POSSIBLE IMPROVEMENTS IN ALFALFA SILAGE THROUGH THE USE OF MUTANTS OF *Lactobacillus plantarum* AS INOCULUM

by

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(ABSTRACT)

Alfalfa is difficult to ensile because of its low water soluble carbohydrate content, low dry matter content, and high buffering capacity. Therefore, the isolation of mutants of L. plantarum was undertaken to develop a strain(s) which could improve the fermentation of alfalfa silage when used as an inoculum.

Efforts to isolate starch- or hemicellulose-utilizing strains proved unsuccessful as did the isolation of high temperature resistant mutants and oxygen resistant mutants. Mutants resistant to plumbagin (an intracellular O2⁻ generator) and citrate were isolated. Of these mutants, some were found to be more sensitive to cadmium than the parent strain. Though these three characteristics point to an enhanced manganese accumulation since O2⁻ is scavenged by manganese, citrate will chelate manganese, making it unavailable to the organism, and cadmium is transported by the manganese transport system, the rates of ⁵⁴Mn transport were no greater than the parent.
When grown aerobically on a glucose-limited medium, the parent strain consumed oxygen and produced high concentrations of acetic acid and low concentrations of lactic acid. By contrast, one mutant (strain MC226), produced high concentrations of lactic acid and demonstrated low rates of $O_2$ consumption and low levels of acetic acid.

The parent strain and two plumbagin- and citrate-resistant mutants were used to inoculate alfalfa for silage production. The inoculated silages showed a lower pH and higher titratable acidity than that of an uninoculated control. Silages inoculated with the parent strain exhibited high levels of acetic acid and low levels of lactic acid, indicative of oxygen consumption by the parent strain. Less acetic acid was evident in silages inoculated with the mutant strains. All silages contained a high level of butyric and isobutyric acids and propionic and isovaleric acids were also detected, indicative of clostridial growth.
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INTRODUCTION AND LITERATURE REVIEW

Silage is the material formed when crops of high moisture content are chopped and placed in an air-tight container, a silo. Carbohydrates and other constituents in the crop are transformed (fermented) by microorganisms and their products (metabolites) cause either preservation or spoilage of the silage. The storage of feedstuffs in silos has been a common practice for many years. The basics of ensiling as now normally practiced can be traced to the beginning of the nineteenth century in northern Europe where Swedes developed the process of ensiling grass and Germans ensiled beet pulps and leaves (McDonald, 1981; McCullough, 1978). In the second half of the nineteenth century the ensiling of corn was enthusiastically adopted in the United States after the publication and translation into English of a book written by Goffart, a French farmer who based his information on his own experience of ensiling green corn. It was stated at the Fifth Ensilage Congress in 1886 that the average farm in the United States was capable of quadrupling its production through the use of ensilage (McDonald, 1981).

Interest in improving silage production was revived around the early 1950's with the advent of increased mechanization on the farm, the need for more intense animal production, and the increasing costs of other feeds such as grain. The importance of
silage production in the United States has continued to increase as evidenced by the fact that annual tonnage production from corn and sorghum has increased from approximately 55 million in 1950 to 150 million in 1976. The latest estimate of total cost of production for silage is 2.25 billion dollars annually (McCullough, 1978).

Advantages and Disadvantages of Silage Production.

The rapid increase in silage production can be attributed to several advantages. Some of these are:

Improved Crop Management: The condition of the crop in the field is less important in silage production than other preservation methods (i.e. hay or grain) since crops for silage production can be harvested at a variety of moisture contents and stages of maturity. This allows the farmer to harvest the crop at a fixed date so a second and third crop can be planted at an optimal time for growth.

Reduced Dependence on Weather Conditions: Since crops for silage production require little or no field curing, losses due to inclement weather are minimized. Reduction in dry matter content constitutes the main loss in field curing due to shattering, leaching, and bleaching (McCullough, 1978). Losses in water soluble carbohydrates and protein nitrogen have also been reported (McDonald, 1981). Field losses with wilted silage
range from 2% to 10%, depending on weather conditions, whereas field losses in hay production can range from 10% to 30% under the same weather conditions (McCullough, 1978).

Maximum Nutrient Utilization: Crops such as corn and sorghum can be used for either silage or grain production. However, harvesting the grain alone will leave about one half of the available nutrients in the field. Silage production not only allows total utilization of the crop but also supplies a good source of roughage in the animal feed.

Increased Range of Crop Selection: Many crops which are unsuitable for haymaking can be utilized in silage production (i.e., sorghum, sudangrass, and sunflowers).

Mechanization: The entire process of silage production from harvesting to feeding can be totally mechanized, thus reducing labor costs.

Improved Ration Development: The nutritional value of silage can be increased by the addition of grain prior to feeding.

However, there are some disadvantages in silage production. These include:

Large Investment: A large initial investment in equipment is required to set up a silage feeding program.

Instability: The quality of silage rapidly deteriorates after removal from the silo. Thus it cannot be transported long
distances and must be fed on a daily basis.

**Silage Unmarketable:** Since silage will not retain its nutritive value long after removal from the silo no established market price can be set. Thus selling excess silage is much more difficult than hay or grain.

**High Storage Costs:** Silage has a high moisture content and consequently the extra weight of water will increase the cost of storage and handling.

Overall, if the initial capital needed to start a silage feeding program is available, the advantages will outweigh the disadvantages. It provides a high quality feed when grazing is not feasible and frees the farmer from variations in market price of other feeds.

**Fundamentals of Ensilage.** There are two main requirements for successful silage production. First there is the rapid development of anaerobic conditions in the silo. The crop is harvested, chopped into small particles (25 mm or less in length), tightly packed and sealed in the silo. If oxygen is allowed to come in contact with the ensiled crop aerobic deterioration will occur from the growth of yeasts, fungi, and aerobic and facultative anaerobic bacteria (Langston, *et al.*, 1962; Edwards and McDonald, 1978; McDonald, 1981). These organisms will compete with lactic acid producing micro-organisms for available carbohydrates, cause extensive losses in dry matter and protein,
and produce an inedible and often toxic silage (McDonald, 1981).

After anaerobiosis has been achieved, the second requirement is the inhibition of clostridial growth. This group of spore-forming soil bacteria grow and produce butyric acid, volatile fatty acids and CO₂ through the fermentation of water soluble carbohydrates and degradation of amino acids resulting in inferior quality silage (Edwards and McDonald, 1978; Beck, 1978). The production of these metabolites will cause losses in dry matter and protein content and reduce the palatability of the silage (McCullough, 1978). Inhibition of clostridial growth is achieved by an accumulation of lactic acid in the silage due to the growth of lactic acid bacteria. Homofermentative lactic acid bacteria will ferment water soluble carbohydrates present in the crop to lactic acid as the sole product by the Embden-Meyers-Parnas pathway. Heterofermentative lactic acid bacteria, on the other hand, produce acetic acid, ethanol and CO₂ in addition to lactic acid by the hexose monophosphate shunt (Edwards and McDonald, 1978). The decrease in pH caused by the lactic acid produced as well as the undissociated acid inhibits the growth of clostridia (Wieringa, 1958). Further, Wieringa (1958) found that increasing the osmotic pressure in the crop will decrease the clostridia's acid tolerance. Thus, a decrease in water content will inhibit clostridial growth (McDonald et al., 1962; Lanigan, 1963) and this is achieved by wilting, the practice of leaving the crop in the
field to dry to approximately 25% to 35% dry matter prior to filling the silo (Cullison, 1975).

Lactic Acid Bacteria in Silage. Keddie (1959) found *Lactobacillus plantarum* becomes the dominant organism in silage made from a variety of crops. Langston, et al. (1962) found that early flora of high quality silage consisted of cocci while the dominant organisms in the completely fermented crop consisted of the homofermentative *Pediococcus* and *L. plantarum*, and the heterofermentative *Lactobacillus brevis*. These three organisms were also found to be the dominant type by Lanigan (1963) although *L. brevis* was less common. Beck (1978) found *Lactobacillus curvatus* and *L. plantarum* were the dominant homofermentative organisms but as the fermentation continued the heterofermentative *L. brevis* and *Lactobacillus buchneri* became most numerous.

Aids in Ensiling. Because of the lack of reproducibility of obtaining high quality silage (McCullough, 1978) and the inherent disadvantages of ensiling certain crops, a number of additives have been examined for their ability to improve silage preservation. McDonald (1981) places these additives into four main groups: (1) nutrients such as urea and ammonia, (2) aerobic deterioration inhibitors such as propionic and sorbic acids, (3) fermentation inhibitors such as formic acid and formaldehyde, and (4) fermentation stimulants such as sugars and bacterial
cultures.

The use of lactic acid bacteria as inoculum for silage has received attention over the past twenty years. Olsen and Voelker (1962) found alfalfa silage treated with Lactobacillus and enzymes from Aspergillus oryzae had a faster pH decline and a lower peak temperature than the uninoculated control. Svensson and Tveit (1964) found no improvements in silage quality when a culture of lactic acid bacteria was added to clover, alfalfa and alfalfa-grass mix silages. However, the genus, species and concentration of inoculum was not mentioned. McDonald, et al., (1964) used an inoculum consisting of eight strains of homofermentative lactobacilli on ryegrass and cocksfoot forage containing 16.2% and 4.3% water soluble carbohydrates, respectively. Their results showed little change in silage quality when the inoculum was applied to ryegrass, but the inoculum did cause an improvement in cocksfoot silage. These same workers found the use of lactobacilli inoculum would improve the quality of red clover silage (McDonald, et al., 1965). Lesin and Schultz (1968) found an improvement in sedge silage quality but none in alfalfa using a mixture of a coccoid lactic acid bacteria isolated from sedge silage and L. plantarum. Whittenbury, et al. (1967) reported a more rapid drop in pH when a 1:1 mixture of Streptococcus faecalis and L. plantarum was used to inoculate cocksfoot and ryegrass for silage. Ohyama, et al. (1975) found
the addition of glucose and \textit{L. plantarum} to ryegrass and cock'sfoot silages which were aerated daily for four days after silo filling produced a lower pH than the control or when glucose was added alone. Carpentero, \textit{et al.} (1979) examined the protein content in ryegrass-clover silage and found that addition of an inoculum of \textit{L. plantarum, S. faecalis} and \textit{Leuconostoc mesenteroides} caused a substantial drop in proteolysis.

