BIOCHEMICAL ANALYSIS OF SPOROGEN AND ASPOROGEN STRAINS OF *B. SUBTILIS*[*]

Jekisiel Szulmajster and Pierre Schaeffer

Laboratoire d'Enzymologie, C.N.R.S., Gif-sur-Yvette (S.O.) and Institut Pasteur, Service de Physiologie Microbienne, Paris.

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We have previously reported (Szulmajster and Schaeffer, 1961) that in the sporogen (Sp^+) strains of *B. subtilis* an increase in the particulate DPNH-oxidase activity occurs during the prespore phase, while the activity of this enzyme remains low and constant during the corresponding period of time in all the asporogen (Sp^-) mutants studied. When in the asporogen mutants sporogenesis is restored through the action of wild type DNA, the behavior of DPNH-oxidase is regained and the specific activity reaches the same value as in the Sp^+ strains.

Since it had previously been shown (Schaeffer and Ionesco, 1960) that these asporogen mutants are genetically different from one another because of their reciprocal transformation behavior, it seemed possible that the separate Sp^- mutations might affect different enzymes in the complex DPNH-oxidase enzyme system. Accordingly, a study of this part of the respiratory chain in the various *B. subtilis* mutants has been undertaken and the present

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communication deals with some of the results obtained in this direction.

Some of the morphological and cytological characteristics of the mutants used for this work and the method of preparation of particles from lysozyme lysed cells have previously been described by Ryter et al (1961) and Szulmajster and Schaeffer (1961). For large scale experiments, particles were prepared from lyophylized cells after treatment in the sonic oscillator (Raytheon, 9 kc/sec) for 20 min.

The results summarized in table 1 were obtained with 20.000 g particles prepared from bacteria collected 5 hours after exponential growth had stopped (T_5). Under our cultural conditions, thermoresistant spores first appear 5½ hours after the cessation of exponential growth ($T_{5.5}$) and represent the majority of the population one hour later ($T_{6.5}$).

In table 1 are given the specific activities of the diaphorases which catalyze the oxidation of DPNH by ferricyanide, 2,6-dichlorophenol-indophenol, mammalian cytochrome c and menadione, together with the overall oxidation of DPNH by oxygen.

In considering these results the mutants should be divided into 3 groups: a) the sporogen strains try^+Sp^+ (SMY wild type) and $try^-Sp_3^+$ (tr); b) the true asporogen mutants $try^-Sp_3^-$ and $try^-Sp_4^-$ and c) the mutants Try^+Sp^- (12 UV, abortive) and try^-Osp (Ryter et al, 1961). It can be seen that at time T_5 the sporogen strains possess relatively high DPNH-oxidase, DPNH-dehydrogenase, diaphorase, cyt.c reductase and cyt.c peroxidase activities. The same enzymes, except the cyt.c peroxidase, are either synthesi-

TABLE 1

Enzymes	Acceptor	Strains					
		Try+Sp$^+$ (w) SMY	Try$^-$Sp$^+_3$ (tr)	Try$^-$Sp$^-_3$	Try$^-$Sp$^-_4$	Try$^+$Sp$^-$ (12UV abortive)	Try$^-$Osp
DPNH-oxidase (A.S. x 10)	Air	4.27	4.1	traces	0.09	0.48	0.4
DPNH-dehydrogenase (A.S. x 10)	$Fe(CN)_6$	3.9	4.1	0.3	0.3	traces	traces
Diaphorase (A.S. x 10)	2-6-Dichlorophenol indophenol	13.2	14.3	2.65	1.7	2.0	3.0
DPNH-Menadione-réductase (A.S. x 10)	Menadione	91	13.4	1.9	-	-	-
DPNH-cyt c-réductase	cyt.c	7200	6940	950	130	traces	75
Succino-cyt c-réductase	cyt.c	-	0	0	-	145	140
cyt c-oxidase	O_2	0	0	0	-	1200	2000
cyt c-peroxidase	H_2O_2	3600	3580	2450	-	3000	2670

zed in small amounts or rapidly inactivated in the asporogen mutants of group b. The sporulation mutants of group c possess a slightly higher DPNH-oxidase activity than the true Sp^- mutants, traces of DPNH-cyt.c-reductase and succinate-cyt.c-reductase and, in addition to the cyt.c-peroxidase an active cyt.c-oxidase. It should be noted that this latter enzyme is completely absent in all other strains of B. subtilis so far studied. It can also be seen in Table 1 that the Sp^+ strains contain very high menadione-reductase

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LEGEND FOR TABLE 1

Comparative specific activities of enzymes involved in electron transport system.

