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Flavour formation from amino acids by lactic acid bacteria: predictions from genome sequence analysis

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Abstract

Flavour development in dairy fermentations is the result of a series of chemical and biochemical processes during ripening. Starter lactic acid bacteria provide the enzymes involved in the formation of specific flavours. Amino acids, and in particular methionine, the aromatic and the branched-chain amino acids, are major precursors for volatile aroma compounds. The recent sequencing of complete genomes of several lactic acid bacteria (i.e. *Lactococcus lactis, Lactobacillus plantarum, Streptococcus thermophilus*) is beginning to provide insight into the full complement of proteins that may be involved in flavour-forming reactions, and hence the potential for formation of specific flavour compounds. Examples are given how bioinformatics tools can be used to search in genomes for essential components, such as proteinases, peptidases, aminotransferases, enzymes for biosynthesis of amino acids, and transport systems for peptides and amino acids. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Flavour development in fermented dairy products is a complex and, in the case of cheese ripening, slow process involving chemical and biochemical conversions of milk components. Flavour compounds are formed by various processes, i.e. the conversions of lactose and citrate (glycolysis and pyruvate metabolism), fat (lipolysis), and caseins (proteolysis). Lactic acid bacteria (LAB) form the main microflora in these dairy products, and they are essential for the biochemical conversions that determine the specific flavour. Although lactose is mainly converted to lactate by LAB, a fraction of the intermediate pyruvate can alternatively be converted to various flavour compounds such as diacetyl, acetoin, acetaldehyde, or acetic acid, some of which contribute to typical yoghurt or butter flavours. An important flavour-generating reaction in some dairy products is the conversion of citrate to diacetyl, which can be performed by some LAB strains (Hugenholtz, 1993). Lipolysis results in the formation of free fatty acids, which can be precursors of flavour compounds such as methyl ketones, alcohols, lactones and esters (Molimard & Spinnler, 1996). Lipolysis is mainly due to mould activity, and much less to LAB activity (Molimard & Spinnler, 1996). Fat hydrolysis is particularly important in soft cheeses like Camembert and Blue cheeses. Proteolysis is undoubtedly the most important biochemical process for flavour and texture formation in hard-type and semi-hard-type cheeses. Degradation of caseins by the activities of rennet enzymes, and the cell-envelope proteinase and peptidases from LAB yields small peptides and free amino acids. A good balance between proteolysis and peptidolysis prevents the formation of bitterness in cheese. Although it is known that peptides can taste bitter (Lemieux & Simard, 1992) or delicious (Yamasaki & Maekawa, 1978) and that amino acids can taste sweet, bitter, or broth-like (Mulder, 1952), the

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direct contribution of peptides and amino acids to flavour is probably limited to a basic taste. Further conversion of amino acids to various alcohols, aldehydes, acids, esters and sulphur compounds is required for specific flavour development.

Using genetic and biochemical tools, numerous flavour-forming routes from amino acids have been identified recently, mainly in lactococci, and the role of various enzymes in these pathways is being elucidated (Gao et al., 1997; Yvon, Thirouin, Rijnen, Fromentier, & Gripon, 1997; Yvon, Berthelot, & Gripon, 1998; Weimer, Seefeldt, & Dias, 1999; Christensen, Dudley, Pederson, & Steele, 1999; Ott, Germond, & Chaintreau, 2000; Smit et al., 2000; Engels et al., 2000; Yvon & Rijnen, 2001).

Flavour formation from amino acids by LAB depends on a very complex network of reactions, and many factors may contribute to the balance of various flavour compounds. In general, the main processes are (1) generation and uptake of amino acids, i.e. formation of the intracellular pool of amino acids; (2) conversion of amino acids; and (3) regulation of these pathways.

2. Amino acid metabolism

2.1. Amino acid biosynthesis and growth requirements

Lactococci have a limited capacity for biosynthesis of amino acids, which explains their complex nutritional requirements. They require essential amino acids for growth, and the number of essential amino acids is strain-dependent (Andersen & Elliker, 1953; Reiter & Oram, 1962; Chopin, 1993; Jensen & Hammer, 1993; Ayad, Verheul, de Jong, Wouters, & Smit, 1999). Most dairy *Lactococcus* strains need glutamate, valine, methionine, histidine, serine, leucine and isoleucine. Industrial *L. lactis* subsp. *cremoris* strains require even more different amino acids for growth (Ayad et al., 1999). Both dairy and non-dairy wild strains need fewer amino acids for growth than the industrial strains. Wild *L. lactis* subsp. *cremoris* and subsp. *lactis* strains generally require only 1–3 amino acids. The absence of some amino acid biosynthethic pathways in dairy lactococci might be a consequence of their adaptation to dairy products. In milk, amino acids are readily available by the proteolysis of caseins. Wild strains are not naturally associated with a rich environment such as milk which makes them more dependent on their own synthesis of amino acids compared to industrial strains.

