CATABOLISM OF AMINO ACIDS TO VOLATILE FATTY ACIDS

BY LACTOCOCCUS LACTIS

by

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of the requirements for the degree

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ABSTRACT

Catabolism of Amino acids to Volatile Fatty Acids by *Lactococcus lactis*

by

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Lactic acid bacteria are essential as flavor producers of cheese and fermented products. They are capable of catabolizing aromatic, branched chain, and sulfur amino acids to flavor compounds. During cheese ripening the numbers of lactococcal colonies decrease, but lactococci survive without replication in culture. This prompted an investigation into possible mechanisms of catabolism of branched chain amino acids into branched chain fatty acids and the physiological relevance of amino acid catabolism to the bacteria. We hypothesized that lactococci catabolize branched chain amino acids to branched chain fatty acids during nonculturability.

Lactococci, lactobacilli, and brevibacteria catabolized both branched chain amino acids and keto acids into branched chain fatty acids. Lactococci survived carbohydrate-limited conditions for over 4 yrs. Their survival was represented by maintaining intracellular ATP, enzyme activity, membrane integrity, capability of ATP- and PMF-dependent substrate transport, transcription, and
catabolism of amino acids to fatty acids. Assays conducted with NMR
spectroscopy coupled with in silico analysis showed that branched chain
substrates are catabolized via keto acids, HMG-CoA, and acetyl-CoA to branched
chain fatty acids. A short list of candidate genes was identified for the pathway
by gene expression analysis coupled to NMR analysis. The expression of these
genes and the presence of the related catabolites were identified in long-term
starved cultures of nonculturable lactococci. This verified that catabolism of
branched chain amino acids to branched chain fatty acids occurred during the
nonculturable state only and in conditions of carbohydrate deprivation. The
pathway also facilitated fixation of carbon by lactococci, revealing the
mechanism of survival of lactococci over 4 yrs in culture without the addition of
external carbon sources. Between strains the availability of carbohydrate and
acid stress played significant roles in modulating their ability to produce
branched chain catabolites.

The ability of lactococci to catabolize branched chain amino acids during
sugar starvation represents a shift in carbon catabolic routes. The identified
pathway also represented a balance between catabolism and anabolism,
suggesting that the bacteria were in a homeostatic state during nonculturability.
We accepted the hypothesis that nonculturable lactococci catabolized branched
chain amino acids to branched chain fatty acids during starvation.
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I dedicate the fruits of this endeavor to my parents, Smt. Prabha Ganesan and Sh. N. Ganesan, my sister, Smt. Gayatri Raja, my late grandmother, Smt. C.S. Narayani, and grandfather, Sh. S. Nagarathnam. Their support has been a constant driving force during my education here.

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LIST OF SYMBOLS, NOTATIONS, DEFINITIONS

Abbreviation Key

3-PG = 3-phosphoglycerate
$\Delta G^\circ$ = standard Gibbs' free energy change
AAT = aromatic amino acid aminotransferase
ADI = arginine deiminase
AP = aminopeptidase
ATase = aminotransferase
ATP = adenosine triphosphate
BAT = branched chain amino acid aminotransferase
BCAA = branched chain amino acid
BCFA = branched chain fatty acid
CDM = chemically defined medium
CE = capillary electrophoresis
CFE = cell-free extract
CFU/g = colony forming units per gram
CFU/ml = colony forming units per milliliter
CoA = coenzyme A
DNA = deoxyribonucleic acid
EC = Enzyme Commission number
FA = fatty acid
GC = gas chromatography
HMGA = 3-hydroxy-3-methylglutaric acid
HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A
HPLC = high performance liquid chromatography
IUPAC = International Union of Pure and Applied Chemistry
JGI = Joint Genomes Institute
KEGG = Kyoto Encyclopedia of Genes and Genomes
KIC = α-ketoisocaproate
KIV = α-ketoisovalerate
KGL = α-ketoglutarate
KMV = α-keto-β-methylvalerate
kJ/mol = kilojoules per mole
LAB = lactic acid bacteria
LABGC = lactic acid bacteria genomes consortium
LE = lipase/esterase
M17G = M17 broth containing 0.5% glucose
M17L = M17 broth containing 0.5% lactose
MCA = monocarboxylic acid
MECC = micellar electrokinetic capillary chromatography
meq = milliequivalents
MES = 2-(N-morpholino) ethane-sulfonic acid-sodium salt
mM = millimolar
MOPS = 3-[N-Morpholino]-propanesulfonic acid-sodium salt
MRS = de Man Rogosa Sharpe
NADH = nicotinamide adenine dinucleotide (reduced)
NAD⁺ = nicotinamide adenine dinucleotide (oxidized)
NC = nonculturable
NH₃ = ammonia
nM = nanomolar
NMR = nuclear magnetic resonance
NSLAB = nonstarter lactic acid bacteria
PEP = phosphoenol pyruvate
Pyr = pyruvate
pM = picomolar
PMF = proton-motive force
Redox = oxidation-reduction potential
RFU = relative fluorescence units
RNA = ribonucleic acid
SAT = sulfur-containing amino acid aminotransferase
SCFA = short chain fatty acid
SLP = substrate level phosphorylation
sn = stereospecific number
TSB = tryptic soy broth
VBNC = viable-but-non-culturable
VFA = volatile fatty acid
VSC = volatile sulfur compound
"Say Cheese" calls the photographer at any part of the world when a photograph is to be taken. This is the extent to which the taste of cheese has influenced people. However cheese flavor is a very complex phenomenon. Even though there is a unique flavor for every cheese, there is a large range of different compounds, in varying quantities in each cheese type that constitute the flavor precursors of each and every cheese. Years of research have not yielded a single unique compound that contributes to any cheese flavor in isolation (13). Hence, the Component Balance Theory of Cheese Flavor (14) that attributes cheese flavor to a delicate balance among a multitude of compounds is widely accepted.

Fat, proteins, peptides, amino acids, volatile sulfur compounds, aldehydes, ketones and volatile fatty acids are some of the classes of compounds that contribute to cheese flavor (24). Volatile sulfur compounds play a major role in cheese flavor (1, 5, 7, 10, 12, 25); but they alone do not lead to the total flavor perception in Cheddar cheese. All attempts to simulate Cheddar cheese flavor have not been successful in producing real cheese flavor (13). This suggests that the major components do not play a single-handed role and the role of minor components must be considered.

Small quantities of volatile fatty acids play a role in cheese flavor (19), especially in cheeses with unique flavors (9). In higher quantities, they lead to off-flavors like rancid, goaty, and lipolytic. Volatile fatty acids can be produced
via three basic metabolic mechanisms of lactic acid bacteria (LAB) – glycolysis, lipolysis and proteolysis. Lipolysis is the dominating mechanism involved in generating the lipolytic flavor of Italian cheeses and flavor defects in milk and other dairy products. But the intriguing factor about the presence of fatty acids in cheese is that starter bacteria used in cheese manufacture possess very limited lipolytic capability. Hard cheeses contain more short chain (volatile) and odd–chain fatty acids, and especially branched–chain fatty acids that are not found as glyceryl esters in milk fat, suggesting that lipolysis is only one of many methods to provide flavor from volatile fatty acids. This fact alone instigates a curiosity to investigate alternate mechanisms for volatile fatty acid production by LAB during cheese ripening.

Lactose, the major carbohydrate in milk, is metabolized to lactic acid during the initial steps of cheese–making. Another portion is removed along with whey in the steps before cheese ripening. Residual lactose in the cheese matrix is utilized by bacteria in the curd within a week of Cheddar cheese manufacture (6). By 30 d the concentration of lactose is near zero. Therefore, there is no available lactose for bacteria to generate glycolytic by-products. Bacteria may utilize lactate to generate acetyl–CoA and eventually acetic acid in the early phase of ripening. But this does not account for higher fatty acids, odd–chain and branched–chain fatty acids produced during ripening. Hence, lactose can not be solely responsible for the increase in fatty acids with time in Cheddar cheese.

Protein metabolism by the associated bacteria is another potential source of fatty acids. During the later phase of ripening, protein breakdown prevails,
and provides peptides and free amino acids. The limited lipolytic capability of
lactic acid bacteria and presence of branched-chain fatty acids suggest that amino
acid catabolism may be involved in fatty acid production. But the catabolic
mechanisms will depend on the genera and their physiological states during
cheese ripening.

During Cheddar cheese ripening, the lactococcal starter population
decreases over time whereas non-starter bacterial population, primarily that of
lactobacilli, increases. Lactococci are added at $10^5$ CFU/ml of milk to initiate
cheese manufacture and increase to $10^8$ to $10^9$ CFU/g of cheese curd at salting.
Depending on the strain, lactococci decline by ~99.9% at the end of 4 weeks to <
$10^5$ to $10^6$ CFU/g cheese (4). The lactobacilli in cheese begin at cell densities of 10
to $10^4$ CFU/g of cheese during initial storage and rise to around $10^8$ CFU/g, and
plateau at $10^6$ to $10^8$ CFU/g (4, 18). The reduction in lactococcal population and
the subsequent increase in non-starter population lead to contradictory views of
possible theories of cheese ripening and the associated mechanisms to produce
flavors during ripening.

One hypothesis attributes the reduction in lactococcal numbers in cheese
during enumeration by cultivation on non-selective media to cell lysis and death
(2, 17, 26). Autolysis releases intracellular enzymes of lactococci that aid non-
starter growth and cheese ripening (3). But this theory neglects other possible
physiological states of bacteria in cheese.

An alternate hypothesis attributes starter bacterial metabolic activity in
the state of non-culturability (NC), also described as the viable-but-non-
culturable state of bacteria, to cheese flavor generation. Lactococci become NC
on laboratory media and remain metabolically active (20). At the onset of lactose starvation, the cells have a sufficient ATP pool (22), and sufficient reserves of glycolytic intermediates for active intake of substrates (23). Since the starter bacteria may also exist in the NC state in cheese, they are still metabolically capable of generating ATP (20) and other substrates essential for viability (21, 22).

The pathways active under such stressful conditions could be multipurpose pathways that help the starter cells survive. Bacteria may synthesize flavor precursors by these pathways and hence, contribute to cheese flavors. In the presence of metabolically active starter cells, the contribution of non-starter bacteria is of questionable importance with respect to ideal flavor production, and hence, ultimately, the quality of cheese (8, 11).

In the absence of lactose, bacteria can utilize proteins in cheese as a source of ATP, carbon, sulfur and nitrogen. Earlier studies partially supported this idea by experimenting with cultures incubated with amino acids or casein digestion products in the absence of any other substrate (21), or by attempting to simulate cheese–like conditions (7, 15, 16) and finding fatty acid production. LAB were unable to produce fatty acids from casein digests or amino acids in presence of lactose (15, 16). But the conversion of amino acids to fatty acids has not been characterized in Cheddar cheese.

Taken together this leads to the hypothesis of this study that some volatile fatty acids found in cheese, especially the branched–chain fatty acids, are formed by the utilization of amino acids by NC bacteria in cheese. The objective of this project is to identify the amino acids that are converted to volatile fatty acids and
branched chain fatty acids. The cumulative effect of sugar starvation and pH on starter activity will be examined to establish the link between environmental conditions and the metabolic mechanisms.

References


CHAPTER 2
LITERATURE REVIEW

Role of microorganisms in cheese flavor

Addition of bacteria is an essential step in cheese-making towards a good-flavored cheese. Cheese without bacteria fails to develop flavor during ripening (3, 89). The flavor profile of cheeses depends on bacteria involved in cheese ripening and their catabolic capabilities leading to the different flavor compounds present in different cheeses (50).

Bacteria are involved in all steps of cheese making. In the initial stages of Cheddar cheese ripening, LAB catabolize lactose to lactic acid. Lactose is reduced to undetectable levels by 30 (28). Proteolysis by bacteria slowly degrades the casein matrix over time. This yields peptides and amino acids that bacteria transport and utilize (25). Peptides are directly related to bitterness in Cheddar cheese (17). Amino acids are catabolized to flavor compounds involved in positive Cheddar flavor in culture and cheese slurries (36, 62, 63). This suggests that the type of bacteria involved may modulate flavor production in Cheddar cheese.

Previous studies focused on identifying the best flora type, either singly or in combination, to produce an acceptable Cheddar cheese flavor (2, 7, 9, 12, 17, 25, 28, 33, 36, 50, 76, 159). The focus is often on nonstarter lactic acid bacteria (NSLAB), predominantly lactobacilli, as the causal agents of flavor on the basis of their population in later stages of cheese ripening (35, 84, 104, 119, 140). The role of different genera in Cheddar cheese flavor continues to be elusive and the
cumulative interpretations from numerous studies add to the controversy. Nonstarter lactobacilli intensify cheese flavor (51, 84, 125), but no specific strains are directly linked to better overall Cheddar flavor. However, lactococci have a unique causative role in cheese flavor (114).

Total bacterial counts are related to flavor development. The lower the number of starter lactococci, more intense the Cheddar flavor; but at when lactococcal plate counts of cheese are $>10^9$ CFU/g, cheese is bitter (95). Lactococci enter into a state of NC (81, 124, 134, 137). This may lead to lower numbers of lactococci by bacterial plate counts during cheese ripening. These studies taken together suggest that catabolism by lactococcal starters may play a role in Cheddar cheese flavor.

Compounds associated with cheese flavor

**Cheese flavor**

Cheese flavor is a combined effect of various compounds at different concentrations and their interactions (105). Each cheese has a specific group of flavor compounds that are responsible for its flavors (Table 2-1). Multiple classes of organic compounds are implicated in cheese flavor (79, 147). But knowledge of the impact is limited to a few groups only (143). Compounds contribute specific flavor attributes based on their physico-chemical properties (143). Some compounds represent typical flavors of certain cheeses, acting as impact compounds for that flavor, but not for the total flavor perception (Table 2-1). Multiple lists of flavor compounds are available in literature (50, 51, 142, 143).
Flavor compounds are generated from substrates available during cheese ripening. The direct role of amino acids and peptides in cheese flavor is limited (2, 43) to contribution to base cheese flavor (129) and acting as substrates for enzymatic modification reactions (142). Volatile fatty acids and volatile sulfur compounds are two major classes that correlate with flavor development during cheese ripening. Volatile fatty acids exist both alone and in combinations with volatile sulfur compounds as thioesters. Volatile sulfur compounds are one of the major classes of flavor compounds that correlate with good Cheddar cheese flavor (36, 99, 153).

**Sulfur compounds in cheese**

Volatile sulfur compounds (VSCs), especially methanethiol, correlate to positive flavor and aroma development in Cheddar cheese (99, 117, 153). VSCs are identified in cheese as flavor precursors at very low concentrations due to their high flavor thresholds (153). Methanethiol is the principal component identified to be responsible for Cheddar aroma. Other compounds like hydrogen sulfide, methyl and dimethyl di- and tri-sulfides are also found in cheese, but their role in cheese flavor is not well-defined (34).

VSCs are highly reactive and may also react with other products of bacterial catabolism. S-Methyl-thioesters, the products of reactions between VSCs and volatile fatty acids, also impact cheese flavor. While microbial mechanisms of thioester production exist, they are yet to be characterized for their contribution to cheese flavor (29, 30).
VSCs alone do not allow Cheddar flavor production. Volatile fatty acids are also necessary to provide ideal Cheddar flavor (94). The balance between VSCs and volatile fatty acids may play a role in ideal cheese flavor. At least H₂S is correlated to quantities of volatile fatty acids in Cheddar cheese flavor (Table 2-2). Hence, the role of volatile fatty acids in cheese flavor seems to be important as they could exist in both oxidized and reduced forms that contribute by interactions with other compounds towards cheese flavor.

**Volatile fatty acids in cheese**

The typical flavors of fatty acids (FAs) in isolation are often considered negative at higher concentrations (Table 2-3). Except for n-valeric acid, none of these compounds possess a flavor that resembles cheese flavor. However, in low concentrations and in combination with other groups of compounds, volatile fatty acids (VFAs) contribute desirable flavors to cheese (115). Therefore, VFAs are considered important in cheese flavor (94, 129) and are also included in synthetic flavor formulations (87).

The role of VFAs in cheese flavor depends on their concentration. VFAs are found in typical aged, good flavored Cheddar cheese at a concentration of ~1000 ppm and in varying amounts in other cheeses (Table 2-4) (156). Most even straight-chain fatty acids and branched chain fatty acids (BCFAs) are at concentrations higher than their reported flavor and aroma thresholds (5). The concentrations of acetic acid, n-butyric acid and n-caproic acid increase during cheese ripening, with a concomitant improved flavor (11, 22, 80). Above these levels, VFAs lead to off-flavor in cheeses (91). Hence, VFAs contribute to off-
flavors at high concentrations, but may aid beneficial Cheddar cheese flavor at low concentrations.

The levels of VFAs change in relation to age of cheese, ripening temperature and cheese composition (22). While VFA concentrations are associated with desirable flavor, individual VFAs modulate the flavor profile of cheeses (Table 2-5) because of their low flavor thresholds and distinctive flavors. Propionic acid, n-butyric acid, n-valeric acid, n-caprylic acid, and thio-esters of these VFAs have aroma properties compatible with participation in cheese flavor development (88). VFAs are vital for development of typical flavor of Blue-veined cheese, both alone and as substrates for oxidation to methyl ketones (88). Italian hard cheeses like Romano, Provolone, and Parmesan cheeses contain VFAs that are attributed to small amounts of deliberately added animal lipase (88), consisting of a large number of BCFAs (60). n-Valeric acid, and BCFAs like 4-methylvaleric acid, 2-ethylcaproic acid and 6-methylheptanoic acid have cheese-like, nutty flavor at concentrations as low as 2.5 ppm (16).

n-Butyric, n-caproic, n-capric and 3-methylbutyric acids are the VFAs among the flavor compounds in cheeses (22, 143). Lauric, myristic, palmitic and stearic acids are present in cheese (146) but not implicated in flavor. n-Butyric acid at concentrations of 45-50 ppm and n-caproic acid at 20-25 ppm are associated with optimum Cheddar flavor (11). n-Butyric acid, isovaleric acid and n-valeric acid are also reported to be absent in commercial Cheddar cheeses (13, 118).

VFAs along with their own individual flavor, also exist as ketones, esters, and lactones in the reduced conditions of cheese (87, 88, 90). Additionally, fatty
acid ethyl esters, especially of caproic and caprylic acids, are involved in cheese flavor (50). But the actual sources of these VFAs, especially BCFAs, in cheese are unknown.

Sources of various fatty acids

**Lipolysis**

Lipolysis is the reaction that cleaves the glycerides of milk fat to produce FAs. Rise in specific FAs is associated with rancid flavor in milk (10). Lipolysis is a source of FA generation in cheese (47-51) and is very important in Blue and Italian type cheeses wherein, *Penicillium roquefortii* sp. and pregastric esterase, respectively, are the principal lipolytic agents (51).

Fresh raw milk contains ~0.1% VFAs prior to lipolysis (82); hence milk itself is not a major contributor to VFAs in cheese. VFAs liberated from lipolytic reactions correspond to the VFAs attached to milk fat as glyceryl esters. The initial FA composition of milk fat is affected by season and milk source (125) and is defined after collection and processing of milk prior to cheese making.

n-Butyric acid, n-caproic acid, n-heptanoic acid, n-caprylic acid, n-nonanoic acid, n-capric acid, 9-decenoic acid and higher BCFAs are found as FAs in milk fat (59). Hence, some of the smaller even chain FAs may arise from milk fat. But this does not account for the levels of smaller BCFAs like isobutyric and isovaleric acids in cheese. This raises the question of factors involved in lipolysis of milk fat.

VFA generation by lipolysis depends on VFA distribution on the glycerol moiety. VFAs in milk fat are stereospecifically distributed among sn–1,2,3 positions of glycerol. The distribution varies inversely with chain length at sn–3
position and directly at sn-1 position. The variation is greater at sn-1 and sn-3 positions than sn-2, and is relatively stable with milk production season (60, 113).

Attempts were made at identifying VFA quantities and associated formulae to identify VFA levels with good cheese flavor. One such study calculated the extent of lipolysis from C-16 value (which is described as 22.3 % of butterfat) using the formula (Eq. 1) (143):

\[
\text{Extent of lipolysis} = \frac{\% \text{ C-16} \times 100 \times 100}{22.3 \times 1 \times 3.3} = \% \text{ C-16} \times 13.6 \quad \text{(Eq. 1)}
\]

The study described good quality cheese to have < 0.52 % lipolysis at 0 months to < 1.6 % at 20 months. Cheeses with a higher extent of lipolysis have off-flavors like soapy, oniony, metallic and vomit (143). Hence, the level of VFAs is important in contributing to a balanced cheese flavor. But this assumes VFA production by lipolysis only. VFAs are not solely derivable from fat. They have other potential sources of origin like lactose and amino acids. This deepens the dogma in importance of milk fat in VFA generation in Cheddar cheese.

A theoretical estimate of amounts of SCFAs that can be derived from milk fat represents the maximum possible VFAs that can be produced. Calculating the amount of SCFAs (≤ C-6) that can be attributed to lipolysis, from FA distribution in milk fat (Table 2-6), the maximum possible VFAs can only be ~150 ppm, which is only ~15% of the total VFAs found in Cheddar cheese and even lower than in many other cheeses. Also, this includes only n-butyric and n-caproic acid which are only straight chain VFAs. None of the BCFAs important towards cheese
flavor (126, 127, 159, 160) are noted. The above calculation neglects action of lactococcal lipases.

Lactococcal lipase and esterases are capable of hydrolysing mono- and di-glycerides more readily than tri-glycerides (93); but the levels of mono- and di-glycerides is < 0.5% of milk fat (10, 82). Even if we consider that some VFAs in cheese are likely to arise from mono- and di- glycerides by lactococcal lipolysis, it amounts to ~0.75 ppm. This does not contribute to VFA quantities in cheese significantly in addition to considering triglyceride hydrolysis. Also, intracellular lipase-esterase activity of bacteria does not positively correlate with Cheddar cheese flavor (152). The known lipase-esterases of lactococci (15) (http://www.jgi.doe.gov/JGI_microbial/html/index.html), and other LAB (40, 44, 45, 75) possess only esterase activity (108). Therefore, the role of fat is questionable and may be less important in generation of VFAs in Cheddar cheese.

Milk contains native lipoprotein lipase and lipases contributed by raw milk microflora. Native milk lipase is destroyed by pasteurization (1, 132) and is not active at the salt content and pH of cheese (76). Lipases from Gram–negative bacteria in raw milk such as Pseudomonas spp. and Achromobacteriaceae possess thermal resistance and cause lipolysis in cheese milk (132). But their role in the reduced conditions in hard cheeses is yet to be characterized. The addition of external lipolytic enzymes from similar raw milk microflora has a negative effect on flavor quality in Cheddar cheese (51). Intracellular lipase-esterase activity of starter bacteria does not positively correlate with Cheddar cheese flavor (152).
This suggests that Cheddar cheese VFAs develop neither due to native milk lipases nor addition of any other external lipase.

The contribution of fat to VFA production in cheese or cheese-like conditions is also experimentally elucidated by other studies. Their approach is to either remove fat from milk and study skim milk cheeses or alternatively substitute milk fat with vegetable lipids (154). Acetic acid concentration is similar in whole milk cheese and skim milk cheese; but the concentration of higher VFAs is reduced in skim milk cheese. This difference is attributed to factors like higher moisture, lower fat and higher salt in fat-free or fat-substituted cheeses (38). Cheddar and Romano cheese slurries from skim milk contain very low concentrations of SCFAs (61) that is attributed to abnormal ripening conditions. While n–butyric acid arises in milk from lipolysis, n–butyric acid in Cheddar cheese is twice the amount of esterified n–butyric acid in milk fat (13). This preempts the conclusion that fat is the sole source of VFAs in Cheddar cheese.

In vegetable fat slurries, VFAs less than C-10 are detected in traces versus milk fat cheese slurries (61). These VFAs do not represent any contribution of vegetable lipid fatty acids in fresh cheese curd. Aging produces SCFAs in vegetable lipid cheese but not VFAs greater than C–12; but SCFAs are at concentrations lower than in milk fat cheese (61). This suggests that other mechanisms are needed to produce VFAs that are not found in milk fat.

Lipases from bacterial and fungal sources are involved directly in fat hydrolysis and cheese flavor as noted by cheese variety and culture addition. SCFAs on the glyceride are preferred over BCFAs by microbial fungal lipases (60). This directs the VFA profile of mold–ripened cheeses; but fungal lipases
may not be present in regular Cheddar cheese as molds are considered contaminants in Cheddar cheeses. This indicates that fat lipolysis is not the only mechanism that leads to VFA formation in cheese. VFAs may be produced by bacterial catabolism of other substrates.

**Carbohydrates**

Carbohydrates are the primary carbon and energy source for LAB and hence, are implicated in VFA and flavor generation. Some genera utilize carbohydrates to produce VFAs. For example, brevibacteria convert glucose to VFAs near pH 7, and galactose to VFAs near pH 8 (69, 70). But their ability to produce VFAs from carbohydrates at a pH of 5.2 is limited (69, 70).

In other genera, VFAs are not directly derived from carbohydrates but from products of carbohydrate catabolism like pyruvate, lactic acid, citrate, and acetyl-CoA (93). Propionibacteria convert lactic acid to acetic and propionic acids, which are important in typical flavor of Swiss cheese (141). NSLAB utilize citrate for energy in absence of carbohydrates and produce formate by the citrate–formate pathway (119). Citrate is reduced to diacetyl and further to acetoin, 2,3-butane diol and 2-butanone, which is important in cheese flavor. (85). However, VFAs are not formed from citrate and its metabolites. Acetyl CoA is converted to straight, even chain fatty acids of length C–2 to C–20 (61), but not BCFAs. There are, therefore, multiple mechanisms that lead to straight chain fatty acids from carbohydrates by microbial metabolism; but not BCFAs.

Lactose is the primary energy source in milk for LAB which utilize sugars as an energy source during growth and acid production. The amount of residual
lactose available to bacteria in cheese depends upon starter activity, washing of curd, and mode of salting of cheese (33). Lactose is reduced to <0.01% after a week of ripening of hard cheeses (28). Hence, it does not account for VFAs beyond the initial week of cheese ripening. Therefore, lactose has little influence on VFAs produced during cheese ripening.

**Amino acids**

The level of n-butyric acid in cheese is higher than the total amount of n-butyric acid found as glyceryl esters with milk fat (13). Hence, other potential sources that can yield n-butyric acid by microbial catabolism may have a role during cheese ripening. One such source is milk protein by the conversion of amino acids to VFAs (67, 106, 107).

Leucine, glutamate, phenyl alanine, valine and lysine are the principal amino acids in Cheddar cheese (43, 157). Multiple mechanisms for production of VFAs and other flavor compounds from amino acids are postulated. Both enzymatic and non-enzymatic degradation of amino acids in cheese yield flavor compounds (148). Deamination of amino acids in cheese produces ammonia and \( \alpha \)-keto acids, and specific VFAs (Table 2-7). Isovaleric and 3-methylbutyric acids found in Livarot and Pont L’Eveque cheeses are produced from leucine and isoleucine, respectively (133). Transamination and Strecker degradation yield aldehydes (50).

A theoretical estimate of maximum possible VFAs from amino acids can be made from data available for amino acid composition of casein (10). The maximum possible total VFAs from this calculation is \(~41,000\ ppm,\) considering
that only known precursors are involved in the contribution and also, only ~30% of protein in cheese is broken down by proteolysis (21). Whereas, the actual total VFA content in Cheddar cheese is around 1,028 ppm (47). The lower amounts of VFAs may be due to the various stress conditions in cheese that slow down metabolic processes and the structural modifications caseins undergo during proteolysis. All peptides that result from casein degradation may not be totally converted to amino acids, and further to VFAs. This suggests that casein degradation to amino acids can produce VFAs.

Catabolism of amino acids to volatile fatty acids

Multiple genera produce VFAs in cheese environmental conditions. Acetic, propionic, isobutyric, n-butyric, isoalvaleric and n-caproic acids are produced by lactococci and lactobacilli, product depending on strain (106). The pH optima for VFA production vary. Lactobacilli produce valeric acid and its isomers from leucine and isoleucine at a lower pH (107). *Lactobacillus delbrueckii* ssp. *bulgaricus* produces propionic and n-butyric acids while *Lactobacillus casei* ssp. *casei*, *Lactobacillus delbrueckii* ssp. *lactis* and *Streptococcus salivarius* ssp. *thermophilus* produce acetic acid (139). In similar experimental conditions, *Lactococcus lactis* ssp. *cremoris* produces more BCFAs than *Lactococcus lactis* ssp. *lactis* (28). *Brevibacterium linens* produces acetic acid from glycine, alanine and leucine, isoalvaleric acid from leucine, and caproic acid from cystine, alanine and serine (69, 70). *Propionibacterium freudenrichi* produces isoalvaleric acid from leucine (135). The catabolism of amino acids in cheese also requires protein breakdown to release free amino acids.
Proteolysis in cheese

The gradual breakdown of proteins in the firm, hard cheese curd to smaller-sized peptides and amino acids, leads to a cheese with softer texture and better flavor than the original rennet curd (50, 51, 92, 149). Hence, the set of reactions, called proteolysis, are important in hard cheeses for flavor and texture development (51). It provides substrates for flavor development by further protein breakdown and indirectly affects the mouth-feel and flavor release during mastication (50).

Starter proteinases act on the residual products of casein breakdown by rennet and plasmin (92). The oligopeptides are then hydrolyzed to yield smaller peptides and amino acids by intracellular peptidases in LAB (25). 25% of proteins in cheese get hydrolysed to peptides and amino acids in ripening (21). Amino acid catabolism leads to production of flavor compounds like VFAs and VSCs.

Proteolysis is important for protein breakdown and substrate release for bacteria to catabolize amino acids to flavor products. It does not directly contribute to flavor except for bitterness (17), because peptides and proteins that possess a flavor close to good cheese flavor are yet to be identified. Hence, amino acid catabolism is important for beneficial flavor development in cheese. In cheese, which is a very complex medium, post carbohydrate exhaustion, amino acids are the simplest molecules available for bacteria to generate ATP for cellular processes. Hence, the conversion of amino acids to VFAs may serve dual purposes in cheese. It may aid both survival of bacteria and flavor development in the product.
Amino acid transport and catabolism

Amino acids are essential for bacterial growth. Glutamate, valine, methionine, leucine, isoleucine and histidine are essential for LAB. Amino acids also enhance bacterial survival in absence of other substrates in *Lactococcus lactis* ssp. *lactis* (136); arginine is as effective as any other group of amino acids in aiding survival under carbohydrate starvation (24, 134, 136). A comparison of the amino acid profiles of a basal chemically defined medium (73) and 6-month-old Cheddar cheese (Table 2-8) shows that Cheddar cheese contains almost all amino acids in excess or in comparable amounts. Hence, it may be deduced that if bacteria can maintain viability within such a minimal medium (134), they will remain viable in milk and Cheddar cheese and contribute to cheese flavor.

Amino acids are transported by lactococci via three different mechanisms. Alanine, serine, the branched chain amino acids (BCAAs) – leucine, isoleucine and valine, and lysine are transported by a proton-motive-force-driven mechanism. Arginine is transported inside by an ornithine antiporter system. Similar antiporter systems also exist for histidine–histamine, tyrosine–tyramine, and aspartic acid–alanine couples (122). Glutamate, glutamine, aspartate and asparagine are transported by a phosphate bond–driven transport system (37), likely to be ATP-driven (15). Hence, transport of most amino acids will require either a potential gradient or energy. This may exist only if cells continue to remain viable and to actively metabolize substrates like amino acids for survival.
Amino acid catabolic pathways

Amino acid catabolism in LAB is important for survival in absence of carbohydrates during cheese ripening. Amino acids are degraded via oxidative deamination and decarboxylation to produce VFAs (106, 107). At the onset of carbohydrate starvation, arginine is utilized by lactococci to increase the extracellular pH and produce ATP via the arginine deiminase (ADI) pathway (24). Histidine is the only other amino acid catabolized by LAB to produce metabolic energy and also used to regulate intracellular pH and redox potential (103).

Aminotransferases (ATases) are the enzymes involved in the first step of the conversion of amino acids to flavor compounds (161). Aromatic ATases (AAT) led to putrefactive compounds like indole and skatole, that cause off–flavor in cheese (53). AAT and branched chain ATases (BAT) of Lactococcus lactis possess overlapping substrate specificities, and transaminate sulfur amino acids, leading to various flavor compounds (20, 126, 127, 159, 160). Lactococci possess more than one BAT or other ATases (20) with overlapping substrate specificities, as mutants of BAT are able to grow on α–ketoisocaproate (4). But a separate sulfur amino acid ATase (SAT) has not been identified in Lactococcus lactis (126, 127, 159, 160).

The catabolic pathways of BCAAs and sulfur amino acids in LAB are yet to be characterized. BAT catabolizes the BCAAs leucine, isoleucine and valine to yield their corresponding α–keto acids (160). Lactococci also produce isobutyric and isovaleric acids (160). But the mechanism is unknown. A number of such
pathways exist in other microorganisms that serve the same or similar purposes of the ADI pathway, i.e. ATP generation by SLP, pH regulation, redox potential maintenance and balancing starvation needs (64, 162). Such pathways may be followed by LAB, especially lactococcal starters, leading to the generation of VFAs.

ATases catalyze the first step in amino acid catabolism to yield the corresponding α–keto acid in Lactococcus lactis (126, 160, 161). Various subsequent amino acid catabolic pathways are described for non-cheese microorganisms. In Enterococcus faecalis, the α–keto acids formed are catabolized to give the corresponding BCFAs (150). Similar pathways may exist in lactococci. This is also related to BCAA utilization as the bkd gene cluster for branched-chain α–keto acid dehydrogenase also exists in Pseudomonas putida, where the transcriptional activator is found to require L-amino acids or D-leucine (96). BCFAs are produced from α–keto acids derived from leucine, isoleucine and valine. Since the genes related to these pathways are present in LAB (14, 15) (http://www.jgi.doe.gov/JGI_microbial/html/index.html), they may be expressed during cheese ripening and allow cell survival. In cheese, BCAAs may be transported faster at a low pH of 5.2 than at pH 7.2 (78). BCAAs may then be utilized by the cell to produce ATP for ATP–driven transport systems. This may be one of the potential roles of BCAA catabolism by starter cells in cheese. Multiple pathways for BCAA catabolism exist in non-LAB genera. A select number of pathways are discussed below with relevance to VFA production from amino acids.
The pathways (Fig. 2-1 and 2-2) illustrate the known catabolic routes from amino acids to VFAs by bacteria. Note that the pathways are all highly exergonic, i.e. produce energy (Table 2-9). A common theme in all pathways is coupling reactions with ATP generation by SLP and/or generation of redox compounds, like NADH. All available amino acids may not be catabolized toward energy requirements, as they are also precursors of nucleotides and proteins, which are essential for bacterial survival and metabolism. These pathways need to be active in absence of fermentable carbohydrates for the bacteria to survive.

Table 2-9 shows Gibbs’ free energy change values ($\Delta G^0$) at pH 7 for the catabolism of amino acids to VFAs. A negative $\Delta G^0$ value means that the pathway produces energy (i.e. is exergonic) and is energetically favorable. From the values of $\Delta G^0$ in the table, the catabolism of most amino acids to VFAs, except threonine, are favorable. Especially, the catabolic mechanisms of BCAAs to BCFAs are favorable. The energy needed to generate 1 ATP is $\sim -30.5$ kJ/mol. Hence, from the $\Delta G^0$ values, we see that multiple ATP molecules can be generated from these pathways.

Eleven genomes of LAB and related bacteria were recently sequenced by the LABGC and JGI (Walnut Creek, CA). Comparative analysis of the draft versions of the sequenced genomes (http://www.jgi.doe.gov/JGI_microbial/html/index.html) and the publicly available Lactococcus lactis subsp. lactis genome (14, 15) revealed the presence of more than 100 genes, that may be directly or indirectly involved in production of FAs or in production of substrates
for catabolism to FAs via SLP to produce ATP. The presence of these genes indicates the feasibility of BCAA catabolism to FAs and survival during substrate exhaustion. One such condition in which these pathways will be relevant is the onset of carbohydrate exhaustion during cheese ripening and growth in culture, commonly referred as carbohydrate starvation.

**Carbohydrate starvation in LAB**

The catabolism of amino acids to VFAs takes place only in the absence of carbohydrates (106, 107). Carbohydrates are the primary energy and carbon source for LAB that grow in laboratory media and natural products such as milk. During fermentation processes LAB are subject to vagaries of stress like water activity, pH, redox potential and substrate availability (77, 112, 124, 128). Lactococci survive stress conditions and remain metabolically active (134). They survive with intracellular glycolytic reserves for moderate periods of starvation (138). During this period, available energy within the cells is utilized towards protein and nucleic acid synthesis. Available proteins can then be degraded by lactococci over time to generate peptides and amino acids that aid survival (136, 137).

Starvation and stress response in LAB lead toward cheese flavor generation mechanisms. During the course of ripening, lactose is depleted to near zero levels by the first week in hard cheeses (28). The pH of cheese drops to ~5.0. Salt in moisture of cheese is around 4% (28). Due to lack of other energy-yielding substrates, lactococci revert to nitrogen compound metabolism and utilize amino acids from protein breakdown. The metabolic activities may
cause the redox potential within the cheese matrix to fall due to low oxygen levels. These conditions cause stimulation of stress responses, activating or inactivating enzymes towards metabolism for bacterial survival (124). Aminopeptidases play an important role during starvation conditions when protein turnover is required and new protein synthesis occurs at transitional states (57).

One such specific physiological condition is the nonculturable physiological state in LAB that is induced by carbohydrate starvation in culture (134). This condition may be induced in cheese also due to depletion of available carbohydrates in the cheese matrix. The induction of VBNC and carbohydrate starvation may be linked with catabolism of amino acids in the later stages of cheese ripening.

