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Phenotypic and Genotypic Analysis of Amino Acid Metabolism in *Lactobacillus helveticus* CNRZ 32

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PHENOTYPIC AND GENOTYPIC ANALYSIS OF AMINO ACID METABOLISM IN

LACTOBACILLUS HELVETICUS CNRZ 32

by

Jason K. Christiansen

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ABSTRACT

Phenotypic and Genotypic Analysis of Amino Acid Metabolism in

Lactobacillus helveticus CNRZ 32

by

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Utah State University, 2007

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This study investigated genetic predictions for amino acid biosynthesis and catabolism by *Lactobacillus helveticus* CNRZ 32, a commercial cheese flavor adjunct that reduces bitterness and intensifies flavor notes. Conversion of amino acids into volatile and nonvolatile flavor compounds by *L. helveticus* and other lactic acid bacteria in cheese is thought to represent the rate-limiting step in the development of mature cheese flavor and aroma. One of the primary mechanisms for amino acid breakdown by these microbes involves the reversible action of enzymes involved in biosynthetic pathways, so our group investigated the genetics of amino acid biosynthesis in *L. helveticus* CNRZ 32. Most lactic acid bacteria are auxotrophic for several amino acids, and phenotypic characterization of *L. helveticus* CNRZ 32 has shown this bacterium requires 14 amino acids. Reconstruction of amino acid biosynthetic pathways from a draft-quality (incomplete) genome sequence for *L. helveticus* CNRZ 32 showed generally

good agreement between gene content and phenotypic amino acid requirements. One

exception involved the requirement of CNRZ 32 for Asp (or Asn) for growth, where

predictions derived from the genome sequence suggested this strain may be able to

synthesize Asp from citrate. This prediction was confirmed as Asp auxotrophy in *L.*

helveticus CNRZ 32 could be alleviated by the addition of citrate to a chemically defined

medium that lacked Asp and Asn. Genome analysis also predicted that *L. helveticus*

CNRZ 32 possessed ornithine decarboxylase activity, and would therefore catalyze the

conversion of ornithine to putrescine, a volatile biogenic amine. Putrescine production in

cheese would be undesirable because this compound may impart a rotting flesh flavor

and can also have adverse effects on human health. Experiments to confirm ornithine

decarboxylase activity in *L. helveticus* CNRZ 32 using a special growth medium, thin

layer chromatography, high performance liquid chromatograph, or ^{13}C nuclear magnetic

resonance were unsuccessful, however, which indicated this bacterium does not

contribute to putrescine production in cheese.

(44 pages)

ACKNOWLEDGMENTS

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INTRODUCTION

Lactobacillus helveticus is a homofermentative, thermo- and acid-tolerant microorganism that is commonly used in the dairy industry (2, 32). *L. helveticus* CNRZ 32 is a commercial cheese adjunct used to intensify flavor and reduce bitterness in several varieties of cheese (8). *L. helveticus*, as a species, requires more amino acids for growth than other LAB (18). *L. helveticus* CNRZ 32 has been shown to require14 amino acids; Arg, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Thr, Trp, Tyr, Val, and either Asp or Asn (9).

Reconstruction of amino acid biosynthesis pathways from a 4X draft genome allowed us to identify many of the enzymes in *L. helveticus* CNRZ 32 thought to be involved in these processes (Table 1). Overall, there was good agreement between the presence of functional genes and prototrophy. One exception was Asp (or Asn), whose pathway was predicted to be functional yet was experimentally determined to be required for growth in a chemically defined medium (CDM) (9).

Analysis of the *L. helveticus* CNRZ 32 genome sequence suggested that auxotrophy for Asp resulted from insertional inactivation by an insertion element, *ISL*2, of the gene that codes for phosphoenolpyruvate carboxylase (*ppc*) which produces oxaloacetate from phosphoenolpyruvate and CO_2 (8). However, it also was predicted that *L. helveticus* CNRZ 32 has citrate lyase, so in theory could produce the oxaloacetate intermediate from citrate and circumvent the need of a functional *Ppc* enzyme (27).

TABLE 1. Genetics and predicted enzymology of amino acid biosynthesis in
Lactobacillus helveticus CNRZ 32.

Amino acid	Genes Present[1]	Predicted Product (EC number)	Pathway	Essential?[2]
Ala	*araT*	aromatic aminotransferase (2.6.1.57)	complete[3]	no
	bcaT	branched-chain aminotransferase (2.6.1.42)		
	ataA-C	aminotransferase (2.6.1.-)		
Arg	*argF*[a,4]	ornithine carbamoyltransferase (2.1.3.3	incomplete	yes
	arcC[a,4]	carbamate kinase (2.7.2.2)		
	arcA[4]	arginine deiminase (3.5.3.6)		
	carA[b]	carbamoyl-phosphate synthase A (6.3.4.16)		
	carB[b]	carbamoyl-phosphate synthase B (6.3.4.16)		
	odcI	ornithine decarboxylase (4.1.1.17)		
	odiC	ornithine decarboxylase (4.1.1.17)		
Asn, Asp	*asnA*	aspartate-ammonia ligase (6.3.1.1)	incomplete	Asn or Asp
	asnB	asparagine synthase (6.3.5.4)		
	ans	asparaginase (3.5.1.1)		
	aspC[a]	aspartic aminotransferase (2.6.1.1)		
	asd[a]	aspartate-semialdehyde dehydrogenase (1.2.1.11)		
	ppc[4]	phosphoenolpyruvate carboxylase (4.1.1.31)		
Glu	*glnA*	glutamate-ammonia ligase (6.3.1.2)	incomplete	yes
	aspC	aspartic aminotransferase (2.6.1.1)		
Gln	*glnA*	glutamate-ammonia ligase (6.3.1.2)	from Glu	no
Cys	*cysK*[a]	O-acetylserine (thiol) lyase (2.5.1.47)	complete	no
	cbl[a]	cystathionine-β-lyase (4.4.1.8)		
	cysE[a]	serine acetyl-transferase (2.3.1.30)		
Met	*metA*[a]	homoserine-O-succinyl transferase (2.3.1.46)	incomplete	yes
	cysK2[a,4]	O-acetylserine (thiol) lyase (2.5.1.47)		
	metE	5-methyltetrahydropteroyl(Glu$_3$)-homocysteine methyltransferase (2.1.1.14)		
	patC[4]	cystathionine-γ-lyase (4.4.1.1)		
Lys	*lysA*[a]	diaminopimelate decarboxylase (4.1.1.20)	complete[3]	yes
	dapD[a]	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase (2.3.1.117)		
	dapE[a]	succinyl-diaminopimelate desuccinylase (3.5.1.18)		
	dapA[a]	dihydrodipicolinate synthase (4.2.1.52)		
	dapB[a]	dihydrodipicolinate reductase (1.3.1.26)		
	aspC[a]	aspartic aminotransferase (2.6.1.1)		
	asd[a]	aspartate-semialdehyde dehydrogenase (1.2.1.11)		
	lysC[b]	aspartate kinase (2.7.2.4)		
	dapF[b]	diaminopimelate epimerase (5.1.1.7)		
	ataA-C	aminotransferase (2.6.1.-)		
Ile, Leu, Val	*bcaT*	branched-chain aminotransferase (2.6.1.42)	incomplete	yes
	ilvB[3]	acetolactate synthase (2.2.1.6)		
Phe, Tyr, Pro	*araT*	aromatic aminotransferase (2.6.1.57)	incomplete	yes
	aspC	aspartic aminotransferase (2.6.1.1)		
His	none	---	incomplete	yes
Pro	none	---	incomplete	yes

TABLE 1. Genetics and predicted enzymology of amino acid biosynthesis in
Lactobacillus helveticus CNRZ 32 (continued)

Amino acid	Genes Present[1]	Predicted Product (EC number)	Pathway	Essential?[2]
Ser	*glyA*	glycine hydroxymethyltransferase (2.1.2.1)	complete	no
	glyK	glycerate kinase (2.7.1.31)		
	serC[a]	phosphoserine transaminase (2.6.1.52)		
	serA[a]	phosphoglycerate dehydrogenase (1.1.1.95)		
	ycsE	phosphoserine phosphatase (3.1.3.3)		
	sdaB[b]	L-serine ammonia-lyase B (4.3.1.17)		
	sdaA[b]	L-serine ammonia-lyase A (4.3.1.17)		
Gly	*glyA*	glycine hydroxymethyltransferase (2.1.2.1)	from Ser	no
Thr	none	---	incomplete	yes

[1]Genes for a common amino acid with the same superscript letter are organized in an operon-like structure and are listed in sequential order of transcription.
[2]As determined by Christensen and Steele (9).
[3]Pathway completion assumes activity for one or more transamination reactions is provided by the aromatic- (*araT*), aspartic- (*aspC*), or branched-chain aminotransferase (*bcaT*), or by one of 3 other predicted amino acid aminotransferases (*ataA-C*) whose genes were found in the *L. helveticus* CNRZ 32 genome.
[4]Probable pseudogene; product is predicted to lack biological activity.

A recent report that showed *Lactobacillus helveticus* ATCC 15807 catabolized citrate to succinate indicates this species can have citrate transport capabilities (32). Consequently, genome predictions (such the presence of citrate lyase) suggest *L. helveticus* CNRZ 32 might be able to utilize citrate to overcome auxotrophy for Asp and Asn.

