Biosynthesis of phenylalanine and tyrosine in the methylotrophic actinomycete amycolatopsis methanolica
Euverink, Gerrit Jan Willem

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Chapter 1

AROMATIC AMINO ACID BIOSYNTHESIS IN ACTINOMYCETES

G.J.W. Euverink
Chapter 1

TAXONOMY OF ACTINOMYCETES

Actinomycetes are bacteria belonging to the order Actinomycetales and are characterized by the formation of branching filaments giving them a fungal appearance (Lechevalier and Lechevalier, 1981). Actinomycetes are widespread in nature and can be separated into two subgroups: oxidative forms, found mostly in soil habitants, and fermentative forms, living in natural cavities of man and animals (Lechevalier and Pine, 1977).

Actinomycetes are very important from a medical point of view and are potential human pathogens affecting both immunocompetent and immunocompromised hosts. The latter number is steadily increasing because modern technology allows the prolonged survival of critically ill patients, e.g. HIV-infected patients (McNeill and Brown, 1994). A number of actinomycetes in the genus Streptomyces are also known as plant pathogens but they play a minor role in plant diseases (Locci, 1994).

Common characteristics of the actinomycetes are formation of substrate- and aerial mycelium on solid media, their Gram-positive character, presence of spores, and a high G+C content of the DNA (60-70 mol%). The aerobic actinomycetes are most easily

![Diagram of actinomycetes relationships](image-url)

Figure 1. Unrooted distance tree, based on 16S rRNA nucleotide sequences, showing the relationships among members of the genera Saccharothrix, Actinokineospora, and members of the family Pseudonocardiaceae. Bar = 10 substitutions per 1,000 bases. Reproduced from Warwick et al. (1994). Inset: Unrooted phylogenetic tree showing the position of the family Pseudonocardiaceae within the actinomycetes. Reproduced from Embley (1991).
separated into different genera via morphological, physical, and chemical criteria. Most actinomycetes possess cell wall type I to IV with peptidoglycans containing L-diaminopimelic acid (DAP) and glycine (type I), meso-DAP and glycine (type II), meso-DAP (type III), or meso-DAP, arabinose, and galactose (type IV) (Goodfellow, 1989). The genus *Streptomyces* is the best known representative with cell wall type I. *Micromonospora* and *Actinoplanes* are genera with cell wall type II. *Thermoactinomyces*, *Microtetraspora*, and *Frankia* are genera with cell wall type III. Cell wall type IV actinomycetes comprise two families with (*Mycobacteriaceae*) or without (*Pseudonocardiaceae*) mycolic acids (Embley, 1991). The family *Pseudonocardiaceae* contains the genera *Amycolatopsis*, *Amycolata*, *Pseudonocardia*, *Saccharomonospora*, and *Saccharopolyspora* (Embley, 1991; Embley and Stackebrandt, 1994; Warwick et al., 1994) (Fig. 1). Previously these genera were referred to as the *Amycolata* wall type IV taxa or as *Micropolysporas* (Embley et al., 1988; Goodfellow and Cross, 1984). *Pseudonocardiaceae* are metabolically diverse and comprise autotrophic, methylotrophic, thermophilic, and halophilic family members.

**SECONDARY METABOLISM**

Actinomycetes are undoubtedly the largest producers of bacterial secondary metabolites (Table 1). Secondary metabolites are, in contrast to primary metabolites, not essential for growth and are synthesized from intermediates or end products of primary metabolism via unique biosynthetic pathways. These pathways may have originated from primary pathways within the same organism but also gene transfer between different organisms is an important factor in the evolution of secondary metabolism (Vining, 1992).

A survey of the literature from 1988-1992 revealed more than one thousand new secondary metabolites detected in actinomycetes. Most of the secondary metabolites identified were produced by *Streptomyces* sp. (Sanglier et al., 1993). The taxonomy of *Streptomyces* is not very clear and more detailed taxonomical studies undoubtedly will result in transfer of organisms previously know as *Streptomyces* sp. into other genera.

