Engineering of Aromatic Amino Acid Metabolism in *Saccharomyces cerevisiae*

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PROEFSCHRIFT

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GENERAL INTRODUCTION

1.0 Amino acids as building blocks for biomass constituents

Amino acids are α-substituted carboxylic acids, the building blocks of proteins. Asparagine was the first amino acid discovered in 1806, whereas threonine was the last one to be identified in 1938. Amino acids carry common names and have sometimes trivial names. The names of the amino acids are given mostly according to the sources they were originally isolated from. For example; glutamate was for the first time isolated from wheat gluten, whereas asparagine was first found in asparagus (66).

The general structure of an amino acid is presented in Fig. 1. All 20 common amino acids have a carboxyl group and an amino group, which are attached to the same carbon atom. The difference between the various amino acids lies in the side chains, which is called "R group" (Fig. 1). The R-groups vary in size, structure and electric charge (66).

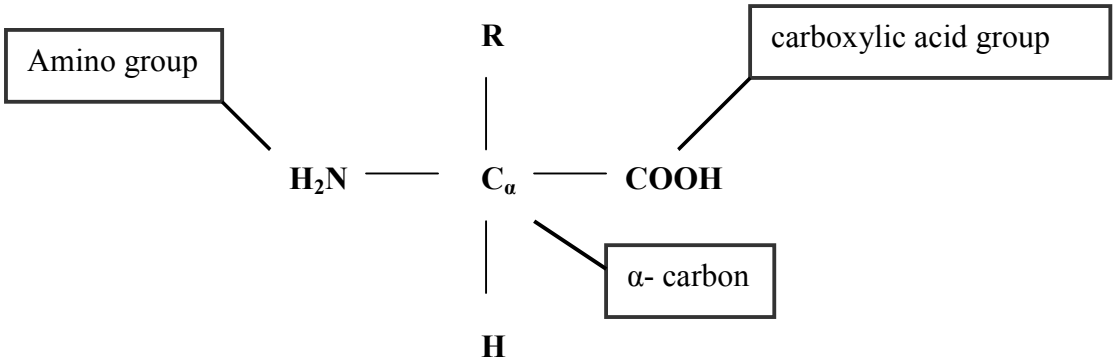

FIG. 1. General structure of an amino acid.

An important property of naturally occurring amino acids is their chirality. Chirality is defined as the ability of a molecule to rotate the plane of polarized light to the right (dextrorotatory) or to the left (levorotatory). These two mirror-image forms of amino acids are called D-isomer and L-isomer respectively (66). Life has developed in a way to favour the use of the L-form instead of D-form in case of proteins. On the other hand, D-forms of amino acids are also natural compounds. They are found in the cell walls of bacteria (58). D-amino acids are important building blocks in the production of pharmaceuticals, food additives, herbicides and other agrochemicals (66).

Apart from 20 common amino acids, there are also amino acids that are modified after the protein is synthesized. For example 4-hydroxyproline and 5-hydroxylysine, which are the derivatives of proline and lysine

respectively, are abundant in collagen, a fibrous protein of connective tissue. In addition to the amino acids synthesized by posttranslational modifications in proteins, there are some other amino acids such as ornithine and citrulline, which are the intermediates in the biosynthesis of arginine and the urea cycle (66).

When a few amino acids are covalently connected through a condensation reaction, the product of this reaction is called a peptide. When more than three or four amino acids make bonds, the product is named as polypeptide. Peptides also have industrial importance such as aspartame, an artificial sweetener, which is L-aspartyl-L-phenylalanine methyl ester. Polypeptides can act as hormones such as oxytocin, which is a vertebrate hormone and secreted by the pituitary gland and glucagon, which is a pancreas hormone are examples (66). Another one is insulin that is nowadays produced with genetically modified *Saccharomyces* (22).

Amino acids have a broad variety of characteristics with respect to nutritional value, taste, and medicinal action and therefore have many potential uses. A summary of the application areas is presented in the following section.

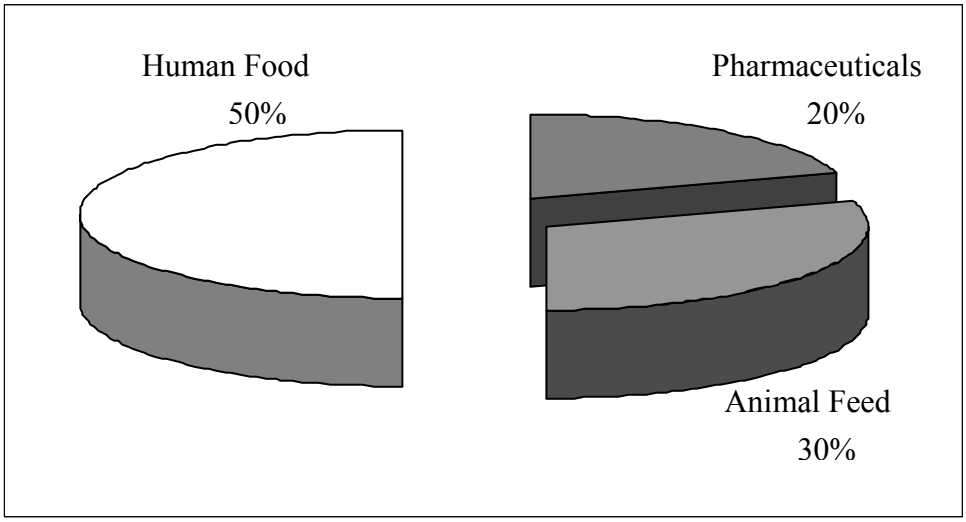

FIG. 2. Representation of amino acid application areas in percentages of amounts used (64)

2.0 Application areas of amino acids

Amino acids are used in a variety of ways and they have extensive industrial applications (12,45) (Table 1). They find applications as feed and food additives, taste and aroma enhancers, pharmaceuticals, components of drugs, dietary formulas, nutraceuticals and as ingredients in cosmetics (7). In addition, fusel alcohols derived from amino acids by fermentation such as 3-methyl-1-butanol, 2-methyl-1-butanol and 2-methyl-1-propanol are compounds which result from the degradation of L-leucine, L-isoleucine and L-valine respectively. They contribute to the characteristic bread flavour (30). Phenylethanol which exhibits a rose-like aroma is formed

from phenylalanine. It has significant industrial value not only in cosmetics but also in the formulation of beverages and food products (27,85). Below the application areas of amino acids are discussed as two categories:

2.1 Food & Feed

The main application field of amino acids nowadays is in food. About fifty percent of amino acids manufactured worldwide are applied in human food production (Fig. 2). The amino acids with the largest market volumes are glutamic acid, lysine and methionine (64) (Table 1).

The microbial production of amino acids dates back to 1957, when a group of scientists isolated a soil bacterium (a Corynebacterium sp.) capable of excreting large amounts of glutamic acid. The main application of glutamic acid in the food industry is in the form of a salt; the flavour enhancer mono sodium glutamate (MSG). A Japanese scientist first discovered MSG in 1908, He observed that MSG is responsible for the characteristic flavour of kelp, which is a traditional Japanese dish; this taste is known as "Umami" in Japan (48). In recent years, special receptor proteins have been identified in the human tongue for sensing the taste of MSG (67).

L-Phenylalanine is a key amino acid in the manufacturing of aspartame, an artificial sweetener. Aspartame is 150-200 times sweeter than sucrose. *Corynebacterium glutamicum* and *E. coli* are the preferred organisms for the microbial production of phenylalanine by fermentation (53). The main producers of L-phenylalanine are Nutrasweet Kelco (USA), Ajinomoto Co (Japan), Amino GmbH(Germany) and Degussa (Germany) (7).

Amino acids such as alanine, arginine and glycine have great significance in the traditional food applications in the Far East. L-Alanine has found application as flavour for the traditional Japanese rice wine Sake and in alitame (Pfizer, Inc., alitame (brand name Aclame™)) as sweetener for cakes and pastries. L-arginine and L-glycine serve as a flavour enhancer in many products (green tea, meat, fish and soy sauce) (64).

About 30% of the worldwide production of amino acids is used for animal feed applications (Fig. 2) because livestock diets, which mainly consist of corn and soy products, are deficient in various essential amino acids. Therefore supplementation of amino acids is an absolute necessity in pig and poultry farming. Methionine, which is the third largest in market volume (Table 1), not only serves as a food additive but also and unlike glutamic acid, it is used as animal feed especially for poultry and pigs (64).

Lysine is also an essential amino acid that is required in the diets of animals. It shows a yearly increase of 7-10 % in production. The supplementation of animal diet is done by adding feed ingredients such as soybean meal, which is very rich in lysine, or by direct addition of lysine. Kyowa Hakko (Japan) and ADM (USA) are the market leaders in lysine production (64). L-threonine is also used for balancing the diets of livestock. In contrast to the other amino acids produced, *E.coli* is exclusively used in the production of threonine (15). Main producers of L-threonine are Ajinomoto, Kyowa Hakko, ADM and Degussa.

TABLE 1. Market, use and production method of some amino acids (Modified from (53))

Amino acid	Market volume* (tons)	Major use	Production method
Arginine	1.000	Pharmaceutical	*In vivo* production via microbial fermentation.
Aspartic Acid	10.000	Sweetener	*In vitro* biocatalysis
Cysteine	3.000	Food Additive	Extraction *In vitro* biocatalysis
Glutamic acid	1000.000	Flavour Enhancer	*In vivo* production via microbial fermentation.
Glycine	22.000	Food Additive	Chemical Synthesis
Leucine	500	Pharmaceutical	*In vivo* production via microbial fermentation.
Lysine	600.000	Feed Additive	*In vivo* production via microbial fermentation.
Methionine	400.000	Feed Additive	Chemical Synthesis
Phenylalanine	10.000	Sweetener	*In vivo* production via microbial fermentation.
Threonine	20.000	Feed Additive	*In vivo* production via microbial fermentation.
Tryptophan	500	Pharmaceutical	*In vivo* production via microbial fermentation.
Valine	500	Pharmaceutical	*In vivo* production via microbial fermentation.

*(Data are average value of 2002-2003)

2.2 Pharmaceuticals

20 % of the amino acids produced are used in medical applications. Amino acids have found applications in the formulation of post and pre-operative nutrition. Standard infusion liquid contains at least eight amino acids, which are essential for the human body.

L-Serine is used in the formulations of antibiotics. L-tyrosine, which has an important biological function as a precursor of thyroid hormone is used for the treatment of the diseases of thyroid gland (http://www.standardvitanet.com/ioandty120ve.html). L-cysteine, a sulphur containing amino acid, is essential for glutathione and taurine synthesis (antioxidants for the liver's detoxification process) (http://www.aminoacidpower.com/osc/product_info.php?cPath=3_23&products_id=180). It is important for

skin, hair and collagen formation, detoxification of toxic compounds & heavy metals, and immune support. Therefore it has found applications in the cosmetics, specifically in hair care products (57,64).

3.0 Production of amino acids

The mode of production of amino acids varies from full chemical synthesis to a microbial fermentation process (Table 1). Three main types of production processes are carried out in the industrial practice: amino acid extraction from protein hydroylsates, chemical synthesis and biochemical synthesis (12,45).

3.1. Extraction

This method is based on the availability, in large quantities of proteinaceous material, from which the amino acid can be obtained via hydrolysis. In the past, this method was prevailing. It is still being used for some amino acids such as L-cysteine, L-leucine, and L-tyrosine. Human hair and chicken feather which are rich in cysteine but also blood meal or soybeans are some of the sources from which these amino acids are extracted (45). First protein hydrolysates are obtained by acid-hydrolysis from the sources mentioned above, active charcoal is used for neutralization and decolourization. Finally electrodialysis is applied to separate amino acids into fractions (73). The combination of extraction with other methods can increase the efficiency and productivity of the production. In the case of L-cysteine for instance, commercial production is performed by enzymatic synthesis in addition to the extraction (45).

3.2 Chemical synthesis

Four out of twenty amino acids are still produced via chemical synthesis (Table 1). Via chemical synthesis, a mixture of D- and L- forms of amino acids is produced. Additional steps are required to get the required L-isomers (45).

3.3 Biochemical methods

Biochemical methods can be summarized under two categories; 1-) *in vitro* biocatalysis and 2-) *in vivo* production via microbial fermentation.

3.3.1 *In vitro* biocatalysis

Enzymes are very helpful catalysts for the enantioselective synthesis of amino acids and are used to obtain optically pure D- and L-amino acids. As compared to chemical synthesis, fewer by products are formed which eases the downstream processing. The enzymes used for this purpose are obtained from microorganisms. However, industrial application of enzymes is limited by thermal and mechanical stability. In order to overcome this hurdle, immobilized enzyme systems have been developed (57). The cost of starting substrates is a very important additional limitation for the applicability of this method.

Enzymatic synthesis of L-aspartic acid can be given as a good example for the enzymatic method. In the case of L-aspartic acid, immobilized *E. coli* cells that express aspartase convert ammonia and fumarate to

aspartic acid in a continuous process. Other amino acids such as L-alanine, L-lysine and L-phenylalanine have also been produced commercially by enzymatic methods. In the case of L-alanine, a decarboxylase from *Pseudomonas dacunhae* is used to convert L-aspartate to L-alanine with a release of carbon dioxide. In this process, immobilized cells are used and a titer of 400 $g.l^{-1}$ L-alanine has been reported (57).

3.3.2 *In vivo* production via microbial fermentation

Most of the amino acids are produced by fermentation. The carbon source, fermentation yield, purification method and productivity in the overall process determine the economy of this method. This method has been proven successful for the bulk production of amino acids such as monosodium glutamate, L-lysine-HCl and L-threonine (45). In the fermentation processes, the production strains are fed with low cost carbon sources such as sucrose or glucose. Batch or fed-batch cultivation is the preferable type of process for amino acid production. L-phenylalanine production by *E. coli* by fed-batch process which resulted in a titer of 34 $g.l^{-1}$ can be given as an example (31).

The strains used in amino acid fermentations are a very important factor for overall process economy. Therefore much emphasis is given on the selection of suitable production organisms and their improvement. Selection of strains with a high yield on the carbon source is never an ending story (45).

The classification of production strains falls into three categories: (1) wild type strains having the ability to produce specific amino acids (2) auxotrophic or regulatory mutants which lost their feed-back inhibition mechanisms, and (3) strains which have gained the ability to overproduce amino acids by overexpression of genes that are responsible for the synthesis of enzymes in rate-limiting steps (45).

Commonly, improvement of amino acid producing microorganisms has been achieved by combining the desired genetic and physiological changes in one host. This results in higher production and lowered by-product formation (45). In the following section, subjects will be discussed in more detail.

4.0 Engineering of amino acid production

Since there is a growing interest in amino acid production, strain and process improvement are of considerable importance. Although strains isolated from nature may produce significant amounts of amino acids, the concentrations, yields and rates are generally much too low for a commercial process. Before the birth of recombinant DNA technology in the 1980s, “classical strain improvement” was the only approach available.

4.1 Classical strain improvement

4.1.1 Selection

Charles Darwin postulated in 1859; "The key is man's power of accumulative selection: nature gives successive variations; man adds them up in certain directions useful to him”. This has been exploited by several researchers to obtain microbial strains with the desired characteristics for amino acid production.

In 1957, a soil bacterium “*Corynebacterium”* that excreted a large amount of glutamate into the medium was discovered (49). Soon similar bacteria were isolated from nature that produced D or L-alanine or L-valine

(*Brevibacterium flavum, B. lactofermentum, B. thiogenitalis, Microbacterium ammoniaphilum*). These discoveries opened the door for a new era in industry: microbial production of amino acids by fermentation (32). Subsequently the application of a large variety of amino acid producing strains commenced. When *Corynebacterium spp*. were started to be used for the large-scale production of glutamic acid, MSG prices dropped from $ 8 to $ 2 worldwide (45).

The optimisation of the production of L-amino acids with *Corynebacterium spp.* has become one of the most popular targets of industrial microbiology. L-glutamic acid and L-lysine are the bulk products but also L-valine, L-isoleucine, L-threonine, L-aspartic acid and L-alanine are commercially produced by fermentation (35). *Corynebacterium spp*. are superior compared to *Enterobacteriaceae* because of the simplicity of their metabolism (52) For example: there is only one aspartokinase enzyme in *C. glutamicum,* whereas in *E. coli,* there are three aspartokinase isoenzymes which are subject to feed back inhibition by lysine, or threonine or isoleucine which very much complicates the overproduction of amino acids with *E.coli* (53).

4.1.2 Mutagenesis and selection

Random mutagenesis and screening

The fundamentals of this method rest on inducing mutations and randomly picking the survivors from the population. A variety of chemical or physical agents, called mutagens, are used to obtain changes in genotype. The goal of the mutagenesis is to get the highest possible number of desired mutations whilst avoiding undesired additional mutations caused by mutagens.

Chemical mutagens can be grouped into three categories depending on their mode of action:

(1) Mutagens which affect non-replicating DNA, (2) analogs showing structural similarity to the bases that are incorporated into replicating DNA and (3) frameshift mutagens.

Mutagens such as HNO_3, hydroxylamine (NH_2OH) and alkylating agents (EMS, NTG, and mustard gas) affect non-replicating DNA. Besides deaminating adenine to hypoxanthine and cytosine to uracil, HNO_3 also induces crosslinks between two DNA strands. EMS and NTG form alkylated bases in DNA. These agents change the DNA through transversions, deletions and frame-shift mutations (12). In several cases, the mutagen nitrosoguanidine (NTG) has been used to provoke the highest mutations per survivor (4). Several examples can be given for overproducing mutants resulting from the use of NTG as a chemical mutagen. Base analogs are another type of mutagens which can be incorporated into the replicating DNA such as 5-bromouracil or 2-amino purine. They incorporate into the replicating DNA by replacing the bases thymine and adenine. Sometimes acridine dyes are used. Their mode of action lies in causing frameshift mutations (12).

The success of random mutagenesis and selection is based on screening strategies. The population obtained after mutagenic treatment is heterogeneous. Therefore individual clones from such populations must be examined to identify the most desired phenotype. The most fundamental techniques for screening are spatial separation and an assay system to characterize the phenotype of interest. High throughput screening is an efficient method to achieve this. An example of high throughput screening is a combination of flow cytometry and cell sorting. This is a very rapid technique to analyse single cells. The analysis and detection is done as cells

flow in a liquid medium through the focus of a laser beam surrounded by an array of detectors. With the help of different fluorescent stains this technique makes it possible to distinguish between different cells in large cell populations. Automated screening is of course a very handy tool to minimize labour cost (74).

In several studies, the aim was to obtain mutants, resistant to feedback inhibition and as a result overproduction of the desired amino acid. The selection was generally done by using amino acid analogues. For example, phenylalanine producers have been constructed as tyrosine auxotrophs of *C. glutamicum* and as mutants of *B. flavum* resistant to phenylalanine analogues such as p-aminophenylalanine and p-fluorophenylalanine. The highest production was 25 g. l^{-1} with a *B. lactofermentum* mutant, which was resistant to PFP, 5- methyltryptophan and sensitive to decoynin, an analogue of purine (79).

Although yeasts are not generally regarded as amino acid producing organisms, *S. cerevisiae* has been studied for amino acid production. In one study (16), *S. cerevisiae* mutants were obtained by applying NTG and EMS mutagenesis. These mutants did not have feedback inhibition of aspartokinase (catalyzing the first reaction of the threonine-methionine biosynthetic route). Two other genes, responsible for the excretion of threonine were mutated as well. As a result of these multiple mutations intracellular threonine concentrations increased up to 20-fold.

4.2 Metabolic engineering of amino acid production

In contrast to classical strain improvement, the metabolic engineering approach tries to understand the metabolic network and use this knowledge for improving strain properties. This is evident from the definition of Bailey; "Metabolic engineering is a field of scientific inquiry aiming at the directed improvement of cellular properties through the modification of specific biochemical reactions or introduction of new ones using recombinant DNA technology"(2). Application of recombinant DNA techniques makes it possible to gain knowledge of the relevant steps and regulation mechanisms in microbial metabolism and to (subsequently) use this knowledge to improve the productivity of conventional strains or to develop new strains (25).

Metabolic engineering has many application areas. Recombinant DNA technology and traditional mutagenesis plus selection have been key factors in the construction of strains that can produce high levels of amino acids. For example, final concentrations (kg. m^{-3}) have been reported for: L-threonine, 100; L-isoleucine, 40, L-leucine 34; L-valine 31, L-phenylalanine 28; L-tryptophan, 55; L-tyrosine 26; L-proline, 100, L-arginine, 100 and L-histidine, 40 (17).

Before developing strategies to improve amino acid production, it is essential to understand the biochemical reactions involved and how these work in cooperation. Key parameters are: substrate uptake, central metabolic pathways, amino acid excretion, global regulatory networks, energy & redox balance, amino acid catabolism pathways etc. (53) (Fig. 3).

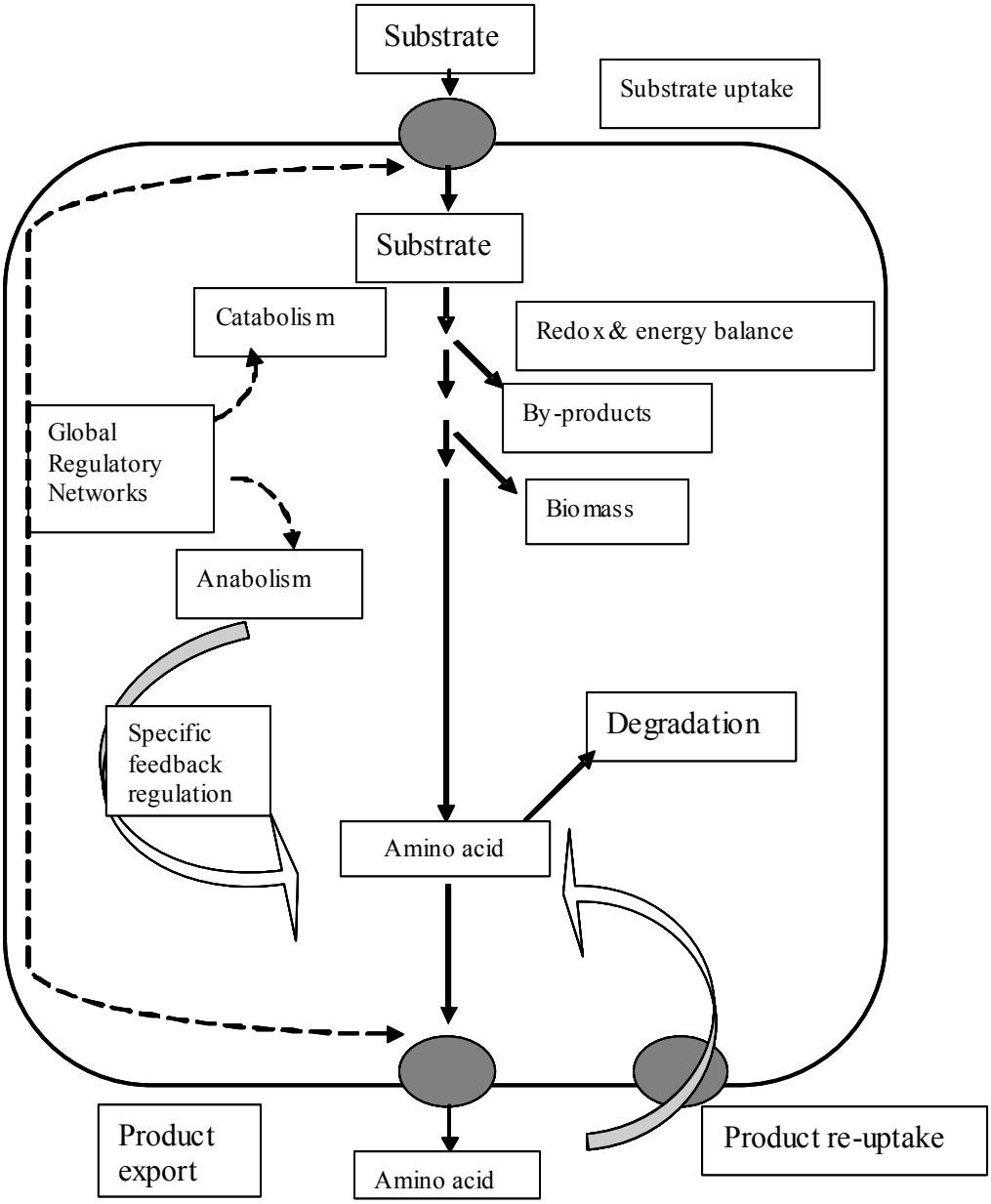

FIG. 3. Basic steps in amino acid production (53)

Several strategies have been followed for improving microbial amino acid production via metabolic engineering:

a-) Modification of product pathways

i) Increasing the flux through the steps in the biosynthetic pathway that exert a high degree of flux control.

ii) Amplification of a branch point enzyme in order to shift the metabolic flow towards the desired direction of amino acid production.

iii) Introduction of heterologous enzymes which have a different catalytic mechanism.

b-) Engineering of the central metabolism

Since the central metabolism provides the precursors for amino acid production, it is essential to engineer central metabolism for channelling the precursors required for amino acid production into the biosynthetic pathways. Therefore calculation of flux distribution, theoretical yield, ATP and redox balances should be determined. Thereafter the redirection of the fluxes can be attempted for constructing strains with desired properties.

c-) Engineering of transport processes

Both the uptake of nutrients and product excretion are possible limitations in amino acid production. Detailed knowledge of these processes is a necessity to tackle these limitations (25,52).

4.3 "OMICS" as a tool for the improvement of amino acid production

In the last decades, straightforward approaches such as deletion and overexpression of genes have been used to improve strains. Nowadays with the availability of complete sequence of more than 80 microbial genomes, powerful analytical tools can be applied to keep track of the results of the genotype changes induced in the organism (68). These tools are: DNA microarrays by which quantification of all mRNA levels is achieved, Proteomics, in which the amount of proteins is analysed by 2D-gel electrophoresis followed by identification of the proteins via MALDI-TOF MS (matrix assisted laser desorption ionization-time of flight mass spectrometry), Metabolomics makes it possible to detect and quantify relevant metabolites by GC-MS or LC-MS (68).

In the field of amino acid production; focus was initially on *Corynebacterium glutamicum.* DNA microarrays were used for analysing the expression of the genes of selected transport systems, central carbon metabolism, product formation and regulatory mechanisms (44).

Via MALDI-TOF-MS-based peptide finger printing, a proteomic map of 200 cytoplasmic and membrane associated proteins of *C. glutamicum* has been obtained (75).

4.4 Optimization of process conditions

The cultivation of microorganisms used in the production of amino acids is performed in a constant environment where parameters are carefully controlled. Parameters like medium composition, pH of the culture,

oxygen supply, feed rate of carbon source, and process temperature are main targets for control and optimisation (35). Three basic methods are available for industrial amino acid fermentations: batch, fed-batch and continuous fermentations.

In batch fermentations, a short fermentation time is applied to avoid accumulation of unwanted metabolites in the stationary phase (35). In fed-batch fermentation, which is the standard process for the production of biochemicals, the parameters that require attention are maintenance of limiting substrate concentration and carefully controlled oxygen supply. When the oxygen supply becomes insufficient, production of unwanted by-products such as lactate or acetate may occur in bacterial cultures (35).

In repeated fed batch, 60-95% of the culture is replaced by a fresh medium. The advantage of this method is that no new inoculum has to be prepared and prior sterilization of the bioreactor is by-passed (70). Another advantage of repeated fed-batch processes is the increased reactor productivity as a result of the shortening of the lag phase and reduction of the preparation times between the batches, thus reducing the variable costs. (36).

Although continuous fermentations have superiority over other methods by offering the highest average volumetric productivity, they have one major disadvantage: the occurrence of variants of the parent production strain by back mutation or loss of expression cassettes (53). Additionally, contaminations and phage infections can be very serious problems. Use of fresh starting material for each run, and the use of stable mutants are required to overcome this hurdle.

In order to improve the process conditions in continuous cultures, sometimes two or more reactors are used in a cascade bioprocess. This involves a growth phase in the first reactor whereas in the second reactor production takes place. Apart from the mode of cultivation, the composition of the medium is a crucial factor for success. For example carbon and phosphate double limitation instead of single limitation increased the production of L-lysine from 3.18 to 3.75 g lysine-HCl $.h^{-1}$ (13).

Separation of amino acids from the culture broth is also an important issue. Centrifugation and filtration are the preferred methods. Poor centrifugation behaviour of cells may be improved by the application of ionic charges on the surfaces of microorganisms. In filtration, parameters such as the properties of the filtrate, characteristics of the solid particles, pressure applied and effects of antifoaming agents on filtration should be taken into consideration. The filtration efficiency may be increased also by the use of filter-aids. These filter-aids improve the porosity of a resulting filter cake leading to a higher flow rate.

For further purification, chromatographic separation and concentration-crystallization methods are applied (45). When the amino acid is used to supplement cattle feed, no further purification is required and the cell-free culture broth can be spray-dried.

5.0 Aromatic amino acid metabolism and its regulation in bacteria

Early studies about the aromatic amino acid biosynthesis generally focused on prokaryotic organisms, mainly on the tryptophan branch. The prokaryotic operon concept of regulatory mechanisms does not apply to eukaryotes (8). There are two major differences between eukaryotic and prokaryotic regulation of amino acid

synthesis, those are: the more complex arrangement of the genetic material and compartmentation of the cellular space.

In bacteria such as *E .coli*, three unlinked genes *aroF, aroG* and *aroH* (Fig. 4) are in charge of the regulation of the common aromatic pathway. Those three genes encode isozymes of the first enzyme, DAHP synthase. Feedback inhibition is the major control mechanism *in vivo* (62). In *E. coli*, all the genes of the tryptophan branch of the pathway, are arranged in the tryptophan operon (Fig. 4) (8). In all eukaryotic organisms, however, the tryptophan genes are scattered over the genome. Every one of them requires its own regulatory signal sequences (42). In the next section, aromatic amino acid biosynthesis and its regulation in *S.cerevisiae* will be discussed in more detail.

In Cyanobacteria, coryneform bacteria (*Brevibacterium spp.* and C*orynebacterium spp*.), and some spore forming Actinomycetes, phenylalanine is produced via phenylpyruvate and tyrosine from arogenate and not from hydroxyphenyl pyruvic acid as in *E. coli* and *S. cerevisiae* (10,79). DAHP synthase enzyme is strongly inhibited by phenylalanine and tyrosine. Furthermore, chorismate mutase also shows sensitivity towards phenylalanine and tyrosine. Prephenate dehydratase and anthranilate synthase are also further targets. They are feedback inhibited by the end products of the pathway (43).

6.0 Aromatic amino acid Metabolism and its regulation in *S. cerevisiae*

6.1 Chorismate pathway (Common genes in phenylalanine, tyrosine and tryptophan pathway)

6.1.1 *ARO3* and *ARO4*

The first reaction of the shikimate pathway is performed by 3-deoxy-D-arabino-heptulosonate (DAHP) synthase that catalysed the condensation of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) (Fig. 5). Two genes (*ARO3, ARO4*) encoding differentially feedback inhibited DAHP synthase are found in *S. cerevisiae.* On one hand, Aro3p is reported as inhibited by phenylalanine and on the other Aro4p is feedback inhibited by tyrosine (8,78).

6.1.2 *ARO1* and *ARO2*

The five reactions leading from DAHP to 5-enolpyruvoylshikimate 3-phosphate (EPSP) are catalyzed by a pentafunctional enzyme, which is encoded by *ARO1* (55,63). *ARO1* presents similarities with the *aroA, aroB, aroD* and *aroE* from *E. coli* that catalyzed the individual step of the EPSP synthesis (8,23,24). The first reaction catalysed by the product of the *ARO1* gene converts DAHP into 3-dehydroquinate (DHQ). This step is performed by the DHQ synthase. Sequentially, the DHQ is converted into 3-dehydroshikimate, shikimate, and shikimate-3-phosphate, 5-enoylpyruvyl shikimate-3-phosphate by the 3-dehydroquinate dehydratase, the shikimate dehydrogenase, shikimate kinase and the 5-enolpyruvylshikimate-3-phosphate synthase respectively. This enzyme has been reported to have applications such as target of herbicide action (56). Finally, the last reaction is catalyzed by the chorismate synthase encoded by *ARO2* that converts EPSP to chorismate (8,55).

