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Sporulation Properties of Cytochrome *a*-Deficient Mutants of *Bacillus subtilis*

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Three classes of cytochrome *a*-deficient mutants of *Bacillus subtilis* have been found to be asporogenic or oligosporogenic. All three classes showed declines in adenosine 5'-triphosphate (ATP) concentrations during early sporulation, at a time when ATP levels in wild-type strains are constant. Class III mutants were found to be deficient in aconitase and isocitric dehydrogenase, and showed reduced maximum growth in nutrient sporulation medium. These mutants also suffered the most rapid decline in ATP concentration in early sporulation, and exhibited neither the biphasic oxygen consumption curve nor the increase in pH normally observed at the end of logarithmic growth in nutrient sporulation medium. Nicotinamide adenine dinucleotide oxidase activities of purified membrane preparations were approximately normal for mutants in all classes, except for two of the class II mutants and one class III mutant. Neither cytochrome *a* nor cytochrome *c* appears to be an obligatory intermediate in cyanide-sensitive nicotinamide adenine dinucleotide oxidation in *B. subtilis*.

A requirement for vigorous aeration is a principal physiological feature of sporulating cultures of *Bacillus subtilis* (8, 24). At least some of the oxygen is needed during sporulation to regenerate adenosine 5'-triphosphate (ATP) by reoxidation of reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and reduced flavine adenine dinucleotide (FADH), which have been reduced during operation of the citric acid cycle. Mutants of *B. subtilis* deficient in the activities of some citric acid cycle enzymes grow but do not exhibit the massive sporulation observed normally after the end of growth; this property has been related to an inability to form ATP during the postexponential developmental period (6, 11). Since oxidative phosphorylation presumably is coupled to the cytochrome system in *B. subtilis*, it is of interest to determine how mutationally induced cytochrome deficiencies affect growth and sporulation.

Among mutants having several types of cytochrome deficiencies (21), cytochrome *a*-deficient strains were investigated in detail, because they were all deficient in sporulation. This paper examines some of their physiological and enzymic properties and relates them to sporulation. An investigation (7) of the ultrastructure of these mutants has revealed grossly abnormal mesosome structures during the postexponential growth phase.

MATERIALS AND METHODS

Strains of B. subtilis. The transformable parent 60317, derived from Spizizen's 168 strain, required L-isoleucine and L-tryptophan (or indole) for growth on glucose minimal medium. All mutants were obtained by ^{61}Co treatment of spores as described elsewhere (21).

Growth media. All strains were routinely cultivated on tryptose blood agar base (TBAB) (Difco). T medium is the liquid equivalent of TBAB. The nutrient sporulation medium (NSM) and its K-phosphate-containing alternative (NSMP) have been described previously (5). Synthetic medium (M) has been described by Spizizen (18); it was always supplemented with 25 μg of L-isoleucine and L-tryptophan per ml. Glucose or Na-L-glutamate concentrations were 28 mM. For solid media 1.7% agar was added.

Sporulation frequency. To measure the formation of heat-resistant spores, cultures were shaken at 37 C in flasks or tubes containing 1/10 of their volume of NSM medium. Appropriate dilutions in phosphate magnesium buffer (same concentration as in minimal medium) were spread on TBAB plates for a determination of the viable titer (V). Samples of the diluted suspensions were heated at 75 C for 15 min and plated onto the same medium for a determination of the spore titer (S).

Antibiotic production. Secretion of the antistaphyloccal antibiotic was determined by growing patches

of *B. subtilis* cells overnight on TBAB, then overlaying with about 10^7 *Staphylococcus aureus* (ATCC 10537) mixed with soft agar. After overnight incubation, clear areas surrounding the patches indicated production of the antibiotic.

