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Utilization of electron acceptors for anaerobic mannitol metabolism by *Lactobacillus plantarum*. Compounds which serve as electron acceptors†

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In a chemically defined medium under anaerobic conditions, Lactobacillus plantarum *grew on glucose, but was unable to grow with mannitol as the carbon source. Mannitol was a suitable carbon source, provided 1 of 11 compounds (pyruvic acid, α-ketobutyric acid, α-ketovaleric acid, α-ketocaprylic acid, acetic acid, acetyl phosphate, acetaldehyde, citric acid, oxaloacetic acid, malic acid, and fumaric acid) was added to the medium as an electron acceptor. The extent of mannitol fermentation was proportional to the concentration of available electron acceptor. The reduced products of the electron acceptor compounds were identified and possible pathways for the metabolism of electron acceptors were proposed. Strains from two other species of lactobacilli which ferment mannitol appear to use different electron acceptor pathways.*

Introduction

Mannitol is a major end product of heterolactic acid fermentations of vegetables such as cabbage (Pederson and Albury 1969) and green beans (Chen et al. 1983a). According to Bergey's Manual (Buchanan and Gibbons 1974), mannitol can be used as a carbon source by eight species of lactobacilli, including *L. plantarum*, the species which generally predominates at the terminal stages of most natural lactic acid fermentations (Pederson and Albury 1969). Based upon limited analytical data, it has been reported that mannitol accumulates early in the sauerkraut fermentation, but it then declines as the *L. plantarum* population builds up (Pederson and Albury 1969). Chen et al. (1983b) found that when *L. plantarum* was inoculated into pH 3·9 green bean juice, which had been first fermented by the heterofermentative organism, *L. cellobiosus*, the accumulated mannitol declined. Accompanying the fermentation of mannitol, a decline in acetic acid and an increase of ethanol was observed. These changes suggested that some acetic acid may have been reduced to ethanol during fermentation.

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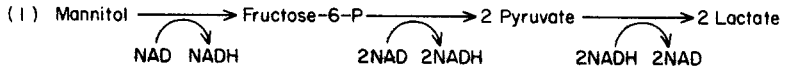
L. plantarum can grow aerobically with mannitol as a carbon source, but not in an anaerobic atmosphere (Brown and VanDemark 1968). This behaviour presumably arises from the fact that NADH will accumulate because 3 mols of NADH are formed by converting mannitol to lactic acid via the glycolytic pathway; however, only 2 mols can be reoxidized anaerobically (Equation 1).



The observation that acetic acid might be converted to ethanol during mannitol fermentation led us to consider whether the acetic acid may serve as an electron acceptor in green bean juice in order for mannitol fermentation to occur (Chen et al. 1983b). It also led us to search for other compounds which might be utilized as electron acceptors by *L. plantarum*.

The objectives of this investigation were: (1) to identify compounds which function as electron acceptors during anaerobic mannitol metabolism by *L. plantarum*; (2) to identify the products formed from the electron acceptors, and (3) to determine quantitative relationships between mannitol fermentation and utilization of acceptors.

Materials and Methods

Organisms

Lactobacillus plantarum (WSO) from this laboratory's culture collection was used in all experiments. Other organisms tested for their ability to utilize certain electron acceptors were: *L. plantarum* C11 from this laboratory, *L. plantarum* ATCC 14917 from the American Type Culture Collection, and *Lactobacillus casei* ssp. *pseudoplantarum* B–4560, *Lactobacillus coryneformis* ssp. *coryneformis* B–4391 and *Lactobacillus coryneformis* ssp. *torquens* B–4390 from the USDA-ARS Northern Regional Research Centre culture collection, Peoria, Illinois.

Growth medium

A chemically defined basal medium was prepared from the five 100-fold concentrated stock solutions listed in Table 1. To the basal medium a carbon source (55·5 mM glucose or mannitol) and a potential electron acceptor (10 mM except as noted) were added. After the pH was adjusted to 6·8 with KOH or HCl, the medium was sterile-filtered through a 0·22 μ Millex filter (Millipore Corporation, Bedford, MA, USA) into sterile test tubes.

Growth conditions

Lactobacillus plantarum was grown anaerobically at 30°C in the defined basal medium with glucose. A BBL Gas Pak (Betcon Dickinson and Co., Cockeysville, MD, USA) with a platinum catalyst was used to produce an anaerobic atmosphere which contained nitrogen, carbon dioxide and hydrogen. A 12-h culture was centrifuged, washed twice with sterile saline (30 ml), and the cell pellet was suspended in sterile saline. For all experiments, each tube was inoculated with washed cells to give 10^6 cfu ml^{-1} and incubated for 2 weeks in an anaerobic jar (Chen et al. 1983a). Duplicate tubes were fermented and analyzed separately in all experiments.

