.* 68: 334–337 (1954).

(*13*) Mandels, G. R., H. S. Levinson and M. T. Hyatt: Analysis of respiration during germination and enlargement of spores of *Bacillus megaterium* and of the fungus *Myrothecium verrucaria*. *J. Gen. Physiol.* 39: 301–309 (1956).

(*14*) Nakada, D., A. Matsushiro, M. Kondo, K. Suga and K. Konishi: Studies on the development of aerobic sporeforming bacteria II. Evolution of the respiratory enzyme system in *Bacillus cereus*. *Med. J. Osaka Univ.* 7: 809–818 (1957).

(*15*) Nakata, H. M. and H. O. Halvorson: Biochemical changes occurring during growth and sporulation of *Bacillus cereus*. *J. Bacteriol.* 80: 801–810 (1960).

(*16*) Nelson, D. L. and A. Kornberg: Biochemical studies of bacterial sporulation and germination XVIII. Free amino acids in spores. *J. Biol. Chem.* 245: 1128–1136 (1970).

(*17*) Schaeffer, P.: Recherches sur le métabolisme bactérien des cytochromes et des porphyrines I. Disparition partielle des cytochromes par culture anaérobie chez certaines bactéries aérobies facultatives. *Biochim. Biophys. Acta* 9: 261–270 (1952).

(*18*) Stewart, B. T. and H. O. Halvorson: Studies on the spores of aerobic bacteria I. The occurence of alanine racemase. *J. Bacteriol.* 65: 160–166 (1953).

(*19*) Tochikubo, K.: Changes in terminal respiratory pathways of *Bacillus subtilis* during germination, outgrowth, and vegetative growth. ibid. 108: 652–661 (1971).

(*20*) Van Gelder, B. F.: On cytochrome *c* oxidase I. The extinction coefficients of cytochrome *a* and cytochrome a_3. *Biochim. Biophys. Acta* 118: 36–46 (1966).