Secondary metabolites are synthesized under suboptimal growth conditions near the end of the exponential growth phase or in the stationary phase. Their formation is directed by organized sets of genes associated with special regulatory mechanisms that control both the timing and the level of gene expression (Horinouchi and Beppu, 1994; Liras et al., 1990; Martin and Demain, 1980; Vining, 1992). Their physiological role is not known. Speculations about their function include a role in overflow metabolism to get ride of excessive amounts of intermediates of primary metabolism. High intracellular concentrations of these intermediates can be toxic for the cell. If export of unmodified
### Table 1. Summary of different classes of secondary metabolites originally isolated from actinomycetes.

<table>
<thead>
<tr>
<th>Class</th>
<th>Common name</th>
<th>Organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aminoglycosides</strong></td>
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<tr>
<td></td>
<td>Streptomycin</td>
<td><em>Streptomyces griseus</em></td>
<td>Crandell and Hamill, 1986</td>
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<tr>
<td></td>
<td>Hygromycin</td>
<td><em>Streptomyces hygroscopicus</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td></td>
<td>Neomycin</td>
<td><em>Streptomyces fradiae</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td><em>Micromonospora</em> spp.</td>
<td>Crandell and Hamill, 1986</td>
</tr>
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<td></td>
<td>Kanamycin</td>
<td><em>Streptomyces kanamyceticus</em></td>
<td>Crandell and Hamill, 1986</td>
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<td></td>
<td>Apramycin</td>
<td><em>Streptomyces tenebrarius</em></td>
<td>Crandell and Hamill, 1986</td>
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<td></td>
<td>Spectinomycin</td>
<td><em>Streptomyces spectabilis</em></td>
<td>Crandell and Hamill, 1986</td>
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<td><strong>Ansamycins</strong></td>
<td>Rifamycin</td>
<td><em>Amycolatopsis mediterranei</em></td>
<td>Embley, 1991</td>
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<tr>
<td></td>
<td>Geldanamycin</td>
<td><em>S. hygroscopicus</em></td>
<td>Crandell and Hamill, 1986</td>
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<tr>
<td></td>
<td>Herbimycin</td>
<td>*S. hygroscopicus var. geldanus var. nova</td>
<td>Crandell and Hamill, 1986</td>
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<tr>
<td></td>
<td>Streptovaricins</td>
<td><em>S. spectabilis</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
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<td><strong>β-lactams</strong></td>
<td>Penicillins</td>
<td><em>Streptomyces spp., Nocardia spp.</em></td>
<td>Cohen et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Cephalosporins</td>
<td><em>Streptomyces spp., Nocardia spp.</em></td>
<td>Cohen et al., 1990</td>
</tr>
<tr>
<td><strong>Macrolides</strong></td>
<td>Oleandomycin</td>
<td><em>Streptomyces antibioticus</em></td>
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</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td><em>Saccharopolyspora erythraea</em></td>
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<td></td>
<td>Spiramycin</td>
<td><em>Streptomyces ambofaciens</em></td>
<td>Crandell and Hamill, 1986</td>
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<td></td>
<td>Tylosin</td>
<td><em>S. fradiae</em></td>
<td>Crandell and Hamill, 1986</td>
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<td><strong>Nucleosides</strong></td>
<td>Sinefungin</td>
<td><em>Streptomyces griseolus</em></td>
<td>Crandell and Hamill, 1986</td>
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<td></td>
<td>Tunicamycin</td>
<td><em>S. griseolus</em></td>
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<td><strong>Peptides</strong></td>
<td>Vancomycin</td>