Since the ultimate test of a feeding program is animal performance, it is important to see how inoculated silages compare to those which rely on inoculation from the normal flora present on the crop or at the site of preparation. In a review of existing data, Bolsen (1978) found when inoculated silage is used in a feeding program it can increase milk fat content, average daily gain, and per cent dry matter digestability above that of uninoculated silage.

Whittenbury (1961, cited by McDonald, 1981) has identified criteria for the selection of strain(s) suitable for use as an inoculum for silage. (1) It should grow vigorously and dominate the fermentation. (2) It must be homofermentative. (3) It must be acid-tolerant and cause a rapid drop in pH to at least 4.0. (4) It must ferment glucose, fructose, sucrose, fructans and pentose sugars. (5) It cannot produce dextran from sucrose or mannitol from fructose. (6) It should have no action on organic acids. (7) It should be able to grow at 50° C. (8) It should be able to grow
Crops for Silage Production. Corn is an ideal crop for ensiling because of its high dry matter content, adequate levels of water-soluble carbohydrates for lactic acid production, and is low in buffering capacity (Cullison, 1975; McDonald, 1981). However, it is low in total protein content and its growth requires high levels of fertilization to obtain high yields. Further, it requires growth temperatures of 70° to 90° F for three to four consecutive months. Consequently, the areas in which it can be cultivated are limited (Chapman and Carter, 1976).

Legumes are known to be good crops for animal feed because of their high protein and mineral content (McDonald, et al., 1965). Further, since they grow in symbiotic relationship with the nitrogen fixing bacteria, Rhizobium spp., less fertilization is needed to obtain optimum growth. Alfalfa is particularly suited for animal feed since it is a perennial and under proper management need not be reseeded for several years (Chapman and Carter, 1976). Further, it can provide three or more cuttings per year and thus has a high yield per acre, it is drought resistant, it is cold weather tolerant and can be grown in areas where corn cultivation is unsuitable. Alfalfa is not considered a good crop for ensiling for three reasons. (1) It is low in water soluble
carbohydrates and thus less substrate is available for lactic acid production. (2) It has a high buffering capacity and therefore more lactic acid is needed to drop the pH to levels necessary for preservation. (3) It has a low dry matter content which favors the growth of clostridia (McDonald, 1981).

Wilting is often used when ensiling alfalfa, since it will increase the concentration of water-soluble carbohydrates and can inhibit clostridial growth (Wieringa, 1958) and also decreases the buffering capacity of the crop (Playne and McDonald, 1966). Wilting, however, increases field losses and dependence on weather conditions and can cause problems during the fermentation due to the inclusion of air in the silo.

Possible Enhancement of Alfalfa Silage

Although it may be advantageous to use alfalfa in a feeding program, storage may present a problem since nutrients can be lost during field curing and alfalfa is difficult to ensile. However, from reviewing work in this area, it appears that the use of bacterial inoculum improves the fermentation of silage. Therefore, if mutants of Lactobacillus plantarum can be isolated that are able to overcome ensiling deficiencies specific to alfalfa, the use of such mutants as an inoculum should make the production of high quality alfalfa silage more reliable.

Starch and Hemicellulose Degradation. Total water soluble
carbohydrates do not account for the total amount of acid produced in silage and evidence exists that hydrolysis products of hemicellulose may be an additional energy source for the lactic acid bacteria (McDonald, et al., 1962; McDonald, et al., 1964). Dewar, et al. (1963) suggests that plant enzymes will breakdown the hemicellulose releasing pentosans and galactans which can be utilized by the bacteria. Two separate studies by Langston (1962) and Goering, et al. (1970) suggested that the silage bacteria themselves are responsible for hemicellulose degradation.

Starch is the main storage polysaccharide in alfalfa (Smith, 1973) and some strains of L. plantarum have been shown to ferment and hydrolyze starch (Langston and Bouma, 1959).

With this in mind it seems reasonable that L. plantarum could be modified by mutation to exhibit high rates of hemicellulose and/or starch degradation if the parent strain does have this potential.

Amino Acid Decarboxylation. Lactobacillus production of amino acid decarboxylases occurs during growth in an acidic environment with a resulting shift in pH towards neutrality. It has been demonstrated that this is a mechanism whereby lactobacilli can stabilize the internal environment against excess acid that is present in the external environment (Gale, 1946; Recsei and Snell, 1972). Amines have been found in low pH
silages (McDonald, 1981) and decarboxylation of amino acids by lactic acid bacteria has been reported by several authors (Lagerborg and Clapper, 1952; Rodwell, 1953; Recsei and Snell, 1972). Therefore, amino acid decarboxylation may retard the drop in pH in the production of silage. It would be desirable to use an inoculum which does not possess amino acid decarboxylases.

Air Incorporation in the Silo. Anaerobiosis, as mentioned earlier, is a key factor in the production of high quality silage. However, due to poor compaction and sealing, air is often incorporated in the silo. This problem is exacerbated by wilting (McDonald, et al., 1966; Henderson, et al., 1972). The incorporation of air in the silo will increase the length of time of aerobic respiration and thus increase CO₂ evolution (and thereby exacerbate dry matter loss) and decrease the amount of carbohydrates available for lactic acid production. Also, aerobic respiration will raise the temperature in the silo to a point which is inhibitory to some lactic acid bacteria, including L. plantarum (Langston and Bouma, 1960; Lanigan, 1965). Further, it is known that these same high temperatures favor clostridial growth (McDonald, et al., 1966).

The presence of oxygen also has a direct effect on L. plantarum. Although the lactic acid bacteria are generally considered to be aerotolerant (Brock, 1979), Archibald and Fridovich (1981) found that oxygen inhibited L. plantarum. In
the same study, they found any limitation of manganese would increase the organism's oxygen sensitivity. Therefore, mutant strain(s) with the ability to transport and accumulate more manganese should be more oxygen tolerant.

Aside from oxygen's toxic effects, there is some evidence that *L. plantarum* may actually gain energy by converting pyruvate to acetate if oxygen is present to act as a reductant and the carbohydrate source is limited. Dirar and Collins (1972) found that *L. plantarum* grown in a medium containing 6 to 17 micromoles galactose/ml under partial anaerobic conditions converted 82% of the galactose to lactic acid and formed 2.51 moles ATP/mole galactose. However, when the galactose concentration was reduced to 1 to 6 micromoles/ml only 58% of it was converted to lactic acid though 2.98 moles ATP/mole galactose were produced. In a separate study by these same authors (Dirar and Collins, 1973), *L. plantarum* grown with 1 to 6 micromoles galactose/ml aerobically resulted in 93% of the galactose being converted to acetic acid with an ATP yield of 3.9 moles/mole galactose. Further, when riboflavin, a precursor for flavin adenine dinucleotide (FAD), was omitted from the growth medium, ATP and acetic acid yields decreased, whereas lactic acid production increased. Therefore, *L. plantarum* may gain more energy per mole of galactose in an aerobic, galactose-limited medium with oxygen serving as an electron acceptor. Flavin mediated electron
transport to oxygen in lactobacilli has also been reported by Strittmatter (1959a, 1959b).

Yousten, et al. (1975) found similar results when L. plantarum was grown aerobically in a glucose limited medium. When the glucose was depleted the cells continued to grow, but when an anaerobic culture under identical conditions consumed all glucose, cell growth ceased. When lactic and acetic acid concentrations were measured along with oxygen consumption, it was found that the concentrations of acetic acid produced and oxygen consumed increased after glucose depletion in the aerobic culture. Hence, it appears that acetate production is linked to oxygen consumption and L. plantarum may gain energy from this activity.

Since oxygen is consumed it should be expected that L. plantarum must have some means of protection against toxic oxygen compounds (i.e. H₂O₂, O₂⁻) that may be produced. Some strains of L. plantarum are known to have catalase (Whittenbury, 1964; Johnson and Delwiche, 1965; Kono and Fridovich, 1983) and in the same study mentioned above, Yousten, et al. (1975) detected low levels of superoxide dismutase and NADH peroxidase. Gotz, et al. (1980) found NADH oxidase and NADH peroxidase in L. plantarum strain ATCC 8014 and Archibald and Fridovich (1981) found that manganese acts as a superoxide dismutase in L. plantarum strains ATCC 8014 and ATCC 14917. Therefore, it seems that L. plantarum
has several methods of protection against toxic oxygen compounds.

Since alfalfa is low in water soluble carbohydrates, oxygen consumption under these limiting conditions by *L. plantarum* may influence the quality of alfalfa silage. Oxygen consumption would increase the population of the organism in the silo but, this would be detrimental to silage quality since acetic acid rather than lactic acid becomes the major fermentation product. Further, if oxygen is consumed then toxic oxygen compounds would be produced which could retard the growth of *L. plantarum*. In order to promote maximum lactic acid production it may be necessary to isolate a strain that cannot utilize oxygen.

The Role of Organic Acids. As stated previously legumes have a high buffering capacity. This has been attributed to protein content (Nillson, 1956) and inorganic ion content (Smith, 1962), but it is now established that organic acids constitute the major portion of buffering capacity in legumes (Playne and McDonald, 1966). As the ensiling process progresses, buffering capacity increases due to the bacterial degradation of organic acids (low pKa) to formic and acetic acids, ethanol, acetylmethylcarbinol, and 2,3 butanediol (Whittenbury, 1967; McDonald, 1981) which have higher pKa values. Campbell and Gunsalus (1944) and Gunsalus and Campbell (1944) found that a number of lactic acid bacteria will ferment citrate to acetic and
formic acids, carbon dioxide, ethanol and acetylmethylcarbinol. Recently, Thornhill and Cogan (1984) found that some strains of *L. plantarum* will also ferment citrate.

Buffering in the silage is due to the neutral products produced (acetylmethylcarbinol, ethanol, and 2,3 butanediol), release of neutralizing cations by decarboxylation of the organic acids and release of salts of organic acids such as acetate (Whittenbury, 1967). Thus, one way to overcome that problem would be to isolate strain(s) unable to degrade organic acids.
Specific Objectives

1. Isolation of a hemicellulose and/or starch utilizing L. plantarum. This will increase the spectrum of carbohydrates (e.g. xylose, arabinose) available for lactic acid production.

2. Isolation of an amino acid decarboxylaseless mutant. This will decrease the buffering capacity and loss of dry matter in the silage.

3. Development of a strain(s) capable of growing at temperatures greater than 45° C. In the event that aerobic respiration causes an increase of temperature in the silo this strain will be able to grow and produce lactic acid.