Another interesting observation was the presence of two genes, *odcI* and *odiC*, in *L. helveticus* CNRZ 32 (Table 1), as well as one copy of the *potABCD* operon. The *odcI* and *odiC* genes are predicted to encode ornithine decarboxylase, which catalyzes the conversion of ornithine to putrescine (1, 4-diaminobutane or butanediamine), while *potABCD* is predicted to code for an ornithine ABC transporter (34). Conversion of ornithine to putrescine, by *L. helveticus* CNRZ 32 has not been shown, and this reaction

would be undesirable in cheese because putrescine is a biogenic amine characterized by the smell of rotting flesh.

Knowledge of whether *L. helveticus* CNRZ 32 can produce putrescine from ornithine may be important in the industrial use of this bacterium, since other cultures added to cheese with *L. helveticus* CNRZ 32 (such as *Lactococcus lactis*) can produce ornithine from arginine, and theoretically lead to putrescine production by *L. helveticus* CNRZ 32. Also putrescine can be a health concern as a precursor for carcinogenic nitrosamine formation (1, 20). It is also involved in hypertensive crises in patients treated with monoamine oxidase inhibitors (MAOI), and a potentiator that enhances the toxicity of histamine (6, 29). The purpose of this study was to determine the validity of genome prediction regarding the ability of *L. helveticus* CNRZ 32 to utilize citrate in place of phosphoenolpyruvate to synthesize Asp, and to investigate ornithine decarboxylase activity in this bacterium.

PREVIOUS WORK-LITERATURE REVIEW

Flavor Development in Cheese

Flavor development in cheese is a very active and intricate process. The compounds thought to be responsible for cheese flavors are formed by enzymes (rennet, native milk enzymes and bacterial), chemical reactions (principally involving modification of products of enzymatic and biological reactions), and living microorganisms (12). The lactic acid bacteria (LAB) that contribute to these processes include deliberately added bacteria (starter and adjunct) as well as nonstarter lactic acid bacteria (NSLAB). The NSLAB enter the cheese through the processing environment and/or the milk (8). Together these organisms change bland cheese curd into flavorful cheese through mechanisms that include lactose fermentation, citrate metabolism, lipolysis/esterification, and proteolysis (6, 12).

Many of the above mentioned reactions in LAB are now moderately well understood, proteolysis in particular (14, 31). These reactions dictate cheese flavor by producing cheese flavor compounds (24). A compiled list of volatile and non-volatile flavor compounds may include over 200 molecules (12, 33). Many of these compounds influence cheese flavor even though they are present at very low concentrations (11). Some flavor compounds of interest are aldehydes, alcohols, acids, hydrogen sulfide, phenol, indole, and cresol (17).

The cooperation between *Lactococcus lactis* and adjunct strains likely contributes to the formation of aroma and flavor compounds in cheese (23). However, our

knowledge concerning the specifics of these interactions remains limited due to the complexity of the microbial population found in cheese.

Proteolysis and Cheese Maturation

Proteolysis is the most complex and, possibly, the most important event during cheese maturation (12). Proteolysis of milk proteins, such as caseins, into peptides and amino acids is performed by proteinases and peptidases. Casein is the major protein involved in proteolysis, and its open, random structure makes it more susceptible than whey proteins to catabolism (11). The casein proteins are first broken down by proteinases into peptides and oligopeptides for cell transport (11). Then, within the cell, peptidases further degrade peptides into amino acids (2). In *L. helveticus*, all the identified peptidases are believed to be intracellular so the acquisition of amino acids is largely dependent on the activity of extracellular proteinase activity and the transport of peptides into the cell (9).

Once free amino acids are liberated from peptides, their direct impact on flavor is believed to be limited. This is because they seem to act as precursors of other flavor compounds (24). Amino acids are catabolized primarily by the reactions of decarboxylation, transamination, deamination, and desulfuration (17). These secondary reactions of proteolysis are thought to be important in flavor and aroma development of cheese (16, 23, 24).

Amino Acid Biosynthesis

LAB have adapted to nutritionally rich environments and, as a result, are very fastidious. Besides a fermentable sugar, they require nucleobases, vitamins, cations, and

amino acids (22). These complex nutritional requirements are considered to be the result of an evolutionary process in which a microbial ancestor with an array of biochemical abilities progressively lost expendable genes following acclimation to a nutrition-rich environment like milk (2, 7, 25).

In LAB, several genes and gene clusters have been implicated in amino acid biosynthesis (35). Minor genetic lesions of these genes have been reported to be the cause of auxotrophy for ten amino acids in *Lactobacillus helveticus* ATCC 15009, because auxotrophy could be reversed by mutagenesis (26). However, work done by Van der Kaaij et al. (35) shows that auxotrophy for L-alanine in *Lactobacillus johnsonii* is due to whole gene loss. Reconstruction of amino acid biosynthetic pathways for *L. helveticus* CNRZ 32 from a 4X draft genome sequence reveals that auxotrophy was primarily due to whole gene loss rather than point mutations or minor genetic lesions (8). These findings appear to contradict the findings of Morishita et al. (26). This is especially interesting because *L. helveticus* ATCC 15009 and CNRZ 32 have been reported to have almost identical amino acid requirements (9, 26). One explanation for this observation is that the predominant mechanisms for gene inactivation may differ in *L. helveticus* ATCC 15009 and CNRZ 32 (e.g., point mutations versus deletion), and their similarities in amino acid auxotrophies are a reflection of both strains' adaptation to a milk environment.

Biogenic Amines

Biogenic amines (BAs) are low molecular weight organic bases with biological activity, such as histamine, putrescine, cadaverine, and tyramine. They are heat stable metabolites of amino acid catabolism, usually formed by decarboxylation reactions. BAs

occur in a wide variety of foods, such as fish products, meat, cheese, wine, and other fermented foods (1).

Putrescine, cadaverine, and other BAs are very pungent and considered undesirable flavor compounds in cheese. It is also known that ingestion of biogenic amines may cause hypertensive crises in patients treated with monoamine oxidase inhibitor drugs (MAOI) (6). Some are used as indicators of spoilage (28). It has been shown that secondary amines (metabolites of putrescine), when in the presence of nitrites, can be converted to carcinogenic nitrosamines (1, 20).

Histamine has been described as a potentially hazardous amine because it can cause scombroid poisoning, histaminic toxicity, and headaches (19). Putrescine and other amines such as cadaverine and tyramine are potentiators that enhance the toxicity of histamine; specifically they are believed to inhibit the metabolizing enzymes diamine oxidase and hydroxymethyl transferase, which are responsible for histamine breakdown (6, 29).

Citrate Catabolism in Cheese

Milk contains 0.15 to 0.2% citric acid. Citrate can be transported into the cells of some LAB by a citrate permease, termed CitP (2). It has been well established that *Leuconostoc* sp. and *Lactoccocus lactis* may collect extra pyruvate for diacetyl production through means of citrate catabolism by citrate lyase (2). Citrate lyase converts citrate into oxaloacetate (OAA), which is then converted to pyruvate by oxaloacetate decarboxylase. Pyruvate can then be converted into diacetyl, which is 65

known to be a major contributor of a "buttery" aroma in cultured milk and cheeses such as Gouda, Edam, and Harvarti (21).

Along with glutamate and pyruvate, citrate is one of three components found in milk that can be catabolized to form α- ketoglutarate (α-KG) (30). It was shown that addition of α-KG to St. Paulin or Cheddar cheese favorably enhances their aromas (3). This aroma development is presumably a result of α-KG's ability to act as the amino group acceptor during enzyme (aminotransferase) catalyzed transaminations, in which amino acids are converted to keto acids (27). The keto acids produced as a result of transamination can be degraded enzymatically to form aldehydes or carboxylic acids or they can undergo spontaneous degradation (37). Aldehydes and carboxylic acids are recognized as key flavor compounds in many cheeses (4, 5).

A specific example concerning α-KG's role in flavor development can be illustrated when considering the production of methanethiol. The formation of methanethiol from methionine is believed to play a significant role in cheddar cheese flavor. This formation has been shown to proceed via a transamination reaction. Interestingly, these studies suggest that α-KG was necessary for the formation of an intermediate for methanethiol production, 2-hydroxyl-4-(methylthio) butyric acid (HMBA), in four of the five lactococcal strains examined. Therefore, α-KG is a key component in methanethiol synthesis by lactococci (13).

MATERIALS AND METHODS

Microorganisms

Lactobacillus helveticus CNRZ 32 was obtained from Dr. Jeff Broadbent's laboratory strain collections (Utah State University, Logan, UT), and the putrescine-producing strain *Lactobacillus* sp. ATCC 33222 (designated as *Lactobacillus* sp. 30a by Garcia-Moruno et al.; 15) was purchased from American Type Culture Collection (Manassas, VA). Stocks of each culture were stored in sterile non-fat milk with 11% (wt/vol) glycerol at -80°C, and working cultures were prepared from frozen stocks by two sequential transfers (1% inoculation) in Man Rogosa and Sharpe medium (MRS) (Difco Laboratories, Sparks, MD) propagated anaerobically for16 h at 37° C.