2.2. The proteolytic system

Since the concentrations of free amino acids and peptides are very low in milk, the LAB depend for growth in milk on a proteolytic system (Fig. 1) for degradation of milk proteins (caseins) into peptides and amino acids, which can subsequently be taken up by cells (Kok & de Vos, 1994; Kunji, Mierau, Hagting, Poolman, & Konings, 1996; Christensen et al., 1988). Proteolysis is initiated by a single cell-wall-bound extracellular proteinase (Prt), that can be either chromosomally or plasmid-encoded (Kok & Venema, 1988; Kunji et al., 1996; Siezen, 1999). While most dairy LAB strains contain such an extracellular proteinase, several do not and these are mainly dependent on other strains

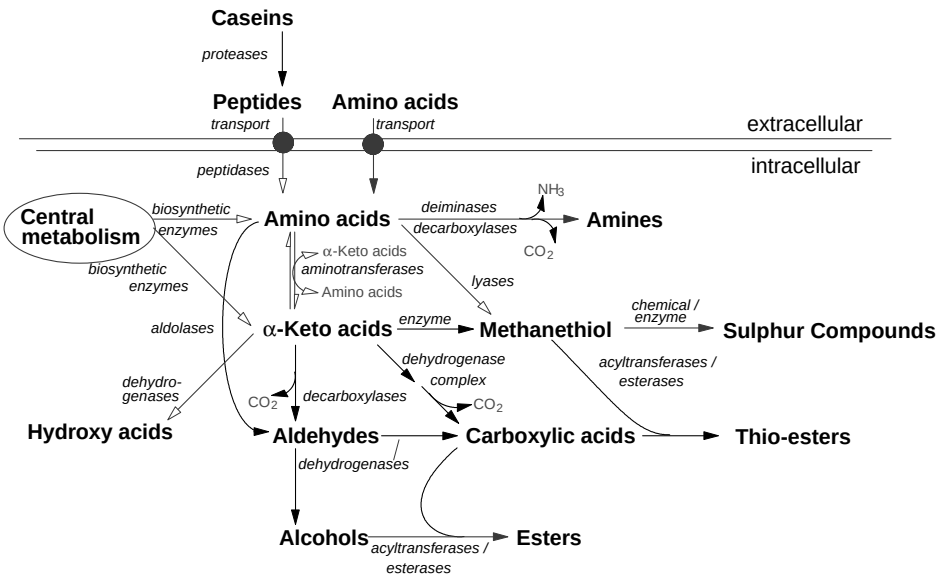

Fig. 1. Summary of general pathways leading to intracellular amino acids and α-keto acids, and their degradation routes to potential flavour compounds. More specifically, pathways from methionine to flavour compounds (methanethiol, thioesters, sulphur compounds) are shown.

in the starter culture for the production of peptides and amino acids. Recently, a house-keeping cell-envelope protease HtrA has been identified in LAB, which mainly plays a role in maturation of secreted proteins, and presumably does not contribute to degradation of caseins (Poquet et al., 2000).

Peptide and amino acid transport systems have been studied extensively in lactococci. Peptide uptake occurs via one or two oligopeptide transport systems (Opp, Opt) and one or two di-/tri-peptide transporters (DtpT, DtpP) (reviewed by Kunji et al., 1996). The oligopeptide transporter 'Opp', is an ABC transporter capable of transporting peptides ranging in size from 4 to at least 18 amino acids (Detmers, Kunji, Lanfermeijer, Poolman, & Konings, 1998). Various amino acid transport systems have been identified with a high specificity for structurally similar amino acids, e.g. Glu/Gln, Ser/Thr, Ala/Gly, Lys/Arg/Orn, branched chain (Ile/Leu/Val), and aromatic (Phe/Tyr/Trp) residues (Konings, Poolman, & Driessen, 1989). Much less is known as yet about peptide and amino acid transporters in lactobacilli.

Following uptake, the peptides are degraded intracellularly by a variety of peptidases, which have been extensively studied in both lactococci and lactobacilli (reviews by Kunji et al., 1996; Christensen et al., 1999). These peptidases of LAB can be divided into endopeptidases, aminopeptidases, di-/tri-peptidases, and proline-specific peptidases. No carboxypeptidases have ever been found in LAB. The specialized peptidases in LAB for hydrolysis of Pro-containing peptides have been postulated to be important for the degradation of casein-derived peptides, since these are known to have a high proline content. Gene inactivation (Guinec, Nardi, Matos, Gripon, & Monnet, 2000) and gene overexpression studies (Courtin et al., 2001) in a cheese model system have shown that several peptidases play an important role in determining the overall level of amino acids at the end of ripening, i.e. endopeptidase PepW, aminopeptidase N (PepN), tripeptidase (PepT), X-prolyl dipeptidylpeptidase (PepX), and prolidase (PepQ).

2.3. Amino acid catabolism

Amino acids are precursors of various volatile flavour compounds. They can be converted in many different ways by enzymes such as deaminases, decarboxylases, transaminases (aminotransferases), and lyases (Fig. 1). Transamination of amino acids results in the formation of α-keto acids that can be converted into aldehydes by decarboxylation and, subsequently, into alcohols or carboxylic acids by dehydrogenation. Many of these compounds are major aroma components. Direct dehydrogenation of α-keto acids results in the formation of hydroxy-acids, which do not contribute to the flavour of the product.

Threonine aldolase, found in many *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains, can convert threonine into acetaldehyde, which is an important flavour compound of yoghurt, but also of cheese, butter and buttermilk (Ott et al., 2000).