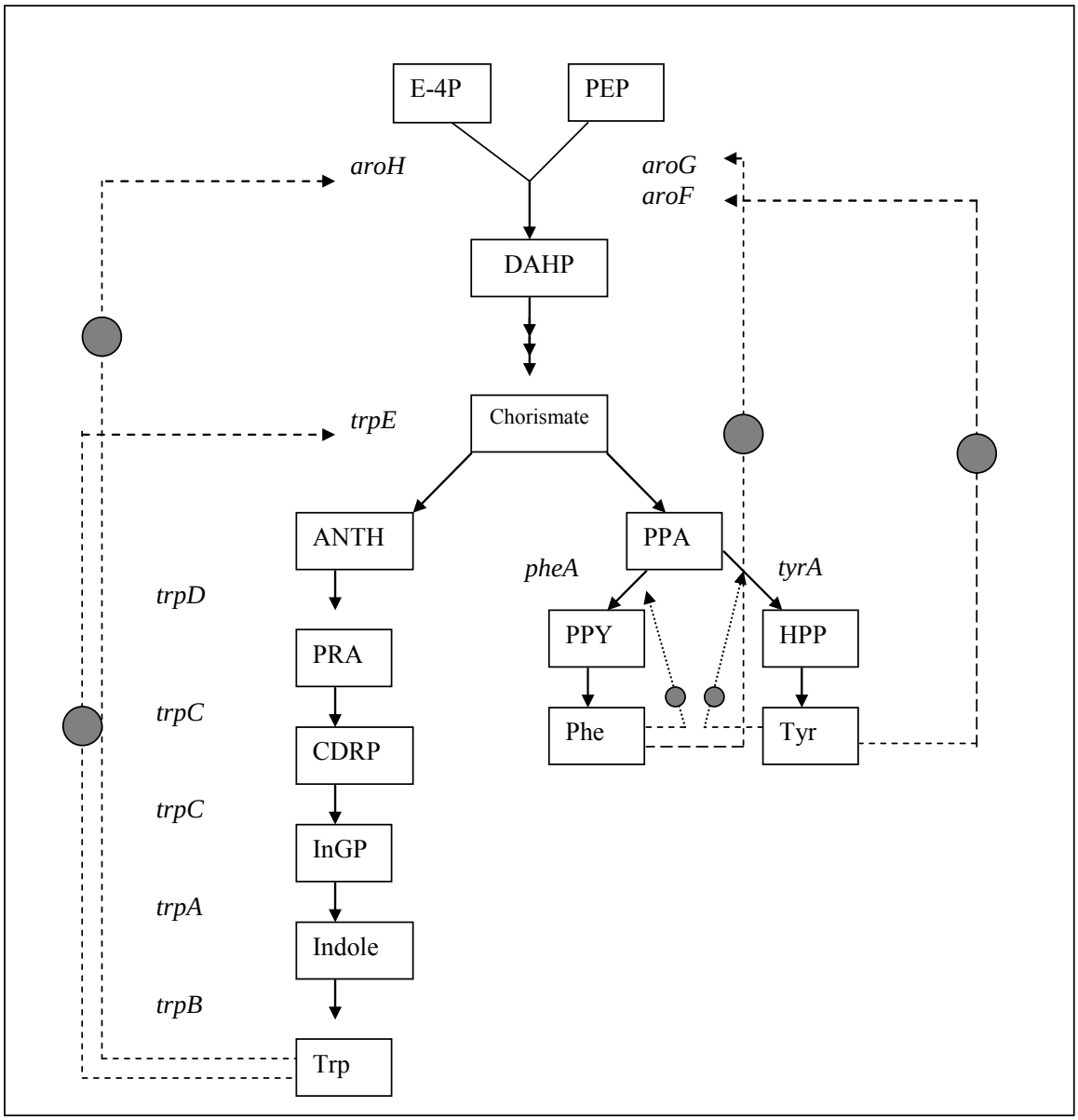

FIG. 4. Aromatic amino acid biosynthesis in *E. coli*. Full circles represent feed-back inhibition. Abbreviations in italics denote the genes. E-4P: erythrose 4-phosphate, PEP: phosphoenol pyruvate, DAHP: 3-deoxy-D-arabino-hetulosonate, ANTH: anthranilate, PRA:phosphoribosyl anthranilate, CDRP: 1-(o-carboxyphenylamino-1-deoxyribulose 5-phosphate, InGP: indole 3-glycerol-phosphate, PPA: prephenate, PPY: phenylpyruvate, HPP: 4-hydroxyphenylpyruvate, Trp: tryptophan, Phe: phenylalanine, Tyr: tyrosine. Modified from (5).

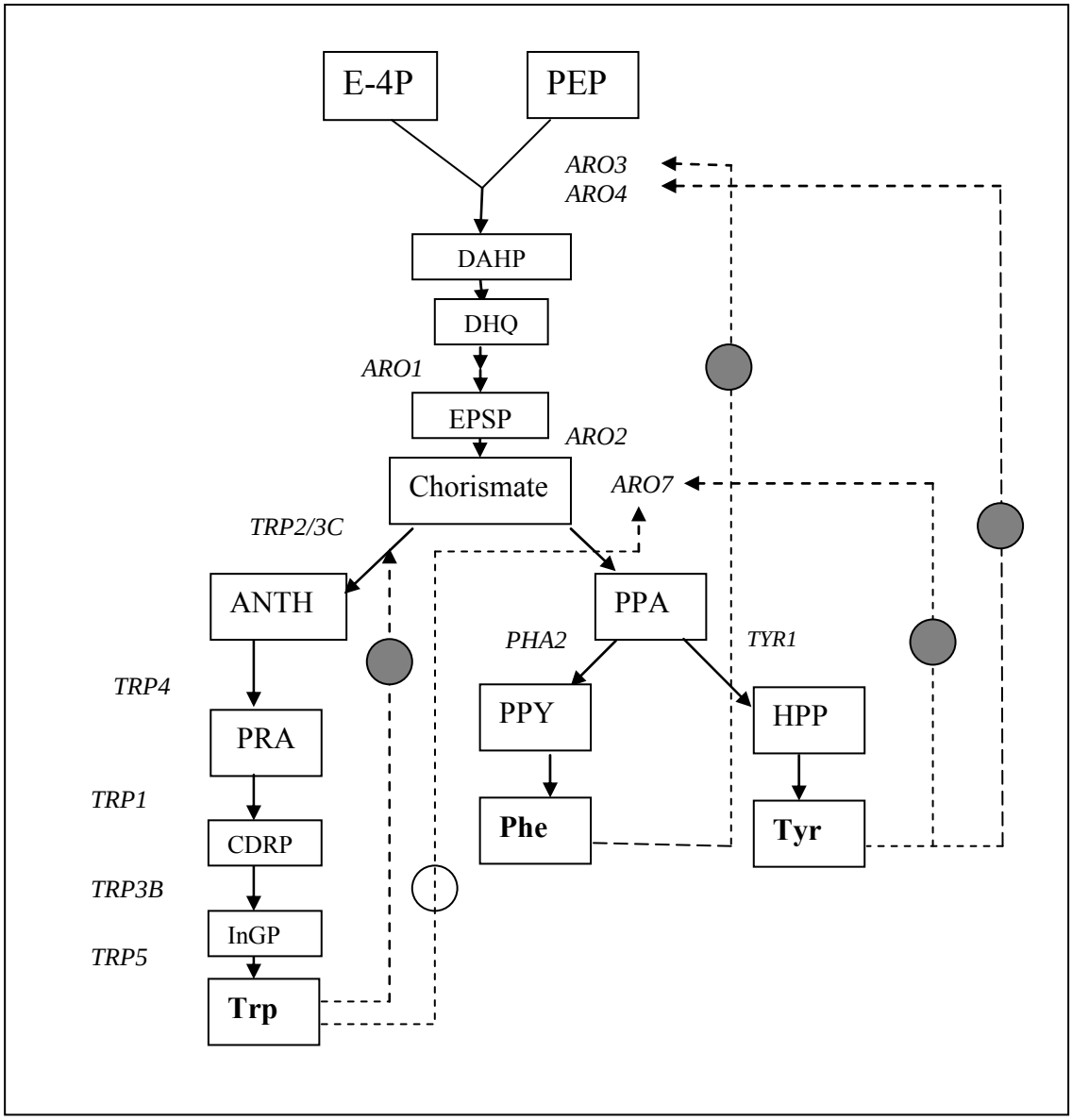

FIG. 5. Aromatic amino acid biosynthesis in *S. cerevisiae.* Full circles represent feed-back inhibition, whereas open circles display activation. Abbreviations in italics denote the genes. E-4P: erythrose 4-phosphate, PEP: phosphoenol pyruvate, DAHP: 3-deoxy-D-arabino-hetulosonate, DHQ: 3-dehydroquinate, EPSP: 5 enolpyruvoylshikimate 3-phosphate, ANTH: anthranilate, PRA:phosphoribosyl anthranilate, CDRP: 1-(o-carboxyphenylamino-1-deoxyribulose 5-phosphate, InGP: indole 3-glycerol-phosphate, PPA: prephenate, PPY: phenylpyruvate, HPP: 4-hydroxyphenylpyruvate, Trp: tryptophan, Phe: phenylalanine, Tyr: tyrosine.Modified from (8).

6.2 Genes common for phenylalanine and tyrosine pathway

ARO7

The first branching point in the pathway for the three aromatic amino acids is chorismate. Chorismate is also of importance for the synthesis of other aromatic compounds such as vitamin K, ubiquinone or p-amino benzoate. Aro7p catalyzes the first step in the phenylalanine-tyrosine branch (Fig. 5). The enzyme is feedback inhibited by tyrosine and strongly activated by tryptophan. Interestingly, in contrast to the other enzymes of the pathway, there is no similarity between yeast chorismate mutase and bacterial (*E. coli*) enzymes (3,8,9,77).

6.3 Genes unique for the phenylalanine pathway

PHA2

Prephenic acid is the branching point in the synthesis of phenylalanine and tyrosine. Pha2p catalyzes the conversion of phenylpyruvic acid (8) (Fig. 5). There is only limited information on the gene product of *PHA2* (prephenate dehydratase), which converts prephenate to phenylpyruvate (8)

6.4 Genes unique for the tyrosine pathway

TYRI

Prephenate dehydrogenase (Tyr1p) catalyzes the oxidative decarboxylation and dehydratation of prephenate to p-hydroxyphenylpyruvic acid. The *TYR1* gene contains an ORF of 441 codons with a molecular weight of 52 kDa. It encodes for prephenate dehydrogenase. There is a consensus sequence for a NAD^+-binding site, which is unique for dehydrogenases (60).

Transaminase *ARO8* and *ARO9*

The ARO8 and *ARO9* genes of *S. cerevisiae* encode aromatic aminotransferases I and II respectively (51) that catalyze the final step in tyrosine and phenylalanine pathway by transferring the amine group from a donor amino acid onto phenylpyruvate and p-hydroxyphenylpyruvate: acceptor of 2-oxo acids. (See section 6.6.2 for the detailed information).

***6.5* Genes unique for the tryptophan pathway**

TRP1, TRP2, TRP3, TRP3B, TRP4, TRP5

In *S. cerevisiae*, there are five steps from chorismate to tryptophan (Fig. 5). The first step of the tryptophan branch is the conversion of chorismate to anthranilate by anthranilate synthase. This enzyme is subject to feedback inhibition by tyrosine. Two genes: *TRP2* and *TRP3* encode this enzyme. *TRP2* encodes anthranilate synthase, which shows a decrease in activity when ammonia is the nitrogen source instead of glutamine (71). The *TRP3* gene encodes for two enzyme activities: the first one supplies nitrogen from glutamine for the synthesis of anthranilate. The second enzyme activity will be discussed below as the gene product of *TRP3B*. *TRP4* is the phosphoribosyl transferase that catalyzes the transfer of a 5-phosphoribosyl moiety from 5-

phosphoribosylpyrophosphate to the amino group of anthranilate, resulting in phosphoribosylanthranilate (PRA). *TRP1* is known as PRA isomerase. The amino glycoside PRA goes through an internal redox reaction, in which carboxyphenylamino-l-deoxyribulose 5-phosphate (CDRP) is formed. The InGP synthase catalyzing the fourth reaction in the pathway is encoded by *TRP3B* the second domain of the bifunctional enzyme which is encoded by the 3' half of the *TRP3* gene. *TRP5* encodes the enzyme, which catalyzes the last step of the tryptophan synthesis. In this reaction, InPG is degraded and tryptophan is produced by tryptophan synthase as a condensation reaction with serine. The enzyme has two active sites: one for the aldol cleavage of InGP to yield indole and glyceraldehyde-3-phosphate, and the other for the synthesis of L-tryptophan from indole and serine (61).

6.6 Regulation of aromatic amino acid metabolism in *S. cerevisiae*

6.6.1 General control

Cells can respond to environmental changes via transcriptional activation and repression. Schematically, transcriptional activators bind to specific promoters DNA sequences controlling expression of the corresponding genes (38). In *Saccharomyces cerevisiae* amino acid biosynthesis is under the control of *GCN4*. Upon starvation of some amino acids or even purines, Gcn4p activates the expression of a large set of genes involved in amino acid synthesis. Indeed the starvation is sensed through level of uncharged t-RNA. Accumulation of uncharged tRNA stimulates Gcn2p kinase activity which in return activates *GCN4* translation. The synthesized factor will activate gene expression by targeting promoter sequences. Besides the activation of the translation of *GCN4* transcript, degradation of the factor itself is an important point of regulation (47).

Historically, *GCN4* has been implicated in the control of 36 genes: all implicated in amino acids metabolism (37). If this list is still recognized as primary markers for *GCN4* response, more recent transcriptomics studies revealed that about 10% of the yeast genome might be controlled by Gcn4p (65). As indicated in Fig. 6, genes involved in pathways as diverse as storage carbohydrates metabolism, vitamin and co-factor biosynthesis, signaling pathway were influenced by the *GCN4* response (Fig. 6). Now the number of genes implicated in amino acid biosynthesis increase up to 73 genes (65). Among these genes, genes of the aromatic amino acid family biosynthesis such as *ARO1, ARO2, ARO3* and *ARO4* which are in charge of chorismic acid synthesis as well as tryptophan synthesis genes such as *TRP2, TRP3, TRP 4* and *TRP5* are in control of *GCN4* (8).

It is interesting to notice here that for instance *ARO7* is not controlled by *GCN4* while the branch leading to tryptophan is under *GCN4* control. As a result of this, a simple overexpression of *ARO7* may lead to a complete deregulation of the pathway and result to partial tryptophan auxotrophy (9) as chorismate is preferentially channeled towards phenylalanine and tyrosine biosynthesis (see Chapter 4).

As will be shown in the following paragraphs in detail, Aro9p is an aromatic aminotransferase, which is induced by tryptophan. Aro10p; a broad substrate specificity decarboxylase, has high transcriptional levels in the presence of leucine, phenylalanine and methionine (82). These two genes (*ARO10* and *ARO9)* are also the targets of *GCN4* as well as Aro8p, which is known as the aromatic aminotransferase I.

6.6.2 Specific control

As shown in the biosynthesis of other amino acids, the general transcriptional control supervised by *GCN4* is not the only regulatory mechanism controlling the transcription. In the case of aromatic amino acid metabolism the activator encoded by *ARO80* was shown to participate in the transcriptional activation of *ARO9* and *ARO10* encoding aromatic amino acid aminotransferase II and a broad substrate 2-oxo acid decarboxylase respectively.

In the case of *ARO9*, the expression was triggered when aromatic amino acids were added to the growth medium. Furthermore as shown by (46), *ARO9* expression would be correlated to the quality of the nitrogen source. The effect of ammonia on *ARO9* expression would be linked to the exclusion of the inducer by lack of import system in presence of a good nitrogen source. Finally the zinc finger transcriptional activator of the Zn_2Cys_6 family Aro80p acts through upstream activating sequence: (UAS_{aro}) that has been reported in only two genes *ARO9* and *ARO10* (46). Aromatic aminotransferase II is active with phenylalanine, tyrosine and tryptophan as amino donors and with phenylpyruvate, hydroxyphenylpyruvate, pyruvate as amino acceptors (51,81). Interestingly, it was reported that the donor acceptor couple formed by methionine and 3methylthio 2-oxobutanoate could be used by Aro9p.

6.7 Regulation of enzyme activity

The two DAHP synthases are regulated by feedback inhibition: phenylalanine feedback inhibits the *ARO3* encoded DAHP synthase, and tyrosine feedback inhibits the *ARO4* encoded DAHP synthase (Fig. 5). For the *ARO3* encoded isoenzyme of DAHP synthase, phenylalanine inhibition is competitive with respect to E4P and non-competitive with PEP, whereas for the *ARO4* encoded isoenzyme of DAHP synthase, tyrosine inhibition is competitive with respect to PEP (8,78).

From the studies which focused on the evolution of two differently regulated DAHP synthases (Aro3p and Aro4p), interesting findings were reported: The exchange of a single amino acid showed different regulatory patterns. Aro3p with (Serine)S219G(Glycine) showed an Aro4p like behaviour and regulation by tyrosine whereas Aro4p with G226S substitution indicated an Aro3p like phenylalanine regulation pattern (34).

DAHP synthases are metal ion-dependent enzymes. In a recent study (50): the crystal structures of several complexes between the tyrosine-regulated form of DAHP synthases from *Saccharomyces cerevisiae* and *E. coli* different metal ions and ligands have been determined. The crystal structures indicated that simultaneous presence of a metal ion (Co^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, Ni^{+2}) and PEP triggers an ordering of the protein into a conformation that allowed the binding of the second substrate erythrose-4-phosphate (E4P) (8,50).

Chorismate mutases that are encoded by *ARO7* are unique enzymatic activities that are found only in microorganisms and plants but not in animals (72). In *S. cerevisiae*, the enzyme competes for a common substrate with anthranilate synthase that catalyses the first step of the tryptophan-specific branch (Fig. 5). The yeast chorismate mutase, encoded by the *ARO7* gene, serves as simple model for an allosteric enzyme (76). Aro7p is a dimer composed of two identical subunits of 30 kDa, each of them can bind one substrate and one

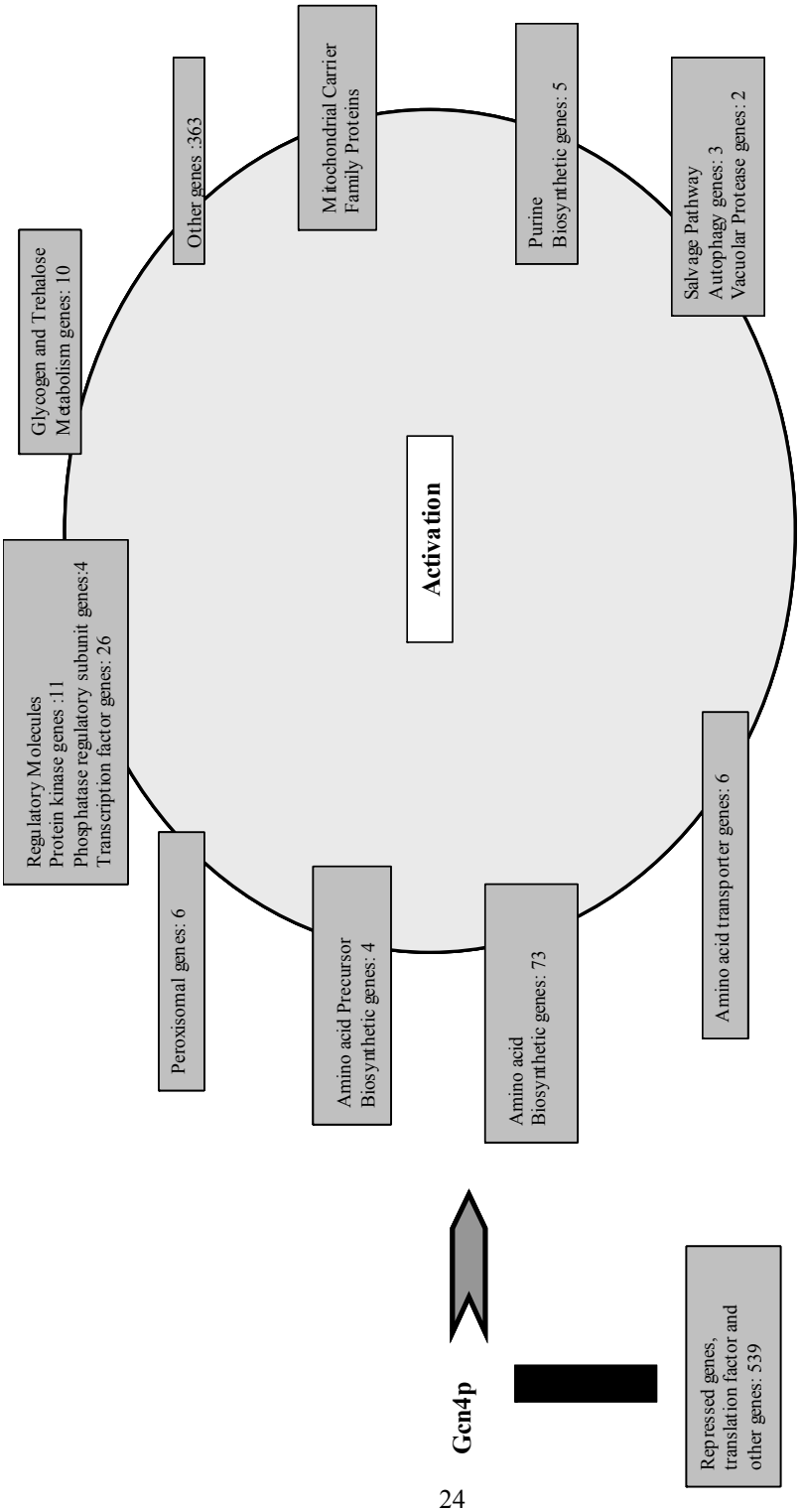

FIG. 6. Target Genes of Gcn4p (modified from (65)).

activator molecule. Additionally each subunit can also bind one inhibitor molecule (76). *ARO7* is known to be allosterically regulated by tyrosine (inhibition) and tryptophan (activation) (Fig. 5) (54).

According to a study by Krapmann et al. (54): Construction of a strain carrying the *ARO7*^{T226I} (*ARO7*^{c}) allele results in the unregulated chorismate mutase, which depletes the chorismate pool and causes growth defects due to tryptophan limitation. This situation is recovered by the induction of *TRP2* that encodes anthranilate synthase. As mentioned in the previous paragraphs, anthranilate synthase competes with chorismate mutase for the same substrate. It was concluded that: *ARO7* is not transcriptionally regulated (not regulated by *GCN4*) and that the transcription of anthranilate synthase-encoding gene is regulated by the availability of the amino acids.

7.0 *S. cerevisiae*

7.1 Applications in biotechnology

For the history of yeast biotechnology, one has to go back to the beginning of human civilization, because yeast applications already started with ancient Egyptians (14,84). Mankind has exploited *S. cerevisiae* for several centuries for the production of food and alcoholic drinks. Being non-pathogenic, having a long history of application and furthermore holding GRAS (Generally Regarded As Safe) status makes this yeast a very attractive organism (69). Today, the application of *Saccharomyces spp.* is not only limited to bread and beer production but also has found its way in the pharmaceutical industry. With the development of metabolic engineering, it has also become a key organism for biotechnology (Fig. 7).

7.2 Fusel alcohols and genetics of fusel alcohol production by *S. cerevisiae*

In addition to the production of ethanol and carbon dioxide during fermentation, *S. cerevisiae* also produces a large variety of low molecular weight flavour compounds. For example, in the case of beer, the chemical components are not only the aromas derived from the barley and hops, but also originate from branched chain amino acid catabolism. These are called higher or fusel alcohols and their acetate esters (33). The fusel alcohols and their acetate esters coming from the branched chain amino acid catabolism (leucine, isoleucine and valine catabolism) such as isoamyl acetate or 3-methlybutyl acetate contribute to beer with characteristic banana and pear flavour whereas 3-methylbutanol is also a very important aroma compounds for beer (13) and other alcoholic beverages (86); including Japanese drink: sake (1).

Ehrlich (26) in 1908 postulated the first principles of fusel alcohol formation (Fig. 8). According to his theory, fusel alcohol formation from amino acids proceeds via three enzyme-catalysed reactions. First, amino acids are transaminated to the corresponding 2-oxo acids, then these are decarboxylated to their corresponding aldehydes and as the last step, the aldehyde is reduced to an alcohol. Some of the fusel alcohols and the amino acids they are derived from are presented in Table 2.

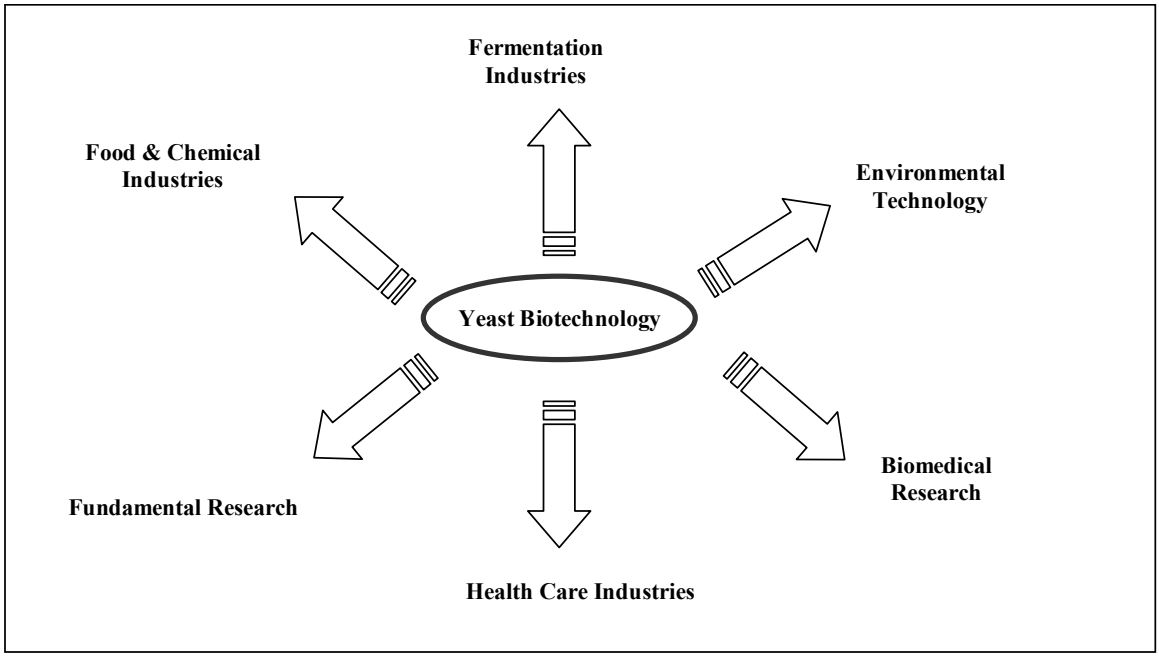

FIG. 7. Application areas of *S. cerevisiae* (Modified from (14)).

The genetics and regulation of Ehrlich Pathway is poorly understood until now. There is very limited information especially on the effects of the expression levels of the decarboxylase genes in fusel alcohol production (82).

The first irreversible step in Ehrlich pathway is the decarboxylation of the 2-oxo acid. Therefore more emphasis was given to this step by scientists. Before looking into the details of those studies, it is important to introduce the candidate decarboxylase genes. There are five TPP dependent decarboxylases that share the same sequence characteristics (39-41). *PDC1, PDC5*, and *PDC6* genes are known to encode pyruvate decarboxylases. *PDC1* and *PDC5* generally have the highest expression levels (29), whereas *PDC6* is specifically expressed under low-sulfur conditions. It encodes a pyruvate decarboxylase that has a low content of sulfur-containing amino acids (6,28).

The other members of the decarboxylases family are *ARO10* (*YDR380w)* and *THI3* (*YDL080c)*. They are thought to encode TPP-dependent decarboxylases (41). Thi3p is a positive regulator of the thiamin biosynthetic pathway. When it was deleted the transcription of all the genes of thiamin biosynthesis were negatively affected (41).

The genetics of the branched chain amino acid catabolism and specifically decarboxylase genes were investigated in detail by Dickinson and co-workers. They reported that in leucine catabolism, *YDL080c* (*THI3, KID1*) is the major decarboxylase (94 % of the decarboxylation), whereas *YDR380w* (*ARO10)* was found to be the minor decarboxylase. In valine catabolism, one of the three isoenzymes of pyruvate decarboxylase appeared

to be sufficient enough to convert α-ketoisovalerate to 2-methylpropanol whereas in isoleucine catabolism any of the five decarboxylases can decarboxylase α-ketomethylvalerate (19-21). In an other study by (80), it was presented that pyruvate decarboxylase *(PDC1* and *PDC5)* can catalyse the decarboxylation of branched chain 2-oxo acids. However, it is not essential for the production of fusel alcohols. Experiments performed with wild type and Pdc^- strains provided results such as the lower rate of fusel alcohol production in Pdc^- strain compared to the wild type strain only in the case of 2-methylpropanol pointing to other genes or mechanisms involved in fusel alcohol production other than *PDC* complex (80).

TABLE 2. Amino acids and derivatives that are metabolized via the Ehrlich pathway

Amino Acid	2-Oxo acid	Acid	Alcohol
Leucine	α-ketoisocaproate	3-methyl 1-butanoate	3-methyl 1- butanol
Valine	α-ketoisovalerate	2-methyl propanoate	2-methyl 1-propanol
Isoleucine	α-ketomethyl valerate	2-methyl butanoate	2-methyl 1-butanol
Methionine	4-methyl thiooxobutanoate	3-methyl thiopropanoate	3-methyl thiopropanol
Phenylalanine	phenylpyruvate	phenylacetate	2-phenylethanol

In case of aromatic amino acids such as phenylalanine and tryptophan; (18) indicated that any of the gene products of *PDC1*, *PDC5*, *PDC6*, or *YDR380w* can catalyse the decarboxylation reaction whereas *YDL080c* has no part in the catabolism of either of these amino acids.

The present literature on the decarboxylation step of the Ehrlich pathway pictured a rather intricate mechanism involving the five members of the decarboxylase family, and that is partially dependent on the nature of the nitrogen source. Indeed, it seems that still two redundant mechanisms could be operate i) a route involving *THI3* and *ARO10* as shown for leucine catabolism, ii) a route dependent of Pdcs . This pictured that the complexity of this single reaction step is not yet resolved.

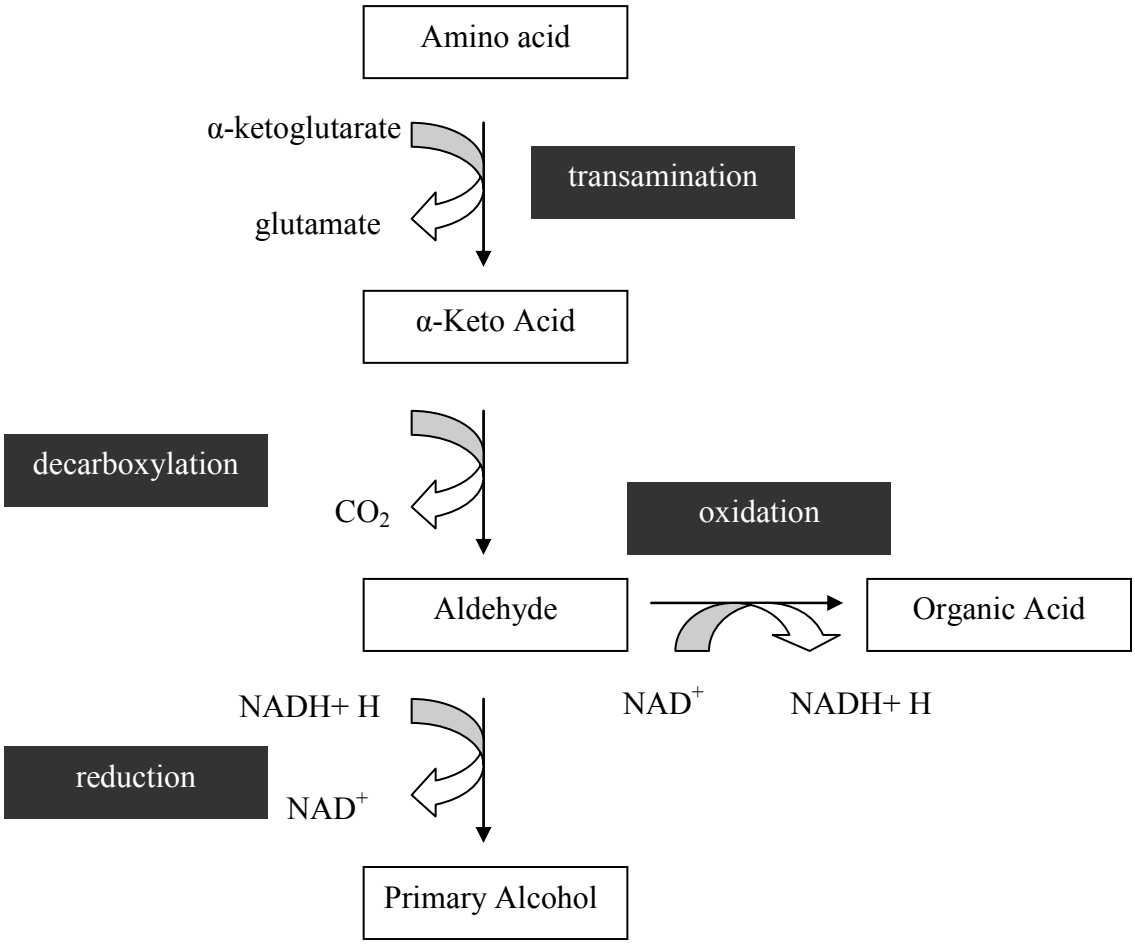

FIG. 8. Schematic representation of Ehrlich Pathway

8.0 The Scope and Outline of the Thesis

The research described in this thesis addresses critical processes in the metabolism of aromatic amino acids by *Saccharomyces cerevisiae*, with the aim to enable metabolic engineering of aromatic compound formation by this yeast. Two research lines were followed.

The first research line focused on the molecular identity and substrate specificity of 2-oxo- acid decarboxylase in *S. cerevisiae*. In Chapter 2, formation of biomass and metabolic products was quantified in aerobic, glucose-limited chemostat cultures of *S. cerevisiae* grown with phenylalanine as the sole carbon source. Phenylacetic acid and 2-phenylethanol were produced in large quantities, indicating that the Ehrlich pathway for phenylalanine catabolism was active under these conditions. To identify candidate structural genes for phenylpyruvate decarboxylase, a key enzyme in the Ehrlich pathway, transcriptome data for these cultures were compared with those of cultures grown with ammonia as the sole carbon source. After identifying *ARO10* as a strong candidate gene, this work was followed up by physiological characterization of deletion mutants. This confirmed that the *ARO10* gene, which exhibits sequence similarity with thiamine pyrophosphate dependent decarboxylases, is indeed involved in phenylpyruvate decarboxylation.

In Chapter 3, the substrate specificity of the Aro10p-dependent decarboxylase and its role in the production of fusel alcohols and acids was studied in more detail. Transcriptome analysis of chemostat cultures grown with different nitrogen sources (leucine, methionine, phenylalanine, asparagine and proline) suggested that the Aro10p-dependent decarboxylase activity may have broad substrate specificity. This was further investigated by overexpression of *ARO10* in an *S. cerevisiae* strain in which all five known and putative structural genes for thiamine pyrophosphate-dependent decarboxylases (*PDC1*, *PDC5, PDC6*, *ARO10*, and *THI3*) had been deleted.