Preparation of lysates and enzyme assays. Cells were grown in NSMP to the measured end of exponential growth. The cultures were centrifuged, washed in 0.05 M tris(hydroxymethyl)aminomethane-chloride, pH 8, and resuspended in this buffer to an A_{600} (extinction at 600 nm; 1 cm light path) of approximately 50. Treatment with 100 μg of lysozyme per ml for 0.5 h at 37 C completely cleared the suspension. This was followed by incubation with 0.1 μg of deoxyribonuclease per ml for 15 min at 37 C, after which the lysate was centrifuged (at 4 C) for 15 min at 30,000 $\times$ *g*. The pellet was washed with 0.05 M K-phosphate, pH 7.3, plus 10^{-3} M $MgCl_2$, resuspended in this buffer, and kept frozen at -20 C until it was used for NADH oxidase measurements.

Protein concentration was measured by the Lowry procedure (12) with bovine serum albumin as standard.

The supernatant fraction was kept in an ice bucket, and used within 3 h for assays of aconitase (EC 4.2.1.3., citrate [isocitrate] hydrolyase) and isocitrate dehydrogenase (EC 1.1.1.42, *threo*-D_s-isocitrate: NADP oxidoreductase [decarboxylating]). Aconitase was measured by the increase in absorbance at 240 nm due to the conversion of isocitrate to *cis*-aconitate (16), and the molar extinction coefficient for *cis*-aconitate was taken to be 3.3×10^3 $cm^{-1}M^{-1}$. Isocitrate dehydrogenase was measured by the increase of absorbance at 340 nm due to reduction of NADP in the presence of isocitrate (15), and the molar extinction coefficient for NADPH was taken to be 6.2×10^3 $cm^{-1}M^{-1}$. Adenosine monophosphate (10^{-3} M) was found to have essentially no stimulatory effect on the isocitrate dehydrogenase activities (10), and was not included in the assays. NADH oxidase activities of the pellet fractions were measured by oxygen consumption in the presence of 10 mM NADH by using a Gilson oxygen electrode thermostatted at 33 C. The aconitase and isocitrate dehydrogenase assays were carried out at room temperature (23 C), and specific activities of these and of NADH oxidase were calculated as nanomoles of substrate consumed or product formed per minute per milligram of protein.

Oxygen consumption of growing or sporulating cultures. Samples (3 ml) were removed from cultures growing in NSM, saturated with air by vigorous shaking, and immediately placed in an oxygen electrode apparatus (Yellow Springs Instrument Co.). Recording was continued until the oxygen concentration reached 65% of saturation. The results were expressed as micromoles of O_2 consumed per minute per liter of culture fluid.

ATP concentration. Samples (0.5 ml) were removed from growing cultures and mixed with 0.5 ml of an ice-cold mixture containing approximately 0.46 M formic acid (1/50 dilution of commercial solution) and 0.002 M sodium ethylenediaminetetraacetic acid. The ATP concentration of these extracts was determined by the sensitive luciferin-luciferase assay described by Klofat et al. (11) with the modification that 10 μliters of the formic acid extract was injected directly into the luciferase mixture by a spring-loaded Hamilton syringe (model CR700). All ATP values belonging to one growth curve were determined with the same luciferase preparation within a short time, so that changes in the sensitivity of luciferase would be negligible.

RESULTS

Distinction of three mutant classes. The isolation and initial characterization of the cytochrome *a*-deficient mutants from the sporulating strain 60317 have been described elsewhere (21). These mutants, which all had very low concentrations of cytochrome *a*, were divided into three classes according to their additional deficiencies in cytochrome *c*, and growth on nonfermentable substrates (Table 1). All mutant numbers will be stated with their class in parentheses, e.g., 60843 (I) is a mutant of class I.

Growth properties. On a synthetic medium containing citrate and either lactate or glutamate, mutants of class I or class II grew at a distinctly lower rate than the parent strain, whereas mutants of class III could not grow at all (21; summarized in Table 1). When malate or oxalate was present together with glutamate, mutants in all classes grew well (21). These properties are reflected by growth data obtained in nutrient sporulation medium (NSMP), as shown in Fig. 1. For all mutants the growth rate started to decline earlier and the maximum absorbancy at 600 nm (A_{600}) reached a lower value than for the standard strain (60317). Apparently, the mutants failed to utilize the nutritional resources of the medium (which lacks glucose) as efficiently as the parent strain. Class III mutants showed a marked lysis after growth had stopped.