Aromatic amino acids, branched-chain amino acids, and methionine seem the most relevant substrates for cheese flavour development (Fig. 1). Conversion of aromatic amino acids can result in formation of undesirable flavours, so-called *off-flavours*, such as *p*-cresol, phenylethanol, phenylacetaldehyde, indole, and skatole, that contribute to putrid, fecal or unclean flavours in cheese (Christensen et al., 1999). Gao et al. (1997) demonstrated that the metabolites formed from tryptophan, under assay conditions that mimic a Cheddar cheese situation, are strain-specific for *L. lactis*. This implies that selection of the starter bacteria can be used to avoid formation of undesirable flavours. Benzaldehyde can also be formed from tryptophan or phenylalanine. This compound is found in various hard-type and soft-type cheeses (Engels, Dekker, de Jong, Neeter, & Visser, 1997; Molimard & Spinnler, 1996). The conversion of phenylalanine to benzaldehyde by *Lactobacillus plantarum* and other LAB is initiated by a pyridoxal 5′-phosphate (PLP)-dependent aminotransferase (Nierop Groot, & de Bont, 1998, 1999). The resulting phenylpyruvic acid is chemically converted to benzaldehyde in the presence of oxygen and manganese. Such a conversion therefore requires an efficient uptake system for Mn^{2+} ions. Recently, two manganese transport systems of *Lb. plantarum* have been characterized (M.N. Nierop Groot, personal communication).

Branched-chain amino acids are precursors of aroma compounds such as isobutyrate, isovalerate, 3-methylbutanal, 2-methylbutanal, and 2-methylpropanal, which are found in various cheese types. Several PLP-dependent enzymes that are able to convert these amino acids have been detected in *L. lactis* (Christensen et al., 1999; Dobric, Limsowtin, Hillier, Dudman, & Davidson, 2000; Gao & Steele, 1998; Rijnen, Bonneau, & Yvon, 1999; Yvon et al., 1997; Engels et al., 2000). The aromatic aminotransferases can convert aromatic amino acids, but also leucine and methionine, while the branched-chain aminotransferases can convert the branched-chain amino acids leucine, isoleucine and valine, but also methionine, cysteine, and phenylalanine.

Volatile sulphur compounds derived from methionine, such as methanethiol, dimethyl sulphide, and dimethyl trisulphide, are regarded as essential components in many cheese varieties (Urbach, 1995). In fact, a Gouda cheese-like flavour can be generated by incubation of methionine with cell extracts of *L. lactis* (Engels & Visser, 1996). Conversion of methionine can occur via

a aminotransferase-initiated pathway by branched-chain or aromatic aminotransferases, or via an α,γ-elimination of methionine by the lyase activities of cystathionine β-lyase (CBL), cystathionine γ-lyase (CGL), or methionine γ-lyase (MGL) (Alting, Engels, van Schalkwijk, & Exterkate, 1995; Bruinenberg, de Roo, & Limsowtin, 1997; Dias & Weimer, 1998a, b; Dobric et al., 2000; Engels et al., 2000; Gao, Mooberry & Steele, 1998; Gao & Steele, 1998; Rijnen et al., 1999; Yvon et al., 1997). Aminotransferase activity requires α-ketoglutarate or other α-keto acids as a co-substrate and results in the formation of 4-methylthio-2-ketobutyric acid (KMBA) which can be converted to methanethiol, probably via a thiamine pyrophosphate (TTP)-dependent decarboxylase that produces 3-methylthiopropanal (methional), and subsequent breakdown (Engels et al., 2000).

Although cystathionine lyases are active under cheese-ripening conditions (Alting et al., 1995; Smacchi & Gobbetti, 1998), their activity towards methionine could not be detected using ^{13}C nuclear magnetic resonance (Gao et al., 1998). With this technique, only the aminotransferase-initiated pathway was observed suggesting that this pathway is most prominent in methionine catabolism to produce methanethiol.

Biosynthesis and degradation of some amino acids are intricately coupled pathways as shown in Fig. 2 for serine, cysteine and methionine, but this also applies for branched-chain and aromatic amino acids (Fig. 3). During cheese ripening, cystathionine β-lyase can convert methionine to various volatile flavour compounds, but in bacteria its physiological function is the conversion of cystathionine to homocysteine, which is the penultimate step of methionine biosynthesis (Fig. 2). In vitro the lactococcal cystathionine β-lyase can also convert cystathionine to cysteine (Alting et al., 1995; Dobric et al., 2000), but it is not known whether this is also part of its in vivo function. The physiological role of branched-chain aminotransferases in bacterial metabolism is to catalyse the last step in the biosynthesis of branched-chain or aromatic amino acids. Several enzymes can thus been considered as being involved in both biosynthesis and degradation of amino acids, and α-keto acids are intermediates in both directions.

2.4. Regulation and complementarity

The growth requirement for specific amino acids can result from either the absence of functional specific biosynthetic genes or from specific regulatory mechanisms (Chopin, 1993). For example, the existence of defects in biosynthesis of histidine and branched-chain amino acids has been established in *L. lactis* strains resulting from accumulated mutations and deletions within the genes coding for the biosynthetic enzymes (Delorme, Godon, Ehrlich, & Renault, 1993; Godon et al., 1993; Bolotin et al., 2001). The involvement of regulatory mechanisms in amino acid requirements has also been demonstrated in *L. lactis*. For instance, the biosynthesis of the amino acids of the glutamate family (Glu, Gln, Arg and Pro) are dependent on the synthesis of glutamate itself which, in turn, can be affected by the ammonium ion concentration in the medium. The finding that many strains have a requirement for valine but not isoleucine (Ayad et al., 1999) cannot result from a defect in biosynthesis, since all genes required for the valine biosynthesis are also required for the synthesis of isoleucine (Fig. 3). Conceivably, an intermediate of the branched-chain amino acid pathway or another anabolic or catabolic pathway interferes with valine biosynthesis (Godon et al., 1993).