4. Examination of the effect of oxygen on L. plantarum and isolation of aerotolerant mutants either by selection of enhanced growth in the presence of oxygen, increased manganese uptake and accumulation, or plumbagin resistance.

5. Isolation of mutants unable to utilize organic acids as a carbon source. This will reduce the production of neutral products and released CO₂ and decrease the loss of dry matter.
MATERIAL AND METHODS

Bacteria. *Lactobacillus plantarum* 32406-2 was donated by George A. Jefferys and Company, Inc., Salem, Virginia. *Lactobacillus* 30a (Recsei and Snell, 1972) was a gift from Dr. Esmond E. Snell, Department of Microbiology, University of Texas, Austin and *Escherichia coli* ATCC 9637 was obtained from stock cultures at VPI and SU.

Media. All cultures were grown in APT medium (Evans and Niven, 1951) adjusted to pH 6.4. Sodium citrate was omitted unless otherwise noted. Iron sulfate was also omitted since *L. plantarum* shows no requirement for it (MacLeod and Snell, 1947; Archibald and Fridovich, 1981; Archibald, 1983). Glucose, MgSO₄, and MnCl₂ were added aseptically from stock solutions (sterilized by autoclaving) after autoclaving. For solid media, 1.5% Bacto agar (Difco) and brom cresol green (.02 g/l) were added. Plumbagin (Sigma Chemical Co.) was solublized in distilled water with heating (approximately 80° C), filter sterilized and added aseptically to the medium to a final concentration of 0.14 mM. Citrate was added directly to solid media before autoclaving to a final concentration of 0.1 M. For citrate utilization and inhibition tests it was added aseptically to the medium after autoclaving from a separately autoclaved stock solution. Ethylenediamine tetraacetic acid (EDTA, Sigma Chemical Co.) and
cadmium were added to APT medium in appropriate amounts and the medium was then filter sterilized.

**Growth Conditions.** All cultures were grown aerobically at 30° C. Inocula were grown overnight in 5.0 ml of APT medium in 13 x 100 mm test tubes (with loose caps) without shaking, centrifuged, washed in buffered saline with gelatin (BSG) and resuspended in APT medium lacking glucose, magnesium and manganese. BSG contains per liter, 8.5g NaCl, 0.3g KH₂PO₄, 0.6g K₂HPO₄, and 10.0 ml of 1% gelatin solution. A 2% (v/v) inoculum was used in all tests unless otherwise noted.

All spectrophotometric measurements were made using a Bausch and Lomb Spectronic 20 spectrophotometer set at 640 nm against a sterile APT medium blank. All centrifugation was done at 10,000 x g for 10 minutes at 4° C unless otherwise noted. Stock cultures were stored at 0° C in a 1:1 mixture of glycerol and APT without added glucose, magnesium or manganese. This suspension in glycerol was used to store strains at 0° C since glycerol is known to protect cells from dessication at freezing temperatures (Brock, 1979).

**Hemicellulose Extraction from Alfalfa.** Alfalfa hay was provided by Dr. Joseph Fontenot of the Department of Animal Science, VPI and SU. Hemicellulose was extracted by the procedure of Whistler and Feather (1965) as modified by Dr. N.
Rukma Reddy, Department of Food Science, VPI and SU. 100 grams of alfalfa hay was washed with 80% ethanol for ten hours to eliminate soluble sugars. The hay was then filtered through 5 layers of cheesecloth and then was washed in 3% NaCl for 10 hours to eliminate proteins and filtered again. Hemicellulose was extracted from the alfalfa by shaking the treated hay in 2.0 liters of NaOH-Na borohydrate solution (10% deoxygenated NaOH, 0.1% Na borohydrate) for 24 hours under an oxygen free atmosphere and the suspension filtered through 5 layers of cheesecloth. The filtrate was centrifuged at 10,000 x g for 15 minutes and the crude hemicellulose was collected and dried.

Mutagenesis. L. plantarum 32406-2 was grown in 10.0 ml APT medium to late log phase at 30°C (approximately 2 to 5 X 10^8 cells/ml) and 0.1 ml of a 1:10 dilution of ethyl methane sulfonate (EMS, Eastman Kodak Co.) was added and the culture incubated at 30°C for one hour. The culture was then centrifuged, washed once in BSG, resuspended in APT medium and grown overnight. That culture was stored at 30°C for later use for selective isolation of mutants.

Mutants of the parental strain, 32406-2, were tested to ensure that they were L. plantarum. The characteristics used were: gram positive rods, acid but no gas from glucose, growth at 15°C, weak or no reaction with rhamnose, acid from cellobiose,
acid from sorbitol, acid from raffinose, acid from amygdalin (Rogosa, 1974) and catalase negative when grown on 1% glucose (Whittenbury, 1964).

**Starch Utilization.** Three approaches were used. Glucose in solid APT medium was replaced with 1% soluble starch (Difco). Mutagenized and unmutagenized cultures of strain 32406-2 were streaked on the soluble starch medium. Also, 0.1 ml of a 10⁴ dilution of the same cultures was spread on the soluble starch medium. APT with glucose and without added glucose were used as controls. Plates were incubated and examined daily for ten days and the largest colonies were picked (possible starch utilizers) and restreaked on the starch medium with strain 32406-2 streaked as the control. For selection by acid production, a 2% (v/v) inoculum (0.1 ml) of mutagenized strain 32406-2 was used to inoculate APT broth containing either 1% soluble starch, 1% glucose or no carbon source. Brom cresol green was the indicator for acid production. An unmutagenized culture was used as a control. Tubes were incubated without shaking up to 10 days at 30° C and examined daily for color change.

**Hemicellulose Utilization.** Isolation of hemicellulose utilizing mutants was attempted by trying to detect high acid producing mutants, as was done for starch. 1% (w/v) crude hemicellulose replaced the soluble starch and after 10 days the
pH was measured.

**Amino Acid Decarboxylation.** *L. plantarum* 32406-2 was examined for the possible presence of amino acid decarboxylases using a modification of the urease test of Hylemon, *et al.* (1973). The test medium consisted of 0.1065% (w/v) BES buffer [N,N bis (2-hydroxyethyl) 2-aminoethane sulfonic acid, Sigma Chemical Co.], 2.0% (w/v) amino acid, and 0.001% (w/v) brom thymol blue, adjusted to pH 6.5. Cells were grown overnight in APT medium with 0.10-0.17% (w/v) of the amino acid to be tested, centrifuged, washed in BSG and resuspended in 5.0 ml BSG. 0.5 ml of this suspension was added to 2.0 ml of the corresponding amino acid test medium and incubated at 37° C for 48 hours. Cultures were examined at 24 and 48 hours for any decarboxylase activity. A change in color from green to blue is a positive reaction for decarboxylation. Positive controls were *Escherichia coli* ATCC 9637, lysine decarboxylase positive (Orskov, 1974) and *Lactobacillus* 30a, histidine decarboxylase positive (Rodwell, 1953). Negative controls were the test medium without added amino acid and uninoculated test medium.

The amino acids chosen were those in highest concentration in alfalfa as determined by Loper, *et al.* (1963). Those chosen were; lysine, glutamine, leucine, isoleucine, valine, histidine, glutamic acid, alanine, arginine, methionine, proline, threonine, serine, aspartic acid, glycine, and
phenylalanine.

Growth at High Temperature. To determine maximum growth temperature for strain 32406-2, 5.0 ml APT broth medium (preheated to the incubation temperature) was inoculated and the culture was incubated at various temperatures (40°-45° C) for 48 hours. The maximum temperature for growth was 44° C. A mutagenized culture was streaked on APT solid medium preheated to 46° C for 24 to 36 hours. Colonies were picked and restreaked on APT solid medium and incubated at 30°, 46°, and 48° C. Colonies showing growth at 46° C were picked and suspended in 1.0 ml BSG buffer. 0.1 ml of of this suspension was used to inoculate replicate tubes of 5.0 ml APT broth medium and incubated at 30° and 46° C for six days. Growth was followed turbidimetrically. During the inoculation the medium was kept at 46° C by placing test tubes in a block heater (Laboratory Supplies Co.).

Oxygen Resistance. APT solid medium with and without 710 micromoles Mn+2 was streaked with a mutagenized culture and incubated under 100% oxygen (1 atm.), room atmosphere, and anaerobically. In separate trials under identical conditions, 0.05 ml of an undiluted mutagenized culture was spread on the plates. To create a 100% O₂ atmosphere plates were placed in a BBL anaerobe jar equipped with a gas valve on the lid. After the lid was secured the jar was evacuated and refilled with oxygen. This procedure was done three times. Anaerobic
conditions were achieved using a BBL Gas Pak anaerobic system. Plates streaked or spread with 0.05 ml with an unmutagenized culture of strain 32406-2 served as controls. Isolation of oxygen resistant mutants was also performed using a modification of the procedure of Gregory and Fanning (1983). 0.1 ml of mutagenized and unmutagenized cultures of strain 32406-2 was spread on APT agar medium with and without 710 micromoles Mn+2. These plates were placed in a stainless steel vessel, pressurized to 100 psi (5 atm), and incubated at 30° C for 24 and 48 hours. After the incubation the plates were removed and incubated at 30° C at room atmosphere for 24-36 hours and examined for colony formation.

**Plumbagin Resistance.** Plumbagin resistance was examined since this compound is known to produce superoxide radicals in *L. plantarum* strains (Archibald and Fridovich, 1981). Therefore, if any mutants are resistant to plumbagin they may be resistant to superoxide radicals. *L. plantarum* 32406-2 was tested for its sensitivity to plumbagin by streaking on the plumbagin medium and incubating under aerobic and anaerobic conditions. The same medium was then streaked with a mutagenized culture and incubated aerobically for 36 to 48 hours. Colonies were picked and restreaked on the same medium to ensure purity. A colony of each strain was picked and grown in APT broth
medium and stored at 0° C until needed.

Organic Acid Utilization. To measure organic acid utilization, 50 mM of citrate, malonate, or malate replaced glucose in APT medium and pH adjusted to 6.4. Controls were APT medium with 55 mM glucose or no carbon source added. A 2% (v/v) inoculum of L. plantarum 32406-2 was added and growth was followed spectrophotometrically by taking measurements every twelve hours. Cultures were incubated for 48 hours.

