Food Microbiology, 1993, 10, 489–499

Evaluation of malolactic-deficient strains of *Lactobacillus plantarum* for use in cucumber fermentations

L. C. McDonald,* D.-H. Shieh, H. P. Fleming,† R. F. McFeeters and R. L. Thompson

Food Fermentation Laboratory, US Department of Agriculture, Agricultural Research Service, and North Carolina Agricultural Research Service, Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624, USA

Received 22 January 1991

In the fermentation of cucumbers, naturally occurring strains of Lactobacillus plantarum *decarboxylate malic acid (MDC$^+$) to form lactic acid and CO_2. Since CO_2 buildup in the brine contributes to bloater damage of the cucumbers, it is desirable to have strains of* L. plantarum *that do not decarboxylate malic acid (MDC$^-$). Two MDC$^-$ mutants, obtained by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis of different MDC$^+$ strains, and their parent strains of* L. plantarum *were evaluated in laboratory fermentations of filter-sterilized cucumber juice and of whole cucumbers as to growth rate, end-products, residual malic acid, and competitiveness with the natural flora. Effects of temperature (15 to 40°C) and NaCl concentration (0–6%) on growth in cucumber juice were determined. One MDC$^-$ strain, designated MOP3-M6, was selected for further development because of its relative dominance in cucumber feremendations, high residual malic acid concentration after fermentation and greater salt tolerance as compared to its closest rival mutant culture. Growth lag and generation times averaged 1·6 and 1·2 times greater, respectively, for the MOP3-M6 mutant than its parent. However, this mutant may still have application as a starter culture for cucumber fermentation, particularly under relatively aseptic conditions.*

Introduction

The use of starter cultures of lactic acid bacteria for vegetable fermentations has been investigated since the early part of this century. However, starter cultures have not been widely used in the commercial fermentation of cucumbers, olives and cabbage, which constitute the largest volume of fermented vegetables. This lack of use has been attributed to the fact that current fermentation practices are not compatible with the use of

*Current address: 1309 Swallow Drive, Raleigh, NC 27606, USA.
†Corresponding author.

0740–0020/93/060489 + 11 $08.00/0

pure cultures, no cultures are available with sufficiently unique and valuable characteristics to justify their use to the exclusion of natural microflora, and other factors (Fleming et al. 1985). Characteristics desired in cultures for potential use in the fermentation of vegetables, particularly cucumbers, have been considered (Daeschel and Fleming 1984).

The fermentation of cucumbers has been shown to result in bloater damage (hollow cucumbers) due to growth by gas-forming yeasts (Etchells et al. 1953) and bacteria (Etchells et al. 1968). It had been assumed that CO_2 from hexose metabolism was the cause of the bloater damage. It was surprising then when *Lactobacillus plantarum*, a homofermenter of hexoses, was shown to cause bloater damage (Fleming et al. 1973). Purging of CO_2 from fermenting cucumber brines was shown to prevent bloater formation. The pickle inudstry now routinely uses nitrogen or air to purge CO_2 from fermenting brines. The source of CO_2 from cucumber fermentation by *L. plantarum* remained a mystery until it was shown that malic acid, a natural component of cucumbers, was degraded to yield the CO_2 (McFeeters et al. 1984a). Bloater damage could be prevented by fermentation of cucumbers with a culture of *L. plantarum* that did not degrade malic acid. However, the culture used, strain 965 which had been isolated from cheddar cheese (Sherwood 1939), was deemed unsuitable for cucumber fermentation because of its reduced ability to ferment the sugars in cucumbers to the same extent as cultures isolated from vegetable fermentations.

Subsequently, *L. plantarum* WSO, a culture with the ability to more completely ferment cucumber sugars and known to decarboxylate malic acid (MDC^+), was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NG) to yield a culture unable to decarboxylate malic acid (MDC^-) (Daeschel et al. 1984). The parent strain (MDC^+) earlier had been evaluated in pilot-scale fermentations for its potential use as a culture for commercial fermentations (Fleming et al. 1988). Although the culture grew early in the fermentation, it was predominated by naturally occurring microflora later in the fermentation. A lactic acid bacterium, tentatively identified as *L. plantarum*, was isolated from a later stage of fermentation (herein identified as MOP3) and chosen for its potential as a starter culture. From the MOP3 parent culture (MDC^+), MDC^- mutants were obtained and provided by M. A. Daeschel, using the procedure cited above.