In *L. lactis* the gene coding for cystathionine β-lyase (*metC*) is clustered together with a gene coding for

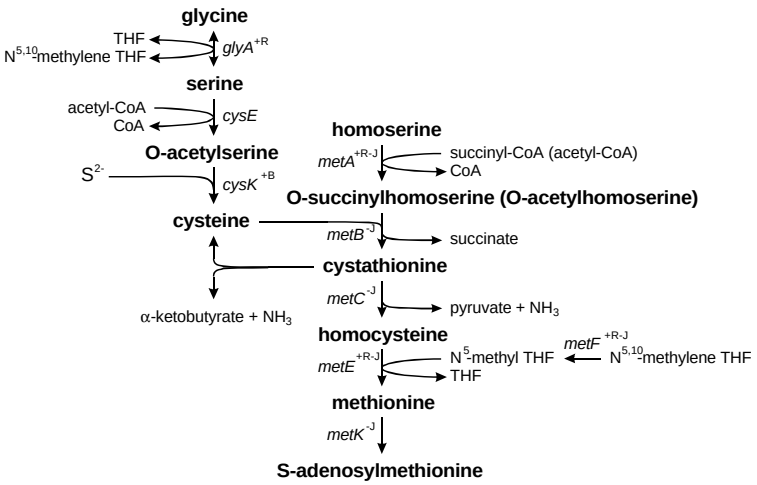

Fig. 2. Cysteine and methionine biosynthesis pathways in *E. coli* and *S. enterica* serovar *typhimurium* and the responsible genes. Activation by CysB ($^{+B}$) or MetR ($^{+R}$) and repression by MetJ ($^{-J}$) are indicated in superscript. The conversion of cystathionine to cysteine is not described for *E. coli*. Cystathionine β-lyase from *L. lactis* is able to catalyse this reaction in vitro (Alting et al., 1995; Dobric et al., 2000).

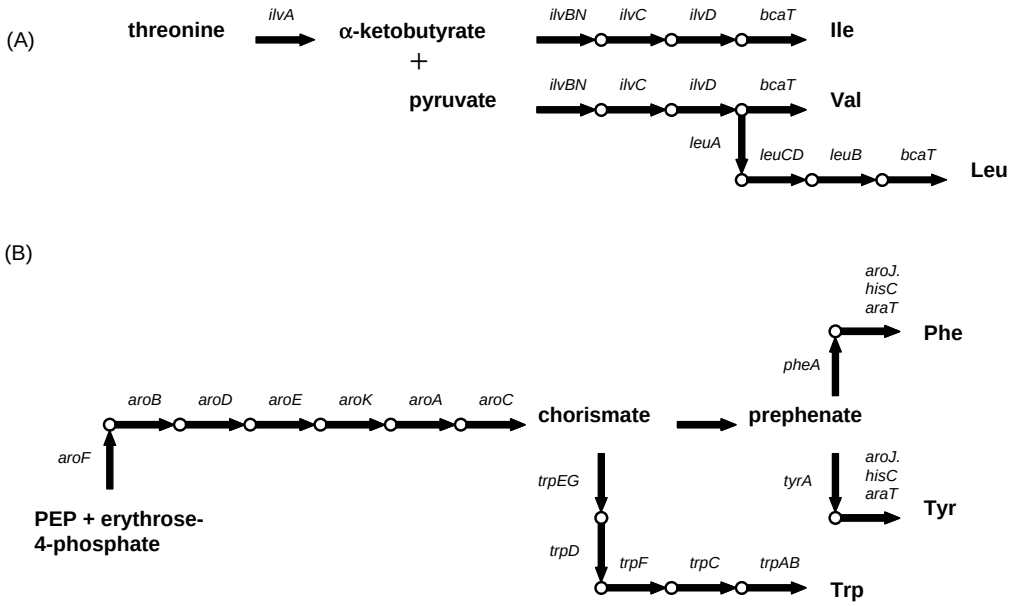

Fig. 3. Schematic view of biosynthetic pathways for (A) branched-chain amino acids, and (B) aromatic amino acids. Only the starting substrates, key intermediates, and end products are shown. Enzymatic steps are shown as arrows with the corresponding genes.

cysteine synthase (*cysK*) (Fernández et al., 2000), thus genetically linking the methionine and cysteine biosynthesis pathways (Fig. 2). Expression of the *metC-cysK* gene cluster is affected by the amounts of methionine and cysteine in the culture medium (M. Fernández et al., unpublished). High concentrations of these amino acids completely abolish transcription and result in *L. lactis* cells almost deficient of cystathionine lyase activity. Regulation of genes involved in methionine and cysteine biosynthesis is well-documented for *Escherichia coli* and *Salmonella typhimurium* (Weissbach & Brot, 1991; Kredich, 1992). Several of the methionine and cysteine biosynthesis genes in *E. coli* and *Salmonella* are under control of the transcriptional regulators MetR and MetJ, or CysB, respectively (Fig. 2). Recently, a LysR-type regulator has been identified in *L. lactis* that is involved in the activation of the *metC-cysK* gene cluster (M. Fernández et al., unpublished). Another lactococcal flavour-forming enzyme that is regulated at the transcriptional level is the branched-chain aminotransferase BcAT. Expression of the *bcaT* gene is repressed by high concentrations of branched-chain amino acids or methionine (Yvon, Chambellon, Bolotin, & Roudot-Algaron, 2000). These examples illustrate that the selection of culture conditions can dramatically influence the flavour-forming capacities of *L. lactis*.