Chapter 4 describes a second line of research: the elimination of feedback inhibition steps in the phenylalanine biosynthetic pathway in *S. cerevisiae*. The reactions focused on were those catalyzed by DAHP synthase (Aro3p and Aro4p, which are subject to feed-back inhibition by tyrosine and phenylalanine, respectively) and chorismate mutase (Aro7p, which is feed-back inhibited by tyrosine). Engineered strains were constructed that exhibited single and combined expression of feedback-insensitive forms of these key enzymes. Subsequently, the impact of feedback inhibition on the aromatic biosynthesis pathway was quantified by analyzing intra- and extracellular concentrations of relevant aromatic compounds in glucose-limited chemostat cultures of wild-type and engineered strains. Moreover, the effect of this deregulation on the intracellular pools of other amino acids was quantified.

REFERENCES

1. **Ashida, S., E. Ichikawa, K. Sugunami, and S. Imayasu.** 1987. Isolation and application of mutants producing sufficient isoamyl acetate, a sake flavor component. Agr. Biol. Chem. **51**:2061-2065.
2. **Bailey, J. E.** 1991. Toward a science of metabolic engineering. Science **252** :1668-1675.
3. **Ball, S. G., R. B. Wickner, G. Cottarel, M. Schaus, and C. Tirtiaux.** 1986. Molecular cloning and characterization of *ARO7-OSM2,* a single yeast gene necessary for chorismate mutase activity and growth in hypertonic medium. Mol.Gen Genet. **205**:326-330.
4. **Baltz, R. H.** 1986. *In* Demain A.L. and Davies J. (ed.), Manual of Industrial Microbiology and Biotechnology. Am. Soc. Microbiol., Washington DC.
5. **Berry A.** 1996. Improving production of aromatic compounds in E.coli by metabolic engineering. Trends Biotechnol **141**:250-256.
6. **Boer, V. M., J. H. de Winde, J. T. Pronk, and M. D. Piper.** The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. J. Biol. Chem. 278, 3265-3274. 2003.
7. **Bongaerts, J., M. Kramer, U. Muller, L. Raeven, and M. Wubbolts**. 2001. Metabolic engineering for microbial production of aromatic amino acids and derived compounds. Metabolic engineering **3**:289-300.
8. **Braus, G. H.** 1991. Aromatic amino acid biosynthesis in the yeast *Saccharomyces cerevisiae:* a model system for the regulation of an eukaryotic biosynthetic pathway. Microbiol.Mol.Biol..Rev. **55**:349-370.
9. **Brown, J., and I. Dawes**. 1990. Regulation of chorismate mutase in *Saccharomyces cerevisiae*. Mol.Gen Genet. **220**:283-288.
10. **Byng, G., A. Berry, and R. A. Jensen.** 1985. Evolutionary implications of features of aromatic amino acid biosynthesis in the genus Acinetobacter. Arch. Microbiol. **143**:122-129.
11. **Cooper, T. G.** 1982. Nitrogen Metabolism in *S.cerevisiae*. In: Strathern J.N, Jones E.W., and Broach J. (ed.), The Molecular Biology of the Yeast *Saccharomyces cerevisiae*. Cold Spring Harbor, NY.
12. **Crueger, W., and A. Crueger.** 1989. Biotechnology: A Textbook of Industrial Microbiology. Science Tech Publishers, Madison, Wisconsin.
13. **de Hollander, J. A., F. R. Eswilder, and J. A. Noordover.** 1998. Amino acid fermentation processes. US Patent 5763230.
14. **de Winde, J. H**. 2003. Functional Genetics of Industrial Yeasts. pp. 1-16. Topics in Current Genetics 2. Springer, Heidelberg.
15. **Debabov, V. G.** 2003. The threonine story. Adv. Biochem. Eng. Biotechnol. **79**: 113-136
16. **Delgado, M. A., J. Guerrero, and J. Conde.** 1982. Genetic and biochemical study of threonine overproducing mutants of *Saccharomyces cerevisiae.* Mol. Cell. Biol. **2**:731-736.
17. **Demain, A. L.** 2000. Microbial Biotechnology. Trends Biotechnol. **18**:26-31.

18. **Dickinson, J. R., L. E. J. Salgado, and J. E. Hewlins**. 2003. The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. J. Biol. Chem. **278**:8028-8034.
19. **Dickinson, J. R., S. J. Harrison, and M. J. E. Hewlins**. 1998. An investigation of the metabolism of valine to isobutyl alcohol in *S.cerevisiae*. J. Biol. Chem. **273**:25751-25756.
20. **Dickinson, J. R., S. J. Harrison, J. A. Dickinson, and J. E. Hewlins**. 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *S.cerevisiae*. J. Biol. Chem. **275**:10937-10942.
21. **Dickinson, J. R., M. Lanterman, D. Danner, B. Pearson, P. Sanz, S. Harrison, and M. Hewlins**. 1997. A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae.* J. Biol. Chem. **272**:26871-26878.
22. **Diers, I. V., E. Rasmussen, P. H. Larsen, and I. L. Kjaersig.** 1991. Yeast fermentation processes for insulin production. Bioprocess Technol. **13**:166-176.
23. **Duncan, K., R. M. Edwards, and J. R. Coggins.** 1987. The pentafunctional arom enzyme of *Saccharomyces cerevisiae* is a mosaic of monofunctional domains. Biochem. J. **246**:375-386.
24. **Duncan, K., R. M. Edwards, and J. R. Coggins.** 1988. The *Saccharomyces cerevisiae ARO1* gene. An example of the coordinate regulation of five enzymes on a single biosynthetic pathway. FEBS Lett. **241**:83-88.
25. **Eggeling, L., and H. Sahm**. 1999. Amino acid production, pp. 153-176. In: Lee S.Y. and Papoutsakis E.T. (ed.), Metabolic Engineering. Marcel Dekker inc., New York / Basel.
26. **Ehrlich, F.** 1907. Uber die Bedingungen der Fuselölbildung and uber ihren Zusammenhang mit dem Eiweissaufbau der Hefe. Ber.Deut.Chem.Gesellsch. **40**:1027-1047.
27. **Fabre, C. E., P. J. Blanc, and G. Goma**. 1998. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. Biotechnol.prog. **14**:270-274.
28. **Fauchon, M., G. Lagniel, J. C. Aude, L. Lombardia, P. Soularue, C. Petat, G. Marguerie, A. Sentenac, M. Werner, and J. Labarre**. 2002. Sulfur sparing in the yeast proteome in response to sulfur demand. Mol.Cell. **9**:713-723.
29. **Flikweert, M., L. van der Zanden, W. M. Janssen, H. Y. Steensma, J. P. van Dijken, and J. T. Pronk.** 1996. Pyruvate decarboxylase: An indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. Yeast **12:** 247-257.
30. **Gassenmeier, K., and P. Schieberle.** 1995. Potent aromatic compounds in the crumb of wheat bread. Z. Lebensm.Unters. Forsch. **201**:241-248.
31. **Gerigk, M., R. Bujnicki, E. Ganpo-Nkwenkwa, J. Bongaerts, G. Sprenger, and R. Takors**. 2002. Process control for enhanced L-phenylalanine production using different recombinant *Escherichia coli* strains. Biotechnol. Bioeng. **80**:746-754.
32. **Glazer, A. N., and H. Nikaido.** 1995. Microbial Biotechnology: Fundamentals of Applied Microbiology. W. H. Freeman and Company, New York, N.Y.
33. **Hansen, J., and M. C. Kielland-Brandt.** 2003. Brewer's yeast: genetic structure and targets for improvement. pp. 143-170. In: de Winde, J. H. Topics in Current Genetics 2. Springer, Heidelberg.

34. **Hartmann, M., T. R. Schneider, A. Pfeil, G. Heinrich, W. N. Lipscomb, and G. H. Braus.** 2002. Evolution of feedback-inhibited beta /alpha barrel isoenzymes by gene duplication and a single mutation. Proc. Natl. Acad. Sci. U S A **100**:862-867.
35. **Hermann, T.** 2003. Industrial production of amino acids by coryneform bacteria. J.Biotechnology. **104**:155-172.
36. **Hermann, T., and R. Kramer.** 1996. Mechanism and regulation of isoleucine excretion in *Corynebacterium glutamicum*. App.Environ.Microbiol. **32**:3238-3244.
37. **Hinnebusch, A. G.** 1986. The general control of amino acid biosynthetic genes in the yeast *Saccharomyces cerevisiae*. Crit. Rev. Biochem. **21**:277-317.
38. **Hinnebusch, A. G., and K. Natarajan.** 2002. Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. Eukaryotic cell **1**:22-32.
39. **Hohmann, S.** 1991. *PDC6*, a weakly expressed pyruvate decarboxylase gene from yeast, is activated when fused spontaneously under the control of the *PDC1* promotor. Curr.Genet. **20**:373-378.
40. **Hohmann, S., and H. Cederberg**. 1990. Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. Eur. J. Biochem. **188**:615-621.
41. **Hohmann, S., and P. A. Meacock**. 1998. Thiamin metabolism and thiamin diphosphatedependent enzymes in the yeast *Saccharomyces cerevisiae:* genetic regulation. Biochim. Biophys. Acta **1385**:201-219.
42. **Hutter, R., P. Niederberger, and J. A. DeMoss.** 1986. Tryptophan biosynthetic genes of eukaryotic microorganisms. Annu.Rev.Microbiol. **40**:55-77.
43. **Ikeda, M.** 2003. Amino acid production processes. Adv. Biochem. Eng. Biotechnol. **79**:1-35.
44. **Ikeda, M., and R. Katsumata.** 1992. Metabolic engineering to produce tyrosine or phenylalanine in a tryptophan-producing *Corynebacterium glutamicum* strain. Appl.Environ.Microbiol. **58**:781-785.
45. **Ikeda, M., and M. Nakagawa.** 2003. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. Appl.Microbiol.Biotechnol. **62**:99-109.
46. **Iraqui, I., S. Vissers, B. Andre, and A. Urrestarazu**. 1999. Transcriptional induction by aromatic amino acids in *S.cerevisiae*. Mol. Cell. Biol. **19**:3360-3371.
47. **Irniger, S., and G. H. Braus.** 2003. Controlling transcription by destruction: The regulation of yeast Gcn4p stability. Curr.Genet. **44**:8-18.
48. **Kimura, E.** 2003. Metabolic engineering of glutamate production. Adv. Biochem. Eng Biotechnol. **79**:37-57.
49. **Kinoshita, S., and S. M. Udaka.** 1957. Studies on the amino acid fermentation. Part I. Production of L-glutamic acid by various microorganisms. J.Gen.Appl. Microbiol. **3**:193-205.
50. **Konig, V., A. Pfeil, G. H. Braus, and S. Schneider.** 2004. Substrate and metal complexes of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase from *Saccharomyces cerevisiae* provide new insights into the catalytic mechanism. J.Mol.Biol. **337**:675-690.

51. **Kradolfer, P., P. Niederberger, and R. Hutter**. 1982. Tryptophan degradation in *S.cerevisiae*: characterization of two aromatic aminotransferases. Arch. Microbiol. **133**:242-248.
52. **Kramer, R.** 1996. Genetic and physiological approaches for the production of amino acids. J. Biotechnol. **45**:1-21.
53. **Kramer, R.** 2004. Production of amino acids: physiological and genetical approaches. Food Biotech. **18**:171-216.
54. **Krappmann, S., W. N. Lipscomb, and G. H. Braus**. 2000. Coevolution of transcriptional and allosteric regulation at the chorismate metabolic branch point of *S.cerevisiae*. Proc Natl Acad Sci U S A 97:13585-13590.
55. **Larimer, F. W., C. C. Morse, A. K. Beck, K. W. Cole, and F. H. Gaertner.** 1983. Isolation of the *AROJ* cluster gene of *Saccharomyces cerevisiae*. Mol.Cell.Biol. **3**:1601-1614.
56. **LaRossa, R. A., and F. C. Falco.** 1984. Amino acid biosynthetic enzymes as targets of herbicide action. Trends. Biotechnol. **2**:158-161.
57. **Leuchtenberger, W.** 1996. Amino Acids : Technical Production and Use, pp. 465-502. *In:* H. J. Rehm and G. Reed (ed.), Biotechnology. VCH, Weinheim.
58. **Madigan, M., J. M. Martinko, and J. Parker.** 2003. Brock Biology of Microorganisms. pp 38-54. Tenth edition. Pearson education inc, New Jersey. USA
59. **Magasanik, B.** 1992. Regulation of nitrogen utilization, pp. 283-318. *In*: Jones E.W., Pringle J.R., and Broach J. (ed.), The Molecular and Cellular Biology of the Yeast *Saccharomyces*. Cold Spring Harbor, NY.
60. **Mannhaupt, G., R. Stucka, U. Pilz, C. Schwarzlose, and H. Feldman.** 1989. Characterization of the prephenate dehydrogenase-encoding gene, *TYR1,* from *Saccharomyces cerevisiae*. Gene **85**:303-311.
61. **Miles, E. W., R. Bauerle, and S. A. Ahmed.** 1987. Tryptophan synthase from *Escherichia coli* and *Salmonella typhimurium*. Methods Enzymol. **142**:398-414.
62. **Moat, A. G., J. W. Foster, and M. P. Spector.** 2002. Microbial Physiology. Wiley-Liss. London
63. **Mortimer, R. K., D. Schild, C. R. Contopoulou and J. A. Kans** 1989. Genetic map of *Saccharomyces cerevisiae*. Yeast **5**:321-403.
64. **Mueller, U., and S. Huebner.** 2003. Economic aspects of amino acids production. Adv. Biochem. Eng. Biotechnol. **79**: 137-170
65. **Natarajan, K., M. R. Meyer, B. M. Jackson, D. Slade, C. Roberts, A. G. Hinnebusch, and M. J. Marton.** 2001. Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast . Mol. Cell. Biol. **21**:4347-4368.
66. **Nelson, D.L., and M. M. Cox**. 2005. Lehninger Principles of Biochemistry. W.H. Freeman and Company, New York.
67. **Nelson, G., J. Chandrashekar, M. A. Hoon, L. Feng, G. Zhao, N. J. Ryba, and C. S. Zucker.** 2002. An amino-acid taste receptor. Nature **416**:199-202.

68. **Nielsen, J., and L. Olsson.** 2002. An expanded role for microbial physiology in metabolic engineering and functional genomics: moving towards systems biology. FEMS Yeast Res. **2**:175-181.
69. **Ostergaard, S., L. Olsson, and J. Nielsen.** 2000. Metabolic engineering of *S.cerevisiae*. Microbiol. and Mol. Biol. Rev. **64**:34-50.
70. **Pfefferle, W., B. Mockel, B. Bathe, and A. Marx**. 2003. Biotechnological manufacture of lysine. Adv. Biochem. Eng. Biotechnol. **79**:59-112.
71. **Prantl, F., A. Strasser, M. Aebi, R. Furter, P. Niederberger, K. Kirschner, and R. Hutter**. 1985. Purification and characterization of the indole-3-glycerolphosphate synthase / anthranilate synthase complex of *Saccharomyces cerevisiae*. Eur.J.Biochem. **146**:95-100.
72. **Romero, R. M., M. F. Roberts, and J. D. Phillipson.** 1995. Anthranilate synthase in microorganisms and plants. Phytochemistry **39**: 263-276.
73. **Sandeaux, J., C. Sandeaux, C. Gavach, H. Grib, T. Sadat, D. Belhocine, and N. Mameri.** 1998. Extraction of amino acids from protein hydrolysates by Electrodialysis. J. Chem. Technol. Biotechnol. **71**:267-273.
74. **Sauer, U.** 2001. Evolutionary engineering of industrially important microbial phenotypes. Adv. Biochem. Eng. Biotechnol. **73**: 129-170
75. **Schaffer, S., B. Weil, V. D. Nguyen, G. Dongmann, K. Gunther, M. Nickolaus, T. Hermann, and M. Bott.** 2001. A high-resolution reference map for cytoplasmic and membrane-associated proteins of *Corynebacterium glutamicum.* Electrophoresis **22**:4404-4422.
76. **Schmidheini, T., H. U. Mosch, J. N. S Evans, and G. H. Braus.** 1990. Yeast allosteric chorismate mutase is locked in the activated state by a single amino acid substitution. Biochemistry. **29**:3660-3668.
77. **Schmidheini T., P. Sperisen, G. Paravicini, R. Hutter, and G. H. Braus.** 1989. A single point mutation results in a constitutively activated and feedback-resistant chorismate mutase of *Saccharomyces cerevisiae*. J. Bacteriol. **171**:1245-1253.
78. **Schnappauf, G., M. Hartmann, M. Kunzler, and G. H. Braus**. 1998. The two 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase isoenzymes from *Saccharomyces cerevisiae* show different kinetic modes of inhibition. Arch. Microbiol. **169**:517-524.
79. **Shiio, I., S. Sugimoto and K**. **Kawamura.** 1988. Breeding of phenylalanine producing *Brevibacterium flavum* strains by removing feedback regulation of both the two key enzymes in its biosynthesis. Agr.Biol.Chem. **52**:2247-2253.
80. **Ter Schure, E. G., M. Flikweert, J. P. van Dijken, J. T. Pronk, and C. T. Verrips**. 1998. Pyruvate decarboxylase catalyzes decarboxylation of branched-chain 2-oxo acids but is not essential for fusel alcohol production by *Saccharomyces cerevisiae*. Appl. Environ Microbiol. **64**:1303-1307.
81. **Urrestarazu, A., S. Vissers, I. Iraqui, and M. Grenson**. 1998. Phenylalanine- and tyrosine auxotrophic mutants of *Saccharomyces cerevisiae* impaired in transamination. Mol.Gen Genet. **257**:230-237.
82. **Vuralhan, Z., M. A. H. Luttik, S. L. Tai, V. M. Boer, M. A. Morais, D. Schipper, M. J. H. Almering, P. Kötter, J. R. Dickinson, J. M. Daran, and J. T. Pronk. 2005.** Physiological characterization of the

ARO10-dependent, broad-substrate specificity 2-oxo-acid-decarboxylase activity of *Saccharomyces cerevisiae*. App.Environ.Microbiol**. 71** (6), 3276-3284.

83. **Vuralhan, Z., M. A. Morais, S. L. Tai, M. D. Piper, and J. T. Pronk.** 2003. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae.* App.Environ. Microbiol. **69**:4534-4541.
84. **Walker, G. M.** 1998. Yeast Physiology and Biotechnology. pp. 265-320.Wiley, London.
85. **Welsh, F. W., W. D. Murray, and R. E. Williams.** 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. Crit Rev.Biotech. **8**:105-169.
86. **Yoshimoto, H., T. Fukushige, T. Yonezawa, and H. Sone**. 2002. Genetic and physiological analysis of branched-chain alcohols and isoamyl acetate production in *Saccharomyces cerevisiae.* Appl. Microbiol. Biotechnol. **59**:501-508.

2

IDENTIFICATION AND CHARACTERIZATION OF PHENYLPYRUVATE DECARBOXYLASE GENES IN *SACCHAROMYCES CEREVISIAE*

ABSTRACT

Catabolism of amino acids via the Ehrlich pathway involves transamination to the corresponding α-keto acids, followed by decarboxylation to an aldehyde and then reduction to an alcohol. Alternatively, the aldehyde may be oxidized to an acid. This pathway is functional in *Saccharomyces cerevisiae*, since during growth in glucose-limited chemostat cultures with phenylalanine as the sole nitrogen source, phenylethanol and phenylacetate were produced in quantities that accounted for all of the phenylalanine consumed. Our objective was to identify the structural gene(s) required for the decarboxylation of phenylpyruvate to phenylacetaldehyde, the first specific step in the Ehrlich pathway. *S. cerevisiae* possesses five candidate genes with sequence similarity to genes encoding thiamine diphosphate-dependent decarboxylases that could encode this activity: *YDR380w/ ARO10*, *YDL080C/THI3*, *PDC1*, *PDC5*, and *PDC6*. Phenylpyruvate decarboxylase activity was present in cultures grown with phenylalanine as the sole nitrogen source but was absent from ammonia-grown cultures. Furthermore, the transcript level of one candidate gene (*ARO10*) increased 30-fold when phenylalanine replaced ammonia as the sole nitrogen source. Analyses of phenylalanine catabolite production and phenylpyruvate decarboxylase enzyme assays indicated that *ARO10* was sufficient to encode phenylpyruvate decarboxylase activity in the absence of the four other candidate genes. There was also an alternative activity with a higher capacity but lower affinity for phenylpyruvate. The candidate gene *THI3* did not itself encode an active phenylpyruvate decarboxylase but was required along with one or more pyruvate decarboxylase genes (*PDC1*, *PDC5*, and *PDC6*) for the alternative activity. The K_m and V_{max} values of the two activities differed, showing that Aro10p is the physiologically relevant phenylpyruvate decarboxylase in wild-type cells. Modifications to this gene could therefore be important for metabolic engineering of the Ehrlich pathway.

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INTRODUCTION

The yeast *Saccharomyces cerevisiae* can use a variety of amino acids as sole nitrogen sources, including three aromatic amino acids, L-tryptophan, L-phenylalanine, and L-tyrosine (10). The primary catabolic products are tryptophol, phenylethanol, and tyrosol, respectively, which are collectively known as fusel oils (32, 34, 40). Fusel oil formation from amino acids is assumed to proceed via the Ehrlich pathway by means of three enzyme-catalyzed reactions. In the case of phenylalanine, the amino acid is deaminated to phenylpyruvic acid and then decarboxylated to phenylacetaldehyde and reduced to phenylethanol (Fig. 1) (16). Phenylethanol, which has a rose-like aroma, is an important fragrance in the cosmetic industry (9, 19) and possesses organoleptic characteristics that contribute to the quality of beverages and foods (19, 22, 52). While chemically synthesized phenylethanol is a valuable compound, phenylethanol that is synthesized biologically is 250- to 300-fold more expensive (17). Various organisms, including *S. cerevisiae*, can produce phenylethanol (2, 18, 50), and optimization of production in *S. cerevisiae* has been the subject of recent research (41). Despite this interest, the production of phenylethanol by *S. cerevisiae* is poorly characterized both genetically and biochemically.

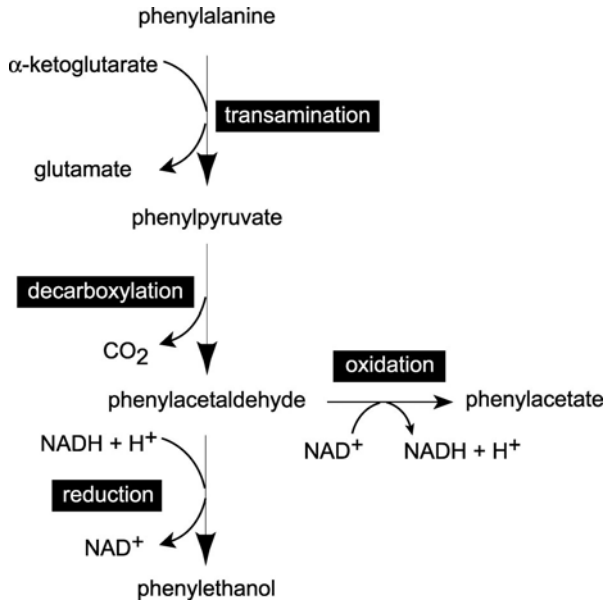

FIG. 1. Catabolism of phenylalanine via the Ehrlich pathway.

A critical step in phenylethanol production is the decarboxylase reaction, which is the first specific step in phenylalanine catabolism (Fig. 1). The *S. cerevisiae* genome contains five candidate genes that could encode phenylpyruvate decarboxylase activity. These are *PDC1*, *PDC5*, and *PDC6*, as well as two open reading frames, *YDR380w* and *YDL080c*, which are also thought to encode thiamine diphosphate-dependent decarboxylases (28). *PDC1*, *PDC5*, and *PDC6* encode the major activity for pyruvate decarboxylation (27). Both the activity

and nature of this enzyme activity in yeast have been extensively studied (for reviews see references 21 and 39). In the catabolism of branched-chain amino acids, the *PDC* genes contribute to fusel alcohol production, but a *PDC*-independent activity also exists (42). The *PDC* homologs Ydl080cp and Ydr380wp contribute to the catabolism of isoleucine (12), and the protein encoded by *YDL080c* is important for leucine catabolism, while valine catabolism involves several pyruvate decarboxylase isozymes (13, 14). For the aromatic amino acids, decarboxylases for the derived α-keto acids have not been described. However, Iraqui et al. (30) found that the *YDR380w/ARO10* open reading frame was transcriptionally induced when cells were grown in the presence of tryptophan with urea as a nitrogen source.

In this study, our objective was to identify the gene(s) that encodes phenylpyruvate decarboxylase(s) in *S. cerevisiae*. We hypothesized that one or more of the five *S. cerevisiae* genes for thiamine diphosphate-dependent decarboxylases encode phenylpyruvate decarboxylation activity. By using a combination of genetic, genomic, physiological, and biochemical approaches, we found that *YDR380w/ARO10* encodes the main physiologically relevant phenylpyruvate decarboxylase activity in wild-type *S. cerevisiae*. Additionally, we partially characterized an alternative activity that requires the presence of both *YDL080c* and one of the pyruvate decarboxylase genes.

MATERIALS AND METHODS

Strains

The *S. cerevisiae* strains used in this study are listed in Table 1. Strains were constructed by using standard yeast media and genetic techniques (3, 51). The kanamycin resistance cassette was amplified by using the pUG vector as the template (24).

Chemostat cultivation

Aerobic chemostat cultivation was performed at 30°C in 1-liter (working volume) laboratory fermentors (Applikon, Schiedam, The Netherlands) at a stirrer speed of 800 rpm and pH 5.0 with a dilution rate of 0.10 h^{-1}, as described by Van den Berg et al. (44). The pH was kept constant by using an ADI 1030 biocontroller (Applikon) and automatic addition of 2 M KOH. The fermentor was flushed with air at a flow rate of 0.5 liter min^{-1} by using a Brooks 5876 mass flow controller (Brooks Instruments, Veenendaal, The Netherlands). The dissolved oxygen concentration was continuously monitored with an Ingold model 34 100 3002 probe (Mettler-Toledo, Greifensee, Switzerland) and was more than 50% of air saturation. Carbon-limited steady-state chemostat cultures of both wild-type and mutant strains were grown on the mineral medium described by Verduyn et al. (47) containing 7.5 g of glucose $liter^{-1}$ as carbon source and either 5.0 g of $(NH_4)_2SO_4$ $liter^{-1}$ or 5.0 g of phenylalanine $liter^{-1}$ as the sole nitrogen source. When phenylalanine was the sole nitrogen source, the amino acid solution was sterilized separately by autoclaving it before addition to the medium, and the absence of $(NH_4)_2SO_4$ was compensated for by addition of equimolar amounts of K_2SO_4. For chemostat cultivation of pyruvate decarboxylase-negative strains, 7.1 g of glucose $liter^{-1}$ and 0.38 g of acetate $liter^{-1}$ (5% acetate on a

carbon basis) were used as carbon sources to overcome the C_2 requirement of *PDC*-negative strains (20). For anaerobic cultivation, media were supplemented with the anaerobic growth factors ergosterol and Tween 80 (10 and 420 mg liter^{-1}, respectively), and the glucose concentration was increased to 25 g liter^{-1} (49). To maintain anaerobic conditions, both the culture vessel and inflowing media were sparged with nitrogen gas at a flow rate of 0.5 liter min^{-1}, and the fermentors were equipped with Norprene tubing and butyl rubber septa to prevent O_2 diffusion into the cultures.

TABLE 1. Strains used in this study

Strain	Genotype	Source/ reference
CEN.PK113-7D	*MAT***a** *MAL2-8^c SUC2*	P.Kötter[a]
CEN.PK555-4A	*MAT***a** *MAL2-8^c SUC2 ydr380w:: loxP-Kan-loxP*	this study
CEN.PK632-3B	*MAT*α *MAL2-8c SUC2 ydl080c::loxP-Kan-loxP ydr380w::loxP-Kan-loxP*	this study
CEN.PK 608-4B	*MAT***a** *MAL2-8^c SUC2 pdc1::loxP pdc5::loxP pdc6::loxP ydl080c::loxP-Kan-loxP*	this study
CEN.PK609-11A	*MAT***a** *MAL2-8^c SUC2 pdc1::loxP pdc5::loxP pdc6::loxP ydr380w::loxP-Kan-loxP*	this study
CEN.PK 689-6C	*MAT***a** *MAL2-8^c SUC2 pdc5::loxP-Kan-loxP ydr380w::loxP-Kan-loxP*	this study

[a] Institut für Mikrobiologie der J.W. Goethe Universität, Marie-Curie-Strasse 9, Biozentrum N250, 60439 Frankfurt, Germany

Shake flask cultivation

Growth rate experiments were performed in 500-ml flasks containing 100 ml of medium, which were incubated at 30°C on an orbital shaker set at 200 rpm. When growth rates on phenylalanine were determined, mineral medium (47) with 5.0 g of phenylalanine liter^{-1} as the sole nitrogen source was used. The pH was adjusted to 6.0 with 2 M KOH, and then the medium was filter-sterilized with a MediaKap-5 filter (Spectrum Europe, Breda, The Netherlands) with a pore size of 0.2 μm. Sterile glucose was added to a final concentration of 2% as the carbon source.

Preparation of cell extracts

For preparation of cell extracts, culture samples were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, concentrated fourfold, and stored at -20°C. Before cell breakage, the samples were thawed at room temperature, washed, and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$ and 2 mM dithiothreitol. Extracts were prepared by sonicating preparations with 0.7-mm-diameter glass beads at 0°C for 2 min at 0.5-min intervals with an MSE sonicator (150 W output, 7-μm peak-to-peak amplitude). Unbroken cells and debris were removed by

centrifugation at 4°C for 20 min at 36,000 x *g*. The purified cell extracts were used for enzyme assays.

Enzyme assays

Pyruvate decarboxylase activity was measured as described by Flikweert et al. (20). Phenylpyruvate decarboxylase activity was measured at 30°C immediately after preparation of cell extracts by using a coupled reaction. Activity was measured by monitoring the reduction of NAD^+ at 340 nm in the presence of excess aldehyde dehydrogenase from yeast. The reaction mixtures (total volume, 1ml) contained 70 mM KH_2PO_4/ K_2HPO_4 buffer (pH 7.0), 2 mM NAD^+, 0.2 mM thiamine diphosphate, 0.35 U of yeast aldehyde dehydrogenase (Sigma-Aldrich, Zwijndrecht, The Netherlands) (dissolved in 1 mM dithiothreitol), and 2 mM phenylpyruvic acid to initiate the reaction. The reaction rates were linearly proportional to the amount of cell extract added. For determination of K_m and V_{max}, the reaction mixture remained the same while the substrate concentration was adjusted from 0.125 to 5 mM.

Analytical procedures

Measurements of biomass, metabolites from culture supernatants, and gasses were obtained as previously described (5). The metabolites of phenylalanine catabolism were analyzed with a high-performance liquid chromatograph fitted with an Alltech Platinum EPS C_{18} column (pore size, 0.01 μm; particle size, 5 μm; Alltech Nederland, Breda, The Netherlands). The mobile phase was phosphate buffer (pH 2.7) with a 5 to 40% acetonitrile gradient at a flow rate of 1 ml min^{-1} at room temperature. The error introduced by the measurement technique was less than 5%.

Microarrays

DNA microarray analyses were performed with S98 Yeast GeneChip arrays from Affymetrix (Santa Clara, Calif.) as previously described (37). Cells were transferred directly from chemostats into liquid nitrogen and processed according to the manufacturer's instructions (Affymetrix technical manual). Data analyses were performed with the following Affymetrix software packages: Microarray Suite v5.0, MicroDB v3.0, and Data Mining Tool v3.0. Microsoft Excel with the Significance Analysis of Microarrays (SAM v1.12) (43) plug-in was used for further statistical analyses.

RESULTS

Physiology of *S. cerevisiae* grown with phenylalanine as the sole nitrogen source

Phenylpyruvate, phenylacetate, and phenylethanol were all detected in the supernatants of aerobic, glucose-limited chemostat cultures grown with phenylalanine as the sole nitrogen source but not in cultures grown with ammonia as the sole nitrogen source (Table 2). These three catabolites and the residual phenylalanine in the

TABLE 2. Characteristics of aerobic and anaerobic glucose-limited chemostat cultures (D=0.1 h^{-1}) of wild-type *S. cerevisiae* CEN.PK 113-7D with phenylalanine or ammonia as the sole nitrogen source[a]

Culture		Physiological characteristics					Metabolite profiles of culture supernatants				
N source	Aeration	Biomass yield (g g of glucose^{-1})	$q_{glucose}$[b]	q_{O2}[c]	$q_{ethanol}$[d]	q_{CO2}[e]	Residual phenyl-alanine concn (mM)	Phenyl-pyruvate concn (mM)	Phenyl-acetate concn (mM)	Phenyl-ethanol concn (mM)	Recovery of phenyl C skeleton (%)[f]
$(NH_4)_2SO_4$ (38 mM)	Aerobic[g]	0.49±0.01	1.1±0.0	2.8±0.3	NA[h]	2.8±0.3	NA	<0.1	<0.1	<0.1	NA
Phenylalanine (30 mM)	Aerobic	0.30±0.01	1.7±0.2	7.1±0.3	NA	7.0±0.4	19.2±4.6	<0.1	11.0±3.1	1.7±0.2	106± 7%
$(NH_4)_2SO_4$ (38 mM)	Anaerobic[i]	0.10±0.00	5.6±0.2	NA	8.5±0.0	8.7±0.6	NA	<0.1	<0.1	<0.1	NA
Phenylalanine (30 mM)	Anaerobic	0.07±0.00	8.1±0.2	NA	12.4±0.3	15.2±0.4	21.7±1.5	0.12±0	<0.1	8.3±0.2	100±5%

[a] All values are means ± maximal deviations derived from two independent experiments.
[b] Rate of glucose consumption, expressed in millimoles of glucose consumed per gram of biomass per hour.
[c] Rate of O_2 consumption, expressed in millimoles of O_2 consumed per gram of biomass per hour.
[d] Rate of ethanol production, expressed in millimoles of ethanol produced per gram of biomass per hour.
[e] Rate of CO_2 production, expressed in millimoles of CO_2 produced per gram of biomass per hour.
[f] Amount of phenylalanine consumed (sum of phenylalanine catabolites in spent medium plus 0.17 mM per biomass for protein synthesis [33]) expressed as a percentage of the phenylalanine in the feed.
[g] Data from reference 5.
[h] NA, not applicable.
[i] Data are from reference 46.

culture medium could account for all the phenylalanine supplied in the feed, indicating that no other metabolites were formed from phenylalanine (Table 2). During anaerobic growth the metabolite profile of the supernatant was different from that during aerobic growth. No phenylacetate was detected, and the phenylalanine supplied could be accounted for by the amount of residual phenylalanine, phenylpyruvate, and phenylethanol (Table 2).