Analysis

Quantitative HPLC analysis of sugars, organic acids, and ethanol was done by the procedure of McFeeters et al. (1984).

Identification of reduced products of electron acceptors

Products formed from the added electron acceptors were identified by comparison of retention times with the retention times of authentic compounds on a C_{18} reversed phase HPLC column (Techsil μ 5 C_{18}, Phenomenex, Rancho Palos Verdes, CA, USA) and on an ion exchange HPLC column (HPX-87H, Bio-Rad Labs., Richmond, CA, USA). The identity of ethanol was also confirmed by gas chromatography using the procedure described by McFeeters and Armstrong (1984) for methanol analysis.

Table 1. Composition of a defined basal medium for determination of electron acceptors in mannitol fermentation[a].

Component	100X Concentration $g\,l^{-1}$	Final concentration in basal medium (mM)
Solution I		
$(NH_4)_2SO_4$	200·0	15·14
$MgSO_4 \cdot 7\,H_2O$	15·0	0·61
$MnSO_4 \cdot 4\,H_2O$	2·0	0·09
$FeSO_4 \cdot 7\,H_2O$	1·0	0·04
Solution II		
Adenine	0·5	$3·7 \times 10^{-2}$
Pyridoxal	0·2	$1·2 \times 10^{-2}$
Nicotinic acid	0·1	$8·1 \times 10^{-3}$
Ca-D-pantothenate	0·1	$2·1 \times 10^{-3}$
Riboflavin	0·1	$2·7 \times 10^{-3}$
Thiamine	0·1	$3·0 \times 10^{-3}$
Vitamin B_{12}	$1·0 \times 10^{-4}$	$7·4 \times 10^{-7}$
Biotin	$1·0 \times 10^{-3}$	$4·1 \times 10^{-5}$
p-Aminobenzoic acid	$5·0 \times 10^{-4}$	$3·6 \times 10^{-5}$
Folic acid[b]	$1·0 \times 10^{-3}$	$2·3 \times 10^{-5}$
Solution III[c]		
Isoleucine	2·0	0·15
Leucine	2·0	0·15
Tyrosine	1·0	0·06
Aspartic acid	7·5	0·56
Tryptophan	2·0	0·10
Methionine	1·5	0·10
Phynylanine	2·0	0·12
Histidine	5·0	0·32
Serine	4·0	0·38
Valine	3·0	0·26
Glutamic acid	7·5	0·51
Alanine	3·0	0·34
Arginine	4·0	0·23
Lysine	5·0	0·34
Proline	5·0	0·43
Threonine	2·0	0·17
Cysteine	2·0	0·17
Solution IV		
Guanine[b]	0·50	0·03
Uracil	0·50	0·05
Xanthine	0·50	0·03
Solution V		
K_2HPO_4	456·0	26·20

[a] The synthetic medium was formulated based upon the media described by Garvie (1967) and Ledesma et al. (1977). The 100× stock solutions were dispensed into small test tubes (5·0 ml $tube^{-1}$) and frozen until use. The frozen solutions have been stored for 3 months without noticeable effect on the growth of *L. plantarum*.

[b] Folic acid and guanine do not dissolve completely in their respective stock solutions. Therefore, these solutions must be stirred as they are dispensed.

[c] Due to the limited solubility of some amino acids in the 100× stock solution, the amino acids must be added into water containing 0·2 N HCl in the order listed to all be completely dissolved.

Chemicals

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA), except as indicated. Mannitol was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). The following compounds were tested as possible electron acceptors for *L. plantarum*: citric acid, isocitric acid, oxalosuccinic α-ketoglutaric acid, succinic acid, fumaric acid, malic acid, oxaloacetic acid, formic acid, acetic acid (Fisher Scientific Company, Fairlawn, NJ, USA), propionic acid, α-ketobutyric acid, α-ketovaleric acid (ICN Pharmaceuticals, Inc., Plainview, NY, USA), α-ketocaprylic acid (ICN), acetoacetic acid, acetone, dehydrochloric acid, acetyl phosphate, acetaldehyde (Fisher), N-acetyl-L-glutamic acid, adipic acid, ascorbic acid, benzaldehyde, benzoic acid, crotonic acid, cysteine, galacturonic acid, gluconolactone, glutamic acid, glutaric acid, lanosterol, maleic acid, methionine, nicotanamide adenine dinucleotide, oxamic acid, sodium nitrate, sodium nitrite, tartaric acid, thioglycolic acid, and yellow No. 5.

