

MUTANTS OF *STREPTOCOCCUS FAECALIS* CONCERNING PYRUVATE DEHYDROGENATION

Akio YAMAZAKI, Kiyoshi WATANABE, Yushi NISHIMURA and Teijiro KAMIHARA

*Laboratory of Industrial Biochemistry, Department of Industrial Chemistry,
Faculty of Engineering, Kyoto University, Kyoto, Japan*

Received 28 February 1976

1. Introduction

The conversion of pyruvate into acetyl-CoA or acetate is recognized to comprise several separate pathways in bacteria. *Escherichia coli* has three pathways; pyruvate dehydrogenation complex (PDH) [EC 1.2.4.1], pyruvate oxidase [EC 1.2.2.2] and pyruvate formate-lyase (PFL). In *Streptococcus faecalis*, PDH is widely accepted to mediate the breakdown of pyruvate to acetyl-CoA [1–3]. PFL is also suggested to occur in this organism under certain conditions on the basis of formate accumulation in the culture [4–7]. Lindemark et al. [8] purified extensively a pyruvate-formate exchange system from this organism, which was believed to be related closely with PFL. In addition, we obtained data suggesting the existence of a lipoic acid-independent pyruvate dehydrogenating system in the cells grown in the presence of ascorbic acid or FeSO₄ [9].

The physiological significance of the existence of these separate pathways for pyruvate dehydrogenation remains obscure. Mutants may provide a useful tool for clarifying this problem.

This communication deals with the isolation of mutants of *S. faecalis* concerning pyruvate dehydrogenation. Partial characterization of the ascorbic acid-induced system and the occurrence of PFL activity are also described.

2. Materials and methods

Organisms: All mutant strains used in this investigation were derived from *S. faecalis* 10C1 (ATCC 11 700).

Cultivation of these strains in a chemically defined medium was carried out, as described previously [9], with supplement of 2 mM sodium acetate, 1 nM DL-lipoic acid or 5 mM ascorbic acid. Galactose (0.5%) was used, when indicated, as carbon source in place of glucose.

Chemicals: All chemicals used were analytical grade.

Mutant selection: Cells collected from a 5 ml exponentially growing culture with sodium acetate were washed with 0.033 M potassium phosphate buffer (pH 7.0) and suspended in 5 ml of the same buffer containing 0.5 mg of *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine. The cell suspension was incubated at 37°C for 30 min. After washed twice with the same buffer, the cells were transferred to the acetate medium and grown to two-third maximal turbidity. At this point, the cells were collected and washed. Penicillin screening was then carried out in the basal medium supplemented with lipoic acid or ascorbic acid. Penicillin G (potassium salt) was added to a final concentration of 1000 units per ml and the incubation was continued for 3 hr. The cells were collected by centrifugation, washed and resuspended in the above buffer. Appropriately diluted aliquots were plated on agar containing the acetate medium. These plates were incubated overnight at 37°C and the resulting colonies were tested for the requirement for growth.

Assay for PDH complex: The PDH complex of cell-free extracts prepared as described previously [10] was assayed according to the method of Leach et al. [1].

Assay for lipoic acid-independent pyruvate dehydrogenation activities: Cells grown with ascorbic acid on glucose or galactose were collected, washed and

suspended in 0.033 M potassium phosphate buffer (pH 7.0). The reaction mixture contained 3.4 mmol of potassium phosphate buffer (pH 7.0), 2.1 mmol of sodium pyruvate and 2 g (wet weight) of cells in a total volume of 50 ml. The reaction was carried out at 37°C for 8 hours in an atmosphere of helium. After centrifugation, the resulting supernatant was analyzed for formate and acetate formed.