The nonculturable state

Cheese lactococcal plate counts on selective media decrease over aging time, while lactobacillic counts increase. But plate counts are measures of cells that are capable of replication only. Lactococci are characterized to be able to go into a nonculturable (NC) state, wherein they do not replicate and hence, do not grow on plates. The cells are capable of survival in even minimal media for over two weeks, without the external addition of an energy source (134). The ability of lactococci to survive under stress conditions (124) and continue protein turnover, RNA synthesis (137), and degradation also indicates their ability to actively metabolize proteins and amino acids. Hence, they may shift toward a
non-lactic, nitrogenous metabolism in their NC state due to lack of lactose and presence of amino acids.

Previous studies focused on the growth and proliferation of starter bacteria. Overnight fermented products result from glycolysis and subsequent metabolism of pyruvate. This led to extensive characterization of the regulation of glycolysis and pyruvate catabolism (18, 19, 26, 109, 158), and acid stress conditions and related genes in LAB (71, 77, 81, 112, 124, 128, 134). Due to their nutritive fastidiousness (6, 73, 145), genes related to amino acid synthesis are also well characterized (32, 55, 86, 110). Amino acid catabolism of LAB is of considerable interest now from the perspectives of Cheddar cheese flavor (123, 159, 161) and bacterial survival (134).

Proteolysis in milk and cheese is important to release free amino acids and peptides (47, 48, 50, 92, 101, 111, 155). The putative role of these compounds in flavor led to extensive research and characterization of protein breakdown (17, 111, 116, 130, 144). Among genes for amino acid metabolism, the ADI gene cluster is characterized in lactococci (24) and lactobacilli (163) but not directly linked to cheese flavor. ATases are the amino acid catabolic enzymes related to good cheese flavor (4, 41, 54, 160, 161); but their physiological role in metabolism and survival during cheese ripening is unknown. VSCs and aromatic compounds impact good and detrimental flavor of Cheddar cheese, respectively (35, 36, 53, 153). The lactococcal genome contains as many as nine ATase genes, among which only four are enzymatically characterized. They are aromatic, branched-chain, sulfur, and aspartic acid ATases, with overlapping substrate specificities (25).
Amino acid to VFA catabolic pathways are identified in both lactic and non-lactic genera (14, 15, 52) (http://www.jgi.doe.gov/JGI_microbial/html/index.html), and characterized in some genera both enzymatically and genetically. The degradation of branched chain α-keto acids to BCFAs is characterized in *E. coli*, *Pseudomonas* spp., *Bacillus subtilis*, *Streptomyces* spp., *Streptococcus* spp., *Enterococcus faecalis*, and archaea (31, 96-98, 150, 151). The catabolism of amino acids to BCFAs for fatty acid biosynthesis via β-keto acids is characterized in *Mycobacterium* spp., *E. coli*, *Pseudomonas* spp., and *B. subtilis* (23, 27, 65, 66, 68, 83, 100, 121). Though the purposes are different, the series of reactions in both cases above are initiated by an ATase (23, 66, 150, 151). A global transcriptional regulator, *codY*, senses intracellular levels of BCAAs and aids in catabolism of amino acids via AAT and BAT (20, 58, 120). The physiological rationale for BCAA catabolism to BCFAs is yet to be explained. Starvation may be a possible condition for this catabolism to occur.

The conditions in which the above pathways occur are analogous to cheese conditions (66, 151). They serve to generate ATP via SLP and modulate redox potential by regeneration of NADH from NAD⁺ under anaerobic conditions. They generate FAs, the purpose yet unknown, except for cell wall FA biosynthesis. Considering that these pathways are not active in presence of sugar, and that lactococci loose sugar catabolic traits via plasmids (26, 46, 102, 131), the evidence towards amino acid catabolism to VFAs is only more convincing, as lactococci will loose glycolytic traits in the absence of sugars and continue with amino acid metabolism.
BCAA catabolism is a plausible approach towards amino acid metabolism in LAB because it yields BCFAs and odd-chain VFAs that are not esters of milk fat or their breakdown products. This would aid us to directly rule out the contribution of lipolysis to VFAs. BATs are necessary for catabolism of the BCAAs. While the genes corresponding to the enzymes are present in the LAB genomes (14, 15) (http://www.jgi.doe.gov/JGI_microbial/html/index.html), the conditions that induce their transcription are unknown. While arginine is co-metabolized with lactose, its catabolism is important only for the onset of starvation, as it is catabolized immediately in lactose limitation or lower pH (24) and arginine residues are present in limited number in casein in comparison to BCAAs (10, 56, 72, 74). Hence, it can be supposed that carbohydrate limitation is necessary to allow for amino acid catabolism to VFAs and generate ATP, because the presence of carbohydrates does not favor amino acid catabolism (106, 107). If lactococci continue to survive either in minimal culture or till end of Cheddar cheese ripening or consumption, it would then point towards BCAAs as the alternate energy sources via catabolism to VFAs.

Hypothesis

In viable-but-nonculturable lactococci, branched-chain amino acids are catabolized to branched-chain fatty acids.

Objectives

1. Screen lactococci, lactobacilli, and brevibacteria for their ability to produce volatile fatty acids from amino acids.
2. Determine the ability of lactococci to survive during long-term carbohydrate starvation and pH changes in a chemically defined medium.

3. Identify the metabolic intermediates of the pathway from branched-chain amino acids to branched-chain fatty acids in culture.

4. Examine lactococcal gene expression in culture with respect to branched-chain amino acid catabolism.

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decarboxylation and electrogenic histidine/histamine antiport in

nonstarter lactobacilli for their influence on Cheddar cheese slurry


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Table 2-1. Flavor compounds formed during cheese ripening (39, 143)

<table>
<thead>
<tr>
<th>Type of cheese</th>
<th>Associated flavor compounds</th>
<th>Impact compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheddar</td>
<td>Lactic acid, acetic acid, amino acids, sulfur compounds, Ammonia</td>
<td>Methanethiol</td>
</tr>
<tr>
<td>Swiss type cheeses</td>
<td>Lactic acid, propionic acid, acetic acid, amino acids</td>
<td>3-Methyl butyric acid</td>
</tr>
<tr>
<td>Blue-veined cheeses</td>
<td>Volatile fatty acids, ketones, amino acids, hydrocarbons, methyl ketones, secondary alcoholes</td>
<td>Heptan-2-one n-Butyric acid</td>
</tr>
<tr>
<td>Italian cheeses</td>
<td>Volatile fatty acids, amino acids, alcohol, ketones</td>
<td></td>
</tr>
<tr>
<td>Gouda cheese</td>
<td>Amino acids, fatty acids</td>
<td></td>
</tr>
<tr>
<td>Tilsit cheese</td>
<td>Methanethiol, methyl thio- acetate &amp; -propionate, hydrogen sulfide</td>
<td></td>
</tr>
</tbody>
</table>
Table 2-2. Relationship of flavor character of commercial Cheddar cheese to ratio of fatty acids (FFA) and hydrogen sulfide ($H_2S$) concentration (80)

<table>
<thead>
<tr>
<th>No. of cheese lots</th>
<th>Flavor character</th>
<th>meq FA/g : mM $H_2S$/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Balanced</td>
<td>14.2 : 1</td>
</tr>
<tr>
<td>4</td>
<td>Sulfide</td>
<td>7.2 : 1</td>
</tr>
<tr>
<td>5</td>
<td>Fermented</td>
<td>27.9 : 1</td>
</tr>
</tbody>
</table>
Table 2-3. Free fatty acids and their related flavors in aqueous solutions (8, 16)

| Fatty acid (IUPAC)
<table>
<thead>
<tr>
<th>Trivial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanoic acid</td>
</tr>
<tr>
<td>Propanoic acid</td>
</tr>
<tr>
<td>n-Butanoic acid</td>
</tr>
<tr>
<td>n-Pentanoic acid</td>
</tr>
<tr>
<td>n-Hexanoic acid</td>
</tr>
<tr>
<td>n-Heptanoic acid</td>
</tr>
<tr>
<td>n-Octanoic acid</td>
</tr>
<tr>
<td>n-Nonanoic acid</td>
</tr>
<tr>
<td>n-Decanoic acid</td>
</tr>
<tr>
<td>2-methyl propanoic acid</td>
</tr>
<tr>
<td>2-methyl butanoic acid</td>
</tr>
<tr>
<td>3-methyl butanoic acid</td>
</tr>
<tr>
<td>2-ethyl butanoic acid</td>
</tr>
<tr>
<td>4-methyl octanoic acid</td>
</tr>
<tr>
<td>4-ethyl octanoic acid</td>
</tr>
<tr>
<td>9-decenioic acid</td>
</tr>
<tr>
<td>Undecanoic acid</td>
</tr>
<tr>
<td>10-undecanoic acid</td>
</tr>
<tr>
<td>Dodecanoic acid</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Flavor threshold (ppm)</th>
<th>Flavor</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA²</td>
<td>pungent, sweaty</td>
</tr>
<tr>
<td>NA²</td>
<td>acid, sharp</td>
</tr>
<tr>
<td>6.2</td>
<td>rancid, sharp, acid</td>
</tr>
<tr>
<td>6.5</td>
<td>nutty, cheese-like</td>
</tr>
<tr>
<td>8.6</td>
<td>acidic, sweaty</td>
</tr>
<tr>
<td>0.28</td>
<td>soapy, fatty, acid-like</td>
</tr>
<tr>
<td>8.7</td>
<td>goaty, waxy, soapy</td>
</tr>
<tr>
<td>2.4</td>
<td>fatty, soapy, waxy</td>
</tr>
<tr>
<td>2.2</td>
<td>soapy</td>
</tr>
<tr>
<td>NA¹</td>
<td>sweaty, fatty acid-like</td>
</tr>
<tr>
<td>1.6</td>
<td>sweet, fruity, waxy</td>
</tr>
<tr>
<td>0.07</td>
<td>sharp, sweaty, sweet, fruity</td>
</tr>
<tr>
<td>NA¹</td>
<td>fruity, pleasant</td>
</tr>
<tr>
<td>0.6</td>
<td>goaty, muttony</td>
</tr>
<tr>
<td>0.006</td>
<td>characteristic goaty</td>
</tr>
<tr>
<td>4.3</td>
<td>sweet, fatty</td>
</tr>
<tr>
<td>0.1</td>
<td>soapy, waxy</td>
</tr>
<tr>
<td>2.3</td>
<td>soapy, sweet</td>
</tr>
<tr>
<td>NA¹</td>
<td>soapy</td>
</tr>
</tbody>
</table>

¹Trivial nomenclature of fatty acids is used in this dissertation

²NA: not available in literature
Table 2-4. Typical concentrations of total fatty acids (TFA) in cheese varieties

<table>
<thead>
<tr>
<th>Variety</th>
<th>TFAs (ppm)</th>
<th>Variety</th>
<th>TFAs (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sapsago</td>
<td>211</td>
<td>Gjetost</td>
<td>1658</td>
</tr>
<tr>
<td>Edam</td>
<td>356</td>
<td>Provolone</td>
<td>2118</td>
</tr>
<tr>
<td>Mozzarella</td>
<td>363</td>
<td>Brick</td>
<td>2,150</td>
</tr>
<tr>
<td>Colby</td>
<td>550</td>
<td>Limburger</td>
<td>4,187</td>
</tr>
<tr>
<td>Camembert</td>
<td>681</td>
<td>Goats' Milk</td>
<td>4,558</td>
</tr>
<tr>
<td>Port Salut</td>
<td>700</td>
<td>Parmesan</td>
<td>4,993</td>
</tr>
<tr>
<td>Monterey Jack</td>
<td>736</td>
<td>Romano</td>
<td>6,754</td>
</tr>
<tr>
<td>Cheddar</td>
<td>1,028</td>
<td>Roqueforti</td>
<td>32,543</td>
</tr>
<tr>
<td>Gruyere</td>
<td>1,481</td>
<td>Blue (US)</td>
<td>32,230</td>
</tr>
</tbody>
</table>
Table 2-5. Fatty acids present in water-soluble fractions of eight cheese types (42)

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Acetic</th>
<th>Propionic</th>
<th>n-Butyric</th>
<th>n-Valeric</th>
<th>n-Caproic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gouda 20+</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Gouda</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Proosdij</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Gruyere</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Maasdam</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Edam</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Parmesan</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Cheddar</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>Positional distribution (mole %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------</td>
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<tr>
<td></td>
<td>original triglyceride</td>
<td>sn-1</td>
<td>sn-2</td>
<td>Sn-3</td>
<td></td>
</tr>
<tr>
<td>n-butyric</td>
<td>11.8</td>
<td>_</td>
<td>_</td>
<td>35.4</td>
<td></td>
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<tr>
<td>n-caproic</td>
<td>4.6</td>
<td>_</td>
<td>0.9</td>
<td>12.9</td>
<td></td>
</tr>
<tr>
<td>n-caprylic</td>
<td>1.9</td>
<td>1.4</td>
<td>0.7</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>n-capric</td>
<td>3.7</td>
<td>1.9</td>
<td>3.0</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>Lauric</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myristic</td>
<td>11.2</td>
<td>9.7</td>
<td>17.5</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>Palmitic</td>
<td>23.9</td>
<td>34.0</td>
<td>32.3</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>Stearic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td>24.0</td>
<td>30.0</td>
<td>18.9</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td>2.5</td>
<td>1.7</td>
<td>3.5</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Linolenic</td>
<td>TR¹</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td></td>
</tr>
</tbody>
</table>

¹Trace
Table 2-7. Possible precursors to volatile fatty acids

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Fatty acids</th>
<th>Aroma threshold (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine, glycine, serine, pyruvate</td>
<td>Acetic acid</td>
<td></td>
</tr>
<tr>
<td>Alanine, aspartic acid, threonine, valine</td>
<td>Propionic acid</td>
<td>40.3</td>
</tr>
<tr>
<td>Pyruvate, lipolysis</td>
<td>Butyric acid</td>
<td>0.3 at pH 5.2</td>
</tr>
<tr>
<td></td>
<td>Isobutyric acid</td>
<td>5.3 at pH 2.0</td>
</tr>
<tr>
<td></td>
<td>4-methyl pentanoic acid</td>
<td>0.61 at pH 2.0</td>
</tr>
<tr>
<td></td>
<td>6-methyl pentanoic acid</td>
<td>0.84 at pH 2.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Isovaleric acid</td>
<td>3.2 at pH 2.0</td>
</tr>
<tr>
<td>Isoleucine, leucine</td>
<td>Isovaleric acid</td>
<td>6.5 at pH 5.2</td>
</tr>
<tr>
<td></td>
<td>n-Valeric acid</td>
<td>1.1 at pH 2.0</td>
</tr>
<tr>
<td></td>
<td>Isocaproic acid</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 2-8. Amino acid composition of Cheddar cheese and basal chemically defined medium (CDM)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration (ppm)</th>
<th>Cheese</th>
<th>CDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>286.84</td>
<td>241.4</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>1001.36</td>
<td>172.92</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>0</td>
<td>91.28</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1328.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>38.517</td>
<td>83.28</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>0</td>
<td>89.67</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2769.52</td>
<td>269.01</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>235.98</td>
<td>153.9</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>0</td>
<td>41.13</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>404.898</td>
<td>90.48</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>2396.59</td>
<td>90.48</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>1006.98</td>
<td>180.74</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>386.745</td>
<td>91.77</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1326.84</td>
<td>176.52</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>286.445</td>
<td>252.46</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>348</td>
<td>252.3</td>
<td></td>
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<tr>
<td>Threonine</td>
<td>382.158</td>
<td>171.87</td>
<td></td>
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<tr>
<td>Tryptophan</td>
<td>0</td>
<td>93.1</td>
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</tr>
<tr>
<td>Tyrosine</td>
<td>611.625</td>
<td>48.93</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>938.477</td>
<td>89.19</td>
<td></td>
</tr>
<tr>
<td>Total AA</td>
<td>13749.1</td>
<td>2680.4</td>
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Table 2-9. Bioenergetics of amino acid catabolism to fatty acids

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Pathway</th>
<th>Fatty acid Products</th>
<th>$\Delta G^\circ$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>Acetic acid</td>
<td>-98.60</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Isovaleric acid</td>
<td>-56.29</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>2–Methyl–valeric acid</td>
<td>-46.38</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>Propionic + formic acid</td>
<td>+53.69</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>Isobutyric acid</td>
<td>-52.74</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Hydroxypropionate</td>
<td>Butyric + acetic acid</td>
<td>-376.60</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Methylaspartate</td>
<td>Butyric + acetic acid</td>
<td>-234.63</td>
</tr>
</tbody>
</table>
Fig. 2-1. Carbohydrate catabolism to some VFAs and other volatile compounds. Dotted lines represent catabolic steps to pyruvate via glycolysis. Known enzymes (corresponding known genes from *L. lactis* IL1403 genome) are: 1, Pyruvate-formate lyase (*pfl*), 2, Pyruvate dehydrogenase (*pdhA, pdhB*), 3, Aldehyde dehydrogenase (*adhE*), 4, Alcohol dehydrogenase (*adhA*), 5, Lactate dehydrogenase (*ldhB, ldhX*), 6, Acyl kinase, 7, Acetyl kinase (*ackA1, ackA2*), 8, Phosphotransacetylase. Note that none of the BCFAs are produced here. Adapted from (26 & 46).
Fig. 2-2. BCAA degradation pathways to BCFAs and straight-chain FAs. Dotted lines represent multiple catabolic steps involved. The enzymes involved are: 1, Aminotransferase, 2, Branched-chain keto acid dehydrogenase, 3, Phosphotransacylase, 4, Acyl kinase. Straight chain amino acids are also involved in VFA production. See Fig. 2-3 for more detail. Note that glycolysis is also a possible source of FAs. Adapted from (96 & 150).
Fig. 2-3. Multiple pathways interconnected for production of VFAs. Note that all VFAs are straight chain as are the amino acids. The pathways involve redox balance, production of ATP by SLP, and production of flavor compounds like VFAs and ammonia.
CHAPTER 3
MONOCARBOXYLIC ACID PRODUCTION BY LACTOCOCCI AND
LACTOBACILLI\textsuperscript{1}

Abstract

Branched chain fatty acids (BCFAs), such as isobutyric and isovaleric acid, contribute to positive cheese flavor at low concentrations and negative flavor at high concentrations. The source of these compounds in Cheddar cheese is controversial. The objective of this study was to determine the capability of lactococci and lactobacilli to produce BCFAs from amino- and $\alpha$-keto- acids. In the assay conditions used in this study, $\alpha$-keto acids spontaneously degraded to monocarboxylic acids (MCA) without any organisms added. When lactococci and lactobacilli were added into the assay with individual $\alpha$-keto acids, they produced MCAs above the level of spontaneous degradation and above the flavor thresholds associated with positive Cheddar cheese flavor. Similar results were observed using amino acids as the substrate. Assays done with equimolar mixtures of amino acids led to lower concentrations of MCAs than from individual amino acids and the flavor thresholds associated with positive Cheddar cheese flavor. Catabolism of amino acids or $\alpha$-keto acids yielded similar MCAs types, but different total concentrations of MCAs. This study

\textsuperscript{1}Co-authored by: Balasubramanian Ganesan, Kimberly Seefeldt, Ramarathna C. Koka, Benjamin Dias, and Bart C. Weimer; reprinted with permission from International Dairy Journal. Copyright held by Elsevier Ltd., Oxford, UK. See Appendix E for Copyright clearance and co-author release letters.
demonstrated that lactococci and lactobacilli were capable of converting amino acids and α-keto acids to MCAs at concentrations associated with positive and negative Cheddar cheese flavor.

Introduction

Lactic acid bacteria (LAB) used to ferment milk produce compounds important in flavor perception of cheese that include volatile sulfur compounds (VSCs) and monocarboxylic acids (MCAs), among others (Ferchichi, Hemme, Nardi, & Pamboukdjian, 1985; Weimer, Seefeldt, & Dias, 1999). Individual VSCs and MCAs, however, do not provide the total flavor perception in Cheddar cheese, while combinations of VSCs and MCAs lead to positive or improved Cheddar cheese flavor (Weimer et al., 1999). The molecular source of these compounds is controversial, as are the role of individual compounds and the specific concentrations associated with positive Cheddar cheese flavor.

Typical flavors associated with MCAs are considered negative at high concentrations (Baldwin, Cloninger, & Lindsay, 1973; Brennand, Ha, & Lindsay, 1989). At low concentrations, these mixtures are considered beneficial or positive for cheese flavor (Sandine & Elliker, 1970), especially in hard Italian varieties (Langsrud & Reinbold, 1973). The exact MCA and concentration for positive flavor depends on the cheese type and aging conditions (Chin & Rosenberg, 1997). While some MCAs are correlated to positive cheese flavor, the occurrence of branched chain MCAs are controversial or unknown. A direct link between these compounds and a specific organism, metabolic mechanism, or flavor benefit is lacking.
The role of lipase to produce MCAs is well known with regard to hydrolysis of triglycerides, but the impact on flavor benefit is less clear. Milk fat contains even-chain and straight-chain MCAs, but no branched-chain fatty acids (BCFA) (Kwak, Jeon, & Perng, 1989). Cheddar cheese often contains even- and branched-chain MCAs at concentrations that are higher than in milk fat (Attaie & Richter, 1996). While MCA concentrations increase during cheese ripening, lipase/esterase activity concurrently decreases (Weimer et al., 1997). Use of lactococci with higher lipase activity does not improve Cheddar cheese flavor (Umemoto & Sato, 1975; Umemoto & Sato, 1977; Kwak et al., 1989), nor does addition of cells that over express the primary lipase in Lactococcus lactis ssp. cremoris (Holland & Coolbear, 1996). Considering these observations, one can conclude that lipolysis from LAB plays a minor role in generating MCAs and BCFAs. Taken together, these observations raise questions about the source of MCAs and BCFAs produced by LAB and lead one to hypothesize that microbial metabolisms play a role in production of these compounds.

MCAs can be produced via glycolysis (Fox, Law, McSweeney, & Wallace, 1993). Glycolysis leads to acetic acid primarily (Harper et al., 1978). Considering the lack of carbohydrate during cheese ripening, glycolysis is likely to be the source of BCFAs by bacteria. Alternatively, MCAs may originate from microbial catabolism of amino acids (Harper, Kristoffersen, & Wang, 1978; Hemme, Bouillane, Metro, & Desmezeaud, 1982). Branched-chain flavor compounds originate from amino acids via microbial metabolism with the initial step being catabolism via aminotransferases (ATases) (Gao, Oh, Broadbent, Johnson, Weimer, & Steele, 1997; Rijnen, Bonneau, & Yvon, 1999a; Yvon, Chambellon,
Bolotin, & Roudot-Algaron, 2000). Metabolism of branched-chain amino acids (BCAAs) may generate ATP via substrate-level phosphorylation, regenerate protons, and help to maintain cellular oxidation-reduction potential (Harwood & Canale–Parola, 1981; Zhang, et al., 1999). The ability of LAB to produce MCAs, especially BCFAs, remains to be elucidated, despite initial studies that link amino acid metabolism to ATases.

The first step of aromatic and BCAA catabolism in lactococci is a transamination (Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997). Rijnen et al. (1999a) found that deletion of the lactococcal aromatic ATase (AraT) also reduces the catabolism of leucine, but presented no evidence for the associated change in BCFA production. Subsequently, Yvon, Berthelot, and Gripon (1999) added α–keto-glutarate to cheese and observed an increase in aroma, but no flavor changes were reported. In spite of these initial findings, further work is needed to define the causal role of amino acids and α–keto acids in BCFA production by LAB.

The aim of this study was to determine the ability and distribution of MCA production in LAB. The hypothesis tested in this study was that lactococci and lactobacilli do not generate BCFAs from amino acids or α–keto acids. To clarify the role of LAB in MCA production, it was necessary to initially determine MCA production in pure culture assays in conditions mimicking the aqueous phase of cheese to establish capability as a single, independent variable.
Materials and methods

Strains and media

Frozen stock cultures of lactococci and lactobacilli used in this study were prepared by growing them twice in 10 mL of the respective broth (Difco Laboratories, Detroit, MI) for 24 h in optimal growth conditions listed in Table 3-1. Before each use or assay, frozen stock cultures (−70°C in 10% non-fat dry milk containing 30% glycerol) were sub-cultured twice for 24 h at their respective optimal growth conditions in 10 mL of broth (Table 3-1). The resulting overnight culture was prepared for MCA assays as listed below.

Cell preparation for MCA assays

The overnight cultures of each individual strain were harvested from 10 mL of broth by centrifugation (3500 × g for 15 min at 4°C), washed twice with 10 mL of sterile 0.05 M potassium phosphate buffer (pH 7.2), and resuspended individually in 5 mL of 0.15 M 2-(N-morpholino) ethane-sulfonic acid-sodium salt (MES) buffer (pH 5.2, containing 4% NaCl) to a final optical density at 600 nm of 0.2. The resuspended cultures were incubated in sterile 0.15 M MES (pH 5.2, containing 4% NaCl) (negative control), individual amino acids, individual α–keto acids, an equimolar mixture of amino acids, and an equimolar mixture of α–keto acids. The individual amino acids (aspartic acid, glutamic acid, isoleucine, leucine, lysine, methionine, proline, serine, threonine and valine) and individual α–keto acids – α–keto-isocaproate (KIC), α–keto-glutarate (KGL), α–keto-isovalerate (KIV), and α–keto-β–methylvalerate (KMV)) were dissolved to a final concentration of 1 mM in 0.15 M MES (pH 5.2, containing 4% NaCl). As an
individual substrate, pyruvate was dissolved to a final concentration of 1 mM in 0.15 M MES (pH 5.2, containing 4% NaCl). Equimolar mixtures of the amino or \( \alpha \)-keto acids (including pyruvate) were made to a final concentration of 1 mM in 0.15 M MES (pH 5.2, containing 4% NaCl). All amino acids, \( \alpha \)-keto acids, MCA standards, and buffer salts were purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

Incubation for each assay was done at the optimal growth temperature of the respective organism (Table 3-1) for 3 h as described by Nakae and Elliott (1965a, 1965b). Subsequently, the supernatant from each assay was collected for MCA analysis after sterilization by passing the liquid through a 0.2 µm syringe filter (Nalge Company, Rochester, NY). The amino acids and \( \alpha \)-keto acids negative controls were incubated in the same conditions as the assays containing the respective organisms. All assays were replicated twice.

Gas chromatography

MCAs in the assay filtrates were extracted and analyzed with gas chromatography as described by de Jong and Badings (1990). Internal standards of n-valeric acid, n-heptanoic acid, n-tridecanoic acid, and n-heptadecanoic acid were added to the supernatant prior to MCA extraction to assess the efficiency. Briefly, the supernatants were acidified with concentrated \( \text{H}_2\text{SO}_4 \), internal standards added, and extracted with ether-heptane (1:1). Subsequently, the extracts were passed through a weak anion-exchange column (Bakerbond solid-phase amino extraction column, Fisher Scientific Inc., Pittsburgh, PA). The neutral lipids were removed from the column with chloroform-isopropanol (2:1). MCAs
were eluted from the column using 2% formic acid in diethyl ether and collected in glass vials.

Quantitation of the extracted MCAs was done using a Shimadzu AOC 20S autosampler connected to a Shimadzu gas chromatograph (GC) model GC17–A, equipped with a flame-ionization detector (Shimadzu, Columbia, MD) as described by de Jong and Badings (1990). Briefly, the samples were injected onto a fused silica DB–FFAP column (J&W Scientific, Folsom, CA), with the injector operating in splitless mode, with a 2 min sampling time, at 225°C. The column temperature was held at 65°C for 1 min, increased linearly to 240°C in 19.5 min, and held for 22 min. Separation was achieved with a carrier gas (Helium) flow velocity of 40 cm s⁻¹ and a flow split ratio of 10. The detector temperature was maintained at 260°C throughout the analysis. The total run time for each sample was 40.5 min.

MCAs were quantified using the internal standards as listed above. All peaks were identified by co-injection of individual MCAs. Peak areas were determined from chromatograms using Shimadzu VP 4.2 software (Shimadzu, Columbia, MD). Results are expressed as concentration (mM) of MCAs using the linear regression equation (R²≥0.99) from the standard curves done with two replications (Appendix A).

**Mole fraction and degradation**

In order to verify that the autodegradation products were not artifacts of analytical technique, we compared the number of carbon atoms per unit mole fraction of the α–keto acids before the start of incubation and of MCAs after the
end of incubation. The extent of degradation of α-keto products acids to MCAs was calculated in three steps: 1) calculate the mole fraction of each substrate/product (Eq. 1), 2) calculate the number of carbon atoms per mole fraction of each substrate/product (Eq. 2), and 3) estimate degradation as a percentage of number of carbons per mole fraction using the following formulae (Eq. 3):

\[
\text{Mole fraction} = \frac{\text{Moles of MCA}}{\text{Total moles of all MCAs}} \quad \text{Eq. 1}
\]

\[
\text{# of C-atoms per mole fraction of MCA} = \frac{\# \text{-} \text{atoms of MCA}}{\text{Mole fraction of that MCA}} \quad \text{Eq. 2}
\]

\[
\text{Degradation (\%)} = \frac{\sum (\# \text{-} \text{atoms per mole fraction of products}) \times 100}{\sum (\# \text{-} \text{atoms per mole fraction of initial substrate})} \quad \text{Eq. 3}
\]

**Statistical analysis**

MCA determinations were replicated twice. The resulting data were used to calculate the average and standard error of the mean for presentation in the figures. Data obtained from individual strains within a genus were not significantly different (p>0.05) and were subsequently averaged within each genus to aid in clarity of interpretation. A Student’s t-test was used to determine significant differences in MCA production among the organisms and substrates. Differences between the treatments were considered significant at \( \alpha = 0.05 \).
Results

α-Keto acid degradation

Amino acids and α-keto acids were used as substrates in an assay format to determine the MCA production capability of each LAB strain. The negative controls of MES buffer only, the individual amino acids, and the amino acid mixture did not contain any MCAs (data not shown). Each α-keto acid spontaneously degraded in the assay conditions used in this study, which mimicked the water phase sugar content, salt concentration, and pH of Cheddar cheese. MCAs were not observed in the buffer or amino acid controls during the incubation time or in the α-keto acid controls prior to incubation. After incubating α-keto acids controls in the same conditions as the assay with cells, various MCAs were observed, with acetic acid being common to all combinations (Fig. 3-2). Presumably, this was due to degradation reactions. The degradation mechanism was beyond the scope of this study; consequently, it was not determined.

The absolute molar concentration of the resulting MCAs was higher than the starting concentration of any individual α-keto acid. Analysis of the number of carbons in mole fraction for each α-keto acid determined that the resulting degradation solutions contained fewer MCAs carbon atoms than did the substrate in the assay buffer at the initial starting point. The amount of α-keto acid auto-degradation ranged from −45 to −79% (Fig. 3-2). Consequently, during all subsequent data analysis of bacterial production of MCAs, the auto-degradation products were deducted before presentation.
Amino acid catabolism by lactococci and lactobacilli

The 10 LAB strains used in this study utilized individual amino acids to produce seven different MCAs (Fig. 3-3). Within a genus, the type and concentration of MCAs were not significantly different ($p > 0.05$); hence, the MCA concentrations within a genus were averaged to aid in the clarity of data presentation. Despite washing away the cells to remove residual nutrients from the previous growth condition, each organism produced MCAs in the control without any substrate (Appendix A), suggesting that the cells used intracellular components during the 3 h incubation to produce a small amount of MCAs. Each organism assayed with individual amino acid substrates produced more MCAs than did the control containing cells only in the assay buffer. Both genera produced less MCAs from the substrate containing equimolar concentrations of each amino acid than they did with individual amino acid substrates (Fig. 3-3).

Each genus produced similar MCA types (Fig. 3-3A & B), but significantly different ($p<0.05$) total MCA concentrations from individual amino acid substrates. Both genera produced acetic, propionic, isobutyric and caproic acids from individual amino acids. Compared to lactobacilli, lactococci produced significantly ($p<0.05$) more acetic acid from lysine, proline and valine; propionic acid from proline; and isobutyric acid from aspartic acid, glutamic acid, methionine, serine and valine.

The MCA profiles of lactococci and lactobacilli were different from the equimolar amino acid mixture compared to the individual amino acid substrates (Fig. 3-3A & B). Acetic and isobutyric acids were the only products from the
amino acid mixture in lactococci (Fig. 3-3A). Lactobacilli produced acetic, propionic, n-caproic and lauric acids from the amino acid substrate mixture (Fig. 3-3B). Lactococci produced significantly more (p<0.05) total MCAs than did lactobacilli from the amino acid mixture. This genus also produced BCFAs from all 11 amino acid substrates, while lactobacilli produced BCFAs only from four of the 11 substrates.

**α-Keto acid catabolism by lactococci and lactobacilli**

Both genera produced similar types, but smaller amounts of MCAs from the α-keto acids than from amino acids, especially lactobacilli (Fig. 3-3D). Acetic acid production was reduced in most of the comparisons among the substrates within each genus. Other MCA products varied between each comparison without a recognizable pattern emerging. One notable observation was that lactococci produced similar MCA types irrespective of providing amino acids or α-keto acids as the substrate.

The total MCA production from α-keto acids was significantly higher (p<0.05) in lactococci than in lactobacilli (Fig. 3-3C & D). Lactococci produced significantly more (p<0.05) of the following MCAs than did lactobacilli: acetic acid from KIV and the α-keto acid substrate mixture; propionic acid from KMV, KGL, α-keto glutaric acid with pyruvate (PKGL), α-keto isocaproic acid with pyruvate (PKIC), and the α-keto acid mixture; and caproic acid from KGL and the α-keto acid mixture. While lactococci produced isobutyric acid from six of
the α–keto acid substrates, lactobacilli did not produce isobutyric acid from any of the α–keto acid substrate tested.

In addition to producing more total MCAs, lactococci produced significantly different (p<0.05) individual MCAs from each α–keto acid substrate compared to lactobacilli (Fig. 3-3C & D). Lactococci produced isobutyric acid from six α–keto acid substrates (Fig. 3-3C), while lactobacilli did not produce this product from any α–keto acid substrate. Isovaleric acid was produced by lactococci from two α–keto acid substrates and lactobacilli produced this acid from four α–keto acid substrates, including pyruvate. Alternatively, lactococci produced lauric acid from PGL only, but lactobacilli produced this MCA in small quantities from all 11 α–keto acid substrates.

Both genera produced BCFAs from pyruvate, but each genus produced significantly different (p<0.05) MCA types when pyruvate was combined with α–keto acids (Fig. 3-3C & D). Lactococci (Fig. 3-3C) produced acetic, propionic, isobutyric, butyric, and caproic acids from KGL; however, with pyruvate added to KGL (PGL) lactococci produced acetic, propionic, caproic and lauric acids – eliminating the production of the BCFAs. Using the same substrates, lactobacilli produced acetic, propionic, isovaleric, and lauric acids from KGL, but addition of pyruvate changed the MCA production to acetic, isovaleric, and caproic acid.

**Use of branched chain substrates to produce branched chain fatty acids**

Lactococci utilized branched–chain α–keto acids (KMV, KIC, and KIV) to produce significantly more (p<0.05) BCFAs than the corresponding
branched-chain amino acids (isoleucine, leucine, and valine) (Fig. 3-3A & C). Conversely, lactobacilli produced significantly fewer (p<0.05) MCAs from branched-chain amino acids compared to branched-chain α-keto acids, but a more diverse set of MCAs was produced from KIC and KIV compared to amino acids (Fig. 3-3B & D). Only lactococci with KIV as the substrate lead to production of isobutyric and isovaleric acid in this study.

Discussion

α-Keto acid auto-degradation

The α-keto acids auto-degraded into MCAs in the assay conditions that mimicked the water phase of Cheddar cheese (pH 5.2, 4% NaCl, no carbohydrate). This observation is similar to reports by Gao et al. (1997), Gao, Mooberry, and Steele (1998), and Yvon and Rijnen (2001). Gao et al. (1997, 1998) found that aromatic α-keto acids completely degraded during a 2-h incubation in similar assay conditions to those used in this study. Therefore, this observation was not surprising. The auto-degradation values were used to adjust the final estimate of α-keto acid metabolism for each organism.

The total MCA concentration from the auto-degradation in the control without cells added increased above the starting substrate concentration of 1 mM in each α-keto acid substrate and the equimolar substrate mixture. This is possible and expected because each substrate produced two or more products of shorter chain length from a single longer chain α-keto acid. When the MCAs produced from degradation are expressed in molar concentration, the resulting total concentration of the products will be at least twice the initial substrate
concentration, depending on the molecular weight of the products. For example, if a compound was present at 1 mM in the initial solution and degraded into two compounds, the total concentration of the products would be at least 2 mM, yet the total mole fraction would be equal to that of the original compound if 100% degradation was achieved. However, the degradation was not 100%.

Consequently, once the data were analyzed as a molar fraction, we found, as expected, fewer carbon molecules in MCAs after degradation, extraction, and GC analysis than the starting substrate. Despite auto-degradation and adjustment of the data to achieve a final estimate, we observed significant $(\alpha \leq 0.05)$ differences in MCA production from $\alpha$-keto acids between the genera.

Considering the $\alpha$-keto acid auto-degradation observed in this study and that from other studies as noted above, perhaps reevaluation of the data from previous studies, where $\alpha$-keto acids (especially KGL) were added to cheese during manufacture (Rijnen, Delacroix-Buchet, Demaizieres, Le Qere, Gripon, & Yvon, 1999b; Rijnen, Courtin, Gripon, & Yvon, 2000), to include a component of KGL auto-degradation is warranted. It is likely that a portion of the reported isobutyric acid increase, attributed to amino acid catabolism by the bacteria, was in part due to auto-degradation of KGL in the physicochemical conditions of cheese during ripening.