Citrate Sensitivity. Preliminary experiments showed possible growth inhibition of strain 32406-2 when citrate (final concentration, 0.05 M) was added to APT medium containing 55 mM glucose and 0.71 mM Mn(II). Since citrate has been shown to inhibit growth of other lactobacilli (Campbell and Gunsalus, 1944), this behavior was examined more closely. An inoculum of L. plantarum 32406-2, after being resuspended in APT broth medium without added glucose, magnesium or manganese, was diluted to an optical density of 0.85 (640 nm). 0.1 ml of this suspension was added to APT broth medium containing 55 mM glucose, 0.71 mM Mn (II), and either 0.1 M or 0.05 M citrate. Medium without citrate served as the control. Optical density was measured hourly.

Citrate Resistance. Citrate resistant mutants were isolated using the same procedure as for plumbagin resistance. APT agar medium containing 55 mM glucose and 0.1 M citrate was used to select mutants.
Mutant Testing.

Efficiency of Plating. After mutants were isolated and purified each strain was tested for its efficiency of plating (EOP) on APT agar medium (control, 1.00 EOP) and APT agar containing either plumbagin or citrate. Strains were grown overnight to a density of approximately 1.5 to 2.0 x 10^8 cells/ml in APT medium at 30° C. 0.1 ml of a 10^6 dilution was spread on the selective and nonselective media in triplicate. Nonselective plates were incubated for 24 to 36 hours and selective plates for 48 to 52 hours. Selective plates which showed no colony formation at this time were allowed to incubate for four days before discarding.

Oxygen Sensitivity. Selected cultures of mutant strains were tested for colony formation following exposure to 100 psi oxygen (5 atm) when starved for manganese. These tests were run concurrently with the isolation of O_2 resistant mutants by growth under 100 psi oxygen. Each strain was grown overnight in 5.0 ml APT medium containing 55 mM glucose with and without 710 micromoles Mn^{2+}, centrifuged, washed once in BSG and resuspended in APT medium lacking glucose, magnesium, and manganese. 0.1 ml of this suspension was used to inoculate the same medium and cultures were grown to approximately 10^8 cells/ml at 30° C and room atmosphere. 1.0 ml of each culture was placed in a 16 X 125 mm pyrex test tube with a loose fitting cap and the tube placed
in a stainless steel pressure vessel. Then the vessel was pressurized to 100 psi with pure oxygen. Colony forming units per ml was determined from the remaining 4.0 ml of culture. The cultures were incubated without shaking for 24 hours after which the vessel was depressurized, serial dilutions made, and cfu/ml determined in duplicate. Strains 32406-2 and 232 were also examined under identical conditions except glucose concentration was reduced to 27.5 mM.

Citrate Utilization. All mutant strains were examined for possible citrate utilization as described previously for organic acid utilization.

Cadmium Sensitivity. To determine the optimum cadmium concentration and duration of incubation for testing, sensitivity of the strains was first examined by minimum inhibitory concentration tests. Cadmium was added to APT broth medium in 13 X 100 pyrex test tubes to a final concentration of 2.0 X 10\(^{-4}\) M and two-fold dilutions made (the lowest being 3.125 X 10\(^{-6}\) M). A 2% (v/v) inoculum of the strains was added and the tubes were examined visually at 12 hour intervals for three days. Cultures grown with 1 X 10\(^{-4}\) M exhibited the greatest variation in turbidity, evident after 24 hours. Therefore, the strains were tested for growth inhibition at this concentration. The control was APT liquid medium without cadmium. The inoculum was added to 5.0 ml of the test medium and degree of sensitivity was
determined by comparing the optical density of the cadmium treated culture to that of the control after a 24 hour incubation period at 30°C. All tubes were vortexed periodically throughout the incubation period.

EDTA Sensitivity. EDTA sensitivity was examined using the same procedure as cadmium sensitivity with the following modifications. For minimum inhibitory concentration tests, .025 M EDTA was added to APT broth medium and two-fold dilutions made (lowest concentration was 7.8 X 10^{-4} M). The largest variation in turbidity between the strains was observed at 6.25 X 10^{-3} M EDTA after 48 hours of incubation at 30°C. Therefore, EDTA sensitivity was measured at this concentration after a 48 hour incubation period.

Oxygen Consumption. Oxygen consumption was measured using a modification of the procedure of Yousten, et al. (1975). A 5% (v/v) inoculum was grown overnight, centrifuged and washed once in BSG. It was resuspended in 3.0 ml APT medium lacking glucose, magnesium and manganese. This suspension was added to 250 ml APT broth medium with 0.5% glucose in a 2 liter flask. Cultures were grown with shaking (New Brunswick shaking water bath Model G76 at 120 rpm) at 30°C and cells were harvested in late stationary phase, centrifuged, washed once with 50 mM K_2HPO_4 buffer (pH 7.0) and resuspended in 5 mM K_2HPO_4 buffer (pH 7.0). Oxygen consumption of cells in a Gilson reaction vessel was measured
with a YSI (Yellow Springs Instrument Co.) oxygen monitor Model 53 and recorded on a Fisher Recordall Series 5000. The reaction chamber was maintained at 30°C. Following equilibration and measurement of endogenous O₂ uptake (6 min), 16 mM glucose (final concentration) was added to the cell suspension. Values reported are for the rates of O₂ uptake during the 2-4 minute period after the initiation of the reaction. KCN and plumbagin (1.9 and 5.9 mM final concentrations) were added 6 minutes after the addition of glucose (in separate tests) and rates derived during the 2-4 minute period following the initiation of the reaction. All values reported were corrected for endogenous uptake. Growth of the cultures was followed spectrophotometrically (640 nm) by taking samples from the growth flasks at 0, 2, 4, and 6 hours and hourly thereafter. Dry weights were calculated in triplicate after heating at 110°C for 48 hours.

Lactic and Acetic Acid Determination. Concentrations of organic acids were determined by high pressure liquid chromatography (HPLC; Dr. R. M. Smibert, personal communication). Supernatants from the centrifuged cell suspensions employed for oxygen consumption tests were collected, acidified with 50% H₂SO₄ and absorbed onto a Clin Elut CE 1001 column (Analytichem International, Inc.) and organic acids extracted with methyl tert butyl ether. The acids were then separated from the ether using 0.2 N NaOH and 20 microliters of the NaOH-extract was injected on
a Bio Rad Aminex ion exclusion column Model HPX - 87H (300 x 7.8 mm) which was immersed in a flowing water bath set at 40° C. Pressure on the column was supplied by a Waters pressure pump set at 1500 psi with a flow rate of 0.8 mm/min. 5 % acetonitrile in .013 N H₂SO₄ was used as the carrier. Concentration of organic acids was measured by absorbance at 214 nm using a Bio Rad UV monitor model 1305 and recorded on a Hewlett Packard integrator model 3380 A.

Manganese Transport. Transport of ⁵⁴Mn was carried out using a modified procedure of Archibald and Duong (1984). 100 ml of APT broth medium lacking manganese and Tween 80 in a 500 ml flask was inoculated with an initial culture absorbance of 0.11 to 0.15. Cultures were incubated for 12 hours in a New Brunswick shaking water bath (Model G76) at 120 rpm. Final culture absorbance was 1.2 to 1.5 at 640 nm. 10 ml of the culture was centrifuged, washed in BSG and resuspended in the same volume of APT broth medium without manganese or Tween 80 and this suspension was added to 100 ml of the same APT medium. This culture was grown under the same conditions as the first and after 10 hours the absorbance leveled off to .32 to .49 with a total cell count of 2.3-3.8 X 10⁸ cells per ml. The cultures were centrifuged, washed in BSG and concentrated 20X in APT salts buffer (Archibald and Duong, 1984) and placed on ice.

To measure manganese uptake, 1.0 ml of the reaction
medium was placed in a 16 x 125 pyrex glass test tube in a 30°C water bath. Reaction media consisted of either APT salts buffer, APT salts buffer with 55 mM glucose and APT salts buffer with 55 mM glucose and 20 mM citrate. 10 microliters of the cell suspension was added to the reaction medium and allowed to equilibrate for 10 minutes and at this time 0.22 micromoles $^{54}$MnCl$_2$ (New England Nuclear, 34.29 mc/mg) was added. For some tests an equimolar mixture of $^{54}$Mn and Mn(II) was added. 50 microliters was removed at 0 and 30 seconds and the cells collected by filtration (Millipore 0.2 microns, 25 mm dia.). Filters were rinsed immediately with 1.0 ml APT salts buffer at 30°C and placed in vials for counting. Total counts were measured by counting 50 microliters of unfiltered cell suspension. Filters through which either cells alone or 50 microliters of cell free, $^{54}$Mn-containing medium was passed served as controls. $^{54}$Mn was counted on a Beckman Biogamma II counter.

Silage Tests. Alfalfa at 10% bloom was harvested with a Gravely mower and shredded with an Ohio shredder to approximately 25 mm lengths. After shredding 9.1 kg was placed in a polyethylene bag and inoculated with $10^6$ cells/g of crop by spraying a cell suspension on the crop. The crop was mixed thoroughly and hand packed in six small silos. The silos were placed indoors at an ambient temperature of 24°C to 26°C. The
material was allowed to ferment for six weeks. Dry matter content of the fresh crop was determined by taking samples and drying them at 100° C for 36 hours (Cullison, 1975).

Inoculum was prepared as discussed in growth conditions and resuspended in M/15 phosphate buffer, pH 7.0. The small silos were 4 liter circular cartons lined with two plastic bags. After filling each bag was sealed separately and tops were taped on the silo.