Analysis of formate and acetate: To the above supernatant was added a solution of perchloric acid in a final concentration of 5%. The mixture was made basic with KOH, then centrifuged. The resulting supernatant was concentrated and extracted with 30 vol of ethylether at pH 1.0. After removal of the ether, methylation was done with a BF_3 -methanol solution at 20°C in a sealed container for 1 h. The methylated acids were analyzed by gasliquid chromatography (GLC) on a Yanagimoto 550T Gaschromatograph equipped with a hydrogen flame ionization detector (165°C) with helium as carrier gas at the flow rate of 17–20 ml per min, by using a glass column (4 mm \times 150 cm) packed with 10% diethylene glycol adipate polyester and 2% H_3PO_4 adsorbed on Chromosorb 101 (60–80 mesh). Initial column temperature was 88°C and the temperature was programmed so as to increase at the rate of 2°C per min. Peak area was determined by triangulation and the amount of acid ester was estimated from GLC tracings with *n*-butyric acid as internal standard.

Protein determination: Protein concentration of the cell-free extracts was measured by the method of Lowry et al. [11].

3. Results

3.1. Growth patterns

Growth characteristics of the parental strain and mutants are shown in table 1. All mutants tested were able to grow exponentially on glucose or galactose with sodium acetate. It was possible to assign all the mutants to one of three arbitrarily chosen groups (A to C). Group A mutants (No. 3 and B-2) were able to grow on glucose with ascorbic acid but not with lipoic acid. On the contrary, group B mutants (B-40 and H-1-12) were permitted to grow with lipoic acid but not with ascorbic acid. Group C mutants (G-97 and H-1-9) could utilize neither lipoic acid nor ascorbic acid. Only acetate sustained the growth of this group of mutants under the conditions described above.

3.2. PDH activity

Table 2 shows a close correlation between the activity of PDH and the lipoic acid-dependent growth. The cell-free extracts of the group B mutants (B-40 and H-1-12) which were able to grow with lipoic acid exhibited the PDH activity comparable to that of the parental strain. The involvement of NAD and lipoic acid in the reaction was confirmed. On the other hand, the PDH activity was not observed in the extracts of the group A mutants (B-2 and No. 3) which were unable to grow with lipoic acid.

3.3. The existence of lipoic acid-independent pathways of pyruvate dehydrogenation

In previous reports [9,10,12,13], it was demonstrat-

Table 1
Growth characteristics of the parental and mutant strains

Additions to the growth medium	Strains						
	Parental	Group A ^a		Group B ^a		Group C ^a	
		No. 3	B-2	B-40	H-1-12	G-97	H-1-9
Lipoic acid (1 nM)	+	—	—	+	+	—	—
Ascorbic acid (5 mM)	+	+	+	—	—	—	—
Na-acetate (2 mM)	+	+	+	+	+	+	+

Organisms were grown as described in Materials and methods. Symbols: + and —, represent the bacterial growth after incubation at 37°C for 24 h.

^a See the text.

Table 2
The activity of PDH complex in cell-free extracts of the
parental and mutant (group A and B) strains

System	Strains				
	Parental	Group A		Group B	
		No. 3	B-2	B-40	H-1-12
Complete	7.7	0.67	0.37	15	12
Minus NAD	0.57	0.55	0.57	0.90	0.72
Minus lipoic acid	0.57	0.63	0.57	0.57	0.57

The enzyme activity was measured as described in Materials and methods with the indicated omissions from the reaction mixture. The values represent specific activities of the enzyme (nmoles of acetyl phosphate formed/min/mg protein).

ed that pyruvate dehydrogenation leading to lipid biosynthesis would be essential for the growth of this organism in a synthetic medium lacking lipid substances and their precursors such as acetate or ethanol. It is, therefore, of interest to investigate the metabolism of pyruvate in the group A mutants. As described above, these mutants were able to grow with ascorbic acid but not with lipoic acid in the lipid-deficient medium. This may imply that there is an alternate pathway(s) for pyruvate dehydrogenation in the cells grown with ascorbic acid. This possibility was hence examined using cell suspension of strain B-2, one of the group A mutants. Fig.1 shows the results of GLC analysis of the products obtained by incubation of pyruvate in the cell suspension. The parental strain and strain B-2 grown on galactose with ascorbic acid gave peaks corresponding to formate and acetate (fig.1 A, C), indicating the presence of PFL activity in these cells. On the contrary, the production of formate was not proved in the cells grown on glucose (fig.1 B, D). This result suggests that the appearance of PFL may be under the influence of glucose repression.