**Amino acid catabolism by lactococci and lactobacilli**

Each of the 10 strains tested produced MCAs from amino and $\alpha$-keto acids (Fig. 3-3). Within a genus, the amount of each MCA produced was not significantly different $(p>0.05)$. Consequently, estimates of the MCA production
were averaged within each genus to aid in presenting the salient differences between the ability of lactococci and lactobacilli to produce MCAs (Fig. 3-3). Nakae and Elliott (1965a, 1965b) determined that a few LAB produce MCAs from amino acids in optimal growth conditions. This study expanded previous work to demonstrate MCA production by 10 phylogenetically diverse LAB in physiochemical conditions that mimicked the aqueous phase of Cheddar cheese (pH 5.2, 4% NaCl, no carbohydrate). Based on previously published results and these observations, it is reasonable to conclude that MCA production is a common trait among LAB.

Lactococci produced BCFAs with all amino acid substrates in the assay conditions used in the study, while lactobacilli produced BCFAs in only four of 11 amino acid substrates. The exact metabolic route for these conversions is not known conclusively, but a compilation of the possible metabolic routes for BCFA production in bacteria is presented in Figure 1 (Harwood & Canale-Parola, 1981; Zhang et al., 1999). This study demonstrated that LAB are capable of producing these products, but further studies to prove the metabolic route in LAB are needed to determine the metabolic route.

Interestingly, production of MCAs decreased when equimolar amino acid mixtures were used as the substrate with both genera. The MCA production in lactococci was restricted to acetic and isobutyric acids in this condition, while in lactobacilli MCA production was nearly abolished. This observation may have many explanations, including: 1) amino acid inter-conversion via biosynthetic pathways (Fig. 1), 2) use of amino acids for cellular protein turnover, 3) conversion of amino acids to non–MCA metabolites (Gao et al., 1997, 1998), or 4)
inhibitory effect of more than one type of substrate present simultaneously in
the mix (Guillouet, Rodal, An, Lessard, & Sinskey, 1999).

Surprisingly, LAB also produced caproic and lauric acid from amino acids
(Fig. 3-3). To our knowledge, no metabolic mechanism is known in LAB that
explains the production of these MCAs. The genomes of lactobacilli, *Lactococcus
lactis* ssp. *cremoris* SK11, *Pediococcus*, *Leuconostoc*, and *Oenococcus*
(http://www.jgi.doe.gov/JGI_microbial/html/index.html) contain the
necessary open reading frames to produce the enzymes that may catalyze the
formation of these compounds from single carbon metabolism. However,
expression of these genes has not been established in LAB. To adequately
describe the molecular details of these observations, a comprehensive metabolic
analysis of these transformations is needed. Irrespective of the mechanism, these
observations verify that LAB, particularly lactococci, are capable of utilizing
amino acids to produce BCFAs in conditions that mimic the water phase of
cheese.

**α–Keto acid catabolism by lactococci and lactobacilli**

Previous studies of α–keto acid catabolism in bacteria as direct substrates
for metabolic products have not been reported. The goal of this approach was to
by pass the initial, and often the limiting step, of the aminotransferase reaction in
amino acid catabolism (Yvon et al., 1997) (Fig. 3-1). However, considering that
there are 12 ATases in the genome of *Lactococcus lactis* IL1403 (Bolotin, Mauger,
Malarme, Ehrlich, and Sorokin, 1999; Bolotin et al., 2001), and that lactococcal
ATases possess overlapping substrate specificity (Rijnen et al., 1999a; Engels et
al., 2000; Yvon et al., 2000), it is unlikely that this is the rate limiting step in this conversion. In this study, α-keto acids were catabolised to MCAs above auto-degradation levels (Fig. 3-2) by all strains tested in these assay conditions, demonstrating that α-keto acids were substrates for MCA production (Fig. 3-3). Lactococci produced significantly (p<0.05) more total MCAs than did lactobacilli (Fig. 3C & D). Each genus produced BCFAs from α-keto acid substrates, but the exact BCFA and the substrate varied between the genera. Lactobacilli produced only isovaleric acid, while lactococci predominantly produced isobutyric acid, and in one case isobutyric and isovaleric acid.

Pyruvate, a common intermediate in carbohydrate and amino acid metabolism, was converted to MCAs by both genera (Fig. 3-3C and D). Addition of pyruvate to individual α-keto acid substrates changed the resulting MCA profile compared to pyruvate or the α-keto acid substrate alone. This indicates that the presence of pyruvate modulates the catabolic route and raises questions about substrate preference for the production of MCAs and gene regulation in LAB. It is reasonable to suspect that LAB utilized pyruvate, as a central glycolytic intermediate, to inter-convert amino and α-keto acid substrates to MCAs (Fig. 3-1). This hypothesis may explain why Nakae and Elliott (1965a, 1965b) and Thompson and Thomas (1977) found MCA production from glycolytic intermediates. This study demonstrated that LAB produced MCAs from amino and α-keto acids in the absence of a sugar source. Taken together, it is reasonable to conclude that LAB can produce MCAs from amino acids or carbohydrates.
The driving force to produce specific MCAs in LAB remains unknown. As depicted in Figure 3-1, the proposed pathway for BCFAs produces ATP via substrate-level phosphorylation and FAD/FADH recycling. It is possible that the cells use these metabolic pathways to produce energy and maintain a reduced environment, especially since LAB do not produce energy from an electron transport system (Konings, Poolman, & Driessen, 1989). MCA production from amino acids and α–keto acids is bioenergetically favourable to LAB in the absence of carbohydrates for the production of ATP and to modulate the oxidation/reduction potential (Fig. 3-1).

Considering that total MCA production with either amino acid and α–keto acid substrates was similar and that the individual products were different, perhaps ATases play a role in directing the resulting intermediates into various pathways that lead to specific MCA products – rather than limiting the total MCA production as a rate-limiting step during catabolism. Experiments using ATase deletion mutants are needed to fully define the role of specific enzymes, metabolic intermediates, and the rate limiting steps in amino acid and α–keto acid catabolism to MCAs.

Conclusions

In this study lactococci and lactobacilli were demonstrated to produce MCAs, specifically BCFAs, from amino and α–keto acids. Acetic acid was a common product for all strains and substrates tested. Each genus produced BCFAs, with isobutyric acid being the most common BCFA from amino acid substrates. Isovaleric acid was produced by each genus during incubation with
α–keto acid substrates. Metabolism of substrate mixtures resulted in lower levels of MCA production except in one case. The mechanism for MCA production is unknown, but a plausible pathway was derived from our data and known literature reports. Observations of this study demonstrated that lactococci and lactobacilli can generate BCFAs from amino and α–keto acids.

References


Table 3-1. Strains and growth conditions used in this study before MCA assays.

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<th>Growth conditions</th>
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<tr>
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<td>30°C, Ellikers</td>
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<td>37°C, MRS</td>
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*Mark Johnson, Wisconsin Center for Dairy Research, University of Wisconsin–Madison, Madison, WI 53706-1565, USA
Fig. 3-1. Proposed metabolic pathways for production of MCAs from amino and \( \alpha \)-keto acids. This diagram was compiled as a compilation from literature sources, but is unproven as the mechanism for LAB MCA production (Harwood & Canale–Parola, 1981; Zhang, et al., 1999). The abbreviations are: \( \alpha \)-KG, \( \alpha \)-keto-glutarate; Glu, glutamic acid; 1, branched–chain aminotransferase; 2, \( \alpha \)-keto acid dehydrogenase; 3, phosphotransacylase; 4, acyl kinase.
L-leucine/ L-isoleucine/ L-valine

α-KG

2-Keto isocaproate/2-Keto-3-methylvalerate/2-Keto isovalerate

CoA

Glu

2-H$_2$CO$_2$

Isovaleryl-CoA/ 2-Methylbutyryl-CoA/ Isobutyryl-CoA

Pi

CoA

Isovaleryl-P/ 2-Methylbutyryl-P/ Isobutyryl-P

ADP

ATP

Isovaleric acid/2-Methylbutyric acid/Isobutyric acid

Aspartate, Glutamate, Lysine, Methionine, Proline, Serine, Threonine

Glycolysis

Pyruvat

3-Methyl crotonyl-CoA/ Tiglyl-CoA/ Methacrylyl-CoA

Degradation

FAD

2-Methylmalonyl-CoA

Acetyl-CoA/ Propionyl-CoA

Acetyl-P/ Propionyl-P

Acetic acid/Propionic acid
Figure 3-2. Mean MCA production from auto-degradation of α–keto acids incubated for 3 h in assay conditions that mimic the water phase of cheese. Values represent average of two replicate experiments. The abbreviations are: Pyr, pyruvate; KIC, α–keto-isocaproate; KGL, α–keto-glutarate; KIV, α–keto-isovalerate; KMV, α–keto-β-methyl-valerate; PIC, pyruvate + α–keto-isocaproate; PGL, pyruvate + α–keto-glutarate; PIV, pyruvate + α–keto-isovalerate; PMV, pyruvate + α–keto-β–methyl-valerate; Mix, equimolar mixture of each α–keto acid.
Mole fraction of carbon recovered after autodegradation

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α-Keto acid

- KMV
- KIC
- KIV
- KGL
- PYR
- PMV
- PIC
- PIV
- PGL
- Mix

- Acetic
- Propionic
- Isobutyric
- Isovaleric
Figure 3-3. Mean MCA production in assay conditions that mimic the water phase of cheese. MCA production from amino acids in lactococci and lactobacilli are presented in panel A & B, respectively. MCA production from α-keto acids in lactococci and lactobacilli are presented in panel C & D, respectively. Values represent the average of two replications for the total MCA production by the lactococci and lactobacilli listed in Table 3-1. The abbreviations for all panels are: Ile, isoleucine; Leu, leucine; Val, valine; Glu, glutamic acid; Lys, lysine; Met, methionine; Pro, proline; Ser, serine; Thr, threonine; Asp, aspartic acid; KMV, α-keto-β-methyl-valerate; KIC, α-keto-isocaproate; KIV, α-keto-isovalerate; KGL, α-keto-glutarate; Pyr, pyruvate; PMV, pyruvate + α-keto-β-methyl-valerate; PIC, pyruvate + α-keto-isocaproate; PIV, pyruvate + α-keto-isovalerate; PGL, pyruvate + α-keto-glutarate; Mix, equimolar mixture of each amino acid for panels A & B or an equimolar mixture of each α-keto acid for panels C & D. The standard deviation for total MCA production was ≤10% of the mean within the strains of each genus.
CHAPTER 4
FATTY ACID PRODUCTION FROM AMINO ACIDS AND $\alpha$-KETO ACIDS
BY BREVIBACTERIUM LINENS BL2

Abstract

Low concentrations of branched chain fatty acids, such as isobutyric and isovaleric acids, develop during the ripening of hard cheeses and contribute to the beneficial flavor profile. Catabolism of amino acids, such as branched chain amino acids, by bacteria via aminotransferase reactions and $\alpha$-keto acids is one mechanism to generate these flavorful compounds; However, metabolism of $\alpha$-keto acids to flavor-associated compounds is controversial. The objective of this study was to determine the ability of Brevibacterium linens BL2 to produce fatty acids from amino acids and $\alpha$-keto acids and determine occurrence of the likely genes in the draft genome sequence. BL2 catabolized amino acids to fatty acids only during carbohydrate starvation conditions. The primary fatty acid end products from leucine were isovaleric acid, acetic acid, and propionic acid. In contrast, logarithmic phase cells of BL2 produced fatty acids from $\alpha$-keto acids only. BL2 also converted $\alpha$-keto acids to branched chain fatty acids after carbohydrate-starvation was achieved. At least 100 genes are potentially involved in five different metabolic pathways. The genome of B. linens

1 Co-authored by: Balasubramanian Ganesan, Kimberly Seefeldt, and Bart C. Weimer; reprinted with permission from Applied and Environmental Microbiology. Copyright held by American Society for Microbiology, Washington, D.C. See Appendix E for Copyright clearance and co-author release letters.
ATCC9174 contained these genes for production and degradation of fatty acids. These data indicate that brevibacteria have the ability to produce fatty acids from amino and α-keto acids, and that carbon metabolism is important in regulating this event.

Introduction

Positive cheese flavor is a result of a balance between different flavor compounds that are produced during ripening. Two important classes of compounds that contribute to positive cheese flavor are volatile sulfur compounds (VSC) and fatty acids (FAs) (7, 21). The exact concentrations of VSCs and FAs that are optimal for the best cheese flavor are variable (22), and the processing events that control flavor compound production are not well understood, despite extensive biochemical details that are well known. Catabolism of sulfur-containing amino acids by brevibacteria is characterized in limited detail, but brevibacteria produce high quantities of VSCs from methionine (3, 5) via a single catalytic step using methionine-γ-lyase, which catalyses the conversion of methionine to methanethiol by demethiolation (4, 21).

Addition of *Brevibacterium linens* to low-fat Cheddar cheese Cheddar cheese significantly enhanced the consumer flavor acceptance, largely due to the sulfur metabolism (20, 21). *B. linens* accelerates the ripening process in Cheddar cheese via its proteolytic capabilities, VSC production, and other unidentified pathways including FA production (20). Production of flavor enhancing compounds, especially branched chain FAs, by lactococci and brevibacteria is
poorly defined. Catabolism of branched chain amino acids (BCAA) to produce FA in lactococci delineated a portion of the mechanism involved in production of these compounds to be modulated by ilvE (8), but the regulation of production is not fully understood. Aminotransferases (ATases) are reported to initiate catabolism of aromatic, branched-chain, and sulfur amino acids to VSCs and FAs in lactococci (24).

Previous studies with lactic acid bacteria and brevibacteria found amino acids are readily converted to FAs (9, 13-15). *B. linens* produces acetic acid from glycine, alanine, and leucine; isovaleric acid from leucine; and caproic acid from cystine, alanine, and serine (13, 14). Interestingly, branched chain fatty acids (BCFAs) do not occur in milk fat, yet BCFAs are found in fermented dairy products. This highlights the holes in understanding the metabolic mechanisms for FA production in these organisms, but it is certain that microbial catabolism is important in the production of FAs in dairy products (9, 12, 15).

Lactococci and lactobacilli produce FAs from amino and α–keto acid substrates (8, 9). This step is initiated by aminotransferases (ATases) that have broad substrate specificities in lactic acid bacteria (8, 9, 23, 24). Ganesan and Weimer (8) found lactococci to produce BCFA from BCAAs even if the branched chain ATase is deleted (ΔilvE) with amino or α–keto acids added as the substrate. The proposed metabolic pathways demonstrate individual pathways (8) for each amino acid substrate for BCFA production. While such a study is not available for *B. linens*, after examination of the draft genome sequence for *B. linens* ATCC 9174 (http://www.jgi.doe.gov/JGI_microbial/html/index.html) it is reasonable
to suspect that this organism also has the ability to produce BCFAs and FAs. Examination of the draft sequence of the genome found 18 ATases, including two homologues of ilvE (E.C. 2.6.1.42). It also contains genes that encode \( \alpha \)-keto acid dehydrogenases, phosphotransacylases, and acyl kinases. However, the conditions for the transcription of these enzymes are unknown.

FA production via amino acid catabolism may be beneficial to brevibacteria, as the associated pathways generate ATP via substrate-level phosphorylation (SLP) and regenerate protons that maintain cellular oxidation–reduction potential (11). *B. linens* also possesses enzymes required for catabolism of FAs via \( \beta \)-oxidation and the utilization of the generated acyl-CoA’s via the TCA cycle. Hence, the catabolism of BCAAs may be beneficial for *B. linens* to generate multiple ATPs and biosynthetic intermediates like \( \alpha \)-keto acids via subsequent pathways. Specific details beyond the initial ATase reaction in the production of BCFA remain to be established in brevibacteria.

Based on these studies, we hypothesize that brevibacteria generate BCFAs from amino acids and \( \alpha \)-keto acids. To test this hypothesis, a buffer assay system was used with individual substrates and their mixtures. This study found that *B. linens* BL2 produced BCFAs from amino acids and \( \alpha \)-keto acids at a pH of 5.2, 4\% NaCl only after carbohydrate starvation was achieved. Subsequently, the FA pool declined, suggesting that the organism metabolized the FAs via \( \beta \)-oxidation as an energy source.
Materials and methods

Strains and media

Stock cultures of *B. linens* BL2 were prepared by growing cultures twice in 100 ml of tryptic soy broth (TSB; Difco laboratories, Detroit, MI) at 25°C with shaking at 225 rpm for 24-48 h. The cultures were frozen at −70°C in TSB containing 30% glycerol. Prior to each experiment a working culture was grown from a frozen stock culture by sub-culturing it twice in TSB at 25°C for 48 h with shaking at 225 rpm in 100 ml of broth in a 250 ml flask. The cells from this culture were used to inoculate specific media for the test conditions after harvest from 10 ml by centrifugation (3500 × g for 15 min at 4°C) and two washing steps with 10 ml of sterile 0.05 M potassium phosphate buffer (pH 7.2). These cells were resupended in the appropriate medium or assay buffer containing the substrates as described below.

FA production assays

Growth in glucose-containing conditions was done using TSB incubated at 25°C for 48 h shaking at 225 rpm in 100 ml of medium contained in a 250-ml flask. Logarithmic phase cells were assayed for FA production in assay buffer and FAs extracted from the spent buffer similar to that for carbohydrate-starved cells described below, after filter sterilization with 0.2-µm-pore-size syringe filters (Nalge Company, Rochester, NY) followed by GC analysis.

The cells exposed to carbohydrate-depleted conditions were grown in a chemically defined medium (CDM) (10), except the culture was incubated at
25°C with shaking at 225 rpm and sampled (10 ml) at 24-h intervals up to 7 d. At each time point cells from 10 ml of CDM were harvested by centrifugation (3500 × g for 15 min at 4°C), washed with 10 ml of sterile 0.05 M potassium phosphate buffer (pH 7.2), and resuspended in the assay buffer containing various substrates.

Carbohydrate-starved cells from incubation in CDM were harvested, washed, and resuspended in 5 ml of 0.15 M 2-(N-morpholino)ethane-sulfonic acid-sodium salt (MES) buffer (pH 5.2) containing 4% NaCl, to a final A₆₀₀ of 0.2. The assay buffer containing the substrate and cells was incubated at 25°C for 3 h without shaking as previously described (13, 15, 16). Subsequently, the entire assay mixture was filter sterilized with 0.2-µm-pore-size syringe filters (Nalge Company) and the FAs were extracted for GC analysis. Additionally, the suspended cells were incubated in sterile buffer (negative control), individual amino acids, individual α-keto acids, and mixtures of the amino acids or α-keto acids. The BCAAs used were leucine and valine. The α-keto acids used were α-keto-isocaprate (KIC), α-keto-glutarate (KGL), α-keto-isovalerate (KIV), and α-keto-β-methylvalerate (KMV), individually, with pyruvate, and as a mixture. All substrates were added to a total final concentration of 1 mM of substrate in the resuspended cell incubation buffer. Amino acids, α-keto acids, FA standards, and buffer salts were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). The control samples did not contain any FAs in freshly prepared solutions (9).

Studies using NMR to confirm FA production also used CDM for carbohydrate starvation followed by incubation in the assay buffer as described
above. The NMR assay mixture consisted of 20 mM L-leucine-2-13C (Isotec Inc. Miamisburg, OH), 20 mg/ml washed BL2 cells that were grown in CDM, and 30 mM sodium phosphate buffer (pH 7.0). This assay mixture was incubated at 25°C with shaking at 225 rpm and sampled at 24-h intervals up to 7 d. Samples were centrifuged (3500 x g for 15 min at 4°C) and the supernatant was frozen at −20°C until NMR analysis was done. A cell suspension with an A650 of 1.0 contained 0.365 mg cells/ml of culture (6), which was used to calculate the weight of the cells.

The frozen samples were thawed and to 0.45 ml sample, 50 µl deuterated water (D2O; Isotec Inc., Miamisburg, OH) was added and the mixture was placed in a 5-mm NMR tube. An insert tube containing chloroform was placed inside the NMR tube to provide a standard around which to calibrate all chemical shifts (17).

**Gas chromatography**

FAs were extracted from the assay buffer that was filter sterilized as described previously (9). The extracted FAs were analyzed by the method described above, except 1 µl was injected during GC analysis by hand. FAs were identified and quantified by the internal standard method as described earlier (2, 9). Results were obtained from two replications of the assays and are expressed as concentration (mM) of FAs.

**NMR spectroscopy**

NMR spectroscopy was done at the Department of Chemistry and Biochemistry, Utah State University, Logan, UT. All 13C-NMR spectra were
obtained with a Bruker model ARX-400 NMR spectrometer operating at a carbon NMR frequency of 100.6 MHz with a 5-mm multinuclear probe at a temperature of 27°C as recommended by the Bruker pulse program, zgdc, by using the following parameters: $^{13}$C spectral window, 225 ppm; 90-degree pulse width, 8 ms; 1-s relaxation delay; 3200 scans per spectrum; and 2 h total acquisition time. D$_2$O was used to obtain the signal lock. In a 7-day sample, a 30,000 scan spectrum with a 20-h total acquisition time was done to identify unknown peaks. All NMR spectra were referenced to an internal chloroform standard with a chemical shift value of 77.2 ppm. Assignments of identity were made on the basis of $^{13}$C chemical shifts (17), and by comparing experimental values to the 3-methyl butyric acid-1-13C standard (Isotec, Inc., Miamisburg, OH).

**Genome analysis**

The genome of *B. linens* ATCC 9174 was sequenced by the Joint Genome Institute (JGI) to produce a draft sequence with at least 10x coverage. This sequence was auto-annotated at Oak Ridge National Laboratory (ORNL) and the draft genome was posted at http://www.jgi.doe.gov/JGI_microbial/html/index.html for public access. In this work the genome was browsed and queried using the tools available at the JGI and ORNL (http://www.ornl.gov/sci/microbialgenomes/organisms.shtml) web sites to determine occurrence of the specific genes related to fatty acid metabolism that were visualized using a Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.ad.jp/kegg) map.
Statistical analysis

FA determination was replicated twice and the data were averaged before presentation. A Student's t-test was used to determine significant differences in FA production among the organisms and substrates. Results of $\alpha = 0.05$ were considered significantly different for FA production. Standard deviation for FA production was $\leq 10\%$ for all products.

Results

End product accumulation

FA production was determined using GC and NMR analysis with cells that were exposed to carbohydrate complete (TSB) or depleted conditions (CDM) in this study. An assay system that mimics the salt and pH of the water phase of Cheddar cheese was used for all substrates primarily because $\alpha$–keto acids are unstable for extended incubation (8). However, this approach was also used to avoid confounding interactions between multiple substrates from a complex medium for the production FAs. All data are reported as incubation times for the culture, not the assay conditions.

Preliminary analysis of culture supernatants from logarithmic phase growth in carbohydrate replete conditions found FA production from $\alpha$–keto acids (Fig. 4-1), but failed to detect FA products from amino acids (data not shown). All substrates resulted in the production of acetic acid predominantly. Propionic acid was detected with KIC and KIC with pyruvate added only. KIC also lead to the production of isovaleric acid. Subsequently, BL2 was incubated
in carbohydrate starvation conditions for ~7 d prior, which proved successful for the production of FAs from all substrates tested. Concurrently, catabolism of $^{13}$C-leucine was determined using NMR spectroscopy.

In consideration of these observations, carbohydrate depleted conditions were used to grow cells and the FA production was assayed. $\alpha$-Keto acids also served as substrates for FA production except the type of products increased (Fig. 4-2A). Acetic acid production declined in comparison to carbohydrate complete conditions, while propionic and isovaleric acid production increased. Isobutyric and caproic acid were also found. The addition of pyruvate along with the $\alpha$–keto acids reduced the quantities of BCFAs produced from KIC and KIV, but not KMV.

To verify the production of FAs from amino acids, a time series experiment was done directly from the carbohydrate depleted CDM. Leucine and valine were added to CDM prior to inoculation with BL2. The inoculated medium was incubated for 5 to 7 d at 25°C with aeration. During the incubation time, BL2 produced FAs from each amino acid (Fig. 4-2A). With Leu as the substrate, isovaleric acid was the predominant end product, with acetic acid being produced at ~1 mM concentrations over the duration of the incubation time (Fig. 4-2B). n-Butyric acid was not found at measurable concentrations during the testing period. Interestingly, isovaleric acid reached the peak concentration of ~1.75 mM at 48 h and then declined to undetectable concentrations at 160 h.
With the addition of Val to CDM as the substrate, BL2 produced the maximum FAs within 24 h (Fig. 4-2C). A new product, 4-methyl-n-valeric acid, was found in this condition, while isovaleric acid was not. All products declined over time, except acetic acid. Compared to Leu as the substrate, n-butyric acid production tripled and propionic acid concentration doubled.

The metabolic end products were verified using NMR analysis. Subsequently, the FA products were metabolized to below the detection limit during 120 to 168 h (5-7 d) of incubation. Identification of intermediate compounds in the metabolic route of Leu to FAs was not achieved. However, end product accumulation was determined to verify the production in time series experiments and α–keto acid catabolism assays.

Pathway estimation via genome Analysis

The draft genome of B. linens ATCC 9174 (http://www.jgi.doe.gov/JGL_microbial/html/index.html) was examined for the presence of the genes required for the conversion of amino acids to FAs as described by various pathways in KEGG. Unlike lactococci (8, 9), B. linens contained all the genes needed for the transformation of amino acids to FAs via FA synthesis via pathway 1 (KEGG map00061), FA biosynthesis pathway 2 (KEGG map00062), and the FA metabolism pathway (KEGG map00071) (Fig. 4-4). Other interacting pathways include butanoate metabolism (KEGG map006650), and one carbon pool by folate (KEGG map00670) that are contained in part by B. linens, but neither by Lactococcus lactis subsp lactis IL1403 (http://www.ncbi.nlm.nih.gov/
genomes/framik.cgi?db=genome&gi=171) nor the *Lactococcus lactis* subsp. *cremoris* SK11 (http://www.jgi.doe.gov/JGI_microbial/html/index.html) genomes. The FA metabolism pathways in *B. linens* have specific genes for each catabolic step that is differentiated by the FA chain length. Considering all the FA metabolic maps, they represent over 100 genes, some of which have multiple homologues. The genome of *B. linens* contains the genes needed for each step in FA biosynthesis and each step in the catabolism for energy. For example, in the FA metabolic pathway, butanoyl-CoA is converted to trans-butanoyl-2-enoyl-CoA via three different acyl-CoA dehydrogenases enzymes (E.C. 1.3.3.6, 1.3.99.2, and 1.3.99.3). All of these genes are present in the *B. linens* genome. These defined pathways suggest that *B. linens* has all the genes needed for the production of FAs from amino acids and for the re-assimilation of the FAs as an energy source and oxidation/reduction regulation mechanism, as demonstrated during the time course experiment (Fig. 4-2A & B).

Discussion

Brevibacteria accelerate flavor development in low fat Cheddar cheese when added with the starter culture via the production of sulfur and FAs (1, 10, 20). This change is associated with the later stages of cheese ripening, which corresponds to lactose depletion in cheese. However, detailed information related to the mechanism of FA production is very limited in bacteria. Coupled to the limited information and the connection to greater influence during the later stages of ripening, we studied the ability of *B. linens* BL2 to produce BCFAs
from BCAAs and α-keto acids after the pure culture entered stationary phase and the carbohydrate was depleted. Our initial observations confirmed the initial observations of Hosono (13) for FA production. Additionally, we demonstrated that FAs were consumed by BL2 during longer incubation times. Our observations, along with previous studies (13), indicate that brevibacteria are capable of producing FAs from amino acids, but only after carbohydrate depletion. This is reasonable based on the need for metabolic energy to activate BCAA catabolism (11) to produce ATP via SLP. Delineation of the metabolic pathway (9) and gene expression regulation is required to determine the mechanism for production of FAs from amino and keto acids.

Catabolism of the BCAAs leucine and valine during the assay incubation produced FAs at different concentrations, though they were of similar types (Fig. 4-2). Leucine was catabolized to isovaleric acid, which is the end product that would be expected from the predicted pathway of BL2 (9), while valine catabolism resulted in n-butyric acid rather than the expected product isobutyric acid. This observation was unexpected and is difficult to explain via known metabolic pathways. One explanation may be due to the lax ATase substrate specificities in lactococci (8, 9). Alternatively, intermediates that lead to isobutyric acid may have been catabolized via alternate pathways to acetyl-CoA that were subsequently condensed to produce n-butyryl-CoA and hence, n-butyric acid. The genes encoding the enzymes for these pathways exist in the genome of BL2 (http://www.jgi.doe.gov/JGI_microbial/html/index.html).
Transcription of these genes is unknown and must be characterized to prove this possible explanation.

Previous studies in lactococci (9, 23, 24) demonstrate that catabolism of BCAAs or their corresponding α–keto acids differ mechanistically due to interaction of complex webs of multiple ATases. Studies in brevibacterial catabolism of amino acids are currently limited to aromatic and sulfur-containing amino acids (19), making it difficult to assess the substrate specificity of ATases in this organism. In order to determine the role of ATases in BCFA production in brevibacteria, this study tested catabolism of α–keto acids by BL2, hence, bypassing the ATase reaction to alleviate the confounding factors in substrate specificity of these enzymes.

Metabolism of α–keto acids did not require carbohydrate starvation and resulted in acetic and propionic acids in logarithmic phase cultures. The α–keto acids were transported and metabolized to FAs within the 3-h incubation, suggesting that either amino acid transport or ATase expression is controlled by the carbohydrate status of the cell. Alternatively, log phase cells may utilize the α–keto acids via other metabolic pathways, while acetic and propionic acids were produced from other energy-yielding intracellular intermediates such as pyruvate or acyl-CoAs. The verification of these explanations is beyond the scope of this study.

Carbohydrate-starved cells of BL2 also catabolized α–keto acids to yield BCFA exclusively, as with amino acids (Fig. 4-3). This is contrary to the metabolism of lactococci and lactobacilli (9). Interestingly, BL2 subsequently
catabolised FAs after their production from amino or keto acids. Presumably, the FAs were catabolized via β–oxidation and channeled toward the TCA cycle and other energy producing pathways, redox reactive compounds, and other shorter chain FAs (Fig. 4-3).

Addition of pyruvate to KIC and KIV reduced the amount of BCFAs produced via α–keto acid catabolism in assays with carbohydrate-starved cells, providing further evidence for the importance of carbohydrate status in this metabolism. A FA product profile change was also observed in assays with logarithmic phase cells. This may be due to the energetic state of the cell modulated by presence of pyruvate, which can be catabolized to products other than FAs (9, 18). The intracellular abundance of these metabolic intermediates may explain why log phase cells of BL2 did not catabolize amino acids to FAs.

Investigation of the genome of B. linens ATCC 9174 found that this organism has all the genes required to produce and degrade FAs (Fig. 4-4). These consist of three primary pathways, two of which are anabolic and the other is catabolic. Each pathway was compared via KEGG between B. linens ATCC 9174, Escherichia coli K12-W3110, Bacillus subtilis, Bacillus cereus, Corynebacterium glutamicum, Corynebacterium efficiens, Corynebacterium diphtheriae, L. lactis subsp. lactis IL1403, Listeria monocytogenes F2365, and Lactobacillus plantarum WCFS1. FA pathway 1 was the most conserved, with all the genes being present except that E. coli K12, B. subtilis, and B. cereus were missing the dehydroxylase (E.C. 4.2.1.58). All the organisms other than B. linens were missing FA pathway two. In the case of the lactic acid bacteria, they were missing all the
enzymes. This was also the case with FA metabolism. None of the organisms than B. linens contained the genes for the complete pathway. This observation makes B. linens somewhat unique among a diverse set of organisms and closely related coryneform bacteria. Further work is needed to define the exact genes and proteins that are used to produce and degrade FAs in this organism.

The observations of this study provide evidence for the production of BCFAs from amino acids and α–keto acids by brevibacteria and their further catabolism to other FAs. It also established the importance of carbohydrate status to control amino acid catabolism and the independence of α–keto acid catabolism to FAs from this control. These observations indicate that brevibacteria have an intricate mechanism that regulates the production of FAs from protein sources. Presumably, the driving force is related to the energy status of the cell.

References


Figure 4-1. Mean FA production from \(\alpha\)-keto-acids by cells from carbohydrate complete TSB. NC, Negative control (cells in plain buffer), KMV, \(\alpha\)-keto-\(\beta\)-methyl-valerate, KIC, \(\alpha\)-keto-isocaproate, KIV, \(\alpha\)-keto-isovalerate, KGL, \(\alpha\)-keto-glutarate, Pyr, pyruvate, PMV, pyruvate + \(\alpha\)-keto-\(\beta\)-methyl-valerate, PIC, pyruvate + \(\alpha\)-keto-isocaproate, PIV, pyruvate + \(\alpha\)-keto-isovalerate, PGL, pyruvate + \(\alpha\)-keto-glutarate, Mix, mix of above 5 \(\alpha\)-keto acids. The standard deviation averaged 4.9% for all analytes tested.
Figure 4-2. FA production from carbohydrate-starved cells of *Brevibacterium linens* BL2. Production of FAs from leucine (panel A) in a time series during incubation in CDM, production of FAs from valine in a time series during incubation in CDM (panel B), and FA production from keto acids during the 3 h assay (panel C). NC, Negative control (cells in plain buffer), KMV, α–keto-β–methyl-valerate, KIC, α–keto-isocaproatue, KIV, α–keto-isovalerate, KGL, α–keto-glutarate, Pyr, pyruvate, PMV, pyruvate + α–keto-β–methyl-valerate, PIC, pyruvate + α–keto-isocaproatue, PIV, pyruvate + α–keto-isovalerate, PGL, pyruvate + α–keto-glutarate, Mix, mix of above 5 α–keto acids. FA concentration was determined using GC analysis in all conditions. The standard deviation averaged 2.6% for all analytes tested.
Acetic
Propionic
Butyric
Isovaleric
4-Methyl n-valeric

A

FA (mM)

0 1 2 3 4

0 25 50 75 100 125 150 175
Time (h)

B

FA (mM)

0 1 2 3 4

0 25 50 75 100 125 150 175
Time (h)

C

FA (mM)

0 5 10 15

NC KMV KIC KIV KGL PYR PMV PIC PIV PGL Mix
Keto acid

Acetic
Isopropionic
Isobutyric
Isovaleric
Caproic
Figure 4-3. Determination of FA production using NMR spectroscopy using Leu as the substrate. The scans (left to right, top to bottom, in that order) represent products detected at 48 h (A), 71 h (B), 96 h (C), 120 h (D), 144 h (E), and 168 h (F), respectively. Peaks on the spectra represent: 1) C-1 of isovaleric acid, 179.98 ppm, 2) C-1 of acetic acid, 178.13 ppm, 3) C-3 of α-ketoglutaric acid, 162.07 ppm, 4) C-1 of propionic acid, 181.12 ppm
Figure 4-4. The genes associated with the metabolic fate of fatty acids via biosynthesis in *B. linens* ATCC 9174 genome. The shaded boxes represent the gene products that are present in the genome.
Figure 4-5. The genes associated with the metabolic fate of fatty acids via biosynthesis through an alternate pathway in *B. linens* ATCC 9174 genome. The shaded boxes represent the gene products that are present in the genome.
Figure 4-6. The genes associated with the metabolic fate of fatty acids during metabolism in *B. linens* ATCC 9174 genome. The shaded boxes represent the gene products that are present in the genome.
CHAPTER 5

ROLE OF AMINOTRANSFERASE ilvE IN PRODUCTION OF
BRANCHED CHAIN FATTY ACIDS BY
LACTOCOCCUS LACTIS
SUBSP. LACTIS

Abstract

The objective of this study was to determine the role of a lactococcal branched chain amino acid aminotransferase gene, ilvE, in the production of branched chain fatty acids. Lactococcus lactis subsp. lactis LM0230 and an ilvE deletion mutant, JLS450, produced branched chain fatty acids from amino and α-keto acids at levels above α-keto acid spontaneous degradation and the fatty acids’ flavor thresholds. The deletion mutant produced the same amounts of branched chain fatty acids from precursor amino acids as the parent. This was not the case however, for the production of branched chain fatty acids from the corresponding precursor α-keto acids. The deletion mutant produced a set of fatty acids different from that produced by the parent. We concluded from these observations that ilvE plays a role in the specific type of fatty acids produced, but has little influence on the total amount of fatty acids produced by lactococci.

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Introduction

Lactic acid bacteria (LAB) are important for flavor production in fermented milk products. Fatty acids (FAs) are an important class of compounds produced by these organisms, contributing to distinctive flavor profiles in cheese (7, 25). Typical flavors of FAs at high concentrations are considered detrimental, but mixtures of FAs at low concentrations are beneficial to cheese flavor (21), especially Italian varieties (17).

FA production occurs via three known mechanisms: 1) glycolysis (14), 2) lipolysis (8), and 3) microbial metabolism of amino acids and α–keto acids (16). Branched chain fatty acids (BCFAs) cannot occur via glycolysis or lipolysis because milk fat lacks BCFAs, yet their concentration increases during aging. The mechanism for their production is unexplained.

The environment of cheese relative to the bacteria is acidic, of sub-optimal water activity and temperature, and lacks fermentable carbohydrates. Stuart et al. (22) demonstrated that lactococci metabolize amino acids during carbohydrate starvation in laboratory conditions that partially mimic cheese. Presumably, this metabolism may produce FAs via amino acid catabolism. This metabolism is relevant because amino acid metabolism generates ATP via substrate–level phosphorylation (SLP) and regenerates protons to maintain the cellular oxidation–reduction potential (15). Amino acid catabolism is one plausible explanation for the occurrence of BCFAs in cheese due the conditions and the metabolism of the associated bacteria.
Aminotransferases (ATases) initiate catabolism of aromatic, branched-chain, and sulfur-containing amino acids to cheese flavor compounds (11, 28). α-Keto acids are the intermediates and amino-group acceptors in an ATase reaction (28); consequently, they are of interest in determining the role of these reactions in FA production. A direct role for the ATases in FA production, except for methionine catabolism (4, 10), is unknown in LAB. Analysis of the genome sequences of Lactococcus lactis IL1403 (2) (GenBank Accession no. AE005176) and Lactococcus lactis subsp. cremoris SK11 (http://www.jgi.doe.gov/JGI_microbial/html/index.html) determined that each contain 12 ATases. However, detailed expression studies of each ATase in these organisms are lacking. Overlapping ATase substrate specificity (6, 20, 27), the large number of ATases in the lactococcal genomes, and lack of defined conditions leading to expression increase the complexity in assigning a specific role for individual ATases.