After the fermentation period the silos were opened and the top 8 cm of silage discarded. Samples were removed for dry matter content analysis. 25 g were removed and blended in 225 ml of distilled deionized water for 2 to 3 minutes. The suspension was then filtered through 4 to 5 layers of cheesecloth and pH readings taken and titratable acidity determined on aqueous extract. 10 ml of the extract was filtered (Millipore 0.45 microns, 25 mm dia.) and the filtrate acidified with 50% H₂SO₄. Concentrations of lactic, acetic and butyric acids were determined on that acidified extract by HPLC as discussed previously, except that the extraction procedure is omitted. Silage quality was determined by titratable acidity (Lesin and Schultz, 1968), the loss of dry matter, pH, and organic acid analysis (McDonald, et al., 1965; Ohyama, et al., 1975).
RESULTS

Isolation of Mutants

Starch and Hemicellulose Utilization. The average colony diameter of the mutagenized and unmutagenized cultures of *L. plantarum* strain 32406-2 when streaked on APT medium containing glucose, starch, or carbon source was 3.0 mm, 2.0 mm, and 1.5 mm, respectively. No large colonies that would indicate starch degraders were evident. The selection test was repeated again with negative results. Since so few single colonies were examined by streaking the cultures on plates (about 50-75), 0.1 ml of a $10^4$ dilution of the mutagenized and unmutagenized cultures of strain 32046-2 were spread on the same agar media. This resulted in approximately $10^4$ cells being plated. Confluent background growth was seen with both cultures after 24-36 hours incubation at 30° C. No larger colonies which could indicate starch utilization were seen. Approximately $10^8$ cells were used to inoculate media for isolation of high acid producing mutants, but this method also failed to isolate any starch degraders. In the APT medium with 1% glucose a deep yellow color appeared after 24 hours. In the medium supplied with 1% soluble starch there was no difference between the mutagenized and unmutagenized cultures even after 10 days incubation. Both showed some acid production probably due to mono- or disaccharides which may have
contaminated the soluble starch.

Results for the isolation of hemicellulose utilizers by acid production was the same as the starch utilization results. No color change was evident in the medium supplied with 1\% (w/v) hemicellulose. After the 10 day incubation period, the pH of the 1\% glucose medium was approximately 3.5, whereas in the hemicellulose-containing medium and that lacking carbon source was 6.1.

Amino Acid Decarboxylation. Strain 32406-2 did not exhibit decarboxylase activity with any of the amino acids tested. By contrast, Lactobacillus 30a and Escherichia coli strain ATCC 9637 decarboxylated histidine and lysine, respectively. Lactobacillus 30a also showed some decarboxylase activity when incubated with lysine. This is in agreement with the results of Rodwell (1953).

Growth at High Temperature. L. plantarum strain 32406-2 grew at 43° C but not at 45° C. Of 25 isolates able to grow on APT medium incubated at 46° C, none grew at 48° C. All isolates grew at 46° C in a repeated test on APT agar medium, but when these isolates were incubated at 46° C in APT liquid medium no growth was evident.

Oxygen Resistance and Growth on Limited Mn(II). The average colony diameter of the unmutagenized culture of strain 32406-2 on APT medium without added manganese was 1.0 mm when incubated in
hyperbaric O₂ or room atmosphere. Under anaerobic conditions the diameter was larger (1.5-2.0 mm). In medium containing 710 micromoles Mn(II), colony diameter was 2.0 mm when grown in hyperbaric O₂ or room atmosphere and 2.5 mm under anaerobic conditions. No larger colonies (compared to majority) were evident in the mutagenized culture. The number of single colonies observed from both cultures was 50-75. Spreading 0.05 ml of the undiluted cultures on APT medium also failed to isolate any oxygen resistant mutants because confluent growth was observed after 24 hours incubation at 30°C in hyperbaric oxygen. Interestingly, no growth was evident on plates incubated at 5 atmospheres O₂ even after 48 hours incubation at 30°C. However, when plates were removed from the pressure vessel and incubated at at room atmosphere at 30°C, both mutagenized and unmutagenized cultures showed confluent growth at approximately 24 hours.

Plumbagin Resistance. The initial tests showed that colony formation of *L. plantarum* strain 32406-2 was prevented by 0.14 mM plumbagin. Plumbagin at that same concentration did not prevent colony formation under anaerobic conditions. 13 plumbagin resistant (PluR) strains were isolated from the mutagenized culture of *L. plantarum* strain 32406-2. All were tested and had the same characteristics as the parent.

Organic Acid Utilization. None of the organic acids tested
were utilized for growth of *L. plantarum* strain 32406-2.

Cultures grown in APT medium containing citrate, malate, or malonate (replacing glucose) reached an absorbance of approximately 0.30 to 0.37 after 12 hours incubation at 30° C which was the same turbidity of cultures which contained neither glucose nor organic acid (0.30-0.40). No increase in absorbance was seen after 48 hours. In contrast, the optical density of cultures with 1% glucose added reached an absorbance of 1.5 after 12 hours and continued to increase to values between 4.0 to 5.0 after 48 hours at 30° C. It is not surprizing that growth occurred in APT medium lacking glucose or organic acids because the medium contains yeast extract.

Citrate Sensitivity. In cultures of strain 32406-2 containing 55 mM glucose and 50 mM citrate, growth as reflected by increases in absorbance at 640 nm was below that of glucose grown cells. Growth inhibition was not evident when glucose was omitted. Since there is evidence that this growth inhibition is due to the chelation of manganese by citrate (MacLeod and Snell, 1947) and manganese affords oxygen resistance to *L. plantarum* strains (Archibald and Fridovich, 1981), I felt that citrate may be involved in oxygen sensitivity. Therefore, citrate sensitivity was examined more closely. Citrate sensitivity is illustrated in Figure 1. Inhibition is evident at 5 hours incubation at 30° C in APT medium containing 55 mM glucose, 0.71
mM Mn (II), and 0.1 M citrate and at 7 hours with 0.05 M citrate. A sharp increase in optical density of the culture containing glucose and 0.05 M citrate starts at 11 hours (Figure 1). Cell clumping was apparent in the culture containing glucose and 0.1 M citrate.

Citrate Resistance. Initial tests showed that 0.1 M citrate completely inhibited colony formation of _L. plantarum_ strain 32406-2 on APT agar medium containing 55 mM glucose and 0.71 mM Mn (II). 13 citrate resistant (CitR) strains were isolated from the mutagenized culture of strain 32406-2 streaked on 0.1 M citrate. All isolates were tested and identified as _L. plantarum_, identical to the parent.

Mutant Characterization

Efficiency of Plating. Results from efficiency of plating on 0.14 mM plumbagin are presented in Table 1. Of the 13 PluR strains originally recovered, some were not as resistant as others (Table 1). Some CitR isolates (MC226, MC229, and MC232) were plumbagin resistant.

The effect of 0.1 M citrate on colony formation is presented in Table 1. Of the 13 original CitR isolates strains MC233 and MC235 were not as resistant as others. The PluR isolate, strain MC210 was also citrate resistant.

Citrate Utilization. Growth of none of the 26 mutants
Figure 1. Citrate sensitivity in *L. plantarum* strain 32406-2. Absorbance was read on a Bausch and Lomb Spectronic 20. Values are the mean of three replicates.
### Table 1. Efficiency of Plating on Plumbagin and Citrate of Parent Strain 32406-2 and isolated mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plumbagin Efficiency</th>
<th>Plumbagin Concentration</th>
<th>Citrate Efficiency</th>
<th>Citrate Concentration</th>
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<td>32406-2</td>
<td>&lt;.01</td>
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<td>.68b</td>
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<td>&lt;.01</td>
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<td>.77b</td>
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<td>.86b</td>
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<sup>a</sup>Efficency of plating is calculated from the ratio of colony counts on selective versus nonselective media. Values are the mean ± s.d. of 3 assays, each assay done in triplicate.

<sup>b</sup>These strains exhibited resistance in one assay. In later assays the efficiency of plating was <.01.
isolated was stimulated by citrate as a carbon source. Results from these tests are similar to the earlier test of organic acid utilization. Optical density of the cultures grown with 50 mM citrate or without carbon source reached a maximum absorbance of 0.32 to 0.40 after 12 hours incubation at 30° C and did not increase.

**Oxygen Sensitivity.** The Plu\textsuperscript{S} Cit\textsuperscript{S} strain 32406-2 (parent), the Plu\textsuperscript{R} Cit\textsuperscript{S} strains MC204 and MC211, the Plu\textsuperscript{R} Cit\textsuperscript{R} strains MC229 and MC232, and the Plu\textsuperscript{S} Cit\textsuperscript{R} strain MC228 showed no differences in cfu/ml when grown under 100 psi oxygen at 30° C for 24 hours at low Mn(II).

**Cadmium Sensitivity.** As seen in Table 2, three groups of mutants can be identified on the basis of the ratio between the absorbance of the cadmium containing cultures to that of the control. The Plu\textsuperscript{S} Cit\textsuperscript{S} strain 32406-2 and the Plu\textsuperscript{S} Cit\textsuperscript{R} strains MC225 and MC234 are the least sensitive as exemplified by an absorbance ratio of 0.45 or greater. The Plu\textsuperscript{R} Cit\textsuperscript{S} strain MC211 and the Plu\textsuperscript{S} Cit\textsuperscript{R} strains MC230, MC231, and MC236 are intermediate with ratios of 0.29, 0.27, 0.29, and 0.30, respectively. Ratios of cadmium-containing cultures to control cultures of the remaining strains are 0.12 or less and thus show the greatest sensitivity to cadmium. Of the six mutants which were more resistant to cadmium than the others, four were also EDTA-resistant.
Table 2. Cadmium and EDTA sensitivity of Parent Strain 32406-2 and isolated mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cadmium</th>
<th>EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>32406-2</td>
<td>0.55±0.14</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td>MC201</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>MC202</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MC203</td>
<td>0.12±0.01</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>MC205</td>
<td>0.05±0.02</td>
<td>0.05±0.02</td>
</tr>
<tr>
<td>MC206</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MC207</td>
<td>0.07±0.06</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>MC208</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MC209</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MC210</td>
<td>0.04±0.02</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>MC211</td>
<td>0.29±0.02</td>
<td>0.14±0.02</td>
</tr>
<tr>
<td>MC212</td>
<td>0.02±0.01</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>MC213</td>
<td>0.09±0.05</td>
<td>0.07±0.00</td>
</tr>
<tr>
<td>MC225</td>
<td>0.61±0.07</td>
<td>0.27±0.19</td>
</tr>
<tr>
<td>MC226</td>
<td>0.10±0.01</td>
<td>0.08±0.02</td>
</tr>
<tr>
<td>MC227</td>
<td>0.09±0.01</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>MC228</td>
<td>0.09±0.00</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>MC229</td>
<td>0.11±0.02</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>MC230</td>
<td>0.27±0.09</td>
<td>0.21±0.03</td>
</tr>
<tr>
<td>MC231</td>
<td>0.29±0.11</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>MC232</td>
<td>0.09±0.02</td>
<td>0.28±0.08</td>
</tr>
<tr>
<td>MC233</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MC234</td>
<td>0.45±0.12</td>
<td>0.29±0.01</td>
</tr>
<tr>
<td>MC235</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MC236</td>
<td>0.30±0.16</td>
<td>0.08±0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values (average ± range) calculated from the ratio of absorbance (640 nm) of cadmium- and EDTA-containing cultures versus control for duplicate or triplicate assays. Each assay done in duplicate.