Recently, it was demonstrated that the absence of parts of the flavour-forming pathways in individual strains can be complemented by using defined strain combinations (Ayad, Verheul, Engels, Wouters, & Smit, 2001). For instance, the combination of *L. lactis* B1157 and SK110 strains in milk resulted in a very strong chocolate-like flavour, due to high levels of 3-methyl butanal. In SK110, a proteolytic strain, the complete pathway from casein via leucine to 3-methyl butanal cannot proceed due to the lack of a decarboxylating enzyme in this strain (Fig. 4B). B1157 is a non-proteolytic strain and therefore unable to produce enough free amino acids that can serve as substrate for the subsequent transamination and decarboxylation steps (Fig. 4C). However, when B1157 and SK110 are incubated together, the strains complement each other with regard to their enzyme activities resulting in a high production of the chocolate flavour component 3-methyl butanal (Fig. 4D).

3. Genome analysis in silico

3.1. Genome sequencing and annotation

Analysis of complete genome sequences of bacteria is beginning to provide an insight into all encoded proteins, and hence into the entire potential of biosynthesis and metabolic routes, transport systems, regulatory systems, etc. of bacteria. One major finding in most bacterial genomes is that genes that are functionally related are often organized into clusters or operons. Examples are the genes encoding enzymes for an entire biosynthetic pathway, or encoding the subunits for protein complexes (e.g. ribosomes, multi-enzyme or transport-protein complexes). This high degree of gene

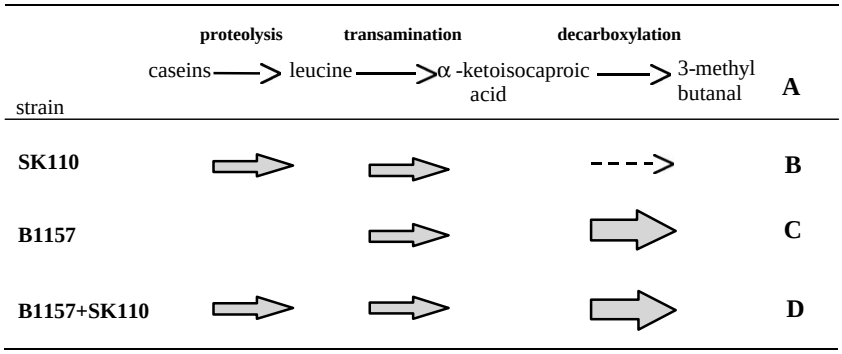

Fig. 4. Proposed pathway of flavour formation from leucine by enzymes from individual and combined lactococcal starter cultures B1157 and SK110 (Ayad et al., 2001). (A) General pathway for the breakdown of caseins to 3-methyl butanal; (B) enzymatic steps in SK110; (C) enzymatic steps in B1157; (D) enzymatic steps in defined culture B1157 + SK110. In the decarboxylation step, the dashed arrow represents very low decarboxylase activity, while the thick arrow represents high decarboxylase activity.

Table 1
Genome sequencing projects of lactic acid bacteria

Species	Strain	Use[a]	Genome size Mb	Institution	Year finished or projected
Lactococcus lactis	IL1403	CF/FF	2.3	INRA, FR	2001
Lactococcus lactis	MG1363	CF/FF	2.6	EU consortium	2001
Lactobacillus plantarum	WCFS1	FF/PR	3.3	WCFS, NL	2001
Lactobacillus acidophilus	ATCC 700396	FF,PR	1.9	Env.Biotech.Inst., California, USA	2001
Lactobacillus bulgaricus	?	FF	2.3	INRA, FR	2001
Lactobacillus helveticus	CNRZ32	FF/PR	2.3	Univ.Wisconsin, USA	2002
Lactobacillus rhamnosus	?	PR	?	NZ Dairy Board	2001
Lactobacillus johnsonii	NCC533	FF/PR	2.0	Nestlé, CH	2000
Streptococcus thermophilus	LMG18311	FF	1.9	UCL, Belgium	2002
Bifidobacterium longum	?	PR	2.3	Nestlé, CH	2001
Bifidobacterium breve	NCIMB8807	PR	2.3	UC Cork, IRL	2002

[a] FF = food fermentation; CF = cell factory; PR = probiotic.

organization facilitates *in silico* predictions of the potential for certain cellular processes, such as flavour formation from amino acids.