Phenylpyruvate decarboxylase activity (53 ± 4 nmol mg of protein^{-1} min^{-1}) was detected in cell extracts of aerobic, glucose-limited chemostat cultures grown with phenylalanine as the sole nitrogen source but not in extracts of cultures grown with ammonia as the sole nitrogen source. Thus, we concluded that phenylpyruvate decarboxylase was regulated and was induced in cultures with phenylalanine as the sole nitrogen source.

Identification of a putative phenylpyruvate decarboxylase gene

We used DNA microarrays to compare the transcriptomes of wild-type cells grown in glucose-limited chemostats with phenylalanine as the sole nitrogen source and the transcriptomes of cells grown with ammonia as the sole nitrogen source. (The entire data set is available at http://www.phepdc.bt.tudelft.nl.) We identified 89 transcripts that were expressed at a significantly higher level when phenylalanine was the sole nitrogen source and 146 transcripts that were expressed at a significantly higher level when ammonia was the sole nitrogen source. Of the five thiamine diphosphate-dependent decarboxylases, *YDR380w/ARO10* was the only transcript whose level increased (it increased 30-fold) when cells were grown on phenylalanine (Fig. 2). *YDR380w/ARO10* was therefore considered a strong candidate to encode phenylpyruvate decarboxylase activity.

To test this hypothesis, we grew an *aro10* deletion strain (CEN.PK 555-4A) in shake flasks with phenylalanine as the sole nitrogen source. The maximum specific growth rate of this strain was three- to fourfold lower than that of the wild-type strain in the same medium (data not shown). In contrast, the growth rates of the two strains were similar when ammonia was the sole nitrogen source, indicating that the reduced growth rate of the mutant was related to phenylalanine catabolism. In cell extracts of the *aro10* knock-out strain grown with phenylalanine as the sole nitrogen source, there was no detectable phenylpyruvate decarboxylase activity. In contrast, there was measurable activity (22 ± 1 nmol mg of protein^{-1} min^{-1}) in wild-type extracts grown under the same conditions. These data suggest that *ARO10* is both necessary and sufficient for phenylpyruvate decarboxylase activity in shake flask cultures of *S. cerevisiae*.

Identification of an alternative phenylpyruvate decarboxylase activity

The supernatant profile and enzyme activities of the *aro10* mutant strain were determined when cells were grown in aerobic, glucose-limited chemostat cultures at a dilution rate of 0.10 h^{-1} with phenylalanine as the sole nitrogen source. The profiles of phenylalanine catabolites in the culture supernatants were similar for wild-type strain CEN.PK113-7D and mutant strain CEN.PK555-4A (Table 3). However, in contrast to the situation in shake flask cultures, the measured phenylpyruvate decarboxylase activity of the *aro10* mutant grown with phenylalanine as the sole nitrogen source was fourfold higher than that of the wild-type strain. Thus, there was an alternative phenylpyruvate decarboxylase activity in chemostat-grown cells that was not expressed during growth in shake flasks. Indeed, a strain in which all thiamine diphosphate dependent decarboxylase genes except

ARO10 were deleted (CEN.PK 608-4B) still exhibited phenylpyruvate decarboxylase activity and had phenylpyruvate catabolites in the culture supernatants when it was grown in chemostat cultures with phenylalanine as the sole nitrogen source (Table 3).

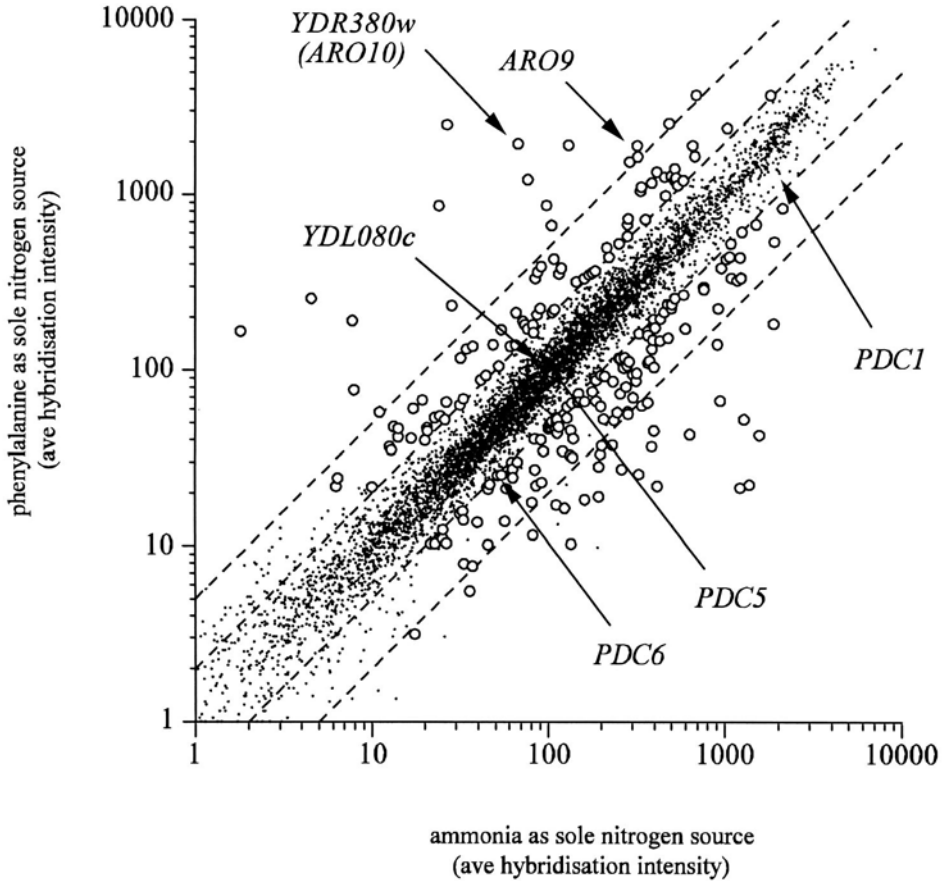

FIG. 2. Genome-wide transcription levels for wild-type *S. cerevisiae* grown on ammonia as the sole nitrogen source plotted versus genome-wide transcription levels for wild-type *S. cerevisiae* grown on phenylalanine as the sole nitrogen source: average signals in microarrays from glucose-limited chemostat cultures grown with ammonia (n = 4) and phenylalanine (n = 3) as the sole nitrogen sources for all 6,383 yeast open reading frames. The open circles represent genes whose expression was significantly different for the two conditions, while the solid dots represent transcripts whose expression was not significantly altered. The diagonal lines are the boundaries for two-and fivefold differences in signals between conditions. The full data set is available at http://www.phepdc.bt.tudelft.nl.

The *S.cerevisiae* genome contains four *PDC*-like open reading frames (*PDC1*, *PDC5*, *PDC6*, and *YDL080c*). Potentially, each of these could encode an enzyme that could decarboxylate phenylpyruvate in the absence of *ARO10*. The *PDC1*, *PDC5*, and *PDC6* gene products are extremely similar (79 to 86% sequence identity for all

pairwise comparisons), and each product has pyruvate decarboxylase activity (25, 26). The fourth homologous open reading frame, *YDL080c*, is less well characterized but has been implicated in the decarboxylation of the branched-chain 2-oxo acids and the regulation of genes involved in thiamine metabolism (12–14, 36).

Both a double-deletion strain (*aro10 ydl080c*; CEN.PK 632-3B) and a quadruple-deletion strain (*pdc1 pdc5 pdc6 aro10*; CEN.PK 609-11A) were grown in aerobic, glucose-limited chemostat cultures with phenylalanine as the sole nitrogen source. Cell extracts from these strains contained no measurable phenylpyruvate decarboxylase activity (Table 3). These assay data were supported by metabolite profile analyses of the culture supernatants, since phenylacetate was undetectable in both cultures and only small amounts of phenylethanol were found in the culture of the double-deletion strain (*aro10 ydl080c*) (Table 3). The quadruple-deletion strain containing only *YDL080c* (CEN.PK 609-11A) showed no evidence of the decarboxylase activities examined (Table 3), demonstrating that Ydl080cp alone could not decarboxylate phenylpyruvate or pyruvate. This result also indicated that it is unlikely that there are any other genes that encode the alternative decarboxylase activity. Instead, the phenotypes of the multiple-deletion strains indicate that the *ARO10*-independent phenylpyruvate decarboxylase activity requires the presence of both *YDL080c* and at least one of the *PDC* genes (Table 3).

Physiological relevance of *S. cerevisiae* phenylpyruvate decarboxylase activities

We determined the K_m and V_{max} values for Aro10p and the alternative phenylpyruvate decarboxylase activity. Cell extracts from a strain containing only the *ARO10*-encoded activity (CEN.PK 608-4B) were compared with extracts from a strain containing only the alternative activity involving Ydl080cp and one or more of the pyruvate decarboxylases (CEN.PK 555-4A). The phenylpyruvate decarboxylase activities of extracts of the wild-type strain and the quadruple-deletion strain (containing only Aro10p) displayed Michaelis-Menten saturation kinetics. Reduction of the substrate concentration in the assay mixture to less than 0.5 mM resulted in altered enzyme activity, from which the K_m and V_{max} values were estimated (Table 4). The V_{max} values for the activities of these two extracts were similar (Table 3). However, the extract from the quadruple mutant (CEN.PK 608-4B) had a slightly higher affinity (K_m, 0.062 ± 0.005 mM) for the substrate phenylpyruvate than the wild type had (K_m, 0.10 ± 0.001 mM). Extracts from the strain lacking *ARO10* alone (CEN.PK 555-4A) resulted in a sigmoidal curve in a plot of substrate concentration versus velocity. This result is consistent with previous observations of cooperativity of pyruvate decarboxylase in the presence of phosphate (6). This *PDC*-like behavior is consistent with the genetic data, according to which at least one *PDC* gene is required for the Aro10p-independent phenylpyruvate decarboxylase activity. In supernatants from wild-type cultures grown with phenylalanine as the sole nitrogen source, all of the phenylalanine consumed was recovered as either phenylacetate or phenylethanol (Table 2). By using the sum of the concentrations of phenylalanine catabolites in the supernatant and the biomass concentration in the culture vessel (2.23 g liter^{-1}), the specific rate of catabolite production was calculated to be 0.57 mmol g of biomass^{-1} h^{-1}. Since the catabolites were derived from phenylpyruvate decarboxylation, the steady-state flux through the decarboxylase enzyme must also have been 0.57 mmol g of biomass^{-1} h^{-1} (or 9.5 nmol mg of biomass^{-1} h^{-1}). Since the estimated soluble protein content is 0.33 g g of biomass^{-1} for yeast (38), this rate was converted to a specific activity of 29 nmol mg of

protein^{-1} min^{-1}. If values were substituted into the Michaelis-Menten equation and the V_{max} and K_m values obtained for wild-type cell extracts were used, the substrate concentration inside the cells was 0.11 mM. This value is the same as the K_m determined for wild-type extracts and is slightly higher than the K_m found for the mutant containing Aro10p only (CEN.PK 608-4B) (Table 4). The K_m of the activity from cells lacking *ARO10* was five- to eightfold higher than that from strains that contained a wild-type *ARO10* allele (Table 4). Therefore, at deduced intracellular phenylpyruvate concentrations of ca. 0.1 mM, this compound is preferentially catabolized by the *ARO10*-encoded activity.

Involvement of *PDC5* in *ARO10*-independent phenylpyruvate decarboxylation

We used a recently compiled transcriptome database for cells grown under four different nutrient limitation regimens (5) to evaluate the correlation between the expression of thiamine diphosphate-dependent decarboxylase genes and phenylpyruvate decarboxylase activity in wild-type *S. cerevisiae* (Table 5) (for the complete data sets accompanying these arrays see reference 5). Phenylpyruvate decarboxylase was detected only during aerobic growth under nitrogen limitation conditions with ammonia as the nitrogen source and glucose as the carbon source and when there was phosphate-limited growth with ammonia as the nitrogen source and glucose as the carbon source (Table 5). Under these conditions, the levels of the *ARO10* transcript were negligible, indicating that the observed phenylpyruvate decarboxylase activity was due to the *ARO10*-independent activity discussed above. In all four cultures, low but significant levels of the *YDL080c* transcript were detected. However, phenylpyruvate decarboxylase was detected in cell extracts when *PDC5* was transcribed at high levels but not when *PDC1* (glucose limitation) or *PDC6* (sulfur limitation) was the predominantly transcribed *PDC* gene. Furthermore, the levels of the *PDC5* transcript in glucose-limited chemostat cultures of the *aro10* strain grown with phenylalanine as the sole nitrogen source were over eightfold higher than the levels in similar cultures of the wild type, while the levels of the *PDC1*, *PDC6*, and *YDL080c* transcripts differed by less than twofold (data not shown).

To test the hypothesis that *PDC5* but not *PDC6* or *PDC1* contributes to the alternative phenylpyruvate decarboxylase activity, we grew a *pdc5 aro10* double-deletion strain (CEN.PK 689-6C) in aerobic, glucose-limited chemostat cultures with phenylalanine as the sole nitrogen source. Cell extracts of steady-state chemostat cultures did not exhibit significant phenylpyruvate decarboxylase activity (<2.5 nmol mg of protein^{-1} min^{-1}), and <10% of the phenylpyruvate formed was converted to phenylacetate. The pyruvate decarboxylase activity in cell extracts of these cultures was 565 ± 45 nmol mg of protein^{-1} min^{-1}, and preliminary experiments indicated that *PDC1* but not *PDC6* was expressed transcriptionally (as determined with a single measurement)

TABLE 3. Enzyme activities and metabolite profiles of aerobic glucose-limited chemostat culture of *S. cerevisiae* strains grown with phenylalanine as the sole nitrogen source (Dilution rate= 0.1 h^{-1})[a]

Strain	Relevant genotype	Enzyme activities (nmol.mg of protein^{-1}.min^{-1})		Conc. of phenylalanine catabolites in culture supernatants (mM)		
		Pyruvate decarboxylase	Phenylpyruvate decarboxylase	Phenyl-pyruvate	Phenyl-acetate	Phenyl-ethanol
CEN.PK 113-7D	*PDC1 PDC5 PDC6 YDR380w YDL080c*[b]	140 ± 2.0	52.5 ± 3.5	< 0.1	11.0 ± 3.1	1.7 ± 0.2
CEN.PK 555-4A	*PDC1 PDC5 PDC6 ydr380w YDL080c*	180 ± 2.0	220 ± 17	0.75 ± 0.40	8.93 ± 0.18	2.5 ± 0.0
CEN.PK 632-3B	*PDC1 PDC5 PDC6 ydr380w ydl080c*	540 ± 55	< 2.5	7.37 ± 0.12	< 0.1	0.81 ± 0.0
CEN.PK 609-11A	*pdc1 pdc5 pdc6 ydr380w YDL080c*	< 10	< 2.5	6.5 ± 1.0	< 0.1	< 0.1
CEN.PK 608-4B	*pdc1 pdc5 pdc6 YDR380w ydl080c*	< 10	40 ± 1.5	0.41 ± 0.1	7.4 ± 2	3.5 ± 0.3

[a] All values are means ± maximal deviations derived from two independent experiments.

[b] *YDR380w* is *ARO10*, and *YDL080c* is *THI3*.

TABLE 4. K_m and V_{max} values[a] determined for phenylpyruvate decarboxylase activity in cell extracts from three *S. cerevisiae* strains grown in aerobic glucose-limited chemostats with phenylalanine as the sole nitrogen source (dilution rate, 0.1 h^{-1})

Strain	Relevant genotype	K_m (mM)	V_{max} (nmol.mg of protein^{-1}.min^{-1})
CEN.PK 113-7D	*PDC1 PDC5 PDC6 YDR380w YDL080c*	0.10 ± 0.001	54 ± 6
CEN.PK 608-4B	*pdc1 pdc5 pdc6 YDR380w ydl080c*	0.062 ± 0.005	38 ± 3
CEN.PK 555-4A	*PDC1 PDC5 PDC6 ydr380w YDL080c*	0.48 ± 0.08	236 ± 28

[a] The values are means ± maximal deviations derived from two independent experiments for CEN.PK 113-7D and CEN.PK 608-4B and from three independent experiments for CEN.PK 555-4A.

(data not shown). However, when the cultures were grown for more than eight generations, the phenylpyruvate decarboxylase activity gradually increased and, after approximately 15 generations, reached specific activities in vitro that exceeded those of wild-type cultures and were similar to that of the *aro10* deletion strain (265 ± 10 nmol mg of protein^{-1} min^{-1}). This increase was reflected in the metabolite profile of the growth medium, which was similar to that of the *aro10* deletion strain growth medium (data not shown).

TABLE 5. Enzyme activities and transcript levels of the thiamine diphosphate-dependent decarboxylases of *S. cerevisiae* under four nutrient limitation regimens in chemostat cultures[a]

	Enzyme activities (nmol.mg of protein^{-1}.min)		Transcript level[b]				
Growth limitation	Pyruvate decarboxylase	Phenyl-pyruvate decarboxylase	*PDC1*	*PDC5*	*PDC6*	*YDR380w*	*YDL080c*
Carbon	580 ± 0.0	<2.5	1946 ± 449	95 ± 5	66 ± 44	67 ± 4	92 ± 9
Nitrogen	2265 ± 25	10.15 ± 0.05	2524 ± 188	1708 ± 87	161 ± 30	< 12	249 ± 34
Phosphorous	1830 ± 50	4.37 ± 0.35	2366 ± 354	836 ± 33	37 ± 7	14 ± 1	120 ± 27
Sulfur	520 ± 30	<2.5	1969 ± 238	169 ± 36	1874 ± 236	< 12	105 ± 16

[a] The cultivation conditions and transcript data are from reference 5.

[b] The transcript levels are data for triplicate arrays sampled from three independent chemostats.

DISCUSSION

Products of phenylalanine catabolism in *S. cerevisiae*

When wild-type *S. cerevisiae* was grown in glucose-limited chemostats with phenylalanine as the sole nitrogen source, phenylethanol and phenylacetate could account for all of the phenylalanine consumed from the feed (Table 2). This result is consistent with the involvement of the Ehrlich pathway in phenylalanine catabolism (Fig. 1). In anaerobic chemostat cultures there was an almost stoichiometric conversion of phenylalanine to phenylethanol (Table 2) that probably reflected the altered redox state of the cells to favor the reductive branch of the Ehrlich pathway over the oxidative, phenylacetate-yielding branch (Fig. 1). The presence of these catabolites in the medium was reflected in the physiological growth parameters measured.

When phenylalanine was used instead of ammonia as the sole nitrogen source, the biomass yield on glucose (expressed in grams [dry weight] of biomass per gram of glucose consumed) of wild-type *S. cerevisiae* was substantially lower (Table 2). In addition, higher rates of carbon dioxide production and, in the aerobic cultures, oxygen consumption accompanied the reduced biomass yield. It has been hypothesized that these changes are indicative of uncoupling caused by enhanced proton cycling via the weak acid phenylacetate (48) and by the stimulating effect of phenylethanol on membrane fluidity (29, 41).

Decarboxylation of phenylpyruvate is not a prerequisite for the transamination of phenylalanine and, hence, utilization of this compound as sole nitrogen source (Fig. 1). Nevertheless, growth with phenylalanine as the sole nitrogen source in shake flask cultures was substantially slower for an *aro10* mutant, which lacked detectable phenylpyruvate decarboxylase activity, than for the wild type. This difference suggests that the physiological role of phenylpyruvate decarboxylation may be to prevent the accumulation of growth-inhibiting concentrations of phenylpyruvate.

Transcriptional regulation of phenylalanine catabolism

Transcriptional regulation of genes in response to phenylalanine occurs via at least two routes in *S. cerevisiae*. The first route involves an intracellular sensor of aromatic amino acids that regulates genes through the transcriptional activator Aro80p (30), and the second route involves a sensor of external amino acids (31). We identified the binding site for Stp1p (a transcriptional regulator downstream of extracellular amino acid sensing [35]), which was overrepresented in the promoters of genes whose transcription was greater when phenylalanine was the nitrogen source (45). An additional element known to bind the GATA family of transcriptional regulators (8) also was found, indicating that there was control via the general response to the use of a nonpreferred nitrogen source (nitrogen catabolite repression [NCR]). Only nine gene promoters in the genome contain an exact match with the proposed binding site for Aro80p (direct repeat of 5′-T[A/T][A/G]CCG-3′ separated by four nucleotides) (30). Among the nine genes, there are four pairs of divergently transcribed genes and one gene without a shared promoter. There were three genes with significantly higher transcript levels in chemostat cultures containing phenylalanine instead of ammonia as the sole nitrogen source (*ARO9*, *ARO10*, and *ESPB6*, exhibiting 6-, 30-, and 2.5-fold changes in transcript levels,

respectively). The promoters of these three genes contained the binding site repeat in the forward direction. If this promoter element operates unidirectionally, it could explain why five of the remaining genes, which have the reverse complement sequence in their promoters, were not regulated in a similar manner. Based on phenotypic analysis of an *aro80* deletion strain, it is not surprising that the domain of Aro80p's control is limited to the degradation of aromatic amino acids (1, 30). Similar regulatory events have also been reported in other microorganisms (4, 11, 23) and probably result from the need to separate phenylalanine biosynthesis from phenylalanine catabolism since the two pathways share phenylpyruvate as an intermediate and are thought to be colocalized in the cytosol.

The wider effects on the transcriptome could be caused by the activity of NCR due to the presence of an aromatic amino acid as the sole nitrogen source. Many genes (including a number of regulated genes according to our data that are required for nutrient transport) are under the control of this regulon. However, previous results have shown that NCR does not directly regulate expression of *ARO9* or *ARO10* (30). Rather, NCR modulates the expression of these genes indirectly by preventing Aro80p-dependent induction by inducer exclusion. Thus, cells cofed ammonia and an aromatic amino acid should preferentially catabolize ammonia by preventing uptake of the aromatic amino acid. Conversely, when ammonia is limiting for growth (or absent), this general repression is relieved, which allows uptake and assimilation of amino acids for use as nitrogen sources (See supplementary material at http://www.phepdc.bt.tudelft.nl for all gene changes).

Substrate specificity of thiamine diphosphate-dependent decarboxylases

Of the five thiamine diphosphate-dependent decarboxylase genes in *S. cerevisiae*, the *ARO10* transcript was the only transcript changed, and the level was 30-fold higher during growth on phenylalanine as the sole nitrogen source than during growth on ammonia as the sole nitrogen source. Two lines of evidence confirm that *ARO10* encodes an active phenylpyruvate decarboxylase: (i) the clear phenotype of an *aro10* null mutant in shake flask cultures grown with phenylalanine as the sole nitrogen source and (ii) the phenotype of a quadruple *pdc1 pdc5 pdc6 ydl080c* mutant in chemostat cultures grown with phenylalanine as the sole nitrogen source.

An alternative, *ARO10*-independent phenylpyruvate decarboxylase activity also was observed in chemostat cultures of the *aro10* null mutant. This activity was not detectable in an *aro10 ydl080c* double mutant. The *YDL080c* product exhibits strong sequence similarity with known thiamine diphosphate-dependent decarboxylases and has a regulatory role in thiamine metabolism (7). Our data show that Ydl080cp cannot decarboxylate phenylpyruvate by itself. However, the combined presence of Ydl080cp and a pyruvate decarboxylase is required for the *ARO10*-independent phenylpyruvate decarboxylase activity. We have recently obtained evidence that a similar situation exists for the branched-chain 2-oxo acids that are formed during the catabolism of leucine, valine, and isoleucine (M. A. Morais and Z. Vuralhan, unpublished data). Analysis of transcript levels in wild-type cultures, as well as physiological analysis of a *pdc5 aro10* strain, indicated that Pdc5p is primarily involved in the *ARO10*-independent, *YDL080c*-dependent phenylpyruvate decarboxylase activity. However the reappearance of phenylpyruvate decarboxylase activity of a *pdc5 aro10* double mutant after prolonged chemostat cultivation suggests that only minor genetic changes allow another *PDC* gene to take

over this role. We have not yet identified the nature of these mutations.

Part of the regulation of the different decarboxylases may occur at the level of transcription through a mechanism in which Thi3p acts as a sensor of intracellular thiamine diphosphate (7). In the presence of phenylalanine, the levels of the *ARO10* transcript were among the highest 3% of the levels of transcripts of transcribed genes in wild-type cells. Deletion of this gene would probably alter the levels of intracellular thiamine diphosphate and trigger a signal via Thi3p to control transcription. However, the poor correlation between the transcript level and enzyme activity (Table 5) indicates that posttranscriptional regulation of the decarboxylase activities also occurs. An attractive model for this posttranscriptional regulation depends upon the in vivo tetrameric form of pyruvate decarboxylase (21). If the Pdc-like proteins can form heterotetramers, the resulting decarboxylase activities may have different substrate specificities. In this model, Aro10p and Thi3p could combine with one or more of the *PDC*-encoded proteins to produce enzymes that decarboxylate the α-keto acids produced during catabolism of the aromatic and branched-chain amino acids. This hypothesis can be tested with reconstitution experiments performed with different amounts and combinations of the purified proteins.

This study increased our understanding of phenylalanine catabolism in *S. cerevisiae* and illustrated the power of combining genome-wide transcript analyses with biochemical and genetic techniques to untangle functionally redundant enzyme activities. To date, analyses of single and multiple knockout mutants have proven to be insufficient to identify singular roles for the thiamine diphosphate-dependent decarboxylases in amino acid catabolism (12–15, 20). Our results provide new insight into the complexity of the regulation of substrate specificity of these decarboxylases, and they also provide a good basis for targeted metabolic engineering of phenylalanine catabolism.

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ADDENDUM

While this manuscript was under review, Dickinson et al. (15) reported on the catabolism of phenylalanine to phenylethanol and the catabolism of tryptophan to tryptophol in *S. cerevisiae*. Using ^{13}C nuclear magnetic resonance spectroscopy, gas chromatography-mass spectrometry, and a range of mutants, these authors showed that Aro10p can catalyse the decarboxylation of phenylpyruvate to phenylacetaldehyde and the decarboxylation of indolepyruvate to indolacetaldehyde and that, in the absence of an active *aro10* gene, pyruvate decarboxylases are involved in phenylpyruvate decarboxylation.

REFERENCES

1. **Akache, B., K. Wu, and B. Turcotte.** 2001. Phenotypic analysis of genes encoding yeast zinc cluster proteins. Nucleic Acids Res. **29:**2181–2190.
2. **Albertazzi, E., R. Cardillo, S. Servi, and G. Zucchi.** 1994. Biogeneration of 2-phenylethanol and 2-phenylethylacetate important aroma components. Biotechnol. Lett. **16:**491–496.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley and Sons, New York, N.Y.
4. **Barrowman, M. M., and C. A. Fewson.** 1985. Phenylglyoxylate decarboxylase and phenylpyruvate decarboxylase from *Acinetobacter calcoaceticus*. Curr. Microbiol. **12:**235–240.
5. **Boer, V. M., J. H. de Winde, J. T. Pronk, and M. D. W. Piper.** 2003. The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus or sulfur. J. Biol. Chem. **278:**3265–3274.
6. **Boiteux, A., and B. Hess.** 1970. Allosteric properties of yeast pyruvate decarboxylase. FEBS Lett. **9:**293–296.
7. **Burrows, R. J., K. L. Byrne, and P. A. Meacock.** 2000. Isolation and characterization of *Saccharomyces cerevisiae* mutants with derepressed thiamine gene expression. Yeast **16:**1497–1508.
8. **Bysani, N., J. R. Daugherty, and T. G. Cooper.** 1991. Saturation mutagenesis of the UASNTR (GATAA) responsible for nitrogen catabolite repression-sensitive transcriptional activation of the allantoin pathway genes in *Saccharomyces cerevisiae*. J. Bacteriol. **173:**4977–4982.
9. **Clark, G. S.** 1990. Phenylethyl alcohol. Perfumer Flavorist **15:**37–44.
10. **Cooper, T. G.** 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*,p. 39–99. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
11. **de Boer, L., W. Harder, and L. Dijkhuizen.** 1988. Phenylalanine and tyrosine metabolism in the facultative methylotroph *Nocardia* sp. 239. Arch. Microbiol. **149:**459–465.
12. **Dickinson, J. R., S. J. Harrison, J. A. Dickinson, and M. J. Hewlins.** 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. **275:**10937–10942.
13. **Dickinson, J. R., S. J. Harrison, and M. J. Hewlins.** 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae.* J. Biol. Chem. **273:**25751–25756.
14. **Dickinson, J. R., M. M. Lanterman, D. J. Danner, B. M. Pearson, P. Sanz, S. J. Harrison, and M. J. Hewlins.** 1997. A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. **272:**26871–26878.
15. **Dickinson, J. R., L. E. J. Salgado, and M. J. E. Hewlins.** 2003. The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. J. Biol. Chem. **278:**8028–8034.

16. **Ehrlich, F.** 1907. Uber die Bedingungen der Fuselölbildung and über ihren Zusammenhang mit dem Eiweissaufbau der Hefe. Ber. Dtsch. Chem. Ges. **40:**1027–1047.
17. **Etschmann, M. M. W., W. Bluemke, D. Sell, and J. Schrader.** 2002. Biotechnological production of 2-phenylethanol. Appl. Microbiol. Biotechnol. **59:**1–8.
18. **Fabre, C. E., P. J. Blanc, and G. Goma.** 1997. Screening of yeasts producing 2-phenylethylalcohol. Biotechnol. Tech. **11:**523–525.
19. **Fabre, C. E., P. J. Blanc, and G. Goma.** 1998. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. Biotechnol. Prog. **14:**270–274.
20. **Flikweert, M. T., L. Van Der Zanden, W. M. Janssen, H. Y. Steensma, J. P. van Dijken, and J. T. Pronk.** 1996. Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. Yeast **12:**247–257.
21. **Furey, W., P. Arjunan, L. Chen, M. Sax, F. Guo, and F. Jordan.** 1998. Structure-function relationships and flexible tetramer assembly in pyruvate decarboxylase revealed by analysis of crystal structures. Biochim. Biophys. Acta **1385:**253–270.
22. **Furia, T. E., and N. Bellanca.** 1971. Fenaroli's handbook of flavor ingredients. CRC Press, Cleveland, Ohio.
23. **Gopalakrishna, Y., T. K. Narayanan, and G. R. Rao.** 1976. Biosynthesis of beta-phenethyl alcohol in *Candida guilliermondii*. Biochem. Biophys. Res. Commun. **69:**417–422.
24. **Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann.** 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. **24:**2519–2524.
25. **Hohmann, S.** 1991. Characterization of *PDC6*, a third structural gene for pyruvate decarboxylase in *Saccharomyces cerevisiae*. J. Bacteriol. **173:**7963– 7969.
26. **Hohmann, S.** 1991. *PDC6*, a weakly expressed pyruvate decarboxylase gene from yeast, is activated when fused spontaneously under the control of the *PDC1* promoter. Curr. Genet. **20:**373–378.
27. **Hohmann, S., and H. Cederberg.** 1990. Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. Eur. J. Biochem. **188:**615–621.
28. **Hohmann, S., and P. A. Meacock.** 1998. Thiamine metabolism and thiamine diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae*: genetic regulation. Biochem. Biophys. Acta **1385:**201–219.
29. **Ingram, L. O., and T. M. Buttke.** 1984. Effects of alcohols on microorganisms. Adv. Microb. Physiol. **25:**253–300.
30. **Iraqui, I., S. Vissers, B. Andre´, and A. Urrestarazu.** 1999. Transcriptional induction by aromatic amino acids in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19:**3360–3371.
31. **Iraqui, I., S. Vissers, F. Bernard, J. O. de Craene, E. Boles, A. Urrestarazu, and B. Andre´.** 1999. Amino acid signaling in *Saccharomyces cerevisiae*:a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the *AGP1* gene, which encodes a broad-specificity amino acid permease. Mol. Cell. Biol. **19:**989–1001.