<sup>b</sup>Not determined.
EDTA Sensitivity. Using the same ratio of absorbance as for cadmium sensitivity, it can be seen in Table 2, 3 groups of mutants are evident. The least sensitive strains exhibit an absorbance ratio of 0.21 or greater. All of these strains (MC225, MC230, MC232, and MC234) are Cit\textsuperscript{R} mutants. One of these, strain MC232, is also plumbagin resistant.

\textit{O}_2 Consumption, Lactic and Acetic Acid Production. \textit{Results from duplicate tests measuring \textit{O}_2 consumption and lactic and acetic acid production are presented in Table 3. Strains MC210 and MC226 were chosen for examination since both are Plu\textsuperscript{R} and Cit\textsuperscript{R}. Peak optical densities (representing late log phase of growth) for the duplicate tests were 3.0 and 3.5 for MC210, 3.2 and 3.4 for 32406-2, and 2.5 and 2.8 for MC226. Though strains 32406-2 and MC210 show a higher rate of \textit{O}_2 consumption than strain MC226, the latter strain produced about 2 times more lactic acid. Not surprisingly, strain MC226 produced less acetic acid. Low levels of formic acid were found only in supernatants of cultures of strains 32406-2 and MC210. Lactic, acetic, and formic acids were not detected in sterile medium. Preliminary results of oxygen consumption and acid production by strain MC232 revealed an \textit{O}_2 consumption rate of 6.7 nm min\textsuperscript{-1} mg\textsuperscript{-1}DCW and the production of 7.4 mM and 1.0 mM of lactic and acetic acids, respectively.}

The addition of cyanide had no effect on the glucose-
Table 3. Oxygen uptake and production of lactic and acetic acids by strains 32406-2, MC210, and MC226.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>( \text{O}_2 ) Consumption(^a)</th>
<th>Lactic acid</th>
<th>Acetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>32406-2</td>
<td>Plu(^S) Cit(^S)</td>
<td>13.4 ± 1.6(^b)</td>
<td>5.1 ± 0.5(^c)</td>
<td>3.2 ± 1.0</td>
</tr>
<tr>
<td>MC210</td>
<td>Plu(^R) Cit(^R)</td>
<td>15.2 ± 3.4</td>
<td>5.7 ± 0.2</td>
<td>4.0 ± 0.9</td>
</tr>
<tr>
<td>MC226</td>
<td>Plu(^R) Cit(^R)</td>
<td>5.7 ± 1.4</td>
<td>8.8 ± 1.1</td>
<td>1.4 ± 0.7</td>
</tr>
</tbody>
</table>

\(^a\) Nanomoles \( \text{O}_2 \) min\(^{-1}\) mg DCW\(^{-1}\).

\(^b\) Average ± range of two assays each run in triplicate.

\(^c\) Average ± range of two assays each run in duplicate.
stimulated oxygen consumption rate. However, the addition of plumbagin increased $O_2$ consumption in all three strains. The increase in strains MC200 and MC210 could not be calculated since the consumption rate was already high. In strain MC226 plumbagin tripled the $O_2$ consumption rate. Preliminary results with strains MC204, MC231, and MC232 also showed a 3-fold increase in oxygen consumption after plumbagin addition.

Manganese Accumulation. Table 4 shows that strain 32406-2 accumulated more $^{54}\text{Mn}$ in 30 seconds than did strains MC210 or MC226. The addition of 20 mM citrate reduced manganese accumulation by strains 32406-2 (68%) and MC210 (70%), but was less inhibitory to strain MC226 (56%). No further conclusions can be drawn because of the rather wide variation. The addition of equimolar concentrations of nonradioactive manganese decreased the accumulation of $^{54}\text{Mn}$ by one half.

Attempts were made to measure $^{54}\text{Mn}$ uptake by strains MC229 and MC232, but severe cell clumping resulted when the strains were grown under manganese limitation. Efforts to break up the clumps using a Branson ultrasonicator proved unsuccessful.

Silage Analysis. Results from the inoculation of alfalfa for silage production are presented in Table 5. There was no significant difference in dry matter content between the control and any of the inoculated silages following 6 weeks incubation, but all of the silages inoculated with \textit{L. plantarum} strains
Table 4. Comparison of $^{54}$Mn accumulation with and without added citrate on strains 32406-2, MC210, and MC226.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>no citrate</th>
<th>20mM citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>32406-2</td>
<td>Plu&lt;sup&gt;S&lt;/sup&gt;  Cit&lt;sup&gt;S&lt;/sup&gt;</td>
<td>18.0 ± 2.0</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>MC210</td>
<td>Plu&lt;sup&gt;R&lt;/sup&gt;  Cit&lt;sup&gt;R&lt;/sup&gt;</td>
<td>13.2 ± 2.5</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>MC226</td>
<td>Plu&lt;sup&gt;R&lt;/sup&gt;  Cit&lt;sup&gt;R&lt;/sup&gt;</td>
<td>8.8 ± 1.3</td>
<td>3.9 ± 1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Per cent of total $^{54}$Mn accumulated after 30 sec. incubation. Values are the average ± range of duplicate or triplicate assays, each done in triplicate, and normalized to the same number of cells.

<sup>b</sup>See material and methods for media preparation.
Table 5. Dry matter, pH, and titratable acidity of silages after 6 weeks of fermentation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%DM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pH&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Titratable acidity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh alfalfa</td>
<td>46.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.4 ± .05</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>39.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.0 ± .04</td>
</tr>
<tr>
<td>32406 - 2</td>
<td>38.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.58</td>
<td>1.6 ± .10</td>
</tr>
<tr>
<td>MC210</td>
<td>38.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.68</td>
<td>1.5 ± .10</td>
</tr>
<tr>
<td>MC226</td>
<td>42.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.58</td>
<td>1.6 ± .03</td>
</tr>
</tbody>
</table>

<sup>a</sup>Analysis by Duncan's multiple range test.
<sup>b</sup>Values are the mean ± s.d. of the amount of 0.1 N NaOH (ml) required for neutralization.
<sup>c</sup>,<sup>d</sup>,<sup>e</sup>,<sup>f</sup>,<sup>g</sup>Means in the same column having different superscripts were significantly different (p<.01).
exhibited a lower pH (Duncan's multiple range test, p < .01). Further, the inoculated silages exhibit a higher titratable acidity than the control. No significant difference in pH or titratable acidity was seen between L. plantarum strains. The final concentrations of lactic and acetic acids are shown in Figure 2. The silage inoculated with strain 32406-2 showed a high acetic acid concentration and a low lactic acid concentration. The silages produced by inoculation of strains MC210 and MC226 also showed higher acetic acid production, although with MC210 it is less pronounced (Figure 2.). Butyric and isobutyric acid production (70 to 52 g/kg DM) was detected in all silages, but there were no differences between silages (data not shown). Small amounts of propionic and isovaleric acids were also present, but again no significant differences in concentrations were seen between the uninoculated and the inoculated silages.
Figure 2. Concentration of lactic and acetic acids in silages after 6 weeks of fermentation. A-uninoculated, B-inoculated with strain 32406-2, C-inoculated with strain MC210, D-inoculated with strain MC226.
DISCUSSION

Mutant Isolation

Starch and Hemicellulose Utilization. Two problems are evident in the procedure of isolation of starch and/or hemicellulose utilizing mutants. The background growth that resulted from spreading 0.1 ml of a $10^{-4}$ dilution of the cultures on the APT agar medium with 1% soluble starch prevented the screening of a large number of cells. Consequently, the isolation of starch utilizing mutants may have failed because an insufficient number of cells was screened. It should be noted, however, that approximately $10^8$ cells were added to the APT broth media containing 1% soluble starch or 1% crude hemicellulose when isolation by acid production was attempted. Still, no high acid producing mutants were recovered. Because the concentration of EMS was 10 times lower than recommended, it may be that the mutant frequency was too low to isolate starch- or hemicellulose-utilizing mutants.

On the other hand, if _L. plantarum_ 32406-2 does not possess the genetic capacity to produce enzymes for starch or hemicellulose hydrolysis then no mutagenic treatment will induce this action. The results presented here do not rule out the possibility of starch and hemicellulose hydrolysis by bacterial action, as other organisms in silage may have this capacity.
Amino Acid Decarboxylation. *L. plantarum* did not decarboxylate amino acids and therefore mutagenesis to isolate a decarboxylaseless mutant was not necessary to reduce buffer formation during fermentation.

Growth at High Temperatures. The failure to isolate true mutants able to grow above 45° C might have required an alteration of various metabolic pathways so that their reactions could occur at elevated temperatures. Although mutagenesis may alter any number of enzymes so their reaction temperature is elevated, the failure of one key enzyme to function at that temperature will restrict growth of the organism. Perhaps a better method of isolating high temperature resistant mutants would be to screen a larger number of colonies over a smaller, stepwise gradation of temperatures.

The fact that the isolates grew at 45° C on agar medium but not in APT liquid medium presents an interesting paradox. As pointed out by Brock (1979), minimum and maximum growth temperatures are not completely fixed and can be modified by environmental factors. Since the cultures in liquid medium were grown without shaking it is probable that the cells were exposed to less air than when grown on agar medium. Hence, exposure to air may have played a role in this phenomenon.

Oxygen Resistance. Since differences were not observed
in colony diameter between suspensions of cells grown in hyperbaric oxygen and room atmosphere, high O₂ concentration had little effect on final cell yield (though perhaps not growth rate). A likely explanation is that the high glucose concentration (1%) prevents oxygen uptake as observed by Gregory and Fridovich (1975) with another strain of *L. plantarum*. *L.* *plantarum* consumes oxygen in appreciable amounts only when the carbohydrate source is limited (Dirar and Collins, 1973; Yousten, *et al.*., 1975). Therefore, if glucose is readily available to the cell (as in this case and that of Gregory and Fridovich, 1975), oxygen will not be consumed and toxic oxygen compounds (i.e. O₂⁻ and H₂O₂) will not be produced to any great extent. Since the glucose concentration was not limiting in the growth medium used, the failure to isolate O₂-resistant mutants could have been due to an absence of respiration and production of toxic oxygen products. The fact that the cultures grown anaerobically did exhibit a slightly larger average colony diameter shows that oxygen did limit growth to some extent. Manganese limitation caused a smaller colony diameter in both the aerobic and anaerobic cultures possibly because it is a cofactor for a number of enzymes (Stettler and Kandler, 1973; Kono and Fridovich, 1983; Wright and Klaenhammer, 1983).