Previous studies focussed on relating lactococcal ATases to flavor compound generation. Deletion of the lactococcal aromatic ATase (araT) reduces catabolism of leucine (20), while addition of α-keto glutarate leads to an increase in aroma compounds (26). These studies independently verified the activities of lactococcal ATases or FA production, but did not associate these two events, making it impossible to define the causal relationship of ATases in FA production. To determine the influence of the IlvE ATase in L. lactis subsp. lactis LM0230 a deletion mutant was previously tested for growth (1).

The role of individual ATases is unclear in the metabolism of amino and keto acids. Use of an ATase deletion mutant allows direct assessment of the
impact of the loss of the ATase on this metabolism. While other studies found IlvE to be involved in anabolism and growth, ATases are bidirectional and they may also be involved in catabolism of amino and α–keto acids. Based on previous studies, we hypothesized that *ilvE* modulates production of BCFAs in lactococci. This study found that deletion of *ilvE* did not significantly (*p* > 0.05) change the total concentration of BCFAs produced from amino acids, but it did significantly (*p* < 0.05) change the type of FAs produced at pH 5.2, 4% NaCl, and no carbohydrate in the assay buffer.

**Strains and media**

*L. lactis* subsp. *lactis* LM0230 was obtained from the USU culture collection. The *ilvE* deletion mutant, JLS450 (1), was kindly provided by Dr. James L. Steele, University of Wisconsin-Madison, Madison WI. Stock cultures were prepared by growing the organisms twice in 10 ml of Elliker broth (Difco laboratories, Detroit, MI) at 30°C for 24 h. Cultures were frozen at ~70°C in 10% non–fat dry milk containing 30% glycerol. Before each experiment, frozen stock cultures were thawed and sub-cultured twice at 30°C for 24 h in 10 ml of Elliker broth.

**Cell preparation for FA assays**

Amino acids, α–keto acids, FA standards, and buffers were purchased from Sigma (Sigma–Aldrich, St. Louis, MO). Cells were harvested from 10 ml of broth by centrifugation (3500 × g for 15 min at 4°C), washed twice with 10 ml of sterile 0.05 M potassium phosphate buffer (pH 7.2), and resuspended in 5 ml of...
0.15 M 2-(N-morpholino)ethane-sulfonic acid-sodium salt (MES) buffer (pH 5.2) containing 4% NaCl to a final cell density of 0.2 at $A_{600}$. The suspended cells were incubated in sterile buffer (negative control), individual amino acids, individual $\alpha$-keto acids, and mixtures of the amino acids or $\alpha$-keto acids as described below. The amino acids used were glutamic acid, isoleucine, leucine, and valine. The $\alpha$-keto acids used were $\alpha$-keto-isocaproate (KIC), $\alpha$-keto-glutarate (KGL), $\alpha$-keto-isovalerate (KIV), and $\alpha$-keto-$\beta$-methylvalerate (KMV). These components were used individually as substrates at a final concentration of 1 mM in the assay buffer containing the cells.

Based on previous studies with LAB (18, 19) and previous work (9), the cells-substrate mixtures were incubated at 30°C for 3 h and filter sterilized with 0.2-µm syringe filters (Nalge Company, Rochester, NY). The collected filtrates were used for FA analysis.

Gas chromatography

FAs were extracted and determined in the sterile buffer filtrates as described by de Jong and Badings (3), and instrument operating conditions were described previously (9). Gas chromatography was done on the Shimadzu gas chromatograph (GC) model GC17-A, equipped with a flame-ionization detector (Shimadzu, Columbia, MD). Samples were separated with a fused silica DB-FFAP column (J & W Scientific, Folsom, CA). Peak areas were determined from chromatograms using the Shimadzu VP 4.2 software (Shimadzu, Columbia, MD). FAs were identified using co-injection of known standards, and
quantified based on internal standards as described previously (9). Analyses were replicated twice and the results were averaged before presentation as FA concentration (mM).

Statistical analysis

Assays were replicated twice and the data were averaged before presentation. A Student’s t-test was used to determine significant differences in FA production between the parent and mutant, and the substrates. Results of $\alpha = 0.05$ were considered significantly different. The standard deviation for FA production was less than 10% between replicates for both strains and all products.

Results and discussion

Previous experiments (9) demonstrated that lactococci produced more FAs than lactobacilli from $\alpha$-keto acids, while the two genera produced similar amounts of FAs from amino acids. These observations, and suggestions from other reports (11, 12), lead us to suppose that the initiation of the catabolic route by an ATase may be the limiting step in FA production. Despite the large number of ATases in lactococcal genomes (2) (http://www.jgi.doe.gov/JGI_microbial/html/index.html) and the overlapping substrate specificity of ATases (6, 20, 27), we hypothesized that deletion of a single enzyme would decrease the total concentration of FAs. Therefore, a direct comparison with a branched chain ATase ($ilvE$) deletion mutant (JLS450) of $L.\ lactis$ subsp. $lactis$
LM0230 in an assay system was used to investigate FA production from branched chain amino acids (BCAAs) and α-keto acids.

As noted previously (9), the cells incubated in buffer without substrate produced FAs (Fig. 5-1). This is probably due to utilization of intracellular proteins and amino acids in the absence of external substrates. We also observed that the absolute concentration of some products was higher than the substrate concentration both in this study, and in previous studies (9). This is plausible due to degradation of amino acids and α-keto acids to shorter-chain FAs and BCFAs. If these data are analyzed as mole fractions, the total amount of carbon is smaller after the assay, despite the FA products having a higher concentration value than amino or α-keto acid substrates (9). Other intracellular intermediates, such as pyruvate, phosphoglycerate or acyl-CoA derivatives, may be incorporated from other pathways into FA production, leading to a higher concentration of products of smaller chain length (24).

The impact of ilvE was assessed using two approaches that allowed analysis of the impact of ATases. First, the initiating ATase reaction was bypassed by adding α-keto acid substrates to a culture of the parent (LM0230) in the assay buffer. Second, the ilvE deletion mutant (JLS450) was incubated with BCAAs in the same conditions as the parent. Additions of amino acid substrate to LM0230 and of the α-keto acid substrate to JLS450 were treated as the controls for these experiments (Fig. 5-1B &C).

The treatment condition using α-keto acids as the substrate for FA production by LM0230 produced approximately the same total FA
concentrations as the control of amino acids, which ranged from 5 mM to 8 mM. LM0230 produced isobutyric acid from all the α–keto acid substrates and isovaleric acid from KIC. Bypassing the initial ATase reaction with the addition of α–keto acids to the parent significantly (p<0.05) changed the type of BCFAs produced compared with the control amino acid substrates (Fig. 5-1A & B). A significantly smaller (p<0.05) amount of acetic acid was produced from all α–keto acids than from the corresponding BCAAs. Isobutyric acid was produced in significantly (p<0.05) higher amounts from KIC and KIV than leucine and valine, respectively. Interestingly, the only condition that produced n-butyric acid was LM0230 with KGL. In the amino acid control, the total FA production ranged from 5 to 10 mM FAs (Fig. 5-1A), and isobutyric acid was the only BCFA produced (Fig. 5-1A). These observations allowed us to suppose that the ATase reactions were not the limiting step in the production of As from BCAAs. However, this approach provided evidence that ATases play a role in directing intermediates into specific pathways that lead to specific FA products. Subsequent to these observations, we also hypothesized that ATases play a role in the types of FAs produced.

To verify the role of ilvE, the deletion mutant (JLS450) was assayed for FA production with amino acids substrates. Deletion of ilvE did not significantly (p>0.05) change the total FA quantities (Fig. 5-1C). However, deletion of ilvE changed the specific FAs produced from amino acids compared with the parent with the same substrates (Fig. 5-1A). With either approach, the total FA production did not decrease significantly (p>0.05). These data agree with the
previous observations that ATases were not the limiting step for producing FAs and lend support to the hypothesis.

Addition of $\alpha$-keto acids to JLS450 allowed comparison of catabolic products independent of ATase reactions. JLS450 produced similar FA products and quantities from all $\alpha$-keto acids, except in the case of KIV where acetic acid was not produced. When the ATase reactions were bypassed, the mutant produced no BCFAs from $\alpha$-keto acids. The mutant also produced significantly less ($p<0.05$) acetic acid than it did from the precursor BCAAs (Figure 1C vs. 1D). These differences suggest that $\alpha$-keto acids are catabolized via multiple routes, and the specific routes are ATase-dependent. This is plausible considering the numerous possible pathways subsequent to the ATase reaction (5, 13, 15, 23, 29).

To further assess the role of IlvE, a comparison was done of the catabolic products from $\alpha$-keto acids by the parent and the mutant (Fig. 5-1B & D). We expected that the deletion of ilvE and the bypassing of the ATase reactions would yield similar FAs from $\alpha$-keto acids. Interestingly, JLS450 produced significantly ($p<0.05$) less BCFAs from $\alpha$-keto acids than did LM0230. Deletion of ilvE also abolished production of n-butyric acid from KGL. While it is unclear why n-butyric acid was produced from KGL by LM0230, it is possible that subsequent metabolism of these components followed different pathways via interconversion of $\alpha$-keto acids and acyl-CoAs leading to different products. These data indicate that ilvE is involved in the production of n-butyric and isobutyric acids. These unexpected differences point toward the interactions of metabolic pathways that occur subsequent to the ATase reaction.
Atiles et al. (1) deleted *ilvE* and found that addition of α-keto acids did not support growth of JLS450 and hypothesized that the deletion blocked BCAA biosynthesis. While this study did not test the role of *ilvE* in growth, we observed that amino acids and α-keto acids were catabolised to FAs in JLS450, suggesting that *ilvE* plays a role in production of specific FAs via a complex web of intermediates in addition to a role in growth on α-keto acids.

Yvon et al. (27) deleted *bcaT* of *L. lactis* subsp. *cremoris*, thought to be involved in BCAA catabolism, and expectedly observed a reduction in BCFA production. Conversely, this study found the *ilvE* deletion of *L. lactis* subsp. *lactis* lead to an increase in isobutyric and isovaleric acids from isoleucine and leucine compared with that produced by the parent. Observations of this study may be explained by the activity of at least 11 other ATases (2) that may catabolise leucine and isoleucine to BCFAs (6).

Considering the observations of this study, the complimentary activity of multiple ATases with BCAAs, and the bidirectional activity of ATases, it is plausible that IlvE and other ATases direct intermediates into different pathways that lead to specific FAs. Deletion or inactivation of any single component of the metabolic web changes the specific products without completely abolishing the catabolic capabilities or reducing the total amount of products.

The observations of this study indicate that the activity of ATases is not the limiting step in producing FAs from amino acids or α-keto acids. However, ATases appear to play a role directing intermediate substrates toward specific FAs. IlvE was directly involved in production of isobutyric, n-butyric, and
isovaleric acids from amino and α-keto acids in *L. lactis* subsp. *lactis*. Additional studies are needed to delineate the role of other ATases in FA production.

References


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Figure 5-1. Mean FA production by *L. lactis* subsp. *lactis* LM0230 from amino acids (A) and from α-keto acids, (B) respectively, and by *L. lactis* subsp. *lactis* JLS450 from amino acids (C) and from α-keto acids (D), respectively, beyond α-keto acid auto-degradation. Values represent average of twice-replicated assays of FA production. NC, negative control (cells in plain buffer), Ile, isoleucine, Leu, leucine, Val, valine, Glu, glutamic acid, KMV, α-keto-β-methylvalerate, KIC, α-keto-isocaproate, KIV, α-keto-isovalerate, KGL, α-keto-glutarate. The standard deviation of the total FA production and individual FAs was ≤ 10% of the mean within both strains.
CHAPTER 6
CARBOHYDRATE STARVATION LEADS TO A NON-CULTURABLE BUT METABOLICALLY ACTIVE STATE IN LACTOCOCCUS LACTIS

Abstract

This study examined the ability of lactococci to become nonculturable and maintain metabolic activity induced by carbohydrate starvation. In this study we determined the changes in physiological parameters and extracellular substrate levels of multiple lactococcal strains during carbohydrate starvation under various environmental conditions that was then compared with a whole genome gene expression profile. At carbohydrate starvation, each lactococcal strain lost the ability to form colonies on solid media, but maintained an intact cell membrane and intracellular ATP for over 2 yrs. Stains that metabolize lactose slowly or not at all, SK11 and II.1403, respectively, required than 3 months to become nonculturable. ML3, a strain that metabolized lactose rapidly, attained nonculturability within 1 week. In all cases, the entire cell population lost the ability to form colonies on solid media, but remained intact and contained at least 100 pM of ATP after 180 d of starvation. Aminopeptidase and lipase/esterase activities decreased below detection limits during the nonculturable phase. During carbohydrate starvation, serine and methionine were produced, while glutamine and arginine were completely metabolized from of the medium. The

1Co-authored by: Balasubramanian Ganesan, Mark Stuart, Adele Cutler and Bart C. Weimer. See Appendix E for Copyright clearance and co-author release letters. We thank Madhavi Ummadi for her expertise with capillary electrophoresis. Manuscript in preparation.
cells retained the ability to transport amino acids and peptides via PMF and ATP-driven translocation, respectively. Addition of branched chain amino acids prior to carbohydrate exhaustion led to higher cellular ATP levels. Gene expression analysis showed that the genes responsible for cellular maintenance such as cell division, transcription and translation were expressed at high levels during logarithmic phase growth. However, cell division genes were repressed at the onset of starvation. Nonculturability induced metabolic abilities that were not observed in logarithmic phase cultures. The genes responsible for autolysis were not expression at any time - starvation or nonculturability. The ability to become nonculturable appears to be a widespread phenomenon in lactococci. Taken together these observations explain the ability of lactococci to maintain nonculturability and metabolic activity during carbohydrate starvation.

Introduction

Carbohydrates are the primary energy and carbon source for lactic acid bacteria (LAB) during growth in laboratory media and fermented products such as milk. During fermentation processes, LAB are subject to the vagaries of stress due to changes in water activity, pH, redox potential, and substrate availability (38). Lactococci respond to stress conditions by regulating many metabolic pathways (62). In response to carbohydrate starvation, lactococci become nonculturable and remain metabolically active for at least 2 weeks (49). Thompson and Thomas (55) suggest that lactococci utilize intracellular glycolytic reserves for moderate periods of starvation to compensate for the lack of sugar.
At the onset of carbohydrate starvation in lactococci, the intracellular levels of the glycolytic intermediates, phosphoenol-pyruvate (PEP), 3-phosphoglycerate (3-PG), and 2-phosphoglycerate increase, all of which constitute the PEP potential (54, 55). During persistent starvation, PEP is metabolized to pyruvate and ATP (54, 55). Presumably, these pools allow enzymatic activity and transport to remain active for survival during the depletion of carbohydrates.

One phenotypic condition that is lost at the onset of carbohydrate starvation is the ability to form colonies on solid media (i.e. the cells become nonculturable) (49). During the nonculturable (NC) state, cells are incapable of undergoing sustained cellular division required for growth on rich nonselective media (39), but do remain metabolically active for extended times. Many genera exhibit the NC state, including *Escherichia coli, Campylobacter jejuni,* and *Brevibacterium linens* (3, 39). However, the duration of survival under the NC state has not been demonstrated past 30 d in lactococci; thereby, leaving an open question about the role of this cellular state to contribute to flavor of fermented foods during storage. While the genes involved in abiotic stresses, such as pH and temperature, are well characterized in lactococci (43-45, 62), the molecular events associated with NC have not been explored.

Macromolecular metabolism dramatically decreases in NC cells similar to the response of marine bacteria to starvation (30). However, starved cultures are not static populations of cells. Changes during or after transition to the NC state include changes in size and shape of the bacteria to minimize energy requirements, decrease in the number of ribosomes, and changes in fatty acid
content of the cell membrane in response to local conditions (39, 58). The NC cells of lactococci retain the ability to synthesize RNA (52). Thus, the cells appear to decrease, or even die, by classical plating techniques, but intracellular processes continue (i.e. they remain metabolically active) (49).

Lactococci are capable of using alternate carbon sources such as RNA, lipids, proteins, peptides, and amino acids for energy during carbohydrate starvation (50). Carbohydrate starvation and energy depletion prompts lactococci to shift their metabolism from glycolysis to amino acid catabolism (49). The arginine deiminase (ADI) pathway is present in lactococci (7, 8) and serves as either the sole or as an additional source of energy, carbon, and nitrogen in LAB, bacilli, *Pseudomonas, Aeromonas, Mycoplasma*, clostridia, and halobacteria (12, 13). This pathway is important because the conversion of arginine to ornithine and citrulline is accompanied by the production of 1 mole of ATP per mole of arginine in carbohydrate-depleted medium (12, 13). The survival time of *L. lactis* increases in the presence of arginine due to this additional source of ATP (49).

Aminopeptidase activity plays an important role during starvation when protein turnover is required and new protein synthesis occurs at transitional states (25). During sugar starvation, the available energy within cells is utilized for protein and biomolecule synthesis, rather than for generation of cell mass. These proteins are then degraded by lactococci over time to generate peptides and amino acids that aid survival (51, 52). While this has been reported, it is reasonable that this may be a limiting factor for survival. As such, the length of survival during starvation is a question that remains to be answered.
The ability of lactococci to survive during carbohydrate starvation and continue protein turnover, RNA synthesis, and degradation also indicates their ability to actively metabolize proteins and amino acids (52). Hence, they shift away for lactic acid production and towards a nitrogenous metabolism in the NC state, which leads to new metabolic end products such as fatty acids (20, 21). We hypothesized that carbohydrate starvation represses specific genes for replication and does not induce lytic genes in lactococci. These changes are manifested as a phenotype of persistent NC. To test this hypothesis, this study compared orthogonal responses in three lactococcal strains during carbohydrate starvation using plate counts, intracellular ATP content, live-dead count, amino acid metabolism, enzyme activity, macromolecule transport, and gene expression profiles to determine multiple metabolic responses to starvation. The findings indicate that lactococci remained metabolically active by maintaining membrane integrity and ATP levels during the NC state. The population remained capable of amino acid catabolism, and yet was NC on solid agar for at least 2 yrs. Gene expression measured across cell phase (logarithmic phase, starvation, and NC) showed that, among many genetic changes, the onset of starvation induced genes for amino acid catabolism and repressed genes associated with cell division. Genes associated cell lysis (N-acetyl muramidases, lysins and holins) were not expressed at any phase of cell growth.
Materials and methods

**Bacterial strains and media**

*Lactococcus lactis* subsp. *lactis* ML3 and IL1403 and *Lactococcus lactis* subsp. *cremoris* SK11 were obtained from the Utah State University culture collection. The *L. lactis* subsp. *lactis* strains were propagated in Elliker’s and M17G broths, respectively, and *L. lactis* subsp. *cremoris* SK11 was grown in M17L broth (Difco Laboratories, Detroit, MI). Stock cultures were prepared by growing the organisms twice in 10 ml of respective broth at 30°C for 24 h. Cultures were frozen at -70°C in 10% non-fat dry milk containing 30% glycerol. Before each experiment, frozen stock cultures were thawed and sub-cultured twice at 30°C for 24 h in 10 ml of respective broth.

The strains were grown overnight at 30°C, harvested by centrifugation (6,000 × g for 15 min at 4°C), washed twice with and resuspended in sterile saline, and inoculated (1%) into sterile chemically-defined basal medium (CDM) (22). For short-term starvation studies, the basal CDM was supplemented with 0.1% lactose. For long-term starvation, the basal CDM contained 0.2% lactose and branched chain amino acids were added at 10 times the original CDM content of 20 mg/l. The CDM was adjusted to either pH 7.2 buffered with 0.19 M of sterile 3-[N-Morpholino]-propanesulfonic acid (MOPS), or to pH 5.2 buffered with 0.19 M 2-[N-Morpholino]-ethanesulfonic acid, and filter sterilized. Amino acids and buffer salts were purchased from Sigma (Sigma Chemical Co., St. Louis, MO).
Starvation period

Lactococcal strains were tested for both short term and long-term starvation survival. Short-term starvation of 42 d was used to identify the organism’s ability to attain the NC state. The long-term starvation response was monitored for over 2 yrs in a batch of three selected strains at neutral (7.2) and cheese-like (5.2) pH conditions. The capability to survive and attain the NC state was determined. *L. lactis* subsp. *lactis* IL1403 is a plasmid-free strain that lacks the ability to use lactose (3). This strain was used to compare against ML3 and SK11, a lactis and a cremoris strain respectively, that are capable of using lactose as a sole sugar source. CDM containing 0.2% glucose at pH 7.2 was also used as a control for IL1403.

Lactose and glucose determination

Lactose or glucose concentration was either determined by the colorimetric method of Dubois et al. (15) or by HPLC as described by Stuart et al. (49).

Culturable cell estimation

Samples were taken at determined time points from the culture suspensions and diluted in sterile saline dilution blanks. These dilutions were either spiral-plated as described by Stuart et al. (49), or the dilutions were plated on the respective solid media using the spread-plate technique in duplicate. The plates were incubated anaerobically for 48 h at 30°C. Colony counts were determined in spiral-plated plates according to the manufacturer’s instructions using duplicate plates.
Cell viability estimation by fluorescence

Samples were collected from the cell suspension and bacterial viability was estimated as described previously by Stuart et al. (49).

ATP determination

Intracellular ATP concentration in cell suspensions was quantified by measuring bioluminescence with an ATP assay kit as described in the manufacturer's instructions (Calbiochem-Novabiochem Corporation, San Diego, CA) and was described earlier (49). Luminescence was measured on an LS6500 Scintillation Counter (Beckman Instruments, Inc.).

Aminopeptidase and lipase/esterase activities

Cell free extracts (CFE) from cultures were prepared as described by Dias and Weimer (14) using sterile 106-micron glass beads (Sigma Chemical Co., St. Louis, MO) to facilitate cell lysis. The samples were vortexed at high speed for 10 min at 1-min intervals with alternate dipping in an ice bath. The supernatant was collected and used as the CFE for intracellular enzyme assays. The protein content of the CFE was determined spectrophotometrically using the bicinchoninic acid assay according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Bovine serum albumin was used to obtain a standard curve.

Aminopeptidase activity was measured with a semi-automated colorimetric method using reflectance colorimetry as described by Dias and Weimer (14). The assay mixtures contained 100 µl of 1 mM chromogenic
substrate in 0.05 M sodium phosphate buffer (pH 7.2) and 100 µl of CFE. Hydrolysis of the chromogenic substrate was measured as the increase in yellowness (\(b^*\)) using a reflectance colorimeter (Omnispec 4000 Bioactivity Monitor, Wescor, Inc., Logan, UT) every 5 min for 3 h at 30°C in duplicate.

Lipase/esterase activity was determined with a semiautomated reflectance colorimetric method using \(p\)-nitrophenyl derivatives (Sigma Chemical Co., St, Louis MO) of butyrate and caprylate (1). Each assay mixture contained 20 µl of 1 mM chromogenic substrate in 80 µl of 0.05 M sodium phosphate buffer (pH 7.2) with 0.2 mM Triton X-100, and 100 µl of CFE. Colorimetric measurements were performed as described for aminopeptidase activity.

Enzyme activities were determined by plotting the change in yellowness (\(\Delta b^*\)) over the assay time (14). The linear portion of the curve was used to calculate the slope. The slope was divided by the amount of protein in the added CFE to give the aminopeptidase and lipase/esterase activity \((\Delta b^*/\text{mg protein/h})\).

**Amino acid determination**

CDM samples were prepared and derivatized with 3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde as described by Stuart et al. and Ummadi and Weimer (49, 56). Nor-leucine was added as an internal standard to each reaction mixture prior to adding the derivatizing agent. Amino acids were monitored by micellar electrokinetic chromatography with capillary electrophoresis and fluorescence (49, 56). Results are presented as amino acid concentrations (mM).
Peptide uptake assay

After 8 months of starvation, peptide uptake using the casein peptide αs1,9 was performed. Cells were collected from CDM by centrifugation on a bench top centrifuge at 12,000 × g for 2 min and washed once with 190 mM MOPS at pH 7.2. The cells were resuspended in the same buffer containing the peptide and the level of peptides in assay buffer was monitored at 0, 1, 3, and 24 h by HPLC as described by Broadbent et al. (4).

Metabolic fingerprint

The ability to utilize different carbon sources at different phases of sugar starvation was analyzed using Biolog Microplates (Biolog, Hayward, CA) as described in the manufacturer’s instructions. The utilization of the different carbon sources was indicated by the reduction of the dye tetrazolium violet to a purple color.

Amino acid metabolism assay

The cells were collected at one specific time point and incubated with [2-13C]-leucine as described by Ganesan et al. (20, 21). Post incubation, the supernatant and cell pellet were collected by centrifugation (12,000 × g for 2 min) and the products were assayed in the supernatant and cell-free extracts by nuclear magnetic resonance spectroscopy (NMR) as described by Ganesan et al. (21).
Gene expression analysis

RNA samples at logarithmic phase, onset of starvation, and NC were extracted, purified, and reverse-transcribed to cDNA as described by Yi et al. (62). The cDNA was sheared enzymatically by the protocol of Nimblegen Systems, Inc. (Madison WI) and biotinylated as described by Yi et al. (62) prior to hybridization. The cDNA samples were then hybridized and detected at Nimblegen on Nimblescreen oligonucleotide microarrays designed and optimized for IL1403.

Statistical analysis and gene expression visualization

All experiments were done in two replicates. Starvation data from lactococcal strains ML3, SK11, and IL1403 were analyzed with time as a repeated measure to determine strain-wise treatment effects (Eq. 2 and Appendix B). Starvation data from the first 6 months of the long-term starvation experiment were analyzed using separate statistical analyses to determine the effect of time, pH, their combined effect on strains, and the differences between any two strains at different pH’s. SAS Statistical Software version SAS 9.0 (SAS Statistical Institute, Cary NC) was used for the analysis. Parameters used in statistical analyses were plate counts, live and dead cell counts, ATP content and AP/LE activities. Significant differences were assigned at $\alpha = 0.05$. All p-values obtained were multiplied by the total number of time points (30) to correct for multiple comparisons.

\[ Y_{ijk} = \text{Overall mean} + \text{pH}_i + \text{Time}_j + (\text{pH} \times \text{Time})_{ij} + \text{(error)}_{ijk} \quad \text{(Eq. 2)} \]
Raw pixel intensities were normalized using the robust multichip average (RMA) package within the R statistical software version 2.1.0. The log₂ values of RMA-normalized data were averaged across replicates and plotted as expression maps in the Hierarchical Clustering Explorer software version 3.0 (47) for visualization. Coloring of the expression maps was based on whole genome expression levels of all samples. A baseline was determined at the software-calculated mode that was color-coded green. Two levels of expression changes were set 2.5-fold apart from each other.

The statistical significance of gene expression changes was determined using the R statistical software suite to analyze the data as a linear mixed effects model with compound symmetry error structure with the lme package. The false discovery rate to adjust against multiple comparisons was done using the q-value package in R. The treatments for comparison were time as a repeated measure, sugar type, and their interaction as fixed effects. The false discovery rate was set at 25%. This means that due to multiple comparisons about 25% genes are likely to be false positives. Significance of fixed effects was based on distribution of the p-values plotted against that of q-values. A uniform distribution indicates insignificance of treatments while a non-uniform distribution indicates significant treatment effects.
Results

Growth and starvation survival

Growth and survival was determined using multiple methods during incubation for 2 yrs to provide orthogonal evidence of NC, survival and metabolic ability induced by carbohydrate depletion. Plate counts were done to determine the culturability of each strain during growth with various lactose concentrations in buffered CDM. IL1403 was used as the control organism because it lacks the ability to metabolize lactose. Its inability to use lactose was compared against IL1403 incubated at pH 7.2 in CDM containing 0.2% glucose. Buffered CDM was used to determine the exact role of carbohydrate starvation in CDM to avoid confounding the effect of pH stress. Lactose was added to ML3 at 0.1% for short-term (i.e. 42 d) starvation assessment, while lactose was added at 0.2% for long-term (i.e. 3.5 yrs) starvation for increased sampling points.

Culturable cell estimation and sugar metabolism. During short-term starvation, the cell density of ML3 significantly (p<0.05) increased. L. lactis subsp. lactis ML3 metabolized 0.1% lactose to below detectable levels within 1 d (Fig. 6-2A), but did not change the pH of the CDM. ML3 became NC at 8 d. When the lactose concentration was raised to 0.2%, ML3 utilized all the available lactose within 1 week (Fig. 6-2A) with a reduction in pH by about 0.5 units at pH 7.2 and 5.2, which agrees with the observations of Chou et al. (8). SK11 utilized all the available lactose at pH 7.2, but did not utilize any lactose at pH 5.2 (Fig. 6-2) and did not alter the pH in either condition. IL1403, being plasmid-free and incapable
of metabolizing lactose, did not utilize lactose at either pH (Fig. 6-2A), as expected. But IL1403 did utilize glucose within 1 d at pH 7.2.

Even with increased lactose ML3 became NC within 9 d at pH 7.2 and 5.2. SK11 and IL1403 at pH 7.2 were culturable 30 d and 112 d, respectively (Fig. 6-1B). When grown at pH 5.2, ML3 and SK11 became NC between 9 and 11 d. IL1403 did not become NC until 240 d at pH 5.2 (Fig. 6-1C). IL1403 in glucose attained NC at 21 d. These data demonstrate the effect of carbohydrate metabolism to influence the culturability of lactococci. These results were expected because carbohydrate metabolism generates ATP and influences cellular growth and density (8, 34). It further suggests that genetic regulation to switch between carbohydrate metabolism and other modes plays a role in NC. This is likely to be regulated by ccpA (23).

Cellular energy. The ATP concentration was determined during growth as another measure of viability. During short-term starvation the ATP concentration of ML3 followed a similar pattern to cell count estimations (Fig. 6-2B), and significantly increased over time (p<0.05). The ATP level rose during lactose metabolism, but declined after depletion lactose to a constant level of about 0.1 pM for 42 d (Fig. 6-2B).

During long-term starvation, cells produced ATP during the growth phase in both pH conditions (Fig. 6-2 C & D) in ML3 and SK11. Interestingly, during long-term starvation, ATP levels varied, but the ATP concentration never dropped below 100 pM. Presumably, ATP was produced and consumed by cellular metabolism during this period, allowing the cells to maintain a basal level of ATP. These data demonstrate that cells contained adequate ATP for metabolic
activities in starvation conditions even though the source of ATP during logarithmic growth (i.e. lactose) was metabolized after 1 d or within 1 week depending on the strain (Fig. 6-2A).

Viability. To verify that the cells were viable without culturing, cellular fluorescence was used to examine membrane integrity as demonstrated by Stuart et al. (49). The relative fluorescence of the live populations for all cultures increased as did the growth curves. Using this measure for the cell populations estimated the viable population without further sub-culturing, to be similar to that of late logarithmic phase.

ML3 exhibited elevated viability defined by increased fluorescence, which was in agreement with the increased cell counts noted with the cell count data (Appendix B). A low level of fluorescence was maintained for 42 d of incubation. During long-term starvation, after attaining the NC state, viability of all strains remained at similar levels, without any change during extended incubation (Appendix B). Further observations for 2 yrs in CDM showed that the cells remained NC with no change in the viability (Appendix B). This indicates that lactococci were capable of surviving long-term starvation with availability of minimal nutrients during the growth phase. These observations are similar to those of Stuart et al. (49), except that this study demonstrated the NC state for at least 2 yrs.

To verify that the cells were metabolically active and contained enough ATP to conduct cellular metabolism after this extended incubation a series of intracellular assays were done that measures various metabolic needs of the cell.
All assays were used to demonstrate the ability of NC cells to remain metabolically active, despite the inability to produce colonies on a solid medium.

**Metabolic activities**

*Aminopeptidase and lipase activity.* During short-term starvation, aminopeptidase (AP) and lipase/esterase (LE) activities were present in ML3 initially at 80-90 units Δb*/mg protein/h (Appendix B); however, these activities significantly (p<0.05) decreased to undetectable activity by 6 d. The decrease in activity of AP and LE may be due to a decrease in the energy needed for these enzymes to function or be transcribed. Alternatively, the lack of triacylglycerols and peptides, or increased free amino acids inhibits LE and AP activity and expression (36). These enzymes were not measured during long-term starvation due to their short time of activity after the cells became NC and the ability of the whole genome arrays to measure the expression of the proteins needed for these processes.

*Peptide transport.* Uptake of peptides derived from casein is dependent upon availability of ATP, as their transport mechanism requires ATP (9) via the Opp system (4). During long-term starvation, peptide uptake was determined with ML3 at 8 mo of incubation in an assay system to verify the presence of adequate ATP as to allow active transport during the NC state. ML3 initiated transport of the αs₁-g-casein peptide within 3 h of exposure to the peptide and completely transported the peptide within 24 h (Appendix B). This confirmed that NC cells contained sufficient ATP (Fig. 6-2), cellular integrity (Appendix B),
and active membrane proteins (oligopeptide transport systems Opt, Opp or Dpp) to transport peptides.

Amino acid transport and production. During short-term starvation, extracellular amino acid concentrations were estimated by capillary electrophoresis using laser-induced fluorescence detection (56). The amino acids glycine/valine, threonine, tyrosine, alanine, histidine, proline, cysteine, isoleucine, phenylalanine, leucine, aspartate, glutamate, and asparagine did not significantly \( (p>0.05) \) change over the incubation time and cellular phase changes.

Arginine decreased significantly \( (p<0.05) \) over time to nondetectable levels after 2 d in CDM with ML3 (Fig. 6-3). Glutamine levels significantly decreased \( (p<0.05) \) over 42 d of incubation for all strains in all media (Fig. 6-3). Conversely, the methionine and serine concentrations significantly \( (p<0.05) \) increased over time in ML3 (Fig. 6-3). The reduction of arginine after lactose depletion is consistent with previous studies (8), as is the production of methionine and serine with studies in cheese and defined media (33, 57). In all, the cells retained the ability to transport peptides and amino acids in all conditions that demonstrate adequate ATP for multiple substrate/product transport during starvation. To complement this observation, we determined the ability of the NC cells to metabolize amino acids that will yield end products, energy, redox control, and a PMF gradient.

Amino acid catabolism in NC cells. To test if NC cells remained capable of catabolism during long-term starvation, NC cells were collected from all strains at pH 7.2 and assayed for fatty acid production from \([2-^{13}C]\)-L-leucine using NMR. At the time of assay, ML3, SK11 and IL1403 were at 3.5, 2, and 2 yrs of
incubation, respectively. All strains catabolized leucine to isobutyric, propionic, and acetic acids. Intracellular leucine was found with the concomitant presence of isobutyric acid in the supernatant, indicating that this amino acid was being converted to a new end product that is only found during starvation (20). This confirmed our hypothesis that amino acids were transported via an ATP-independent mechanism and served as substrates for metabolism of end products not found in logarithmic phase cells. Interestingly, this was occurring even after 2 yrs of starvation.

Branched chain amino acids (BCAAs) are imported via a PMF-dependent transport system, which requires an intact membrane to maintain the potential (32). We observed BCAA transport and metabolism during this study, further supporting previous observations of intact membranes using fluorescence (Fig. 6-4). Taken together, these experiments verify that NC cells are intact, capable of transport via at least two different mechanisms, and able to metabolize amino acids to new end products that result in ATP generation during carbohydrate starvation.

**Gene expression analysis**

Biochemical data to support the hypothesis provided evidence that the cells were capable of survival. However, gene expression changes at key phenotypic points during the cycle from growth to NC were used in combination with the biochemical data to test the hypothesis. This was done using whole genome expression profiles during logarithmic phase and during the NC state, with the initial inoculation time as the control. Only genes
annotated that are functionally involved in cell replication, recombination, and repair, transcription, translation, substrate transport, ATP synthesis, sugar and protein catabolism are represented in the expression data (Fig. 6-4 A-D). Using a repeated measures statistical analysis, 29 out of 300 were significantly (q-value = 0.25) regulated due to starvation during NC from these functional categories (Table 6-1).

Sugar catabolism. Many of the glucose catabolic genes were repressed or did not change (< 2-fold change) at glucose depletion (Fig 6-4 A). This was expected as glucose exhaustion represses the genes associated with sugar metabolism (16). Interestingly, the use of or non-metabolizable carbohydrates in place of glucose produced the same results as glucose depletion. One such gene that was significantly (q-value = 0.25) induced with the replacement of glucose with lactose in IL1403, which was not catabolized, was the catabolite control protein (ccpA). This gene was induced 1.5-fold at NC in glucose, but was continually expressed during all phases with lactose addition, just as one would expect with a lactose negative strain. This observation supports the role of ccpA in regulating the genetic control to switch between sugar and protein for catabolic processes.

The repression of butB (2,3-butanediol dehydrogenase), and induction of ptsH (phosphocarrier protein Hpr) were significantly (q-value = 0.25) different between glucose availability and starvation. The induction of ptsH indicates the rapid exhaustion of glucose that leads to the deprivation of PEP potential for transport by Hpr in the PTS system causing sugar starvation due to lack of transport. Genes that were constitutively expressed during the three
physiological phases were enoB (2-phosphoglycerate dehydratase), yjhF (phosphoglyceromutase), citR (citrate lyase regulator), pyk (pyruvate kinase), pdhB (PDH E1 component, β subunit) and pdhC (dihydrolipoamide acetyltransferase component of PDH complex). Notably, the constitutively expressed genes are all associated with either ATP or redox generation, indicating that metabolic mechanisms that scavenge substrates for redox and ATP generation remain active during starvation.

Replication and cell division. Genes that were significantly (q-value = 0.25) repressed in glucose starvation were ftsX (cell division protein), gidB (glucose inhibited division protein), and dnaC (replicative DNA helicase), all of which were highly expressed during glucose availability despite a 2- to 4-fold repression of dnaC. Notably, five of eight genes directly involved in cell division (fts) did not change in expression. The Fts proteins are intermembrane proteins that bind each other in normal conditions and are critical for cell division (27, 29). However, the details of their exact role in cell division are unknown (27, 29). These data indicate that ftsX, gidB, and dnaC are directly responsible for the loss of colony formation.