Development of a suitable fermentation culture lacking the ability to produce CO_2 from malic acid could eliminate the need for purging, with considerable economic savings to the pickle industry. Since no genetically or phenotypically altered lactic acid bacterium has been previously introduced for cucumber fermentation, it was deemed appropriate to develop a protocol for culture evaluation prior to commercial introduction. This study was designed to evaluate selected MDC^- and MDC^+ parent cultures of *L. plantarum* as to: (1) generation and lag times in broth culture at 0 to 6% NaCl and 15 to 40°C; (2) malic acid decarboxylation, sugar use, and acid formation; and (3) ability of MDC^- cultures to dominate cucumber fermentations.

Materials and Methods

Organisms

The following *L. plantarum* strains were evaluated herein: WSO (MDC^+) and a mutant therefrom, WSO-M35 (MDC^-; Daeschel et al. 1984), and MOP3 (MDC^+; Fleming et al. 1988) and a mutant therefrom, MOP3-M6 (MDC^-), obtained from MOP3 by the procedure of Daeschel et al. (1984). WSO-M35 and MOP3-M6 will be designated herein simply as M35 and M6, respectively.

Buffered cucumber juice

The juice from fresh cucumber was extracted and diluted to 60% by volume (McFeeters et al. 1984b). The juice was supplemented with 53 mM acetic acid, 18 mM $Ca(OH)_2$ and NaCl at specified concentrations and adjusted to pH 4·75 to simulate conditions earlier suggested for controlled cucumber fermentations (Fleming et al. 1988). This buffered cucumber juice broth (BCJ) was then filter-sterilized (0·22 μm filter). It was used to cultivate inocula, for growth kinetic studies, and for fermentation balance studies. Unless indicated otherwise, all BCJ for inocula preparation was supplemented with 2% NaCl.

Preparation of inocula

Laboratory cultures (LC) of the parent and mutant bacteria for kinetic studies were prepared from stock cultures frozen and stored at –70°C in MRS broth (Difco Laboratories, Detroit, MI) containing 16% glycerol. As needed, the frozen stock cultures were streaked onto MRS agar (MRS broth + 1·5% agar), and an isolated colony was picked and transferred into MRS broth. Then, the cultures were transferred (1%, 0·1 into 10 ml) through BCJ two times, each transfer being made at the late log phase of growth. After the second growth in BCJ, the cells were harvested at late log phase by centrifugation and washed twice in 0·85% sterile saline. The washed cells were adjusted to $O.D._{650}$ = 0·2 (approximately 2×10^8 cells ml^{-1}). All laboratory inocula were grown at 30°C.

The commercial cultures (CC) of the above four bacteria were grown in broth similar in composition to MRS broth (differing in the source of individual components) at 37–40°C, maintained at pH 5·5 with NH_4OH and harvested at early stationary phase of growth. The cells were concentrated to about $3·5 \times 10^{10}$ cells ml^{-1} by centrifugation and frozen and stored under liquid nitrogen in the growth medium without addition of cryo-protectant. They were shipped to our laboratory in 18 ml aluminium cans on dry ice and then held at –70°C until needed for growth kinetic studies. They were evaluated within 1 month of receipt. The cans of culture were thawed at 30°C, opened and diluted to 2×10^6 cells ml^{-1} in the designated growth medium.

Growth kinetics

Growth kinetics, including lag time and generation (doubling) time (Monod 1949), were determined in BCJ (containing 0 to 6% NaCl in increments of 2%) for each parent and mutant grown in the laboratory, and for the commercial culture concentrates of each micro-organism. The cultures were incubated in 10 ml of BCJ contained in 16 × 150 mm, disposable, borosilicate tubes (Corning Glass Works, Corning, NY, USA) with Kim-Kap (Kimble Glass, Inc., Vineland, NJ, USA) closures that were placed in a water bath adjusted to 15 to 40°C (in increments of 5°C). Growth was measured as optical density at 650 nm in a Lumetron Colorimeter (Photovolt Corporation, Indianapolis, IN, USA). The $O.D._{650}$ readings were plotted on a logarithmic scale (y-axis) versus incubation time (x-axis, linear scale) and a calculated regression line based on 4 to 5 points in the exponential phase of growth was extrapolated to the level of cells at the time of inoculation. The growth lag time (h) was the horizontal distance from the extrapolated regression line to the y-axis point representing the actual initial cell number. The actual cell number was established by plate count enumeration. The generation time (g) was calculated from the relationship:

$$g = \frac{\ln 2}{k} = \frac{0·693}{k}$$

where k is the specific growth rate (Ingraham et al. 1983). The specific growth rate was calculated from the slope of the regression line above multiplied by 2·303. Data presented for lag and generation times are means of duplicate determinations.