Genome sequencing of several lactic acid bacteria has begun in the past few years (Table 1), and at least two genomes have now been completed, i.e. *L. lactis* ssp *lactis* IL1403 (Bolotin et al., 2001; http://spock.jouy.inra.fr), and *Lactobacillus plantarum* WCFS1 (M. Kleerebezem et al., unpublished). Bioinformatics tools can be used to search in genomes for the different components that could contribute to flavour formation from amino acids as described above. Some examples will be given below, focussing on the genome of *L. lactis* IL1403, which is the only LAB genome publicly available at this moment. Each analysis generally begins with automated database searching, using the sequences of all encoded proteins in the genome to search in databases for homologs with known or predicted function. The function of newly identified protein sequences can often accurately be predicted with the help of database similarity search tools such as BLAST or FASTA. This generally leads to a prediction of function (or "annotation") for over 50% of the encoded proteins.

However, several cases exist in which additional tools are needed to assign protein function. For instance, a family of proteins with identical function may share too little sequence similarity to allow correct assignment with global or local sequence alignment tools. The definition of short sequence patterns or "fingerprints" including highly conserved amino acid residues can assist in predicting protein function. This is exemplified by the subtilase protein family to which also the extracellular proteases of LAB belong (Siezen & Leunissen, 1997). Three subtilase-specific patterns have been defined containing the conserved residues surrounding the three catalytic residues. By searching bacterial genomes with these three patterns several subtilases were identified, which were previously not recognized as members of this family (R.J. Siezen et al., unpublished).

Alternatively, it may be that proteins with a high structural and sequence similarity perform very diverse

functions. Here, the different functions may only depend on small differences that can be recognized by the presence of only a few highly conserved amino acid residues. The definition of patterns for a functional subfamily which include these highly conserved residues can be of crucial importance in identifying subfamily members. An example within the aminotransferase protein family will be discussed below (Section 3.5).

3.2. Biosynthesis routes of amino acids

Since branched-chain amino acids (Val, Ile, Leu), aromatic amino acids (Trp, Tyr, Phe) and sulphur-containing amino acids (Cys, Met) are important precursors of flavour compounds, the genome of *L. lactis* IL1403 was screened for genes encoding enzymes of the biosynthetic pathways for these amino acids. All of the enzymes of the cysteine and methionine biosynthesis pathways, as shown in Fig. 2 for *E. coli*, are also found to be encoded in the *L. lactis* IL1403 genome sequence. Obvious homologues of the *cysB, metR* and *metJ* genes, encoding LysR-type regulators, have not been identified in this genome yet, but they may be present since sequence homology is known to be low amongst LysR-type regulators.

All but one of the genes required for biosynthesis of Val, Ile and Leu are found to be present in a gene cluster *leuABCD-ilvDBNCA* (Chopin, 1993), and the final step in the pathway can be catalysed by a branched-chain aminotransferase, encoded by the *bcaT* gene (Fig. 3A). This clearly indicates that this *L. lactis* strain has at least the potential to synthesize all the branched-chain amino acids. The fact that most *L. lactis* strains nevertheless require branched-chain amino acids in the growth medium has been ascribed to mutations in biosynthesis genes making them inactive (Godon et al., 1993).

Biosynthesis of the three aromatic amino acids, starting from erythrose-4-phosphate and PEP, follows a common pathway up to chorismate (Fig. 3B). The seven *aro* genes required for this pathway are all present in *L. lactis* IL1403. From chorismate, the branching point, the seven genes required for the Trp biosynthesis pathway are also all present in this strain, and organized in a single cluster in the order *trpEGDCFBA*. In the *L. lactis* IL1403 genome, a gene for chorismate mutase, which converts chorismate to prephenate, has not been found yet, while the genes for further conversion of prephenate to both tyrosine and phenylalanine are present (Fig. 3B). This suggests that the pathway for biosynthesis of tyrosine and phenylalanine may be interrupted in *L. lactis* IL1403, but the interconversion of tyrosine and phenylalanine is still possible. Such predictions of course need to be supported by experimental studies of amino acid growth requirements.

3.3. Transport systems for peptides and amino acids

The number of transport systems for peptides and amino acids encoded in the genome of *L. lactis* IL1403 is shown in Table 2. Two oligopeptide ABC transport systems have been identified, i.e. OppABCDF and OptSABCDF. In both cases an operon of 5–6 genes encodes 2 intracellular ATP binding subunits, 2 membrane spanning subunits and 1–2 extracellular substrate binding subunits. In addition, a single di-/tripeptide transporter DtpT was found.

At least 25 different transport proteins for amino acids are encoded in the *L. lactis* genome (Bolotin et al., 2001). The substrate specificity of many of these amino acid transport systems cannot be predicted as yet, since the encoded proteins are only annotated as amino acid permease or transporter. However, some transport systems specific for charged amino acids (e.g. Arg, Lys, Glu), including two ABC transporters, and branched-chain amino acids are predicted (Table 2).

3.4. Peptidases

The different types of peptidases encoded in the *L. lactis* IL1403 genome are summarized in Table 3. These predictions are fairly sound, since the amino acid sequence identity is relatively high for peptidases, making it easy to find good matches in databases. These good matches in databases are generally to peptidases of lactococci and lactobacilli of which the function has often been experimentally verified with respect to substrate specificity (Kunji et al., 1996; Christensen et al., 1999).