Cell wall remodeling and repair is facilitated by the acm gene family, which also plays a role in lysis in lactococci (5, 28, 48). Additionally, lactococci contain bacteriophage and prophage elements that are activated during stress (2) that also cause lysis. As such, the genes associated with these events are thought to be induced during stationary phase and cellular stress and lead to lysis (5, 27, 46). The N-acetyl muramidases (acmABCD), which are directly responsible for autolysis in lactococci, were not expressed at any phase in this study (5, 28, 48).
Additionally, during the same cellular phase in glucose starvation a 2.5-to 4-fold repression of *acmB* occurred. The cell wall biosynthetic *mur* operon was not expressed during the NC state. Additionally, holins from three different prophage families (pi148, pi251, and pi306) were not expressed during this experiment. Taken as a whole, these data indicate that cell division and cell wall repair slowed or stopped, yet the autolytic genes were not induced, which led to a cell that stopped dividing without lysing or dying.

**Recombination and repair.** Two genes (*grpE* - stress response protein; and *hexB* - DNA mismatch repair protein) in this COG were significantly (q = 0.25) induced between glucose availability and starvation by more than 2-fold. The genes involved in the SOS response (*umuC*) and DNA binding proteins for DNA repair (*mutX* and *mutT*) were highly expressed during glucose availability and starvation. The chaperone genes of *groES*, the *rec* operon, and the phosphate starvation inducible *phoL* were also expressed in glucose availability and starvation. Conversely, *groEL* was repressed during the three physiological phases. These observations suggest that proteins responsible for DNA repair and protein folding were being expressed and regulated while genes known to be responsive for stress (*dna* operon, *groEL*, *groES*) were repressed. This suggests that the cells acclimated to starvation and NC while maintaining some DNA repair activity.

**Transcription and translation.** Genes from these categories produce RNA and proteins for maintaining metabolic activity. Nine genes in this COG were differentially expressed between glucose availability and starvation (Table 6-1); however, many genes were expressed in both glucose availability and starvation.
(including ycfI, rarA, tuf, mtlR, rliB, yjaJ, ahrC, gntR, yrbl, rmaA, greA, and rcfA). The *rpo* genes relate to RNA polymerases (2). Their induction indicates that transcriptional events were occurring in the cells that produced mRNA required for protein synthesis. The regulator *ahrC* responsible for arginine metabolism was also expressed indicating that arginine metabolism was induced during glucose starvation (34), as was observed with the depletion of arginine from the media (Fig. 6-3).

Similar observations were also noted in the category of genes responsible for translation, where *infA, rpmC, rpsN, rpsN2, and rplR* were significantly repressed in glucose availability, while *rplC, rplK*, and *rpsU* were significantly induced in glucose availability (*q = 0.25*) versus starvation. While nine ribosomal protein genes (*rpsK, rplP, rpsT, rpmH, rplL, rpmH, rpsO, rpmJ*, and *rpmA*) were expressed during glucose availability and starvation. This again verified that protein synthesis was occurring during starvation and NC conditions, which explains the metabolic capabilities of cells despite the lack of colony formation.

*Substrate transport.* We monitored expression of transporter genes in IL1403, of which three genes were significantly (*q < 0.25*) repressed between glucose availability and starvation. But amino acid transport-related, ABC transporters, and unknown transporter genes (*yshA, ydgB, ydgC, yaiE, yfcA*, and *yvdF*) were expressed in both conditions. This indicates that short chain substrate transport and specifically amino acid transport systems were active during sugar starvation and NC, as were observed in the biochemical studies (Fig. 6-3). Additionally, the induction of *codY*, the transcriptional regulator that senses BCAA pools and is repressed by high BCAA pools (26), also verifies that these
transporters were involved in amino acid transport and that active gene regulation was occurring during starvation and NC. Interestingly genes that related to transport of another sugar, maltose (*malE* and *malF*; maltose ABC transporter permease protein and substrate binding protein), were expressed at high levels even in absence of extraneous sources of maltose.

*Protein metabolism.* Protein metabolism, which includes proteases, oligopeptide transporters, and aminopeptidases, provides amino acid substrates from intracellular proteins or from transport of oligopeptides. Among all peptidase and peptide transport genes, *optC* was the only gene significantly repressed by at starvation and NC. The oligopeptide transporters of *optB*, *optC*, *optD*, and *optS* were expressed across the three phases. None of the genes of the *opp* operon was expressed in any condition due to mutations in the regulator sequences up stream of the structural genes (61). The *dpt* genes were also expressed in both of the conditions.

Among aminopeptidase genes, the gene expression of *pepXP* (X-prolyl-dipeptidyl aminopeptidase) was high in both glucose availability and starvation. Notably, *pepN*, the gene for the enzyme used as the hallmark of lactococcal lysis, was not expressed in any phase. Enzyme activities of APs are typically associated with cellular lysis of lactococci in the cheese matrix (6, 10, 11). Enzyme activities of APs in this study were inhibited during starvation, as was the gene expression. This is verified by the high level of expression of the pleiotropic regulator *codY*, which when expressed, senses availability of pools of BCAAs and represses aminopeptidase gene expression (26). The lack of enzyme activity was hence confirmed by gene expression.
ATP Synthase. ATP synthesis is essential as an energy macromolecule required for most cellular processes. The F₀F₁ ATP synthase of lactococci acts as a proton pump that maintains intracellular proton-motive force gradient by utilizing energy from ATP hydrolysis to pump protons out of the cell (31). Two genes of the F₁ unit of ATP synthase, that of the ε subunit, atpE, were significantly induced at starvation, while gene expression was low and remained unchanged for the α, β, γ, and δ subunits. The genes for the F₀ unit responsible for proton channel activity, atpB and atpF, were expressed during glucose availability and starvation. The α, β, and γ subunits are responsible for ATP hydrolysis for proton translocation while ε is responsible for connecting channel activity to proton translocation (46). The low expression of α, β, and γ subunit genes suggests that the cells are reducing ATP hydrolysis during starvation to minimize ATP loss by proton translocation, yet expressing these proteins at a sufficient level to maintain the PMF, as indicated by amino acid transport.

Discussion

Starvation and NC is a complex and controversial topic. Hence, orthogonal evidence is needed to prove the changes and existence of these events opposed to cell death and lysis. The orthogonal evidence in this study demonstrated how the interactions between strain, time, carbohydrate metabolism, and medium pH modulate metabolism during starvation and the existence of the NC state in lactococci. Incubation time significantly (p<0.05) influenced pH and strain after the initial day of incubation as compared to the
initial incubation point. This is demonstrated in all the biochemical assays in all strains tested. The medium pH significantly (p≤0.05) shortened the metabolism of lactose and onset of NC in SK11, but it did not impact the ability of ML3 to become NC and remain metabolically active during long-term starvation (Fig. 6-1). IL1403 was also significantly (p≤0.05) impacted by the medium pH, as pH 5.2 delayed the onset of NC (Fig. 6-1C). The pattern of pH response within strains differed by their sugar utilization indicating that the ability to use carbohydrates is the primary factor in the response to starvation and impact on survival, irrespective of the pH conditions of the media. This observation is consistent with the results of Chou et al. (8) where ML3 only metabolized arginine after the cell depleted lactose. AhrC and CcpA need to be induced before arginine is metabolized rather than lactose (34). Interestingly, all strains behaved in a similar way after the initial response during the first month of starvation. This leads one to believe that once the strains attain the NC state, their cellular responses are likely to be similar, suggesting there is a minimal set of metabolic abilities that allow the cell to survive long-term carbohydrate starvation. Varying the pH only modified the initial response to enter NC, but did not change the long-term biochemical capabilities during NC.

Glucose metabolism genes were repressed during starvation and NC, presumably due to the lack of metabolic substrates. This was also verified by induction of the *pts* and *butB* genes along with other genes to generate redox and energy precursor molecules, suggesting that active metabolism of short chain molecules exists during starvation and NC as shown in *E. coli* (24, 37, 40).
By comparing the cell count to the ATP concentration (Fig. 6-1A & 5A) obtained during the 42 d starvation, it appeared that the cultures became NC on solid agar after the ATP concentration dropped below 0.5 pM. We presumed that this represented the maintenance energy required for lactococci to remain culturable on solid agar. However, this supposition was not true during long-term starvation, where ATP consistently remained above 100 pM throughout the NC period of at least 2 yrs. The difference in the minimal ATP during long-term starvation may be accounted for by the presence of BCAAs at 10 times higher levels in comparison to short-term starvation. This change was made for long-term starvation experiments to test if these amino acids were able to increase ATP levels. The positive result led us to conclude that BCAAs may support the carbon and energetic needs of the cells during long-term starvation at levels that were similar to those during lactose utilization. The genes for amino acid transport such as yshA, ydgB, ydgC, yaiE, yfcA, and yvdF were expressed, further supporting the evidence amino acid transport and metabolism resulting in new end products and more ATP. Presumably the growth phase requires amino acid for translation and cell division whereas in starvation and NC amino acids acted as an energy source since cell division ceased.

Lactococci maintained constant levels of ATP for long periods of carbohydrate starvation, even after becoming NC within 7 d of carbohydrate depletion. This suggests that the cells have enough energy to conduct reactions that require energy such as enzyme activity and transport, which was verified by expression of genes for transcription, translation, and substrate transport, and phenotypic determination of amino acid and peptide transport. One possible
explanation for maintenance of ATP levels during starvation is that the cells are conserving ATP by reducing the activity of the ATP synthase and reducing proton pumping. This would channel ATP for intracellular energetic processes. This is also verified by observations of ATP synthase subunits responsible for ATP hydrolysis being poorly expressed (Table 6-1; Fig. 6-4) that would conserve ATP for cellular requirements.

The depletion of arginine, with lactose also being depleted, is in agreement with previous studies done in starvation conditions and ripening cheese, in which the arginine concentration decreased during ripening (42, 50, 57). Arginine is used for growth requirements (32), maintaining pH homeostasis (8), and maintenance of ATP. The transport of glutamine is energy driven via phosphate, presumably through the use of ATP or other energy-rich phosphate-intermediates (32). The consistent decrease of glutamine over time in all conditions (Fig. 6-7) indicates that alternate energy sources are being utilized to generate ATP, which is used in transport. Considering that the cells contain low levels of ATP - \(\sim\)pM - (Fig. 6-2A), it appears that a low level of ATP is sufficient to maintain transport and other cellular events that need energy. This is also verified by expression of amino acid transport genes by IL1403, to show that transport mechanisms are active over long-term starvation (Table 6-1; Fig 6-4 – gene expression maps).

The presence of BCAAs at a 10-fold higher level during the long-term starvation experiment than short-term starvation led to 1,000- to 10,000-fold higher levels of ATP (Fig. 6-5) even though the NC state was attained within the same time interval (Fig. 6-1). The medium supported survival and metabolic
activity for at least 2 yrs. This indicates that BCAAs are involved in providing additional energy during starvation survival of lactococci independent of lactose. Logarithmic phase cells of lactococci utilize BCAAs to produce straight and branched chain fatty acids (20). These pathways also allow production of ATP by substrate level phosphorylation and generation of NAD that can subsequently be used in redox mediated biosynthetic pathways for additional ATP (20, 21). *Brevibacterium linens* utilizes BCAAs only after carbohydrate starvation (21). These observations provide evidence that BCAAs extend survival via additional ATP and support precursor generation for other pathways to be functional during starvation. Arginine was depleted before the onset of the NC state (Fig. 6-3). In such energy-depleted conditions, BCAAs may be the substrates that support energy, carbon, and nitrogen requirements in long-term starvation and regulate the expression of *codY* (41) to control the peptidase system (26).

The production of methionine occurs during cheese ripening (33, 57), as well as at the onset of starvation (Fig 6-3). Methionine is associated with desirable sulfur notes in Cheddar cheese and is linked to the production of volatile sulfur compounds (59, 60). It is not known why methionine increases during carbohydrate starvation, but may linked to nucleic acid metabolism via S-D-riboyl-L-homocysteine or S-adenosyl-L-methionine, which lactococci produce (2). It is unclear how the cell acquires the sulfur atom in methionine, but it is clear that the increase in this amino acid is not due to protein recycling during starvation and NC. This is also in accord with the release of serine during the ripening of cheese (33, 57), during starvation (19), and during NC (Fig. 6-3). In our observations and those with cheese ripening (19, 33, 57), serine consistently
increased over time. These observations suggest that lactic acid bacteria may not possess pathways that allow serine degradation, or alternatively, mechanisms to import serine may not be active during starvation. Hence, methionine and serine production may be the biomarker of starvation or NC in lactococci.

The presence of viable cells up to 2 yrs, as measured by spectrofluorometry, in combination with low expression of lytic, cell division, and cell wall repair genes, denotes the maintenance of a cellular membrane that did not undergo lysis and aided the preservation of nucleic acids. This is contrary to the current dogma that states that lactococci die, lyse and lose viability due to harsh environments (18, 35, 53). Estimation of culturable cells on solid media is a common way to determine cell numbers. This process relies on the ability of cells to replicate on solid media. The absence of any culturable counts only indicates the inability of cells to replicate, not necessarily their death, as demonstrated in this study in combination with transcription and translation events identified with gene expression analysis.

The protease system was regulated in mixed directions during the incubation. Oligopeptide transporter (Table 6-1; Fig. 6-4) expression was regulated as the cell entered starvation and proceeded to NC. Some components were repressed while others were induced (Fig. 6-4). Three peptide transport systems exist in lactococci – opp, dpt, and opt (9). Only the regulation and action of opp is well characterized (17), while the role of the other systems remains to be defined. In this study, optBCS were induced upon entry into starvation and NC, as was dpt (Fig 6-4). Considering the transport of $\alpha_{s_1}$-casein and the induction of
opt and dpt, it is likely that these redundant protein transporters and used in place of opp.

The transport of peptides and amino acids regulate aminopeptidases via codY (41). During this study, only pepCDTO were induced during NC. The remaining aminopeptidases were repressed. This observation verifies the activity of aminopeptidases increasing during growth but terminated over starvation, as with the biochemical data. This also indicates that peptides are not the source of amino acids in the medium as opposed to amino acids, for which transporters were also expressed.

To our knowledge the use of gene expression to demonstrate metabolically active but NC cells has not been done previously. While the 29 genes that were significantly induced or repressed (Table 6-1) constituted only 10% of the total genes among the categories that were discussed in this study, another 50 genes were expressed in all conditions. The expression of transcription- and translation-related genes verified that many genes that were responsive for cellular repair, nucleic acid and protein synthesis and turnover, and substrate transport were expressed, and also verified by substrate transport and metabolic activity. The cell division genes were not actively transcribed, verifying our inability to culture the cells in solid media. The lytic and cell wall repair genes were also not transcribed, confirming that cellular membrane integrity is preserved and that lactococci did not lyse upon substrate starvation. Low levels of aminopeptidase enzyme activity and gene expression additionally verify this observation during starvation and NC. Cumulatively, these observations show that lactococci did not lyse, but remained metabolically active
to perform macromolecular synthesis and substrate metabolism during starvation and NC.

Conclusions

After carbohydrate depletion, all the strains tested became NC, despite the variation in the onset time that was modulated by presence of lactose in the CDM. Once the cells became NC, they remained intact and retained the ability to transport substrates via ATP- and PMF-dependent mechanisms. However, starvation altered cellular metabolism to produce new products that were not produced in logarithmic phase. The AP and LE activities became non-detectable after 21 d of starvation, while the ATase activity remained for at least 3.5 yrs. Intracellular ATP concentration was consistently maintained. NC cells transported and metabolized glutamine and arginine, but produced serine and methionine during the same time period. Leucine was transported and catabolized to fatty acids, demonstrating that NC cultures remain metabolically active for at least 2 to 3.5 yrs. Presence of higher amounts of BCAAs did not prevent cells from attaining the NC state but allowed maintenance of 1,000 to 10,000 fold higher levels of ATP compared to cultures that lacked these amino acids. Gene expression information allowed the identification of major cellular processes that were still active during starvation and nonculturability.
References


Table 6-1. Significantly differentially expressed genes (q-value ≤ 0.25) between glucose availability and starvation

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Annotation</th>
<th>Expression change compared to logarithmic phase in IL1403 with glucose over time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sugar Catabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>butB</td>
<td>2,3-butanediol dehydrogenase (EC 1.1.1.4)</td>
<td>Repressed</td>
</tr>
<tr>
<td>ptsH</td>
<td>phosphocarrier protein Hpr</td>
<td>Repressed</td>
</tr>
<tr>
<td><strong>Recombination and repair</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hexB</td>
<td>DNA mismatch repair protein MutL</td>
<td>Induced</td>
</tr>
<tr>
<td>grpE</td>
<td>stress response protein GrpE</td>
<td>Induced</td>
</tr>
<tr>
<td><strong>Cell division and replication</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ftsX</td>
<td>cell division protein</td>
<td>Repressed</td>
</tr>
<tr>
<td>gidB</td>
<td>glucose-inhibited division protein GidB</td>
<td>Repressed</td>
</tr>
<tr>
<td>dnaC</td>
<td>replicative DNA helicase</td>
<td>Repressed</td>
</tr>
<tr>
<td><strong>Transcription and regulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rpoA</td>
<td>DNA-directed RNA polymerase alpha chain (EC 2.7.7.6)</td>
<td>Induced</td>
</tr>
<tr>
<td>rpoC</td>
<td>DNA-directed RNA polymerase beta-chain (EC 2.7.7.6)</td>
<td>Induced</td>
</tr>
<tr>
<td>rpoD</td>
<td>major RNA polymerase sigma factor</td>
<td>Induced</td>
</tr>
<tr>
<td>ccpA</td>
<td>catabolite control protein A</td>
<td>Induced</td>
</tr>
<tr>
<td>codY</td>
<td>transcriptional regulator</td>
<td>Induced</td>
</tr>
<tr>
<td>rdrA</td>
<td>transcriptional regulator</td>
<td>Induced</td>
</tr>
<tr>
<td>rmaB</td>
<td>transcriptional regulator</td>
<td>Induced</td>
</tr>
<tr>
<td>rmaH</td>
<td>transcriptional regulator</td>
<td>Repressed</td>
</tr>
<tr>
<td>rmeC</td>
<td>transcriptional regulator</td>
<td>Repressed</td>
</tr>
<tr>
<td><strong>Translation</strong></td>
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<tr>
<td>rpsN</td>
<td>&quot;30S ribosomal protein S14&quot;</td>
<td>Induced</td>
</tr>
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<td>rpsN2</td>
<td>&quot;30S ribosomal protein S14&quot;</td>
<td>Induced</td>
</tr>
<tr>
<td>rpsU</td>
<td>&quot;30S ribosomal protein S21&quot;</td>
<td>Induced</td>
</tr>
<tr>
<td>rplK</td>
<td>&quot;50S ribosomal protein L11&quot;</td>
<td>Repressed</td>
</tr>
<tr>
<td>Protein</td>
<td>Description</td>
<td>Regulation</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>rplR</td>
<td>&quot;50S ribosomal protein L18&quot;</td>
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</tr>
<tr>
<td>rpmC</td>
<td>&quot;50S ribosomal protein L29&quot;</td>
<td>Induced</td>
</tr>
<tr>
<td>infA</td>
<td>&quot;translation initiation factor IF-1&quot;</td>
<td>Repressed</td>
</tr>
<tr>
<td>Transport</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ydcE</td>
<td>ABC transporter ATP binding protein</td>
<td>Repressed</td>
</tr>
<tr>
<td>ysaC</td>
<td>ABC transporter ATP-binding protein</td>
<td>Repressed</td>
</tr>
<tr>
<td>ycdH</td>
<td>transporter</td>
<td>Repressed</td>
</tr>
<tr>
<td>Protein metabolism</td>
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<td></td>
</tr>
<tr>
<td>optC</td>
<td>oligopeptide ABC transporter permease protein</td>
<td>Repressed</td>
</tr>
<tr>
<td>F$_0$F$_1$ ATP synthase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>atpE1</td>
<td>ATP synthase epsilon subunit (EC 3.6.1.34)</td>
<td>Induced</td>
</tr>
<tr>
<td>atpE2</td>
<td>ATP synthase epsilon subunit (EC 3.6.1.34)</td>
<td>Induced</td>
</tr>
</tbody>
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FIG. 6-1. Cell counts during growth and carbohydrate starvation in buffered CDM. Plate counts of ML3 in buffered CDM containing 0.1% lactose (□) (panel A), Plate counts of ML3 (□), SK11 (◇), and IL1403 (O) in CDM at pH 7.2 (panel B) and within the first 15 d of starvation (panel B1). Plate counts of ML3 (□), SK11 (◇), and IL1403 (O) in CDM at pH 5.2 (panel C) and within the first 15 d of starvation (panel C1). ML3 data shown up to 2 yrs, but was NC up to 3.5 yrs. Coefficient of variation ranged between 0.1-9% for all strains at each time point and pH condition.
(Diagram A) Plate count (Log$_{10}$ CFU/ml) over time (days) ranging from 0 to 14 days.

(Diagram B) Plate count (Log$_{10}$ CFU/ml) over time (days) ranging from 0 to 15 days with an inset showing data from 0 to 12 days.

(Diagram C) Plate count (Log$_{10}$ CFU/ml) over time (days) ranging from 0 to 200 days with an inset showing data from 0 to 15 days.

NC at 8 months
FIG. 6-2. Lactose utilization during growth and NC in buffered CDM and intracellular ATP concentration of cells. Initial lactose level was 0.2-0.25% for all media. Lactose levels of ML3 at pH 7.2 (hatched squares) and pH 5.2 (open squares), SK11 at pH 7.2 (hatched diamonds) and pH 5.2 (open diamonds), and IL1403 at pH 7.2 (hatched circles) and pH 5.2 (open circles) (Panel A). Data shown for 93 d while lactose levels were maintained for 3.5 yrs. ATP levels of ML3 in CDM containing 0.1% lactose □) (panel B) and ATP levels within the first 7 d of starvation (panel B1). ATP levels of ML3 (□), SK11 (◇), and IL1403 (◇) in CDM at pH 7.2 (panel C) and within the first 7 d of starvation (panel C1). ATP levels of ML3 (□), SK11 (◇), and IL1403 (◇) in CDM at pH 5.2 (panel D) and within the first 7 d of starvation (panel D1). ML3 data shown up to 2 yrs, but contained 100 pM ATP up to 3.5 yrs. Coefficient of variation ranged between 0.8-11% for all strains at all time points and pH conditions tested.
FIG. 6-3. Extracellular amino acids' profile for ML3 grown in CDM with 0.1% lactose. Serine concentrations are depicted on the yy-axes, and all other amino acids in the y-axis. Coefficient of variation ranged between 1-8% over all time points.
A diagram showing the changes in amino acid levels over time.

- Glutamine
- Methionine
- Arginine
- Serine

The x-axis represents time (days) from 0 to 42, and the y-axis represents amino acid levels in mM from 0 to 250.

- Glutamine decreases sharply in the first few days and then remains low.
- Methionine shows an initial rise followed by a plateau.
- Arginine increases steadily over time.
- Serine levels remain low throughout the time period.

Time (days):
0 6 12 18 24 30 36 42

Amino acid (mM):
0 50 100 150 200 250
FIG 6-4. Expression maps of different functional categories of genes involved in starvation and nonculturability. A color change from green to black denotes a 2.5-fold increase in gene expression. A color change from black to red denotes a ≥ 2.5-fold change in gene expression. 1, IL1403 in glucose, T0, 2, IL1403 in glucose, sugar exhaustion (1 day), 3, IL1403 in glucose, nonculturability (21 d)
CHAPTER 7
CATABOLISM OF LEUCINE TO 2-METHYLBUTYRIC ACID BY
LACTOCOCCUS LACTIS: A POSSIBLE
PATHWAY AND ITS GENE
EXPRESSION PROFILE

Abstract

Lactococci remain intact and metabolically active during carbohydrate starvation for periods of at least 2 yrs. After carbohydrate exhaustion, the cell population enters a nonculturable state and is no longer capable of growth on solid media; however, the cells gained the ability to catabolize amino acids to fatty acids. Previously, we showed (Chapter 6) that sugar catabolism induces the nonculturable state in Lactococcus lactis. Further, we showed that lactococci use amino acids as an energy source. This study determined a possible conversion route from leucine to branched chain fatty acids that was induced during starvation. The phenotypes were associated with the production of isovaleric acid from branched chain amino acids available in the growth medium. When tested using NMR spectroscopy the catabolism of leucine resulted in the production of α-ketoisocaproate, 3-hydroxy-3-methyl-glutaric acid, glutamate, 2-methylbutyric acid, citrate, and α-ketosiovalerate. To gain insights into a pathway for branched chain amino acid catabolism we used gene expression profiles and in silico pathway analysis coupled with NMR spectroscopy data to develop a potential metabolic route. We propose a complete metabolic route for
L. lactis that utilizes leucine via transamination, hydroxymethyl-glutaryl-CoA, and acetyl-CoA to produce 2-methylbutyric acid, as well as incorporating all the other end products observed. This pathway is also found in Pseudomonas and Clostridium, giving it some credibility as a proposed pathway. Gene expression profiles demonstrated that lactococci have redundant genes to carry out the entire pathway, which were selectively regulated to modify leucine metabolism during starvation.

Introduction

The catabolism of amino acids by lactic acid bacteria is a constant source of interest for production of positive flavors and suppression of negative flavors in cheese (12). The catabolites resulting from aromatic amino acids lead to off-flavors and odors (17, 20, 42). Products from sulfur-containing amino acids such as thiols, and from branched chain amino acids (BCAAs) such as isobutyric and isovaleric acids, are implicated in positive flavor (43, 45, 48).

Lactic acid bacteria catabolize amino acids to survive the harsh milieu of cheese during the ripening stage (6). This leads to secondary end products that are important in cheese flavor (8, 13, 49). High quantities of fat and protein, high osmotic conditions, low oxidation-reduction potential, low pH, and lack of available sugar cumulatively affect the ability of starter and non-starter lactic acid bacteria to continue surviving in cheese. While most of these conditions are consistent from the onset of cheese ripening, carbohydrate depletion occurs within the first month (11, 41) adding to the abiotic stress conditions on the cell during cheese ripening (4, 41).
Catabolism of amino acids such as arginine and BCAAs is implicated for survival during long-term starvation (4, 5, 14). While addition of arginine extends survival during early post-carbohydrate depletion (4, 41), in the later stages, the consumption of other amino acids, such as BCAAs and α-keto acids, produces branched chain fatty acids (BCFAs) (13). Known pathways in bacteria for this catabolism yield ATP via substrate-level phosphorylation and reducing equivalents (i.e. NAD) (13). The availability of BCAAs and α-keto acids provides lactococci with an alternate source of nutrients for generating cellular energy and metabolites that aid cellular maintenance during stress.

A completed genome of *Lactococcus lactis* IL1403 is publicly available (1). A draft genome of *L. cremoris* SK11 is under the finishing process (http://www.jgi.doe.gov/JGI_microbial/html/index.html), while a third genome, *L. lactis* MG1363 is being sequenced and annotated (27). Availability of microbial genomes offers a new level of capability to relate metabolic profiles to changes in gene expression profiles in culture. The genome is a source of information about the existence of genes whose products are related to a particular pathway or set of pathways. Genome-wide expression profiling yields putative targets from the homologous genes for identifying and modulating metabolism of well-characterized and unknown routes. Combining the gene expression studies with intermediary metabolite data will provide new insights into cellular processes that will enable new mechanisms of control for food fermentations.

Based on the ability of lactococci to catabolize amino acids to fatty acids (13), and their ability to survive extended periods of starvation (15), we
hypothesized that lactococci produce BCFAs during carbohydrate starvation from BCAAs via one of the known lipid metabolic routes in bacteria, despite the lack of genomic evidence. To prove this hypothesis, we determined the intermediary metabolic products of \textit{L. lactis} IL1403 from leucine while simultaneously measuring the whole genome expression profile using a microarray designed and optimized with the IL1403 genome. Specific attention was given to relevant phenotypic milestones (sugar depletion, onset of nonculturability, and BCFA production) to determine the biochemical and gene expression profiles that enable the elucidation of a putative metabolic route of BCAA catabolism during sugar exhaustion and starvation.

Materials and methods

\textbf{Strains and media}

\textit{Lactococcus lactis} subsp. \textit{lactis} IL1403 was obtained from the Utah State University culture collection and propagated in M17G broth (Difco Laboratories, Detroit, MI). Stock cultures were prepared by growing the cells twice in 10 ml of M17G broth at 30°C for 24 h. Cultures were frozen at −70°C in 10% non-fat dry milk containing 30% glycerol. Before each experiment, a frozen stock culture was thawed and sub-cultured twice at 30°C for 24 h in 10 ml of M17G broth. The culture was harvested by centrifugation (6,000 \times g for 15 min at 4°C), washed twice with and resuspended in sterile saline, and inoculated (1%) into sterile chemically-defined basal medium (CDM) (22). The CDM contained 0.2% glucose and BCAAs were added to a final concentration of 200 mg/l, 10 times the
original CDM content. The pH of the CDM was adjusted to 7.2. CDM was buffered with 0.19 M of sterile 3-[N-Morpholino]-propanesulfonic acid (MOPS) and filter sterilized with a 0.2 µ sterile bottle-top filter (Corning Inc., Corning NY) before use.

**Culturability and viability**

Culturability of the cells was estimated on M17G agar medium that contained 0.5% glucose using the spread plate technique. Viability of the culture was simultaneously determined using the Baclight Live-Dead viability kit (Molecular Probes Inc., Eugene, OR) as described previously (41).

**Glucose determination**

Glucose in CDM was quantified by the spectrophotometric assay of Dubois et al (9). Concentrations were estimated from a standard curve that was linear over the detection range with R² ≥ 0.98 (Appendix C).

**Metabolic assays**

Substrate utilization assays were done at sugar exhaustion and nonculturability as described previously (13). The substrate for BCAA catabolism was 2-13C-leucine at a concentration of 20 mM in 190 mM 2-(N-morpholino)propane-sulfonic acid-sodium salt buffer at pH 7.2. Leucine assays were done in presence and absence of 20 mM pyruvate for comparison of products. The cells were incubated with the substrates at 30°C for 3 h. After incubation, the filter-sterilized assay buffer was analyzed for products from 13C-leucine by NMR spectroscopy, fatty acids (FAs) by gas chromatography and
α-keto acids by capillary electrophoresis as described below. Cell-free extracts were prepared from cell pellets for NMR spectroscopy as described previously (8).

**Gas chromatography of FAs**

FAs were estimated from supernatants of assay buffer and cell-free extracts as described previously (13). Results are expressed as the average FA concentrations (mM) of two replicate experiments.

**α-Keto acid analysis**

α-Keto acids were estimated from cell-free extracts prepared as described previously (8). The cell-free extracts were desalted using a C-18 column (Alltech Associates), concentrated 100-fold using an AES1000 speed vac system (Savant Instruments Inc., Farmingdale KY), and derivatized with 1.16 mM 3-(methyl)-2-benzo-thiazolinone hydrazone in sodium acetate buffer. The α-keto acids were separated by micellar electrokinetic capillary chromatography (MECC) in a P/ACE 2100 capillary electrophoresis unit (Beckman-Coulter Inc., Palo Alto CA) with UV detection at 340 nm as described previously (7). Corrected peak areas obtained from the electropherograms were used to calculate concentrations of individual α-keto acids from standard curves that were linear over the detection range (R²≥0.98; Appendix C). Standard solutions of KIV and α-keto-β-methylvalerate separated as two distinct peaks from all other α-keto acids (Appendix C). The corrected peak areas of both peaks were added to determine the concentrations of these α-keto acids.
NMR spectroscopy

The products from $^{13}$C-labeled leucine in assay buffer and cell-free extracts were identified using NMR spectroscopy as described previously (14). Briefly, NMR spectroscopy was done at the NMR facility at the Utah State University Department of Chemistry and Biochemistry (14). Chloroform was used as an external standard with a $^{13}$C-spectral shift of 77.2 ppm to reference spectral shifts of products. Assignments to the observed spectral shifts were done based on natural $^{13}$C abundance spectra of the observed intermediates and known spectral shifts (36).

Pathway identification

Based on the products observed from NMR spectroscopy, a possible pathway was computed using the Pathcomp feature of KEGG (24, 25). Pathcomp does not allow assignment of labels for guidance; rather it directly computes the pathway from starting substrate to end product by matching enzymes for each reaction to its database of reference pathways of multiple organisms. The metabolic pathway of leucine catabolism was reconstructed for pictorial depiction in the PathwayTools software v8.5 (Fig. 7-2 - 7-11) (28). After computation and pictorial depiction the $^{13}$C labels of the identified products were marked in the figures (solid black circles). The location of the $^{13}$C labels in identified intermediates was used to track potential label positions in the intermediates that were not identified by NMR (gray circles) to speculate how the products were labeled at the identified positions.
Protein homology identification

KEGG maps for leucine metabolism were produced using the public IL1403 genome (http://www.genomes.co.jp/kegg; map00280) and the closed genome sequence for L. cremoris SK11 at Oakridge National Laboratories (http://genomes/ornl.gov/microbial) (24, 25). A number of genes for the enzymes of the proposed pathway were present in the genome of L. lactis subsp. lactis IL1403 (1). In cases where a functionally annotated gene for a particular enzyme was not present, a list of respective enzymes (i.e. genes) capable of catalyzing the reaction was identified by their putative assigned EC numbers and COG classification. This list was examined further for gene candidates by comparing the domains of the primary protein structure to other known protein domains using the Conserved Domain database CDDv2.03 from NCBI which encompasses the protein domains’ information from Pfam, COG, and Smart databases (32). Significance of structural homology was assigned at an E-value $\geq 10^{-6}$ Gene expression data for all such identified homologues were mapped to the pathway reactions to monitor the expression profiles of the possible enzymes involved in the pathway.

Gene expression profiles

Gene expression was determined at the start and the end of the NMR assay. RNA was collected and reverse-transcribed to cDNA from cells as described by Yi et al. (47). Prior to biotinylation, the cDNA was sheared with DNase1 according to the protocol of Nimblegen Systems (Madison, WI). The cDNA was subsequently biotinylated as described by Yi et al. (47). Hybridization
of target cDNA was done at Nimblegen Systems using a custom Nimblescreen expression chip optimized for the genome of *L. lactis* subsp. *lactis* IL1403. The chip contained 5-6 probes per ORF after optimization. The raw data were obtained as pixel intensities that were normalized using the R statistical package by the robust multichip average (RMA) method (21) at Nimblegen Systems. Where possible, the gene expression data have been added to support, confirm or verify the genes involved in a specific step of the identified pathway. The annotations of genes used in this study are listed in Table C-1 (Appendix C).

Statistical analysis and data visualization

The NMR assay experiments were conducted in biological two replicates. The RMA-normalized data were averaged across replicates before visualization. The log₂ transformed values from the normalized data were depicted as changes over time by expression maps drawn by Hierarchical Clustering Explorer (HCE) software version 3.0 (39, 40). The cutoff value for minimum levels of gene expression was based on the whole genome expression profiles generated by HCE. The minimum cutoff value was set at the mode that was determined by the software and based on the entire data set of expression signals. Two levels of expression changes were set to a 2.5-fold change apart from each other, which allowed color depiction of the data set (26). The data were further subdivided into classes of enzymes identified by protein homology identification as described above. The heat map for each class was drawn separately and the expression cutoff values were applied to generate colored expression maps for each individual class for visualization. Fold changes for each gene at the end of
assay were calculated with data from start of assay as control. Statistical significance for differential gene expression was analyzed using the SAM statistical package. The false discovery rate was assigned at 20%. In other words we expect 20% of all genes called statistically significant to be false positives.

Results

Culturability, sugar exhaustion, and viability

*L. lactis* subsp *lactis* IL1403 utilized 0.2% glucose during exponential growth and attained maximum cell number of ~10⁹ CFU/ml within 24 h of inoculation. Glucose was depleted within 24 h; however, IL1403 did not reach the nonculturable (NC) state until 21 d of incubation. During this time and further incubation in the NC state the cells remained intact and at a constant level as determined using fluorescent cell counts (Fig. 7-1).

BCFA production

IL1403 initially produced acetic and propionic acid during the incubation. Production of 0.1 mM isovaleric acid was identified in culture at 21 d of incubation. This coincided with the attainment of NC in IL1403, but was unusual in comparison to the initiation of BCFA production in ML3 and SK11 (15), which required additional time for production of BCFA end products.

Presuming that lactococci use similar pathways to that of brevibacteria for production of isovaleric acid from leucine, we looked for the presence of related genes in the lactococcal genome. Notably, using KEGG to determine the genes in the reconstructed pathways, none of the homologous genes were present for the
established catabolic pathways for leucine in *L. lactis* IL1403 or *L. cremoris* SK11, except the aromatic and BCAA aminotransferases (ATases), which catalyze the first step in the metabolic route (18, 31, 38, 48). Both lactococcal genomes contained seven homologues of ATases capable of catabolizing BCAAs. Further, abolishing the activity of the BCATase (*ilvE*) alone did not reduce BCFA production, but rather changed the FA product profile (16). Therefore, IL1403 was found to be capable of BCFA production from amino acids, but the genes responsible for the known catabolic routes were not assigned using genome reconstruction tools. Taken together, these observations indicated that lactococci may use an unknown pathway for the production of BCFA products or that the gene annotations in IL1403 are not complete, thereby causing the reconstruction program to fail in assigning the correct gene to the correct metabolic step. To test this hypothesis we used NMR spectroscopy coupled with genome-wide gene expression profiles to determine the metabolic route and gene expression during the entire incubation. The focus for gene expression was limited to specific assays during NMR spectroscopy; thereby providing controlled conditions for the determination of the metabolic route.

