RELATIONSHIP BETWEEN THE TRICARBOXYLIC ACID CYCLE ENZYMES AND SPORULATION OF B.SUBTILIS

by

R.S. Hanson[+], J. Blicharska and J. Szulmajster

Laboratoire d'Enzymologie du C.N.R.S, Gif-sur-Yvette, France

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The enzymes of the tricarboxylic acid (TCA) cycle are repressed during vegetative growth of several species of aerobic sporeforming bacilli in a complex medium containing glucose (Hanson et al.,1961; Hanson et al. 1963). It has been postulated that derepression of these enzymes is necessary for sporulation to take place. It has also been shown that sporulation in B.subtilis,Marburg, is associated with a high rate of $NADH_2$ oxidation as compared to the rate observed during vegetative growth and to the rate observed in the asporogenic mutants during the entire growth cycle (Szulmajster and Schaeffer; 1961). Because a relationship between $NADH_2$ oxidase activity, TCA cycle and sporulation apparently exists, studies were therefore undertaken on several asporogenic mutants of B.subtilis, some of which are blocked in a step in the TCA cycle.

Experiments were first carried out to investigate the growth, sporulation, glucose utilization and the evolution of the pH in the wild type and in several asporogenic mutants (Sp^-) of B.subtilis, Marburg, in G medium by the active culture procedure described by Halvorson (1957)

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These results are very similar to those obtained with B.cereus (Nakata et al.,1960). During vegetative growth, the pH drops as a consequence of acids produced from the utilization of glucose. Following glucose exhaustion, these acids are utilized and the pH of the medium then increases. Further results indicate that the evolution of the pH is a reflexion of the repression (during growth) and the derepression (after growth stopped) of the tricarboxylic acid cycle enzymes. The nature of this repression phenomenon will be the subject of a separate paper now in preparation.

It is important that similar curves for growth, pH evolution, glucose utilization and acetoin formation were obtained with several Sp mutants of B.subtilis (except C_{4-4}), some of them blocked at an early morphological stage of spore development (Schaeffer et al.,1963).

The activities of some enzymes of the TCA cycle and the sporulation capacities of two different wild type strains and of several mutants of B.subtilis are given in Table I. It can be seen that except the C_{4-4} mutant, all the strains tested demonstrate normal levels of these enzymes with some variations from one strain to another. The C_{4-4}, as shown by Ramos et al.,(1962), is unable to synthetize aconitase (aconitase$^-$) and consequently requires glutamate for growth, In addition this mutant, unlike the parent strain (C_4), is unable to sporulate$^+$).

To eliminate the possibility that conditioning of the growth medium might play an inhibitory role in the expression of

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+) It should be noted that in some other glut$^-$ mutants tested, the requirement for glutamate can be satisfied by aspartate. These mutants, however are able to synthetize the TCA cycle enzymes and have been found to be sporogenic.

sporulation of the aconitase$^-$ mutant, we have grown mixed cultures of this mutant and the wild type SMY in nutrient broth in the conditions for maximum sporulation. The two types of colonies could be easily distinguished by color (see TableI) and size. The results of these experiments have shown that the wild type strains sporulated normally (100%), while the aconitase$^-$ (glut$^-$) mutant formed no spores. (The sensitivity of this method would not allow us to detect less than 2-3% spores).

Further evidence showing that the block in the aconitase activity leads to the suppression of the sporulation capacity of the mutant comes from transformation expe-

Table I. Some properties of the strains of B.subtilis used in this investigation.

Strain	Phenotype	Aconitase	Isocit. deh-ase	Malic deh-ase	% Spores after 24 h.
		Specific activities			
SMY	wt, Sp$^+$ brown	380	0,240	0,968	100
C-4$^{+)}$	wt, Sp$^+$ albino	450	—	—	100
$C_{4-4}$$^{+)}$	Glut$^-$, Sp$^-$	0	0,268	0,258	<0,1
168	try$^-$, Sp$^+$	420	0,175	0,305	100
186$^{++)}$	try$^-$, leu$^-$, Sp$^+$	400	0,175	0,275	85
168$^{+++)}$ (4)	try$^-$, Sp$^-$	615	0,430	0,810	<0,1

Cells were grown in Nutrient Broth (Difco) supplemented with 10^{-3}M $CaCl_2$, 10^{-6}M $FeSO_4$, 10^{-5}M $MnCl_2$, 0,1% KCl, 0,025% $MgSO_4$ and 0,1% glucose. Extracts were prepared by lysing cells in Tris buffer M/20, pH 7,4 and 0,1 NaCl with 100 µg/ml lysozyme. Sporulation was determined by plating cells after heating 10 mn. at 80°C on nutrient broth agar. Enzyme activities were determined by the standard methods. Specific activities are expressed in µmoles of $NADPH_2$ or $NADH_2$/mg prot./min. (isocitric deh-ase and malic deh-ase or in OD_{240}/mg prot./min. (aconitase)

Strains kindly supplied by $^{+)}$Dr. Wiame, $^{++)}$Dr. Anagnostopoulos, $^{+++)}$Dr. Schaeffer.

riments summarized in Table II.