32. **Kradolfer, P., P. Niederberger, and R. Hutter.** 1982. Tryptophan degradation in *Saccharomyces cerevisiae*: characterization of two aromatic aminotransferases. Arch. Microbiol. **133:**242–248.
33. **Lange, H. C., and J. J. Heijnen.** 2001. Statistical reconciliation of the elemental and molecular biomass composition of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **75:**334–344.
34. **Lingens, F.** 1967. The biosynthesis of aromatic amino acids and its controls. Angew. Chem. Int. Ed. Engl. **6:**811.
35. **Nielsen, P. S., B. van den Hazel, T. Didion, M. de Boer, M. Jorgensen, R. J. Planta, M. C. Kielland-Brandt, and H. A. Andersen.** 2001. Transcriptional regulation of the *Saccharomyces cerevisiae* amino acid permease gene *BAP2*. Mol. Gen. Genet. **264:**613–622.
36. **Nishimura, H., Y. Kawasaki, Y. Kaneko, K. Nosaka, and A. Iwashima.** 1992. A positive regulatory gene, *THI3*, is required for thiamine metabolism in *Saccharomyces cerevisia*e. J. Bacteriol. **174:**4701–4706.
37. **Piper, M. D. W., P. Daran-Lapujade, C. Bro, B. Regenberg, S. Knudsen, J. Nielsen, and J. T. Pronk.** 2002. Reproducibility of oligonucleotide microarray transcriptome analyses: an interlaboratory comparison using chemostat cultures of *Saccharomyces cerevisiae*. J. Biol. Chem. **277:**37001–37008.
38. **Postma, E., C. Verduyn, W. A. Scheffers, and J. P. van Dijken.** 1989. Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **55:**468–477.
39. **Pronk, J. T., H. Y. Steensma, and J. P. van Dijken.** 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast **12:**1607–1633.
40. **Sentheshanmuganathan, S.** 1960. The mechanism of the formation of higher alcohols from amino acids by *Saccharomyces cerevisiae*. Biochem. J. **74:**568–576.
41. **Stark, D., T. Munch, B. Sonnleitner, I. W. Marison, and U. von Stockar.** 2002. Extractive bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. Biotechnol. Prog. **18:**514–523.
42. **ter Schure, E. G., M. T. Flikweert, J. P. van Dijken, J. T. Pronk, and C. T. Verrips.** 1998. Pyruvate decarboxylase catalyzes decarboxylation of branched-chain 2-oxo acids but is not essential for fusel alcohol production by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **64:**1303–1307.
43. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA **98:**5116–5121.
44. **Van den Berg, M. A., P. de Jong-Gubbels, C. J. Kortland, J. P. van Dijken, J. T. Pronk, and H. Y. Steensma.** 1996. The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. J. Biol. Chem. **271:**28953–28959.
45. **Van Helden, J., B. Andre´, and J. Collado-Vides.** 2000. A web site for the computational analysis of yeast regulatory sequences. Yeast **16:**177–187.
46. **Van Hoek, P., J. P. van Dijken, and J. T. Pronk.** 2000. Regulation of fermentative capacity and levels of glycolytic enzymes in chemostat cultures of *Saccharomyces cerevisiae*. Enzyme Microb. Technol.

26:724–736.

47. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken.** 1990. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J. Gen. Microbiol. **136:**395–403.
48. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken.** 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast **8:**501–517.
49. **Visser, W., W. A. Scheffers, W. H. Batenberg-van der Vegte, and J. P. van Dijken.** 1990. Oxygen requirements of yeasts. Appl. Environ. Microbiol. **56:**3785–3792.
50. **Vollbrecht, D., and F. Radler.** 1973. Die Bildung höherer Alkohole bei Aminosaüremangelmutanten von *Saccharomyces cerevisiae*. Arch. Microbiol. **94:**351–358.
51. **Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen.** 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10:**1793–1808.
52. **Welsh, F. W., W. D. Murray, and R. E. Williams.** 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. Crit. Rev. Biotechnol. **8:**105–169.

3

PHYSIOLOGICAL CHARACTERIZATION OF THE *ARO10*-DEPENDENT, BROAD-SUBSTRATE-SPECIFICITY 2-OXO ACID DECARBOXYLASE ACTIVITY OF *SACCHAROMYCES CEREVISIAE*

ABSTRACT

Aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* CEN.PK113-7D were grown with different nitrogen sources. Cultures grown with phenylalanine, leucine, or methionine as a nitrogen source contained high levels of the corresponding fusel alcohols and organic acids, indicating activity of the Ehrlich pathway. Also, fusel alcohols derived from the other two amino acids were detected in the supernatant, suggesting the involvement of a common enzyme activity. Transcript level analysis revealed that among the five thiamine-pyrophospate-dependent decarboxylases (*PDC1*, *PDC5*, *PDC6*, *ARO10*, and *THI3*), only *ARO10* was transcriptionally up-regulated when phenylalanine, leucine, or methionine was used as a nitrogen source compared to growth on ammonia, proline, and asparagine. Moreover, 2-oxo acid decarboxylase activity measured in cell extract from CEN.PK113-7D grown with phenylalanine, methionine, or leucine displayed similar broad-substrate 2-oxo acid decarboxylase activity. Constitutive expression of *ARO10* in ethanol-limited chemostat cultures in a strain lacking the five thiamine-pyrophosphate-dependent decarboxylases, grown with ammonia as a nitrogen source, led to a measurable decarboxylase activity with phenylalanine-, leucine-, and methionine-derived 2-oxo acids. Moreover, even with ammonia as the nitrogen source, these cultures produced significant amounts of the corresponding fusel alcohols. Nonetheless, the constitutive expression of *ARO10* in an isogenic wild-type strain grown in a glucose-limited chemostat with ammonia did not lead to any 2-oxo acid decarboxylase activity. Furthermore, even when *ARO10* was constitutively expressed, growth with phenylalanine as the nitrogen source led to increased decarboxylase activities in cell extracts. The results reported here indicate the involvement of posttranscriptional regulation and/or a second protein in the *ARO10*-dependent, broad-substrate specificity decarboxylase activity.

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INTRODUCTION

Saccharomyces cerevisiae has a narrow range of carbon sources that support growth (1) but is considerably more flexible with respect to the utilization of nitrogen sources (2). Most amino acids can be utilized as sole nitrogen sources but not as sole carbon sources for growth (28). The most common mechanism for utilizing amino acids as nitrogen sources is transamination, using 2-oxoglutarate or other 2-oxo acids as amino acceptors. This process leaves the carbon skeleton of the amino acid intact, in the form of a 2-oxo acid. For some amino acids (e.g., alanine), the resulting 2-oxo acid, pyruvate, can be readily co-metabolized in central metabolism. In other cases, such as for the aromatic and branched-chain amino acids, the 2-oxo acids resulting from transamination are not intermediates of central metabolism. Even though they cannot be used as auxiliary carbon sources, these compounds are often transformed by the yeast cells before they are excreted into the growth medium.

An important and common pathway for catabolism of amino acids by yeasts is called the Ehrlich pathway (7–12, 37). This pathway is initiated by transamination of the amino acid to the corresponding 2-oxo acid. This 2-oxo acid is then decarboxylated to the corresponding aldehyde. Depending on the redox status of the cells (44), the aldehydes can then be reduced by alcohol dehydrogenases (yielding a group of compounds commonly referred to as fusel alcohols) or be oxidized to the corresponding organic acid ("fusel acids") by aldehyde dehydrogenases (Fig. 1). The fusel alcohols and their esters are especially important contributors to the flavor and aroma of fermented beverages (6, 16, 45). Phenylethanol, which has a typical rose-like flavor, can be produced by biotransformation of phenylalanine with *S.cerevisiae* cell suspensions (38, 39).

The identity of the decarboxylase(s) that catalyzes the initial step of the Ehrlich pathway has recently been investigated in our laboratories (7, 9–11, 44). The *S.cerevisiae* genome contains five genes that share sequence similarities with genes encoding thiamine pyrophosphate (TPP)-dependent decarboxylases (19, 20, 27) (for a review, see reference 21). Three of these genes (*PDC1*, *PDC5*, and *PDC6*) encode pyruvate decarboxylases. *PDC1* and *PDC5* encode the major pyruvate decarboxylases under most cultivation conditions (15, 20); *PDC6* is specifically expressed under low-sulfur conditions and encodes a pyruvate decarboxylase that has a low content of sulfur-containing amino acids (4, 14). Mutants in which all three *PDC* genes have been inactivated, and which completely lack pyruvate decarboxylase activity, still express branched-chain and aromatic 2-oxo acid decarboxylase activities (7, 40, 44). The other two members of this gene family are *ARO10* and *THI3*. Based on studies with deletion mutants, both have been implicated in the decarboxylation of branched-chain and aromatic 2-oxo acids (7, 10, 44). In addition, Thi3p has been assumed to be a positive regulator of the thiamine biosynthetic pathway. Upon its deletion, the transcription of all the genes of thiamine biosynthesis was negatively affected (13, 21). An *aro10 thi3* double-deletion mutant completely lacks phenylpyruvate decarboxylase activity, whereas the single-deletion mutants in these genes retain this enzyme activity (44). This might lead to the simple conclusion that both genes encode active phenylpyruvate decarboxylases. However, the situation is more complicated, as *pdc1 pdc5 pdc6 thi3* quadruple-deletion mutants, but not *pdc1 pdc5 pdc6 aro10* mutants, express phenylpyruvate decarboxylase activity (7, 10, 44). These and other observations have led to the proposal that *THI3* may not by itself encode an active phenylpyruvate decarboxylase but requires the

simultaneous expression of one of the *PDC* genes to contribute to phenylpyruvate decarboxylase activity (44). This provided a first indication that the regulation and substrate specificities of the TPP-dependent decarboxylases in *S.cerevisiae* may be more complicated than a simple situation in which substrate specificity is determined by a mixture of five decarboxylases with defined—if overlapping—substrate specificities and kinetics.

With the exception of the transcriptional regulation of *ARO10* by aromatic amino acids modulated by the positive transcription factor *ARO80* (24), comparatively little is known about the regulation of fusel alcohol production in *S.cerevisiae* and the impact of the expression levels of the decarboxylase genes on the rates of production of the different decarboxylases.

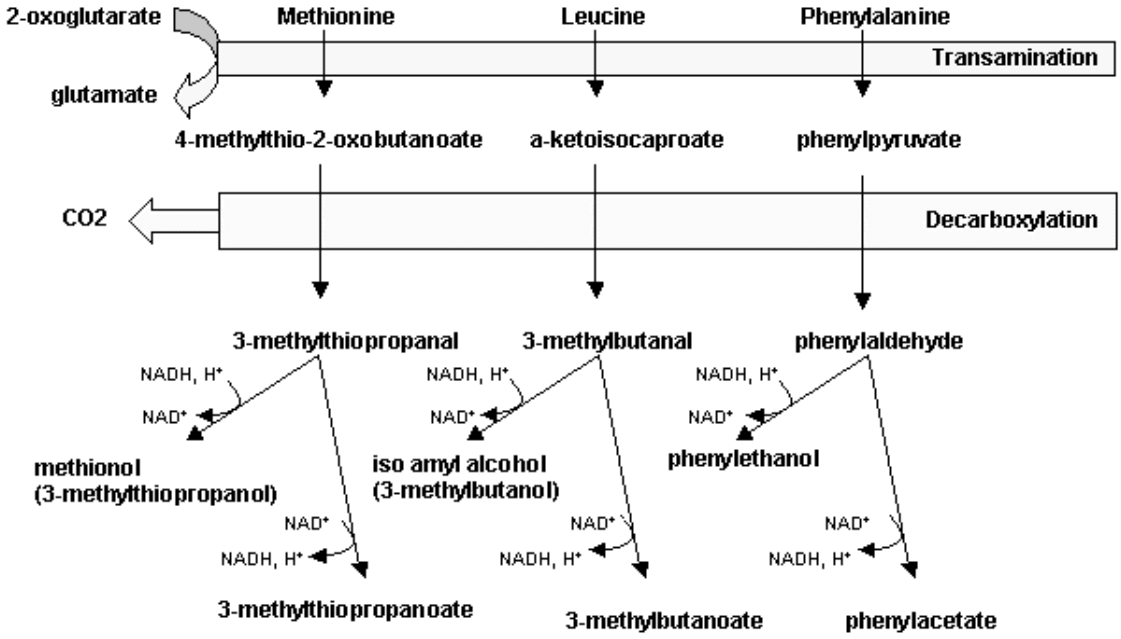

FIG. 1. Formation of fusel alcohols and fusel organic acids during the catabolism of the amino acids leucine, phenylalanine, and methionine

The aim of the present study was to analyze the substrate specificity of the *ARO10*-dependent decarboxylase activity in *S.cerevisiae*, its impact on the production of fusel alcohols and acids, and the importance of transcriptional regulation in controlling its in vivo activity. To this end, we correlated the expression of *ARO10* (as well as that of the other decarboxylase genes) with the levels of fusel alcohols and acids in chemostat cultures of *S.cerevisiae* grown with different nitrogen sources. Subsequently, we investigated the substrate

specificity of the *ARO10*-dependent decarboxylase activity and the impact of transcriptional regulation of *ARO10* on this activity by constitutively expressing *ARO10* in a wild-type *S.cerevisiae* strain, as well as in a *pdc1 pdc5 pdc6 aro10 thi3* quintuple-null mutant.

MATERIALS AND METHODS

Strains

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1.

Recombinant-DNA techniques

Standard protocols were followed for plasmid isolation, restriction, ligation, transformation, and gel electrophoresis (30). Yeast chromosomal DNA was isolated by a method described previously (22). *S.cerevisiae* strains were transformed using the lithium acetate–single-stranded carrier DNA–polyethylene glycol method (17).

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
CEN.PK113-7D	*MAT**a** MAL2-8c SUC2* isogenic prototrophic strain	P. Kötter[a]
CEN.PK 113-5D	*MAT**a** MAL2-8c SUC2 ura3*	P. Kötter[a]
CEN.PK 555-4D	*MAT**a** MAL2-8c SUC2 aro10*Δ	(44)
CEN.PK 711-7C	*MAT**a** MAL2-8c SUC2 ura3 pdc1*Δ*pdc5*Δ*pcd6*Δ*aro10*Δ*thi3*Δ	This study
IMZ001	*MAT**a** MAL2-8c SUC2 ura3 pdc1*Δ*pdc5*Δ*pcd6*Δ*aro10*Δ*thi3*Δ p426GPD (*URA3*)	This study
IMZ002	*MAT**a** MAL2-8c SUC2 ura3 pdc1*Δ*pdc5*Δ*pcd6*Δ*aro10*Δ *thi3*Δ pUDe001 (*URA3 TDH3*p-*ARO10*)	This study
IME003	*MAT**a** MAL2-8c SUC2 ura3* pUDe001 (*URA3 TDH3*p-*ARO10*)	This study
IME004	*MAT**a** MAL2-8c SUC2 ura3* p426GPD (*URA3)*	This study

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Overexpression of *ARO10*

The *ARO10* (*YDR380W*) open reading frame was PCR amplified from CEN.PK113-7D genomic DNA using primers *ARO10*-fwd(GGTCTAGAATGGCACCTGTTACAATTGAAAAG) and *ARO10*-rev (GGCTCGAGCTATTTTTTATTTCTTTTAAGTGCCGC), designed to introduce restriction sites (underlined) for endonuclease XbaI upstream of the ATG and XhoI downstream of the stop codon, respectively. The PCR product and the vector p426GPD (31) were digested by XbaI and XhoI. The XbaI-XhoI PCR fragment was directionally cloned behind the glyceraldehyde-3-phosphate dehydrogenase promoter (*TDH3p*) into p426GPD, resulting in plasmid pUDe001. The *ARO10* open reading frame sequence was confirmed by sequencing. The plasmid pUDe001 was transformed by the lithium acetate–single-stranded carrier DNA–polyethylene glycol method (17) into the *S.cerevisiae* CEN.PK 113-5D strain, resulting in strain IME003, and into strain CEN.PK 711-7C, resulting in strain IMZ002. Similarly, CEN.PK 113-5D and CEN.PK 711-7C were transformed with p426GPD (31), resulting in IME002 and IMZ001, respectively.

Chemostat cultivation

Aerobic chemostat cultivation was performed at 30°C in 1-liter working volume laboratory fermentors (Applikon, Schiedam, The Netherlands) at a stirrer speed of 800 rpm, pH 5.0, with a dilution rate (D) of 0.10 h^{-1} as described previously (42), with the exception of the strains IMZ001 and IMZ002, which were grown at a dilution rate of 0.05 h^{-1} . The pH was kept constant, using an ADI 1030 biocontroller (Applikon, Schiedam, The Netherlands), via the automatic addition of 2 M KOH. The fermentor was flushed with air at a flow rate of 0.5 liter min^{-1} using a Brooks 5876 mass-flow controller (Brooks Instruments, Veenendaal, The Netherlands). The dissolved-oxygen concentration was continuously monitored with an Ingold model 34 100 3002 probe (Mettler-Toledo, Greifensee, Switzerland) and was above 50% of air saturation.

Carbon-limited steady-state chemostat cultures of *S.cerevisiae* strains were grown as described previously (43) on synthetic medium containing 7.5 g of glucose $liter^{-1}$ or 5.7 g $liter^{-1}$ of ethanol, keeping molar carbon equivalence constant at 0.25 M, and either 5.0 g $liter^{-1}$ $(NH_4)_2SO_4$, 5.0 g $liter^{-1}$ of L-phenylalanine (44), 10 g $liter^{-1}$ L-leucine, 11.3 g $liter^{-1}$ L-methionine, 5 g $liter^{-1}$ L-asparagine, or 8.8 g $liter^{-1}$ L-proline as the sole nitrogen source. The absence of $(NH_4)_2SO_4$, was compensated for by the addition of equimolar amounts of K_2SO_4 when phenylalanine, leucine, methionine, proline, or asparagine was used as the only nitrogen source.

Culture dry weight

Culture dry weights were determined via filtration as described previously (35).

Extracellular-metabolite analysis

For the determination of phenylalanine, leucine, and methionine catabolism products and carbon recovery, culture supernatants and media were analyzed by high-performance liquid chromatography (HPLC), fitted with an AMINEX HPX-87H ion-exchange column (300 by 7.8 mm; Bio-Rad) mounted in a Waters Alliance 2690 HPLC apparatus, at 60°C using H_2SO_4 as the mobile phase with a flow rate of 0.6 ml · min^{-1}. Metabolites were

detected by a dual-wavelength absorbance detector (Waters 2487) and a refractive-index detector (Waters 2410) and integrated with Chrompack Maitre 2.5 software.

Identification of metabolites by NMR spectroscopy

After lyophilization, samples of culture supernatants were dissolved in D_2O. ^{1}H, ^{1}H-^{1}H TOCSY, and ^{1}H-^{13}C correlation spectra (direct and long range) were measured at 300 K on a Bruker Avance 600 nuclear magnetic resonance (NMR) spectrometer equipped with an inverse triple-resonance probe and a pulse field gradient system. Quantitative ^{1}H-NMR experiments were also performed at 600 MHz. To 0.5 ml of supernatant, an equal amount of a standard solution containing maleic acid and EDTA was added. After lyophilization, the residue was dissolved in D_2O and the ^{1}H -NMR spectrum was measured using a relaxation delay of 30 seconds, ensuring full relaxation of all the hydrogen atoms between pulses. The integrals of the characteristic resonance for each component and the internal standard (singlet at 6.1 ppm) were measured, and the contents of the individual components were calculated.

Preparation of cell extracts

For the preparation of cell extracts, culture samples were harvested by centrifugation; washed twice with 10 mM potassium-phosphate buffer, pH 7.5, containing 2 mM EDTA; concentrated fourfold; and stored at -20°C. Before cell breakage, the samples were thawed at room temperature, washed, and resuspended in 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 2 mM dithiothreitol. Extracts were prepared by sonication with 0.7 mm glass beads at 0°C for 2 min at 0.5-min intervals with an MSE sonicator (150-W output; 8-μm peak-to-peak amplitude). Unbroken cells and debris were removed by centrifugation at 4°C (20 min; 36,000 *x g*). The purified cell extract was used for enzyme assays.

2-Oxoacid decarboxylase assays

2-Oxo acid decarboxylase activity was measured at 30°C immediately after preparation of cell extracts using a coupled reaction. Activity was measured by following the reduction of NAD^+ at 340 nm in the presence of excess aldehyde dehydrogenase from yeast. The reaction mixtures contained, in a total volume of 1 ml, 100 mM KH_2PO_4 / K_2HPO_4 buffer, pH 7.0; 2 mM NAD^+ ; 5 mM $MgCl_2$; 15 mM pyrazole; 0.2 mM thiamine diphosphate; 1.75 U of yeast aldehyde dehydrogenase (Sigma-Aldrich, Zwijndrecht, The Netherlands) (dissolved in 1 mM dithiothreitol); and 2 mM phenylpyruvic acid, α-ketoisocaproate, α-ketoisovalerate, α-ketomethylvalerate, 3-methylthio-α-ketobutyrate, or pyruvate to initiate the reaction. Reaction rates were linearly proportional to the amount of cell extract added.

Activity data normalization

The per-strain normalization accounts for the difference in detection efficiency between 2-oxo acid decarboxylase activities. It also allows comparison of the relative change in activity levels, as well as displaying

these levels in similar scales on the same graph. GeneSpring (Silicon Genetics, Redwood City, CA) uses the following formula to normalize to the median for each strain: (activity of strain X on substrate Y) / (median of every measurement of strain X).

Protein determination

Protein concentrations in cell extracts were determined by the Lowry method (29). Bovine serum albumin (fatty acid free; Sigma, St. Louis, Mo.) was used as a standard.

Microarray analysis

DNA microarray analyses were performed with the S98 Yeast GeneChip arrays from Affymetrix as previously described (34). Cells were transferred directly from chemostats into liquid nitrogen and processed according to the manufacturer's instructions (Affymetrix technical manual; Affymetrix, Santa Clara, CA.). Data analyses were performed with the Affymetrix software packages Microarray Suite v5.0, MicroDB v3.0, and Data Mining Tool v3.0. The Significance Analysis of Microarrays (SAM version 1.12) (41) add-in to Microsoft Excel was used for comparisons of replicate array experiments.

RESULTS

Measurement of phenylalanine and phenylethanol in chemostat cultures of *S.cerevisiae* grown on various nitrogen sources

The *S.cerevisiae* reference strain CEN.PK113-7D was grown on synthetic medium in aerobic, glucose-limited chemostat cultures with different nitrogen sources: ammonium sulfate, phenylalanine, leucine, methionine, proline, or asparagine. During growth on phenylalanine as the nitrogen source, HPLC analysis of culture supernatants revealed the presence of high concentrations of phenylethanol and phenylacetate, consistent with the operation of the Ehrlich pathway. Surprisingly, low but significant concentrations of these metabolites were also observed when leucine or methionine was the sole nitrogen source (Table 2). The concentrations of phenylethanol and phenylacetate in leucine-and methionine-grown cultures were 20- to 50-fold higher than in cultures grown with ammonium sulfate as the nitrogen source (Table 2). Similarly, 2-methylpropanoate and *p*-hydroxyphenylacetate, which are Ehrlich pathway-derived catabolites of valine and tyrosine, respectively, were also detectable when phenylalanine, leucine, or methionine was used as the sole nitrogen source. Although 3-methylbutanol, an expected product of leucine catabolism, could not be detected in our HPLC setup, the compound was detected by ^{1}H-NMR in leucine, phenylalanine, and methionine cultures (data not shown). Conversely, none of these metabolites were detected in cultures grown with ammonium sulfate, proline, or asparagine as the nitrogen source (Table 2).

These results can be explained in two different ways. First, growth on amino acids whose catabolism involves the Ehrlich pathway may lead to coordinate induction of Ehrlich pathway enzymes with different substrate specificities. Alternatively, these amino acids may induce Ehrlich pathway enzymes with broad

TABLE 2. Concentrations of fusel alcohols and corresponding organic acids[a]

N source	Phenyl-ethanol [mM]	Phenyl-acetate [mM]	3-Methyl-butanoate [mM]	2-Methyl-propanoate [mM]	3-Methylthio propanol [mM]	3-Methyl-thio-propanoate [mM]	*p*-Hydroxy-phenylacetate [mM]	*p*-Hydroxy-phenylethanol [mM]
Ammonia	0.003 ± 0.000	0.003 ± 0.000	ND[b]	ND	ND	ND	ND	ND
Leucine	0.059 ± 0.013	0.137 ± 0.036	4.538 ± 0.351	0.310 ± 0.000	ND	ND	0.135 ± 0.031	ND
Methionine	0.225 ± 0.071	0.180 ± 0.045	ND	0.204 ± 0.000	0.757 ± 0.309	ND	0.190 ± 0.017	0.054 ± 0.025
Phenylalanine	1.261 ± 0.141	9.915 ± 0.681	ND	0.161 ± 0.021	ND	ND	0.076 ± 0.020	ND
Proline	ND	ND	ND	ND	0.037 ± 0.007	ND	ND	ND
Asparagine	ND	ND	ND	ND	0.026 ± 0.004	ND	ND	ND

[a] In aerobic, glucose-limited chemostat cultures (D = 0.10 h^{-1}) of *S. cerevisiae* CEN.PK 113-7D grown with different nitrogen sources. Data are presented as average ± mean deviation of metabolite quantification from two independent chemostat cultures. 3-Methylbutanol (derived from leucine), 2-methylpropanol (derived from valine), 2-methylbutanol, and 2-methylbutanoate (derived from isoleucine) were not detected by the HPLC setup used in the present study.

[b] ND, not detected.

substrate specificities. To further investigate this phenomenon, we focused on the irreversible decarboxylase reaction.

Decarboxylation of 2-oxoacids by cell extracts of wild-type *S.cerevisiae* grown on various nitrogen sources

2-Oxo-acid-decarboxylase activities involved in the Ehrlich pathway were analyzed in cell extracts of *S.cerevisiae* CEN.PK113-7D grown in aerobic carbon-limited chemostat cultures with different amino acids as the sole nitrogen source (Table 3). Phenylpyruvate, α-ketoisovalerate, α-ketoisocaproate, α-ketomethylvalerate, and 3-methylthio-α-ketobutyrate were selected as substrates based on the observed metabolite profiles (Table 2). Significant activities with all five substrates were detected in cultures grown with leucine, methionine, or phenylalanine as the nitrogen source (Table 3). Conversely, no activity was measured in cell extracts from cultures grown on ammonium, asparagine, or proline, in good agreement with the absence of alcohols and acids in the corresponding culture supernatants (Table 2). When activities were expressed relative to the activity with phenylpyruvate, the substrate specificity did not differ markedly as a function of the nitrogen source for growth. This suggested involvement of a single common decarboxylase activity in the catabolism of leucine, methionine, and phenylalanine (Fig. 2).

Transcript levels of TPP-dependent decarboxylase genes in wild-type *S.cerevisiae* grown on various nitrogen sources

The pyruvate-decarboxylase genes *PDC1*, *PDC5*, and *PDC6* and the related genes *THI3* and *ARO10* have all been implicated in the production of fusel alcohols and fusel acids by *S.cerevisiae* in the literature (7, 9–11, 44), but their substrate specificities and catalytic contributions remain unknown. To check whether the induction of a "broad-substrate-specificity decarboxylase activity" observed in cell extracts could be correlated with the transcriptional induction of a single gene, expression of the five decarboxylase genes was analyzed.

The levels of the *ACT1* transcript, a commonly used "loading standard" for mRNA analysis (32), were the same for all six nitrogen sources (Table 4). *PDC5*, *PDC6*, and *THI3* were transcribed at a constant, very low level. *PDC1* showed much higher transcript levels, but they did not significantly differ for the six nitrogen sources (*t* test analysis at $P < 0.01$). Only *ARO10* was differentially transcribed for the different nitrogen sources (Table 4). In cultures grown with leucine, phenylalanine, or methionine as the nitrogen source, the transcript level was at least 15-fold higher than in cultures grown with ammonium sulfate as the nitrogen source. Moreover, cultures grown with proline or asparagine as the nitrogen source yielded the same very low *ARO10* transcript levels as ammonium sulfate-grown cultures (Table 4).

ARO10* encodes a broad-substrate-specificity 2-oxo-acid-decarboxylase in *S.cerevisiae

The transcriptional regulation of *ARO10*; the similar substrate specificities of decarboxylase activities in cell extracts of leucine-, methionine-, and phenylalanine-grown cultures; and the metabolite profiles in these cultures all suggested that Aro10p is responsible for a broad-substrate-specificity decarboxylase activity involved in the

production of fusel alcohols and acids. To test this hypothesis, an *S.cerevisiae* strain lacking all five TPP-dependent decarboxylase genes (CEN-PK711-7C *pdc1Δ pdc5Δ pdc6Δ thi3Δ aro10Δ ura3Δ*) was constructed. The *ura3* genotype was complemented by transformation either with the empty expression vector p426GPD (strain IMZ001) or with the same vector carrying *ARO10* under the control of the constitutive *TDH3* promoter (strain IMZ002). Strains IMZ001 and IMZ002 could not grow on glucose synthetic media as a result of the *pdc1Δ pdc5Δ pdc6Δ* genotype (15). Therefore, ethanol was used as a carbon source.

TABLE 3. Specific activities of 2-oxo acid decarboxylation by cell extracts[a]

	Specific decarboxylase activity [nmol.min^{-1}.(mg protein)$^{-1}$]				
Nitrogen source	Phenylpyruvate [phenylalanine]	α-Keto-isovalerate [valine]	α-Keto-isocaproate [leucine]	α-Keto-methylvalerate [isoleucine]	3-Methylthio α-ketobutyrate [methionine]
Ammonia	BD[b]	BD	BD	BD	BD
Leucine	13.5 ± 0.7 (100%)	4 ± 0.01 (29%)	6.5 ± 0.7 (48%)	4.5 ± 0.7 (33%)	5.5 ± 0.6 (41%)
Methionine	22.25 ± 1.8 (100%)	8.5 ± 0.5 (38%)	9.25 ± 0.5 (42%)	5.5 ± 0.9 (25%)	9 ± 0.01 (40%)
Phenylalanine	67.5 ± 0.7 (100%)	19 ± 0 (28%)	29.5 ± 0.7 (43%)	25.7 ± 3.8 (38%)	22 ± 0 (32%)
Proline	BD	BD	BD	BD	BD
Asparagine	BD	BD	BD	BD	BD

[a] Prepared from aerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK 113-7D grown with different amino acids as the sole nitrogen source. Data are the average ± mean deviation of assays from two independent chemostat cultures. The relative 2-oxo acid activities, expressed as a percentage of phenylpyruvate activity, are in parentheses. The column headings include in parentheses the amino acid which the 2-oxo acid used as a substrate is derived from.

[b] BD, below detection limit.

Cell extracts of the quintuple-deletion strain IMZ001, grown in aerobic, ethanol-limited chemostat cultures at a dilution rate of 0.05 h^{-1} and with ammonium sulfate as the nitrogen source, did not exhibit any decarboxylase activity (Table 5). Constitutive expression of *ARO10* in this genetic background (strain IMZ002) restored decarboxylase activity with the 2-oxo acids derived from leucine, phenylalanine, and methionine. Interestingly, no activity could be measured with pyruvate as a decarboxylase substrate (Table 5). The relative specific activities with the non-pyruvate substrates were similar to those observed in cell extracts of the reference strain CEN.PK 113-7D grown with phenylalanine, leucine, or methionine as the nitrogen source (Fig. 2 and Tables 3 and 5).

The quintuple-deletion strain IMZ001 was unable to grow in ethanol-limited chemostat cultures (D = 0.05 h^{-1}) when phenylalanine was the sole nitrogen source. This ability was recovered in strain IMZ002, which constitutively expresses *ARO10* from the *TDH3* promoter. Unexpectedly, decarboxylase activities in cell extracts of strain IMZ002 grown with phenylalanine as the nitrogen source were 4.5-fold higher than in cultures

grown with ammonium sulfate as the nitrogen source (Table 5). Reintroduction of *ARO10* in the quintuple-deletion strain also restored the production of fusel alcohols and acids. HPLC analysis of ethanol-limited, ammonium-grown chemostat cultures of IMZ002 revealed low but significant concentrations of phenylethanol (0.11 ± 0.01 mM) and phenylacetate (0.20 ± 0.03 mM). The concentrations of these compounds were below the HPLC detection limit (0.003 mM) in chemostat cultures of the quintuple-deletion strain IMZ001. When the *ARO10*-expressing strain was grown with phenylalanine as the nitrogen source, high concentrations of phenylethanol (2.69 ± 0.06 mM) and phenylacetate (7.29 ± 0.34 mM) were observed in culture supernatants. Furthermore, low concentrations of 3-methylthiopropanol (0.67 mM) and *p*-hydroxyphenylacetate (0.16 mM) were identified, confirming the involvement of Aro10p in the synthesis of a broad range of fusel alcohols and acids in vivo.

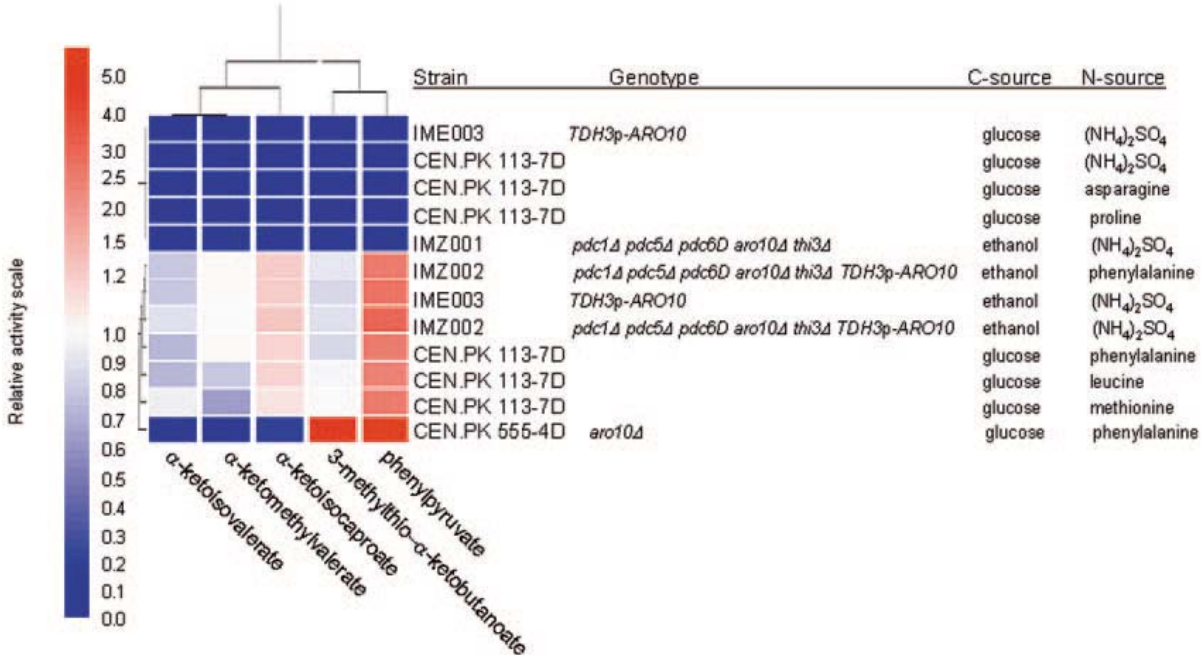

FIG. 2. Eisen representation of relative 2-oxo acid decarboxylase activity. Cell extracts of CEN.PK113-7D, CEN.PK 555-4D, IME003, IMZ001, and IMZ002 grown in aerobic carbon-limited (glucose or ethanol) chemostat cultures with different nitrogen sources were measured for 2-oxo-acid decarboxylase activity. Each cell extract was tested for conversion of phenylpyruvate, α-ketoisovalerate, α-ketoisocaproate, α-ketomethylvalerate, and 3-methylthio-α-ketobutyrate. The activity data were normalized to the mean and clustered by hierarchical clustering using Genespring (Silicon Genetics, Redwood City, CA). The so-called normalized data were displayed on a scale from 0 to 5 (see Materials and Methods).