Effects of MRS broth and BCJ inoculum media and NaCl concentration during growth of the inoculum on growth kinetics of the M6 culture were determined. For this study M6 was grown at 30°C in either MRS broth or BCJ with 0, 2, 4 or 6% NaCl. Cells were transferred (1%) twice through the respective media, harvested at late log phase, washed twice in saline, and adjusted to $O.D._{650}$ = 0·2, as described above. Next, the washed M6 cells were inoculated into either MRS broth with 6% NaCl or BCJ with 6% NaCl, and incubated at 30°C. Lag and generation times were calculated as described above.

Cucumber fermentations

Growth and predominance of cultures in brined cucumbers were determined. Cucumbers in good condition and free of mechanical damage were washed and used at a 50/50 pack-out ratio (w/v), cucumbers/brine, in

fermentations with laboratory cultures. A 55/45 pack-out ratio (w/v) was used in studies with commercially concentrated cultures. All brines contained 18 mM $Ca(OH)_2$ and 53 mM acetic acid (when equilibrated with cucumbers) and initially were pH 4·75 ± 0·02. In addition, one set of jars (1 gal. each) contained 4% NaCl (equilibrated level) and nonheated cucumbers. A second set of jars contained cucumbers which were heated for 3 min at 77°C, thus eliminating naturally occurring bacterial vegetative cells, prior to packing into the fermentation jars. NaCl was not used in fermentations with heated cucumbers. The jars (in duplicate) were inoculated with one of the strains listed above at a level of 10^6 to 10^7 colony-forming units/ml (cfu/ml). In fermentations with laboratory cultures, the inoculum was grown in BCJ before use in the fermentation studies. The population of lactic acid bacteria (LAB) was followed during the course of the fermentation by plating with modified MRS agar (MRS broth + 1·5% agar + 0·02% NaN_3). Approximately 50 colonies were selected from each jar at each plating interval and inoculated into individual microtiter wells containing MD broth (Daeschel et al. 1984), which allows differentiation between MDC^+ and MDC^- cultures.

Plasmid isolation

Plasmids were extracted from the four *Lactobacillus* strains under study, and electrophoretically separated and visualized following the procedures of Anderson and McKay (1983). Cells were grown overnight in MRS broth at 30°C. A 2% inoculum of the overnight culture was added into 600 ml of MRS broth. After a 2 to 3 h incubation at 37°C, cell pellets were collected for plasmid extraction. Plasmid preparations were loaded directly onto agarose gels without digestion with restriction enzymes.

Determination of substrates and products

Malic acid, acetic acid, lactic acid, succinic acid and hexoses were measured using high performance liquid chromatography (HPLC) (McFeeters et al. 1984b). An Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA) with a cation guard column was used for separation of acids, and an Aminex HPX-87C column (Bio-Rad) with cation and anion guard columns was used for separation of sugars. A refractive index detector was used for quantification of sugars, and a UV detector (210 nm) was used for quanitification of acids. In order to detect malic acid using HPLC, reduction of fructose in the sample was necessary. The procedure of McFeeters et al. (1993) was used for fructose reduction. CO_2 was determined according to the method of Fleming et al. (1974).

Statistical analyses

A randomized complete block was the experimental design for determining effects of salt and temperature on lag and generation times of cultures. The response surfaces were generated by the General Linear Models procedure in PC-SAS (version 6·03, SAS, Cary, NC, USA).

Results

A preliminary screening of seven *L. plantarum* MDC^- mutant cultures, based on growth vigor in BCJ, resulted in the selection of two cultures and their parents for further evaluation. These four cultures were compared as to their growth kinetics and fermentation patterns in broth media and their predominance in cucumber fermentations.

Growth kinetics in broth media

Growth lag times of both mutants were greater, in general, than their parent cultures over all temperatures and salt concentrations tested (Table 1). Overall, the lag times for the M35 and M6 mutants were significantly greater (1·4 and 1·6 times, respectively; $P \leq 0.05$) than their corresponding parent cultures. Generation times of both mutants were significantly greater (1·3 and 1·2 times, respectively; $P \leq 0.05$) than their parent cultures (Table 1).