Asp and Asn Auxotrophy in
L. helveticus CNRZ 32

Chemically defined medium (Table 2) was prepared according to the method described by Christensen and Steele (9), with the following modifications: One sample lacked citrate, Asp, or Asn (CDM); a second contained 2 g per L sodium citrate but lacked Asp or Arg (CDM-Cit); and a third, which served as the positive control, lacked citrate but contained 200 mg per L of L-aspartic acid and 400 mg per L of L-asparagine (CDM-Asp/Asn). Medium ingredients were all obtained from Sigma-Aldrich, Inc. (St. Louis, MO), except that sodium acetate (trihydrate), manganese sulfate (monohydrate), sodium chloride, and glucose were obtained from Mallinckrodt, Inc, (Hazelwood, MO); potassium phosphate (dibasic) was obtained from Fisher Scientific, Inc. (Pittsburgh, PA);

TABLE 2. Chemically defined medium for analysis of Asp and Asn
auxotrophy in *Lactobacillus helveticus* CNRZ 32.

Component	Addition per liter
Salts:	
Sodium acetate (trihydrate)	5 g
Potassium phosphate (monobasic)	1 g
Potassium phosphate (dibasic)	1 g
Sodium chloride	200 mg
Calcium chloride (dihydrate)	200 mg
Magnesium sulfate	200 mg
Magnesium sulfate (monohydrate)	50 mg
Components dissolved in 5 ml of 1 M HCL:	
L-Glutamic acid	400 mg
L-Tryptophan	25 mg
Carbohydrate:	
D-(+)-Glucose (anhydrous)	20 g
Components dissolved in 6 ml of 1 M NaOH	
L-Phenylalanine	20 mg
L-Tyrosine	70 mg
Adenine, guanine, uracil, xanthine	25 mg each
Other amino acids added	
L-Alanine	400 mg
L-Arginine	200 mg
L-Cysteine	80 mg
L-Glutamine	400 mg
Glycine	400 mg
L-Histidine	200 mg
L-Isoleucine	80 mg
L-Leucine	80 mg
L-Lysine	400 mg
DL-Methionine	80 mg
L-Proline	800 mg
DL-Serine	400 mg
DL-Threonine	400 mg
L-Valine	400 mg
Other ingredients	
Tween 80 (polyoxyethylenesorbitan monooleate)	1 ml
Tween 20 (polyoxyethylenesorbitan monolaurate)	1 ml
Glycerol	1 ml
DL-Mevalonic acid lactone	10 µl
Pyridoxal HCL	10 mg
RPMI 1640 vitamin solution (100x)	20 ml

and potassium phosphate (monobasic) was obtained from MP Biomedical, Inc. (Salon,

OH). All components except the RPMI 1640 vitamin solution were dissolved in the

order listed in Table 2 to maintain relatively moderate pH. After adding and solubilizing

all ingredients except the vitamin solution, the medium was adjusted to pH 6.50 and

autoclaved at 121° C for 10 min. The vitamin solution was sterilized by passage through

a 0.2 μm cellulose acetate membrane (VWR International, West Chester, PA) and

aspetically added to the cooled medium immediately prior to inoculation.

To eliminate carryover of any essential nutrients (18), 10-ml working cultures of

L. helveticus CNRZ 32 were harvested by centrifugation (5,500 rpm for 15 min) and

washed twice in 10 ml of sterile sodium phosphate (50 mM, pH 7.0). Cell samples were

split into three equal parts and each washed in one of the variations of CDM. Then, a 1%

dilution of the cells was made in a fresh 10 ml tube of the corresponding CDM. Growth

in each medium was determined by measuring optical density spectrophotometrically at

660 nm (A_{660}) after 0, 19, 24, and 48 h of incubation.

**Screening for Ornithine Decarboxylase
Activity**

Several methods were employed to detect putrescine production by *L. helveticus*

CNRZ 32. The first technique involved an improved decarboxylase medium described

by Bover-Cid and Holzapfel (6), which is reported to have a limit of detection for

biogenic amine production of 350 mg per liter. Next, thin layer chromatography (TLC)

and high performance liquid chromatography (HPLC) were used to obtain greater

sensitivity in assays to detect putrescine production from ornithine. TLC was performed

as described by Garcia-Moruno et al. (15), and HPLC was done as outlined by

Hernandez-Jover et al. (19). Both procedures required a dansyl-chloride derivitization step with *L. helveticus* CNRZ 32 supernatant, which was collected after 3 and 7 d incubation at 30°C in MRS supplemented with 3 mM L-ornithine.

Finally, ^{13}C-nuclear magnetic resonance (NMR) analysis of *L. helveticus* CNRZ 32 supernatant was performed to gain greater assay sensitivity without the need for sample derivitization. Prior to NMR, a 300 mM concentration of [U-^{13}C5] L-ornithine (Cambridge Isotope Laboratories Inc., Andover, MA) and 2 mM pyridoxal 5-phosphate (PPL) (Sigma-Aldrich) was prepared in sterile double distilled H_2O and filter sterilized through a 0.2 μm cellulose acetate membrane (VWR International). The ^{13}C ornithine solution was stored in the dark at 4°C until needed.

In an effort to promote induction of ornithine decarboxylase activity before NMR (6), *L. helveticus* CNRZ 32 and *Lactobacillus* sp. ATCC 33222 were subcultured twice in MRS containing 0.1% L-ornithine monohydrochloride, and 0.005% pyridoxal-5-phosphate (PPL). Cell samples were prepared in three 1.5 ml, sterile, centrifuge tubes. One served as the positive control, and contained 1 ml MRS, 1% inoculation of *Lactobacillus* sp. ATCC 33222, and 100 μl [U-^{13}C5] L-ornithine solution. The cell free negative control contained 1 ml MRS and 100 μl [U-^{13}C5] L-ornithine solution. The test sample consisted of 1 ml MRS, 1% *L. helveticus* CNRZ 32, and 100 μl [U-^{13}C5] L-ornithine solution. The tubes were incubated 3 d at 37°C, or 7 d at 30°C, then centrifuged at 12,000 rpm for 5 min, and 0.6 ml of sample supernatant was transferred to 5-mm high-pressure NMR tubes (Wilmad-Labglass, Buena, NJ). To address the possibility that ornithine decarboxylase activity might be induced at low pH, similar

experiments were performed using cells suspended in MRS buffered to a pH of 5.1 or 6.0 (10).

All NMR spectra were collected on a Bruker DMX400 NMR spectrometer (Bruker Analytik GmbH, Ettlingen, Germany) operated at a carbon frequency of 100.6 MHz. The probe temperature was between 13 and 37°C. NMR spectra were referenced for carbon by a capillary insert tube that contained chloroform-d (Sigma Chemical Company, St. Louis, MO). Five thousand scans were recorded for each sample. In addition, 50,000 scans were run on *L. helveticus* CNRZ 32 samples to increase sensitivity for putrescine detection. [13]C chemical shifts for ornithine and putrescine were identified by NMR analysis of unlabeled standards.

RESULTS

Alleviation of Asp Auxotrophy

L. helveticus CNRZ 32 cells incubated in CDM did not show any significant

growth (Fig. 1). This observation was expected because the gene encoding

phosphoenolpyruvate carboxylase was interrupted by an insertion element, ISL2 (Table

1), and the absence of citrate and Asn eliminated each of the remaining pathways

predicted for Asp formation in L. helveticus CNRZ 32 (Fig. 2). In contrast, cells

incubated in CDM-Cit grew to an OD_{660} of 1.0 ± 0.1 within 48 h, while cells inoculated

into CDM-Asp/Asn grew to a slightly higher final cell density ($OD_{660} = 1.4 \pm 0.2$) (Fig.

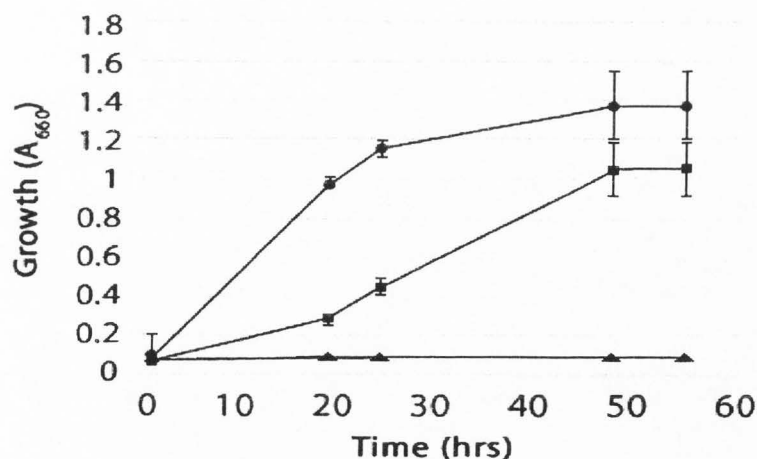1). This outcome supported our hypothesis that auxotrophy for Asp in L. helveticus

CNRZ 32 can

FIG. 1. Growth of Lactobacillus helveticus CNRZ 32 in chemically defined medium supplemented with Asp and Asn (●), or citrate (■), or that lacked Asp, Asn, and citrate (▲). Values represent the mean (± SE) from triplicate experiments.