It is interesting to note that when isolation of O₂ resistant mutants was attempted under 100 psi (5 atm) oxygen, no
growth was evident on the agar plates even after 48 hours incubation. However, when the agar plates were removed from the pressure vessel and incubated at room atmosphere (30° C), confluent growth was seen in 24 hours. Therefore, it seems high oxygen concentrations inhibited the growth of \textit{L. plantarum} strain 32406-2, but had little effect on the viability of the cells. This is in agreement with Archibald and Fridovich (1981) who found that \textit{L. plantarum} strains ATCC 8014 and ATCC 14917 showed little growth at 5 and 10 atmospheres of pure oxygen but also showed very little loss of viability.

Mutant Characterization

In the following discussion, all tests performed on \textit{L. plantarum} strain 32406-2 and mutant strains will be described and the possible basis for each characteristic (i.e. resistance or sensitivity to plumbagin, citrate, cadmium, and EDTA) will be presented. This is followed by a discussion of the strains as classified by observed phenotypes in Table 6.

Plumbagin Resistance. Plumbagin resistance could be caused by a number of different alterations. (1) A mutation could occur in the diaphorase which reduces plumbagin. Its activity may be completely lost or reduced to such an extent that little $O_2^-$ is produced and there is no growth inhibition. (2) Plu$^R$ mutants may have a higher concentration of polyphosphate or pyrophosphate
which allows them to accumulate more manganese than the parental strain. Archibald and Fridovich (1982) reported that manganese in *L. plantarum* is bound to polyphosphate. They also found O$_2^-$-scavenging ability was similar between crude cell extracts and prepared manganese polyphosphate and the scavenging activity of the manganese polyphosphate preparation was increased when pyrophosphate was added. (3) Plu$^R$ mutants could transport Mn(II) or other cations able to substitute for Mn (II) (e.g. Mg$^{+2}$) at a rate higher than Plu$^S$ strains. (4) They could be impermeable to plumbagin. The fact that plumbagin-resistance in strains MC201, MC202, MC208, and MC209 was not reproducible may mean that these strains have reverted to the wild type genotype since EMS causes transition base-pair substitutions. It is known that mutants which revert to the wild type generally have base-pair substitutions (Fincham, 1983). On the other hand, they may be intermediate in their resistance. That is, they are more plumbagin-resistant than the parent strain 32046-2 but less resistant than other plumbagin resistant strains (Table 1).

Organic Acid Utilization. Since none of the strains could utilize the organic acids tested, their inoculation in forage should not increase the buffering capacity in silage by the formation of neutral end products. Although the *L. plantarum* strains tested in this study did not utilize malate as a carbon source, some *L. plantarum* strains have been shown to possess a
malate decarboxylase (McDonald, 1981; Daeschel, et al., 1984). This will cause buffering in silage by a release of neutralizing cations (McDonald, 1981). Since I did not test for this activity, the absence of buffering increases in silages inoculated with any of these strains cannot be assumed.

Citrate Sensitivity. As seen in Figure 1, when *L. plantarum* strain 32406-2 was grown on .05 M citrate, inhibition lasted for approximately 11 hours at which point growth increased. This could be due to lactic acid production since metalocplexes of citrate decrease in stability as pH decreases (MacLoed and Snell, 1947). Cell clumping rather than lysis, seen in the 0.1 M citrate culture may explain the drop in optical density seen at 8 hours.

Citrate Resistance. *Lactobacillus plantarum* has been shown to possess a specific, active transport system for manganese (Archibald and Duong, 1984). Therefore, CitR mutants may have a lower Km for manganese transport than strain 32406-2 and thus transport more manganese when grown in the presence of citrate.

MacLoed and Snell (1950) reported growth inhibition due to manganese limitation in some lactobacilli can be counteracted by the addition of magnesium, calcium, or strontium to the medium. This could be due to a release of Mn(II) from chelators in the medium, though not all the divalent cations could relieve Mn(II) limitation in all organisms (MacLoed and Snell, 1950). In their
study, MacLoed and Snell (1950) found that magnesium or calcium, but not strontium, could relieve manganese limitation in *Lactobacillus pentosus* whereas in *L. arabinosis* all three divalent cations separately could relieve manganese-limitation. In *Lueconostoc mesenteroides*, however, only magnesium could overcome manganese-limitation. MacLoed and Snell (1950) concluded that although all three organisms required manganese, other divalent cations may be able to replace manganese in certain enzyme systems and this behavior varies between different organisms. Hence, another possible explanation for citrate resistance could be that these mutants may have a lower requirement for manganese by being able to replace it with magnesium (present in APT medium) in some metaloenzyme systems.

**Oxygen Sensitivity.** The fact that there was no reproducible difference in oxygen sensitivity (cfu/ml) between the parental strain 32406-2 and any of the mutants tested was probably due to growth inhibition at 100 psi oxygen without killing. As seen in the results from the isolation of O₂-resistant mutants, 100 psi oxygen inhibited growth, but had little effect on the viability of the cultures. Perhaps a better method to examine possible oxygen sensitivity in the strains would be to expose the cultures to several periods of high pressure O₂, incubating them at room atmosphere between each period. This may increase the amount of time the cells are actively growing when
exposed to high pressure oxygen and thus, increase the selection for oxygen-tolerant mutants.

Cadmium Sensitivity. Cadmium transport in *Staphylococcus aureus* occurs by a Mn(II) active transport system (Weiss, et al., 1978; Perry and Silver, 1982) and Archibald and Duong (1984) have found this to be the case in *L. plantarum*. Thus, increased cadmium sensitivity may indicate a higher rate of manganese transport and mutants that are more cadmium sensitive might transport manganese at higher rates. As stated in the results, there appears to be 3 classes of cadmium sensitivity (Table 2). The least sensitive (absorbance ratio of 0.45 or greater) strains, 32406-2, MC225 and MC234 are plumbagin-sensitive as would be expected since increased cadmium sensitivity should indicate increased Mn(II) uptake. An intermediate class (absorbance ratio of 0.27-0.29) consists of the plumbagin-sensitive strains MC230, MC231, MC236 and the plumbagin-resistant strain MC211. The increased sensitivity of this intermediate group would suggest that they may be more efficient at Mn(II) transport than strains 32406-2, MC225, and MC234. Also, they should be more resistant to plumbagin, but at the concentration of plumbagin used (0.14 mM) this was not evident for strains MC230, MC231, or MC236. Further tests using plumbagin at lower concentrations would prove or disprove this possibility. Since strain MC211 is plumbagin-resistant, it may possess an altered
diaphorase which will no longer reduce plumbagin. The third class, which shows the greatest sensitivity (absorbance ratio of 0.12 or less), should have an increased rate of manganese transport.

EDTA Sensitivity. In order to see if growth inhibition of \textit{L. plantarum} by chelation of manganese was unique to citrate, the sensitivity of strains to EDTA was examined. Results show that EDTA does inhibit growth of \textit{L. plantarum} (Table 2). It should be noted that the strains with the least sensitivity are all CitR mutants. Of these strains, MC225, MC230, and MC234 are also plumbagin-sensitive whereas strain MC232 is plumbagin-resistant. Consequently, it seems likely that a lower sensitivity to EDTA, like citrate-resistance, indicates a smaller amount of manganese required for growth, or increased manganese transport. However, some discrepancies are evident in the data for the EDTA sensitive strains (Table 2). For example, parent strain 32406-2, which is citrate-sensitive, is more resistant to EDTA than some of the citrate-resistant mutants (i.e. MC227 and MC229). Further, strain MC211, also citrate-sensitive, is more resistant to EDTA than the parent strain and all citrate-resistant strains with the exception of strains MC225, MC230, MC232, and MC234.

When grown in EDTA-containing medium, the cultures were incubated in test tubes without shaking. Therefore, they were exposed to less oxygen than when grown on citrate-containing agar medium and
thus, may have required less Mn(II) under the conditions of the EDTA sensitivity tests.

Mutant Classification

Class 1. Parental strain 32406-2 is sensitive to plumbagin, citrate, and EDTA and resistant to cadmium by definition.

Class 7. Strain MC211 is the only member of this class and differs from 32406-2 only in its resistance to plumbagin. As stated earlier, it seems likely that it possesses an altered diaphorase which will not catalyze the reduction of plumbagin. Alternatively, it may contain higher concentrations of polyphosphate or pyrophosphate and thus is able to retain a higher concentration of Mn(II) than 32406-2. In order to determine if there is a functioning diaphorase, it will be necessary to examine oxygen uptake and the effect of plumbagin on the oxygen uptake rate. Since it shows an intermediate cadmium sensitivity however, a third possibility exists. It may be able to transport higher levels of Mn(II) than the parent strain which would make it plumbagin resistant.

Class 8. These strains are more plumbagin resistant and cadmium sensitive than the parent. Thus, they may have a Mn(II) transport system whose Km is lower than 32406-2. Interestingly, strain MC203 appears to be less plumbagin-resistant (EOP = .77) than the other members of this class. Further, although not
Table 6. Classification of *L. plantarum* 32406-2 and all isolated mutants in accordance to Plumbagin, Citrate, EDTA and Cadmium sensitivity.

<table>
<thead>
<tr>
<th>Class</th>
<th>Plumbagin</th>
<th>Citrate</th>
<th>EDTA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cadmium&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Strain(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>32406-2</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>none</td>
</tr>
<tr>
<td>3</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>none</td>
</tr>
<tr>
<td>4</td>
<td>S</td>
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<td>R</td>
<td>R</td>
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</tr>
<tr>
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<td>R</td>
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<td>R</td>
<td>S</td>
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</tr>
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<td>6</td>
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<td>R</td>
<td>R</td>
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<td>7</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>MC211</td>
</tr>
<tr>
<td>8</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>MC203, MC204, MC205, MC207, MC212, MC213</td>
</tr>
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<td>9</td>
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<sup>a</sup>Strains showing an absorbance (640 nm) ratio of 0.21 or greater are considered resistant.