No extracellular PrtP proteinase is encoded on the chromosome, while there are two genes encoding intracellular endopeptidases, i.e. PepF and PepO. Four aminopeptidases are encoded, two of which are rather specific, i.e. PepM and PepA, while the encoded PepC and PepN are more general aminopeptidases. Further-

Table 2
Predicted peptide and amino acid transport systems encoded in the genome of *L. lactis* IL1403

Transported peptide or amino acid	*L. lactis* IL1403	
	General transporter	ABC transporter
Oligopeptide	—	2
Di-/Tripeptide	1	—
Arginine	2	—
Lysine	2	—
Glutamine/glutamate	1	2
Branched-chain	1	—
Unknown amino acids	11	2

Table 3
Predicted peptidases encoded in the genomes of *L. lactis* IL1403

Peptidase	Specificity	Gene(s)	Number of Genes
Cell-envelope proteinase	$(X)_n \downarrow (X)_n$	*prtP*	0
		htrA	1
Endopeptidases			
Oligoendopeptidase F	$(X)_n \downarrow (X)_n$	*pepF*	1
Endopeptidase	$(X)_n \downarrow (X)_n$	*pepO*	1
Aminopeptidases			
Aminopeptidase M (Met)	$Met \downarrow (X)_n$	*pepM*	1
Aminopeptidase A (Glu/Asp)	$Glu/Asp \downarrow (X)_n$	*pepA*	1
Aminopeptidase C	$X \downarrow (X)_n$	*pepC*	1
Aminopeptidase N	$X \downarrow (X)_n$	*pepN*	1
Tri-/di-peptidases			
Tripeptidase	X↓X-X	*pepT*	1
Dipeptidase	X↓X	*pepD*	2
Dipeptidase	X↓X	*pepV*	1
Proline-specific peptidases			
Aminopeptidase P	$X \downarrow Pro\text{-}(X)_n$	*pepP*	1
Xaa-Pro dipeptidyl aminopeptidase	$X\text{-}Pro \downarrow (X)_n$	*pepX*	1
Proline iminopeptidase	$Pro \downarrow X\text{-}(X)_n$	*pepI*	0
Pro-Xaa dipeptidase (prolinase)	Pro↓X	*pepR*	0
Xaa-Pro dipeptidase (prolidase)	X↓Pro	*pepQ*	1
Total			14

more, four general di- and tripeptidase genes are found, encoding PepDA, PepDB, PepT and PepV (Table 3). The complement of proline-specific peptidases is not very high compared to lactobacilli (Kunji et al., 1996; M. Kleerebezem et al., unpublished) and it appears that *L. lactis* IL1403 has no specific enzymes like PepR and PepI for cleavage of N-terminal Pro residues from peptides.

3.5. Amino acid catabolism

Aminotransferases catalyse a key step in both the biosynthesis and catabolism of amino acids (see above). At least 12 aminotransferases are encoded in the genome of *L. lactis* IL1403 (Bolotin et al., 2001), some of which can catalyse transamination of aromatic, branched-chain and sulphur-containing amino acids. This specificity has been experimentally determined for the branched-chain aminotransferase BcAT and the aromatic aminotransferase ArAT (Yvon et al., 2000; Yvon & Rijnen, 2001), but not for several other encoded aminotransferases yet. Since their specificity is not directly obvious from database sequence comparisons, it was investigated whether more specific ''fingerprint'' patterns can be assigned to assist in predicting specificity.

The aminotransferase superfamily consists of PLP-dependent enzymes that catalyse the reversible transamination reaction with a broad variety of substrates and acceptor molecules. Moreover, some of these enzymes exhibit partially overlapping substrate specificities. Two excellent reviews are available that discuss the evolutionary relationships in the context of biochemical function and substrate specificity (Alexander, Sandmeier, Mehta, & Christen, 1994; Jensen & Gu, 1996). These have supported the classification of aminotransferases into at least 4 distinct families.

The family I aminotransferases is of particular relevance with respect to flavour formation since the aromatic aminotransferase (ArAT) of *L. lactis* belongs to this subfamily. Some other reactions catalysed by enzymes of this subfamily are transamination reactions with aspartate, alanine or histidine. The situation is further complicated by the fact that the substrate specificities of only a limited number of enzymes have been experimentally determined. Proteins belonging to this family I can be identified with the help of similarity searches or a conserved sequence pattern, i.e. PDOC00098 in the Prosite database (Hofmann, Bucher, Falquet, & Bairoch, 1999). In an extensive study by Jensen and Gu (1996) seven distinct subfamilies were defined that differ in both substrate specificity and protein sequence. Rijnen et al. (1999) found that the lactococcal ArAT contains 80% of the highly conserved residues of the aminotransferase subfamily Iγ and it was therefore classified as a member of this subfamily. The authors also suggest that this subfamily should be divided into at least two subclasses consisting of the enzymes specific for either aromatic amino acids or those specific for aspartate, respectively. By looking in

(A) **Aromatic amino acid aminotransferases**

```
                    ▼
TYRB_SALTY   134 NPTGADLTPSQWDAVIEIVKA
TYRB_ECOLI   134 NPTGADLTNDQWDAVIEILKA
PHHC_PSEAE   134 NPTGFDLSHDDWRRVLDVVRR
TYRB_PARDE   130 NPTGANLTLDQWAEIASILEK
ARAT_LACLA   129 NPTGVTYNREQIKDLAEVLKK

Pattern
                 NPTGA LN DDI  AA AAEA
                     V YS EEL  II IIKK
                     F  T SQW  LL LLRR
                               VV VV
```