Citate ⟶ Oxaloacetate ⟵ Phosphoenolpyruvate

1

3

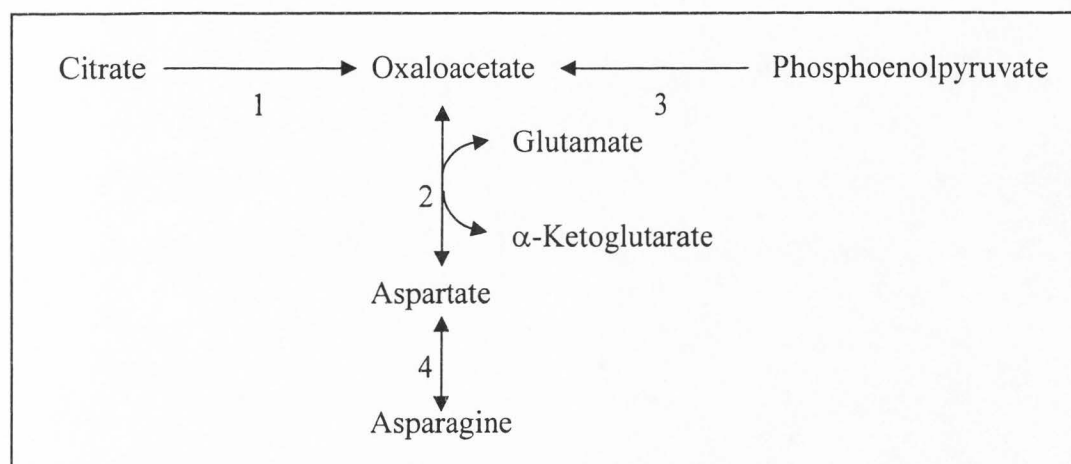Glutamate

2

α-Ketoglutarate

Aspartate

4

Asparagine

FIG. 2. Predicted pathways for Asp and Asn biosynthesis in *Lactobacillus helveticus* CNRZ 32. Enzymes involved in these conversions include citrate lyase (1), aspartate aminotransferase (2), phosphoenolpyruvate carboxylase (3), and asparaginase (4). Reversible reactions are indicated by a double-headed arrow.

be alleviated by citrate because citrate permease and citrate lyase activities generate the

oxaloacetate intermediate required for de novo Asp biosynthesis (Fig. 2). Interestingly,

growth curves for *L. helveticus* CNRZ 32 in CDM-Cit showed an extended lag phase as

compared to cells incubated in CDM-Asp/Asn (Fig. 1). The basis for this observation is

unknown, but it could reflect delayed induction of genes for citrate utilization, or a low

level of in vivo activity for one or more of those enzymes.

**Screening for Ornithine Decarboxylase
Activity in Lactobacilli**

The formation of putrescine has been shown by Arena and Manca de Nadra (1) to

proceed in other organisms through three potential pathways (Fig. 3). Protein homology

searches against the translated protein database for the *L. helveticus* CNRZ 32 genome

Arginine ——→ Agmatine
            3

1 ↓   ↓ 5

Citrulline   4   N-Carbamoylputrescine

2 ↕   ↓ 6
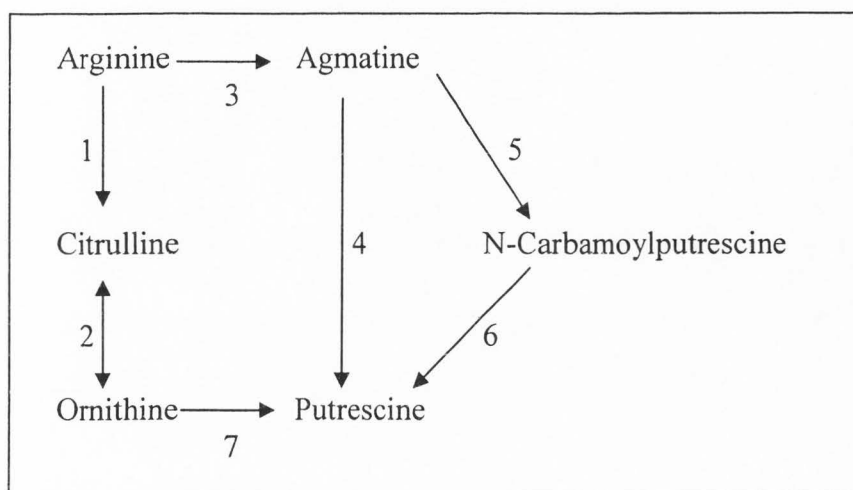
Ornithine ——→ Putrescine
            7

FIG. 3. Potential pathways for putrescine synthesis by lactobacilli. Enzymes that may be involved in these conversions include arginine deiminase (1), ornithine transcarbamylase (2), arginine decarboxylase (3), agmatine deiminase (4), agmatinase (5), N-carbamoylputrescine hydrolase (6), and ornithine decarboxylase (7). Reversible reactions are indicated by a double-headed arrow. Adapted from Arena and Manca de Nadra (Ref. 1).

sequence did not detect any orthologs to arginine decarboxylase, agmatine deiminase, agmatinase, or N-carbamoylputrescine hydrolase. Moreover, arginine deiminase and ornithine transcarbamylase are pseudogenes in *L. helveticus* CNRZ 32. Therefore, we predict this bacterium cannot produce putrescine from Arg, but the presence of two genes encoding paralogs to ornithine decarboxylase in the *L. helveticus* CNRZ 32 genome (Table 1) suggested the bacterium may be able to produce putrescine from exogenously supplied ornithine (Fig. 3). To test this hypothesis, we assayed *L. helveticus* CNRZ 32 for the ability to convert ornithine into putrescine using several different techniques.

Lactobacillus sp. ATCC 33222, a bacterium originally isolated from horse stomach, has been shown to rapidly convert ornithine into putrescine (15) and was used as a positive control for this activity. As expected, colonies from this strain gave a faint

purple color indicative of ornithine decarboxylase activity when streaked on improved decarboxylase medium (6). However, colonies of *L. helveticus* CNRZ 32 did not produce any purple color on this medium.

Efforts to detect ornithine decarboxylase activity in *L. helveticus* CNRZ 32 by TLC or HPLC also proved unsuccessful. As was the case for observations from the improved decarboxylase medium, which is reported to have a limit of detection for biogenic amine production of 350 mg per liter (6), TLC and HPLC results were deemed inconclusive because they might be attributed to inadequate assay sensitivity. The limit of detection for biogenic amines by TLC and HPLC is reported to be 10 mg and 3 mg per liter, respectively (19, 28), but both techniques require a derivitization (dansyl chloride) step that theoretically can reduces assay sensitivity.

To overcome the need for sample derivitization, experiments to detect putrescine production from ornithine were also performed using ^{13}C-NMR. The limit of detection for putrescine by this method was determined experimentally to be .08 mM or 7.6 mg per liter. Moreover, putrescine can readily distinguished from ornithine on ^{13}C-NMR spectra by the presence of a peak at 24 ppm (parts per million) (Fig. 4). As shown in Fig. 5, ^{13}C-NMR scans for the positive control, *Lactobacillus* sp. ATCC 33222, showed two major peaks; one was a chemical shift at 24 ppm (putrescine), and the other was at 39 ppm (Fig. 5). The cell-free [U-^{13}C5] L-ornithine solution (negative control) showed five major peaks at 23, 27, 39, 54, and a carbonyl signal at 174 ppm (Fig. 6). Spectra from ^{13}C-NMR scans of unlabeled ornithine (Fig. 7) and from *L. helveticus* CNRZ 32 supernatant (Fig. 8) were identical to that obtained for the negative control. Similar overall results were obtained with samples were incubated at 30° C for 7 d (Figs. 9, 10, and 11).
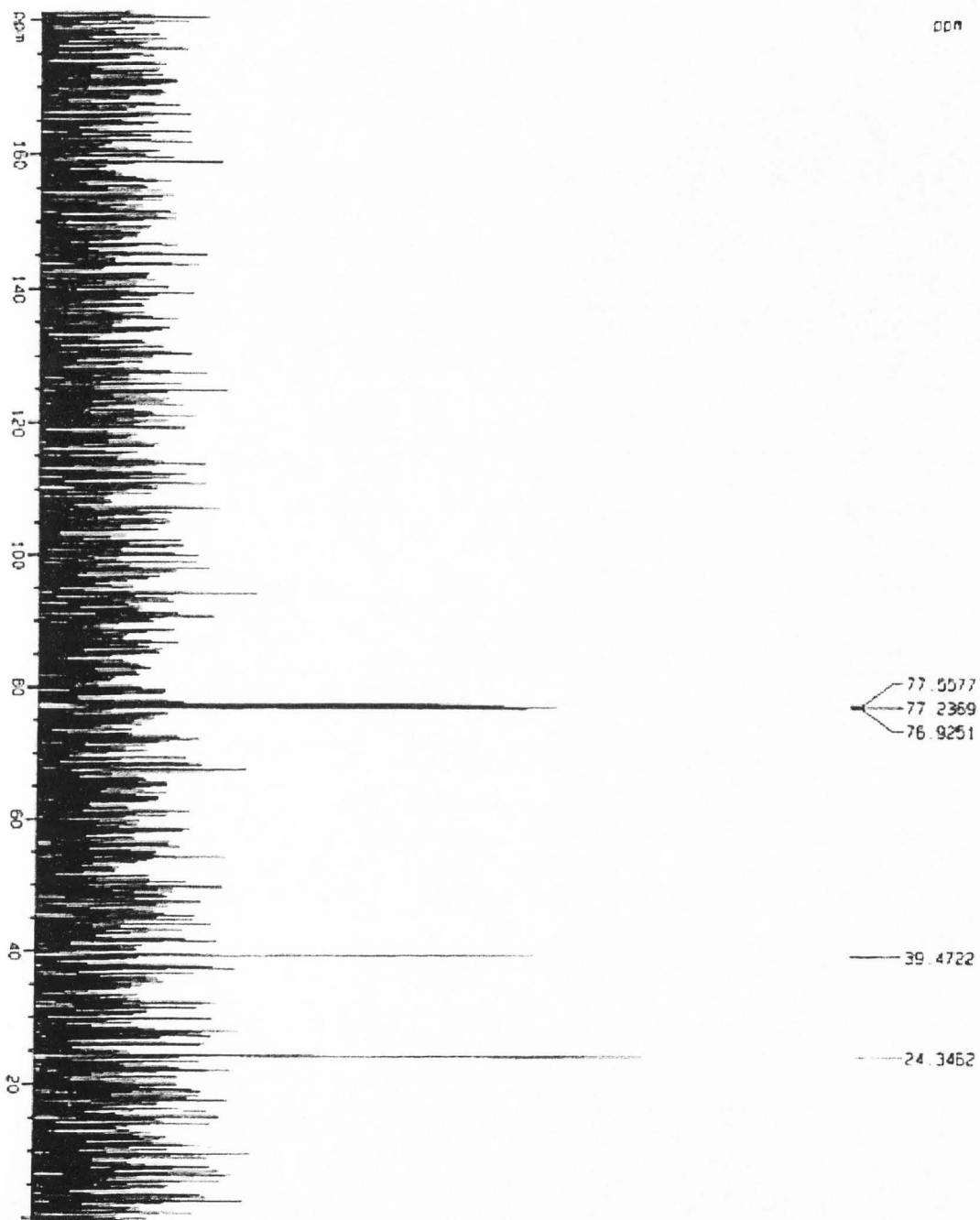
FIG. 4. ^{13}C-nuclear magnetic resonance scans of 8.7 mM putrescine in MRS broth. Putrescine was unlabeled so natural ^{13}C is shown, which results in higher background noise. The peaks show the resonating ^{13}C atoms within a compound in parts per million (ppm). Carbon reference d-chloroform was added by capillary insert and is indicated by the peak at 77 ppm.