Three-dimensional response surface graphs were generated for lag and generation times of the four cultures over the range of both salt (0–6%) and temperature (15–40°C) tested. The results for the M6 culture are illustrated in Figs 1 and 2 for lag and generation times, respectively. Figs 3 and 4 show cut-away

Table 1. Comparison of mutant and parent cultures of *L. plantarum* for lag and generation times in cucumber juice.[a]

Culture[b]	Lag time		Generation time	
	Hours	Ratio[c]	Hours	Ratio[c]
M6/MOP3	5·66/3·53	1·60*	1·87/1·54	1·21*
M35/WSO	5·47/4·07	1·39*	1·85/1·41	1·31*
WSO/MOP3	4·07/3·53	1·15*	1·41/1·54	0·92*
M35/M6	5·47/5·66	0·97	1·85/1·87	0·99

[a]Growth comparisons were determined at 25, 30, 35 and 40°C and 0, 2 and 4% NaCl at each temperature. Lag and generation times are in hours and are means of overall temperatures and NaCl concentrations. Ratios of lag and generation times were calculated from these means.
[b]WSO (parent) and M35 (mutant); MOP3 (parent) and M6 (mutant).
[c]*Indicates a statistically significant difference ($P \leq 0.05$) between the means of the cultures compared.

views of the response surfaces at 4% salt for all four cultures. The coefficients of determination, R^2, shown in Figs 3 and 4 indicate the goodness-of-fit of the data to the predictive equations over the entire ranges of salt and temperature tested. In developing the predictive equations, only terms which significantly contributed to the fit were retained in models. The general form of prediction equations for lag time and generation time was:

$$\hat{LT} \text{ or } \hat{GT} = \text{Intercept} + b\,(\text{temperature}) + b_2\,(\text{temperature})^2 + b_3\,(\text{temperature})^3 + b_4\,(\%\text{salt}),$$

where $\hat{LT}$ is predicted lag time and $\hat{GT}$ is predicted generation time.

In general, lag times for all cultures increased as salt concentrations increased (data not shown), and temperature decreased (Fig. 3). The lag times for MOP3 parent and mutant (M6) cultures were shorter than those for the WSO parent and mutant (M35) cultures at all salt concentrations at 25 and 30°C, but were similar at 35 and 40°C. The M35 mutant did not grow within 48 h at 6% NaCl and at any of the temperatures, while its parent culture did not grow within 48 h at 6% NaCl and 40°C (data not shown). The MOP3 parent and mutant grew at all salt concentrations and temperatures. Generation time increased as temperature decreased for all four cultures (Fig. 4).

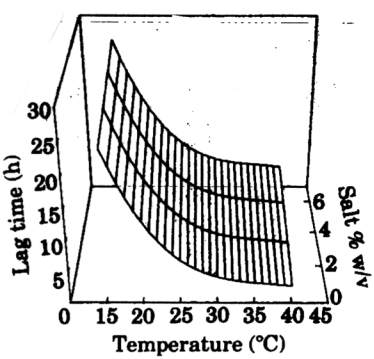

Fig. 1. Effect of salt concentration and incubation temperature on lag time of *L. plantarum* MOP3-M6.

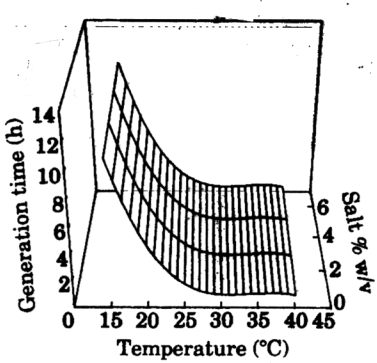

Fig. 2. Effect of salt concentration and incubation temperature on generation time of *L. plantarum* MOP3-M6.

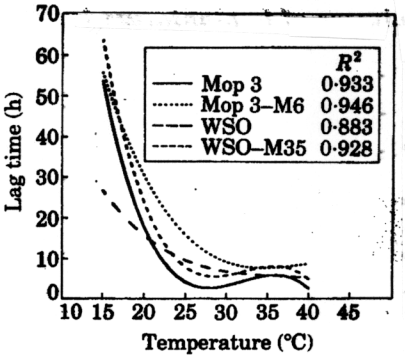

Fig. 3. Effect of temperature on lag time of parent and mutant cultures of *L. plantarum* grown in BCJ at 4% NaCl. The R^2 values, coefficients of determination, indicate the goodness-of-fit of the data used to generate the prediction equations over all salt concentrations tested.

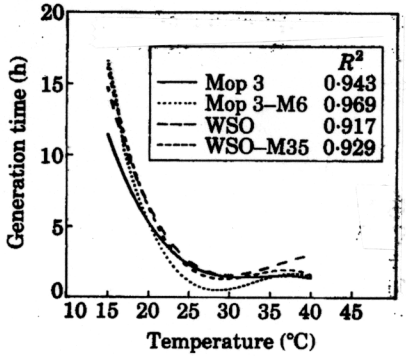

Fig. 4. Effect of temperature on generation time of parent and mutant cultures of *L. plantarum* grown in BCJ at 4% NaCl. The R^2 values, coefficients of determination, indicate the goodness-of-fit of the data used to generate the prediction equations over all salt concentrations tested.