Overexpression of *ARO10* in the isogenic reference strain CEN.PK 113-5D

To investigate whether overexpression of *ARO10* can be used to modify fusel alcohol production by wild-type *S.cerevisiae* strains, the expression vector carrying *ARO10* under the control of the *TDH3* promoter was introduced into the reference strain CEN.PK113-5D (resulting in strain IME003 [Table 1]). Surprisingly, except for pyruvate decarboxylase, no 2-oxo acid decarboxylase activity was detectable in cell extracts of this strain when it was grown in glucose-limited chemostat cultures with ammonium sulfate as the nitrogen source (Table 6). Monitoring of the *ARO10* transcript level by quantitative PCR in strain IME003 grown in a glucose-limited chemostat with ammonium sulfate as the nitrogen source revealed expression of the *TDH3*-driven construct.

TABLE 4. Transcript levels of genes with sequence similarity to thiamin-pyrophosphate-dependent decarboxylases in aerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D grown with different amino acids as the sole nitrogen source[a]

Nitrogen source	*ACT1*	*ARO10*	*PDC1*	*PDC5*	*PDC6*	*THI3*
Ammonia	2488 ± 81	67 ± 3	1946 ± 449	95 ± 4	66 ± 4	92 ± 9
Leucine	2149 ±204	1045 ± 167	1311 ± 90	73 ± 7	31 ± 8	128 ± 11
Methionine	2831 ±624	1335 ± 130	1459 ±226	87 ± 14	17 ± 3	126 ± 22
Phenylalanine	2917 ±575	1996 ± 201	894 ±319	87 ± 32	25 ± 5	109 ± 17
Proline	2294 ±127	37 ± 6	1505 ± 173	81 ± 8	76 ± 8	115 ± 12
Asparagine	2416 ±122	61 ± 11	1170 ± 109	64 ± 6	46 ± 8	105 ± 47

[a] Transcript levels were determined with Affymetrix Gene Chips. Data are the average ± standard deviation of three independent chemostat cultures. The *ACT1* transcript is included as a reference

The level of expression was equivalent to half of the *ACT1* reference transcript signal. In the meantime, no *ARO10* transcript was detected in strain IME004 grown under similar conditions. Furthermore, decarboxylase activities in glucose-limited chemostat cultures grown with phenylalanine as the nitrogen source were the same as those of the empty-vector reference strain IME004 (Table 6). When ethanol instead of glucose was used as the carbon source, the presence of the *ARO10* expression vector did result in increased decarboxylase activities relative to an empty-vector reference strain (Table 6). These results contradict the simple view that *ARO10* encodes a fully functional decarboxylase whose expression is primarily regulated at the level of transcription.

DISCUSSION

Formation of fusel alcohols by *S.cerevisiae*

In brewery and wine fermentations, *S.cerevisiae* is responsible for the production of a variety of metabolites that contribute to flavor and aroma. Among the volatile flavor compounds, an important class consists of higher alcohols that are less volatile than ethanol (18). These higher alcohols are derived from the carbon skeletons of amino acids, which can in theory be synthesized de novo but in brewery and wine fermentations are generally

taken up from the wort or grape must. It is commonly accepted that branched-chain (9–11) and aromatic (7, 44) amino acid-derived alcohols originate from the Ehrlich pathway (12) (Fig. 1). Our results support the notion that this pathway is also involved in the production of 3-methylthiopropanol (methionol) and 3-methylthiopropanoate from methionine. These sulfur-containing compounds are relevant to the production of alcoholic beverages. Methionol, which has a raw-potato odor, is commonly measured in wine and is known to negatively affect white wine and red wine aroma above 0.6 mg/liter and 2 to 3 mg/liter, respectively (3) (Table 1).

TABLE 5. Substrate specificity of the *ARO10*-dependent 2-oxo-acid-decarboxylase activity in *S. cerevisiae*[a]

Substrate	Enzyme activity nmol.min^{-1}.(mg protein)$^{-1}$			
	IMZ001 [$(NH_4)_2SO_4$[c]]	IMZ002 [$(NH_4)_2SO_4$[c]]	IMZ002 [Phenylalanine[c]]	Ratio[b]
Phenyl-pyruvate	BD[d]	61.75±1.71 (100%)	270 ± 6.98 (100%)	4.37
α-Keto isovalerate	BD	16.75 ±2.21 (27%)	78.25 ± 4.1 (29%)	4.67
α-Keto isocaproate	BD	25±0.82 (40%)	118 ± 7.53 (44%)	4.72
α-Keto methylvalerate	BD	21±1.63 (34%)	97 ± 6.68 (36%)	4.62
3-Methylthio α-ketobutyrate	BD	18.5±1.29 (30%)	87.7 ± 7.69 (32%)	4.74
Pyruvate	BD	BD	BD	-

[a] Strain IMZ001 is *pdc1Δ pdc5Δ pdc6Δ aro10Δ thi3Δ* carrying the empty expression vector p426GPD (2μ *URA3 TDH3p*). Strain IMZ002 is the same strain carrying the plasmid pUDe001 (2μ *URA3 TDH3p-ARO10*). Both strains were grown in aerobic, ethanol-limited chemostat cultures with ammonia as the nitrogen source. Enzyme activities were assayed in cell extracts. Data are the average ± average deviation of the mean from assays of two independent chemostat cultures. The relative 2-oxo acid activities, expressed as a percentage of phenylpyruvate activity, are in parentheses.

[b] Ratio of phenylalanine versus $(NH_4)_2SO4$.

[c] N source.

[d] BD, below detection limit.

Our results indicated that induction of an Ehrlich pathway for catabolism of one amino acid leads to the formation of significant amounts of fusel alcohols and acids from other amino acids. This suggested that conversion of branched-chain, aromatic, and sulfur-containing amino acids to the corresponding fusel alcohols and acids, via an Ehrlich pathway, involves common broad-substrate-specificity enzyme activities. Furthermore, as our experiments were performed with synthetic media to which only single amino acids were added, these results indicated that the decarboxylase activity involved in the Ehrlich pathway could compete for 2-oxo acids with the transaminases involved in de novo amino acid biosynthesis. The chemostat conditions used in this study were designed to reveal the molecular nature of the decarboxylase step of the Ehrlich pathway. Although these conditions are different from typical alcoholic fermentation processes, the conclusion drawn about the role of *ARO10* is relevant for interpreting the patterns of flavor production in wine and beer fermentation.

Aro10p is involved in a broad-substrate-specificity Ehrlich pathway decarboxylase activity

Transcript analysis demonstrated that the induction of Ehrlich pathway activity by the amino acids leucine, phenylalanine, and methionine coincided with the transcriptional up-regulation of *ARO10*, but not with that of the other four genes encoding (putative) thiamine pyrophosphate-dependent decarboxylases. Indeed, overexpression of *ARO10* in a strain in which the five chromosomal decarboxylase genes had been deleted was sufficient to restore a broad-substrate-specificity decarboxylase activity. The substrate specificity profile of this strictly *ARO10*-dependent activity was the same as those of the activities induced by leucine, phenylalanine, and methionine in wild-type cells.

Previous research in *S.cerevisiae* with *aro10* null mutants has indicated the presence of an *ARO10*-independent decarboxylase activity (44). This alternative activity has been reported to require the simultaneous expression of at least one of the three pyruvate decarboxylase genes (*PDC1*, -*5*, and -*6*) and the putative decarboxylase gene *THI3*. Based on previous work by Dickinson and coworkers, the last gene has also been implicated in the decarboxylation of the 2-oxo acids derived mainly from leucine (11) and to a lesser extent isoleucine (10). This *ARO10*-independent 2-oxo acid decarboxylase activity exhibited a completely different substrate specificity profile. In particular, the decarboxylase activity observed in cultures of an *aro10* null mutant grown with phenylalanine as the nitrogen source showed no activity with α-ketoisovalerate and α-keto-methylvalerate as the substrate (Fig. 2, strain CEN.PK 555-4D).

The results described here support the notion that the *ARO10*-dependent, broad-substrate-specificity decarboxylase is primarily responsible for the Ehrlich pathway decarboxylation reaction in wild-type *S.cerevisiae*. The molecular basis and substrate specificity of the *ARO10*-independent activity that is detected in *aro10* null mutants (44) (Table 5), as well as its possible involvement in (off-) flavor production by wild-type strains, require further research.

Transcriptional regulation of *ARO10*

Previous work has shown that transcription of *ARO10* is induced by tryptophan (24) and phenylalanine (44) and is dependent on the transcriptional regulator Aro80p (24). Other studies (7, 9–11) suggested that expression of *ARO10* might also be up-regulated by valine and isoleucine, based on metabolite profiling; however, this conclusion was not backed up by expression analysis. Our results clearly show that *ARO10* expression was strongly up-regulated in the presence of leucine and methionine (Table 3), consistent with its proposed role as a broad-substrate-specificity decarboxylase.

Further analysis of the transcriptome data revealed that *ARO9* (aromatic amino transferase II) (23) was co-expressed with *ARO10* in cultures grown with different nitrogen sources (data not shown). This suggested that the transaminase activity of Aro9p might not be restricted to aromatic amino acids (24) but, similar to the Aro10p-dependent decarboxylase activity, might have a broad substrate specificity. It remains to be in vestigated whether and to what extent Aro80p is involved in the transcriptional up-regulation of *ARO9* and *ARO10* by the non aromatic amino acids leucine and methionine. This question could not be resolved by the microarray analyses, since *ARO80* transcript levels were extremely low and did not differ significantly for the

TABLE 6. Regulation of decarboxylase activities in the reference *S. cerevisiae* strain IME004 (CEN.PK113-5D, p426GPD) and in an isogenic strain expressing a multicopy plasmid-borne *ARO10* gene from a constitutive *TDH3* promoter, strain IME003[a]

	IME004 [*ura3* p426GPD (*URA3*)]				IME003 [*ura3* pUDe001 (*URA3 TDH3p-ARO10*)]			
Substrate for decarboxylase Assay	Ethanol		Glucose		Ethanol		Glucose	
	$(NH_4)_2SO_4$	Phenylalanine	$(NH_4)_2SO_4$	Phenylalanine	$(NH_4)_2SO_4$	Phenylalanine	$(NH_4)_2SO_4$	Phenylalanine
Phenylpyruvate	BD[b]	64 ± 1.2	BD	67.5 ± 0.7	35 ±5.7	76.5 ± 1.5	BD	82.75 ± 26.73
α-ketoisovalerate	BD	17 ± 2.2	BD	19 ± 0	19 ± 0	21.75 ± 1.7	BD	22.4 ± 6.4
α-ketoisocaproate	BD	28 ± 3.1	BD	29.5 ± 0.7	15 ± 1.4	30.5 ± 3	BD	31.5 ± 9.8
α-ketomethylvalerate	BD	23 ± 0.7	BD	25.7± 3.8	12.5 ± 0.7	28 ± 4.0	BD	28.3 ± 13.6
3-methylthio-α-ketobutyrate	BD	20.5 ± 1.2	BD	22 ± 0	11 ± 0	23.5 ± 1.2	BD	24.25 ± 9.1

[a] Both strains were grown in aerobic, carbon-limited chemostat cultures with glucose or ethanol as a carbon source and ammonia or phenylalanine as a carbon source and ammonia or phenylalanine as a nitrogen source. Enzyme activities were assayed in cell extracts of independent duplicate cultures and are expressed as nmol . min^{-1} (mg protein)$^{-1}$. Data are presented as average ± average deviation of the mean of two chemostat cultures.

[b] BD, below detection limit.

nitrogen sources studied (data not shown). Further research with *aro80* null strains is required to investigate whether the regulatory role of Aro80p extends beyond aromatic amino acid metabolism expression control or, alternatively, another regulatory protein or proteins control the upregulation of *ARO9* and *ARO10* in leucine and methionine-grown cultures. A comprehensive discussion of the genome-wide transcriptional responses of *S.cerevisiae* to the six nitrogen sources used in this study will be published elsewhere (V. M. Boer, S. L. Tai, Z. Vuralhan, Y. Arifin, M. C. Walsh, M. D. W. Piper, J. H. de Winde, J.-M. Daran, and J. T. Pronk, unpublished data).

Involvement of other factors in the activity and regulation of Aro10p

Earlier works (7, 9–11, 15, 40, 44) on the decarboxylation of branched-chain and aromatic 2-oxo acid decarboxylation were based on the implicit assumption that single proteins (e.g., Aro10p and/or Thi3p) would act as thiamine pyrophosphate-dependent decarboxylase enzymes. While the present study proves that Aro10p plays a key role in broad-substrate-specificity decarboxylase activity, it also provides several clear indications that additional factors are involved in this activity and its regulation.

Our attempt to overexpress *ARO10* under the control of the *TDH3* promoter in order to uncouple its expression from environmental parameters, such as the presence of phenylalanine, yielded unexpected results. In cultures grown with ammonium sulfate as the nitrogen source, the *TDH3p-ARO10* construct yielded activity in ethanol-grown cultures but, surprisingly, not when glucose was the carbon source. This unexpected dependency on the carbon source was independent of the expression of the other four decarboxylase genes. As the *TDH3* promoter is known to give very high transcript levels in glucose as well as ethanol-grown cultures, this observation suggests that transcription of the *ARO10* gene is not sufficient to yield an active broad-substrate-specificity decarboxylase activity. Furthermore, in ethanol-grown cultures of the "*ARO10* constitutive" strains, addition of phenylalanine to culture media caused a strong increase in the broad-substrate-specificity decarboxylase activities in cell extracts.

These observations may indicate that the functional expression of the *ARO10* gene is regulated at a posttranscriptional level in a carbon and nitrogen source-dependent manner. Alternatively, the catalytic activity and/or stability of Aro10p may require the presence of one or more additional proteins whose expression is carbon and nitrogen source dependent.

Recent protein interactome studies based on the two-hybrid approach (25) identified two potential Aro10p interaction partners. Fit2p is possibly involved in iron uptake (33, 36), and Ena5p is an ATP-driven sodium transporter, a member of the Na^+ transporting ATPase family in the superfamily of P-type ATPases (5, 26). Taking into account the subcellular localization of Fit2p and Ena5p (the cell wall and plasma membrane, respectively), it is difficult to envision them as key factors in controlling the activity or stability of Aro10p. Of these two putative partners, only *FIT2* would show an expression profile that would corroborate our assumption (data not shown).

The present study has clearly established the importance of Aro10p in the key decarboxylation step of the Ehrlich pathway. At the same time, it has raised new and important questions about the additional factors

involved in the molecular composition, posttranscriptional regulation, and/or stability of the Aro10p-dependent decarboxylase activity. These questions need to be resolved before strategies can be devised to rationally modify the production of volatile flavor compounds by *S.cerevisiae* in beverages and fine-chemical production, e.g., via genetic modification of *in vivo* decarboxylase activity. Purification and characterization of the broad-substrate-specificity decarboxylase from cell extracts is likely to be essential to resolve the outstanding issues.

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REFERENCES

1. **Barnett, J. A.** 1992. Some controls on oligosaccharide utilization by yeasts: The physiological basis of the Kluyver effect. FEMS Microbiol. Lett. **100:** 371–378.
2. **Barnett, J. A., R. W. Payne, and D. Yarrow.** 1983. Yeasts: characteristics and identification, 3rd ed. Cambridge University Press, Cambridge, United Kingdom.
3. **Baumes, R., R. Cordonnier, S. Nitz, and F. Drawert.** 1986. Identification and determination of volatile constituents in wine from different vine cultivars. J.Sci. Food Agric. **37:**927–943.
4. **Boer, V. M., J. H. de Winde, J. T. Pronk, and M. D. Piper.** 2003. The genome-wide transcriptional responses of *Saccharomyces cerevisiae* grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. J. Biol. Chem. **278:**3265–3274.
5. **Catty, P., D. A. de Kerchove, and A. Goffeau.** 1997. The complete inventory of the yeast *Saccharomyces cerevisiae* P-type transport ATPases. FEBS Lett. **409:**325–332.
6. **Clark, G. S.** 1990. Phenylethyl alcohol. Perfumer Flavorist **15:**37–44. 7. **Dickinson, J. R., L. E. J. Salgado, and J. E. Hewlins.** 2003. The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. J. Biol. Chem. **278:**8028–8034.
7. **Dickinson, J. R., and I. W. Dawes.** 1992. The catabolism of branched-chain amino acids occurs via 2-oxoacid dehydrogenase in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **138:**2029–2033.
8. **Dickinson, J. R., S. J. Harrison, and M. J. E. Hewlins.** 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. **273:**25751–25756.
9. **Dickinson, J. R., S. J. Harrison, J. A. Dickinson, and J. E. Hewlins.** 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. **275:**10937–10942.

10. **Dickinson, J. R., M. Lanterman, D. Danner, B. Pearson, P. Sanz, S. Harrison, and M. Hewlins.** 1997. A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. **272:**26871–26878.
11. **Ehrlich, F.** 1907. Uber die Bedingungen der Fuselölbildung and uber ihren Zusammenhang mit dem Eiweissaufbau der Hefe. Ber. Dtsch. Chem. Ges. **40:**1027–1047.
12. **Enjo, F., K. Nosaka, M. Ogata, A. Iwashima, and H. Nishimura.** 1997. Isolation and characterization of a thiamin transport gene, *THI10*, from *Saccharomyces cerevisiae*. J. Biol. Chem. **272:**19165–19170.
13. **Fauchon, M., G. Lagniel, J. C. Aude, L. Lombardia, P. Soularue, C. Petat, G. Marguerie, A. Sentenac, M. Werner, and J. Labarre.** 2002. Sulfur sparing in the yeast proteome in response to sulfur demand. Mol. Cell **9:**713–723.
14. **Flikweert, M. T., L. van der Zanden, W. M. Janssen, H. Y. Steensma, J. P. Van Dijken, and J. T. Pronk.** 1996. Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. Yeast **12:**247–257.
15. **Furia, T. E., and N. Bellanca.** 1971. Fenaroli's handbook of flavor ingredients, synthetic flavors. CRC Press, Cleveland, Ohio.
16. **Gietz, R. D., and R. A. Woods.** 2002. Transformation of yeast by the Liac/SS carrier DNA/PEG method. Methods Enzymol. **350:**87–96.
17. **Harrison, G. A. F.** 1970. The flavor of beer: a review. J. Inst. Brew. **76:**486–495.
18. **Hohmann, S.** 1991. *PDC6*, a weakly expressed pyruvate decarboxylase gene from yeast, is activated when fused spontaneously under the control of the *PDC1* promotor. Curr. Genet. **20:**373–378.
19. **Hohmann, S., and H. Cederberg.** 1990. Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*. Eur. J. Biochem. **188:**615–621.
20. **Hohmann, S., and P. A. Meacock.** 1998. Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae:* genetic regulation. Biochim. Biophys. Acta **1385:**201–219.
21. **Holm, C., D. W. Meeks-Wagner, W. L. Fangman, and D. Botstein.** 1986. A rapid, efficient method for isolating DNA from yeast. Gene **42:**169–173.
22. **Iraqui, I., S. Vissers, M. Cartiaux, and A. Urrestarazu.** 1998. Characterisation of *Saccharomyces cerevisiae ARO8* and *ARO9* genes encoding aromatic aminotransferases I and II reveals a new aminotransferase subfamily. Mol.Gen. Genet. **257:**238–248.
23. **Iraqui, I., S. Vissers, B. Andre, and A. Urrestarazu.** 1999. Transcriptional induction by aromatic amino acids in *S. cerevisiae*. Mol. Cell. Biol. **19:**3360–3371.
24. **Ito, T., T. Chiba, R. Ozawa, M. Yoshida, M. Hattori, and Y. Sakaki.** 2001. A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc. Natl. Acad. Sci. USA **98:**4569–4574.
25. **Jesus Ferreira, M. C., X. Bao, V. Laize, and S. Hohmann.** 2001. Transposon mutagenesis reveals novel loci affecting tolerance to salt stress and growth at low temperature. Curr. Genet. **40:**27–39.

26. **Kellermann, E., P. G. Seeboth, and C. P. Hollenberg.** 1986. Analysis of the primary structure and promoter function of a pyruvate decarboxylase gene (*PDC1*) from *Saccharomyces cerevisiae*. Nucleic Acids Res. **14:**8963–8977.
27. **Large, P. J.** 1986. Degradation of organic nitrogen compounds by yeasts. Yeast **2:**1–34.
28. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randell.** 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193:**265–275.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Mumberg, D., R. Muller, and M. Funk.** 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene **156:**119–122.
31. **Ng, R., and J. Abelson.** 1980. Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77:**3912–3916.
32. **Philpott, C. C., O. Protchenko, Y. W. Kim, Y. Boretsky, and M. Shako Elizeh.** 2002. The response to iron deprivation in *Saccharomyces cerevisiae*: expression of siderophore-based systems of iron uptake. Biochem. Soc.Trans. **30:**698–702.
33. **Piper, M. D., P. Daran-Lapujade, C. Bro, B. Regenberg, S. Knudsen, J.Nielsen, and J. T. Pronk.** 2002. Reproducibility of oligonucleotide microarray transcriptome analyses. An interlaboratory comparison using chemostat cultures of *Saccharomyces cerevisiae.* J. Biol. Chem. **277:**37001–37008.
34. **Postma, E., C. Verduyn, W. A. Scheffers, and J. P. Van Dijken.** 1989. Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **55:**468–477.
35. **Protchenko, O., T. Ferea, J. Rashford, J. Tiedeman, P. O. Brown, D. Botstein, and C. C. Philpott.** 2001. Three cell wall mannoproteins facilitate the uptake of iron in *Saccharomyces cerevisiae*. J. Biol. Chem. **276:**49244–49250.
36. **Sentheshanmuganathan, S.** 1960. The mechanism of the formation of higher alcohols from amino acids by *Saccharomyces cerevisiae*. Biochem. J. **74:**568–576.
37. **Stark, D., D. Zala, T. Munch, B. Sonnleitner, I. W. Marison, and U. Von Stockar.** 2003. Inhibition aspects of the bioconversion of phenylalanine to 2-phenylethanol by *Saccharomyces cerevisiae*. Enzyme Microbiol. Technol. **32:**212–223.
38. **Stark, D., T. Munch, B. Sonnleitner, I. W. Marison, and U. Von Stockar.** 2002. Extractive bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. Biotechnol. Prog. **18:**514–523.
39. **Ter Schure, E. G., M. Flikweert, J. P. van Dijken, J. T. Pronk, and C. T.Verrips.** 1998. Pyruvate decarboxylase catalyzes decarboxylation of branched-chain 2-oxo acids but is not essential for fusel alcohol production by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **64:**1303–1307.
40. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci.USA **98:**5116–5121.

41. **van den Berg, M. A., P. de Jong-Gubbels, C. J. Kortland, J. P. Van Dijken, J. T. Pronk, and H. Y. Steensma.** 1996. The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. J. Biol. Chem. **271:**28953–28959.

42. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. Van Dijken.** 1990. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J. Gen. Microbiol. **136:**395–403.

43. **Vuralhan, Z., M. A. Morais, S. L. Tai, M. D. Piper, and J. T. Pronk.** 2003. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **69:**4534–4541.

44. **Welsh, F. W., W. D. Murray, and R. E. Williams.** 1989. Microbiological and enzymatic production of flavor and fragrance chemicals. Crit. Rev. Biotech

4

ALLEVIATION OF FEEDBACK INHIBITION IN *SACCHAROMYCES CEREVISIAE* AROMATIC AMINO ACID BIOSYNTHESIS: QUANTIFICATION OF METABOLIC IMPACT

ABSTRACT

A quantitative analysis of the impact of feedback inhibition on aromatic amino-acid biosynthesis was performed in chemostat cultures of *Saccharomyces cerevisiae*. Introduction of a tyrosine-insensitive *ARO4* allele (encoding DAHP synthase) caused a three-fold increase of intracellular phenylalanine and tyrosine concentrations. These amino acids were not detected extracellularly. However, an over 50-fold increase of the extracellular levels of phenylacetate, phenylethanol and their para-hydroxyl analogues was observed. The total increase of the flux through the aromatic pathway was estimated to be 1.9 fold. Overexpression of a tyrosine and tryptophan insensitive allele of *ARO7*, either alone or in combination with the Tyr-insensitive *ARO4* allele, had little impact on the synthesis of aromatic compounds. Elimination of allosteric control on these two key reactions in aromatic amino acid metabolism significantly affected intracellular concentrations of several non-aromatic amino acids. This broader impact of amino-acid biosynthesis presents a challenge in rational optimisation of the production of specific amino acids and derived flavour compounds.

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INTRODUCTION

The aromatic amino acids phenylalanine and tryptophan are mainly used in food and feed applications. They are produced on an industrial scale via bacterial fermentation processes using *Escherichia coli* and *Corynebacterium glutamicum* (16,29). Interest in phenylalanine has increased proportionally with the increased demand for low caloric food and soft drinks, as phenylalanine is a precursor for the low caloric sweetener aspartame (12). While *Saccharomyces cerevisiae* is not under consideration for the industrial production of amino acids, aromatic amino acid metabolism by this yeast is of interest for other industrial applications.

In *Saccharomyces cerevisiae* and other yeasts (7, 45) the phenylalanine biosynthesis pathway is involved in the synthesis of phenylethanol. This molecule has interesting sensory properties (including a rose-like aroma) and is of increasing economical interest. Phenylethanol can be produced via bio-transformation of phenylalanine with *S. cerevisiae* (6). This conversion involves the reactions of the Ehrlich pathway (5), which is involved in the catabolism of several amino acids by *S. cerevisiae* (Fig. 1). In the case of phenylalanine, the Ehrlich pathway is initiated by its transamination to phenylpyruvate. This 2-oxo acid is then decarboxylated to phenylacetaldehyde (42). Depending on the redox status of the cells, phenylacetaldehyde is then reduced by alcohol dehydrogenases (yielding phenylethanol) or oxidized to phenylacetic acid by (43). Phenylpyruvate, the precursor for phenylethanol production by *S. cerevisiae*, is also an intermediate in the *de novo* biosynthesis of phenylalanine from sugars.

In bacteria, extensive strain improvement programmes, involving a combination of random mutagenesis and targeted metabolic engineering, have been applied to improve aromatic amino acid biosynthesis (17, 18). In these strain improvement programmes, a first and essential step is invariably the elimination of feedback inhibition on key enzymes in the biosynthetic pathway. In *S. cerevisiae*, two reactions in the phenylalanine biosynthesis pathway are known to be subject to feedback inhibition (Fig. 1). The first committed step in aromatic amino acid metabolism is catalysed by 3-deoxy-D-arabinoheptulonate-7-phosphate (DAHP) synthase, for which two isoenzymes exist in *S. cerevisiae*, encoded by the *ARO3* and *ARO4* genes (21, 36). Aro3p and Aro4p are feedback inhibited by phenylalanine and tyrosine, respectively. Besides, both DAHP synthases show considerable tryptophan regulation as recently reported (15). A single lysine-to-leucine substitution in Aro4p at position 229 results in a deregulated enzyme that is no longer feedback inhibited by tyrosine (13). Chorismate mutase, encoded by *ARO7*, has been identified as a second reaction subject to allosteric regulation (2, 3), being subject to feedback inhibition by tyrosine as well as activation by tryptophan. Substitution of the serine residue 141 by a glycine abolished effects of both tyrosine and tryptophan, thus leading to a non-allosterically regulated chorismate mutase (20, 33). Although considerable knowledge is available on the molecular basis for feedback inhibition of aromatic amino acid metabolism (14), no quantitative studies have yet been performed on its impact on product formation by growing *S. cerevisiae* cultures.

The aim of this study is to quantify the combinatorial effects of deregulation of DAHP synthase and chorismate synthase in *S. cerevisiae*. To this end, a tyrosine- feedback insensitive DAHP synthase and a non-allosterically regulated chorismate mutase were expressed in an *aro3Δ* genetic background. Subsequently, the production of aromatic amino acids and the corresponding fusel alcohols and 'fusel acids' were quantified in

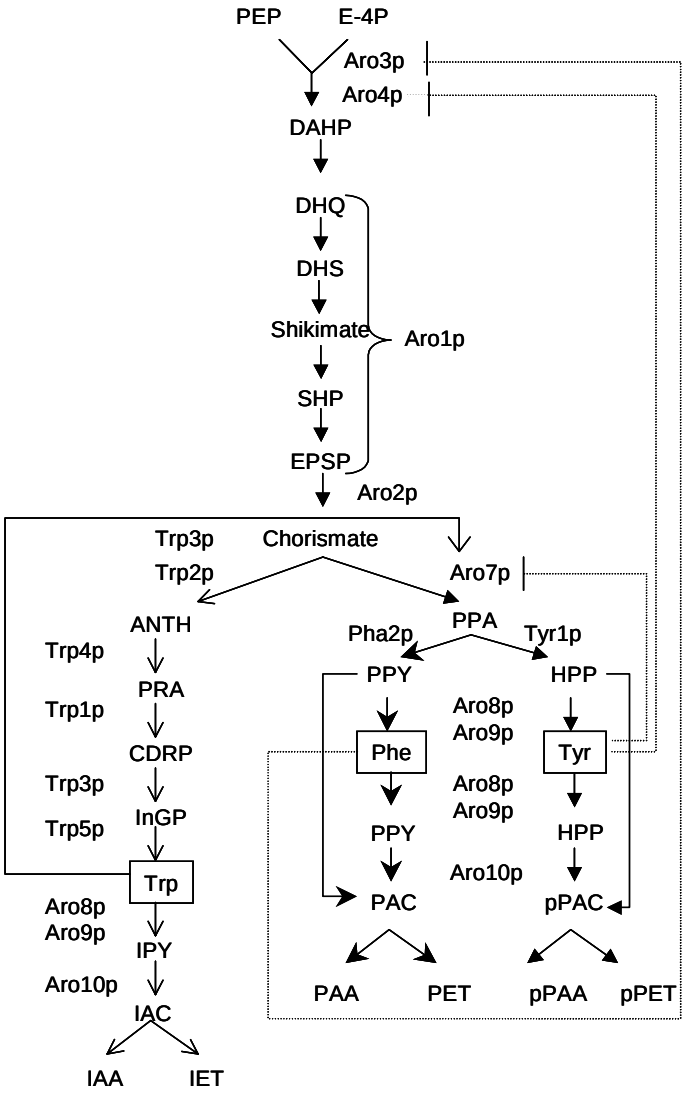

FIG. 1. Pathways of aromatic amino acid biosynthesis and catabolism in ***S. cerevisiae***. The dashed lines indicate feedback inhibition of Aro4p and Aro7p by tyrosine and feedback inhibition of Aro3p by phenylalanine. The solid line indicates activation of Aro7p by tryptophan. E-4P: erythrose 4-phosphate, PEP: phosphoenol pyruvate, DAHP: 3-deoxy-D-arabino-heptulosonate, DHQ: 3-dehydroquinate, DHS: SHP: EPSP: 5 enolpyruvoylshikimate 3-phosphate, ANTH: anthranilate, PRA:phosphoribosyl anthranilate, CDRP: 1-(o-carboxyphenylamino-1-deoxyribulose 5-phosphate, InGP: indole 3-glycerol-phosphate, PPA: prephenate, PPY: phenylpyruvate, HPP: *para*-hydroxy-phenylpyruvate, PAC: phenylacetaldehyde, PAA: phenylacetate, PET: phenylethanol, *p*PAC: *para*-hydroxy-acetaldehyde *p*PAA:para-hydroxy-acetate, *p*PET: *para*-hydroxy-phenylethanol, IAC: indole-acetaldehyde, IAA: indole-acetate, IET: indole-ethanol, Trp: tryptophan, Phe: phenylalanine, Tyr: tyrosine.

aerobic, glucose-limited chemostat cultures. To assess the specificity of this approach to deregulate aromatic amino acid metabolism, we also analysed intracellular concentrations of other amino acids in reference and engineered strains.

MATERIALS AND METHODS

Strain construction and maintenance

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Stock cultures were grown at 30°C in shake flasks on synthetic medium (26) supplemented with 20 g of glucose l^{-1}. When stationary phase was reached, sterile glycerol was added to 30% (vol/vol), and 2-ml aliquots were stored in sterile vials at –80°C.

The construction of the *ARO3* and *ARO4* null mutants was done using the PCR based method using short flanking homologies (SFH) (26). SFH deletion cassettes were obtained by PCR using the primers ARO3-S1 (GAATCTCCAATGTTCGCTGCCAACGGCATGCCAAAGGTAACAGCTGAAGCTTCGTACGC)/ARO3-S 2 (TCAAGGCCTTTCTTCTGTTTCTAACACCTTCTGCCAATAGGCATAGGCCACTAGTGGATCTG) and ARO4-S1(GAATCTCCAATGTTCGCTGCCAACGGCATGCCAAAGGTAACAGCTGAAGCTTCGTACGC) /ARO4-S2 (TTGTTAACTTCTCTTCTTTGTCTGACAGCAGCAGCCAATTGCATAGGCCACTAGTGGAT CTG) and pUG6 as template (11). The deletion cassettes were transformed in the prototrophic diploid yeast strain CEN.PK122. After sporulation and tetrad analysis the corresponding segregants were isolated resulting in strains CEN.PK532-1A and CEN.PK437-1C. Both strains were crossed to obtain after tetrad analysis the *aro3 aro4* double deletion strain CEN.PK557-5A.