FIG. 5. Putrescine production by *Lactobacillus* sp. ATCC 33222 incubated at 37°C for three days in MRS with [U-[13]C5] L-ornithine. Peaks show resonating ^{13}C atoms within a compound in parts per million (ppm) after 5,000 NMR scans at room temperature. Carbon reference (d-chloroform) is shown at 77 ppm.
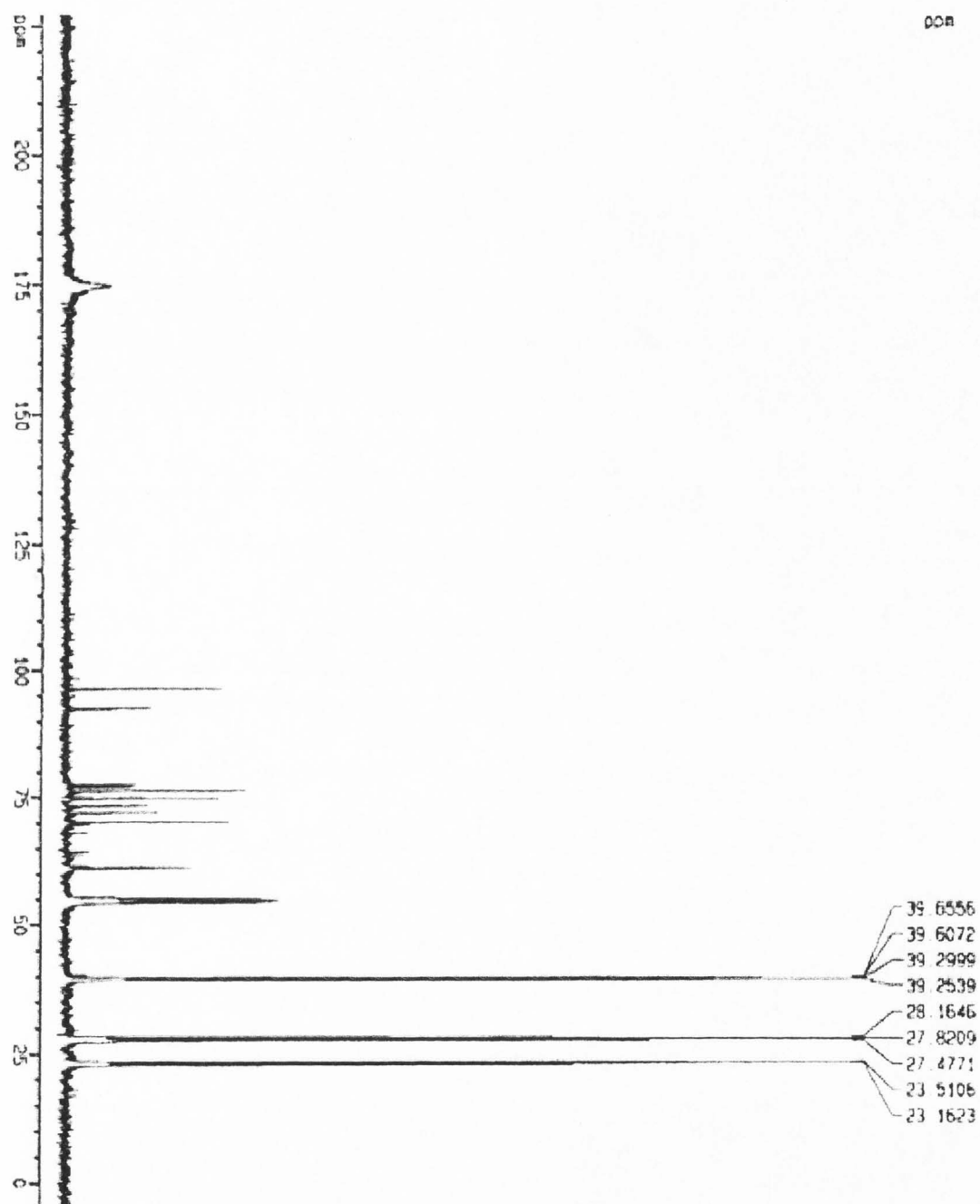
FIG. 6. ^{13}C-nuclear magnetic resonance scans of cell free control incubated at 37°C for three days in MRS with [U-^{13}C5] L-ornithine. Smaller peaks from about 60-100 ppm are probably due to residual alcohol from cleaning.
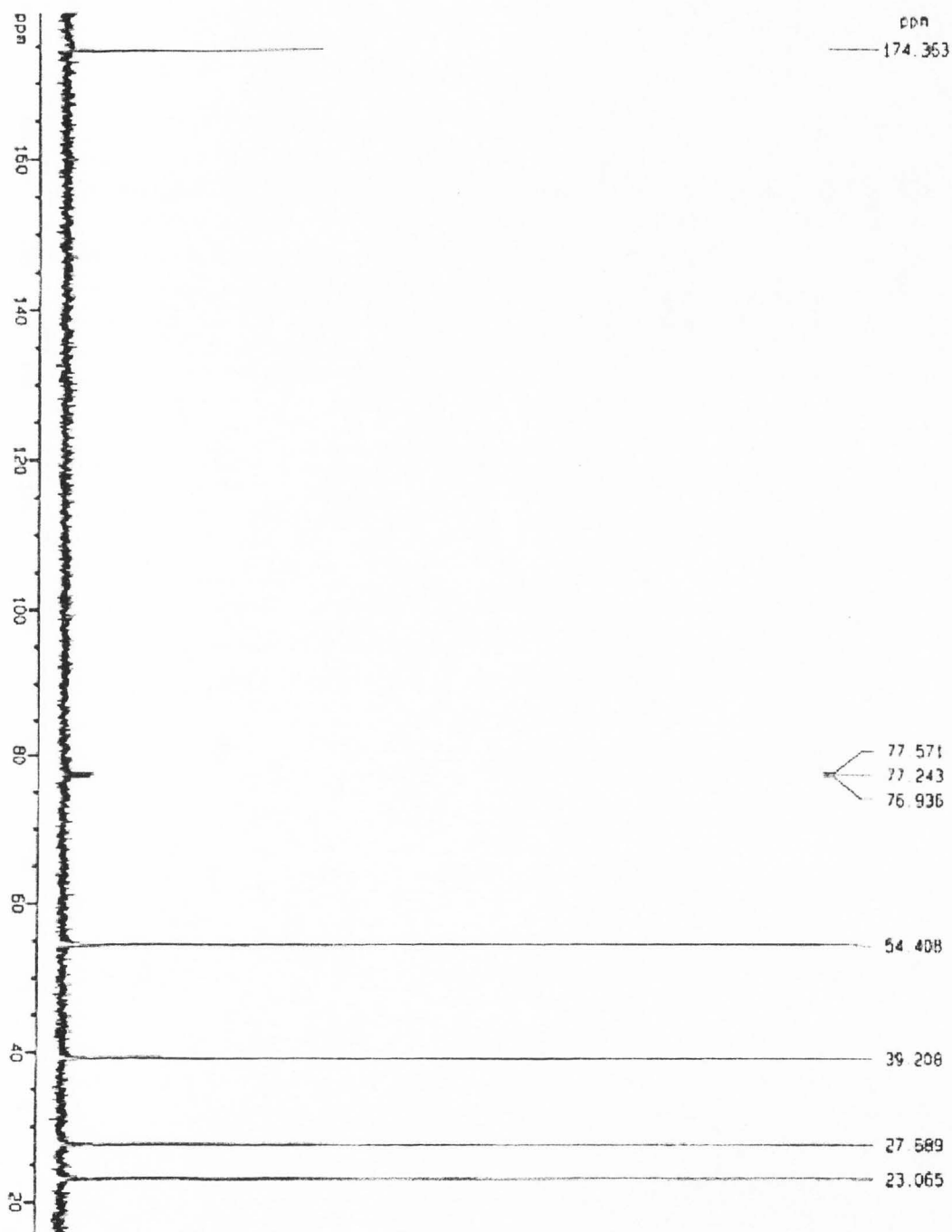
FIG. 7. ^{13}C-nuclear magnetic resonance scans of 870 mM ornithine in MRS broth. Ornithine was unlabeled so detection is based on naturally-occurring ^{13}C in the sample. Since the minimum level of detection for ornithine by NMR was not of interest to this study, a high concentration of this compound was included in the assay for clarity (less noise). Carbon reference (d-chloroform) is shown at 77 ppm