**NMR spectroscopy**

Taking small samples from the incubating culture and adding 2-$^{13}$C-leucine in assay identified the products of intermediary metabolism. Use of whole genome gene expression studies at the same assays determined a possible set of genes needed for the metabolic route of BCFA’s in IL1403. Six different metabolic intermediates and products were identified using 2-$^{13}$C-leucine in
NMR assays (Table 7-1). The expected transamination product, 
\( \alpha \)-ketoisocaproate (KIC) with the \(^{13}\)C label at the \( \alpha \)-keto group, was detected. 
The primary BCFA product was 2-methylbutyric acid, which contained the \(^{13}\)C 
labels on the alkyl side chain at the C3 and C4 carbons (Table 7-1). Another 
product was 3-hydroxy-3-methylglutaric acid (HMGA) derived from 3-hydroxy-
3-methylglutaryl-CoA (HMG-CoA). The presence of pyruvate did not alter the 
products that were obtained from leucine catabolism (Table 7-1). IL1403 shuffled 
the label to form 2-methylbutyric acid rather than isovaleric acid (4-
methylbutyric acid) during starvation. GC analysis confirmed this product. This 
observation is a primary difference between metabolism in assay during log 
phase growth and starvation.

The other products that were \(^{13}\)C-labeled were \( \alpha \)-ketoisovalerate (KIV), 
citrate, and glutamate (Table 7-1). The presence of these intermediates indicated 
that lactococci utilize leucine by pathways different from those of brevibacteria 
(14). To test the hypothesis of alternate pathways, we used \textit{in silico} analysis with 
the Pathcomp feature in KEGG to compute possible biological routes to 
catabolize leucine to 2-methylbutyric acid, citrate, glutamate, and KIV (24, 25).

Pathcomp pathway computation matched two intermediates that were 
related to products identified by NMR to produce 2-methylbutyric acid. While 
KIC, the expected transaminated product of leucine (Fig. 7-2A), was found, 3-
hydroxy-3-methylglutaric acid (HMGA), the product of 3-hydroxy-3-
methylglutaryl-CoA (HMG-CoA) hydrolysis, was also found (Fig. 7-2B). The 
pathway from leucine to citrate shared common intermediates until the
formation of acetyl-CoA from HMG-CoA (Fig. 7-2A, B, D, G, and I). Pyruvate derived from acetyl-CoA subsequently yielded oxaloacetate and citrate (Fig. 7-2G). The computed pathway for glutamate intersected with the formation of citrate, which was further catabolized in five steps to glutamate (Fig. 7-2H and 7-2I). Leucine was potentially catabolized to KIV via the generation of pyruvate with 3 further steps (Fig. 7-2G and 7-J). These results from the in silico analysis were used to identify the possible enzymes and their homologues in lactococci.

**Identifying gene candidates**

A few steps in the computed pathway used enzymes that are functionally characterized in lactococci. The first step in the catabolic route used A Tases, which are extensively characterized in lactic acid bacteria (37, 48). The pyruvate and citrate catabolic enzymes are part of the incomplete Krebs’ cycle variant of lactococci (44). The enzymes involved in KIV production are part of the ilv operon for BCAA biosynthesis (19). For the unidentified enzymes needed for the pathway we used protein domain analysis to infer the possible involvement of uncharacterized genes.

Functional protein domains were determined from the genome sequence using the CDSearch and CDArt features from NCBI. Subsequently, the protein homologies were used to search for other proteins and genes that contained the same or similar protein domains and structure. This search revealed considerable differences in gene annotations across multiple organisms (Appendix C) compared to the annotation in the genome at NCBI. In some cases this analysis led to genes with the same gene names but different functional descriptions,
even with significant sequence homology (E-value < 10^{-6} to 10^{-100}) or to genes in other bacteria that encoded enzymes for leucine degradation. These observations supported the assertion that the IL1403 genome contains mis-annotations and gene descriptions for genes that are associated with leucine catabolism. This explains the lack of automated pathway reconstruction using KEGG. Additionally, paralogues and other unknown genes that fall in the functional categories that may catalyze steps in the proposed pathway were uncovered with this analysis. To further understand these complex metabolic webs of pathways candidate genes for expression studies of leucine degradation were determined based on known enzymes or structurally related homologues that were predicted by domain homology (Appendix C). Identification of these new candidate genes were included in the analysis of gene expression for pathway reconstruction.

**Gene expression profiles of candidate enzymes**

*Statistical significance of gene expression.* Whole genome expression analysis of the NMR assays showed that none of the genes involved in FA metabolism significantly changed during the NMR assay at sugar exhaustion. At the onset of NC only four unknown genes related to FA production — *ycfD* (2.5-fold), *yteC* (2-fold), *yuiC* (3-fold), and *ynjF* (2.5-fold) - that had domain similarity to dehydrogenases, significantly decreased (qvalue = 0.2) in gene expression. This suggests that the cells were primed to produce FAs from amino acids and did not need to change genes to initiate FA production. If a particular gene for a specific enzyme was already expressed at a higher level then there would not be many genes that would be differentially expressed. The lack of more significantly
different genes was due to variability across replicates and the stringent level of significance testing used to minimize the false discovery of genes that were not associated with this metabolic route. We have described the data here in an exploratory fashion to verify if a particular subset of genes some are expressed while others are not irrespective of differential gene regulation and gene expression variability. Currently there is no statistic that can be attached to such an analysis and developing this statistic is beyond the scope of this study. However, use of heat maps for visualization of expression at key phenotypic points allows conclusions to be made about the relative expression level and direction of expression. To fully explore the additional genes implicated in the catabolism of AAs, additional testing was done to look for enzyme activities and gene expression trends.

Aminotransferases. The first step in catabolizing amino acids is an exchange of the amino group with an α-keto acid, preferably KGL (49). The genome of IL1403 has nine ATases (EC 2.6.1.42), of which six are uncharacterized. IL1403 also contains an amidase that catalyzes amino group removal (1).

Three ATase genes (*yeiG*, *nifZ*, or *aspB*) were expressed at over 2.5-fold in both assays at sugar starvation and nonculturability. One of these genes (*yeiG*) was repressed during the incubation by 3.5-fold at sugar exhaustion and was not up-regulated during NC but was expressed above baseline expression (Fig. 7-2A) while *aspB* was expressed at the same levels at sugar exhaustion and induced 3-fold during NC. Expression of *ybgE*, *araT* and *bcaT* did not increase in relationship to leucine catabolism, and were not expressed to above baseline
levels. As a class, ATases possess multiple substrate specificities (23, 34, 46), presumably the production of BCFAs from BCAAs can be routed through alternate ATases that were responsive to starvation conditions, such as *yeiG*, *nifZ*, or *aspB*, which may be primary candidates for this reaction. The synthesis of glutamate from KGL derived via citrate also requires ATase activity (Fig. 7-2I). Any ATase that is active can use KGL to produce glutamate. Hence, *yeiG*, *nifZ*, or *aspB* would also be favorable candidates for glutamate synthesis directly from citrate.

*Dehydrogenases and acyl transferases.* The conversion of the α-keto acid to an acyl-CoA is catabolized in two different steps by dehydrogenases (EC 1.2.1.25 and 1.3.99.3) or acyltransferases (EC 1.2.1.25). The reaction of 1.2.1.25 may be catabolized in one step or also in two steps that require a dehydrogenase (EC 1.2.4.4) and an acyl tranferase (EC 2.3.1.-). The two-step reaction is carried out through α-keto acid dehydrogenases that are structurally very similar to the pyruvate dehydrogenase (PDH) complex of *L. lactis* subsp. *lactis* IL403 (Appendix C). The genes for the subunits were induced in both assay conditions by 1.5-fold, except for *pdhC* at NC (Fig. 7-2A). This allows one to speculate that the lactococcal PDH complex may be involved in catabolism of other α-keto acids such as KGL, KIV, and KIC, assuming that the substrate specificity is this broad.

The *L. lactis* subsp. *lactis* IL1403 genome contains 38 uncharacterized dehydrogenases (1). Any biological activity that catalyzes short chain molecules would be favorable for four reactions in the pathway – α-keto acid dehydrogenase (EC 1.2.1.25) and three different acyl-CoA dehydrogenases (EC
1.3.99.3, 1.1.1.35, and 1.3.1.44) (Fig. 7-2A and 7-2C). Comparing across the assays, ten genes from this subset (yphC, ygcA, ypfF, yugB, - repressed by 2-fold but higher than baseline expression; yteC, ysjB, ypal, ypfH, ycgD, menD - induced 2-2.5-fold) were expressed at higher levels or were up regulated during the incubation time (Fig. 7-2A and 7-2C). Any of these may be involved in dehydrogenase reactions. The gene ywjF is annotated as 3-hydroxyisobutyrate dehydrogenase (1) and is a good candidate to catalyze this reaction, but the gene expression did not change and expression was low, near baseline expression.

An acyl transferase catalyzes the reaction of EC 1.2.1.25 entirely by itself or that of EC 2.3.1.-, which involves the removal of the C1 carbon (Fig. 7-2A). It is also involved in the reaction of EC 2.3.1.16 where it adds an acetyl group onto the propionyl-CoA backbone (Fig. 7-2C). Among the 12 potential acetyl transferases found in the genome, the expression of three of them (yhfG, ycjC, and ycjD) increased by 1.5- to 3-fold over the incubation time (Fig. 7-2A and 7-2C). These three enzymes are proposed to be involved in production of 2-methylbutyric acid.

Carboxylases. Multiple reactions in the proposed pathway result in fixation or removal of carbon via carboxylases. These enzymes are involved in two reactions, EC 6.4.1.4 and EC 6.4.1.1 (Fig. 7-2B and 7-2F). There are four homologous genes that may aid in carboxylation for EC 6.4.1.4 - all were expressed above baseline and induced by 1.5- to 3-fold across the incubation time. Hence, all four known carboxylases are candidates to catalyze this reaction. EC 6.4.1.1 is specifically attributed to the pyruvate carboxylase gene pycA (Fig. 7-
The carboxylation of pyruvate may be possible since \textit{pycA} was expressed at levels that were adequate to form the end products.

\textit{Hydratases}. Hydratases catalyze the addition of a water molecule to or its removal from short chain molecules. In production of 2-methylbutyric acid they are involved in hydrolysing coenzyme A from HMG-CoA (Fig. 7-2B) and in the reaction of EC 4.2.1.17 (Fig. 7-2D). While no specific hydratases are known for these reactions the IL1403 genome contains nine known hydratases that have similar action (1) and two homologues to the \textit{Bacillus} genome (Appendix C).

Increase in gene expression over 2-fold was noted for \textit{fabZl} and \textit{aroD}, while \textit{enoB} was expressed above baseline throughout the incubation even after 3-fold repression (Fig. 7-2B and 7-2D). There was a reduction in gene expression of \textit{menB} by 2-fold in both conditions and no change for \textit{ysiB}, suggesting that these genes are not involved in this catabolic step.

\textit{HMG-CoA synthase}. This enzyme catalyzes the formation of HMG-CoA from or its degradation to acetyl-CoA and acetoacetyl-CoA. The gene \textit{hmcM} was down regulated by 2-fold in both assay conditions (Fig. 7-2C). This suggests that this gene may not be involved or may be repressed by product accumulation. Alternatively an undefined homologue of this gene may be involved in this reaction.

\textit{Phosphotransacylase and acyl kinases}. The action of phosphotransacylases (EC 2.3.1.19) on acyl-CoA intermediates combined with acyl kinases (EC 2.7.2.7) yields FAs via substrate-level phosphorylation (Fig. 7-2E). This is one of the mechanisms of producing ATP from acyl-CoA yielding FAs. The lactococcal genome also contains genes for enzymes that hydrolyze the phosphate molecule
from short chain acyl phosphates without generating ATP \((\text{apl}, \text{yfjC})\) that were also monitored in this study \((1)\). The expression of the transacylase \(\text{pta}\) and acyl kinases \(\text{ackA1}\) and \(\text{ackA2}\) was higher than the lesser known phosphatases in spite of a 2.5-fold reduction in \(\text{pta}\) expression level at sugar exhaustion (Fig. 7-2E). There were no changes in expression of the phosphatases. This clearly points to the role of the known enzymes in producing BCFAs.

**Metabolism of acetyl-CoA.** Acetyl-CoA is one of the possible products of leucine catabolism in \(L. \text{lactis}\) subsp \(\text{lactis}\) IL1403 (Fig. 7-2F). It is also a central molecule in carbon metabolism that can be shunted via pyruvate and citrate to biosynthetic or catabolic pathways. There are at least three different mechanisms by which acetyl-CoA is converted to pyruvate in lactococci (Fig. 7-2F), which can be further converted to other \(\alpha\)-keto acids for amino acid biosynthesis.

Many of the genes related to pyruvate and citrate metabolism were expressed throughout or were up regulated over the incubation in both assay points (Fig. 7-2F and 7-2H). Genes that were an exception were those of isocitrate dehydrogenase (EC 1.1.1.42) (Fig. 7-2I), malate dehydrogenase (EC 1.1.1.38), and aspartate ATases (EC 2.6.1.1; Fig. 7-2G). These genes were repressed by 2-fold or did not change except \(\text{aspB}\) (increase by 2-fold), suggesting that they might be regulated by an excess of oxaloacetate and aspartate produced by asparagine synthases (EC 3.5.1.1; EC 6.3.5.4) as \(\text{asnB}\) was up regulated by 1.6- to 3-fold.

**Branched chain amino acid biosynthesis.** The \(\text{ilv}\) operon plays a definitive role in lactococci for valine biosynthesis \((19)\). While we observed production of KIV, the precursor for valine, no production of valine was found (Table 7-1). To
further verify this observation the gene expression profiles of the ilv operon and the ATases were overlaid for comparison. There was no coordinated expression of the genes of the operon in this study. While ilvC and ilvD were either up regulated by 1.5- to 2-fold or down regulated by 2.5 to 3-fold together (Fig. 7-2J), ilvB matched them in one condition while ilvN and als had exactly opposite expression profiles (1.5-fold repression or 2-fold induction respectively)(Fig. 7-2F). Even with multiple ATases being expressed (Fig. 7-2J), valine was not produced. This may be explained by the down regulation of the ATases specifically involved in BCAA biosynthesis (bcaT, yjiB, araT, and ytjE – no change or 1.5-fold repression). Deletion of the BCATase (16) resulted in the same observation as down regulation of the four ATases found in this study. These data verify the coordinated expression was needed for valine production, which did not occur.

Discussion

The catabolism of BCAAs in lactococci is of interest commercially for the potential benefits for the production of compounds associated with improved flavored, control of off flavors, and accelerated ripening (11, 13). However, the exact biochemical mechanisms for most flavor compounds are not known in lactococci. A number of pathways have been proposed for probable mechanisms to generate FAs, but none have been verified to be involved in end product formation for straight or BCFAs (12, 43). The postulated mechanisms for the involved pathways have primarily relied on single or a limited combination of
reactions, such as an ATase to derive the \( \alpha \)-keto acid, dehydrogenases to reduce it to acyl-CoA, and a decarboxylase to produce BCFAs (12).

This work observed both straight and branched chain intermediates and products (KIC, HMGA, 2-methylbutyric acid, glutamate, citrate, and KIV). A shift in the metabolic end products from short, straight chain fatty acids to BCFAs occurred at the onset of the NC state 21 d after sugar depletion. Based on NMR studies that revealed specific intermediates, accumulation of multiple end products, and gene expression profiles that provided insight into the genes involved in the biotransformation we proposed a new metabolic mechanism for the utilization of leucine that has not be observed in lactic acid bacteria (6). This pathway was previously identified in *Pseudomonas* and *Clostridium* spp. for the production of BCFAs (10, 33).

Four steps in the proposed pathway are catalyzed by dehydrogenases, including an \( \alpha \)-keto acid dehydrogenase (EC 1.2.1.25) and three different acyl-CoA dehydrogenases (EC 1.3.99.3, EC 1.1.1.35, and EC 1.3.1.44) (Fig. 7-2 and 7-4). The *L. lactis* subsp. *lactis* IL1403 genome contains 38 uncharacterized dehydrogenases and 12 acyltransferases (1). Their specific catalytic domains were not found using homology matching, but the dehydrogenase domains were similar to the general classes of short chain dehydrogenases or aldo-keto reductases with unidentified or varying substrate specificities (Appendix C). Many of the dehydrogenases contained binding domains for acceptors (NADH) and cofactors like thiamine pyrophosphate, biotin, and dinucleotides. Twelve dehydrogenases were expressed suggesting that they may be involved in leucine
catabolism (Fig. 7-2A and 7-2C). Such determination of functionality of dehydrogenases based on structural homology has been shown by Zhang et al. (50).

One type of dehydrogenase that has been partially characterized is pyruvate dehydrogenase (PDH). The PDHs of lactococci have not been characterized previously (1) except for their enzyme activity (2). Analysis of their protein homology determined that the four subunits were highly similar [E-value < $10^{-6}$] to that of other α–keto acid dehydrogenases (Appendix C). The subunits also contained domains that require or bind co-factors for α–keto acid dehydrogenase activity, such as NAD(P)H, FADH, and lipoate. (Appendix C). The PDHs of Bacillus subtilis, to which lactococci are structurally similar (Appendix C), are capable of catabolizing α–keto acids to form acyl-CoAs (30). Lactococci do not have a functional Krebs' cycle (44) and predominantly produce lactate than acetyl-CoA from pyruvate. Carbon flux studies in lactococci did not identify a flux through the partial Krebs' cycle (35). Hence, acetyl-CoA from PDH transformations may not drive the Krebs' cycle, but rather the PDH complex may act as an α–keto acid dehydrogenase (KDH) complex that catabolizes multiple α–keto acids including pyruvate. Taken together these observations indicate that PDH in lactococci may also catabolism pyruvate, an α–keto acid, to acyl-CoA in addition to other characterized activities.

HMG-CoA allows the branching of leucine catabolism to pathways other than FA production. HMGA was observed to be present in this study using the assay system with NMR. This was unusual since HMGA and HMG-CoA are
precursors of sterol biosynthesis pathways, which are not found in lactococci. HMGA may not be formed unless there is an excess of HMG-CoA that is not catabolized to acetyl-CoA. This may be attributed to possible product inhibition by either 2-methylbutyric acid or acetyl-CoA and acetoacetyl-CoA (Fig. 7-7). This enzyme is also inhibited by acetate, a possible product of acetyl-CoA catabolism, and glutamate, produced by ATases (33). As the HMG-CoA-synthase reaction is reversible the reaction may proceed in a direction driven by substrate or energy requirement. The reversibility of the reaction may also explain the observation that HMGA carried labels at alternate positions (Fig. 7-2B). Acetyl-CoA labeled at C-2 may combines with acetoacetyl-CoA labeled at C-3 to form HMG-CoA with alternate labels. Acetoacetyl-CoA may be labeled from 2 molecules of acetyl-CoA with one of them having a $^{13}$C label at its C-2 position.

The activities of acyltransferases, carboxylases, and dehydrogenases produce 2-methylbutyryl-CoA which is then further converted to 2-methylbutyric acid. The transacylases and acyl kinases catabolize 2-methylbutyryl-CoA to 2-methylbutyric acid, coupled with the production of ATP. The genes for these proteins were expressed in this study and shown to be involved in leucine degradation, despite the presence of competing genes for phosphatase action that could prevent ATP formation. 2-Methylbutyric acid was labeled at both carbon moieties (Fig. 7-2E). This suggests that acetyl-CoA gets labeled at both of its carbons because it forms the aceto-branch of 2-methylacetoacetyl-CoA (Fig. 7-2C) which is then further reduced by a series of steps to the alkyl group of 2-methylbutyric acid (Fig. 7-2D and 7-2E). As none of the subsequent steps involve addition of a carbon moiety that could possibly
introduce a label further metabolism of acetyl-CoA becomes of interest as it determines the BCFA produced.

The inter-conversion of acetyl-CoA to pyruvate does not account for $^{13}$C labeling in adjacent positions of 2-methylbutyric acid (Fig. 7-2F) (44). Acetyl-CoA needs to be labeled at both C-1 and C-2 positions to produce 2-methylbutyric acid with adjacent $^{13}$C labels. Tracking the labeling patterns that lead to the keto acids and fatty acids by known biochemical pathways showed that acetyl-CoA existed in three distinct labeled forms. Apart from labels at both carbons, acetyl-CoA labeled at C-1 only generated KIV while label only at C-2 eventually generated citrate, glutamate, and HMGA. The possible inter-conversions among acetyl-CoA, pyruvate, oxaloacetate or acetolactate that also result from glycolytic products and ketogenic amino acids (35) do not account for the $^{13}$C label redistribution to any position among these intermediates. An alternate possibility is one carbon metabolism via tetrahydrofolate and S-adenosylmethionine, which could redistribute carbon into the observed products. For example $^{13}$C-glutamate synthesized from Krebs' cycle intermediates is labeled at all positions except the C-1 carbon (29). Such a distribution of carbon labels across catabolic and anabolic pathways has been previously demonstrated in lactococci (35). The ability to synthesize glutamate via the Krebs' cycle is discordant from observations of Wang et al. (44). This suggests that glutamate synthesis may be induced during starvation based on carbon availability. The accurate determination of labeling patterns is currently beyond the scope of available biochemical and bioinformatic tools.
The genes of the BCAA biosynthetic ilv operon were not coordinated in expression, which limited the cell’s ability to produce known end products, such as valine. While their penultimate product KIV was identified the subsequent product valine was not observed. Neither were isovaleric and isobutyric acids, other possible catabolic products of KIV. Such lack of coordinated expression during carbohydrate starvation was also observed in arginine metabolism (3). Chou (3) observed specific intermediates, but not the known end products as well. The role of KIV seems to be ambiguous here, as it remains an α-keto acid, suggesting that the enzymes required for its metabolism were not expressed. KIV may only serve to maintain the α-keto acid pool in lactococci.

The suggested pathway involves steps of carbon fixation (EC 6.4.1.4, E.C. 2.3.1.6 and enzymes of pyruvate metabolism). This pathway suggests how lactococci may survive carbon (i.e. sugar) limitation and requirement for specific molecules during starvation and the NC state by fixing carbon from the medium. Their capability to survive for over 3 yrs of incubation and maintain metabolic capability during this cellular state (15) may be associated with their ability to obtain carbon moieties for necessary biosynthetic molecules via carbon fixation pathways (Fig. 7-2B, 7-2C, 7-2G, and 7-2J). Wang et al. (44) observed carbon fixation but found that glutamate synthesis was limiting due to lack of isocitrate dehydrogenase activity. Based on our results, it appears that carbon fixation may play a predominant role during starvation than exponential growth.

An interesting biochemical feature of the pathway from leucine transamination to 2-methylbutyric acid is the molecular efficiency in production
and use of the intermediates and co-factors while generating energy and redox co-factors. The products from earlier reactions are subsequently consumed in the later steps or vice versa, as with many biochemical pathways. For example, NADH generated earlier by dehydrogenases is used by subsequent dehydrogenase reactions (Fig. 7-2A and 7-2C). CO₂ released by the dehydrogenase re-enters the pathway by the carboxylase reaction (Fig. 7-2A, 7-2B, 7-2F, 7-2G, and 7-2I). The splitting of HMG-CoA into three molecules of acetyl-CoA generates three ATP from the substrate level phosphorylation reaction of acyl kinase (Fig. 7-2E) of which one ATP is used during carbon fixation (Fig. 7-2B). Coenzyme A used in earlier steps is also regenerated later. The generation of acetyl-CoA may facilitate rerouting of carbon in lactococci in absence of specific pathways to catabolize FAs. This suggests that during substrate starvation and the NC state the cells may exist in a homeostatic condition in which they reroute and reuse their cofactors and continue to be able to fix carbon for survival during cellular stress due to substrate limitation while generating energy and redox-related co-factors.

Conclusions

IL1403 consumed leucine to produce KIC, KIV, HMGA, glutamate, citrate, and 2-methylbutyric acid during carbohydrate starvation. Production of these compounds was not observed until the cell shifted to the NC state. Gene expression profiles coupled with determination of the metabolic intermediates resulted in the production of a bioconversion pathway that incorporated all chemical intermediates. Gene lists of possible catalysts were narrowed to a
handful of possible candidate enzymes for all reactions, except those involving dehydrogenases. This work shows that lactococci do not lyse upon nutrient limitation, but rather enter a NC state, and are metabolically active during these cellular phases and produce BCFAs and FAs via a complex pathway after induction due to carbohydrate exhaustion.

References


Table 7-1. Location of $^{13}$C labels found in products of samples analyzed by NMR spectroscopy and their putative candidate genes and enzymes

<table>
<thead>
<tr>
<th>Product</th>
<th>Label Location</th>
<th>Catalytic Enzyme</th>
<th>Number of possible genes in the genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Ketoisocaproate</td>
<td>C-2</td>
<td>Aminotransferase</td>
<td>9</td>
</tr>
<tr>
<td>2-Methyl Butyric acid$^1$</td>
<td>C-3, C-4</td>
<td>Acyl kinase</td>
<td>2</td>
</tr>
<tr>
<td>3-Hydroxy-3-methylglutaric acid</td>
<td>C-3, C-5</td>
<td>Hydrolase</td>
<td>9</td>
</tr>
<tr>
<td>$\alpha$-Ketoisovalerate</td>
<td>C-2, C-3</td>
<td>Dehydratase</td>
<td>1</td>
</tr>
<tr>
<td>Citrate</td>
<td>C-2 or C-4</td>
<td>Citrate synthase</td>
<td>4</td>
</tr>
<tr>
<td>Glutamate$^1$</td>
<td>C-2</td>
<td>Aminotransferase</td>
<td>9</td>
</tr>
</tbody>
</table>

$^1$Product was found in inside and outside the cell; other products were found only inside the cell
Figure 7-1. Plate counts and live dead counts of *L. lactis* subsp. *lactis* IL1403 during incubation in glucose CDM. Plate counts (□, y-axis), fluorescent live counts (◇, yy-axis), and fluorescent dead counts (○, yy-axis)
Figures 7-2A to 7-2J. Putative pathway inferred from Pathcomp computation for catabolism of leucine to 2-methyl butyric acid, citrate, glutamate and KIV. The black circles on the compound structures reflect the actual peaks that were identified by NMR. The gray circles are overlaid to track possible paths of the $^{13}$C label in intermediates that were not identified by NMR. The gene expression data is overlaid as expression maps drawn by HCE. Color changes within the expression maps are: green to black, 2.5-fold increase in gene expression, black to red, ≥ 2.5-fold increase in gene expression and vice versa 1, 0 h of IL1403 at sugar exhaustion, 2, 3 h after incubation of IL1403 at sugar starvation in 2-$^{13}$C-leucine, 3, 0 h of IL1403 at nonculturability, 4, 3 h after incubation of IL1403 at nonculturability in 2-$^{13}$C-leucine. The boxed region of the glyph on the right hand side top corner indicates the part of the whole pathway that is depicted in the figure. Catabolism of leucine to 3-methylcrotonyl-CoA (A), catabolism of 3-methylcrotonyl-CoA to 3-hydroxy-3-methylglutarate (B), catabolism of 3-hydroxy-3-methylglutaryl-CoA to 2-methylaceto-acetyl-CoA and acetaldehyde (C), catabolism of 2-methylaceto-acetyl-CoA to 2-methylbutyryl-CoA (D), catabolism of 2-methylbutyryl-CoA to 2-methylbutyric acid (E), catabolism of acetyl-CoA to 2-aceto-lactate and oxaloacetate (F), catabolism of oxaloacetate to pyruvate and anabolism of L-asparagine from oxaloacetate (G), anabolism of oxaloacetate to isocitrate (H), anabolism of L-glutamate from isocitrate (I), and anabolism of 2-aetolactate to 2-ketoisovalerate (J), respectively.
\[
\begin{align*}
\text{L-leucine} & \rightarrow \text{\(\alpha\)-ketoglutarate} &\text{Branched chain aminotransferase} & 2.6.1.42 \\
\text{L-glutamate} & \rightarrow & & \\
\text{2-keto-4-methyl-pentanoate} & & & \\
\text{coenzyme A} & & & \text{\(\alpha\)-Keto acid Dehydrogenase} & 1.2.1.25 \\
\text{NAD} & & & \\
\text{CO}_2 & & & \\
\text{NADH} & & & \\
\text{CoA} & & & \text{isovaleryl-CoA} \\
\text{FAD} & & & \text{Acyl-CoA Dehydrogenase} & 1.3.99.3 \\
\text{FADH}_2 & & & \\
\text{CoA} & & & \text{3-methylcrotonyl-CoA} \\
\end{align*}
\]
3-methylcrotonyl-CoA

Carboxylase 6.4.1.4

HCO₃⁻ ATP

phosphate ADP

trans-3-methylglutaconyl-CoA

H₂O spontaneous

-3-hydroxy-3-methyl-glutaryl-CoA

Acceptor/H₂O 3-Hydroxy-3-methyl GlutarylCoA Hydrolase/CoA Transferase CoenzymeA/Acceptor-CoA

3-hydroxy-3-methylglutarate
3-hydroxy-3-methyl-glutaryl-CoA Synthase

Acetoacetyl-CoA

CoA

acetyl-CoA

propionyl-CoA

Acyl transferase/Thiolase

Methionine, Threonine metabolism

2-methylaceto-acetyl-CoA

2.3.1.16

CoA

NADH NAD

coenzyme A

onlineen A

acetaldehyde
2-methylaceto-acetyl-CoA → 3-Hydroxy-Acyl-CoA Dehydrogenase (1.1.1.35)

H⁺
NADH

2-methyl-3-hydroxybutyryl-CoA → Enoyl-CoA Hydratase (4.2.1.17)

H₂O

2-methylbutyryl-CoA

Acyl-CoA Dehydrogenase (1.3.1.44)

2-methylaceto-acetyl-CoA
2-methylbutyryl-CoA

Phosphat

2-methylbutanoyl-phosphate

ADP

Acyl Kinase

2-methyl butyric acid
Phosphotransacetylase: 2.3.1.8; 2.3.1.19

Acetyl Phosphate

Pyruvate Oxidase: 1.2.3.3

CO₂, H₂O₂

PO₄³⁻, O₂, H₂O

Pyruvate Carboxylase: 6.4.1.1

ATP, HCO₃⁻

ADP, phosphate

Pyruvate Formate Lyase: 2.3.1.54

CO₂

NADH

Formate

Pyruvate Formate Dehydrogenase: 1.2.4.1

NAD coenzyme A

CO₂

CoA acetyl-CoA

CoA acetyl-CoA

Pyruvate

Acetolactate Synthase: 2.2.1.6

2-aceto-lactate

Oxaloacetate

ATP

HCO₃⁻

ADP, phosphate

7-2F
Citrate Synthase/Lyase
4.1.3.6
4.1.3.7

Aconitate Hydratase
4.2.1.3

isocitrate
isocitrate

\[ \text{NADP} \rightarrow \text{Isocitrate Dehydrogenase 1.1.1.42} \]

\[ \text{CO}_2 \rightarrow \text{NADPH} \]

\[ \rightarrow \alpha\text{-ketoglutarate} \]

an amino acid

\[ \text{Aminotransferase 2.6.1.42} \]

a 2-oxo acid

\[ \rightarrow \text{L-glutamate} \]
2-aceto-lactate →

\[ \text{H}^+ \quad \text{NADPH} \]

Ketol-Acid Reductoisomerase
1.1.1.86

2,3-dihydroxy-isovalerate →

\[ \text{H}_2\text{O} \]

Dihydroxy acid Dehydratase
4.2.1.9

2-keto-isovalerate
CHAPTER 8
GENE EXPRESSION DURING THE CATABOLISM OF BRANCHED CHAIN AMINO ACIDS AND α-KETO ACIDS TO BRANCHED CHAIN FATTY ACIDS IN LACTOCOCCUS LACTIS

Abstract

Lactococci remain intact and metabolically active during carbohydrate starvation for at least 2 yrs. After carbohydrate exhaustion the population enters a nonculturable state that is defined by the lack of growth on solid media. The cells utilize amino acids to produce end products associated with energy, redox, and flavor compounds during this cellular state. This study determined the ability of Lactococcus lactis subsp. lactis and Lactococcus lactis subsp. cremoris to produce branched chain α-keto acids and fatty acids during starvation using biochemical evidence and gene expression profiles. Straight chain fatty acids were produced throughout the 2-yr period, but the branched chain fatty acids isovaleric and isobutyric acid, were produced only after the carbohydrate source was depleted and the cells attained the nonculturable state. Initial growth on glucose led to similar branched chain end products compared to growth on lactose, but the metabolic routes were different between the sugar sources. Sugar exhaustion and the achievement of the nonculturable state differed between the sugar sources and were affected by acid stress. Gene expression profiles identified genes related to enzymes that catabolized branched chain amino acids.
Differential gene expression between acid stress, sugar exhaustion, nonculturability and fatty acid production was observed for 40 genes. These data provide conclusive evidence that lactococci genetically regulate amino acid catabolism during long-term starvation in a nonculturable state that lead to the production of secondary metabolites of branched chain fatty acids.

Introduction

Lactic acid bacteria catabolize amino acids during limited availability of carbohydrate for energy and cellular intermediates (6, 18, 42). This ability allows lactococci to remain intact and survive extended periods of nutrient starvation (42) (Chapter 6). However, the survival mechanisms of lactococci after sugar depletion are poorly characterized. It is widely held that lactococci die and lyse with the lack of sugar during cheese ripening (8, 31). While controversially, increasing evidence exists to demonstrate that lactococci remain physiologically capable of metabolism in minimal media without a sugar source (5, 42). This is demonstrated by the amount of ATP and the detection of intact cells (18). Lactococci become nonculturable (NC) on optimal solid media with the lack of sugars (18, 42). Their continued survival encourages a search for alternate mechanisms involved in energy, metabolic precursor production, and biochemical routes that lead to compounds that will protect the cell from energy starvation and oxidation.

Catabolism of amino acids such as arginine and branched chain amino acids (BCAAs) is implicated during long-term starvation survival (6, 18, 42). While arginine extends survival after carbohydrate depletion (6, 42), BCAAs also
provide energy and reducing potential (18). Branched chain α-keto acids and fatty acids (BCFAs) are the products of BCAAs metabolism. Known pathways in bacteria for this mechanism indicate that this metabolic ability yields ATP via substrate-level phosphorylation, biosynthetic precursors such as acetyl-CoA, and reducing co-factors such as NAD+ (16).

Lactococci catabolize amino acids to fatty acids in the absence of carbohydrate and at low pH (16). This observation prompts an interest in characterizing the effect of sugar depletion mechanisms and the importance of these events for practical applications. Studying this trait in pure culture allows monitoring of a limited set of substrates and parameters for elucidating the induction and prevalence of amino acid catabolites from lactococci during starvation. Eventually, application of this information to complex systems will be of use in controlling and improving fermented products.

The genes required for subsequent catabolism of the α-keto acids produced from BCAAs are yet to be characterized in lactococci. Genome-wide expression profiling yields putative targets for optimization and represents a method to verify pathways directly related to known mechanisms as well as associated pathways (46). It was established in a previous study that the strains incubated in lactose survived for over 2 yrs without losing cellular integrity and continued maintenance of ATP levels (18). Lactococci also catabolized amino acids during assays done with long term starved cells (18). Based on the ability of lactococci to catabolize amino acids to fatty acids (FAs) (16) and their ability to survive extended periods of carbohydrate starvation (18), we hypothesized that
nonculturable lactococci produce BCFAs during carbohydrate starvation in culture via genes that are induced at the onset of starvation. This study characterized the cell’s ability to produce BCAA-derived catabolites in concordance with their gene expression profiles.

Materials and methods

Strains and media

*Lactococcus lactis* subsp. *lactis* ML3 and IL1403 and *Lactococcus lactis* subsp. *cremoris* SK11 were obtained from the Utah State University culture collection. The strains were propagated in Elliker’s or M17G broth (Difco Laboratories, Detroit, MI). Stock cultures were prepared by growing the organisms twice in 10 ml of respective broth at 30°C for 24 h. Cultures were frozen at −70°C in 10% non-fat dry milk containing 30% glycerol. Before each experiment, frozen stock cultures were thawed and sub-cultured twice at 30°C for 24 h in 10 ml of respective broth.

The strains were grown overnight at 30°C, harvested by centrifugation (6,000 × g for 15 min at 4°C), washed twice with sterile saline, and resuspended and inoculated (1%) into sterile chemically-defined basal medium (CDM) (19). The basal CDM contained 0.2% lactose or glucose and branched chain amino acids were added at 200 mg/l, 10 times the original CDM content. The CDM was adjusted to either pH 7.2 buffered with 0.19 M of sterile 3-[N-Morpholino]-propanesulfonic acid (MOPS), or to pH 5.2 buffered with 0.19 M 2-[N-Morpholino]-ethanesulfonic acid, and filter sterilized prior to use. Amino acids
and buffer salts were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). While *L. lactis* subsp. *lactis* ML3 and *L. lactis* subsp. *cremoris* SK11 were tested for starvation on lactose-containing CDM, *L. lactis* subsp. *lactis* IL1403 was tested on CDM containing lactose and glucose separately. This served to establish the role of sugar catabolism in inducing amino acid catabolism.

Plate counts, live-dead counts, ATP content, lactose utilization, and ATase activities were all monitored in previous studies (Chapter 6 and 7). This study was extended to measure gene expression and FA profiles.

**Gas chromatography of fatty acids**

Fatty acids were estimated from supernatants of CDM at different time points over the 2-3 yr incubation. The procedure used to analyze fatty acids described previously (16) was slightly modified to include a C-18 column (Alltech Inc. CA) in addition to the amino-propyl column. This was done in order to negate the effect of Tween 80 present in CDM in preventing extraction of FAs. There was no significant reduction in efficiency of FA extraction with the additional use of the C-18 column (Appendix D).