<td><em>Amycolatopsis orientalis</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td></td>
<td>Actinomycin</td>
<td><em>S. antibioticus</em></td>
<td>Crandell and Hamill, 1986</td>
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<td></td>
<td>Thiolactone</td>
<td><em>Amycolatopsis azurea</em></td>
<td>Crandell and Hamill, 1986</td>
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<tr>
<td></td>
<td>Avoparcin</td>
<td><em>Amycolatopsis coloradensis</em></td>
<td>Labeda, 1995</td>
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<td></td>
<td>Orientisin A and B</td>
<td><em>A. orientalis</em></td>
<td>Embley, 1991</td>
</tr>
<tr>
<td></td>
<td>Muraceins</td>
<td><em>A. orientalis</em></td>
<td>Embley, 1991</td>
</tr>
<tr>
<td></td>
<td>Macrobicyclic peptide</td>
<td><em>Amycolatopsis fastidiosa</em></td>
<td>Embley, 1991</td>
</tr>
<tr>
<td></td>
<td>Azureomycin A and B</td>
<td><em>A. azurea</em></td>
<td>Embley, 1991</td>
</tr>
<tr>
<td><strong>Polyenes</strong></td>
<td>Amphoterin B</td>
<td><em>Streptomyces nodosus</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td><em>Streptomyces novossii</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td></td>
<td>Cândicin</td>
<td><em>S. griseus</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td><strong>Polyether antibiotics</strong></td>
<td>Monensin</td>
<td><em>Streptomyces cinnamononis</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td></td>
<td>Salinomycin</td>
<td><em>Streptomyces albua</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td></td>
<td>Narasin</td>
<td><em>Streptomyces aureofaciens</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td><strong>Tetracyclines</strong></td>
<td>(Chlor-) Tetracycline</td>
<td><em>S. aureofaciens</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td></td>
<td>Oxytetracycline</td>
<td><em>Streptomyces rimosus</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td>Cetocyline</td>
<td><em>Amycolatopsis sulphurea</em></td>
<td>Embley, 1991</td>
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<tr>
<td></td>
<td>Chloramphenicol</td>
<td><em>Streptomyces venezuelae</em></td>
<td>Crandell and Hamill, 1986</td>
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<tr>
<td></td>
<td>Cycloserine</td>
<td><em>Streptomyces sp.</em></td>
<td>Crandell and Hamill, 1986</td>
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<td></td>
<td>Rapamycin</td>
<td><em>S. hygroscopicus</em></td>
<td>Paiva et al., 1993</td>
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<tr>
<td></td>
<td>Lincomycin</td>
<td><em>Streptomyces lincolensis</em></td>
<td>Crandell and Hamill, 1986</td>
</tr>
</tbody>
</table>
metabolites is not possible, their conversion into less toxic, or more easily exported metabolites, may provide a way to prevent build up of toxic intermediates (Malik, 1980). Several of the enzymes in secondary metabolism are also regulated via repression or feedback inhibition making such a role in overflow metabolism less likely (Martin and Demain, 1980). Another role for secondary metabolites may be in defence mechanisms. Inhibiting other, competing cells, would leave more nutrients for the survival of the secondary metabolite producing strain (Malik, 1980; Maplemmote et al., 1992). Indeed many secondary metabolites show anti-bacterial or other inhibitory activities (anti-tumour, anti-fungal) or may function as herbicides (Demain, 1983; Malik, 1980; Sanglier et al., 1993). Various groups of secondary metabolites can be distinguished depending on the primary intermediate from which they are derived. This division,
however, is not absolute because more than one primary metabolite may be involved in
the biosynthesis of a particular secondary metabolite. The search for new secondary metabolites continues and if a more detailed understanding of the physiology of actinomycetes is combined with increased automatization of detection equipment, the list of known secondary metabolites undoubtedly will grow even further (Bevan et al., 1995).

