Mutation and Selection of *Lactobacillus plantarum* Strains That Do Not Produce Carbon Dioxide from Malate†

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A differential medium was developed to distinguish between malate-decarboxylating (MDC^+) and -non-decarboxylating (MDC^-) strains of *Lactobacillus plantarum*. MDC^- strains produced a visible acid reaction in the medium, whereas MDC^+ strains did not. Use of the medium allowed for rapid screening and isolation of mutagenized cells that had lost the ability to produce CO_2 from malate.

Bloating of brine-fermented cucumbers has been attributed to the accumulation of CO_2 and other gases within the cucumbers (7). Fermentative yeasts (3), members of the *Enterobacteriaceae* (5), and heterolactic acid bacteria (4) that occur during fermentation have been implicated as major sources of CO_2. Fleming et al. (9, 10) found that CO_2 is also formed by brined cucumber tissue and by the homofermentative *Lactobacillus plantarum* present during the fermentation of brined cucumbers. The combination of these two sources of CO_2 caused significant bloating even in controlled fermentations in which other microorganisms were excluded. McFeeters et al. (14, 15) found that malate is the major organic acid in pickling cucumbers and that malate decarboxylation can account for most of the CO_2 produced during fermentation of cucumber juice by *L. plantarum*. Schultz and Radler (17) showed that *L. plantarum* has an active malolactic enzyme which decarboxylates malate to lactate and CO_2 according to the reaction 1 malate + $H^+ \rightarrow$ 1 lactate + 1 CO_2. These results suggest that malate decarboxylation is an important source of CO_2 in cucumber fermentations.

It would be desirable to obtain strains of *L. plantarum* which lack the ability to produce CO_2 from malate, but which retain desirable characteristics, for use in cucumber fermentations. However, a simple selection system for the rapid screening of malate-non-decarboxylating (MDC^-) strains of *L. plantarum* is not available. Several methods have been used to test lactic acid bacteria for their ability to degrade malate. These entailed using tubed media with agar seals and observing CO_2 production from malate (12, 22), observing a pH increase in tubed media containing malate (22), and assaying for the disappearance of malate and the production of lactic acid with paper chromatography (2, 21) or high-pressure liquid chromatography (2a). These methods would not be practical for mutant screening or selection because of the large numbers of colonies that would have to be examined during routine mutagenesis. Recently, Subden et al. (19) introduced a plating medium for detecting microbial colonies performing a malolactic fermentation. The system is based upon the enzymatic detection of L-lactate, the decarboxylation product of L-malate. However, the method would only work with species (e.g., *Leuconostoc* spp.) that do not produce L-lactic acid from glucose (18). Glucose is necessary in the medium as an energy source since the malolactic reaction does not yield energy for growth (11, 13). *L. plantarum* produces DL-lactic acid from glucose (1), which would result in a false-positive reaction on Subden's medium if the strain was MDC^-.

We have developed a differential medium (Table 1) to select colonies of *L. plantarum* which do not produce CO_2 from malate. The key components of this medium, designated MD medium, are a pH indicator (bromocresol green; pH range, 3.8 to 5.4), a relatively low amount of glucose (0.5% [wt/vol]), which serves as an energy source, and a high concentration of malate (2.0% [wt/vol]). When malate is decarboxylated, there is uptake of a proton. Therefore, an *L. plantarum* strain that decarboxylates malate will neutralize the lactic acid produced by the fermentation of glucose so the pH will not decrease and the medium will remain dark blue. Strains which do not decarboxylate malate will decrease the pH of the medium because the lactic acid from glucose will not be neutralized. As a result, the medium will become yellow-green in color.

In developing the medium, we employed two strains of *L. plantarum*, WSO and 965, which are malate decarboxylating (MDC^+) and MDC^-, respectively. The MDC^- strain, *L. plantarum* 965, gave an acid reaction in broth medium, turning the color from dark blue to light green within 2 days at 30°C. On overlaid pour plates and streak plates, the MDC^- strain formed yellow-green halos around the colonies within 3 to 4 days at 30°C. The MDC^+ strain, *L. plantarum* WSO, did not change the color of broth or agar MD medium within the same time period or during prolonged incubation (21 days). Both the MDC^+ and the MDC^- strains gave an acid reaction to the medium when it was prepared without malic acid.