Growth kinetics of laboratory cultures (LC) freshly grown in BCJ and those commercially grown and concentrated (CC) were compared. Both laboratory and commercially prepared cultures grew well in BCJ containing 0 to 4% NaCl at 35°C. However, growth lag time was 1·59 times greater for the commercially prepared M35 culture than for the laboratory grown culture. Conversely, growth lag time was 1·56 times greater for the laboratory grown M6 culture than for the commercially prepared culture. Generation times of cultures

Table 2. Effect of inoculum growth medium and salt concentration on subsequent generation time (GT) and lag time (LT) of *L. plantarum* MOP3-M6 in 6% NaCl.

Inoculum medium		Growth medium			
Basal	NaCl (%)	Basal	NaCl (%)	GT (h)	LT (h)
MRS	0	MRS	6	1·0[a]	7·5
	2		6	0·9	7·0
	4		6	0·9	6·8
	6		6	1·0 (10)[b]	6·6
MRS	0	BCJ	6	1·9	7·9
	2		6	2·1	6·8 (11)
	4		6	2·0	6·1
	6		6	1·9	5·4 (11)
BCJ	0	MRS	6	1·1	10·2
	2		6	1·0	9·3
	4		6	1·0	8·5
	6		6	1·1	8·0
BCJ	0	BCJ	6	2·1	10·6
	2		6	2·2	8·5
	4		6	2·5	6·5
	6		6	2·5	5·9

[a]Value listed is the mean of duplicates.
[b]Number in parentheses is coefficient of variation (CV). If no value is given, the CV was <10.

were not significantly affected by method of preparation.

The M6 mutant was further evaluated as to effects of inoculum medium composition on its growth kinetics upon transfer to another medium (Table 2). The lag time increased for growth in 6% NaCl when the culture was grown at lower NaCl concentrations, regardless of the basal medium. Lag time also was greater when the inoculum was grown in BCJ than in MRS. Generation time was not appreciably affected by NaCl concentration in the inoculum medium, except for the slightly longer time when the inoculum and growth media were BCJ. Generation times were longer when BCJ was the growth medium, regardless of the inoculum medium composition.

Fermentation balances

Fermentation balances of the four cultures grown in BCJ were determined (Table 3). The BCJ was allowed to ferment until termination by the cultures due to acid production and low pH. Residual hexose remained in all fermentations, but more remained for the mutant cultures. Malic acid was decarboxylated by the parent but not the mutant cultures. The terminal pH was slightly higher for the mutant cultures, which is consistent with the higher residual hexose and the lower concentration of lactic acid produced. Carbon recoveries were 110 and 106% for the WSO parent and mutant, respectively, and 101 and 103% for the MOP3 parent and mutant (data not shown).

Plasmid profiles

Plasmid profiles of the mutant and parent cultures were compared (Fig. 5). There was no evidence that mutation altered the profile of either culture. The two parent cultures, however, had different profiles.

Cucumber fermentation

The two mutant cultures were compared as to their potential for cucumber

Table 3. Balances for fermentation of buffered cucumber juice by parent and mutant cultures of *L. plantarum*.

	Culture			
	WSO		MOP3	
	Parent	M35	Parent	M6
Substrates (mM)				
Initial				
Hexoses	79·2	79·2	79·2	79·2
Malic acid	11·4	11·4	11·4	11·4
Residual				
Hexoses	12·2	19·4	11·2	20·3
Malic acid	0	12·9	0·3	10·3
Final pH	3·28	3·32	3·28	3·32
Products, mm				
Lactic acid[a]	155·6	122·0	146·2	118·6
Acetic acid[b]	4·2	3·1	2·8	2·8
Succinic acid[c]	0·2	2·2	0·3	1·4
CO_2[a]	11·2	4·1	8·0	2·6

[a]It was assumed that malic acid was converted to lactic acid and CO_2 by both WSO and MOP3 since no malic acid was present at the end of the fermentation.
[b]This represents a net increase in acetic acid over that added initially to the cucumber juice.
[c]Succinic acid was not included in calculating elemental recoveries from hexoses.