Results

Identification of electron acceptors

A defined medium was used which supported growth of *L. plantarum* on glucose, but in which no detectable growth occurred when mannitol was used as the carbon source. Compounds were initially identified as electron acceptors for mannitol fermentation by the fact that *L. plantarum* showed visible growth when an appropriate compound was added with mannitol in the medium. Among the 46 compounds tested as possible electron acceptors, 11 presumptive acceptors were identified. Four of the compounds were TCA cycle intermediates (citric acid, fumaric acid, malic acid, and oxaloacetic acid). In addition, acetic acid, acetyl phosphate, acetaldehyde, and four α-keto acids (pyruvic α-ketobutyric, α-ketovaleric, and α-ketocaprylic) allowed the fermentation of mannitol. With glucose as a carbon source, *L. plantarum* grew when each of the 46 compounds was added to the defined medium. This result indicated that the lack of growth in the presence of mannitol for 35 of the compounds was not caused by specific toxic effects of the compounds on the organism.

Metabolism of glucose and mannitol with electron acceptors

Two mols of lactic acid were formed when 10 of the 11 presumptive electron acceptors was added to the growth medium containing either glucose or mannitol. More than 2 mols of lactic acid were found when oxaloacetate was added as an acceptor.

The effect of acceptor concentration on the amount of mannitol fermented was studied for acetic acid. The amount of mannitol fermented was directly related to the amount of acetate added to the medium over a concentration range of 5 to 25 mM (Fig. 1.) As indicated by the slope of the line, 2 mols of mannitol were fermented per mol of acetate utilized. Since 55·5 mM mannitol was available for fermentation in each tube, the mannitol fermentation appeared to be strictly limited by acetate availability.

Table 2 shows the relationship between

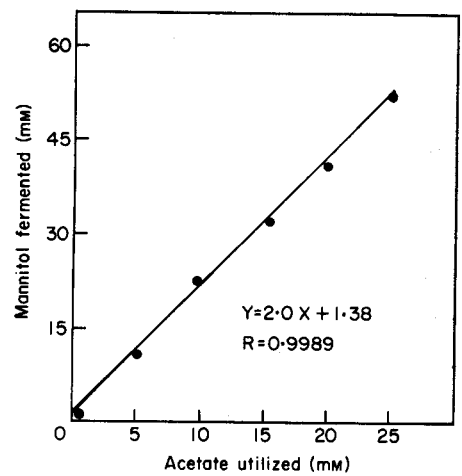


Fig. 1. Relationship between acetic acid utilization and the amount of mannitol fermented by *L. plantarum*.

Table 2. Relationship among electron acceptor utilization, mannitol utilization and electron acceptor product formation by *L. plantarum*.

Electron acceptor	Electron acceptor concentration (mM)	Products formed	Product concentration (mM)	Mannitol used (mM)	Mannitol used / acceptor used
Pyruvic acid	10·0	Lactic acid	10·2	10·40	1·04
α-Ketobutyric acid	10·0	α-Hydroxybutyric acid	9·8	10·70	1·07
α-Ketovaleric acid	5·0	-[a]	–	4·82	0·96
α-Ketocaprylic acid	5·0	-[a]	–	4·72	0·95
Oxaloacetic acid	10·0	Lactic acid; ethanol	5·9; 3·9	14·10	1·41
Malic acid	10·0	Succinic acid	10·6	9·57	0·96
Fumaric acid	10·0	Succinic acid	10·2	9·90	0·99
Citric acid	10·0	Succinic acid; ethanol	9·8; 10·4	39·30	3·93
Acetic acid	10·0	Ethanol	10·8	20·80	2·08
Acetyl phosphate	10·0	Ethanol	10·7	20·32	2·03
Acetaldehyde	6·9	Ethanol	7·01	8·00	1·16

[a] Products from these compounds were not observed by HPLC chromatography.