(B) **Aspartate aminotransferases**

```
AAT_BACSP    155 NPTGMVYTREELEDIAKIALE
ASPC_BACST   132 NPTGMIYTAEELKALGEVCLA
ASPC_PSEAE   134 NPSGASLPRATWEALAELCMA
AAT_ECOLI    159 NPTGIDPTLEQWQTLAQLSVE
```

(C) **Class I aminotransferases in *B. subtilis***

```
PATA_BACSU   121 NPTGVTLSEEELKSIAALLKG
YUGH_BACSU   123 NPTGSVYSKEELNEIAEFAKK
ASPB_BACSU   124 NPTGVMYTEEELSALGEVCLE
YWFG_BACSU   138 NPTGAVADAAFYAKAAAFAKE
YHDR_BACSU   129 NPTGTVLSQKNIDDLGALLKE
PATB_BACSU   119 NPSGRSWSREDLLKLGELCLE
```

Fig. 5. Conserved sequence pattern in aromatic amino acid aminotransferases and target sites with aminotransferases. The asparagine residue that is involved in binding in the PLP cofactor is indicated with ▼. Residues differing from the pattern are shaded. (A) The target sequence in an alignment of 5 aromatic amino acid aminotransferases. The aromatic amino acid aminotransferase activity of TYRB_ECOLI, TYRB_PARDE, TYRB_SALTY, and TYRB_LACLA has been experimentally verified. (B) The target sequence in an alignment of 4 experimentally verified aspartate-specific aminotransferases. (C) The target sequence in an alignment of 6 class I aminotranferases found in the *B. subtilis* genome. *Note*: TYRB_SALTY, aromatic amino acid aminotransferase in *S. typhimurium* (Accesion number S71928); TYRB_ECOLI, aromatic amino acid aminotransferase in *E. coli* (P04693); PHHC_PSEAE, putative aromatic amino acid aminotransferase in *Ps. aeruginosa* (P43336); TYRB_PARDE, aromatic amino acid aminotransferase in *P. denitrificans* (P95458); ARAT_LACLA, aromatic amino acid aminotransferase in *L. lactis* (AF146529); AAT_BACSP, aspartate aminotransferase in *Bacillus* sp. (P23034); ASPC_BACST, aspartate aminotransferase in *B. stearothermophilus* (Q59228); ASPC_PSEAE, putative aspartate aminotransfrease of *Ps. aeruginosa* (F83024); AAT_ECOLI, aspartate aminotransferase in *E. coli* (P00509); PATA_BACSU (P16524), YUGH_BACSU (BG12362), ASPB_BACSU (P53001), YFGH_BACSU (P39643), YHDR_BACSU (BG13024), PATB_BACSU (Q08432) are putative aminotransferases found in the *B. subtilis* genome.

more detail at this subfamily, we were able to define a sequence pattern that is specific for the aromatic amino acid aminotransferases. The software PRATT (Jonassen, 1997) was used to find conserved patterns in a set of aromatic amino acid aminotransferases that were either experimentally verified with respect to substrate specificity, or well-annotated (see Fig. 5). The patterns were further refined in an iterative process using sequence alignments of these proteins together with a reference set of aspartate aminotransferases. This procedure generated the sequence pattern **N**-P-T-G-[AVF]-x-[LY]-[NST]-x-[DES]-[DEQ]-[ILW]-x(2)-[AILV]-[AILV]-x-[AILV]-[AILV]-[EKR]-[AKR] that is specific for aromatic amino acid aminotransferases. The N-terminal part of the pattern is highly conserved among class I aminotransferases and contains the essential asparagine (bold) that is involved in binding of the PLP cofactor. The C-terminal part of the pattern contains the information for discriminating the aromatic amino acid-specific enzymes (Fig. 5A) from the aspartate-specific enzymes (Fig. 5B).

The generated sequence pattern was tested on the genome of *Bacillus subtilis* 168 (Kunst et al., 1997), which contains at least 6 genes that may encode an aromatic amino acid aminotransferase. The aromatic amino acid aminotransferase pattern (Fig. 5A) was used to predict which of these genes encodes the aromatic amino acid aminotransferase. PatA appears to be the most likely candidate, since it contains all of the conserved residues with the exception of a Gly residue that is found at position 142 (Fig. 5C). Expanding such analyses to other genomes, including those of LAB, combined with experimental verification of the predicted substrate specificities will show the potential of such patterns for predicting aminotransferase function. Furthermore, these profiles may serve as targets for specific probes that can be used in the screening of culture collections for the presence of strains carrying or expressing the desired genes.

4. Conclusions

In summary, amino acid catabolism is important in flavour development of dairy products as it generates desired, and sometimes also non-desired, flavour compounds. Recently, genetic techniques have been applied to unravel the effects of specific enzymes on amino acid formation and catabolism, and on the formation of specific flavour compounds.

Whole genome analysis should expand our knowledge of flavour-forming pathways and mechanisms in different bacteria. It will soon allow prediction of the flavour-forming capacity of various lactic acid bacteria, and secondly lead to the design of probes for high-throughput screening and strain selection in the future. Since flavour production is highly species- and strain-specific amongst various lactic acid bacteria this offers a new potential for industrial applications.

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