The prospects of isolating new anti-bacterial, -fungal, -tumour compounds, herbicides, or pesticides, has stimulated interest in research of secondary metabolite synthesis. Despite this, little attention has been paid thus far to primary metabolism in actinomycetes and its regulation. Poor growth characteristics and difficulties encountered in growing actinomycetes, have most likely contributed to this neglect. Identification of primary metabolites involved in secondary metabolite biosynthesis and information about the regulation of their synthesis is necessary to further increase the yield of secondary metabolite production.

The first steps in secondary metabolite biosynthesis usually involve a series of very specific enzymatic steps to synthesize a common building block. This compound is then derivatized via nonspecific dehydrogenases, transferases, methyl-transferases, oxidases, or other activities, acting in various combinations or sequences, resulting in an enormous diversity of chemical compounds as end products. The exchange of genes coding for these nonspecific enzymes, via natural recombination or recombinant DNA techniques, indeed has lead to the formation of new hybrid secondary metabolites (metabolic pathway engineering) (Decker et al., 1995; Ghisalba et al., 1984; Hopwood, 1995; McDaniel et al., 1995; Piepersberg, 1994; Vournakis and Elander, 1983).

Mutagenesis and selection of strains overproducing secondary metabolites is necessary to overcome the initially low rate of secondary metabolite synthesis. In the initial strain improvement procedures, the flux of the specific secondary metabolite biosynthetic pathways themselves may be increased in the production strains. Later, the availability of precursors from primary metabolism will be of influence. At this stage one may expect that increasing the flow towards these building blocks will also increase the productivity of secondary metabolite formation.

An interesting group of secondary metabolites containing aromatic rings is derived from intermediates of aromatic amino acid biosynthesis or the aromatic amino acids themselves (Fig. 2). Many of these compounds are clinically important, especially vancomycin and teicoplanin, glycopeptide antibiotics active against the methicillin resistant Staphylococcus aureus (MRSA) (Brogden and Peters, 1994; Spencer and Bryson, 1995).

Knowledge of the biosynthesis and regulation of aromatic amino acids in actinomycetes is limited but will be of importance for further production strain improvement. Aromatic amino acid biosynthesis is generally regulated very well due to
the high energetic costs to synthesize these amino acids. Among the amino acids, aromatic amino acids are the most expensive to synthesize (L-Phe: 65, L-Tyr: 62, and L-Trp: 78 ATP metabolic equivalents, respectively) (Atkinson, 1977).

**THE SHIKIMATE PATHWAY**

Shikimate is an organic acid first isolated from the fruits of *Illicium religiosum* and *Illicium anisatum* where it is present in large quantities (up to 20%) (Eykmann, 1885, 1891). The name shikimate was derived from the Japanese name for the tree *Illicium religiosum*, shikimi-no-ki (anise tree). About 60 years later it was discovered that shikimate was an intermediate in aromatic amino acid biosynthesis in plants and microorganisms (animals cannot carry out de novo synthesis of aromatic amino acids) (Davis, 1955; Gibson and Pittard, 1968; Sprinson, 1961).
Biosynthesis of aromatic amino acids starts with a common pathway, the shikimate pathway, leading to the branchpoint intermediate chorismate (Fig. 3). Chorismate can be converted into anthranilate (L-Trp) or prephenate (L-Phe and L-Tyr). The sequence of enzymatic steps and the intermediates in the shikimate pathway and the L-Trp pathway are consistent throughout nature. The pathways to L-Phe and L-Tyr, however, show some variations. L-Phe is synthesized via either phenylpyruvate and/or arogenate while L-Tyr is synthesized via 4-hydroxyphenylpyruvate and/or arogenate. In some organisms both pathways to L-Phe and L-Tyr are present (see below).

CHEMISTRY OF THE SHIKIMATE PATHWAY

Aromatic amino acids are synthesized from the central metabolites phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P). In the first step of the shikimate pathway E4P and PEP are condensed into 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP). The phosphate group of PEP is released as inorganic phosphate after C-O cleavage leaving an active pyruvyl group which reacts with E4P into DAHP. DAHP is converted into 3-dehydroshikimate via ring closure through formation of a C-C bond between C-2 and C-7 and release of inorganic phosphate. The formation of a double bond in 3-dehydroshikimate via the elimination of water between C-1 and C-2 results in 3-dehydroshikimate. Shikimate is synthesized via the reduction of the C-3 keto-group in 3-dehydroshikimate. A phosphate group is transferred from ATP to the C-3 hydroxyl in the synthesis of shikimate-3-phosphate. A molecule of PEP is transferred to the C-5 position of shikimate-3-phosphate to yield 5-enolpyruvyl-shikimate-3-phosphate (EPSP) and inorganic phosphate via, again a C-O cleavage of PEP and release of inorganic phosphate. This pyruvyl group later will be the sidechain in L-Phe and L-Tyr and will be lost as pyruvate in the formation of anthranilate, the precursor of L-Trp biosynthesis. Chorismate is synthesized from EPSP via elimination of the C-6 hydrogen introducing the second double bond and the release of inorganic phosphate from C-3 (Fig. 3).