FIG. 8. Assay for putrescine production by *Lactobacillus helveticus* CNRZ 32 after incubation at 37° C for three days in MRS with [U-[13]C5] L-ornithine. Peaks show the resonating ^{13}C atoms within a compound in parts per million (ppm) after 5,000 NMR scans at room temperature.
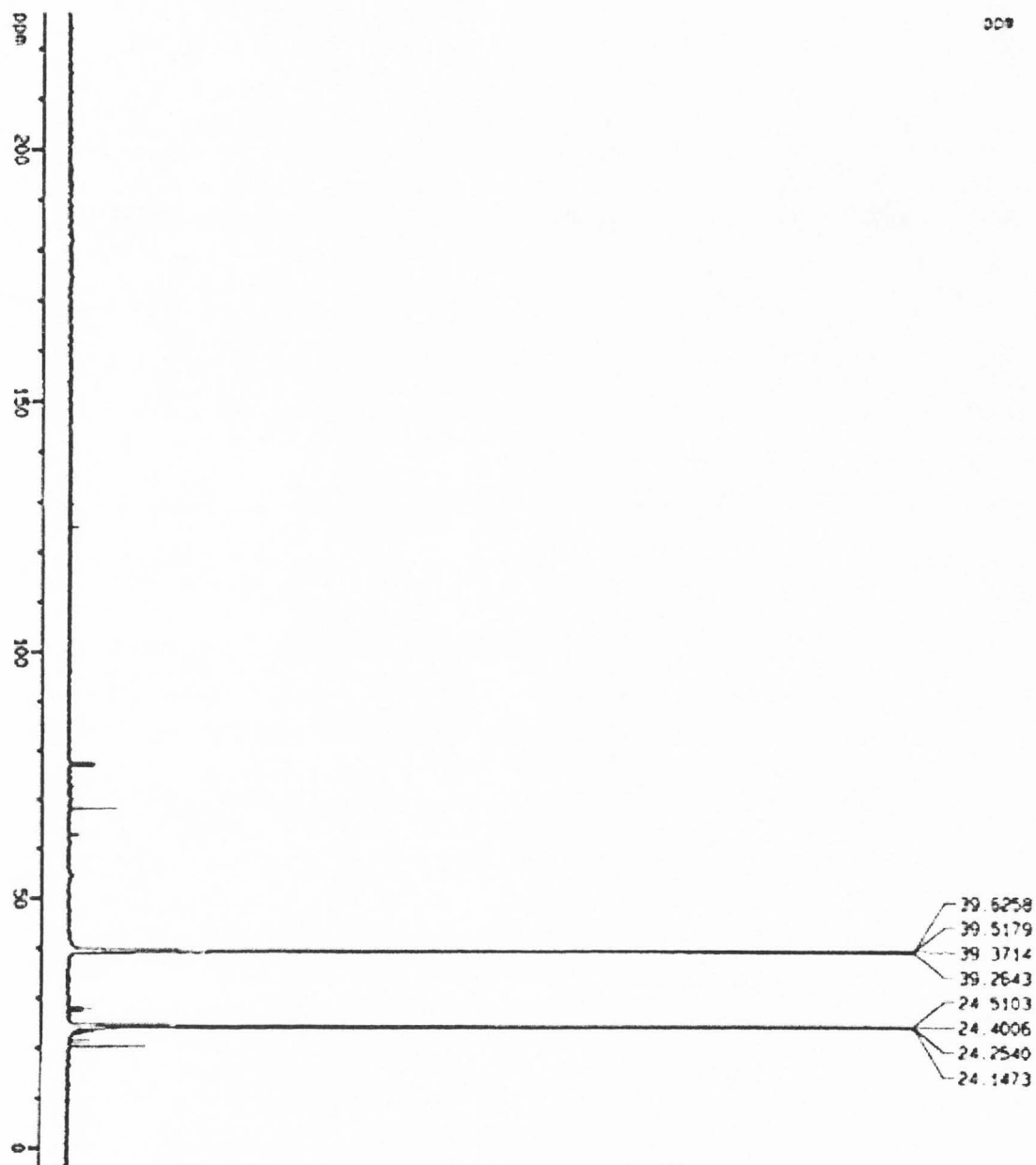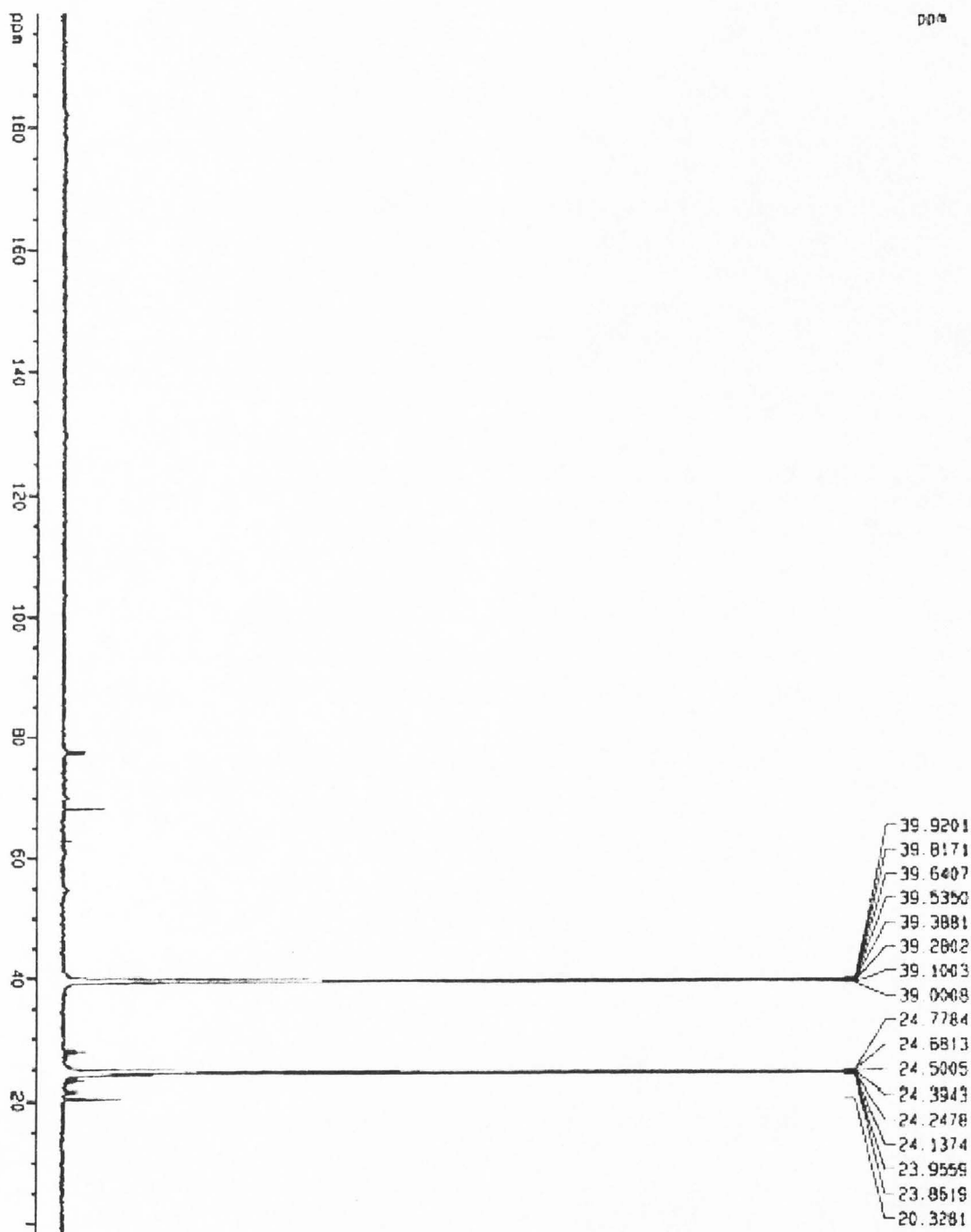
FIG. 9. Putrescine production by *Lactobacillus* sp. ATCC 33222 incubated at 30°C for seven days in MRS with [U-[13]C5] L-ornithine. Peaks show the resonating ^{13}C atoms within a compound in parts per million (ppm) after 5,000 NMR scans at room temperature.