<sup>b</sup>Strains showing an absorbance (640 nm) ratio of 0.29 or greater are considered resistant.
significantly different from strain MC213, MC203 is less sensitive to cadmium than strains MC204, MC205, MC207, and MC212 (Table 2). Therefore, if plumbagin-resistance and cadmium-sensitivity is caused by increased Mn(II) uptake, strain MC203 may be able to transport Mn(II) at a higher rate than strain 32406-2, but not at the rate of the other strains in this class. Since the standard deviation of efficiency of plating on plumbagin-containing medium is high, more tests are needed to determine if there are significant differences in plumbagin-resistance between these strains.

If these strains are plumbagin-resistant and cadmium-sensitive due to a lower Km for manganese transport, then they should also be resistant to citrate. This is not the case when 0.1 M citrate is added to APT medium (Table 1). However, this does not rule out the possibility that these strains could be CitR when compared to strain 32406-2, but at lower concentrations of citrate. There is probably some variation in citrate-resistance among strains as is evident in cadmium- and EDTA-sensitivity (Table 2). In order to get a more precise mutant characterization, citrate-resistance should be examined with several concentrations of citrate added to the APT medium.

Class 9. Since these strains are both PluR and CitR it seems likely that they can accumulate more Mn(II) than the parent 32406-2. As discussed for strain MC203 in Class 8, MC226 appears
less plumbagin-resistant than strains MC210 and MC229 and thus may have a Km for Mn(II) transport between these strains and strain 32406-2. MC226 is also slightly less cadmium sensitive than MC210 (Table 2). The possibility that manganese transport is higher is supported by the fact that these strains are also cadmium sensitive. However, this is in opposition to the results of the $^{54}$Mn uptake experiments in which both strains MC210 and MC226 transported less manganese than strain 32406-2. It may be that 30 seconds is too short or too long a time period for examining Mn(II) uptake. Also, as mentioned earlier, manganese limitation can cause clumping of the cells and this was evident in strains 32406-2, MC210, and MC226, though not as severe as it was in strains MC229 and MC232. This could produce an artificially low Mn(II) uptake rate by reducing the net surface area of the cell suspension and consequently fewer sites of Mn(II) transport would be exposed to the manganese in the reaction medium.

Class 11. On the basis of its relative plumbagin-, citrate-resistance and cadmium-sensitivity strain MC232 should be able to accumulate more manganese. This could not be measured by $^{54}$Mn uptake due to the extensive clumping of that strain upon Mn-limitation.

Class 13. Since strains MC231 and MC236 were only Cit$^R$, they could possibly metabolize citrate and alleviate
Mn-limitation. However, results from the citric acid utilization tests do not support this theory. As mentioned previously, magnesium will promote growth of some lactobacilli in manganese-limited medium and this may be caused by a replacement of manganese with magnesium as a cofactor in some enzyme systems (MacLoed and Snell, 1950). Since the addition of citrate creates a manganese-limitation, strains MC231 and MC236 may be able to utilize the magnesium present in APT medium to replace Mn, while citrate-sensitive strains cannot. This could be tested by growing these strains on a Mn-limited medium and examining the effects of adding several different concentrations of magnesium.

Class 14. Since strains MC227 and MC228 are sensitive to plumbagin, it would first appear that they do not accumulate higher concentrations of Mn(II). However, they are also cadmium sensitive and citrate resistant. Therefore, they may possess a lower Km for Mn(II) but have a fault in the polyphosphate system. Archibald and Fridovich (1982) found when L. plantarum ATCC 14917 was grown on a phosphate-deficient medium the concentration of cell polyphosphate decreased and this prevented accumulation of Mn(II). Possibly then, although strains MC227 and MC228 transport Mn(II) more efficiently than strain 32406-2, a mutation in the polyphosphate formation system will prevent accumulation of enough manganese to effectively scavenge superoxide radicals produced by plumbagin.
Class 15. On account of the plumbagin sensitivity and cadmium resistance exhibited by strains MC225, MC230, and MC234, this class probably does not have any mutation in the Mn(II) transport system. However, they are both citrate and EDTA resistant and thus may be better able to utilize magnesium to replace manganese.

Oxygen Consumption and Acid Production. The facts that culture yields for strains 32406-2 and MC210 were consistently higher than that for MC226 (based on absorbance at 640 nm) and that the former two produce significantly higher amounts of acetic acid (Table 3), may possibly indicate that energy is being produced during acetic acid production as suggested by Yousten, et al. (1975). The higher $O_2$ consumption rates of these two strains indicate that oxygen rather than pyruvate is being used as the electron acceptor for NADH oxidation. The mechanism of acetic acid production is unclear. There may be a functioning pyruvate dehydrogenase complex as suggested by Dirar and Collins (1973), but since some formic acid was detected, pyruvate formate lyase may also be active in these strains as was seen in Lactobacillus casei L3 by de Vries, et al (1970).

Strain MC226 is of interest since even under limited glucose concentrations it still produced a high concentration of lactic acid with respect to acetic acid. Fructose-1,6-diphosphate (FDP)
is required for lactate dehydrogenase activity (Wolin, 1964; de Vries, et al., 1970) and when glucose is limited, FDP concentration decreases (Hensel, et al., 1977). Consequently, there is a decrease in lactic acid production and a possible increase in acetic acid production. This activity has been reported in streptococci (Yamada and Carlsson, 1975; Thomas, et al., 1979) and in L. casei L3 (de Vries, et al., 1970). Some of the Streptococcus strains in the reports mentioned above retained a high LDH specific activity under glucose-limited conditions and thus did not reduce lactic acid production. The LDH activity in these strains did not require FDP. With this in mind, I feel that strain MC226 may possess an altered lactate dehydrogenase (LDH) which is less dependent on FDP or other effectors as compared to strains 32046-2 and MC210. This may explain the citrate resistance of strain MC226. As stated previously, citrate sensitivity in some lactobacilli is decreased when the pH decreases (MacLoed and Snell, 1947). Also, it has been shown that manganese acts as a positive effector of LDH at neutral pH (de Vries, et al., 1970; Hensel et al., 1977). Thus, if MC226 possesses an altered LDH which does not require positive effectors such as FDP or Mn(II), it may be able to produce lactic acid at a rate higher than citrate-sensitive strains and consequently be relieved from citrate inhibition through the production of low pH in the medium. It is difficult to say
whether this has any bearing on plumbagin resistance or not. If the LDH of strain MC226 has a higher rate of reaction because it does not require some positive effectors, the enzyme may be able to oxidize NADH more effectively than the diaphorase. This would decrease the rate at which $O_2^-$ was produced. However, as mentioned in the Results, plumbagin causes an increase in $O_2$ consumption in strain MC226. In order to see if there is a relationship between plumbagin resistance and lactic acid production, it will be necessary to compare the increase in $O_2$ consumption due to plumbagin and determine the rate of lactic acid production between strains which produce high and low concentrations of lactic acid.

Oxygen consumption by homfermentative lactobacilli could have a profound effect in forages of low soluble carbohydrate. Lactic acid bacteria that can utilize $O_2$ to produce more ATP under limited glucose conditions could grow to a higher cell density in low carbohydrate crops. However, the concentration of lactic acid in the silage would be reduced. Acetic acid production would be especially detrimental, if the pyruvate formate lyase system was active, since even under anaerobic conditions less lactic acid would be produced resulting in a higher final pH. Therefore, a lactic acid bacteria with a lactate dehydrogenase independent of FDP or other effectors could be more effective as an inoculum for alfalfa silage since it would remain homolactic.
even under limited carbohydrate conditions.

Manganese Accumulation. In conformation of results of Archibald and Duong (1984), my results show that citrate inhibits manganese accumulation. This is probably due to the ability of citrate to chelate Mn(II) (MacLeod and Snell, 1947). Strains 32046-2 and MC210 both show about a 70% drop in $^{54}$Mn accumulation when 20 mM citrate was added to the reaction medium. The inhibition of $^{54}$Mn accumulation in strain MC226 appears to be less (56%), but the deviation in the $%^{54}$Mn accumulation (3.9±1.7) prevents this conclusion. The fact that there are no significant differences in citrate inhibition of the rate of $^{54}$Mn uptake between the three strains may mean that the concentration of citrate in the reaction mixture was too high and it will probably be necessary to examine inhibition of $^{54}$Mn uptake at a number of different citrate concentrations.

In their report on manganese transport Archibald and Duong (1984) showed that the limit of Mn(II) accumulation in L. plantarum was 32 mM and this limit was attained when the final concentration of manganese in the medium was 200 micromolar. Since APT medium contains 710 micromolar Mn (II), it would be interesting to see if any mutants possessed a higher Mn(II) accumulation limit. If a mutant strain can accumulate more manganese than the parent strain 32406-2, then there may be more Mn(II) available to scavenge O$_2$ and thus afford higher plumbagin
resistance.

It is clear strains 32406-2, MC210, MC226, MC229, and MC232 require manganese for the dechaining activity as evidenced by the clumping seen when they were grown without manganese (Wright and Klaenhammer, 1983).

Silage Inoculation. The fact that a lower pH and higher titratable acidity was evident in the inoculated silages as compared to the control (Table 5) shows the use of L. plantarum as an inoculum is beneficial in alfalfa silage production. However, the pH dropped only to 4.5 and the production of butyric, isovaleric and propionic acids suggests that clostridial growth might have occurred. Though there was no increase in lactic acid concentration due to the use of the inocula, a more rapid production during the initial stages of the fermentation might have occurred. This has been seen by other workers (McDonald, et al., 1965; Ohyama, et al., 1975).

Since neither of the mutant strains used showed a better fermentation than the parent, it appears that citrate and plumbagin resistance does not confer superior ensiling characteristics but, this does not take into account the possibility that the rates of pH decrease and organic acid production may have differed.

The relationship between lactic and acetic acid production presents an interesting point. As shown previously,
carbohydrate limitation can cause the normally homofermentative L. plantarum to produce acetic acid and consume oxygen (Table 3). High acetic acid production is exemplified by the silages inoculated with 32406-2 (Figure 2). Since the dry matter of the fresh alfalfa was high it is quite possible that air was incorporated into the silos. It is known that silage crops with greater than 35% dry matter do not pack well and air is often incorporated during filling (Cullison, 1975). This could present ideal conditions for oxygen consumption by the organisms since the addition of an inoculum would increase the competition for available carbohydrates.
LITERATURE CITED


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