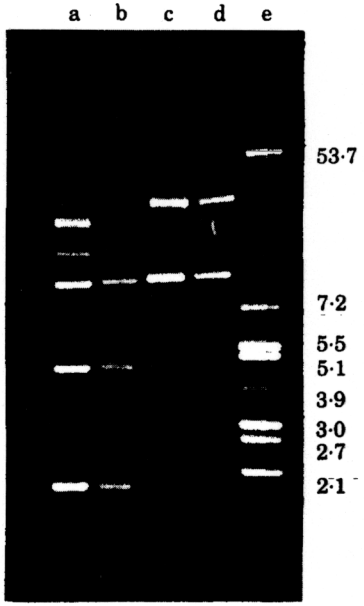

Fig. 5. Agarose gel electophoresis of plasmid DNA from *L. plantarum* strains and mutants. (a) MOP3-M6 mutant, MDC⁻; (b) MOP3-parent, MDC⁺; (c) WSO-M35 mutant, MDC⁻; (d) WSO-M35 parent, MDC⁺; (e) *E. coli* V17 reference mobility plasmids. Numbers indicate size in kb.

fermentation by two different methods. In one method, the cucumbers were heated before brining and no salt was added to the brine (HNS). In the other method, the cucumbers were not heated and NaCl (4%) was present (SNH). Thus, the ability of the cultures to predominate in an aseptic procedure with low salt stress was compared to a non-aseptic procedure with a moderate concentration of salt.

In experiment no. 1, the culture inocula were grown in BCJ. Both cultures grew rapidly in the HNS treatment, but more slowly in the SNH treatment (Fig. 6). The growth lag was less for the M6 than the M35 mutant, particularly in the SNH treatment. Both mutants predominated the HNS fermentation, while only the M6 predominated the SNH fermentation, based upon the percentage of MDC⁻ colonies enumerated through the 144 h test period (Fig. 6). Residual malic acid after fermentation of the cucumbers (Table 4) support these data. The M35 culture did not predominate after 96 h in the SNH treatment, and there was no residual malic acid.

In experiment no. 2, the culture inocula were commercially grown concentrates. The growth lag of both cultures was less than previously observed with cultures grown in BCJ, but otherwise the growth profiles were similar to Fig. 6 (data not shown). Both mutant cultures

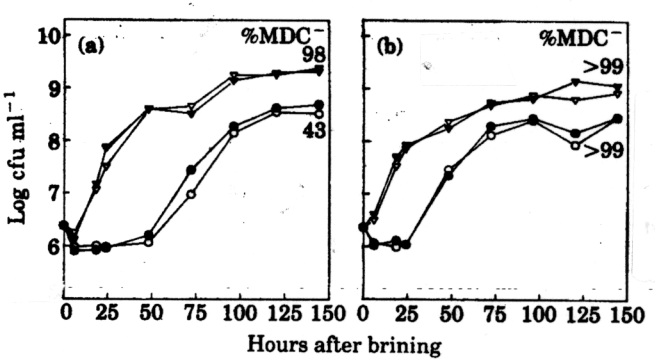

Fig. 6. Growth of lactic acid bacteria in cucumber brines inoculated with laboratory-grown MDC⁻ mutant cultures of *L. plantarum*. For cucumber treatments: HNS, heated, no salt; SNH, salt, not heated. The prominence of the added culture after 144 h is indicated as % MDC⁻. The remaining isolates were determined to be MDC⁺. The symbols Δ and ▲ (HNS) or ○ and ● (SNH) represent duplicate fermentations of the treatments. See related information for experiment no. 1 in Table 4. (a) M35. (b) M6.

Table 4. Final pH and residual substrate concentrations following fermentation of cucumbers inoculated with MDC⁻ mutant cultures.

Treatment	Culture	Malic acid (mM)	Hexose (mM)	Final pH
		Experiment no. 1[d]		
HNS[a]	M35	2·75	1·14	4·03
	M6	3·09	0·55	3·99
SNH[a]	M35	0	5·10	3·87
	M6	2·53	0·78	3·83
Raw cucumber[b]		5·50	58·5	
		Experiment no. 2[d]		
HNS	M35	0	0·75	3·48
	M6	3·69	6·27	3·34
SNH	M35	0·90	14·51	3·38
	M6	2·92	16·34	3·41
Raw cucumber[c]		7·85	72·28	

[a]Experiment no. 1 contained size 3b cucumbers and had an inoculum size of 10^6 cfu ml^{-1} of jar contents; inoculum prepared in the US Food Fermentation Laboratory. Experiment no. 2 contained size 3a cucumbers and had an inoculum size of 10^7 cfu ml^{-1} jar contents; inoculum prepared by Chr. Hansen's Laboratory, Inc. For cucumber treatments: HNS, heated, no salt; SNH, salt, not heated. See Materials and Methods for details of treatments.

[b]Values listed for malic acid and glucose are 50% of the actual value obtained from the raw cucumber, since the pack-out ratio (w/v), cucumbers/brine, was 50/50.

[c]Values listed for malic acid and glucose are 55% of the actual value obtained from the raw cucumber, since the pack-out ratio (w/v), cucumbers/brine, was 55/45.