174 658

96 356
92 539
76 922
76 321
76 277
74 681
73 277
71 994
71 835
70 130
70 088
61 231
61 081
55 165
54 817
54 640
54 288
39 710
39 665
39 356
39 306
28 203
27 861
27 517
23 560
23 212
22 853

FIG. 10. ^{13}C-nuclear magnetic resonance scans of a cell free control incubated at 30°C for seven days in MRS with [U-^{13}C5] L-ornithine. Peaks show the resonating ^{13}C atoms within a compound in parts per million (ppm) after 5,000 NMR scans at room temperature.
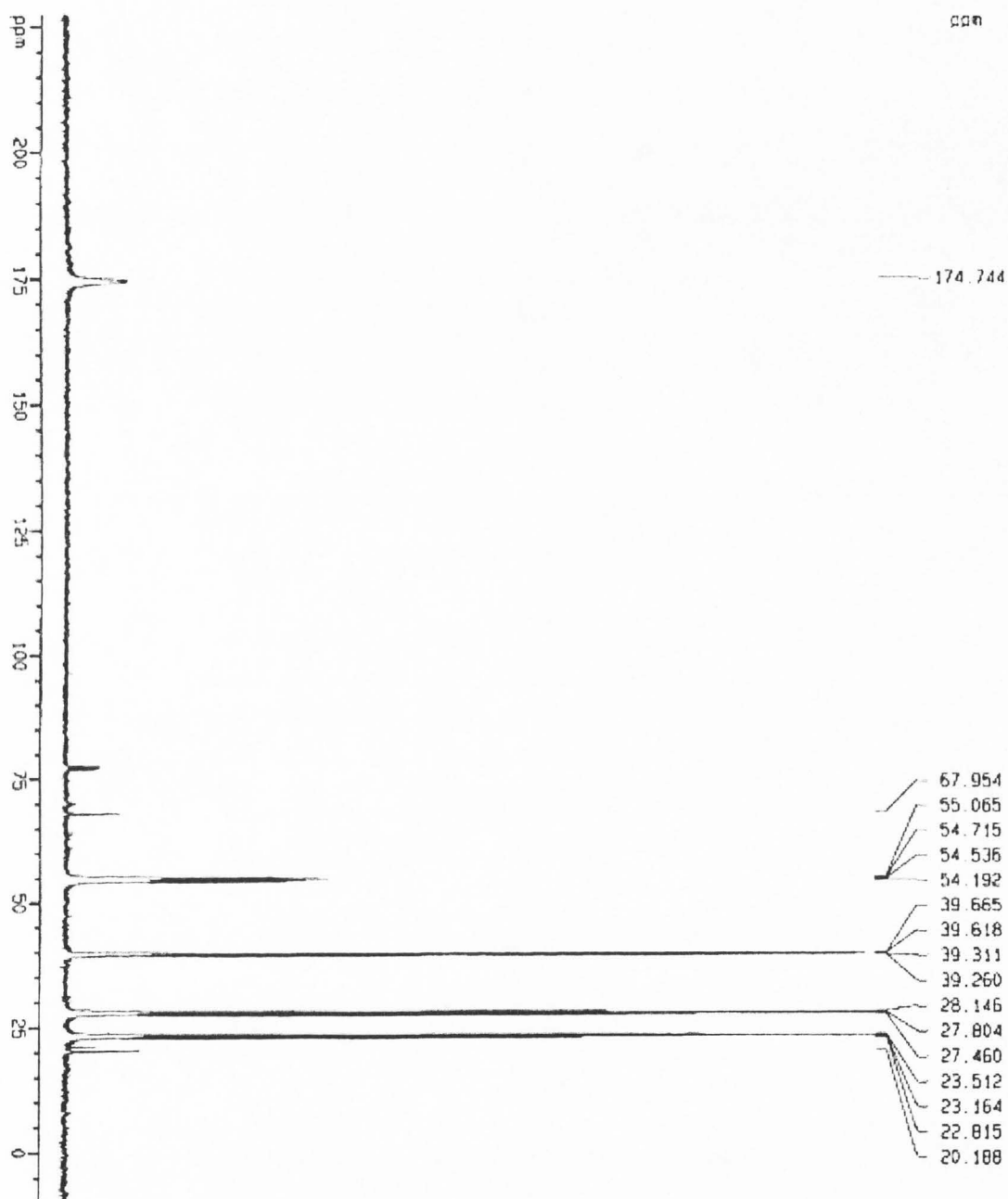
FIG. 11. Assay for putrescine production by *Lactobacillus helveticus* CNRZ 32 after incubation at 30°C for seven days in MRS with [U-[13]C5] L-ornithine. Peaks show the resonating ^{13}C atoms within a compound in parts per million (ppm) after 50,000 NMR scans at room temperature.
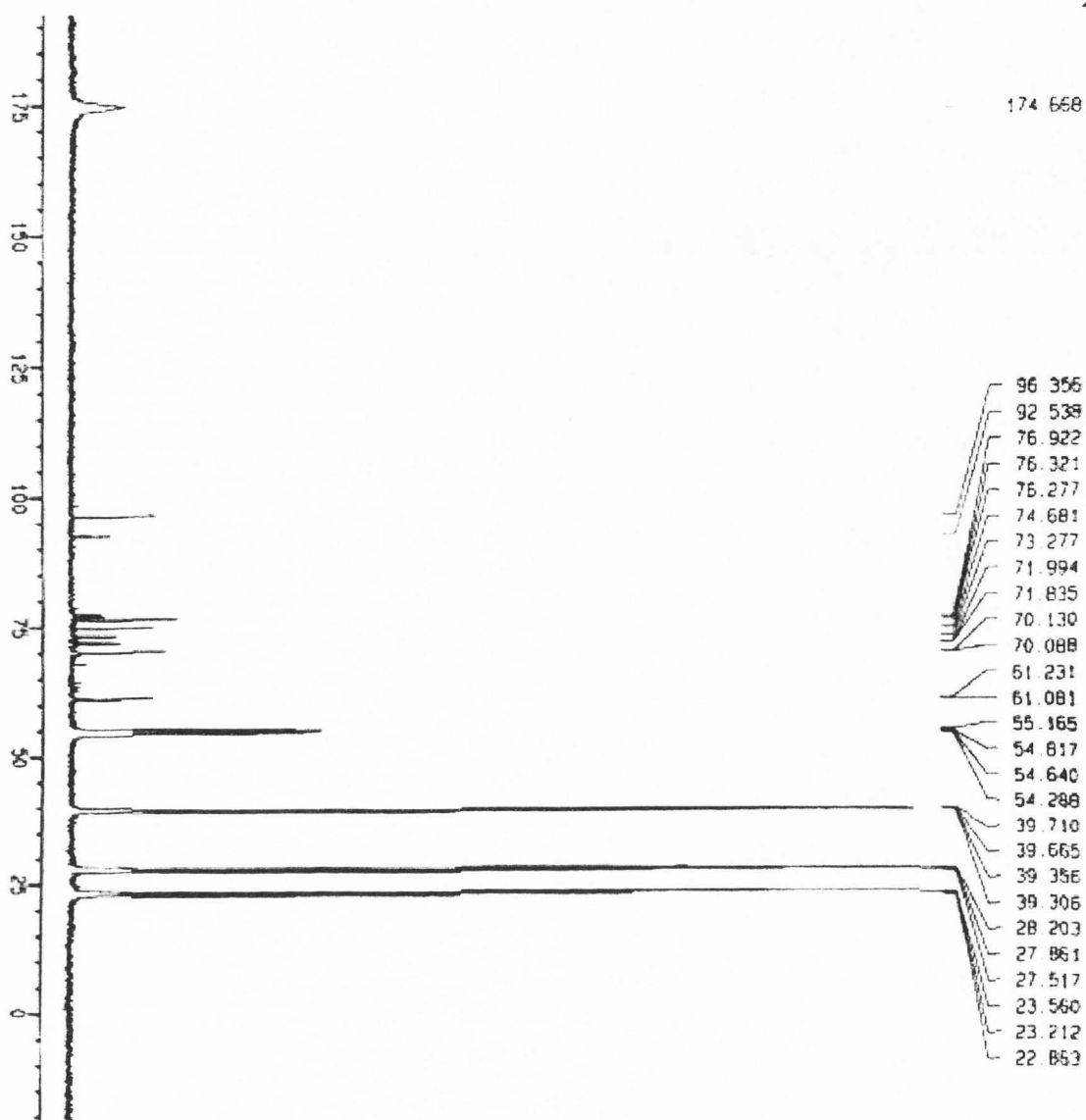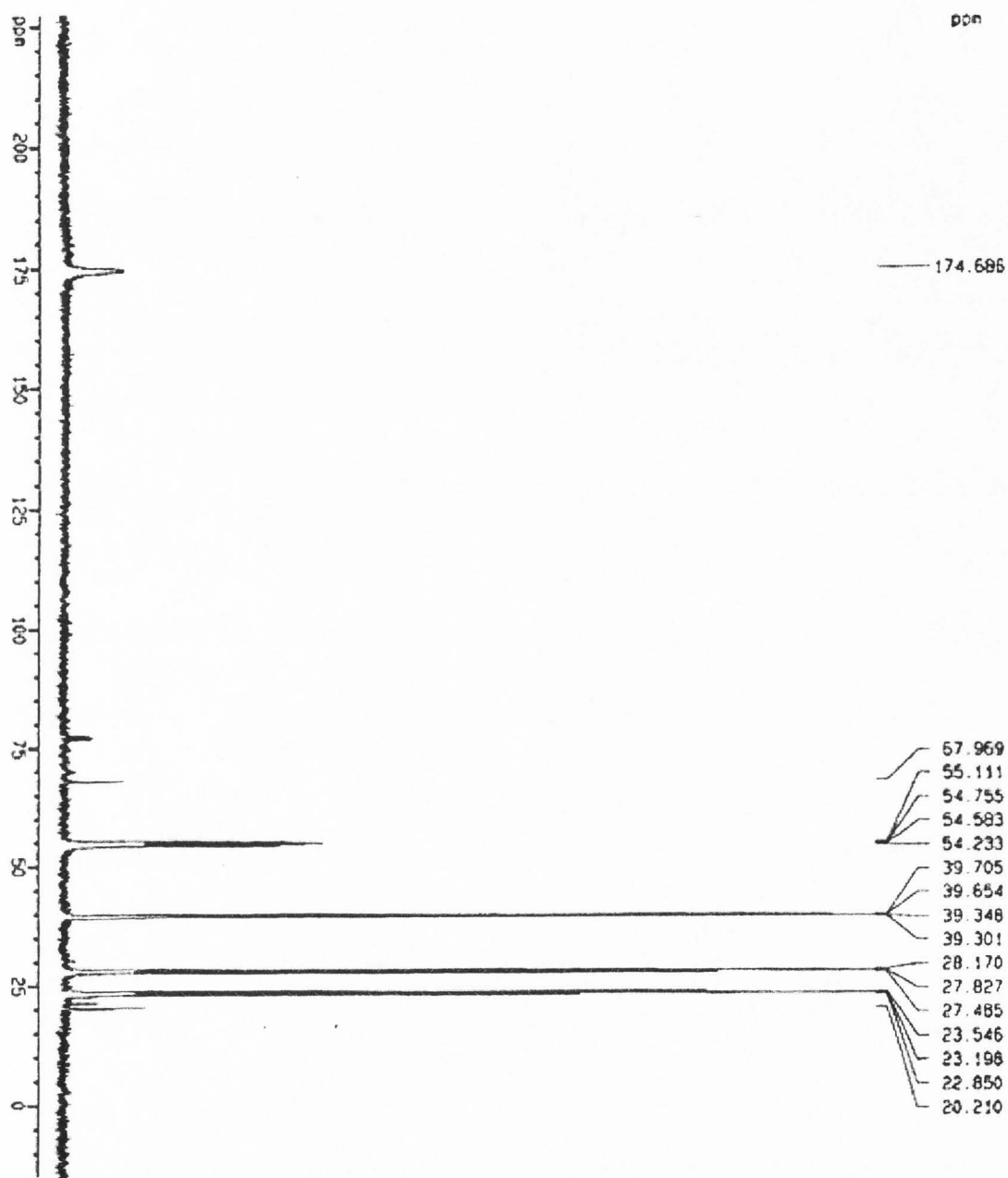
DISCUSSION