The plasmid pME2027 harbouring the $ARO4^{K229L}$ (13) was PCR-amplified using the primers ARO4-A8 (TTGTTAACTTCTCTTCTTTGTCTG)/ARO4-A9 (GAATCTCCAATGTTCGCTGCC) yielding a fragment starting at nucleotide 7 to 1106 of the *ARO4* coding region. The resulting PCR product was transformed in the *aro3Δ aro4Δ* double deletion strain CEN.PK557-5A and transformants were selected on SM plates. Sequencing using primer ARO4-A11 (CGAATCTCAACTGCA CAGAG) proofed the *ARO4* point mutation in the obtained strain CEN.PK718-5A.

The addition of the *ura3-52* allele in the *aro3Δ* $ARO4^{K229L}$ strain CEN.PK718-5A was achieved by crossing it with the strain CEN.PK113-13D (*ura3-52)*. After tetrad analysis the corresponding segregants were identified on the respective media (YEPD, YEPD+G418, SCD-ura, SCD+trp+tyr+phe, SCD+trp+tyr, SCD+ura+trp+tyr and SCD+ura+trp+tyr+phe) and the $ARO4^{K229L}$ point mutation of resulting strain CEN.PK790-2D was confirmed again by sequencing using primer *ARO4*-A11.

To express the *ARO4* gene under the control of the *TPI1* promoter the $TPI1_{promter}$-loxP-KanMX-loxP cassette was amplified using primers ARO4-T1 (TGGCATGCCGTTGGCAGCGAACATTGGAGATTCACTCATTCT AGTTTATGTATGTGTTTTTTGTAG) and ARO4-P2 (GTAACGGTCTCACGGAACACTGTGTAGTTGCAT TACTGTCGCATAGGCCACTAGTGGATCTG) and plasmid pPK261 that carries the$TPI1_{promoter}$-loxP-KanMX4-loxP integration cassette as template.

TABLE 1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference
CEN.PK122	MATa / MATα prototrophic reference diploid strain	P. Kötter[a]
CEN.PK113-7D	MATa Prototrophic reference haploid strain, *ARO3 ARO4 ARO7*	P. Kötter[a]
CEN.PK113-5D	MATa *ura3 ARO3 ARO4 ARO7*	P. Kötter[a]
CEN.PK113-13D	MATα *ura3 ARO3 ARO4 ARO7*	P. Kötter[a]
CEN.PK532-1A	MATa *aro3Δ ARO4*	This study
CEN.PK532-1C	MATα *aro3Δ ARO4*	This study
CEN.PK437-1C	MATα *ARO3 aro4Δ*	This study
CEN.PK557-5A	MATa *aro3Δ aro4Δ*	This study
CEN.PK790-2D	MATa *ura3 aro3Δ* $ARO4^{K229L}$	This study
CEN.PK718-5A	MATa *aro3Δ* $ARO4^{K229L}$	This study
CEN.PK621-1B	MATa *TPI1p-ARO4*	This study
CEN.PK661-1C	MATa *aro3Δ TPI1p-ARO4*	This study
IMZ014	MATa *ura3 aro3Δ* $ARO4^{K229L}$ pUDe003 *(2μ URA3 TDH3p-ARO7)*	This study
IMZ018	MATa *ura3 aro3Δ* $ARO4^{K229L}$pUDe004*(2μ URA3 TDH3p-*$ARO7^{G141S}$*)*	This study
IMZ016	MATa *ura3 ARO3 ARO4* pUDe003 *(2μ URA3 TDH3p-ARO7)*	This study
IMZ017	MATa *ura3 ARO3 ARO4* pUDe004 *(2μ URA3 TDH3p-*$ARO7^{G141S}$*)*	This study

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The resulting PCR product was transformed in the diploid strain CEN.PK122 and transformants were selected on YPD+G418 plates. After sporulation and tetrad analysis the corresponding segregants were isolated resulting in strain CEN.PK621-1B. Correct integration of the $TPI1_{promoter}$-loxP-KanMX-loxP cassette was confirmed by diagnostic PCR. For the construction of the *aro3* $TPI1_{pro}$*-ARO4* strain CEN.PK661-1C, the two strains CEN.PK532-1C and CEN.PK621-1B were crossed and segregants were isolated showing in tetrad analysis a 2:2 segregation of the kan-marker.

The *ARO7* wild type and mutant $ARO7^{G141S}$ open reading frame were PCR-amplified from CEN.PK113-7D genomic DNA and pME1463 plasmid DNA (33) respectively using primers *ARO7*-fwd (GGGCTAGCATGG ATTTCACAAAACCAGAAACTG) and *ARO7*-rev (GGCTCGACTTACTCTTCCAACCTTCTTAGCAAG) designed to introduce restriction sites (underlined) for endonuclease *Nhe*I, upstream of the ATG and *Xho*I, downstream of the stop codon respectively. The PCR product and the 2μm-based expression vector p426GPD (27) were digested by *Xba*I and *Xho*I. The *Nhe*I-*Xho*I PCR fragments were directionally cloned behind the glyceraldehyde-3-phosphate dehydrogenase promoter (*TDH3*p) into p426GPD resulting in plasmids pUDe003 (WT *ARO7*) and pUDe004 ($ARO7^{G141S}$). The *ARO7* open reading frame sequences were confirmed by sequencing. The plasmids pUDe003 and pUDe004 were transformed by the LiAc/SS/PEG method (10) into the *S. cerevisiae* CEN.PK 113-5D strain resulting in strain IMZ016 and IMZ17 respectively. Similarly, the

plasmids pUDe003 and pUDe004 were transformed into the *S. cerevisiae* CEN.PK 790-2D strain resulting in strains IMZ014 and IMZ18 respectively.

Growth conditions

Shake flask cultures were performed as described previously (39). The synthetic medium (SM, reference) contained 20 $g.l^{-1}$ glucose as carbon source and 5 $g.l^{-1}$ ammonium sulphate as nitrogen source. Where mentioned, filter-sterilized L-tryptophan was added to a concentration of 50 $mg.l^{-1}$. To test growth inhibition by L-tyrosine, 24 h SM-shake flask cultures were streaked out on SM-agar plates with 2% glucose and L-tyrosine concentrations ranging from 0 to 1 mM. After inoculation, the plates were incubated at 30^{o}C for 52 h.

Steady-state chemostat cultures were grown in Applikon laboratory fermenter of 1-liter working volume (Applikon, Schiedam, The Netherlands), as described in detail elsewhere (38). The cultures were fed with a defined synthetic medium containing glucose as the growth-limiting nutrient (41). The dilution rate (which equals the specific growth rate) in the steady-state cultures was 0.10 h^{-1}, the temperature was 30°C, and the culture pH was 5.0. Aerobic conditions were maintained by sparging the cultures with air (0.5 $liter.min^{-1}$). The dissolved oxygen concentration, which was continuously monitored with an Ingold model 34-100-3002 probe (Mettler-Toledo, Greifensee, Switzerland), remained above 50% of air saturation.

Culture dry weight

Culture dry weight was determined via filtration as described previously (30).

Extracellular metabolite analysis

Culture supernatants and media were analysed by high-performance liquid chromatography (HPLC), fitted with an AMINEX HPX-87H ion-exchange column (300 by 7.8 mm; Bio-Rad) mounted in a Waters Alliance 2690 HPLC apparatus, at 60°C using H_2SO_4 as the mobile phase having a flow rate of 0.6 $ml \cdot min^{-1}$. Aromatic amino acid metabolites were analysed by a dual-wavelength absorbance detector (Waters 2487) and integrated with Chrompack Maitre 2.5 software.

Intracellular metabolite Analysis

30 ml of cell broth was collected at steady state. The cells were spun down at 4^{o}C, 5000 rpm for 5 min. The pellet was resuspended in 10 ml of boiling water and incubated above 90^{o}C in a thermo-bath for 10 min. Subsequently, cells were spun down. The supernatant was collected and then evaporated in a Speedvac® under full vacuum conditions. The sample was resuspended in 300 µl of H_2O before further processing.

Phenomenex amino acid kit for GC-MS (Phenomenex Inc, Torrence, CA, USA) was used to measure intracellular amino acid concentrations. The amino acids were derivatized according the supplier's recommendations after a solid phase extraction on a strong cation exchanger to remove proteins and other interfering sample components; the amino group was alkylated with alkyl chloroformate while the carboxylic group was simultaneously esterified. The volatile alkoxycarbonyl alkyl esters of amino acids were injected into

a GC-MS TRACE DSQ™ (Dual-Stage Quadrupole) (ThermoFinnigan, San Jose, CA, USA). The GC temperature program as proposed by Phenomenex was: initial temp 110°C, ramped to 320°C at 30°C.min^{-1} (hold 1 min) with a column flow of 1.1 ml.min^{-1}. To compare genotype-dependent concentration profiles for different amino acids, concentrations were normalized relative to the concentration in the reference strain CEN.PK113-7D:

Relative $[AA_j]^{\text{strain i}} = ([AA_j]^{\text{strain i}} / [AA_j]^{\text{CEN.PK113-7D}})$ x 100 %

This normalized data set was submitted to hierarchical data clustering and the data presented as a heat map using GeneSpring® software (Silicon Genetics, Redwood City, CA).

Enzyme Assays

Chorismate mutase: Cell extract was prepared as described previously (43) Chorismate mutase (EC 5.4.99.5) was assayed according to Schmidheini et al. (31) with minor modifications. A 1 ml incubation mix with Tris-HCl pH 7.6 (50 mM), dithiothreitol (DTT, 1mM), and EDTA (0.1 mM) was incubated with cell extract (20-200 µl) at 30˚C. At t=0 min, 1 mM (final concentration) of barium chorismate was added. In order to check allosteric control of the various alleles of *ARO7* used in this study, either 0.5 mM of tyrosine or 0.5 mM of tryptophan were used to test inhibition or activation respectively. For the duration of 5 minutes, a 100 µl sample was taken every minute, and added to 100 µl of 1 M HCl. These samples were each incubated at 30˚C for 10 minutes, after which 800 µl of 1 M NaOH was added. Absorbance was measured at 320 nm, an experimentally determined extinction coefficient of 13.165 mM^{-1} ·cm^{-1} was used to calculate the phenylpyruvate concentration.

Blank measurements and controls with prephenic acid or phenylpyruvic acid showed no detectable substrate decay or chemical decay of the product formed. Specific activities were linearly proportional to the amount of cell extract added.

DAHP synthase: Biomass samples from chemostat cultures (ca. 3.4 mg dry weight) were washed and re-suspended in 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). Cell extracts were prepared using a French pressure cell operated at 2 kbar. After disruption, cell extracts were centrifuged as described previously (43). Prior to DAHP-synthase assays, the cell extract was desalted using Pharmacia PD-10 Sephadex G-25 M columns (9.1 ml bed volume, 5 cm bed height). The DAHP synthase assay was modified from (35). The assay mixture of 1 ml contained 100 mM KPB buffer, 0.5 mM PEP and E-4P and the reaction was started with the addition of cell extract (volume). After 5, 10, 15 minutes of reaction times, 200 µl of the reaction mixture was transferred to 280 µl of 2.85 % w/v trichloroacetic acid solution. The resulting mixture was centrifuged for 5 min in an eppendorf table centrifuge to remove proteins. 125 µl of the supernatant was removed into eppendorf tubes for assaying DAHP. DAHP (0.01 to 0.05 µmole in 0.125 ml of solution) was treated with 0.125 ml of periodic acid having a final concentration of 2 mM in 0.125 N H_2SO_4. This was then left in room temperature for 45 minutes. 0.25 ml of 1.33 % (w/v) sodium arsenite in 0.5 N HCl was added to destroy excess periodate (2 min at room temperature). 1 ml of thiobarbituric acid (2%, w/v, pH 2.0) was added to the tubes, which were subsequently placed in a boiling-water bath for 5 min. The mixture

was cooled in a water bath for 10 min at 40°C and the pink colour developed was measured immediately at 549 nm in an Amersham Pharmacia Novaspec II spectrophotometer against a blank run with water under the same conditions. One μmole of DAHP gives an OD_{549} of 13.6 in the above assay.

Protein Determination

Protein concentrations in cell extracts were determined by the Lowry method (25). Bovine serum albumin (fatty acid free; Sigma, St. Louis, Mo.) was used as a standard.

RESULTS

Elimination of allosteric control on DAHP synthase and chorismate mutase

To confirm that the expression of the *ARO4* ^{K229L} and *ARO7*^{G141S} alleles resulted in the elimination of allosteric control on DAHP synthase and chorismate mutase, respectively, plate assays and in vitro enzyme activity measurements were performed. Plate assays in the presence of 1 mM tyrosine completely inhibited growth of *aro3Δ* strain CEN.PK532-1A (Fig. 2), thus confirming the tyrosine-feedback sensitivity of the Aro4p-encoded DAHP synthase in the CEN.PK strain background. This tyrosine sensitivity could not be overcome by overexpression of the wild-type *ARO4* allele (strain CEN.PK666-1C, Fig. 2). However, introduction of the *ARO4* ^{K229L} allele completely eliminated the tyrosine sensitivity (Fig. 2), thus confirming the lack of tyrosine sensitivity of the DAHP synthase encoded by this mutant allele (13). Allosteric control of DAHP synthase in the strains used in this study was further analysed by measuring enzyme activities in cell extracts, prepared from glucose-limited chemostat cultures. Although levels of DAHP synthase activity in cell extracts varied among replicate experiments, they confirmed the complete absence of tyrosine inhibition in the strains carrying the *ARO4* ^{K229L} allele (data not shown).

Allosteric control of chorismate mutase was analysed in cell extracts of glucose-limited chemostat cultures. In strains carrying a single chromosomal copy of the wild-type *ARO7* allele, activities of chorismate mutase were close to the detection limit (Fig. 3), which made it difficult to quantify allosteric effects of tyrosine and tryptophan, the two known allosteric regulators of Aro7p (31, 32). Overexpression of the wild type *ARO7* allele led to an over 50-fold increase of chorismate mutase activities relative to reference strains carrying a single chromosomal copy and confirmed both activation by tryptophan and inhibition by tyrosine (Fig. 3). Finally, overexpression of the *ARO7*^{G141S} allele led to the complete elimination of both tryptophan activation and tyrosine inhibition. Although the chorismate mutase activities in strains overexpressing the *ARO7*^{G141S} allele were lower than those in strains overexpressing the wild type *ARO7* allele, they were still over ten-fold higher than in strains that carried a single chromosomal copy of *ARO7* (Fig. 3). Plate assays showed that feedback inhibition of Aro7p by tyrosine was less critical than for Aro4p DAHP, as no growth defect was recorded for strains IMZ014 and IMZ016. This suggests that the inhibition constant of Aro7p for tyrosine is lower than the K_i reported for Aro4p (K_i = 0.9 μM tyrosine) (31).

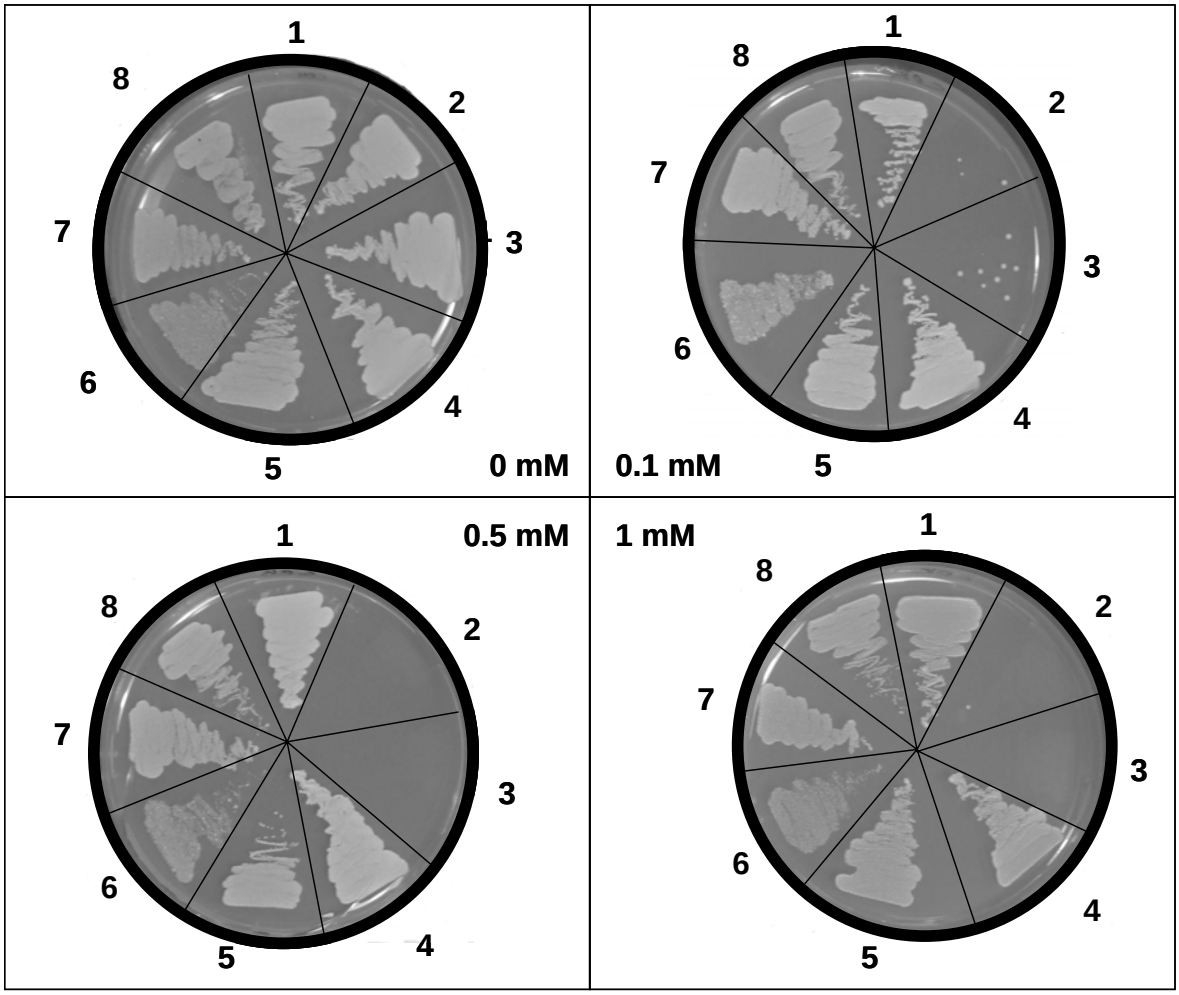

FIG. 2. Growth inhibition by L-tyrosine of the reference *S. cerevisiae* strain CEN.PK113-7D and strains engineered in key steps of aromatic amino acid biosynthesis. The yeast strains were streak on SM medium with 2 % glucose containing either 0 (left top panel), 0.1 mM (right top panel), 0.5 mM (left bottom panel) or 1 mM (right bottom panel) L-tyrosine. Plates were incubated at 30°C for 52 h. **1**: CEN.PK 113-7D (prototrophic reference strain) **2:** CEN.PK532-1A *(aro3Δ ARO4)* **3:** CEN.PK666-1C *(aro3Δ TPI1p-ARO4)* **4:** CEN.PK718-5A *(aro3Δ ARO4^{K229L})* **5:** IMZ014 *(aro3Δ ARO4^{K229L} TDH3-ARO7)* **6:** IMZ018 *(aro3Δ ARO4^{K229L} TDH3- ARO7^{G141S})* **7:** IMZ016 *(TDH3-ARO7)* **8:** IMZ017 *(TDH3- ARO7^{G141S})*

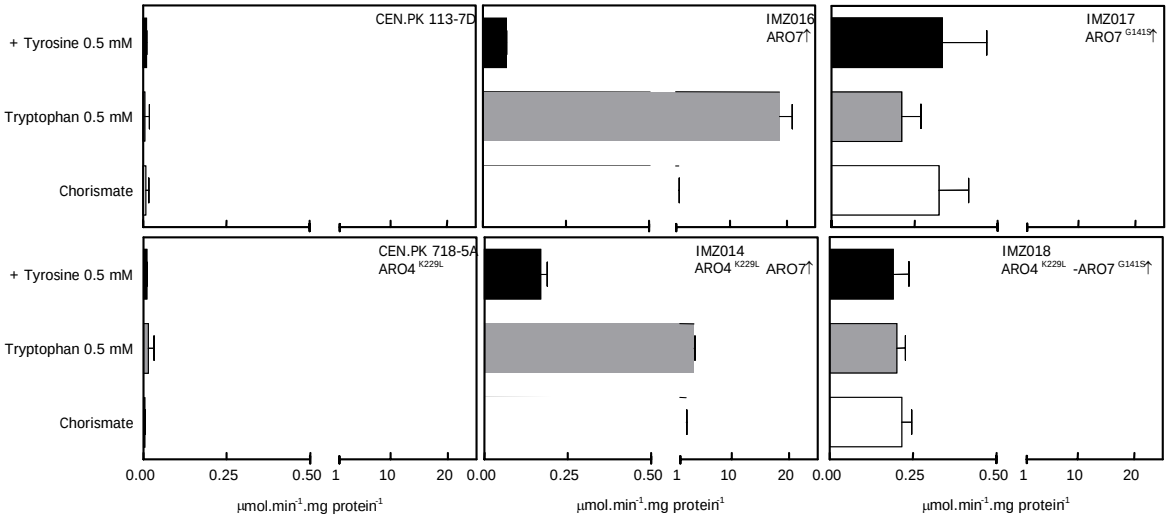

FIG. 3. Activities of chorismate mutase in the presence and absence of L-tryptophan and L-tyrosine in the reference *S. cerevisiae* strain CEN.PK113-7D and in strains engineered in key steps of aromatic amino acid biosynthesis. Enzyme activities were assayed in cell extracts of aerobic, glucose-limited chemostat cultures. Data represent the average ± standard deviation of results from two independent chemostat cultures.

Partial tryptophan auxotrophy of strains overexpressing different *ARO7* alleles

It has previously been reported that overexpression of *ARO7* leads to tryptophan auxotrophy by preferentially channelling chorismate towards phenylalanine and tyrosine biosynthesis (20). Although CEN.PK strains that overexpressed the wild-type *ARO7* allele (strains IMZ016, IMZ014, Table 1) exhibited a reduction of their specific growth rate, this could not be restored by adding tryptophan (Table 2). Strains overexpressing the tryptophan-insensitive allele *ARO7*^{G141S} (IMZ017, IMZ018) (33) grew even slower than strains overexpressing the wild-type *ARO7* allele (Table 2). In this case, a partial restoration of growth was observed upon the addition of tryptophan. This partial tryptophan auxotrophy was most pronounced in the strain that expressed both *ARO4* ^{K229L} and *ARO7*^{G141S} (Table 2). Even in the latter strain, the specific growth rate in the absence of tryptophan was 0.15 h^{-1}. This enabled us to quantitatively analyse product formation by the engineered strains in chemostat cultures grown at a dilution rate of 0.10 h^{-1} without the need for tryptophan supplementation.

Analysis of extracellular product formation in chemostat cultures

The impact of the expression of the *ARO4* ^{K229L} and the overexpression of the *ARO7*^{G141S} alleles on biomass yields and on the production of extracellular products was analysed in aerobic, glucose-limited chemostat cultures grown at a dilution rate of 0.10 h^{-1}. In all of the chemostat cultures, the extracellular concentrations of

phenylalanine and tyrosine were below the detection limit of the analysis method (10 µM for both amino acids). However, significant concentrations of the fusel alcohols phenylethanol and p-hydroxyphenylethanol, as well as of the corresponding organic acids were detected in several of the cultures (Table 3). The expression of the *ARO4*^{K229L} allele resulted in an over 50-fold increase of the total extracellular concentration of these compounds relative to the isogenic reference strain CEN.PK113-7D, in which concentrations of these compounds were close to or below the detection limit (Table 3). Overexpression of the *ARO7*^{G141S} allele did not by itself have a significant impact on the production of the aromatic fusel alcohol and corresponding acids (Table 3). When both the *ARO4* ^{K229L} and *ARO7*^{G141S} alleles were expressed, the resulting extracellular concentrations of aromatic metabolites were only marginally higher than in the strain that expresses only the *ARO4* ^{K229L} allele (Table 3).

The strains that combined the expression constructs for *ARO4* ^{K229L}, *ARO7* or *ARO7*^{G141S} all exhibited a ca. 10 % lower biomass yield than the isogenic reference strain CEN.PK113-7D (Table 3). This reduction of the biomass yield was not clearly correlated to the concentrations of aromatic metabolites in the cultures and may therefore reflect an effect of protein burden (44) rather than a specific metabolic effect related to aromatic amino acid metabolism.

TABLE 2. Specific growth rate µ (h^{-1}) determination of chorismate-mutase-overexpressing strains mutants in presence and absence of 50 $mg.l^{-1}$ tryptophan in SM medium with 2% glucose. Data are presented as average ± mean deviation of metabolite quantification from two independent shake flask cultures.

		Maximum specific growth rate μ_{max} (h^{-1})	
Strain	Genotype	-Trp	+Trp
CEN.PK113-7D	*ARO3 ARO4*	0.46 ± 0.01	0.45 ± 0.01
IMZ016	*ARO3 ARO4 TDH3p-ARO7*	0.25 ± 0.01	0.26 ± 0.01
IMZ017	*ARO3 ARO4 TDH3p-ARO7*^{G141S}	0.22 ± 0.01	0.27 ± 0.00
IMZ014	*aro3Δ ARO4*^{K229L}*TDH3p-ARO7*	0.31 ± 0.01	0.33 ± 0.01
IMZ018	*aro3Δ ARO4*^{K229L}*TDH3p-ARO7*^{G141S}	0.15 ± 0.00	0.39 ± 0.01

Intracellular amino acid concentrations in chemostat cultures

As the absence of significant extracellular concentrations of aromatic amino acids may reflect a limitation in their export from the cells, intracellular amino acid concentrations were analysed in the reference and engineered strains. The Expression of the *ARO4* ^{K229L} allele led to a *circa* 3-fold increase of the intracellular concentrations of phenylalanine and tyrosine (Fig. 4). As observed for the extracellular aromatic metabolites, the simultaneous overexpression of the *ARO7*^{G141S} allele did not cause a clear further increase of the intracellular phenylalanine and tyrosine concentrations. However, in contrast to the effects observed for the extracellular

TABLE 3. Biomass yields on glucose and extracellular concentrations of fusel alcohols and corresponding organic acids in aerobic, glucose-limited chemostat cultures (D = 0.10 h^{-1}) of different *S. cerevisiae* strains. Data are presented as average ± mean deviation of results from two independent chemostat cultures for each strain.

Strain	Genotype	Biomass yield g (g glucose)$^{-1}$	Phenyl-ethanol (μM)	Phenyl-acetate (μM)	*p*-hydroxy-phenyl-ethanol (μM)	*p*-hydroxy-phenyl-acetate (μM)	Σ^{a} *[metabolites]* (μM)
CEN.PK 113-7D	*ARO3 ARO4*	3.60 ± 0.02	3 ± 0	3 ± 0	<2	<2	< 10
CENPK718-5A	*aro3Δ ARO4^{K229L}*	3.45 ± 0.03	220 ± 20	110 ± 10	150 ± 10	17 ± 2	497
IMZ016	*ARO3 ARO4 TDH3pr-ARO7*	3.35 ± 0.05	<2	<2	<2	<2	< 8
IMZ017	*ARO3 ARO4 TDH3pr-ARO7^{G141S}*	3.33 ± 0.03	<2	<2	<2	<2	<8
IMZ014	*aro3Δ ARO4^{K229L}TDH3pr-ARO7*	3.43 ± 0.14	170 ± 20	220 ± 60	160 ± 50	<2	550
IMZ018	*aro3Δ ARO4^{K229L}TDH3pr-ARO7^{G141S}*	3.27 ± 0.03	205 ± 5	210 ± 0	200 ± 0	<2	615

[a] corresponds to the sum of the phenylethanol, phenylacetate, p-hydroxyphenylethanol and p-hydroxyphenylacetate concentration measured in each culture

TABLE 4. Specific fluxes through the aromatic amino acid biosynthesis pathway after the chorismate branch. Total fluxes were calculated by combining (i) specific rates of production of aromatic metabolites (fusel alcohols and acids) derived from phenylalanine and tyrosine, (ii) specific rates of production of phenylalanine and tyrosine in cellular protein and (iii) specific rates of production of intracellular free phenylalanine and tyrosine.

Strain	Genotype	Fluxes ($mmol.g^{-1}.h^{-1}$)				Normalized flux %
		Aromatic metabolites[a]	Phe and Tyr in protein[b]	Phe and Tyr intracellular[c]	Total	
CEN.PK 113-7D	*ARO3 ARO4*	<0.001	0.024	0.001	0.025	100
CENPK718-5A	*aro3Δ ARO4^{K229L}*	0.014	0.024	0.004	0.042	168
IMZ016	*ARO3 ARO4 TDH3pr-ARO7*	<0.001	0.024	0.002	0.026	104
IMZ017	*ARO3 ARO4 TDH3pr-ARO7^{G141S}*	<0.001	0.024	0.002	0.026	104
IMZ014	*aro3 Δ ARO4^{K229L}TDH3pr-ARO7*	0.016	0.024	0.004	0.044	176
IMZ018	*aro3Δ -ARO4^{K229L}TDH3pr-ARO7^{G141S}*	0.019	0.024	0.004	0.047	188

[a] sum of the phenylethanol, phenylacetate, p-hydroxyphenylethanol and p-hydroxyphenylacetate concentrations measured multiplied by the dilution rate and divided by the biomass concentration in each culture

[b] concentrations of phenylalanine and tyrosine in protein were calculated from published data on protein content and amino acid composition of *S. cerevisiae* (22, 29), multiplied the dilution rate and divided by the biomass concentration in each culture

[c] sum of the free intracellular phenylalanine and tyrosine concentrations in the cultures were calculated based on an intracellular volume of 2 ml.g biomass^{-1} (23), multiplied by the dilution rate and divided by the biomass concentration in each culture

metabolites, the individual overexpression of either the wild type *ARO7* allele or the *ARO7*^{G141S} alleles caused a significant, ca. 50 % increase of the intracellular concentrations of phenylalanine and tyrosine relative to the reference strain CEN.PK113-7D.

Intracellular concentrations of tryptophan were low in the reference strain CEN.PK113-7D (ca. 0.2 mM, Fig. 4). Elimination of feedback inhibition on DAHP synthase caused a ca. 3-fold increase of the intracellular tryptophan concentration. Interestingly, this increase was still present when a tryptophan- and tyrosine insensitive chorismate mutase (encoded by *ARO7*^{G141S}) was simultaneously overexpressed. Conversely, overexpression of the wild-type *ARO7* allele reduced the intracellular tryptophan in a strain expressing a feedback insensitive DAHP synthase to the level observed in the reference strain. This difference may be related to the observation that the maximum chorismate mutase activity in strains overexpressing the wild-type *ARO7* allele (measured in the presence of the activator tryptophan) was higher than those in strains overexpressing the tryptophan-insensitive *ARO7*^{G141S} allele (Fig. 3).

The effects of the expression of the *ARO4* ^{K229L} and overexpression of *ARO7*^{G141S} alleles on intracellular amino acids were not confined to the aromatic amino acids (Fig. 4). Based on their intracellular concentration profiles, two classes of non-aromatic amino acids could be identified. A first group of amino acids did not show a clear correlation with the genotype of the strains and included valine, alanine, glycine, leucine, serine, glutamate, isoleucine, proline and aspartate (Fig. 4). Concentration changes between the different strains were below 50 % for this group of 'non-responsive' amino acids. Interestingly, a second class of amino acids was responsive to the genotype of the strains. This class included asparagine, histidine, ornithine, threonine, lysine and methionine, which consistently showed a genotype-dependent concentration profile that closely mirrored that of phenylalanine and tyrosine (Fig. 4). This result demonstrates that deregulation of the amino acid biosynthesis pathway has more widespread repercussions on amino acid biosynthesis.

Quantification of metabolic fluxes through the phenylalanine-tyrosine branch of aromatic amino acid biosynthesis

In order to quantify the overall effect of the expression of the *ARO4*^{K229L} and *ARO7*^{G141S} alleles on the metabolic fluxes through the phenylalanine-tyrosine branch of amino acids (i.e. excluding the flux towards tryptophan and related metabolites), three components need to be taken into account:

(i) The specific production rates of the aromatic metabolites phenylethanol, phenylacetate and their p-hydroxy derivatives. In calculating the specific production rates mentioned in Table 4, it was assumed that these metabolites do not accumulate inside the cells.

(ii) The specific production rate of phenylalanine and tyrosine incorporated into cellular protein. Based on the assumption that the protein content in all strains is identical to that of the reference strain CEN.PK113-7D grown at a dilution rate of 0.10 h^{-1} (22) and that the amino acid composition of the cellular protein is equal to that reported by Oura (1972) (28), this rate is identical for all strains (Table 4).

(iii) The specific production rates of free intracellular phenylalanine and tyrosine, which can be calculated from the data presented in Fig. 4 by assuming an intracellular volume of 2 ml (g biomass)$^{-1}$ (Table 4).