**α-Keto acid analysis**

α-Keto acids were estimated from cell-free extracts prepared as described previously. The cell-free extracts were desalted using a C-18 column (Alltech Associates, Fullerton, CA), concentrated 100-fold using an AES1000 speed vac system (Savant Instruments Inc., Farmingdale, KY), and derivatized with 1.16 mM 3-(methyl)-2-benzo-thiazolinone hydrazone in sodium acetate buffer. The
α-keto acids were separated by micellar electrokinetic capillary chromatography in a P/ACE 2100 capillary electrophoresis unit (Beckman-Coulter Inc., Palo Alto CA) with UV detection at 340 nm as described previously (9). Corrected peak areas obtained from the electropherograms were used to calculate concentrations of individual α-keto acids from standard curves that were linear over the detection range ($r^2 \geq 0.98$; data not shown).

**Genomic DNA hybridization**

We used a macroarray that was designed for the genome of IL1403 to compare the metabolic capability of the three strains using a single gene expression platform. The array was printed on nylon membranes with a single probe per ORF for 1920 genes with annotated known and putative functional assignments. Probe design for the array has been described previously (46). The genomic DNA of *L. lactis* subsp. *lactis* ML3 and *L. lactis* subsp. *cremoris* SK11 were isolated from overnight grown cultures using the WizardGenomic DNA extraction kit (Promega Inc., Madison, WI) as per the manufacturer’s protocol. Prior to hybridization genomic DNA was biotinylated using the Psoralen-Biotin DNA labeling kit (Ambion Inc., Austin, TX). Probe design, spotting, DNA hybridization, chemiluminiscent detection, and image acquisition were performed as per the macroarray protocol described previously (46).

**Gene expression profiles**

Gene expression of *L. lactis* subsp. *lactis* IL1403 was monitored at the starting time point, sugar exhaustion, attainment of the nonculturable state, and
production of BCFAs. RNA was extracted from cells and reverse-transcribed to cDNA as described by Yi et al. (46). Prior to biotinylation, the cDNA was sheared with DNaseI according to the in-house protocol of Nimblegen Systems (Madison WI). The cDNA was subsequently biotinylated as described by Yi et al. (46). Hybridization of target cDNA, fluorescent labeling, and detection by scanning was done at Nimblegen Systems (Madison, WI) on Nimblescreen chips optimized for the genome of *L. lactis subsp. lactis* IL1403. The NimbleScreen chip contained 5-6 probes for each open reading frame to include a total of over 13,600 features for the whole genome in 1 well of a 12 well-chip, allowing us to analyze 12 samples on one chip.

**Statistical analysis and data visualization**

The data from fatty acid and keto acid production was analyzed using a repeated measures model with time as a repeated measure (Eq. 2 and Appendix B) to determine the effect of time, pH, their combined effect on strains, and the differences between any two strains at different pH’s. SAS Statistical Software version SAS 9.0 (SAS Statistical Institute, Cary NC) was used for the analysis. The adjustment for multiple comparisons was done by assigning significant differences at $\alpha = 0.05/n$ where $n$ is the number of timepoints tested.

$$Y_{ijk} = \text{Overall mean} + \text{pH}_i + \text{Time}_j + (\text{pH}\times\text{Time})_{ij} + \text{error}_{ijk} \quad (\text{Eq. 2})$$

The cDNA hybridization data was obtained as pixel intensities and normalized by the R Statistical package using the robust multichip average (RMA) method (24) at Nimblegen Systems (Madison, WI). Expression maps were
drawn with RMA-normalized data after averaging across replicates by Hierarchical Clustering Explorer software version 3.0 (38, 39) from log$_2$ values of the normalized data depicted over events. The cutoff value for minimum levels of gene expression was based on the whole genome expression profiles generated by HCE. The minimum cutoff value was set at the software-calculated mode of gene expression. The fold change was set to a 2.5-fold increase each in gene expression for visualization. The data was then further subdivided into enzyme classes that were identified by protein homology (Chapter 7 and Appendix E). Fold changes were calculated for analysis. Each enzyme class was clustered separately and the determined cutoff values were applied to generate colored expression maps for each individual class for ease of visualization.

The normalized data was statistically analyzed for significant changes in gene expression in the R statistical package using a linear mixed effects model with a compound symmetry error structure. The fixed effects tested were the effect of time, pH and their interaction or time, sugar type and their interaction. Adjustment for significance of multiple comparisons was done using the q-value package as described by Storey and Tibshirani (41). Significance of treatments was based on plots of the p-values against q-values. A uniform distribution signifies no effect whereas a non-uniform distribution indicates significant effects due to that treatment (41). For significant effects the p-value cut-off was 0.1 and the false discovery rate was chosen at 25%. This means that among all genes identified to be significant 25% are likely to be false positives.
Results

Fatty acid profiles

Three lactococcal strains, ML3, SK11 and IL1403, were tested for their ability to produce BCFAs from amino acids during starvation. Each culture was observed for acid stress, sugar exhaustion, attainment of nonculturability (NC), and BCFA production during the incubation period. The effect of acid stress was tested in media that were buffered to pH 7.2 and 5.2. IL1403, which is Lac\(^{-}\), was used as a negative control culture for the Lac\(^{+}\) strains *L. lactis* subsp. *lactis* ML3 and *L. lactis* subsp. *cremoris* SK11 to determine the effect of sugar source on BCFA production. The effect of metabolizable sugar without acid stress on NC and BCFA production was compared by incubating IL1403 in CDM with lactose and glucose separately at pH 7.2.

ML3 and SK11 produced straight chain fatty acids (SCFAs) during the logarithmic through sugar exhaustion, but subsequently utilized these compounds. BCFAs were observed only after ML3 and SK11 attained the NC state, but IL1403 produced BCFAs before attaining NC (Fig. 8-1). ML3 produced and accumulated isovaleric acid along with other FAs at similar levels and initiated BCFA production when it was NC (51 d) in both pH conditions (Fig. 8-1 A and B and 8-2 A and B). SK11 produced isobutyric acid at 3 months of incubation and after it had utilized lactose and attained NC at pH 7.2. SK11 did not utilize lactose at pH 5.2, but did produce acetic and propionic acids before and after attaining NC (Fig. 8-2 C and D).
When IL1403 was incubated in CDM containing lactose it produced SCFAs and BCFAs (Fig. 8-1 and 8-2). BCFA production was initiated at 15 d in both pH conditions and preceded the attainment of NC in CDM containing lactose (Fig. 8-1E and F and 8-2E and F). Subsequently, IL1403 attained NC at 3 months of incubation at pH 7.2 and 8 months of incubation at pH 5.2 (Chapter 6). During NC IL1403 utilized the BCFAs and SCFAs in the medium. In CDM containing glucose IL1403 depleted the sugar and subsequently attained NC (Chapter 7). While this culture produced SCFAs during logarithmic growth, production of BCFAs was initiated with the attainment of NC (Fig. 8-2G).

All three strains produced and utilized SCFAs irrespective of growth phase but produced BCFAs only when they were sugar-deprived irrespective of acid stress or NC. With the observation of BCFA production, we further proceeded to investigate intracellular pools of the metabolic precursors, specifically α–keto acids, to gain insight into the metabolic route of production.

α–Keto acid profiles

SK11 and IL1403 possessed similar overall α–keto acids pools during starvation, which ranged from 20-100 nM (Fig. 8-3 A-D). The α–keto acid pool was dynamic during the starvation time, but these intermediates were not limiting compounds in the production of BCFAs. The cells also contained pyruvate during BCFA production and NC (Fig. 8-3). These observations demonstrated that the cells contained adequate metabolic intermediates to produce energy and co-factors for the production of short chain carbon compounds during starvation and NC.
While BCFA production was dependent on sugar deprivation, intracellular α-keto acids were present in cells during all growth stages, including BCFA production and NC. However, it was not clear why BCFA production varied by 10 fold in different strains. We hence used comparative genome hybridization and gene expression profiling to identify specific genes to the attainment of NC and BCFA production and gain insight into the genomic differences between the strains.

Genomic DNA hybridization

The genomic DNA of SK11 and ML3 were hybridized to a macroarray that was designed for the genome of IL1403 with a single 22-mer probe for each ORF as described by Xie et al. (46), except the probes were increased from 375 genes to 1920 genes. If the ML3 and SK11 genes were homologous to the IL1403 genome the DNA from the other two cultures would hybridize to the spotted probes. This provided a measure of the homology across the entire genome for each strain compared to IL1403. It also indicated the probes that would hybridize from ML3 and SK11 in gene expression studies. Genomic DNA from SK11 hybridized to 45% of probes for the 106 genes of interest for BCAA catabolism and ML3 hybridized to 36% of the probes (Fig. 8-4). These data indicate substantial differences in homology between the genes capable of participating in BCAA metabolism between the strains, irrespective of any regulatory differences that may be found between the strains. Further, this low level of homology precluded the use of an array designed for IL1403 to determine the
gene expression profile for ML3 and SK11. Therefore, gene expression profiles were measured in IL1403 only.

**Gene expression profiles**

Expression profiles were used to identify the genes associated with the observed cellular responses and BCFA production. The specific aim was to unravel the influence of sustained acid stress from that of sugar type, the onset of starvation, attainment of NC, and production of BCFA. Gene expression was determined in three conditions, IL1403 grown in lactose buffer to pH 7.2, grown in lactose buffered to pH 5.2 and grown in glucose buffered to pH 7.2. This approach allowed study of acid stress on attaining the NC state and producing BCFAs by comparing the expression profiles at the time of phenotypic and metabolic shifts when BCFA production occurred. The current annotations of genes whose expression profiles were monitored are listed in Appendix D (Table D-1).

The genome of IL1403 contains multiple genes that are capable of catalyzing each of the 12 steps of BCFA production. As such genes capable of producing a protein with metabolic potential for a specific reaction were grouped for comparison during each cellular event that were associated with BCFA production. Gene expression was substantially (i.e. greater that a 2.5-fold change and statistically significant) different between growth on glucose and lactose for the genes associated with BFCA production (Fig. 8-5). The profiles also varied between the pH conditions when grown with lactose as the sugar source. In some cases, a specific gene was expressed in all the cellular conditions,
such as the ATase yeiG. In other cases, a gene was induced at a specific cellular condition and further induced at the onset of BCFA production, such as ybgE. Lastly, the profile allowed determination of the genes that were repressed at specific conditions, and therefore not involved with BCFA production, such as bcaT. These observations point out the importance of the regulatory mechanisms and gene redundancy in BCFA production for expression in different environmental conditions. To identify the exact gene(s) responsible for every step additional studies are needed to characterize the regulatory mechanisms and the enzymology of each implicated gene product. The detailed profiling of gene regulation and redundancy is beyond the scope of this work, but the gene expression profiles adequately point to this area as a worthy focus to unravel the complexity of metabolism in future studies.

Statistical significance of gene expression. Gene expression analysis among the treatments tested (pH, time, and pH-time interaction, or sugar type, time, and sugar-time interaction) showed that the main treatments – pH and sugar type – affected the gene expression. Gene expression profiles did not significantly change over time. The expression of 15 genes that related to FA production was significantly different (p-value = 0.1, q-value = 0.25) across pH and sugar type. The possibility of multiple genes catabolizing a reaction has forced comparison of the gene expression levels with the biochemical data from the metabolic analysis. The development of such a statistic is beyond the scope of this study.

Aminotransferases. ATases (EC 2.6.1.42) are the primary enzymes for activating amino acid catabolism in lactococci and the focus of recent studies to
improve cheese flavor via end products and α-keto acid intermediate increases 
(4, 12, 48). ATase action contributes to the pool of intracellular α–keto acids that 
can subsequently be catabolized to BCFAs. The gene yeiG was significantly 
induced by 1.5-2.5-fold, while nifZ was constantly expressed with no significant 
change at BCFA production and NC at IL1403 for growth on lactose. Expression 
of two ATase genes (yeiG and nifZ) corresponded with BCFA production in 
glucose, as with lactose at pH 7.2 (yeiG, nifZ, and bcaT) and two in lactose at pH 
5.2 (yeiG and yjiB). The expression of yeiG was significantly different across pH 
and sugar type. On attainment of NC in lactose at pH 7.2, IL1403 induced 
expression of nifS and repressed the expression of aspB, while aspB was induced 
at pH 5.2. This demonstrated that additional ATases to those previously 
described (36, 47) were involved in BCFA production during NC and in acid 
stress compared to those involved in log phase growth and SCFA production. 
Specifically, yeiG, yjiB, and aspB were implicated in BCFA production, rather than 
bcaT, which did not significantly change in expression (47).

Dehydrogenases and acyl transferases. The reaction that succeeds ATases 
converts the α–keto acid to an acyl-CoA (Fig. 8-3). This reaction is facilitated by 
any number of the 42 dehydrogenases (EC 1.2.1.25 and 1.3.99.3) and 11 
acyltransferases (EC 1.2.1.25) in the IL1403 genome. Twelve dehydrogenase 
genes that corresponded to BCFA production were expressed by IL1403 in 
growth on glucose (yphC, ypal, ybiE, yteC, ysjB, yrfB, ypfF, ycdG, ypfH, yphA, ybjA, 
and ygcA) (Fig. 8-3) of which three were significantly induced (yteC, ysjB, and 
yrfB) in glucose at pH 7.2 and lactose at pH 5.2 versus lactose at pH 7.2. The other
genes did not change significantly but were expressed constitutively. Among these genes, except for *ypjF*, *ybjA*, and *yrjB*, all others were previously implicated in leucine catabolism (Chapter 7). In growth on lactose at pH 7.2, *yrfB* was significantly induced by at BCFA production in comparison to pH 5.2; however, only *yteC* expression was common with growth on glucose. Growth on lactose at pH 5.2 resulted in the expression of *yphC*, *yteC*, *yrjB*, and *ypjF*. Considering that *yphC* and *yteC* were common in all three conditions for BCFA production, these genes may be involved in BCFA production during sugar deprivation in IL1403 while the other dehydrogenases may be associated specifically with sugar starvation, acid stress, and NC.

We previously hypothesized that the pyruvate dehydrogenase (PDH) complex was involved in catabolizing α-keto acids to acyl-CoAs based on metabolic intermediates and gene expression profiles (Chapter 7). In this study the genes of the PDH complex lacked coordinated expression in glucose during BCFA production (Fig. 8-3). While the expression of genes of the subunits was induced (*pdhCBA*) in lactose at pH 7.2, the genes were not transcribed together as an operon (*pdhDCBA*) in either glucose or lactose at pH 5.2. Neither were the expression changes significantly different. This suggests that the other dehydrogenases and acyltransferases that were expressed were involved in BCFA production during starvation (Fig. 8-3).

Three acyltransferases (*yhjG*, *ycjC*, and *ycjD*) were transcribed in glucose but repressed in lactose at pH 7.2. The gene *ycjD* was significantly induced in glucose at pH 7.2 and lactose at pH 5.2 (Fig. 8-3). The expression of
dehydrogenases and acyltransferases verified that a keto acid is catabolized when BCFAs were produced. The acyltransferases are also involved in catabolism of acetyl-CoA to produce a keto-acyl-CoA that is then further reduced to finally obtain a FA. The expression of an acyltransferase also verified the possibility of their catabolizing acetyl-CoA to form FAs.

Carboxylases. The carboxylases (EC 6.4.1.4 and EC 6.4.1.1) are predominantly involved in carbon fixation (Fig. 8-3). The regulation of these genes was different in each sugar and pH condition (Fig 8-3). Growth in glucose resulted in expression of all the carboxylase genes at the onset of BCFA production. While ipd was significantly expressed at higher levels in lactose at pH 7.2, it was significantly repressed at pH 5.2. Conversely, accA was induced at pH 5.2 compared to pH 7.2 while pycA and pdc expression were no significantly different. This suggests that accA and pycA are used for carbon fixation during BCFA production, while ipd is used for this function during growth on sugar. This observation clearly defines the metabolic shift during specific cellular events to a different set of redundant genes in response to environmental conditions.

Dehydratases. For the production of BCFAs, hydrolysing coenzyme A from HMG-CoA and enoyl-CoA by dehydration (Fig. 8-5) is likely catalyzed by a number of the 11 structurally homologous dehydratases (EC 4.2.1.17) in the genome of L. lactis subsp. lactis IL1403 (Appendix D). Seven of the 11 dehydratases were expressed at BCFA production in glucose (Fig. 8-3), while five genes (pheA, aroD, leuD, hisB and menB) were induced during BCFA production in lactose at pH 7.2. Interestingly, at pH 5.2 with lactose only three genes were expressed, leuD, enoB and hisB. The expression changes for these genes except
menB were not significantly different confirming their high level of expression. The pH of 5.2 repressed menB in comparison to pH 7.2. These data also clearly demonstrate the shift in expression for sugar source, pH, onset of NC, and BCFA production. Acid stress shifts the possible genes involved in BCFA production to hisB and enoB, with a lesser role for menB.

*Hydroxymethylglutaryl-CoA synthase.* The metabolism of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) has not been previously identified in lactococci. However, the in silico analysis for the complete pathway for BCFA production (Chapter 7) found this reaction a possible intermediary step. The gene hmcM for HMG-CoA synthase (EC 2.3.3.10) was expressed in lactose at pH 7.2 at a constant level with no significant expression change. However, in lactose at pH 5.2 this gene was significantly repressed (Fig. 8-3). The expression of hmcM also correlated with higher levels of BCFA production in lactose at pH 7.2 (Fig. 8-1E, 4, and 5) and lower levels of expression at pH 5.2 with lower amounts of BCFAs produced (Fig. 8-1E, 8-4, and 8-5). The expression of this gene appears to be regulated by sugar source and acid condition. This enzyme may be the rate-limiting step for BCFA production as noted by the quantitative differences in the BCFAs produced.

*Phosphotransacylase and acyl kinases.* The last two steps of BCFA production may rely on phosphotransacylase (EC 2.3.1.19) and acyl-kinase (EC 2.7.2.7) reactions (Fig. 8-5). These enzymes were expressed at BCFA production only when lactose was the sugar source. With lactose at both pH conditions only apl was significantly induced in lactose at pH 7.2 in comparison to pH 5.2 (Fig. 8-4).
The expression changes of *pta* and *yfjC* were not affected by pH or sugar type suggesting that *apl* may be the phosphotransacylase involved in FA production.

The acylkinases were not significantly induced over time or affected by pH or sugar source in any of the conditions tested (Fig. 8-4). This suggests that the enzymes may have a low level of expression in comparison to other genes.

**Discussion**

The metabolism of amino acids by lactococci is of interest due to its importance in flavor production and the intermediates of energy and reducing compounds needed for survival during carbohydrate limitation (2, 13, 19, 20, 47). We monitored the ability of lactococci to survive long-term starvation with specific attention to the production of BCFAs. The use of different sugar sources established the role of sugar to vary the expression pattern of the 80 possible genes that may be involved in BCFA production. Acid stress differentiates lactococci by their adaptive response (26, 33, 37) and was monitored at two different pH conditions.

Acid stress tolerance, the NC state, and the sugar source affected BCFA production and utilization and gene expression. ML3 was the most adapted for survival in acid stress and sugar limitation as it attained the NC state within a week (Fig. 8-1). It produced and maintained a pool of FAs over starvation and NC at approximately 5 to 15 mM in all conditions (Fig. 8-1 and 8-2). *L. lactis* subsp. *cremoris* SK11 metabolized lactose only at pH 7.2 (Chapter 5), but it attained the NC state in a longer time, maintained pool of intracellular keto acids and took a longer incubation time to produce isobutyric acid only in pH 7.2 as
compared to ML3 (Fig. 1 C and D). SK11 also produced a lower amount of FAs compared to either of the other two strains. When IL1403 was incubated with a non-metabolizable sugar NC and BCFA production were reversed in order and the events coincided when incubated in CDM containing glucose (Chapter 5 and Fig. 8-2 E-G). These observations suggest that the three strains have different mechanisms of regulation underlying their ability to adapt, survive, and metabolize carbon during sugar limitation, acid stress, and the NC state.

The production of SCFAs and BCFAs from BCAAs is known to occur in brevibacteria (17), propionibacteria (44), clostridia (11, 23), Pseudomonas (30), enterococci (45), Bacteroides (1), Flavobacterium (34), and other ruminant and caecal microflora. While these reports did not monitor the effect of acid stress, they did show that BCFA production occurs after sugar exhaustion in many genera. BCFA production after sugar exhaustion seems to be a widespread phenomenon in different bacterial genera.

When IL1403 attained NC the FA levels in the CDM decreased (Fig. 8-1 C and D) suggesting that IL1403 utilized FAs during carbon deprivation. While SCFA utilization was observed in SK11 and ML3 (Fig. 8-2 A-D), the BCFA pool of ML3 remained at similar levels over starvation. The FAs possibly act as carbon stores for L. lactis subsp lactis IL1403. Lactococci shifted between arginine and lactose metabolism to regulate environmental pH during availability of both substrates (6). Such a metabolic switch is also possible between SCFAs leading to BCFA in lactococci. While brevibacteria catabolized BCFA to SCFAs in shorter time periods (17), FA utilization may be a long-term phenomenon in lactococci as FAs were present at similar levels over months of starvation. To our knowledge
the ability of lactococci to utilize FAs, especially BCFAs, has not been described in literature. But the production of BCFAs from acetate via acetyl-CoA has been described for myxobacteria and other ruminant bacteria (28). Hence IL1403 may be using acetate for BCFA production during starvation.

Mining the genome using bioinformatics and comparative genomics reveals their metabolic capability for previously unidentified pathways (40). Comparative genomic hybridization is a useful technique that has been demonstrated in other bacteria in identifying their genetic diversity (10, 25). We tried to compare the ability of BCFA production in lactococci by analyzing their genetic composition. While the limited similarity between IL1403, ML3, and SK11 genomes (Fig. 8-4 and Appendix D) precluded the comparison of their gene expression profiles it also suggested the possibility of divergent genetic compositions for BCAA catabolism leading to differences in physiological responses to acid stress, sugar catabolism, NC, and BCFA production. This diversity of bacterial genomes at the species level is also in agreement with findings in other bacteria (10, 25). The molecular mechanisms underlying stress adaptation in ML3 have been studied for short starvation periods (5, 27). Gene expression studies for long term starvation and FA metabolism of ML3 and SK11 would be of future interest in understanding the diversity in stress adaptation and flavor compound generation mechanisms of lactococci.

Previously we identified a pathway for the catabolism of BCAAs to FAs and BCFAs (Chapter 7). The proposed pathway involved a complicated 12-step process for producing BCFAs than previous investigations have allowed for but not identified in lactococci (7, 14, 15). BCAA catabolism proceeds through the
same set of enzymes irrespective of which BCAA is catabolized (Appendix D; map00280 for BCAA catabolism). The same genes are likely to be involved in catabolism of BCAAs to BCFAs even during starvation and NC. In this study we focused on understanding the role of genes identified as potential candidates for BCAA catabolism (Chapter 7) during starvation in culture rather than in an assay system. Gene expression data were used in combination with BCFAs and keto acid production to make the association between transcription and metabolite occurrence.

ATases were active for the entire duration of long-term starvation (Chapter 6), indicating that the responsible genes were being transcribed. This was confirmed by gene expression profiles showing ATase genes being constitutively expressed (Fig. 8-5). Their expression is required for the initiation of amino acid utilization. ATase genes were expressed at BCFA production and NC (Fig. 8-4 and 8-5) in both lactose pH conditions and glucose suggesting that ATase activities of yeiG and nifZ were not eliminated by acid stress or sugar sources. Keto acids were also present at consistent levels during starvation. This brings into question the role of the characterized ATases bcaT (47) and araT (36) which were not significantly induced in this study. Other ATases that transcribed differently may be associated with sugar deprivation and NC.

Dehydrogenases other than those identified in Chapter 7 were also transcribed over starvation. The genes yteC and yphC may be directly implicated due to their expression in all three conditions. The relation of many dehydrogenases such as ybiE and ycdG that were induced and repressed in different conditions could not be definitely established. But their homology to
short chain alcohol/aldehyde dehydrogenases (Appendix D) in concordance with the production of different FAs and α–keto acids over starvation suggests that there may be an active turnover of specific short chain carbon metabolites over starvation. The acyltransferase ycjD also is involved in catabolizing short chain metabolites at different physiological conditions. The role of multiple acyltransferases during BCAA catabolism is known in *Pseudomonas* (22). The studies of these enzyme classes in lactococci have not been reported thus far. Lactococci have homologues to acyltransferases of *Pseudomonas* (Appendix D). Hence the expressed genes may relate to enzymes needed for BCAA catabolism.

HMG-CoA synthase is predominantly involved in isoprenoid biosynthesis in *Bacillus* (3), *Enterococcus* (43), and many other bacteria (3, 21, 29), but not known previously in lactococci. The involvement of this enzyme for isovaleric acid production is shown in myxobacteria (28). The HMG-CoA synthase gene hmcM was repressed by acid and salt stress (Table 8-1) (46). Hence it is plausible that it was repressed in IL1403 at pH 5.2 due to acid stress, hence reducing BCFA production. The low expression of transacylases and acylkinases together with the perennial availability of α–keto acids suggests that unknown proteins may be involved in culture for catabolizing amino acids to BCFA in place of the known enzymes for these reactions.

Metabolic shifts were effected by sugar, acid, and NC and also verified by gene expression. For example, carboxylases are primarily involved in carbon fixation. Among the three known carboxylases all were expressed in the glucose CDM during NC. In lactose *ipd* was significantly induced by 8-fold and *pycA* was
repressed at NC with pH 7.2 and ipd was repressed and pycA induced at pH 5.2. The enzymatic co-expression of carboxylases is known in *Pseudomonas* (22) but no mechanisms were determined. A similar reversal of expression was also noted for all other enzyme classes and particularly dehydratases. Sugar metabolism, acid stress, and NC cumulatively induced these differences in gene expression. These three factors inevitably are linked with each other as acid stress tolerance facilitates NC attainment and sugar exhaustion (Fig. 8-1), and sugar limitation positively affects BCFA production (e.g. ML3 and IL1403) but not if inhibited by acid stress, which then also prevents attainment of NC (e.g. SK11 and IL1403 at pH 5.2). Genes whose expression was induced or repressed also matched previous studies done with IL1403 to monitor the effect of acid stress (Table 8-1) (46) suggesting that mechanisms that were induced in introduction of acid stress possibly pervaded over time in order to allow survival in an acid environment. The interaction of sugar starvation, acid stress, and NC primarily affected the BCFA production and genes expressed for their catabolism in this study.

Based on BCFA production and gene expression profiles in this study, and previous studies in assay, sugar metabolism primarily determined the ability to produce BCFAs. Log phase cells catabolized amino acids in assay when sugar was absent (16). In culture when sugar was present and the cells were metabolizing sugar no BCFAs were produced (Fig. 8-1 and 8-2). Artificially limiting sugar by supplying a non-metabolizable sugar induced BCFA production as did sugar exhaustion from the medium leading to NC (Fig. 8-2 E and F). Sugar exhaustion controlled attainment of NC. Sugar source also affected
which genes were induced to facilitate carbon fixation, BCFA production, and FA utilization. Switches in metabolism controlled by sugar occur in multiple ways in lactococci (5, 35) and even in yeast sugar metabolism (32). This may be a common mechanism in lactococci to regulate their metabolism in response to sugar availability and deprivation.

Conclusions

Lactococci metabolized BCAAs and keto acids to BCFAs after sugar starvation in culture. IL1403 also utilized SCFAs produced during log phase growth for survival during starvation. BCFA production was facilitated by lack of sugar and NC negatively affected by an inability to tolerate acid stress. Genes responsible for BCAA catabolism were induced and repressed during BCFA production and NC when analyzed in culture. This study demonstrated that the NC cells were capable of transcription and gene regulation irrespective of being incapable of reproduction on solid media (Fig. 8-1 and 8-5). The expression and regulation of these genes play a role in catabolizing amino acids during starvation.

References


summaries of high density oligonucleotide array probe level data.
Biostatistics 4:249-64.


Figure 8-1. Production of total short chain fatty acids (TSCFAs) and total branched chain fatty acids (TBCFAs) over incubation time in minimal CDM over different cellular phases by *L. lactis* subsp. *lactis* ML3 at pH 7.2 (A) and pH 5.2 (B) [TSCFAs (□) on the y-axis, TBCFAs (◊) on the yy-axis], by *L. lactis* subsp. *cremoris* SK11 at pH 7.2 (C) and pH 5.2 (D) [TSCFAs (□) on the y-axis, TBCFAs (◊) on the yy-axis], and by *L. lactis* subsp. *lactis* IL1403 in lactose at pH 7.2 (E) and pH 5.2 (F) [TSCFAs (□) on the y-axis, TBCFAs (◊) on the yy-axis]. Bars on the top represent different cellular phases. White bars, logarithmic phase; light spotted bars, post sugar exhaustion; dense spotted bars, nonculturability; cross etched bars, inability to utilize sugar.
Figure 8-2. Production of fatty acids over incubation time in minimal CDM by *L. lactis* subsp. *lactis* ML3 at pH 7.2 (A) and pH 5.2 (B) [acetic acid (☐) on the y-axis, propionic(◇), isobutyric (○), butyric (△), isovaleric (☆) and caproic (☒) acids on the yy-axis], by *L. lactis* subsp. *cremoris* SK11 at pH 7.2 (C) and pH 5.2 (D) [acetic (☐), propionic(◇), and butyric (△) acids on the y-axis, isobutyric acid (○) on the yy-axis], by *L. lactis* subsp. *lactis* IL1403 in lactose at pH 7.2 (E) [acetic (☐) and propionic(◇) acids on the y-axis, isobutyric acid (○) on the yy-axis] and at pH 5.2 (F) [acetic (☐), propionic(◇), and isobutyric (○) acids on the y-axis, butyric (△), isovaleric (☆) and caproic (☒) acids on the yy-axis] and by *L. lactis* subsp. *lactis* IL1403 in glucose at pH 7.2 (G) [acetic (☐) and propionic (◇) acids on the y-axis, isobutyric (○), isovaleric (☆) and caproic (☒) acids on the yy-axis]. T0, 0 d of incubation, NS, time point of sugar exhaustion = 1 d, NC, time point of attainment of nonculturability = 21 d. White bars, logarithmic phase; light spotted bars, post sugar exhaustion; dense spotted bars, nonculturability; cross etched bars, inability to utilize sugar.
Figure 8-3. Intracellular α–keto acids in by *L. lactis* subsp *cremoris* SK11 at pH 7.2 (A) and pH 5.2 (B) and *L. lactis* subsp. *lactis* IL1403 at pH 7.2 (C) and pH 5.2. (D) over incubation time in minimal CDM. The abbreviations are Pyr, pyruvate, KIC, ketosiocaproate, KGL, ketoglutarate, KIV, ketosiovalerate, KMV, keto-methyl-valerate, and KBU, ketobutyrate.
Fig. 8-4. Genomic hybridization data for genes related to BCFA production. Gray bars are positive for probe hybridization while white bars denote negative.
Figure 8-5. Pathway and gene expression map of different enzyme classes for *L. lactis* subsp. *lactis* IL1403. Color changes within the expression maps are: green to black, 2.5-fold increase in gene expression, black to red, ≥ 2.5-fold increase in gene expression. 1, IL1403G – 0 d, 2, IL1403G – time point of sugar exhaustion = 1 d, IL1403G – time point of nonculturability + BCFA production = 21 d, 4, IL1403L – pH 7.2 – 0 d, 5, IL1403L – pH 7.2 – time point of BCFA production = 15 d, 6, IL1403L – pH 7.2 – time point of nonculturability = 93 d, 7, IL1403L – pH 5.2 – 0 d, 5, IL1403L – pH 5.2 – time point of BCFA production = 15 d, 6, IL1403L – pH 5.2 – time point of nonculturability = 240 d.
Table 8-1. Comparison of differential gene expression due to acid stress to results from literature. Underlined genes did not match previous work (46).

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Annotation</th>
<th>This study</th>
<th>Yi et al. (46)</th>
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<td></td>
<td></td>
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<td>Repressed</td>
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<td>menD</td>
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<td>fadA</td>
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LAB produced FAs from amino acids. This phenomenon occurred only after sugar depletion and at low pH in assay buffer with amino acid substrates. The types and total quantities of FAs produced were similar among the lactococci and lactobacilli tested. From previous observations ATases appeared to be a key step in the production of FAs. Hence, we compared the ability of LAB to catabolize $\alpha$–keto acids with that of amino acids in order to biochemically bypass the initial ATase reaction. Notably, in acid conditions the $\alpha$–keto acids autodegraded to various FAs, suggesting that free $\alpha$–keto acids could also contribute to FA profiles as observed in other studies. Lactococci produced more BCFAs than lactobacilli from $\alpha$–keto acids, but both genera catabolized $\alpha$–keto acids beyond autodegradation levels. Addition of pyruvate to the $\alpha$–keto acids substrates changed the resulting types of BCFAs and FAs produced by the LAB. Addition of pyruvate also modified the cells’ energy state. Hence, we concluded that $\alpha$–keto acids were the limiting factor in production of FAs and that the catabolism of $\alpha$–keto acids, and that pyruvate availability also determines the exact type of BCFA and FA product pool in LAB.

*B. linens* produced FAs only when it depleted the carbohydrate. We concluded that this was required in order to switch from carbohydrate catabolism to amino acid catabolism for energy via a complex regulatory mechanism that is yet to be defined. Otherwise no FA products were observed when *B. linens* was incubated with amino acids in assay buffer. However, *B.*
*B. linens* did catabolize α-keto acids to acetic and propionic acids in assay buffer at pH 5.2. The genetic mechanisms required for activating the production of FAs by this organism are a worthy field of study for the future.

Assays using NMR spectroscopy showed that carbohydrate-starved *B. linens* produced the BCFA isovaleric acid from leucine and subsequently degraded it to acetic acid and propionic acid. To analyze the molecular mechanism of these products to be produced and utilized we queried the draft genome of *B. linens* to verify the presence of genes required for BCFA production and utilization. All the genes required for BCAA catabolism were present in *B. linens*. We found at least three different pathways for further catabolism of FAs to SCFAs in the *B. linens* genome. Hence, we have shown that brevibacteria catabolize amino acids only during carbohydrate starvation and they subsequently utilize the FAs they produce for further metabolism to generate cellular energy. This demonstrates a recycling mechanism for carbon that is induced after carbohydrate depletion, which is not found in LAB.

A similar genome query on the draft genome of *L. lactis* subsp. *cremoris* SK11 and the finished genome of *L. lactis* subsp. *lactis* IL1403 yielded only the presence of the initial ATase gene in the metabolic pathway for FA production. None of the other enzymes required for producing BCFAs was identified in the genome. The lack of genes coupled with the observation of FA production initiated the effort to characterize the ability of lactococci to catabolize BCAAs, despite the apparent lack of needed genes. The lactococcal genomes had at least nine different homologous genes for ATase action, suggesting a complex
regulatory system for transcription and a unique level of redundancy for aminotransferase reactions. At least nine different enzymes may be expressed in absence of one ATase to catabolize amino acids to FAs pathways.

To verify the role of the a single ATase enzyme in FA production from amino acids a BAT (ilvE) deletion mutant in lactococci was made. This strain and its parent were assayed with three BCAAs and glutamate and the corresponding α–keto acids of each amino acid for the production of FAs. Surprisingly, the BCAAs were still catabolized by both the parent and the mutant to produce the same total quantity of FAs but BCAAs were catabolized to different BCFAs. Also interesting was the fact that the deletion of IlvE affected the FAs produced from glutamate and KGL, its transamination product. The deletion also modified the products that were obtained from α–keto acids but did not abolish catabolism of BCAAs and α–keto acids. These observations showed that deletion of a single ATase only modified the pool of products, but did not abolish the activity. This result highlights the ability of redundant genes to carry out reactions that are required for the cell with slight modifications in the final end products. With this observation in mind, we sought to investigate the molecular details for the ability of lactococci to produce BCFAs. We also hypothesized that carbohydrate starvation was specifically involved in BCFA production and that other ATases were being induced to carry out the initial reaction in the metabolic pathway to FA production.

Initially we monitored the ability of L. lactis strains to survive long term carbohydrate starvation since this is important in the application of FA
production in fermented foods. This was done in *L. lactis* subsp. *lactis* ML3, *L. lactis* subsp. *cremoris* SK11, and the lac *L. lactis* subsp. *lactis* IL1403 to monitor the effect of available sugar on survival. All three strains were inoculated into a minimally defined medium containing lactose to monitor their ability to remain metabolically active during long term starvation. While ML3 metabolized lactose rapidly and attained the NC state within a week, SK11 utilized lactose slowly at pH 7.2 and attained NC within a month. At pH 5.2 SK11 did not utilize lactose at all and attained the NC state within 15 d. IL1403 did not use lactose, as anticipated, but required 3 months and 8 months at pH 7.2 and 5.2, respectively, to attain the NC state. These observations demonstrate the complex role of sugar use and strain variability in inducing the NC state in LAB. SK11 is an example of the role of pH in lactose utilization diversity. Clarification of these effects are beyond the scope of this work, but are a worthy area of study in light of the ability of LAB to survive starvation and metabolize amino acids to important flavor compounds.

While strains became NC they remained intact, as demonstrated by fluorescent staining, consistent basal level of intracellular ATP for at least 2 yrs. The sufficient availability of ATP was also verified by monitoring the ability of one of the strains to transport ATP-dependent substrates (e.g. casein derived peptides). ML3 transported and metabolized αs₁,α-casein during long-term starvation. This confirmed that the cells retained ATP-dependent substrate transport and that they were intact so that this peptide was removed from the medium. We also verified their ability to maintain an intact membrane by
determining transport of BCAAs, which is PMF-dependent and can only occur if the membrane is intact. All strains utilized BCAAs to produce BCFAs during this experiment, verifying that the cells remained intact and retained an adequate amount of ATP to drive transport as well as enough energy to produce PMF-dependent transport. The strains remain metabolically active even until today, 2-3.5 yrs after inoculation into CDM. Taken together these data show that the cells survive starvation and retain mechanisms for generating substrate transport and turnover of metabolites.