Experiments outlined in this thesis were designed to test genome predictions that auxotrophy for Asp in *L. helveticus* CNRZ 32 could be alleviated by the addition of citrate, and that this organism could convert ornithine to putrescine. As is shown in Fig. 1, the inability of *L. helveticus* CNRZ 32 grow in CDM was restored in CDM-Cit. These results, and the observation that growth is supported in CDM-Asp/Asn, confirmed that *L. helveticus* CNRZ 32 is dependant on Asp or Asn for growth, and that citrate can alleviate Asp/Asn auxotrophy in *L. helveticus* CNRZ 32. These findings underscore the usefulness of genotypic analysis as a tool for discovery and support our hypothesis that *L. helveticus* CNRZ 32 has citrate permease and citrate lyase activities which convert citrate into the oxaloacetate (OAA) needed for Asp biosynthesis (Fig. 2).

Production of Asp from oxaloacetate results in the formation of α-ketoglutarate (α-KG), a compound known to favorably enhance aroma development in cheese (3). This contribution results from use of α-KG as the amino group acceptor by aminotransferases, which carry out the first step of many amino acid catabolic reactions in lactic acid bacteria (27). The contribution of citrate catabolism and Asp biosynthesis to α-KG production and aroma development by *L. helveticus* CNRZ 32 in cheese is unknown and would require future analysis of the mechanisms for regulation of the genes encoding all of the enzymes involved in these conversions. Nonetheless, results from CDM growth studies indicated *L. helveticus* CNRZ 32 does possess at least one mechanism for α-KG production.

In contrast to data for citrate use, experiments to confirm ornithine decarboxylase activity in *L. helveticus* CNRZ 32 were unsuccessful. After initial experiments using chromogenic agar gave negative results, the cells were assayed using TLC and HPLC methods, but again no evidence for biogenic amine formation was noted. Both chromatogenic procedures provide substantially greater sensitivity than is available with the improved decarboxylase medium. The limit of detection for the agar was reported to be 350 mg per liter, while that of TLC is about 0.1 mM (~ 10 mg per liter) and while the HPLC method that is about 3X more sensitive than TLC (6, 15, 19, 28). However a derivitization step is required for the TLC and HPLC methods, so the absence of detectible putrescine may theoretically result from inefficient derivitization, rather than a lack of putrescine biosynthesis by *L. helveticus* CNRZ 32.

In an effort to resolve this question, we turned to ^{13}C-NMR which is more sensitive than TLC and not as invasive as HPLC. Visual examination of the chemical shifts of the putrescine standard (Fig. 4) showed that the peak which most clearly separates putrescine from L-ornithine occurs at 24 ppm. This peak is caused by the second and third carbons of putrescine and is clearly present in samples that contained the *Lactobacillus* sp. ATCC 33222 positive control (Figs. 5 and 9), even though the carbon-carbon splitting is dividing the signal. The five chemical shifts that describe L-ornithine (Figs. 6, 7, and 10) are entirely missing in Figs. 5 and 9, except for the peak at 39 ppm, suggesting that *Lactobacillus* sp. ATCC 33222 converted most of this substrate into putrescine. As expected, the negative controls (Figs. 6 and 10) simply showed the presence of labeled ornithine, thereby ruling out the possibility of false positives.

Unexpectedly, ^{13}C-NMR spectra from *L. helveticus* CNRZ 32 (Figs. 8 and 11) samples looked very similar to those from the negative control and the ornithine standard (Figs. 6, 7 and 10). The limit of detection with unlabeled putrescine for our instrument and conditions is approximately 0.087 mM (based on the detection of 8.7 mM unlabeled putrescine, and considering the natural abundance of ^{13}C is 1%). Moreover, increasing the number of scans to 50,000 lowers the limit of detection (Fig. 11). In water, putrescine has an odor threshold of 0.1 mM and 0.5 mM in a food matrix (2 % soybean flour solution; 36). Based on these values, one can conclude that ornithine decarboxylase activity in *L. helveticus* CNRZ 32 is either absent or so low it will not affect the odor or flavor of cheese products containing this bacterium.

Though it remains possible that *L. helveticus* CNRZ 32 is able to generate putrescine at a concentration below the limit of detection for ^{13}C-NMR, a more plausible conclusion is that *L. helveticus* CNRZ 32 does not possess ornithine decarboxylase activity and that *odiC* and *odcI* encode alternate and as yet unknown enzyme function(s). The finding that annotations for *odiC* and *odcI* are likely incorrect was somewhat surprising because the *L. helveticus* CNRZ 32 genome also contains the *potABCD* operon, which is predicted to code for an ornithine ABC transporter. Recent discovery of *potABCD*, two ornithine decarboxylases, and an atypical *potBCAD* operon in the genome sequence of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC was interpreted to reflect a physiological need for polyamines in this species (34). Our finding that a closely-related bacterium (*L. helveticus* CNRZ 32) with similar gene content does not convert ornithine to putrescine indicates this hypothesis requires further exploration.

In summary, this study provided examples of both the value and pitfalls associated with phenotypic predictions from genome sequence information for bacteria. First, we confirmed a genome sequence prediction that citrate can alleviate Asp/Asn auxotrophy in *L. helveticus* CNRZ 32 grown in CDM, and propose that this metabolic pathway may provides a mechanism for α–KG production by *L. helveticus* CNRZ 32 in cheese. However, our inability to substantiate putrescine synthesis from ornithine by this bacterium also underscored the need to experimentally confirm genome sequence predictions. Future studies to determine factors that regulate the expression of *L. helveticus* CNRZ 32 genes under the environmental conditions associated with cheese maturation would provide valuable insight into the contribution of citrate catabolism to α–KG production, and may also provide new hints on the physiological role of *odiC*, *odcI*, and *potABCD*.

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