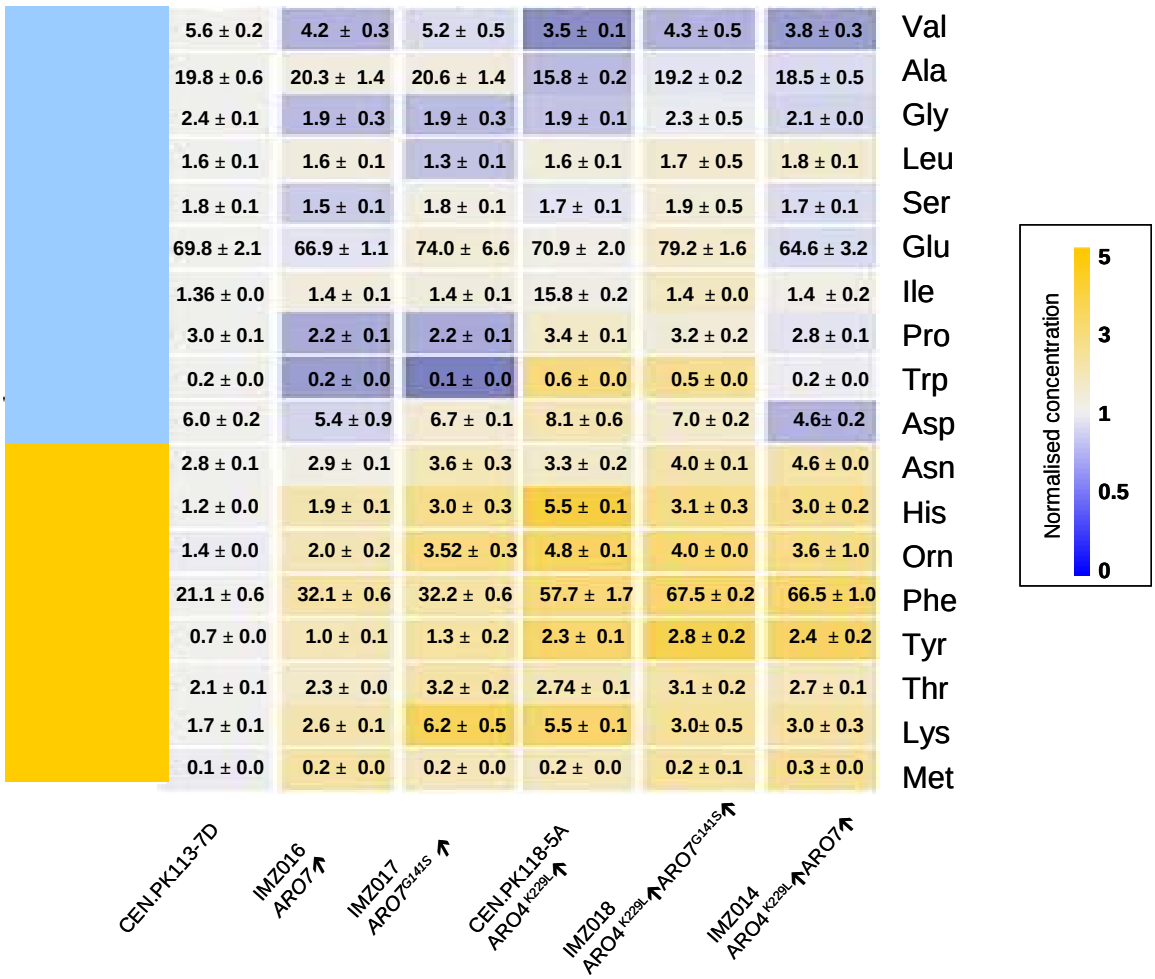

FIG. 4. Intracellular amino acid concentrations in the reference *S. cerevisiae* strain CEN.PK113-7D and in strains engineered in key steps of aromatic amino acid biosynthesis. Numbers indicated the average ± mean deviation of results from two independent chemostat cultures. Colors represent a concentration heat map normalized for the concentration of each amino acid in cultures of the reference strain CEN.PK113-7D.

In the reference strain CEN.PK113-7D, the flux towards phenylalanine and tyrosine was virtually completely incorporated into cellular protein. In the strains that expressed the *ARO4* ^{K229L} allele, the overall flux through this branch of aromatic amino acid metabolism increased by almost two-fold (Table 4). This was

primarily due to the increased synthesis of the fusel alcohols and corresponding acids. The increased concentrations of free intracellular phenylalanine and tyrosine had little impact on the overall fluxes.

DISCUSSION

This study presents a quantitative analysis of the impact of allosteric control of two key enzymes in aromatic amino acid metabolism on product formation by *S. cerevisiae*. The results demonstrate that DAHP synthase exerts a strong degree of control on the synthesis of aromatic compounds by *S. cerevisiae*. This result is not surprising: it has previously been demonstrated that non-defined feedback insensitive mutants of *S. cerevisiae* obtained by ethyl methyl-sulfonate mutagenesis and screening on phenylalanine analogues produce increased levels of phenylethanol (8, 9). Moreover, elimination of feedback regulation is a classical first step in improving amino acid production by prokaryotes. In stark contrast with the situation in bacteria such as *E. coli* and *C. glutamicum* (1, 23, 24), in *S.cerevisiae* the alleviation of feed-back inhibition on DAHP synthase did not lead to measurable extracellular concentrations of phenylalanine or tyrosine. Apparently, even in situations where the intracellular concentration of phenylalanine reaches levels of over 60 mM (Fig. 4), export across the plasma membrane does not occur. So far the only system reported to catalyse the export of amino acids from *S.cerevisiae* cells involved the *AQR1* gene product (40), which mediates the export of several amino acids via an exocytosis-like mechanisms. However, phenylalanine and tyrosine are not among the reported substrates for this export system. If *S. cerevisiae* is ever to be considered for the production of amino acids, engineering of product export is clearly a priority target. The steep gradient of phenylalanine that exists across the plasma membrane of *S. cerevisiae* strains that express the $ARO4^{K229L}$ and $ARO7^{G141S}$ alleles make them ideally suited as a model system to explore strategies for introducing amino acid export into *S. cerevisiae*.

In *S. cerevisiae*, chorismate mutase (Aro7p) is known to be subject to allosteric regulation by tyrosine (inhibition) and tryptophan (activation). Our hypothesis that, especially in strains expressing a feed-back insensitive DAHP synthase, this allosteric regulation would have a strong effect on aromatic amino acid biosynthesis was not borne out by the experimental results. In fact, the product concentrations and specific aromatic product formation rates observed in strains with a combined expression of the feed-back insensitive $ARO4^{K229L}$ and the non-allosterically controlled $ARO7^{G141S}$ alleles showed only small differences in the formation of intracellular and extracellular aromatic products (Fig. 4, Table 3). This indicates that, in strains with a deregulated DAHP synthase, control of the synthesis of aromatic compounds resides elsewhere. Since we have not analysed levels of all intermediates of the aromatic amino acid biosynthesis pathway, a discussion on the actual rate-controlling step(s) in these strains is by necessity speculative. Still, three possibilities may be of special interest in further work.

Firstly in the strains that express the $ARO4^{K229L}$ allele the activity of DAHP synthase may exert a high degree of flux control. In that case, overexpression of $ARO4^{K229L}$ might lead to increased fluxes toward aromatic metabolites (Table 4). Secondly, formation of these compounds requires the conversion of phenylpyruvate and *p*-hydroxylphenylpyruvate, the penultimate compounds in phenylalanine and tyrosine biosynthesis, respectively,

by a TPP-dependent decarboxylase activity (4, 42, 43). Measurements of the activity of this decarboxylase activity in cell extracts of the chemostat cultures of the wild-type and engineered strains yielded only very low activities of this key enzyme activity. We are currently attempting to overexpress phenylpyruvate decarboxylase in *S. cerevisiae*. However, these studies are complicated by the fact that the molecular nature of this decarboxylase activity is not yet fully understood (42). A final possibility involves a reaction outside the main pathway for synthesis of aromatic amino acids. The pentose phosphate pathway is essential for aromatic amino acid biosynthesis as it provides erythrose-4-phosphate, an essential precursor for the shikimate pathway (Fig. 1). It has been demonstrated that transketolase, the enzyme that catalyses erythrose-4-phosphate formation, is inhibited by *p*-hydroxyphenylpyruvate, the penultimate intermediate in tyrosine biosynthesis (34). Although no *p*-hydroxyphenylpyruvate-insensitive mutant forms of yeast transketolase have been described, it may be of interest to exchange the yeast *TKL1 / TKL2* genes for a heterologous transketolase gene.

The deregulation of the aromatic amino acid biosynthesis pathway had a strong impact on the intracellular concentrations of several non-aromatic amino acids (Fig. 4). We are not aware of regulatory networks that might account for the observation that intracellular concentrations of asparagine, histidine, ornithine, threonine, lysine and methionine all showed genotype-dependent concentration profiles similar to those of phenylalanine and tyrosine (Fig. 4). Involvement of transaminases with overlapping substrate specificities in the synthesis of these amino acids may contribute to this phenomenon by connecting a number of intracellular amino acid 2-oxo acid equilibrium reactions. For example, it is known that the aromatic amino transferase encoded by *ARO8* can also transaminate methionine (19, 37). However, a complete overview of the substrate specificities of the *S. cerevisiae* transaminases is currently not available. The perturbance of non-aromatic amino acid biosynthesis pathways upon the deregulation of the shikimate pathway represents an important challenge for further research on metabolic engineering of aromatic metabolism in *S. cerevisiae*.

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REFERENCES

1. **Baez-Viveros, J. L., J. Osuna, G. Hernandez-Chavez, X. Soberon, F. Bolivar, and G. Gosset.** 2004. Metabolic engineering and protein directed evolution increase the yield of L-phenylalanine synthesized from glucose in *Escherichia coli*. Biotechnol. Bioeng. **87**:516-524.
2. **Ball, S. G., R. B. Wickner, G. Cottarel, M. Schaus, and C. Tirtiaux**. 1986. Molecular cloning and characterization of *ARO7-OSM2*, a single yeast gene necessary for chorismate mutase activity and growth in hypertonic medium. Mol. Gen. Genet. **205**:326-330.
3. **Brown, J. F., and I. W. Dawes**. 1990. Regulation of chorismate mutase in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **220**:283-288.
4. **Dickinson, J. R., Salgado L. E. J., and J. E. Hewlins**. 2003. The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. J. Biol. Chem. **278**:8028-8034.
5. **Ehrlich, F.** 1907. Uber die Bedingungen der Fuselölbildung and uber ihren Zusammenhang mit dem Eiweissaufbau der Hefe. Ber. Deut. Chem. Gesellsch. **40**:1027-1047.
6. **Etschmann, M. M., W. Bluemke, D. Sell, and J. Schrader**. 2002. Biotechnological production of 2-phenylethanol. Appl. Microbiol. Biotechnol. **59**:1-8.
7. **Fabre, C. E., P. J. Blanc, and G. Goma**. 1998. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. Biotechnol. Prog. **14**:270-274.
8. **Fukuda, K., M. Watanabe, K. Asano, K. Ouchi, and S. Takasawa**. 1991. A mutated *ARO4* gene for feedback-resistant DAHP synthase which causes both o-fluoro-DL-phenylalanine resistance and beta-phenethyl-alcohol overproduction in *Saccharomyces cerevisiae*. Curr. Genetics **20**:453-456.
9. **Fukuda, K., M. Watanabe, K. Asano, K. Ouchi, and S. Takasawa**. 1991. Isolation and genetic study of p-fluoro-DL-phenylalanine-resistant mutants overproducing beta-phenethyl-alcohol in *Saccharomyces cerevisiae.* Curr. Genetics **20**:449-452.
10. **Gietz, R. D., and R. A. Woods**. 2002. Transformation of yeast by the LiAc/SS carrier DNA/PEG method. Methods Enzymol. **350**:87-96.
11. **Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann**. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. **24**:2519-2524.
12. **Gunby, P.** 1983. FDA approves aspartame as soft-drink sweetener. J. A. M. A. **250**:872-873.
13. **Hartmann M., Schneider T.R., Pfeil A., Heinrich G., W. N. Lipscomb, and G. H. Braus.** 2003. Evolution of feedback-inhibited beta /alpha barrel isoenzymes by gene duplication and a single mutation. Proc. Natl. Acad. Sci. U. S. A. **100**:862-867.
14. **Helmstaedt, K., S. Krappman, and G. H. Braus**. 2001. Allosteric regulation of catalytic activity*: Escherichia coli* aspartate transcarbamoylase versus yeast chorismate mutase. Microbiol. Mol. Biol. Rev. **65**:404-421.

15. **Helmstaedt, K., A. Strittmatter, W. N. Lipscomb, and G. H. Braus**. 2005. Evolution of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase-encoding genes in the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U. S. A. **102**:9784-9789.
16. **Hermann, T.** 2003. Industrial production of amino acids by coryneform bacteria. J. Biotechnol. **58**:781-785.
17. **Ikeda, M.** 2003. Amino acid production processes. Adv. Biochem. Eng. Biotechnol. **79**:1-35.
18. **Ikeda, M., and R. Katsumata.** 1992. Metabolic engineering to produce tyrosine or phenylalanine in a tryptophan-producing *Corynebacterium glutamicum* strain. Appl. Environ. Microbiol. **58**:781-785.
19. **Iraqui, I., S. Vissers, M. Cartiaux, and A. Urrestarazu.** 1998. Characterisation of *Saccharomyces cerevisiae ARO8* and *ARO9* genes encoding aromatic aminotransferases I and II reveals a new aminotransferase subfamily. Mol. Gen. Genet. **257**:238-248.
20. **Krappman, S., W. N. Lipscomb, and G. H. Braus.** 2000. Coevolution of transcriptional and allosteric regulation at the chorismate metabolic branch point of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U. S. A. **97**:13585-13590.
21. **Kunzler, M., G. M. Paravicini, C. Egli, S. Irniger, and G. H. Braus.** 1992. Cloning, primary structure and regulation of the *ARO4* gene, encoding the tyrosine-inhibited 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase from *Saccharomyces cerevisiae*. Gene **113**:67-74.
22. **Lange, H. C., and J. J. Heijnen**. 2001. Unpublished data.
23. **Lange, H. C., and J. J. Heijnen**. 2001. Statistical reconciliation of the elemental and molecular biomass composition of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **75**:334-344.
24. **Li, K., M. R. Mikola, K. M. Draths, R. M. Worden, and J. W. Frost.** 1999. Fed-batch fermentor synthesis of 3-dehydroshikimic acid using recombinant *Escherichia coli*. Biotechnol. Bioeng. **64**:61-73.
25. **Liao, H. F., L. L. Lin, H. R. Chien, and W. H. Hsu**. 2001. Serine 187 is a crucial residue for allosteric regulation of *Corynebacterium glutamicum* 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase. FEMS Microbiol. Lett. **194**:59-64.
26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randell.** 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**:265-275.
27. **Luttik, M. A., K. M. Overkamp, P. Kotter, S. de Vries, J. P. Van Dijken, and J. T. Pronk**. 1998. The *Saccharomyces cerevisiae NDE1* and *NDE2* genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. J. Biol. Chem. **273**:24529- 24534.
28. **Mumberg, D., R. Muller, and M. Funk**. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene **156**:119-122.
29. **Oura, E.** The effect of aeration on the growth energetics and biochemical compostion of baker's yeast 1972, PhD Thesis. University of Helsinki, Finland.
30. **Pittard, A. J.** 1996. Biosynthesis of aromatic amino acids, p. 458-484. *In* R.C.F.C.Neidhardt (ed.), *Escherichia coli* and *Salmonella*, Cellular and Molecular Biology. Am. Soc. Microbiol, Washington DC.

31. **Postma, E., C. Verduyn, W. A. Scheffers, and J. P. Van Dijken**. 1989. Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae.* Appl. Environ. Microbiol. **55**:468-477.
32. **Schmidheini, T., P. Sperisen, G. Paravicini, R. Hutter, and G. H. Braus**. 1989. A single point mutation results in a constitutively activated and feedback-resistant chorismate mutase of *Saccharomyces cerevisiae*. J. Bacteriol. **171**:1245-1253.
33. **Schmidheini, T., H. U. Mosch, J. N. S. Evans, and G. H. Braus**. 1990. Yeast allosteric chorismate mutase is locked in the activated state by a single amino acid substitution. Biochemistry **29**:3660-3668.
34. **Schnappauf, G., S. Krappman, and G. H. Braus**. 1998. Tyrosine and tryptophan act through the same binding site at the dimer interface of yeast chorismate mutase. J. Biol. Chem. **273**:17012-17017.
35. **Solovjeva, O. N., and G. A. Kochetov**.1999. Inhibition of transketolase by p-hydroxyphenylpyruvate. FEBS Lett. **426**:246-248.
36. **Sprinson, D., P. R. Srinivasan, and M. Katagiri.** 1962. 3-deoxy-D-arabino-hepturosonate-7-phosphate synthetase from *E.coli.* Methods Enzymol. **5**:394-398.
37. **Teshiba, S., R. Furter, P. Niederberger, G. H. Braus, G. Paravicini, and R. Hutter**. 1986. Cloning of the *ARO3* gene of *Saccharomyces cerevisiae* and its regulation. Mol. Gen. Genet. **205**:353-357.
38. **Urrestarazu, A., S. Vissers, I. Iraqui, and M. Grenson**. 1998. Phenylalanine- and tyrosine auxotrophic mutants of *Saccharomyces cerevisiae* impaired in transamination. Mol. Gen. Genet. **257**:230-237.
39. **Van den Berg, M. A., P. de Jong-Gubbels, C. J. Kortland, J. P. Van Dijken, J. T. Pronk, and H. Y. Steensma**. 1996. The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. J. Biol. Chem. **271**:28953-28959.
40. **Van Maris, A. J., J. M. Geertman, A. Vermeulen, M. K. Groothuizen, A. A. Winkler, M. D. Piper, J. P. van Dijken, and J. T. Pronk**. 2004. Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae,* yielding a C(2)-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. Appl. Environ. Microbiol. **70**:159-166.
41. **Velasco, I., S. Tenreiro, I. L. Calderon, and B. Andre**. 2004. *Saccharomyces cerevisiae* Aqr1 is an internal-membrane transporter involved in excretion of amino acids. Eukaryot. Cell **3**:1492-1503.
42. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. Van Dijken**. 1990. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J.Gen.Microbiol. **136**:395-403.
43. **Vuralhan, Z., M. A. Luttik, S. L. Tai, V. M. Boer, M. A. Morais, D. Schipper, M. J. Almering, P. Kötter, J. R. Dickinson, J. M. Daran, and J. T. Pronk.** 2005. Physiological characterization of the *ARO10*-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*. App. Environ. Microbiol. **71**:3276-3284.
44. **Vuralhan, Z., M. A. Morais, S. L. Tai, M. D. Piper and J. T. Pronk.** 2003. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae.* App. Environ. Microbiol. **69**:4534-4541.

45. **Wijker, J. E., P. R. Jensen, J. L. Snoep, G. A. Vaz, M. Guiral, A. P. Jongsma, A. de Waal, S. Hoving, S. van Dooren, and C. C. van der Weijden**. 1995. Energy, control and DNA structure in the living cell. Biophys. Chem. **55**:153-165.

46. **Wittmann, C., M. Hans, and W. Bluemke**. 2002. Metabolic physiology of aroma-producing *Kluyveromyces marxianus*. Yeast **19**:1351-1363.

SUMMARY

Saccharomyces cerevisiae is a popular industrial microorganism. It has since long been used in bread, beer and wine making. More recently it is also being applied for heterologous protein production and as a target organism for metabolic engineering. The work presented in this thesis describes how *S. cerevisiae* may be used as a metabolic-engineering platform to produce aromatic compounds such as phenylalanine or its catabolites, phenylethanol and phenylacetate.

S. cerevisiae is capable of using many amino acids as sole nitrogen source via deamination or transamination. In case of transamination the amino group may be transferred to a variety of (acceptor) keto acids, which then become amino acids. In this process the nitrogen-donating amino acid becomes a 2-oxo-acid itself. When this 2-oxo-acid cannot be further converted into cell constituents, it is catabolized via the Ehrlich pathway: upon transamination of the amino acid to the corresponding 2-oxo-acid, the latter compound is decarboxylated to the corresponding aldehyde. The redox state of the cells (i.e. the availability of oxygen) determines the fate of the aldehyde: it can be reduced by alcohol dehydrogenases (yielding a group of compounds commonly referred to as fusel alcohols) or be oxidized to the corresponding organic acid ("fusel acids") by aldehyde dehydrogenases (Chapter 1). In the case of nitrogen assimilation from phenylalanine, the product of phenylalanine transamination, phenylpyruvic acid, is decarboxylated to phenylacetaldehyde which, in turn, is reduced to phenylethanol or oxidized to phenylacetic acid.

Chapter 2 of this thesis focuses on the identification of genes that code for phenylpyruvate decarboxylase. According to the literature, *S. cerevisiae* has five candidate genes that may encode this activity. These are *PDC1, PDC5, PDC6 and ARO10,* and *THI3*.

In this Chapter it is reported that phenylpyruvate decarboxylase activity is present in cells from glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK 113-7D, growing with phenylalanine as N-source. This activity could not be detected in cultures grown with ammonia as N-source. Transcriptome analysis demonstrated that *ARO10* transcript levels increased 30-fold when phenylalanine replaced ammonia as N-source. Metabolite profile analysis and enzyme activity measurements on cells from chemostat cultures indicated that *ARO10* alone is sufficient for phenylpyruvate decarboxylase activity when other candidate genes are absent. However, extracts of cells lacking Aro10p *(PDC1 PDC5 PDC6 YDL080c aro10Δ)* still exhibited phenylpyruvate decarboxylase activity. Therefore, it seems that one or more of the other candidate genes can replace *ARO10*. One of the candidate genes; *THI3 (YDL080c)* seemed to require one of the other decarboxylase genes *(PDC1, PDC5, PDC6)* for phenylpyruvate decarboxylase activity. *In vivo* Aro10p is probably the relevant enzyme in phenylalanine catabolism. This conclusion is based on an analysis of the kinetic parameters of phenylpyruvate decarboxylase activity in cells with or without Aro10p.

Although *ARO10* is not the only gene encoding 2-oxoacid decarboxylase activity, it is crucial to the formation of fusel alcohols and acids (Chapter 3). In aerobic glucose- limited chemostat cultures growing with ammonia, proline, asparagine, leucine, methionine or phenylalanine as N-source, only with the latter three amino acids formation of fusel alcohols and acids was observed. Transcriptome analysis revealed that under the latter three growth conditions *ARO10* was up-regulated, whereas the expression levels of the other four candidate genes, encoding putative thiamine pyrophosphate-dependent decarboxylases, remained unchanged. Measurements of decarboxylase activity in cell-free extracts confirmed that only when the yeast was grown with phenylalanine, leucine or methionine as nitrogen source, phenylpyruvate decarboxylase activity was present. Not only phenylpyruvate, but also α-ketoisovalerate, α-ketoisocaproate, α-ketomethylvalerate and 3-methyl-thio-α-ketobutyrate served as substrate for the decarboxylase activity. In a decarboxylase-negative background (*pdc1, pdc5, pdc6, thi3*), overexpression of *ARO10* resulted in decarboxylase activities with the 2-oxoacids derived from phenylalanine, namely leucine and methionine. The ratio between these activities in the mutant overexpressing *ARO10* was the same as in the wild type. Therefore, Aro10p is probably a broad-substrate decarboxylase. This enzyme is subject to posttranscriptional regulation. In spite of constitutive expression of *ARO10* under the control of the *TDH3* promotor, enzyme levels were strongly affected by the nature of the carbon and nitrogen sources for growth.

Microbial production of aromatic amino acids requires insight into the regulation of the synthesis of these compounds and in the mechanism and regulation of (unwanted) catabolism to fusel alcohols and acids, as well as into the mechanism of product export.

In the last chapter of this thesis initial attempts are described to overproduce phenylalanine and tyrosine with *S. cerevisiae*. To this end the combination of deregulated DAHP synthase and chorismate mutase was investigated. The introduction of a tyrosine-insensitive allele of *ARO4* led to a three-fold increase in intracellular levels of phenylalanine and tyrosine. However, these amino acids were not excreted into the culture broth. This probably results from the absence of an adequate export system. On the other hand, extracellular concentrations of phenylacetic acid, phenyl ethanol and their para-hydroxy-analogues increased fifty-fold, thus confirming the necessity of preventing amino acid catabolism in a production process for aromatic amino acids.

Production of aromatic amino acids by *S. cerevisiae* will influence other parts of the metabolic network. For example, *ARO7* overexpression combined with the tyrosine-insensitive *ARO4* allele did not only affect the intracellular levels of aromatic amino acids, but also resulted in enhanced levels of other amino acids. Thus, changing the concentration of non-aromatic amino acids, regulated via the shikimate pathway, might provide new targets for engineering of the metabolism of aromatic compounds in *S. cerevisiae.*

SAMENVATTING

Saccharomyces cerevisiae wordt veel gebruikt voor industriële toepassingen. Deze gist wordt reeds lang benut voor de bereiding van brood, bier en wijn. Meer recente toepassingen zijn productie van heterologe eiwitten en gebruik van de gist voor metabolic engineering. Dit proefschrift beschrijft hoe *S. cerevisiae* kan worden gebruikt als metabolic engineering platform voor de productie van aromatische verbindingen zoals fenylalanine, of de afbraakproducten daarvan: fenylalcohol en fenylazijnzuur.

S. cerevisiae is in staat om vele aminozuren te gebruiken als enige stikstofbron, via deaminering of transaminering. In het geval van transaminering kan de aminogroep aan diverse ketozuren overgedragen worden. Deze ketozuren worden dan de overeenkomstige aminozuren. Het aminozuur dat de aminogroep afstaat wordt daarbij zelf een ketozuur. Wanneer dit ketozuur niet verder omgezet kan worden tot celbestanddelen, wordt dit gedeeltelijk afgebroken via de Ehrlich route: na transaminering van het aminozuur tot het corresponderende ketozuur, wordt deze verbinding gedecarboxyleerd tot het overeenkomstige aldehyde. De redox-status van de cel (de aan of afwezigheid van zuurstof) bepaalt de vervolgreactie. Het aldehyde kan gereduceerd worden door alcohol dehydrogenasen (tot zogenaamde foezelalcoholen) of geoxideerd worden door aldehyde dehydrogenasen tot het overeenkomstige zuur (foezelzuren) (Hoofdstuk 1).

In het geval van fenylalanine wordt het transaminerings product, fenylpyruvaat, gedecarboxyleerd tot fenylacetaldehyde dat vervolgens gereduceerd wordt tot fenylalcohol of geoxideerd tot fenylazijnzuur.

Hoofdstuk 2 van dit proefschrift richt zich op de genen die coderen voor fenylpyruvaat decarboxylase . In de literatuur is beschreven dat vijf genen kunnen coderen voor deze activiteit te weten *PDC1, PDC5, PDC6, ARO10 en THI3*.

Hoofdstuk 2 laat zien dat fenylpyruvaat decarboxylase activiteit aanwezig is in *S. cerevisiae* CEN.PK113-7D, wanneer de gist gekweekt wordt in glucose- gelimiteerde chemostaat cultures met fenylalanine als stikstofbron. Deze activiteit is afwezig wanneer ammonium als stikstof dient.

Transcriptoomanalyse toonde aan dat het *ARO10* transcriptieniveau met een factor 30 toeneemt wanneer, in plaats van ammonium, fenylalanine als stikstofbron dient. Analyse van extracellulaire metabolieten en metingen van enzymactiviteiten met cellen van chemostaat cultures gaven aan dat *ARO10* op zich voldoende is voor het genereren van fenylpyruvaat decarboxylase wanneer de andere decarboxylase genen afwezig zijn.

Echter, extracten van cellen zonder Aro10p (*PDC1,PDC5, PDC6, YDL080C, aro10Δ)* vertoonden toch fenylpyruvaat decarboxylase activiteit. Dit wijst erop dat één of meerdere genen *ARO10* kunnen vervangen. Een van deze genen, *THI3 (YDL080C)* leek één van de andere genen *(PDC1, PDC5, PDC6)* nodig te hebben voor het bewerkstellingen van fenylpyruvaat decarboxylase activiteit. Aro10p is waarschijnlijk het belangrijkste

enzym *in vivo* voor de afbraak van fenylalanine. Deze conclusie is gebaseerd op een analyse van de kinetiek van de fenylpuruvaat decarboxylase activiteit in cellen met en zonder Aro10p.

Hoewel *ARO10* niet het enige gen is dat codeert voor 2-ketozuur decarboxylase activiteit is het wel cruciaal voor de vorming van foezelalcoholen en -zuren. Dit wordt beschreven in hoofdstuk 3. In aërobe glucose-gelimiteerde chemostaatcultures gekweekt met ammonium, proline, asparagine, leucine, methionine of fenylalanine als stikstofbron werd alleen met de laatste drie aminozuren vorming van foezelalcoholen en -zuren waargenomen. Transcriptoom analyse wees uit dat bij groei met deze stikstofbronnen de transcriptie van *ARO10* toenam, terwijl de expressie van de andere vier genen die coderen voor mogelijke thiamine pyrofosfaat (TPP)-afhankelijke decarboxylasen niet veranderde. Metingen van decarboxylase-activiteit in celvrije extracten bevestigde dat alleen bij groei met leucine, methionine of fenylalanine als stikstofbron fenylpuruvaat decarboxylase activiteit aanwezig was. Niet alleen fenylpyruvaat maar ook α-ketoisovaleraat, α-ketoisocaproaat, α-ketomethylvaleraat en 3-methylthio-α-ketobutyraat fungeerden als substraat voor de decarboxylase-activiteit. In een decarboxylase-negatieve achtergrond *(pdc1, pdc5, pdc6, thi3)* veroorzaakte overexpressie van *ARO10* decarboxylase activiteit met de van fenylanaline afgeleide 2-ketozuren , leucine en methionine. De verhouding tussen de drie decarboxylase-activiteiten in de mutant met *ARO10* overexpressie was gelijk aan die in het wild type. Aro10p is dus waarschijnlijk een decarboxylase met een brede substraatspecificiteit. Dit enzym wordt waarschijnlijk ook gereguleerd op post-transcriptioneel niveau. Niettegenstaande constitutieve expressie van *ARO10* onder controle van de *TDH3* promotor werd toch een sterke invloed van de koolstof- en stikstofbron op de enzymniveau's waargenomen.

Microbiële productie van aromatische aminozuren vereist inzicht in de regulatie van de synthese van deze verbindingen, in mechanisme en regulatie van de vorming van ongewenste bijproducten zoals foezelalcoholen en –zuren, en ook in het mechanisme van productexport. In het laatste hoofdstuk van dit proefschrift worden experimenten beschreven die tot doel hadden overproductie van phenylalanine en tyrosine te bewerkstellingen in *S. cerevisiae* via een combinatie van gereguleerd DAHP synthase en chorismaat mutase. De introductie van een tyrosine-ongevoelig allel van *ARO4* had een drievoudige verhoging van de intracellulair concentratie van fenylalanine en tyrosine tot gevolg. Deze aminozuren werden echter niet uitgescheiden, hetgeen waarschijnlijk z'n oorzaak heeft in de afwezigheid van een adequaat export systeem. Wel werd een 50-voudige verhoging van de extracellulaire concentraties van fenylalcohol, fenylazijnzuur en hun para-hydroxy-analogen waargenomen.

Deze waarneming bevestigt de noodzaak, vorming van bijprodukten in een productieproces voor aromatische aminozuren te voorkomen.

Productie van aromatische aminozuren door *S. cerevisiae* beïnvloedt ook andere delen van het metabole netwerk. Bijvoorbeeld, overexpressie van *ARO7* in combinatie met de tyrosine-ongevoelige *ARO4* allel beïnvloedde niet alleen de intracellulaire concentraties van aromatische aminozuren, maar ook die van andere aminozuren. Dit fenomeen, gevolg van de regulatie van de shikimaatroute, zal mogelijk nieuwe aangrijpingspunten opleveren voor het manipuleren van het metabolisme van aromatische aminozuren.

PUBLICATIONS

1. ***Vuralhan Z.,*** M. A. Morais, S. L. Tai, M. D. Piper, and J. T. Pronk. 2003. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae.* App.Environ.Microbiol. **69**:4534-4541

2. ***Vuralhan Z.,*** M. A. Luttik, S. L. Tai, V. M. Boer, M. A. Morais, D. Schipper, M. J. Almering, P. Kötter, J. R. Dickinson, J. M. Daran, and J. T. Pronk. 2005. Physiological characterization of the *ARO10*-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*. App.Environ.Microbiol. **71**:3276-3284.

3. ***Vuralhan Z.,*** M. A. Luttik, G.H. Braus, J.M. Daran, and J.T. Pronk. 2005. Alleviation of feedback inhibition in *Saccharomyces cerevisiae* aromatic amino acid biosynthesis: quantification of metabolic impact. *Manuscript in preparation*

4. Boer, V. M., S. L. Tai, ***Z. Vuralhan,*** Y. Arifin, M. C. Walsh, M. D. Piper, J. H. de Winde, J. M. Daran, and J.T. Pronk (2005). Transcriptional responses of *Saccharomyces cerevisiae* to growth on preferred and non-preferred nitrogen sources in glucose-limited chemostat cultures. *Submitted for publication.*

Curriculum Vitae

Zeynep Vuralhan obtained her diploma as Food Engineer in July 1995 from the Faculty of Chemical & Metallurgy Engineering at Istanbul Technical University (ITU). In 1995-1996, she completed an Executive MBA study on a joint program between Marmara University, Istanbul and the University of Maine-USA, while working as a junior engineer for an international company in Istanbul. In the same year (1996), she obtained a scholarship to continue with academical studies in Denmark. In 1999, she received her M.Sc. degree in Food Science and Technology from the Technical University of Denmark (DTU). Her M.Sc thesis focused on the molecular biology of lactic acid bacteria. In December 1999, she became research / teaching assistant at the University of Arkansas, Food Microbiology Department, Fayetteville, USA. In 2000, she returned to Europe by joining Industrial Microbiology Group as a Ph.D. student at the Department of Biotechnology, TU-Delft where she studied aromatic amino acid metabolism in *S. cerevisiae.*

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