Enzyme activities. The growth requirement for glutamic acid exhibited by class III mutants

TABLE 1. *Summary of properties of cytochrome a-deficient mutants*[a]

Class	Relative cytochrome concentrations		Relative growth rates on minimal medium plus		
	Cytochrome *a*	Cytochrome *c*	Glucose	Lactate	L-glutamate
Parent	1.0	1.0	1.0	1.0	1.0
I	0.32–0.50	0.78–1.0	0.80	0.30	0.35
II	0.23–0.43	0	0.80	0.25	0.27
III	0.14, 0.06	1.0, 1.0	1.0	0	0

[a] From reference 21.

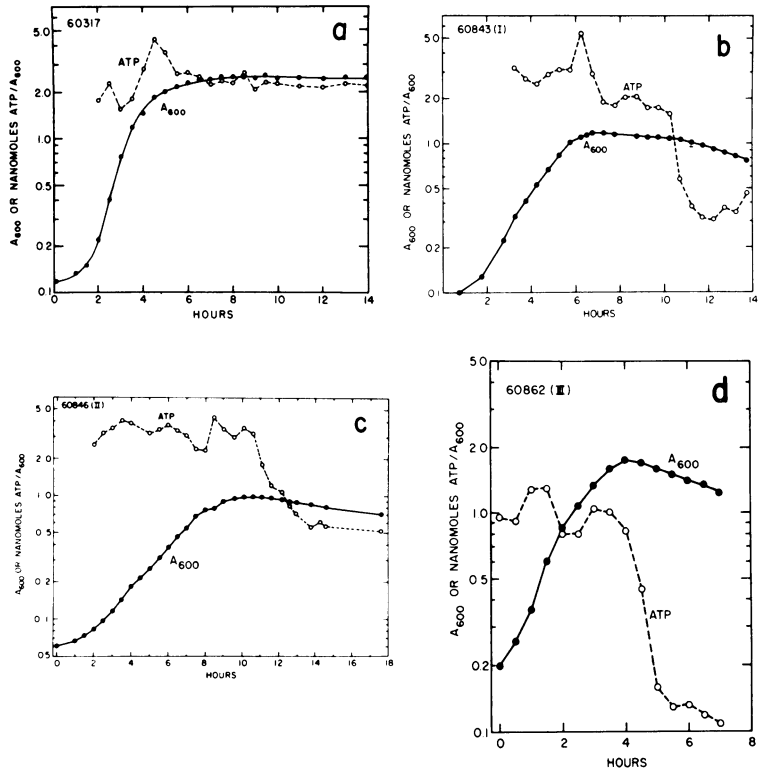

FIG. 1. *Growth characteristics and ATP concentrations of the parent strain (60317) and of cytochrome a-deficient mutants grown in NSMP medium. Nanomoles of ATP/A_{600} are indicated by open circles and dashed lines (ATP); growth of the cultures is indicated by closed circles and solid lines (A_{600}).*

(21) suggested deficiencies of the citric acid cycle enzymes. Table 2 shows that these mutants lacked aconitase and isocitric dehydrogenase activities, even when measured at the end of growth in NSMP. Partial deficiencies of the two enzymes were exhibited by some members of both classes I and II, but these apparently were not serious enough to create the requirement for glutamate previously noted (21).

Cytochrome a concentrations of citric acid cycle mutants. To determine whether a severe cytochrome *a* deficiency (as in class III mutants) could result secondarily from the mutational loss of aconitase or isocitric dehydrogenase, low-temperature spectra (21) were measured in mutants primarily lacking these enzyme activities (5, 20). The results in Table 3 show that such mutants have normal cytochrome *a* concentrations relative to the concentrations of cytochrome *b* and cytochrome *c*.

Change of ATP concentration during growth in NSMP. Klofat et al. (11) have shown that in a sporulating strain (60015) of *B. subtilis* the amount of ATP per A_{600} (ATP/A), which is a measure of the amount of ATP per cell, remained rather constant during growth and development in NSMP medium. In citric acid cycle mutants, the ATP/A value remained al-

TABLE 2. *Aconitase and isocitrate dehydrogenase activities of cytochrome a-deficient mutants*

Class	Strain numbers 60...	Sp act (nmol/min/mmg of protein)	
		Aconitase	Isocitric dehydrogenase
Parent strain	317	90	81
I	843	32	55
	848	8	150
	860	27	166
	861	15	212
II	845	22	171
	846	50	148
	847	15	111
	863	37	15
	864	ND[a]	14
III	844	<2	<2
	862	<2	<2

[a] Not determined.