[d]Data shown for Experiment 1 are after 144 h (6 days) and for Experiment 2 after 47 days.

predominated (>99% MDC⁻) both the HNS and SNH treatments, based upon percentages of MDC⁻ colonies enumerated (data not shown). However, malic acid was depleted, or nearly so, with the M35 mutant in both the NHS and SNH treatments, but was retained at greater levels with the M6 culture after 47 days (Table 4).

Discussion

The MDC⁻ mutants evaluated in this study grew less vigorously over a wide range of salt concentrations and temperatures than their parent cultures. Thus, such mutants may be less useful than preferred for cucumber fermentations using traditional brining procedures because of their unlikely ability to predominate over naturally occurring lactic acid bacteria. Since most naturally occurring lactic acid bacteria on ccucumbers are MDC⁺ (unpublished observations), their growth would result in CO_2 production and, thus, compromise the unique value of the added MDC⁻ culture. Purging of the brine still would be required to prevent bloater formation. The fact that the M6 mutant predominated all fermentations in this study during the 144 h test period (both HNS and SNH treatments) is encouraging. We are unsure why malic acid was depleted with the M35 mutant and partially so by the M6 mutant. The reversion frequency of the MDC⁻ mutation was found to be less than 10^{-10} (Breidt and Fleming 1992). Therefore, it is unlikely that the MDC⁻ mutants reverted to their MDC⁺ phenotype. Gradual depletion by naturally occuring MDC⁺ lactic acid bacteria is a likely possibility. Controlling the numbers of such bacteria in commercial operations may require more aseptic conditions than currently employed in the pickle industry. However, benefit could be derived

from an MDC⁻ culture that reduced the rate of CO_2 production by natural microflora. Cucumbers were shown to be less susceptible to bloater formation after extended storage in brine (e.g. 49 days) than during the first 32 days (Fleming et al. 1978). Also, MDC⁻ cultures would be expected to be inconsequential as to bloater formation if they should enter and ferment substrates within the cucumber tissue. Lactic acid bacteria have been shown to be capable of entry into and growth within brined cucumbers (Daeschel and Fleming 1981).

The MDC⁻ mutants could be useful for cucumber and other vegetable fermentations under aseptic conditions such as the HNS treatment described herein. By blanching the cucumbers and sanitizing the fermentation vessel, naturally occurring lactic acid bacteria could be greatly reduced or eliminated. Or, perhaps sufficiently high concentrations of mutant (MDC⁻) culture could be added to overwhelm the naturally occurring lactic acid bacteria. The ratio of MDC⁻ mutant to natural MDC⁺ bacteria to effect the desired predominance has yet to be determined. An important advantage of such MDC⁻ cultures in this type fermentation is that the fermentation vessel could be sealed after inoculation, and very little pressure would result as a consequence of fermentation.

It was clearly shown that growth lag times of the mutant cultures were reduced by growth of the inoculum in NaCl concentration approximating that in the subsequent growth medium. Also, the inoculum basal medium influenced subsequent growth lag times. Although not tested, parent cultures would likely exhibit similar traits. Thus, conditions for inoculum preparation are important in its subsequent predominance over naturally occurring microflora in vegetable fermentations.

Aside from practical implications, the MDC⁻ mutants may be useful in studying the physiological role of the malolactic reaction in *L. plantarum*. Cox and Henick-Kling (1989) suggested that the reaction may be a source of energy. Another function would be the uptake of a proton from the medium (Henick-Kling 1986), with resultant increase in pH. MDC⁺ cultures of *L. plantarum* and *Leuconostoc mesenteroides* have been shown to have higher specific growth rates in media supplemented with L-malate (Daeschel 1988), apparently because of the more favorable pH.

If the malolatic reaction provides a competitive advantage to *L. plantarum*, it may be futile to seek MDC⁻ mutants that will predominate traditional cucumber fermentations. It is not clear from our studies of the MDC⁻ mutants in our collection what causes their varying growth responses. The two cultures studied herein were not greatly impaired in growth compared to some that were eliminated in screening tests. We are unsure if the relatively slight impairment is due to deletion of the malolactic reaction or to other changes that the NG mutagen may have induced. It is known that NG mutagenesis has a tendency to produce multiple mutations (Birge 1981). We have observed other responses in the MDC⁻ mutants that may or may not be associated with malolactic activity. For example, the MDC⁻ cultures do not grow as well as their parents when the MRS medium we use for cultivation is supplemented with 0·02% sodium azide. We are continuing studies to determine the role of the malolactic reaction in *L. plantarum*. Also, we are attempting to obtain an MDC⁻ mutant by site deletion of the gene encoding for the malolactic reaction. Such a mutant may prove beneficial in understanding the role and value of the malolactic reaction.