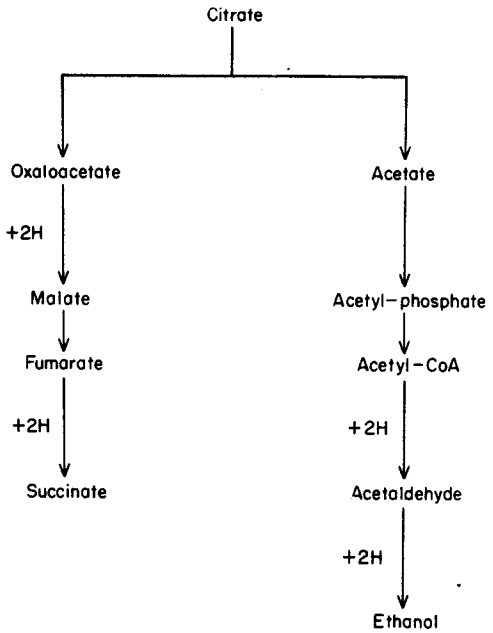


Fig. 2. Proposed pathway for citrate utilization during mannitol fermentation.

the amount of each electron acceptor added to the defined medium and the amount of mannitol fermented during a 2-week incubation period. The results indicated that the ratio of the mols of mannitol fermented per mol of electron acceptor added was close to a small whole integer in every case, except oxaloacetic acid. When the α-keto acids (pyruvic, α-ketobutyric, α-ketovaleric, and α-ketocaprylic) were added, 1 mol of mannitol was fermented for each mol of electron acceptor. A ratio close to 1·0 was also observed for malic acid, fumaric acid, and acetaldehyde. When acetic acid and acetyl phosphate were added to the medium, 2 mol of mannitol were fermented per mol of electron acceptor. Finally, 4 mols of mannitol were fermented per mol of citric acid added.

Identification of the products of acceptor metabolism

Table 2 shows both the products and quantity of the products formed from each acceptor during mannitol fermentation. *L. plantarum* did not produce these products, with the exception of lactic acid, either from glucose under anaerobic conditions or from mannitol in the presence of air. Quantitative product formation was observed in each case where authentic compounds were available for comparison. Formation of lactic acid from pyruvate was assumed because 3 mols of lactic acid were formed for each mol of mannitol fermented when pyruvate was added to the medium. α-Hydroxybutyric acid was produced from α-ketobutyric acid. Products from the metabolism of α-ketovaleric acid and α-ketocaprylic acid were not identified. Both malic acid and fumaric acid were quantitatively converted to succinic acid. Acetic acid, acetyl phosphate and acetaldehyde all were quantitatively reduced to ethanol. On a molar basis, citric acid was quantitatively converted to succinic acid and ethanol. Oxaloacetic acid was the only compound not converted to an equimolar amount of a product. However, the sum of lactic acid and ethanol was 9·8 mM compared to 10·0 mM oxaloacetate added to the medium.

Acceptor utilization with other lactobacilli

Two other strains of *L. plantarum*, the type strain and a strain recently isolated from a cucumber fermentation, showed the same pattern of metabolism for citrate, malate and acetate as WSO. On the other hand, *L. casei* ssp. *pseudoplantarum*, *L. coryniformis* ssp. *coryniformis*, and *L. coryniformis* ssp. *torquens* all grew anaerobically on mannitol without the addition of an electron acceptor. These organisms produced ethanol with or without the addition of an external electron acceptor. None of them produced succinate from citrate or malate.

Discussion

L. plantarum was previously found to grow with mannitol as a carbon source in the presence of oxygen, but not in the absence of oxygen (Brown and VanDemark 1968). These results confirmed these observations and showed that at least 11 compounds in addition to oxygen could serve as electron acceptors for the organism during mannitol fermentation. These compounds fall into three groups: α-keto acids; intermediates found in the heterofermentative pathway, such as occurs in the heterofermentative lactic acid bacteria; and citric acid cycle intermediates.

A requirement for an external electron acceptor to support mannitol fermentation has previously been reported for other organisms, including *Streptococcus faecalis* (Gunsalus 1947), *Clostridium lacto-acetophilum* (Bhat and Barker 1947) and *L. casei* (Brown and VanDemark 1968). However, strains of *L. casei*, which do not require an external electron acceptor, have been found by DeVries et al. (1970) and in this study. Compounds found to serve as electron acceptors during mannitol fermentation for these organisms include: acetic acid for *C. lacto-acetophilum* (Bhat and Barker 1947), fumarate for *S. faecalis* (Gunsalus 1947) and acetic acid, pyruvic acid, methylene blue, potassium ferricyanide, and 2,6-dichlorophenol indophenol for *L. casei* (Brown and VanDemark 1968, DeVries et al. 1970).

The specificity of electron acceptor utilization in *L. plantarum* was indicated by the inability of the organism to utilize structural analogs of several acceptors. For example, only acetic acid, among the aliphatic carboxylic acids from C_1 to C_8, supported mannitol fermentation. Four α-ketocarboxylic acids tested were electron acceptors, but α-ketoglutaric acid, acetone and acetoacetic acid did not allow the organism to ferment mannitol. The inability of the organism to utilize maleic acid , the cis-isomer of fumaric acid, also indicates a high degree of specificity in the metabolism of electron acceptors. At present, there is no evidence for the existence of a relatively nonspecific electron acceptor system in *L. plantarum* which can utilize a broad range of compounds to reoxidize NADH under anaerobic conditions.