ENZYMEOLOGY OF THE SHIKIMATE PATHWAY

The enzymology of the shikimate pathway is covered in a range of excellent reviews which, however, mainly deal with the biosynthesis of aromatic amino acids in Gram-negative bacteria, (mostly *Escherichia coli*), yeast and plants (Bentley, 1990; Braus, 1991; Byng *et al.*, 1982; Camakaris and Pittard, 1983; Dewick, 1989,1995; Ganem, 1978; Garner and Herrmann, 1983; Haslam, 1993; Herrmann, 1983; Jensen *et al.*, 1991; Pittard, 1987).
All the genes coding for enzymes of L-Phe and L-Tyr biosynthesis have been cloned from *E. coli* and their nucleotide sequence determined. From *Bacillus subtilis* and *Saccharomyces cerevisiae* most genes have been cloned and sequenced. In Table 2, a short summary is given about current knowledge of these genes, proteins, and the main effectors of enzyme activity in these organisms.

In yeast and fungi a special arrangement of five of the seven enzymes of the shikimate pathway is present. Five enzymatic activities, converting 3-dehydroquinate into EPSP, are localized on a pentafunctional enzyme, the *arom* protein (Haslam, 1993). Most of the research on the *arom* protein has been carried out in *S. cerevisiae*, *Aspergillus nidulans* and *Neurospora crassa*. The *arom* protein consists of five domains, each showing homology to the individual enzymes of for instance *E. coli* (Coggins et al., 1987b; Duncan et al., 1987). The gene, *aroI*, encoding the *arom* protein can be split into five different genes with the ability to complement corresponding *E. coli* mutants (Hawkins and Smith, 1991). Initially, channelling of substrates to the next active site was thought to be the physiological role of this *arom* protein. In view of the substantial leakage of shikimate pathway intermediates from the protein, this appears less likely (Lamb et al., 1991, 1992). The function of this protein possibly is in the coordinate expression of a single gene instead of five different genes (Duncan et al., 1988).

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**Table 2. Summary of genes and some properties of enzymes in the biosynthesis of aromatic amino acid biosynthesis in *E. coli*, *B. subtilis*, and *S. cerevisiae***

<table>
<thead>
<tr>
<th>Enzyme Gene</th>
<th>Escherichia coli</th>
<th>Bacillus subtilis</th>
<th>Saccharomyces cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS-tyr</td>
<td>aroF</td>
<td>38804</td>
<td>2</td>
</tr>
<tr>
<td>DS-phe</td>
<td>aroG</td>
<td>38009</td>
<td>4</td>
</tr>
<tr>
<td>DS-trp</td>
<td>aroH</td>
<td>38602</td>
<td>2</td>
</tr>
<tr>
<td>DS-cha/ppa</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>DHQS</td>
<td>aroB</td>
<td>38880</td>
<td>1</td>
</tr>
<tr>
<td>DHQD</td>
<td>aroD</td>
<td>26407</td>
<td>2</td>
</tr>
<tr>
<td>SAdh</td>
<td>aroE</td>
<td>29380</td>
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</tr>
<tr>
<td>SKI</td>
<td>aroK</td>
<td>19500</td>
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</tr>
<tr>
<td>SKII</td>
<td>aroL</td>
<td>18937</td>
<td>1</td>
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<tr>
<td>EPSPS</td>
<td>aroA</td>
<td>46112</td>
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</tr>
<tr>
<td>CS</td>
<td>aroC</td>
<td>38183</td>
<td>4</td>
</tr>
<tr>
<td>T-protein</td>
<td>tynA</td>
<td>43111</td>
<td>2</td>
</tr>
<tr>
<td>P-protein</td>
<td>pheA</td>
<td>42042</td>
<td>2</td>
</tr>
<tr>
<td>CM</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>PDT</td>
<td>nd</td>
<td>nd</td>
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</tr>
<tr>
<td>PDH</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>aro AT</td>
<td>tyrB</td>
<td>43537</td>
<td>?</td>
</tr>
</tbody>
</table>

* a deduced from gene sequence; b not detectable; c no information available; d see text. For abbreviations and references, see text.