Acknowledgements

We thank Chr. Hansen's Laboratory, Inc., of Milwaukee, WI, particularly D. McCoy, for providing culture concentrates of test micro-organisms. We thank F. G. Giesbrecht for advice on statistical analyses.

This investigation was supported in part by a research grant from Pickle Packers International, Inc., St. Charles, IL.

References

Anderson, D. G. and McKay, L. L. (1983) Rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Environ. Microbiol.* **46**, 546–552.

Birge, E. A. (1981) *Bacterial and bacteriophage genetics*. Springer-Verlag, New York.

Breidt, F. and Fleming, H. P. (1992) Competitive growth of genetically marked malolactic-deficient *Lactobacillus plantarum* in cucumber fermentations. *Appl. Environ. Microbiol.* **58**, 3845–3849.

Cox, D. J. and Henick-Kling, T. (1989) Chemiosmotic energy from malolactic fermentation. *J. Bacteriol.* **171**, 5750–5752.

Daeschel, M. A. (1988) A pH control system based on malate decarboxylation for the cultivation of lactic acid bacteria. *Appl. Environ. Microbiol.* **54**, 1627–1629.

Daeschel, M. A. and Fleming, H. P. (1981) Entrance and growth of lactic acid bacteria in gas-exchanged, brined cucumbers. *Appl. Environ. Microbiol.* **42**, 1111–1118.

Daeschel, M. A. and Fleming, H. P. (1984) Selection of lactic acid bacteria for use in vegetable fermentations. *Food Microbiol.* **1**, 303–313.

Daeschel, M. A., McFeeters, R. F., Fleming, H. P., Klaenhammer, T. R. and Sanozky, R. B. (1984) Mutation and selection of *Lactobacillus plantarum* strains that do not produce carbon dioxide from malate. *Appl. Environ. Microbiol.* **47**, 419–420.

Etchells, J. L., Bell, T. A. and Jones, I. D. (1953) Morphology and pigmentation of certain yeasts from brines and the cucumber plant. *Farlowia.* **4**, 265–304.

Etchells, J. L., Borg, A. F. and Bell, T. A. (1968) Bloater formation by gas-forming lactic acid bacteria in cucumber fermentations. *Appl. Microbiol.* **16**, 1029–1035.

Fleming, H. P., McFeeters, R. F. and Daeschel, M. A. (1985) The lactobacilli, prediococci, and leuconostocs: vegetable products. In *Bacterial starter cultures for foods* (Ed Gilliland, S. E.) pp 97–118. Boca Raton, FL, CRC Press.

Fleming, H. P., McFeeters, R. F. and Daeschel, M. A., Humphries, E. G. and Thompson, R. L. (1988) Fermentation of cucumbers in anaerobic tanks. *J. Food Sci.* **53**, 127–133.

Fleming, H. P., Thompson, R. L. and Etchells, J. L. (1974) Determination of carbon dioxide in cucumber brines. *J. AOAC* **57**, 130–133.

Fleming, H. P., Thompson, R. L., Etchells, J. L., Kelling, R. E. and Bell, T. A. (1973) Bloater formation in brined cucumbers fermented by *Lactobacillus plantarum. J. Food Sci.* **38**, 499–503.

Fleming, H. P., Thompson, R. L and Monroe, R. J. (1978) Susceptibility of pickling cucumbers to bloater damage by carbonation. *J. Food Sci.* **43**, 892–896.

Henick-Kling, T. (1986) *Growth and metabolism of* Leuconostoc oenos *and* Lactobacillus plantarum *in wine*. Ph. D. Thesis. University of Adelaide, Australia.

Ingraham, J. L., Maaloe, O. and Neidhardt, F. C. (1983) *Growth of the bacterial cell*. Sinauer Associates, Inc., Sunderland, MA.

McFeeters, R. F., Fleming, H. P. and Daeschel, M. A. (1984a) Malic acid degradation and brined cucumber bloating. *J. Food Sci.* **49**, 999–1002.

McFeeters, R. F., Thompson, R. L. and Fleming, H. P. (1984b) Liquid chromatographic analysis of sugars, acids and ethanol in lactic acid vegetable fermentations. *J. AOAC* **67**, 710–714.

McFeeters, R. F., Thompson, R. L. and Fleming, H. P. (1993) Malic acid analysis in cucumber juice and fermentation brines in the presence of interfering fructose. *J. Food Sci.* **58**, 832–834.

Monod, J. (1949) The growth of bacterial cultures. *Annu. Rev. Microbiol.* **3**, 371–394.

Sherwood, I. R. (1939) The bacterial flora of New Zealand cheddar cheese. *J. Dairy Res.* **10**, 426–448.