TABLE 3. *Cytochrome concentrations of aconitase-deficient (aco⁻) isocitrate dehydrogenase-deficient (idh⁻) mutants*

Class	Strain numbers	Absorbance ratios	
		cyto *a*/ cyto *b*	cyto *c*/ cyto *b*
Parent strain	60317	1.06[a]	1.18[a]
aco⁻	RB30	1.18	1.07
idh⁻	60991	1.05	1.13
aco⁻idh⁻	60525	1.66	1.66

[a] From reference 21.

most constant during exponential growth but declined to a much lower value during the developmental period. All cytochrome mutants examined in this study also showed an almost constant value of ATP/A during exponential growth as well as during the first part of the developmental period; but at some later time the value decreased. Examples of each mutant class are shown in Fig. 1.

For mutants of class I and II the decrease in ATP/A occurred within 4 h after the time (T_o) at which growth had declined from the exponential rate, as can be seen in Fig. 1b and c. The time of the ATP decrease was mutant specific and may reflect the completeness of the block in the electron transport system. For example, mutant strain 60860 (I), which formed about 50% of the wild-type level of cytochrome *a*, maintained a normal ATP level for a longer time than strain 60861 (I), which formed only about 30% of the wild-type level of cytochrome *a*.

Class III mutants had a somewhat lower average ATP/A value during exponential growth and exhibited a rapid decline as growth terminated (Fig. 1d). For comparison, results obtained with an aconitase-deficient strain (RB30) that contained normal cytochrome *a* are shown in Fig. 2. Since the ATP concentration curves of the class III mutant and of the aconitaseless mutant were nearly identical, the early decline of ATP could be explained by the lack of aconitase alone.

Sporulation. All cytochrome *a*-deficient mutants exhibited low sporulation frequencies in NSMP or NSM. Table 4 lists the spore (S) and vegetative (V) titers and the sporulation frequencies (S/V) for all mutants grown 24 h in NSM. Class III mutants produced less than three heat-resistant spores per milliliter of culture. For several of the mutants, sporulation frequencies (S/V) were not meaningful because the extensive lysis at the end of growth in NSM or NSMP (cf. Fig. 1) diminished viable counts and yielded artificially high S/V values in some cases.

Antibiotic production. Table 4 shows that all mutants produced antibiotic activity against *S. aureus* ATCC 10537.

pH change and oxygen demand during post-exponential growth. In early stages of *B. cereus* sporulation a rise in oxygen consumption has been observed which was correlated with a pH increase of the culture (8). A similar response has been observed for *B. subtilis* (Fig. 3a) for the parent strain 60317 grown in NSM. Actually, there were two maxima of oxygen consumption that occurred with a time interval of 1 to 1.5 h. The deficiency of cytochrome *a* alone, in class I mutants, reduced the rate of oxygen consumption (per A_{600}) at all times during the culture cycle, but it did not markedly affect this pattern, as is shown for strain 60843 (I) in Fig. 3b. Class II mutants (e.g., 60846), which lacked both cytochrome *a* and *c*, also showed reduced oxygen consumption and exhibited a smaller second peak of oxygen consumption than strain 60317; but they still showed a rise in pH during the stationary period (Fig. 3c). For class III mutants no increase of the pH was noted (e.g., strain 60862 in Fig. 3d). The oxygen consumption declined after the initial rise and did not show a second maximum.