MD medium was used to detect mutants of *L. plantarum* which had lost the ability to decarboxylate malate. *L. plantarum* WSO was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the procedure of Miller (16) with the following modifications. Log-phase cells (4 h) grown in MRS broth (Difco Laboratories; Detroit, Mich.) were diluted with saline to give an optical density of 0.2 at 650 nm ($\approx 10^9$ CFU/ml). A nitrosoguanidine concentration of 500 μg/ml

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TABLE 1. Formulation and preparation of MD medium for the detection of MDC^- mutants of *L. plantarum*[a]

Component	Manufacturer	Amt per liter
L-Malic acid	Sigma Chemical Co.	20 g
Trypticase	BBL Microbiology Systems	10 g
D-(+)-Glucose	Sigma Chemical Co.	5 g
Casamino Acids	Difco Laboratories	3 g
Phytone	BBL Microbiology Systems	1.5 g
Yeast extract	Difco Laboratories	1.0 g
Tween 80	Atlas Chemical Industries	1.0 g
Bromocresol green	Fisher Scientific Co.	20 ml[b]
Agar (when desired)	Difco Laboratories	20 g

[a] Adjust the pH to 7.0 with 10 N KOH. Autoclave at 15-lb pressure for 15 min. Can be stored at room temperature.
[b] Stock solution (solubilize 0.1 g in 30 ml of 0.01 N NaOH).

with an exposure time of 90 min at 30°C was used. This condition consistently gave a 90% kill based on viability counts. After exposure, the cells were washed and resuspended in MRS broth for 3 h to allow for mutant expression before pour plating with MD medium. Colonies giving a positive reaction (yellow halos on blue background) were picked and tested for CO_2 production and for pH in MD broth. The results of these tests for two WSO mutants, the MDC^+ parent culture (WSO), and the MDC^- strain 965, are shown in Table 2. Each strain and mutant completely metabolized the glucose in the medium. The cultures giving acid reactions did not produce significant amounts of CO_2, indicating a loss in ability to decarboxylate malate. *L. plantarum* WSO, which did not give a color change, produced a large amount of CO_2. Strains WSO, WSO-M35, and 965, when inoculated into MD broth prepared without malic acid, gave acid reactions and did not produce significant amounts of CO_2.

The medium has proven to be accurate in distinguishing between MDC^+ and MDC^- strains of *L. plantarum*. In addition, it is useful for rapidly screening large numbers of mutagenized cells for variants that have lost the ability to produce CO_2 from malate. Such mutants may be useful as starter cultures in the fermentation of cucumbers and could reduce the need for purging of CO_2 from fermenting brines to prevent bloater damage, as is now common commercial practice (6).

The MDC^- strain of *L. plantarum* WSO, designated herein as M35, has been deposited with the Northern Regional Research Laboratory, Peoria, Ill. as NRRL B-15422, in accordance with the Budapest Treaty.

TABLE 2. pH reaction, CO_2 produced, and reducing sugar concentration in MD broth fermented by strains and mutants of *L. plantarum* at 30°C for 7 days

Strain	pH[a]	CO_2 (mg/100 ml)[b]	% Reducing sugar (wt/vol)[c]	Broth color
965[d]	5.19	16.9	<0.02	Light green
WSO[e]	6.82	596.6	<0.02	Dark blue
WSO-M34[f]	5.28	25.3	<0.02	Light green
WSO-M35[f]	5.23	18.9	<0.02	Light green
Uninoculated control	6.96	15.5	0.48	Dark blue

[a] Mean of two replicate cultures.
[b] Mean of three replicate cultures. Method of Fleming et al. (8).
[c] Mean of two replicate cultures. Method of Sumner and Somers (20).
[d] Obtained from the National Institute for Research in Dairying, Reading, England.
[e] Obtained from U.S. Department of Agriculture, Agricultural Research Service, Food Fermentation Laboratory culture collection.
[f] Mutants of WSO.

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