Table II. Properties of glutamate requiring mutants obtained by transformation.

Exp	DNA donor	Recipient cells	Isolate designation	Aconitase	Isocit. deh-ase	% Spores+) after 24 h.
				Specific activities		
I	C_{4-4}	168 (try^-, Sp^+)	TT_1	0	+	<0,1
II	C_{4-4}	168	TT_2	0	+	<0,1
III	TT_1	168	TT_3	0	+	<0,1
IV	TT_1	186 (try^-, leu^- Sp^+)	TT_{4a}	0	0,141	<0,1
	TT_1	186	TT_{4b}	0	0,138	<0,1
	TT_1	186	TT_{4c}	0	0,201	<0,1
	TT_1	186	TT_{4d}	0	0,190	<0,1

Growth, sporulation and the determination of enzymes activities were given in Table I. Sporulation for some strains was also determined in nutrient broth cultures supplemented with 0,15% glycerol and neutralized with sterile potassium hydroxide. The strains were asporogenic under these conditions also.

+). The sporulation frequency in some TT strains was found to be between $1/10^4$ and $1/10^5$.

By introduction of the DNA from the aconitase$^-$ mutant (C_{4-4}) into the try^- Sp^+ mutant (168, Marburg) followed by selection of try^+ transformants on minimal medium containing 0,05 M glutamate, we have been able to isolate some double transformants try^+ $glut^-$. Although these $glut^-$ clones are unstable and yield $glut^+$ clones at high frequency, stable glutamate$^-$ clones were isolated after four or five repeated selections of auxotrophic clones from spread plates. On examination, all the stabilized $glut^-$ clones were found to be unable to form spores. The $glut^+$ clones were sporogenic although they retained the other

characteristics of the DNA donor strain (albino try^+ colonies).

Extracts prepared from the different $glut^-$ Sp^- transformants (TT mutants) were found to be completely devoid of aconitase activity.

Our attempts for reciprocal transformation using the $aconitase^-$ mutants as recipient cells and the wild type DNA as donor were all unsuccessful. All these mutants fail to develop competence for transformation under a variety of conditions.

Experiment IV in Table II shows one of the attempts to isolate double auxotrophic strains by using $glut^-$ ($aconitase^-$) DNA and leu^- try^- Sp^+ mutant as recipient cells. The try^+ transformants were isolated on minimal medium supplemented with leucine and glutamate and the leu^- $glut^-$ clones identified by replication. The proportion of double auxotrophs leu^- $glut^-$ among the try^+ transformants was very low. Five leu^- $glut^-$ clones were examined and they all have been found to be unstable and yielded $glut^+$ colonies during purification. It was however possible to isolate $glut^-$ leu^+ clones from this experiment and the properties of these transformants are shown in Table II (TT_4, a, b, c, d).

Attempts were also made to obtain revertants of $glut^-$ ser^- and $glut^-$ ad^- mutants obtained by ultraviolet irradiation of $glut^-$ TT_3 strain. No $glut^+$ or Sp^+ revertants were found even after plating about 10^{10} cells. Therefore we have been unable to test $glutamate^+$ derivatives of these strains for their ability to sporulate. We cannot completely rule out the possibility that a gene involved in sporulation may be located very near the aconitase gene and that strain

C_{4-4} has two closely linked mutation sites.

By plating a heavy suspension of TT_3 cells after heating 10 mn. at 80°C, it was possible to isolate a few clones that survived this treatment. The progeny of these clones retain their glut$^-$ phenotype but form again heat resistant colonies at a frequency of about $1/10^5$ cells after several successive isolations. This frequency of survival is more than 100 times higher than that obtained by heating cells of $Sp^-_{(3)}$ strains under the same conditions.

These results rather indicate that the aconitase$^-$ mutants are genotypically Sp^+ and their inability to sporulate may be the result of a physiological disturbance in the events occuring during the sporulation process. The block in the tricarboxylic acid cycle appears to be the cause of this disturbance. The evidence reported here indicate that in a sporogenic strain the operation of the tricarboxylic acid cycle is indispensible for the expression of the spore genes.

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- BIBLIOGRAPHY -

Hanson R.S., Srinivasan V.R. and Halvorson H.Orin, Biochem. Biophys. Res. Com. 5,457,1961.
Hanson R.S., Srinivasan V.R. and Halvorson H.Orin, J; Bact. 86,45;1963.

Szulmajster J. and Schaeffer P., C.R. Acad. Sc., 252,220,1961.
Halvorson H.Orin, J.Appl.Bact., 20,305,1957.
Nakata H.M. and Halvorson H.Orin, J. Bact. 80,801,1960.
Ramos F., Wiame J.M., Wynants J. and Bechet J., Nature,193, 70,1962.