Enzyme assays. The oxidation using air as the acceptor was carried out as previously described (Szulmajster and Schaeffer, 1961). With ferricyanide as oxidant the incubation mixture contained per ml: ferricyanide, 0.4 umoles, K-phosphate buffer pH 7.2, 100 umoles; enzyme protein, 40-80 ug and DPNH, 0.2 umoles. With 2.6 dichlorophenol-indophenol about 17 ug of dye per ml of incubation mixture was employed. Menadione was used at a final conc. of 10^{-4} M. The assay mixture for cyt.c-reductase contained (in 3 ml): 120 umoles of "Tris" buffer, pH 8.0; 0.35 ml of 10^{-4} M cyt.c (Sigma) about 20 ug of enzyme protein and 0.25 umoles of DPNH. Readings at 550 mu (KCN is not necessary in this assay, since no cyt.c-oxidase activity is present). For succinate-cyt.c-reductase, 50 umoles of Na-succinate per ml of incubation mixture was used in place of DPNH. In the cyt.c-oxidase assay 40 umoles of phosphate buffer, pH 7.2 95% reduced cyt. c, with an initial O.D. of about 400-500 (reduction carried out by means of electron-exchange resin (Chantrenne, 1955) and the usual amounts of enzyme were used in an incubation mixture of 1 ml. Cyt.c-peroxidase was measured anaerobically in Thunberg-Beckman test tubes containing in a final vol. of 3 ml : 60 umoles of phosphate buffer pH 7.2, reduced cyt.c (OD about 500), 5 umoles of N_3Na 10^{-2} M, 0.75 umoles of H_2O_2 and 10-20 ug of particles protein. In order to run the peroxidation anaerobically, all reagents, except H_2O_2 and enzyme were first incubated in the tube and a stream of nitrogen bubbled through the mixture for 3 min. The enzyme was added, the side bulb containing the H_2O_2 is connected and the tube is evacuated and refilled with nitrogen, 3 times. A control without enzyme was included for each assay. Specific activities (A.S.) are expressed either in umoles of DPNH oxidized/min/mg protein (DPNH-oxidase, DPNH-deshydrogenase, diaphorase, menadione reductase) or in ΔE 550/min/mg protein (DPNH-cyt.c-reductase, succinate-cyt.c-reductase, cyt.c-oxidase, cyt.c-peroxidase).

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activities which are about 10 to 50 times higher than those obtained with the asporogen mutants Sp_3^- and Sp_{51}^-. (the activity of the latter is not shown in this table).

The lack of a functional cyt.c-oxidase in the strains of groups a and b has been confirmed by the absence of cyanide inhibition of the oxygen uptake by cell suspensions in the presence of glucose. Table 2 shows the Q_{O_2}-glucose of some of the mutants listed in Table 1. With the Sp^+ and true Sp^- strains KCN stimulates oxygen uptake during both the active growth and the prespore phase. With the oligosporogen mutant there is no effect of cyanide during exponential growth; however, sensitivity to this inhibitor appears about 3 hours after this phase ceases, a time which corresponds with the appearance of cytochrome c-oxidase activity. It seems, therefore, that a good correlation exists between cyt.c-oxidase activity and cyanide sensitivity. It should be noted that there is no effect of KCN or azide (4×10^{-3}M) on DPNH oxidation in any of the particula-

Table 2

Q_{O_2}-glucose of Sp^+ and Sp^- strains of B.subtilis

	Try^+Sp^+(w)		$Try^-Sp_3^+$ (tr)		Try^-Sp^-3		Try^+Osp	
		+KCN		+KCN		+KCN		+KCN
T	46	60	66.5	76.0	62	70	52	40
T_3	52	86	-	-	59	65	52	12
T_5	60	110	50	59	54	60	-	-

Cell-suspensions were prepared either from bacteria collected during exponential growth (T), 3 hours (T_3) and 5 hours (T_5) after this phase. The cells were washed with 0.2 M phosphate buffer pH 7.2 containing 0.02% $MgSO_4$ and 0.01% $CaCl_2$ and resuspended in the same buffer. Oxygen uptake was measured by conventional Warburg.manometric methods. Glucose was used at the final concentration of 0.02 M and KCN at 4×10^{-3}M. Q_{O_2} = umoles O_2/mg d.w/h.

te systems studied. However, Antimycin A, which is known to inhibit the electron flow from DPNH to cytochrome c, inhibits the DPNH-oxidase in our system.

As shown above, the Marburg strain, as well as its Sp^- mutants, lack a functional cyt.c-oxidase although they show the spectral characteristics of cytochrome a. A similar observation has been made with other strains of B. subtilis by Smith (1954). Only the two mutants of group c (thus far tested) have shown cytochrome c-oxidase activity in addition to their cytochrome c-peroxidase activity. These results eliminate partially the objection that these bacteria may show preferential activity toward their own cytochromes and would not use the mammalian cytochrome tested in our experiments.

At the present stage it can only be said that activity of the particulate multienzyme DPNH-oxidase present in the Sp^+ strains of B. subtilis is considerably depressed in the Sp^- mutants. However, the real significance of this finding for sporulation is not yet clear. It is reasonable to assume that the relation might be an indirect one and that the deficiency of the above enzymatic activities are a secondary consequence of an important disturbance brought about by a mutation in genes directly involved in sporogenesis.

The second problem arising from the present results concerns the nature of the respiratory mechanism itself in B. subtilis. The fact that all of the strains tested except the mutants of group c, lack a functional cyt.c-oxidase but have a cyt.c-peroxidase would suggest a cytochrome-peroxidase mediated respiration with hydrogen peroxide as the ultimate oxidant of the cytochrome chain. The overall

sequence of reactions involved in such respiration would be similar to that suggested for P. fluorescens by Moss et al (1956). It should be noted that the relatively high menadione-reductase activity(tab. 1) might be of special interest in these bacteria, since by virtue of its autoxidizability, menadione could link the oxidation of DPNH to oxygen reduction. Furthermore menadione has been directly implicated in the respiratory chain of some bacteria and may be coupled to phosphorylation (Brodie et al, 1957).

ABBREVIATIONS

The following designations were used in describing the various strains: Try-tryptophane requiring, Sp^--asporogenic, SMY-strain Marburg-yale, (w)-wild type, (tr)-transformed, Osp-oligosporogene.

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