Gene expression analysis during starvation compared the effect of sugar metabolism on genes expressed during starvation. While 29 genes of 300 that related to replication, transcription, translation, substrate transport, sugar and protein metabolism, and ATP synthesis were significantly differentially expressed across sugar type, 50 additional genes were constitutively expressed in culture in either or both sugar type conditions at pH 7.2. Specifically the genes that related to cell wall division and replication were repressed or expressed at baseline levels, as were genes that related to producing lytic enzymes such as muramidases, lysins, or holins. The expression of these genes was not induced during starvation. These observations along with the observation that genes were being transcribed during starvation, verified that lactococci become NC as they lose their ability to divide but remain metabolically active, as they induced genes for transcription, translation, substrate transport, and metabolism.

To ascertain the ability of lactococcal strains to metabolize BCAAs for generating the needed energy during starvation we monitored the ATase activity, ATP, and metabolic end products. Over long periods of starvation
lactococci maintained ATases activity for BCAAs, sulfur-containing, and aromatic amino acids. Hence, the cells retained the ability to metabolize amino acids completely to energy rich compounds and flavor intensive end products. While this result is possible if the cells have lysed, we proceeded to verify that they produced BCFAs in culture. While all strains produced BCFAs there were many differences among them based on the incubation conditions. ML3 attained NC and produced BCFAs concurrently and was not affected by the incubation pH for the amount of BCFAs produced. Conversely, SK11 did not produce BCFAs at all at pH 5.2 and IL1403 produced 10 times less BCFAs at pH 5.2 than at 7.2. There was also a reversal in events in IL1403 in that it produced BCFA before becoming NC. To verify that this was not just an attribute of strain variation we verified the sequence of NC and BCFA production for IL1403 in CDM containing glucose.

IL1403 utilized glucose within 1 d but took 21 d to attain NC and also initiated BCFA production simultaneously. It hence appeared that the mechanisms for BCFA metabolism induction were very different across the three strains. Two of them were affected strongly by acid stress and they were all affected by availability of metabolizable sugar to attain the NC state or to produce BCFAs. Investigation a single gene at a time is unrealistic to determine the complex pathways and stress responses needed to induce NC and FA production. As such, we turned to a whole genome microarray constructed based on the IL1403 genome. While whole genome profiles may be informative about the genes that are coordinately transcribed it may not point us directly toward the right targets for specific metabolic pathways of interest unless the
target genes have already been characterized or annotated previously. With ~35% of the IL1403 genome unannotated, we anticipated that uncharacterized genes were involved in FA production. We hence decided to couple this with NMR spectroscopy to determine the metabolic compounds that were subsequently correlated to gene expression.

NMR spectroscopy with IL1403 during glucose starvation allowed identification of at least six different metabolites that were labeled with $^{13}\text{C}$ from 2-$^{13}\text{C}$-leucine. Some metabolites were also labeled at two different positions. It was intriguing that the BCFA produced was not isovaleric acid (4-methylbutyric acid), but rather 2-methylbutyric acid with the $^{13}\text{C}$ labels at its alkyl chain and not at the carboxylic acid group, as anticipated from the results for \textit{B. linens}. It indicates that there were totally different pathways associated with BCFA production in lactococci compared to that of brevibacteria. The same BCFA was observed from assays with long term starved ML3, SK11, and IL1403. Hence, all strains likely metabolized leucine via the same pathway. We also found metabolites labeled with $^{13}\text{C}$ included $\alpha$-keto acids, citrate, HMG, and glutamate; none of which were expected via BCAA catabolism to BCFA. This observation strongly indicates that the pathways for these products share common intermediates with BCFA production from leucine. Once a picture of the NMR-determined metabolites was developed, we computed a proposed pathway for leucine catabolism in lactococci by correlating the microarray data with the metabolite picture.
Initial efforts to construct the metabolic route based on the genome alone were unsuccessful. It was also not clear as to why the gene homologues were not identified for BCFA production in the IL1403 genome during the initial annotation effort by Bolotin et al. (1, 2). To overcome this problem, we constructed protein sequences and queried the genome for possible homologues that were uncharacterized or mis-annotated in IL1403 based on protein domain structure. We found at least 38 dehydrogenases that had putative protein domains for catabolizing short chain aldehyde/keto-group-containing substrates that were unannotated. Many other reactions for the possible pathway had protein homologues of very high similarity to other organisms but were annotated differently in the IL1403 genome. Since 200 more bacterial genomes have become available after IL1403 was sequenced, including the SK11 genome, it is possible to better estimate the homologous genes (i.e. proteins) that have common structures for uncharacterized genes in the IL1403 genome. From the list of genes that were assigned probable functions in the IL1403 genome we analyzed and selected all the putative homologues that could contained the catalytic domains for the reactions of interest and monitored their expression profile.

Many genes were induced and repressed during the NMR studies with NC cells that were associated with the putative genes for involvement in FA production. There was at least one gene for either the known or uncharacterized reactions of the pathways that were active and transcribed during the assay, suggesting involvement in the proposed pathway for FA production. While it is not clear why HMGA was accumulating, it is possible that it acts as a carbon sink
in conditions of excess substrate availability, as was case in the NMR assay, so that it can be recovered under carbon deprivation. Based on this information we proceeded to examine the expression of the putative gene homologues in culture along with production of BCFAs.

In culture many more genes that were identified by correlation with NMR were expressed compared to the assay conditions used in the NMR study. Some of the genes that were identified in NMR were expressed at lower levels while some were expressed constitutively irrespective of condition. Hence, many more genes are involved during BCFA production in culture from the putative homologues than were found in NMR. Notably many of the uncharacterized genes were putative dehydrogenases and acyltransferases that would be critical for action on short chain aldo-/keto- groups to produce FAs. Therefore, it appears that the metabolism of short chain molecules such as BCFAs during starvation involves a large group of genes that are yet to be functionally characterized in lactococci. The cells still contained a pool of α–keto acids that were consistently maintained across starvation suggesting that the α–keto acids alone may not be limiting the production of BCFAs. This observation is consistent with the deletion of IlvE, which suggested that the ATase reaction was not the limited step in FA production.

This study demonstrated that LAB and brevibacteria require carbon starvation to induce FA production from AAs. We used orthogonal approaches to demonstrate that lactococci remain in the NC state during starvation in culture and retain the ability to produce energy needed for active transport. Lactococci
also produced BCFAs from amino acids and keto acids in assay and in higher quantities than by lactobacilli. These observations suggest that the role of lactococci in producing BCFAs may supersede the role of lactobacilli. Yet, the question remains about the relative role of these two populations and the proportion of the population of lactococci that may lyse during the transition from active growth to the NC state in a fermented food such as cheese.

Based on plate count data available in literature (Fig. 9-1) (3, 4) culturable lactococci are present in Cheddar cheese at higher numbers than lactobacilli initially, yet appear to reduce (i.e. dye and lyse) to a constant level of \(~10^4\) cfu/g during cheese ripening. While lactobacilli begin at non-detectable amounts and grow to \(~10^9\) cfu/g during the same time. Overlaying the likelihood that lactococci remain NC during cheese ripening, rather than lyse, as demonstrated in this study, a theoretical estimate to determine the relative proportion of the possible metabolic states—actively growing, NC, and lysed. This analysis found that over \(10^9\) cfu/ml cells remained as NC after reaching a plateau of cell numbers during growth, the fluorescence live and dead counts maintained that plateau while cell numbers reduced. In other words, lysis was observed during active growth while post sugar exhaustion, no further lysis occurred.

Extrapolating this calculation to cheese, where lactococcal plate counts decrease from \(10^9\) cfu/g to \(10^4\) cfu/g, there is still a population of \(~10^9\) cells/g cheese \((10^9-10^9)\) are NC, meaning that only 0.001% of the total lactococcal population is estimated to be culturable. Therefore, almost the entire population of lactococci remains NC in cheese, despite the appearance that the cells die and lyse. The limited amount of lysis that occurs during cell growth in cheese would explain
the limited amount of intracellular components that is found in cheese during ripening (6). It may also be due to the growth of lactobacilli (5).

Based on FA production data by log phase cells under identical assay conditions and equal cell numbers (Chapter 3) lactococci produce FAs in quantities ranging from equal amounts to five times higher than lactobacilli. While even the same numbers of log phase cells ensures that lactococci produce more FAs there is also the possible presence of $10^9$ cfu/g lactococcal cells that are NC and culturable, compared to $\sim 10^7$ cfu/g lactobacilli. The cumulative effect of NC and log phase lactococci in cheese would lead to FA production that is around 100-500 times higher than lactobacilli. Considering the higher population of lactococci and of the increased production of FAs compared to lactobacilli (Chapter 8), it is likely that lactococci are the primary source of secondary metabolites found in cheese that are only produced during the NC phase.

We hypothesized that nonculturable lactococci catabolized BCAAs to BCFAs. We demonstrated that lactococci produce BCFAs from amino acids, but only after carbohydrate starvation, which persisted for at least 3 yrs. From these data a metabolic pathway for the production of BCAAs was proposed for lactococci and brevibacteria that contained genetic redundancy in all the steps. Genes needed for these metabolic routes were controlled by many environmental conditions including: 1) carbohydrate limitation, 2) pH, 3) pyruvate status, and 4) intracellular metabolite pool. Collectively, this work demonstrated the ability of lactococci and brevibacteria to catabolize BCAAs in the nonculturable state, rather than lyse. We hereby accept the hypothesis, and conclude that these bacteria utilize AAs to produce FAs and BCFAs that are not found in milk fat.
References


Fig. 9-1. Plate count data in Cheddar cheese from literature (1,2) for *Lactococcus lactis* (open squares) and adventitious lactobacilli (open diamonds) and possible nonculturable lactococci represented as cell numbers (dotted line). Data are averages of multiple strains and species/subspecies cell counts adapted from literature (3-6).
Appendix A. Supplementary data for Chapter 3
Figure A-1. Standard curves for estimation of FAs.
Figure A-2. FAs produced in plain buffer by lactococci (NC-Llac) and lactobacilli (NC-Lacb)
Appendix B. Supplementary data for Chapter 6
Figure B-1. Standard curve for estimation of α-ketoglutarate by spectrophotometry for ATase activity

\[ y = 6.132x - 0.001 \quad r^2 = 0.992 \]
Figure B-2. Transport of the αs₁,9 casein peptide by L. lactis subsp. lactis ML3 at 8 months of incubation in the nonculturable state.
Appendix B-1. SAS code used for repeated measures analysis of starvation data from Chapters 6 and 8

data cheese;
    input ph $ rep time a;
    cards;
    [input data]
    ;
    proc mixed;
    class time rep ph;
    model a=ph time ph*time/ddfm=satterthwaite outp=residuals;
    repeated/subject=rep(ph) Type=ar(1);
    run;
    proc univariate normal plot data=residuals;
    var resid;
    run;
    Model statement:
    Yiijk = Overall mean + pH_i + Time_j + (pH*Time)ij + (error)ijk
    #The error term has an autoregressive covariance structure
Table B-1. P-values from repeated measures analysis of IL1403 parameters over starvation corrected for multiple comparisons

<table>
<thead>
<tr>
<th>Treatments</th>
<th>pH</th>
<th>Time</th>
<th>pH*Time</th>
</tr>
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<tbody>
<tr>
<td>Plate Counts</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Live</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Dead</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>ATP</td>
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<td>&lt;0.003</td>
<td>0.024</td>
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<tr>
<td>AAT</td>
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<td>0.6162</td>
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<td>BAT</td>
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<td>0.9976</td>
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<tr>
<td>SAT</td>
<td>0.5593</td>
<td>&lt;0.003</td>
<td>0.0522</td>
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<tr>
<td>Acetic</td>
<td>0.2087</td>
<td>0.0976</td>
<td>0.5869</td>
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<tr>
<td>Propionic</td>
<td>0.0811</td>
<td>0.024</td>
<td>0.1797</td>
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<tr>
<td>Isobutyric</td>
<td>0.5164</td>
<td>0.171</td>
<td>0.5663</td>
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<tr>
<td>Butyric</td>
<td>0.4867</td>
<td>0.4871</td>
<td>0.3693</td>
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<tr>
<td>Isovaleric</td>
<td>0.1932</td>
<td>0.2739</td>
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<td>Caproic</td>
<td>0.3647</td>
<td>0.4563</td>
<td>0.459</td>
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Table B-2. P-values from repeated measures analysis of ML3 parameters over starvation corrected for multiple comparisons

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<td>ND*</td>
<td>ND*</td>
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<td>Live</td>
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<td>&lt;0.003</td>
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<td>&lt;0.003</td>
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<tr>
<td>AAT</td>
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<td>&lt;0.003</td>
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<td>BAT</td>
<td>0.8464</td>
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<td>0.84</td>
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<td>SAT</td>
<td>0.5605</td>
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<td>0.1907</td>
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<td>ND*</td>
</tr>
<tr>
<td>Butyric</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Isovaleric</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
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<tr>
<td>Caproic</td>
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<td>ND*</td>
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Table B-3. P-values from repeated measures analysis of SK11 parameters over starvation corrected for multiple comparisons

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<td>&lt;0.003</td>
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<td>Live</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Dead</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
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<td>ATP</td>
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<td>BAT</td>
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<td>SAT</td>
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<td>0.027</td>
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<td>Isobutyric</td>
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<td>ND*</td>
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<tr>
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Table B-4. P-values from repeated measures analysis of ML3 vs IL1403 parameters over starvation corrected for multiple comparisons

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<tr>
<td>Dead</td>
<td>0.1264</td>
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Table B-5. P-values from repeated measures analysis of IL1403 vs SK11 parameters over starvation corrected for multiple comparisons

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<th>Time</th>
<th>pH*Time</th>
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<tr>
<td>Plate Counts</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Live</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Dead</td>
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<td>&lt;0.003</td>
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Table B-6. P-values from repeated measures analysis of ML3 vs SK11 parameters over starvation corrected for multiple comparisons

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<th>Treatments</th>
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<th>Time</th>
<th>pH*Time</th>
</tr>
</thead>
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<td>Plate Counts</td>
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<td>&lt;0.003</td>
<td>0.015</td>
</tr>
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<td>Live</td>
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<td>&lt;0.003</td>
</tr>
<tr>
<td>Dead</td>
<td>0.8535</td>
<td>&lt;0.003</td>
<td>&lt;0.003</td>
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*ND - not determined due to too many zero values in either of the data sets resulting in infinite likelihood in iterations
FIG. B-3. Estimation of viability with fluorescence in spent CDM. Live counts of ML3 in CDM containing 0.1% lactose (■), and no lactose (□), and S2 in CDM containing 0.1% lactose (●), and no lactose (○) (panel A). Live and dead counts of ML3 (■), SK11 (◆, ◊), and IL1403 (●, Os) in CDM at pH 7.2 (panel B). Live and dead counts of ML3 (■, □), SK11 (◆, ◊), and IL1403 (●, ○) in CDM at pH 5.2 (panel C). Inset panels A1, B1, and C1 depict live and dead counts within the first 15 days of starvation for short-term starvation (A), pH 7.2 (B), and pH 5.2 (C), respectively.
FIG. B-4. Enzyme activities measured during starvation. AP and LE activity measured by the change in b* for arginine (open squares), methionine (open triangles), lysine (open circles), and butyrate (etched squares) and caprylate (etched diamonds) for strains ML3 (A) and S2 (B). The SEM was 12.5 and the LSD was 43.3 for AP activity, and the SEM was 4.5 and the LSD was 15.7 for LE.
FIG B-5. Expression maps of different functional categories of genes involved in starvation and nonculturability. A color change from green to black denotes a 2.5-fold increase in gene expression. A color change from black to red denotes a $\geq$ 2.5-fold change in gene expression. 1, IL1403 in lactose, T0, 2, IL1403 in lactose, BCFA production (15 d), 3, IL1403 in lactose, nonculturability (93 d), respectively.
Sugar Catabolism

ldhB
ndjF
dhal
lacZ
galT
yrj1
mleR
lacR
dhalM
citB
pfK
galK
galM
citD
mleS
tpiA
tkt
dhalK
lacC
citF
glpD
ptdD
zuf
butA
pfA
ptdA
pfK
ldh
adhA
ptdB
psk
adhE
cnoA
gpdA
ptdC
butB
gapB
ldhX
citR
gapA
ptsH
yjhF
citE
efoB

cell division, replication, lysis, recombination and repair

radA
dinF
estA
hexB
mutS
groES
cpaA
dhal
phal
recA
hexA
fba
groEL
radC
recN
recM
gyrE
yjbe recF
relA
dnaK
umuC
mutX

transcription and regulation

rpoB
ydcG
yabA
ynrE
busR
yhf1
copR
ycfD
ybeD
yaeil
tcrB
tenA
rcfA
rmaA
codZ
ybdG
kdgR
rhaB
rmaB
yabB
rmaH
ysgA
ydhF
yohC
yxeB
rmaB
yeA
rpoE
rnuA
ylnA
rliDA
codY
abwC
rliB
yja1
yjaE
yjaA
yjaD
yeF
ydfD
rpoD
rhrG
mtlR
rmeC
purR
rmaG
gadR
rliC
tuf
yllA
yebF
yhgC
rarA
ychI
Appendix C. Supplementary data for Chapter 7
Table C-1. Genes monitored in this study and their original annotations (Bolotin et al., 2001)

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<th>Description</th>
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<tr>
<td><strong>Aminotransferases</strong></td>
<td></td>
</tr>
<tr>
<td>araT</td>
<td>aromatic amino acid specific aminotransferase</td>
</tr>
<tr>
<td>aspB</td>
<td>aspartate aminotransferase (EC 2.6.1.1)</td>
</tr>
<tr>
<td>aspC</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>bcdT</td>
<td>branched-chain amino acid aminotransferase (EC 2.6.1.42)</td>
</tr>
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<td>nifS</td>
<td>pyridoxal-phosphate dependent aminotransferase NifS</td>
</tr>
<tr>
<td>nifZ</td>
<td>pyridoxal-phosphate dependent aminotransferase</td>
</tr>
<tr>
<td>yeiG</td>
<td>Amino acids and amines aminotransferase</td>
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</table>
Phosphatases/Phosphotransacylases

*apl* alkaline phosphatase

*pta* phosphate acetyltransferase (EC 2.3.1.8)

*yjCM* phosphatase

*yjFC* acylphosphate phosphohydrolase (EC 3.6.1.7)

**Acyl kinases**

*ackA1* acetate kinase (EC 2.7.2.1)

*ackA2* acetate kinase (EC 2.7.2.1)

**Pyruvate catabolism**

*pfl* pyruvate-formate lyase (EC 2.3.1.54)

*pflA* pyruvate-formate lyase activating enzyme (EC 1.97.1.4)

*poxL* pyruvate oxidase (EC 1.2.3.3)

**Branched chain amino acid biosynthesis**

*als* alpha-acetolactate synthase

*ilvB* acetolactate synthase large subunit (EC 4.1.3.18)

*ilvC* ketol-acid reductoisomerase (EC 1.1.1.86)

*ilvD* dihydroxy-acid dehydratase (EC 4.2.1.9)

*ilvN* acetolactate synthase small subunit (EC 4.1.3.18)

**Asparagine synthases**

*ansB* L-asparaginase

*asnB* asparagine synthetase B

*asnH* asparagine synthetase (EC 6.3.5.4)

**Citrate and aldehyde metabolism**

*adhA* alcohol dehydrogenase (EC 1.1.1.1)

*adhE* alcohol-acetaldehyde dehydrogenase (EC 1.2.1.10)

*citB* aconitate hydratase (EC 4.2.1.3)

*citD* citrate lyase acyl-carrier protein (EC 4.1.3.6)

*citE* citrate lyase beta chain (EC 4.1.3.6)

*citF* citrate lyase alpha chain (EC 4.1.3.6)

*gltA* citrate synthase (EC 4.1.3.7)

*icd* isocitrate dehydrogenase (EC 1.1.1.42)

**Malate dehydrogenase**

*mae* malate oxidoreductase (EC 1.1.1.38)

*mleR* malolactic fermentation system transcriptional activator

*mleS* malolactic enzyme
Table C-2. Full protein domain matches of *L. lactis* subsp. *lactis* IL403 putative genes

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<th>Gene ID</th>
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<td>AdhC - Alcohol dehydrogenase Class III, Zn-dependent</td>
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*Contains in this family, 3-HydroxybutyrylCoA dehydratase, enoylCoA hydratase, naphthoate synthase, dodecanolCoA delta-isomerase, and carnitine racemase

<sup>1</sup>This family includes acetate kinase, butyrate kinase and 2-methylpropanoate kinase
Table C-3. Partial protein domain matches of *L. lactis* subsp. *lactis* IL1403 genes

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<td>COG0451</td>
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<td>ypgB</td>
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<td>FabI, Enoyl Carrier protein</td>
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<td>Short chain dehydrogenase involved in D-alanine esterification of lipoteichoic acid</td>
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<td>WbbJ, Acetyltransferase</td>
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<td>Gene</td>
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<td>Serine-Pyruvate aminotransferase</td>
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<td>COG0076</td>
<td>GadB, Glutamate decarboxylase and related PLP-dependent enzymes</td>
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<td></td>
<td>COG2873</td>
<td>Met17, O-acetylhomoserine sulfhydrylase</td>
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<td>COG3844</td>
<td>Kynureninase</td>
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<td></td>
<td>COG0626</td>
<td>MetC, Cystathionine beta-lyase</td>
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<td>nifZ</td>
<td>COG0076</td>
<td>GadB, Glutamate decarboxylase and related PLP-dependent enzymes</td>
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<td>Serine-Pyruvate aminotransferase</td>
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<td>Aro8, transcriptional regulators containing DNA-binding HTH domain and aminotransferase domain</td>
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<td>Succinate dehydrogenase/fumarate reductase flavoprotein subunit</td>
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<td>COG0644</td>
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<td>pfam01039</td>
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<td>COG4799</td>
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Table C-4. Comparison of genes available in other organisms and L. lactis for branched chain amino acid degradation based on amino acid sequence homology

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<th>EC #</th>
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<th>L. lactis ID - annotation</th>
<th>Homology (%)</th>
<th>E-value</th>
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<td>E.C.-2.3.1._</td>
<td>bkdA2, bfmBAB, bfmB1b</td>
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<tr>
<td>E.C.-2.3.1._</td>
<td>bkdB - branched-chain alpha-keto acid dehydrogenase E2 subunit</td>
<td>pdhC - dihydrolipoamide succinyltransferase</td>
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<td>E.C.-2.3.1._</td>
<td>ysnE - acetyl transferase</td>
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<td>E.C.-2.6.1.42</td>
<td>ywaaA - branched chain aminotransferase</td>
<td>ilvE - branched chain aminotransferase</td>
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<td>E.C.-2.6.1.42</td>
<td>ybgE - branched chain aminotransferase</td>
<td>ybgE - amidase</td>
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<td>yusK - similar to acetyl-CoA C-acetyltransferase</td>
<td>thL - acetylCoA-acetyltransferase</td>
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<td>E.C.-2.3.1.-</td>
<td>pksF - putative polyketide beta-ketoacyl synthase</td>
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<td>yunH - phosphinothricin acetyltransferase</td>
<td>bar - acyltransferase</td>
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<td>E.C.-4.2.1.17</td>
<td>ysiB - enoylCoA hydratase</td>
<td>ysiB - permease</td>
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<td>E.C.-4.2.1.17</td>
<td>yngF - enoylCoA hydratase</td>
<td>ysiB - permease</td>
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<td>39/63</td>
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<td>E.C.-4.2.1.17</td>
<td>yufP - BCAA ABC permease (no yufP in L. lactis)</td>
<td>yngF - Sugar ABC transporter permease</td>
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<td>E.C.-4.2.1.17</td>
<td>yusL - 3-hydroxyacyl-CoA dehydrogenase; enoylCoA hydratase</td>
<td>ysiB, yngF homolog in B. subtilis; no yusL in L. lactis</td>
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<td>EC 6.4.1.4</td>
<td>accA - acetylCoA carboxylase - Vibrio parahaemolyticus</td>
<td>accA - acetylCoA carboxylase</td>
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<td>EC 4.2.1.18</td>
<td>ttc0182 - <em>Thermus thermophilus</em></td>
<td>pycA - <em>L. lactis</em> C2 menB - dihydroxynaphthoic acid synthase enoylCoA hydratase domains of <em>Bacillus cereus</em></td>
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<td>CD match</td>
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Figure C-1. KEGG pathway map of branched chain amino acid degradation pathways in *B. subtilis*. Shaded EC numbers depict the enzymes for which genes are present.
Figure C-2. KEGG pathway map of branched chain amino acid degradation pathways in *Pseudomonas putida*. Shaded EC numbers depict the enzymes for which genes are present.
Figure C-3. KEGG pathway map of branched chain amino acid degradation pathways in the draft genome of *Brevibacterium linens*. Shaded EC numbers depict the enzymes for which genes are present.
Figure C-4. KEGG pathway map of branched chain amino acid degradation pathways in *L. lactis* subsp. *lactis* IL1403. Shaded EC numbers depict the enzymes for which genes are present.
Figure C-5. Standard curve for estimation of glucose concentration using the spectrophotometric assay of Dubois et al.

\[ y = 7.264x - 0.057 \quad r^2 = 0.970 \]
Appendix D. Supplementary data for Chapter 8
Appendix D-1. Summary for extraction of FAs from CDM

Initial efforts to extract CDM from protocol used previously (Chapter 3) did not yield any fatty acids from CDM samples. A problem during this analysis was also the absence of internal standards added externally that should have extracted out if there were no FAs in samples and extraction protocol worked. To identify the problem by ingredient, the three surface-active agents glycerol, Tween-20, and Tween-80 were dissolved in water at the concentrations found in CDM. Repeating the FA extraction with these solutions identified that Tween-80 was the responsible ingredient. To overcome this problem, a C-18 column was tried in combination with the amino-propyl column in use for FA extraction. The C-18 column was conditioned in two different ways, (a) methanol and water in that sequence (AQ-C18) and (b) water and methanol (ORG-C18). ORG-C18 gave optimal extraction of internal standards with negligible losses and high repeatability. The effect of Tween-80 in preventing FA extraction may be due to its complex structure providing high surface-active effects.
Figure D-1. Extraction of internal standards from different solutions and columns to determine effect of surface active ingredients of CDM on FA extraction. CDM, chemically defined medium, Mix, equimolar mix of Tween-80, Tween-20, and glycerol, Tween-80, 1 ml/l Tween-80, Tween-20, 1 ml/l Tween-20, Glycerol, 1 ml/l glycerol, AQ-C18, extraction from Mix using a C-18 column initially conditioned with methanol and water in that order, ORG-C18, extraction from Mix using a C-18 column initially conditioned with water and methanol in that order.
Appendix D-2. Summary for analysis of branched chain α-keto acids

Initial use of a five-keto acid mixture (1 mM ea. of Pyr, KGL, KIC, KIV and KMV) for CE column optimization showed no improvement in background with ten runs. At this point we suspected that there were some keto acids that degraded during the run (alkaline conditions cause degradation of MBTH-derivatives; Dias, Ph.D. dissertation, 1998). So we derivatized individual keto acids and analyzed them separately. Pyr, KG, and KIC gave very sharp peaks at different migration times. The runs also had very low background (compared to previous runs). KIV and KMV gave two small but distinct peaks, consistently with 8 different runs, each peak eluting between 1-1.5 min of each other, but the runs had substantially high background. We also extended the run time for 35 min and confirmed that no bigger peaks were eluting. Our suspicions at this time were that these degraded too quickly during the CE run, or did not derivatize completely. Derivatization efficiency may be associated with the keto acids' structure. Access to the keto group for the amino group of MBTH to form the hydrazone derivative may be weakened or blocked by methyl groups in proximity. To further verify this issue we queried the structures of all keto acids tested by Dias and us. All keto acids tested by Dias with the exception of KIC had straight chain R-groups. The R-group of KIC (tested by both Dias and us) has the methyl group on the fourth carbon, i.e. two carbons from the keto group, whereas KIV and KMV have the branched methyl group on the adjacent carbon itself. This structure might probably (a) slow the derivative formation (needs more time to derivatize) or (b) prevent derivative formation completely (not
possible, as we did see two distinct peaks in a 30-min derivatization reaction),
acting by steric hindrance. The high background is likely to be due to
underivatized MBTH, which will also be detected during the run. As keto acid
derivatives are stable for at least 5 d at pH 5 at which they are derivatized, it is
unlikely that they degrade to levels lower than background during a 25-min run
in which they elute within 10 min. The two-peak phenomenon is likely to be the
separation of stereo-isomers of these keto acid derivatives (KIV, KMV). Why we
do not see the stereo-isomers of other keto acids is not clear, the possibilities
being that only one stereo-isomer is present (or preferably derivatized) in their
case (Pyr, KG, KC, KB, KMTB, KIC), or that the reaction happens much rapidly
without steric hindrance, preventing the formation of stereo-isomers in solution,
due to the large structure of the hydrazone. Whereas in a slower reaction, stereo­
isomers form at an equilibrated rate, reacting at equal rates with MBTH. We
tested this hypothesis by increasing the reaction time for pure KIV and KMV
solutions to 1 h and 2 h or 3 h, and then testing the samples. Further extension of
the derivatization time led to improved derivatization but no change in elution
pattern of these two keto acids. The ability of capillary electrophoresis to
separate stereo-isomers is well known, but has not been shown previously for
keto acids.
Figure D-2. Replicate runs each of 1 mM KIV and 1 mM KMV showing separation of 2 distinct peaks from standard solutions separated by MECC.
Figure D-3. Graph showing the effect of derivatization time on efficiency of detection of KIV and KMV. KIV1 and KIV2 are the two peaks for KIV and KMV1 and KMV2 are the two peaks for KMV respectively.
Pyruvate $y = 0.001x + 0.011$  $r^2 = 0.999$

Ketoisocaproate $y = 0.001x + 0.011$  $r^2 = 0.999$

Ketoglutarate $y = 0.001x + 0.005$  $r^2 = 0.997$

Ketoisovalerate $y = 0.001x - 0.001$  $r^2 = 0.998$

Ketomethylvalerate $y = 0.001x - 0.002$  $r^2 = 0.989$

Figure D-4. Standard curves for estimation of α-keto acids. Data are average of two replicates. Abbreviations are Pyr, pyruvate, KIC, α-Ketoisocaproate, KGL, α-ketoglutarate, KIV, α-ketoisovalerate, KMV, α-keto-β-methylvalerate
Appendix D-4. Summary for genomic DNA hybridization of lactococci

Lactococcal gene expression analysis would reveal valuable clues to its metabolism and hence is of commercial and scientific value. To analyze the similarity of their gene content and to verify the usability of a single probe per open reading frame macroarray, the genomic DNA from each strain of interest was isolated and hybridized against a macroarray built for IL1403. The results are presented as a heat map for presence (gray) or absence (black) of spots. Some strains shared high similarity while some were extremely divergent from each other and from IL1403. Close resemblance to IL1403 was restricted to MG1363, SK11, and FG2.

<table>
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<th>Strain</th>
<th>% Genes hybridized to probes</th>
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<td>FG2</td>
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<td>SK11</td>
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<td>LM0230</td>
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<td>JLS450</td>
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<td>ML3</td>
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Appendix E. Multiple comparisons correction information
Note on multiple comparison statistics for Chapters 3, 4 and 5

In the Student's T-tests described in the aforementioned chapters, corrections for multiple comparison were necessitated, as there were multiple FA products in each condition tested. So for each condition a correction was applied wherein the obtained p-values were corrected by multiplying them with the total number of FA products. This correction did not in anyway affect the statistical significance of the results at $\alpha = 0.05$. 
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Dear Dr. Koka

I am in the process of preparing my dissertation titled "Catabolism of amino acids to fatty acids in Lactococcus lactis" at Utah State University. I hope to complete my degree in the spring of 2005.

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Two articles, "Monocarboxylic acid production in lactococci and lactobacilli", published in International Dairy Journal and "Fatty acid production from amino acids and α-keto acids by Brevibacterium linens BL2", published in Applied and Environmental Microbiology, of which you are one of the co-authors, compose an integral part of my dissertation research. I hereby request your permission to reprint the article as a chapter in my dissertation. Please note that USU sends the dissertation to Bell and Howell Dissertation Services to be made available for reproduction.

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Date 4-4-05
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12/20/2004

Dear Mr. Stuart

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Date 1/7/05
Balasubramanian Ganesan

Nationality: Indian

Date of Birth: 16th November 1975

Place of Birth: Salem, Tamil Nadu State, India

Language Skills:
- Writing and reading: English, Tamil, Hindi, and Sanskrit
- Spoken languages: English, Tamil, and Hindi

Scientific skills:
- Microbiological aseptic technique and studies of microbial physiology
- Designing of microarrays and analyzing microbial genomes using arrays for studying genome-wide expression and genome cross-comparison, including using software for design, data acquisition, and analysis
- Genome annotation of bacteria – Lactococcus lactis and Brevibacterium linens – as part of the Lactic Acid Bacteria Genomes Consortium
- Instruments involved in genomic analysis such as RNA Bio-analyzer, imager, and spotter
- Genome Bioinformatic analysis with multiple software suites, especially many major freeware sources – representative suites are the MUMmer suite from The Institute for Genomic Reseach, USA, Artemis and ACT from the Sanger Research Institute, UK, STRING, VISant, MGView, EMBOSS, Blast, T-Coffee, and ClustalW
- Trained in PathwayTools database building and curation of genomes for pathway-based representation and analysis of genomic data
- Commercial diagnostic and analytical kits for metabolite analysis and genomic sample extraction and manipulation
• Other analytical techniques – Nuclear Magnetic Resonance spectroscopy analysis for tracking metabolic pathways, gas chromatography – static headspace sampling and volatile compound separation and analysis, High Performance Liquid Chromatography, capillary electrophoresis – micellar electrokinetic capillary chromatography of amino acids and keto acids
• Statistical analysis using the SAS, JMP, and SAM software suites
• Familiar with pathogen detection techniques and ELISAs
• Functional familiarity with the PERL programming language scripting
• Functional familiarity with all 3 major computer Operating Systems – Apple, Windows, and Linux

Scientific Publications:

• 6 in preparation
• Data and literature review generated for parts of grants and proposals

Scientific Conferences and Presentations:

• Ganesan, B., Seefeldt, K., and Weimer, B.C. Volatile fatty acid production by starter and adjunct bacteria: Poster presented at the Cheese Technology and Ripening Conference organized by the International Dairy Federation, 2000, Banff, Alberta, Canada

• Ganesan, B., and Weimer, B.C. Fatty acid production by *Lactococcus lactis* during carbohydrate starvation in a chemically defined medium. Poster presented at the American Society of Microbiology General Meeting, 2002, Salt Lake City, Utah, USA

• Ganesan, B., and Weimer, B.C. Production of fatty acids by lactococci during carbohydrate starvation. Poster presented at the Federation of Europe for Microbiological Sciences, Lactic acid bacteria Division Conference, 2002, Ede, The Netherlands

• Attended the Institute for Food Technologists conference, 2002, Anaheim, California, USA

• Weimer, B.C. and Ganesan, B. Comparative genomics of dairy lactococci. Lecture presented at the symposium for the Lactic Acid Bacteria Genome Consortium held at the American Society of Microbiology General Meeting, 2004, New Orleans Louisiana, USA

Professional Memberships and Subscriptions:

• Member of the American Society of Microbiology, 2000-present
• Member of the Institute of Food Technologists, 2001-present
• Active subscriber of the molecular techniques journal *Biotechniques*

Work Experience:

• Research Assistant under Dr. Bart C. Weimer, Department of Nutrition and Food Sciences, Utah State University, Logan UT, 1999-current
• Involved in laboratory management and technique validation during the last five years, along with basic science research
• Marketing Executive at The Gujarat Co-operative Milk Marketing Federation Ltd., India’s largest co-operative Milk Marketing organization, 1998-99

Technical training and Experience:
Training in genome-based database building and curation for metabolic reconstruction of genomes using the PathwayTools software suite at Menlo Park, California, USA, 2004.

Involved actively during employment at The Gujarat Co-operative Milk Marketing Federation Ltd. in implementation of Total Quality Management programs in daily official and personal life.

Trained in Enterprise Resource Management systems operation implementation during employment at The Gujarat Co-operative Milk Marketing Federation Ltd.

One month in-plant training post-Bachelor’s degree at the Modern Bread Factory, Ahmedabad, India, as extra-curricular experience, 1998

One year in-plant student employee at Vidya Dairy, Anand, India as part of curricular practical experience during Bachelor’s degree, 1996-1997

Academic Awards and Honors:

Recipient of the Gandhi Scholarship Award from the Department of Nutrition and Food Sciences, Utah State University, Logan for two consecutive years, 1999-2000

Recipient of the Inez Waldron Biotechnology scholarship, 2000

Academic Background:

Currently enrolled as a Ph.D. student at the Department of Nutrition and Food Sciences, Utah State University, Logan, USA

Current GPA: 3.62 / 4.00

First class degree in B. Tech. (Dairy Technology) from Sheth M.C. College of Dairy Science, affiliated to The Gujarat Agricultural University, S.K. Nagar, India

GPA in B.Tech.: 7.08 on a 10.0 GPA basis

XII grade from P.S. Senior Secondary School, Madras, India, affiliated to the Central Board of Secondary Education, India with an aggregate of 89%

Extracurricular activities:

Service at the Shri Ganesha Hindu Temple, West Jordan, Utah, USA
• Representative of Graduate Student senate for the Department of Nutrition and Food Sciences, 2003-2004
• Member of Food Science College Bowl team, 2002 – West Zone Champions, and attended the Food Science College Bowl National Championships at the Institute of Food Technologists’ Conference, 2002, Anaheim, CA
• The Vice-President of cultural activities of the Indian Students’ Association at the Utah State University, Logan, 1999-2000
• Organized, participated and won prizes in quizzes and word games, at National level competitions in India
• Organized many inter- and intra-cultural events at high school and university level at India
• Member of trivia quiz fora

Hobbies:
• Expert in Indian Classical Music - Carnatic style - vocal
• Sing, listen to Indian classical and cine-music, and collect songs
• Read a variety of books, ranging from classical literature and history to science fiction
• Play chess, quiz, especially trivia, Word Games and Word Power, Crossword and Mathematical puzzles
• Cooking, especially Indian vegetarian dishes