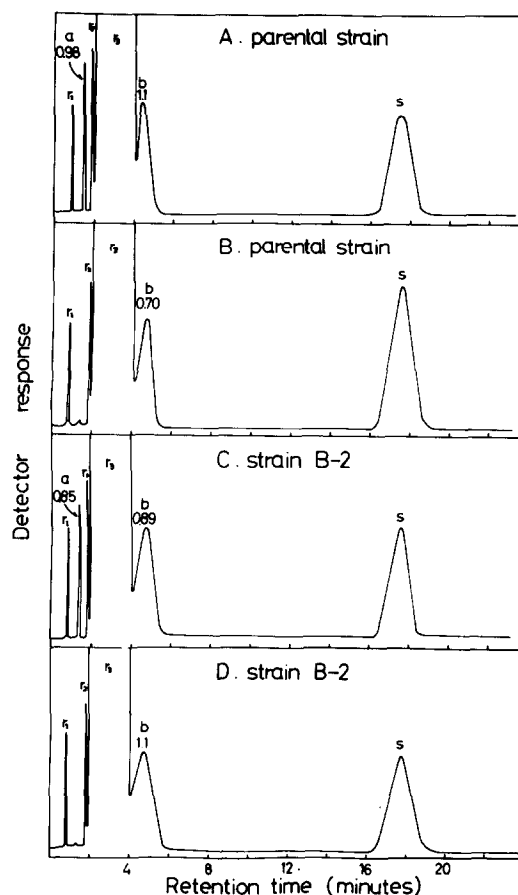


Fig.1. Gas-liquid chromatograms of methyl esters of formate and acetate produced from pyruvate by the parental and B-2 strains. The organisms were grown on glucose (A, C) or on galactose (B, D) with ascorbic acid. GLC analysis was carried out as described in Materials and methods. The methyl esters are marked as follows: (a) formate, (b) acetate, and (s) *n*-butyrate (internal standard). The amount of acids (mmoles) are indicated below the identifying letters. Peaks (r_1), (r_2) and (r_3) represent reagent blanks.

The possibility of the decomposition of formate once produced is ruled out, since it was confirmed manometrically that sodium formate added to the cell suspension was not decomposed.

In addition, the above results suggest the existence of an alternate enzyme system which would be insensitive to the glucose repression and catalyze the conversion of pyruvate to acetate (or acetyl-CoA) without concomitant production of formate. The cells growing with ascorbic acid under derepressed conditions would utilize either PFL or this alternate pathway for the dehydrogenation of pyruvate.

To confirm that PFL undergoes a glucose repression, an attempt was made to isolate a mutant which was able to grow with ascorbic acid only under derepressed conditions. A mutant strain, No. 109, was isolated to meet this requirement. The strain was able to grow with lipoic acid and sodium acetate in either glucose or galactose medium, whereas ascorbic acid permitted the growth to occur only when galactose was used in place of glucose (fig.2). As judged by GLC analysis, the production of formate from pyruvate was confirmed in the cells of this strain grown with ascorbic acid on galactose (fig.3). The results of fig.2 and 3 clearly demonstrate that PFL functions and hence supports the bacterial growth under derepressed conditions. The failure to grow with ascorbic acid on glucose can be account for by the absence of the alternate enzyme system in this mutant strain.

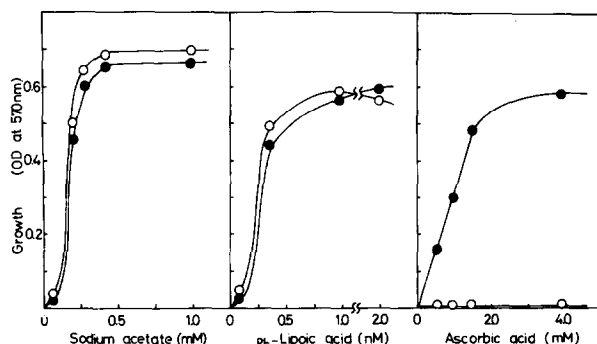


Fig.2. Growth of strain No. 109. Cultivation was carried out on glucose (—○—○—) or on galactose (—●—●—) as described in Materials and methods.