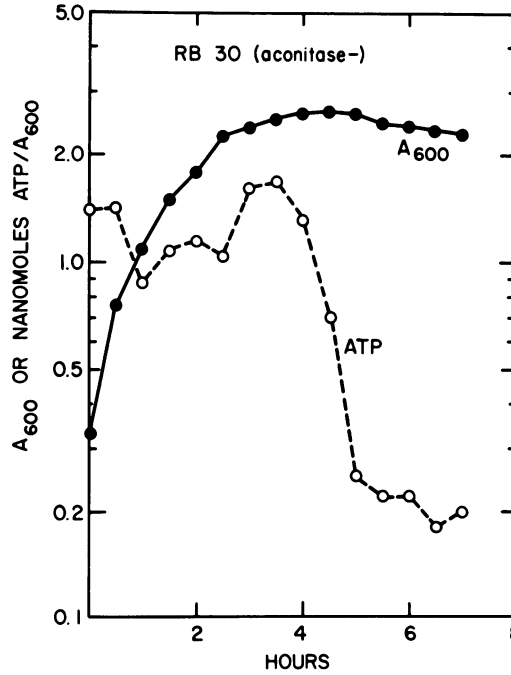

FIG. 2. *Growth characteristics and ATP concentration of an aconitase-deficient strain grown in NSMP medium. Symbols are as in Fig. 1.*

TABLE 4. *Sporulation of cytochrome a-deficient mutants on NSM medium*

Class	Strain numbers 60...	S	V	S/V	Antibiotic production
Parent strain	317	2.0×10^8/ml	2.3×10^8/ml	0.85	+
I	843	2.9×10^3	7.2×10^8	4.0×10^{-4}	+
	860	3.0×10^3	1.9×10^8	1.6×10^{-5}	+
	861	1.2×10^3	3.5×10^7	3.4×10^{-5}	+
	848	1.3×10^2	2.0×10^6	6.5×10^{-5}	+
II	845	3.5×10^6	7.4×10^7	4.7×10^{-2}	+
	846	1.9×10^3	1.0×10^5	1.9×10^{-2}	+
	863	5.3×10^2	8.8×10^6	6.0×10^{-5}	+
	847	1.8×10^5	1.0×10^8	1.8×10^{-3}	+
	864	1.5×10^4	2.8×10^7	5.3×10^{-4}	+
III	844	<10	8.2×10^6	$<1.2 \times 10^{-6}$	+
	862	<10	6.2×10^6	$<1.6 \times 10^{-6}$	+

NADH oxidase in cell envelope preparations. The oxygen consumption of whole cells depends both on the production of reduced compounds, such as NADH, and on the rate at which these compounds can be reoxidized. The rate of NADH oxidation was measured in cell envelope preparations (see Materials and Methods) made from cultures grown in NSMP and harvested at T_0. The results in Table 5 show that most cytochrome-deficient mutants had NADH oxidase activities equivalent to or greater than that of the parent strain. Only one mutant strain (of class III) produced a significantly reduced activity. It was interesting that the mutants of class II could be further subdivided into two types of four mutants examined, two produced normal levels of NADH oxidase, whereas the other two produced a four to five times higher specific activity.

In both the parent strain and all of the cytochrome *a*-deficient mutants, NADH oxidase was 70 to 80% inhibited by 10 mM KCN.

DISCUSSION

Cytochrome *a* deficiencies have been found associated with a variety of *B. subtilis* mutant phenotypes (14, 19, 21, 22). The cytochrome *a* deficiencies are two types (22): (i) derepressed/cytochrome *a*-deficient (i.e., showing a normal stationary growth phase elevation of cytochromes *b* and *c*, and loss of cytochromes c_1 and *o*); this type is exemplified by *citD* mutants, which do not sporulate, and by *strC* mutants, which do; (ii) repressed/cytochrome *a*-deficient (cytochromes *b* and *c* are not elevated in stationary growth, and cytochromes c_1 and *o* are retained in the cell membrane); this type includes the *mar-3* mutants (22) and the three classes described in the present communication; none of the mutants of the repressed/cytochrome *a*-deficient type appear to sporulate normally. We conclude tentatively that cytochrome *a* deficiency alone is not sufficient to block sporulation, and that there must be in addition improper regulation of the cytochrome system, caused by a mutation in a controlling gene, to give rise to a Spo$^-$ phenotype.