In the presence of the 11 electron acceptors studied, *L. plantarum* quantitatively fermented 1 mol of glucose and mannitol to 2 mols of lactic acid, except oxaloacetate. This indicated that energy was produced by the glycolytic pathway when mannitol was fermented. Also, reactions which result in the formation of products other than lactate from pyruvate were minimal or absent. Oxaloacetic acid is unstable in solution (Wilcock and Goldberg 1972). It was degraded into pyruvate and other unknown compounds in the defined medium at 30°C (data not shown). Presumably, pyruvate formed from oxaloacetate was reduced to lactic acid. This might be the reason for more than 2 mols of lactic acid produced per mol of mannitol degraded.

Two α-keto acids, pyruvic acid and α-ketobutyric acid, were quantitatively reduced to α-hydroxy acids (Table 2). The reduction of pyruvic acid to lactic acid was inferred from the fact that 30 mM of lactic acid was produced when 10 mM of mannitol and pyruvic acid were metabolized. Purified lactate dehydrogenase (LDH) from *L. plantarum* can reduce both pyruvic acid and α-ketobutyric acid (Dennis and Kaplan 1960). LDH from various muscle tissues can catalyze the NAD-dependent reduction of both α-ketovaleric acid and α-ketocaprylic acid (Meister 1950), but the ability of *L. plantarum* to utilize these substrates has not been determined. Recently, Schutte et al. (1984) reported that L-2-hydroxyisocaproate dehydrogenase from

Lactobacillus confusus could catalyze the NAD-dependent reduction of several α-keto acids, including α-ketovaleric acid. Therefore, it is possible that *L. plantarum* contains enzymes other than LDHs, which catalyze the reduction of α-keto acids other than pyruvate. The enzymes responsible for the reduction of α-keto acids will be discussed in a subsequent paper.

If we assume that α-keto acids are reduced by LDH or other enzymes specific for α-keto acids, the other electron acceptors found in this study can be organized into the proposed pathway shown in Fig. 2. With the exception of the result obtained when oxaloacetic was added as an acceptor, this sequence of reactions can explain both the products formed from each acceptor and the ratio of mannitol fermented per mol of acceptor metabolized. For example, 2 mols of NADH, would be oxidized to NAD on each branch of the pathway from citric acid. This can account for fermentation of 4 mols of mannitol per mol of citric acid. We believe that oxaloacetate formed within the cell from citrate would be metabolized via these reactions. However, the instability of this compound in solution probably resulted in its decomposition before entering the cell when it was added to the medium. Experiments to provide further evidence to support the proposed pathway for reduction of the electron acceptors identified herein will be presented in a subsequent paper

Strains from two other species of lactobacilli, *L. casei* and *L. coryniformis*, which utilize mannitol as a carbon source and which were able to grow in the defined medium used in this study, appear to have different electron acceptor capabilities than *L. plantarum* since they grew anaerobically on mannitol without an electron acceptor. DeVries et al. (1970) previously found that *L. casei* produced lactate, acetate, ethanol, and formate from mannitol under anaerobic conditions without addition of an acceptor. Neither organism produced succinate from either malate or citrate, indicating that the sequence of reactions for succinate formation shown in Fig. 1 is not present.

On the other hand, two other strains of *L. plantarum* were found to be like the WSO strain in that they were unable to grow on mannitol in the absence of an added acceptor, and they produced the same fermentation products when citrate, malate and acetate were added to the mannitol medium. This suggests that the proposed acceptor pathways may be characteristic for *L. plantarum* and that *L. plantarum* is different in its electron acceptor capabilities than some other mannitol-utilizing lactobacilli. This is significant in food fermentations since *L. plantarum* is the usual terminal organism in natural lactic acid fermentations. In cases such as sauerkraut fermentations where mannitol is formed during the period when heterofermentative organisms dominate the fermentation, it would be expected that some ethanol would be produced by *L. plantarum* at the expense of acetic acid as it ferments mannitol. If citrate were a major organic acid in the fermented food, substantial amounts of succinic acid, as well as ethanol, could be formed. Malic acid could also be converted to succinic acid, but in natural fermentation it could also be decarboxylated by a malolactic enzyme to lactic acid and CO_2. Thus, if the proposed pathways are confirmed, it will help to better understand the metabolism of organic acids in food fermentations.

Acknowledgements

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