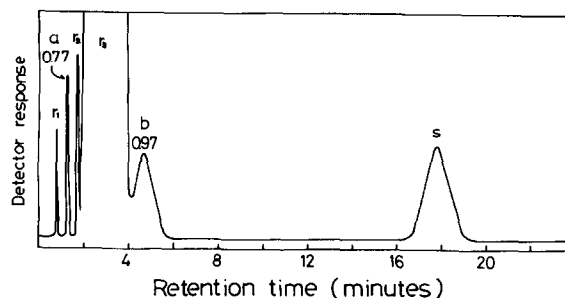


Fig.3. Gas-liquid chromatograms of methyl esters of formate and acetate produced from pyruvate by strain No. 109. The organism was grown on galactose with ascorbic acid. GLC analysis was made as described in Materials and methods. Symbols are the same as in fig.1.

4. Discussion

As mentioned above, we have already pointed out that there would exist a pyruvate dehydrogenase other than PDH complex in *S. faecalis* grown in the presence of ascorbic acid. The results of this study using mutants would provide unambiguous evidence for the existence of PFL and another pyruvate dehydrogenating system. Studies are now in progress on the interrelationship and mutual regulation among PDH and these two enzymes. The use of mutants concerning the individual pathways reported here may be essential for these studies.

The appearance of PFL and the third enzyme system was undoubtedly caused by the addition of ascorbic acid to the growth medium. However, ascorbic acid was not specific in this ability; it could be replaced by FeSO_4 . The addition to the medium of these substances caused a marked decrease in oxidation-reduction potential (ORP) as we reported previously [9]. It would be hence deduced that a lowering of ORP of the culture is responsible for the appearance of the activities of these enzymes.

Several reports have suggested that existence of PFL activity in *S. faecalis* as mentioned above. In our investigation, more definitive evidence was presented by identifying formate as product of pyruvate dehydrogenation in resting cells.

The third enzyme would not be classified into any type of pyruvate dehydrogenase reported hitherto.

The enzyme would be distinguishable from the pyruvate oxidase in the lack of participation of oxygen in the reaction. This enzyme and PFL appear to have some fundamental significance in anaerobic metabolism since it can be assumed as mentioned above that a lowered ORP of culture would result in the appearance of the enzymes, although PFL may function only under derepressed conditions.

Acknowledgment

We wish to express our deep gratitude to Prof. Saburo Fukui for his guidance and continuous encouragement during the course of this investigation.

References

- [1] Leach, F. R., Yasunobu, K. and Reed, L. J. (1955) *Biochim. Biophys. Acta* 18, 297–298.
- [2] Reed, L. J., Leach, F. R. and Koike, M. (1958) *J. Biol. Chem.* 232, 123–142.
- [3] Gunsalus, I. C. and Razzell, W. E. (1957) in: *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 3, pp. 941–946, Academic Press, N.Y.
- [4] Gunsalus, I. C. and Niven, C. F. (1942) *J. Biol. Chem.* 145, 131–136.
- [5] Campbell, J. J. R. and Gunsalus, I. C. (1944) *J. Bacteriol.* 48, 71–76.
- [6] Deibel, R. H. and Niven, C. F. Jr. (1960) *J. Bacteriol.* 88, 4–10.
- [7] Fukuyama, T. T. and O'Kane, D. J. (1962) *J. Bacteriol.* 84, 793–796.
- [8] Lindemark, D. G., Paolella, P. and Wood, N. P. (1969) *J. Biol. Chem.* 244, 3605–3612.
- [9] Yamazaki, A., Kamihara, T. and Fukui, S. (1972) *J. Agr. Chem. Soc. Japan* 46, 21–26 (in Japanese).
- [10] Kamihara, T. (1969) *Arch. Biochem. Biophys.* 133, 137–143.
- [11] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275.
- [12] Kamihara, T. (1969) *J. Bacteriol.* 97, 151–155.
- [13] Kamihara, T., Yamazaki, A., Yamaoka, K. and Fukui, S. (1969) *J. Agr. Chem. Soc. Japan* 43, 159–164 (in Japanese).