Class I and class III mutants, as defined previously (21), may be altered in such controlling genes, whereas the cytochrome *a* deficiency in class II mutants may result indirectly from the cytochrome *c* deficiency. In certain cytochrome *c*-deficient mutants of yeast, cytochrome *a* synthesis is extremely sensitive to catabolite repression (17). Although we have not yet succeeded in locating the loci governing the cytochrome *a* phenotypes on the *B. subtilis* genetic map, we have isolated revertants from each mutant class in which normal cytochrome complements and ability to sporulate are restored (H. Taber and G. Halfenger, unpublished observations). Therefore, we believe the phenotypes are consequences of a single mutational events.

All of the present mutants are of the repressed/cytochrome *a*-deficient type, and the sub-classification (21) is based on (i) a complete lack of cytochrome *c* in class II strains, and (ii) aconitase and isocitrate dehydrogenase deficiencies in class III strains. The latter deficiencies appear to be secondary to the cytochrome *a* deficiency (rather than the reverse), since *aco*$^-$ and *idh*$^-$ strains (Table 3; 22) are not cytochrome *a* deficient. In addition, aconitase and isocitrate dehydrogenase are normally controlled independently (4, 5, 9, 13). The Aco$^-$ Idh$^-$ phenotype of class III mutants may result from repression of synthesis of these two enzymes due to accumulation of *a*-ketoglutarate or glutamate (4) by cells growing in NSM or NSMP. Inefficient coupling of NADH-linked

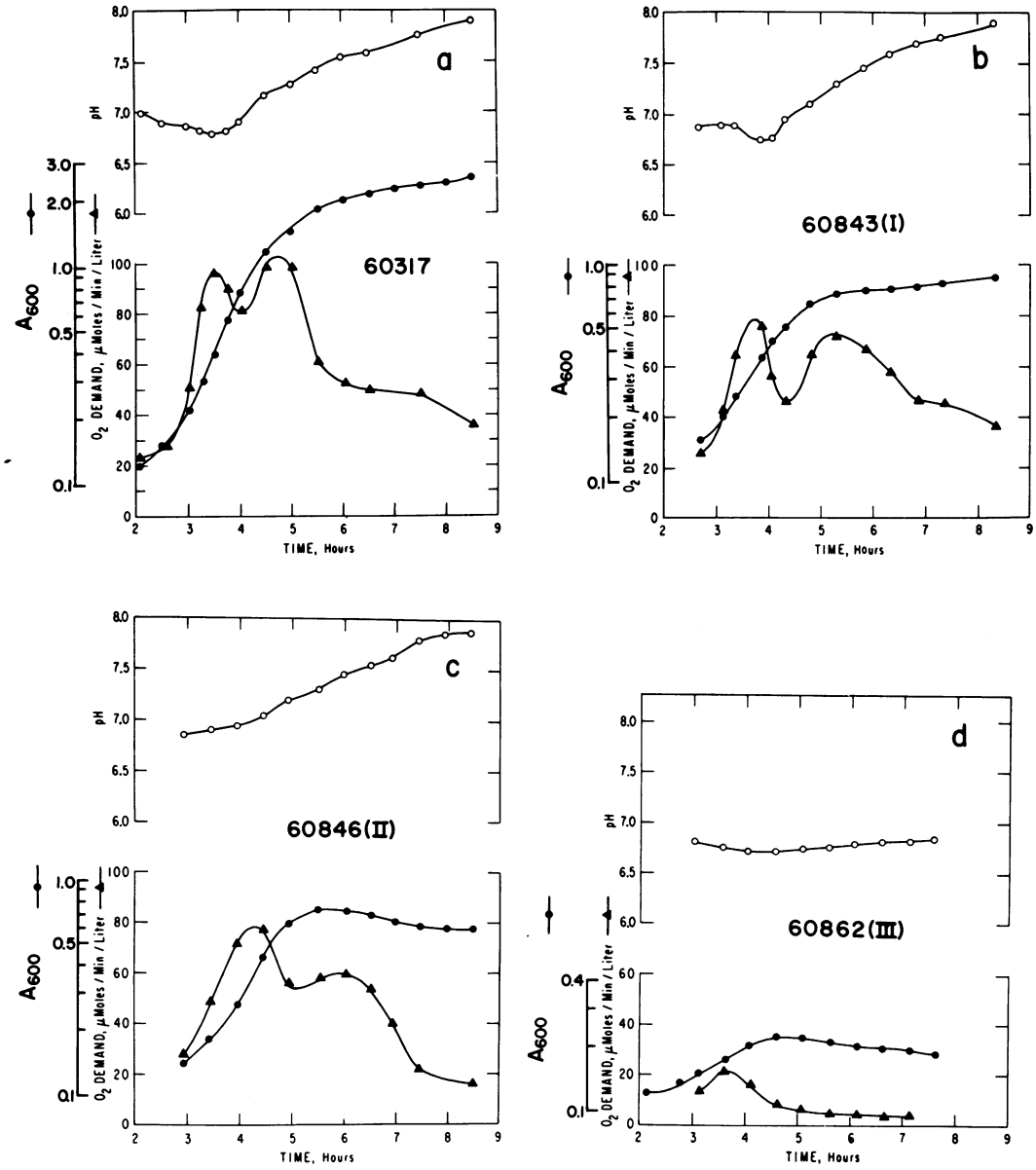

FIG. 3. *Changes in oxygen demand and pH in cultures of strain 60317 and of cytochrome a-deficient mutants during growth in NSM medium.*

dehydrogenases to oxygen, via the electron transfer system, is indicated by the inability of class III mutants to grow on lactate or glutamate. However, this interpretation is not supported by the maintenance of NADH oxidase activities in class III mutants. A systematic effort to find conditions for derepression of aconitase and isocitrate dehydrogenase, as Ohné (13) has made for aconitase, will be necessary to resolve this inconsistency.

The rate of oxygen consumption by whole cells is reduced in most cytochrome *a* mutants (21). However, the time course of oxygen consumption during growth in sporulation medium is altered only in class III mutants. Cultures of the latter strains also do not exhibit any increases in pH after growth has stopped. In wild-type bacilli, this pH rise appears to be due to oxidative deamination of amino acids (1), and it may be that deamination in class III mutants is restricted by the severe cytochrome *a* deficiency characteristic of these strains. Again, the

TABLE 5. *NADH oxidase activity of cell envelope preparations from cytochrome a-deficient mutants*

Class	Strain number 60...	nmoles of O_2/min/mg of protein
Parent	317	47
I	843	44
	848	100
	860	53
II	846	221
	847	203
	863	37
	864	37
III	844	24
	862	42

interpretation is complicated by the fact that cyanide-inhibited NADH oxidase is not reduced significantly in class III mutants. In fact, since NADH oxidase activities in the mutants are (except for strain 60844) at least as high as in the parent strain, and even elevated in certain class II mutants (60846, 60847), the results suggest that cytochromes *a* and *c* are not essential for reoxidation of NADH in *B. subtilis*, although they are necessary for normal growth yields and for sporulation. A cytochrome absorption maximum at 557 nm has been observed in intact *B. subtilis* cells (3, 21) which has been identified by Tochikubo (23) as cytochrome *o*, a terminal oxidase. *B. megaterium* has a similar enzyme (2). Whether an alternate path for electron transfer from NADH utilizes this cytochrome and what the identities are of other components of this path will have to be established. The elevated NADH oxidase activities of some class II mutants do not result from higher concentrations of cytochromes *o* or c_1, because class I and class II mutants show the same relative amounts of these cytochromes as the parent strain (21) and also because the mutants of class III do not show elevated NADH oxidase activities, although they do exhibit increased amounts of both cytochromes *o* and c_1 (21).

In contrast to the parent strain, the concentration of ATP in all mutants decreases within 4 h after growth has stopped. For mutants of class III this decrease occurs almost immediately after the end of growth, and can be attributed to the Aco$^-$ Idh$^-$ phenotype, which alone can cause such an effect (Fig. 2; 11). The inability of the cytochrome mutants to sporulate normally might thus be caused by the decrease in the amount of ATP per cell, at least for the mutants of class III. For the mutants of class I or class II, however, the cytochrome deficiency may affect some other process, required for sporulation, earlier than the production of ATP.

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