All the genes coding for enzymes of L-Phe and L-Tyr biosynthesis have been cloned from *E. coli* and their nucleotide sequence determined. From *Bacillus subtilis* and *Saccharomyces cerevisiae* most genes have been cloned and sequenced. In Table 2, a short summary is given about current knowledge of these genes, proteins, and the main effectors of enzyme activity in these organisms.
In the following, current knowledge about the individual enzyme steps in aromatic amino acid biosynthesis will be briefly reviewed. Where possible, available information about Gram-positive bacteria and in particular actinomycete enzymes will be highlighted.

3-Deoxy-d-arabino-heptulosonate 7-phosphate synthase

The first enzyme in the shikimate pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DS), converts PEP and E4P into DAHP. DS requires the presence of divalent metal ions for activity (Fe, Co, or Mn). DS proteins possess the largest variety of allosteric regulatory patterns thus far described for any protein (Bentley, 1990). They can be divided into five major isoenzymes based on their sensitivity towards feedback inhibition by L-Phe (DS-phe), L-Tyr (DS-tyr), L-Trp (DS-trp), chorismate and prephenate (DS-cha/ppa), or no feedback inhibition (DS-o). Activity is usually inhibited by a single aromatic amino acid or by a combination of L-Phe, L-Tyr, or L-Trp. In addition, DS activity is inhibited by phenylpyruvate or 4-hydroxyphenylpyruvate in some organisms.

In *E. coli* three different DS isoenzymes are present. Each isoenzyme is effectively feedback inhibited by a single aromatic amino acid. The DS isoenzymes are not expressed equally. The ratio of the activities of DS-phe, DS-tyr, and DS-trp is approximately 75:25:<1 and is regulated via repression by the corresponding aromatic amino acid. The three proteins are 41% identical, suggesting a common evolutionary origin. DS-phe is considered evolved recently because it is present in the enteric lineage of the Gram-negative Superfamily B only (Ahmad and Jensen, 1989; Ahmad *et al.*, 1986). Feedback inhibition of DS activity is a major control point for the carbon flow through the shikimate pathway in *E. coli* (Ogino *et al.*, 1982).

In *S. cerevisiae* two DS proteins are present, DS-phe and DS-tyr encoded by *aro3* and *aro4*, respectively. Both proteins have a high degree of similarity with the *E. coli* isoenzymes. DS-phe and DS-tyr are also present in the yeasts *Candida maltosa* and *Hansenula henricii* and in the fungus *N. crassa* an additional DS-trp is present (Bentley, 1990).

The *B. subtilis* and *Bacillus licheniformis* DS enzymes are inhibited by chorismate and prephenate (Jensen and Nester, 1966a, 1966b; Nasser *et al.*, 1969). In *B. subtilis* strain 168 both DS and chorismate mutase (CM) activity are located on a bifunctional protein. Both the *B. subtilis* bifunctional and the *B. licheniformis* monofunctional DS proteins are tetramers of M₈ 180,000. CM activity on the bifunctional DS-CM protein arose from a mutation in the prephenate feedback inhibition binding site to allow also binding of chorismate and preferentially the transition state analog, thereby converting chorismate into prephenate (Llewellyn *et al.*, 1980). The gene, *aroA-G*, encoding the bifunctional DS-CM from *B. subtilis* 168 encodes a protein with a M₈ of 39,539 (Bolotin *et al.*, 1992).
In *Brevibacterium flavum* DS activity is synergistically inhibited by L-Phe and L-Tyr and forms a bifunctional complex with CM. Activity of DS, a tetramer with a subunit Mr of 55,000 is stimulated by the CM component (Shiio and Sugimoto, 1979; Shiio et al., 1974).

In *Corynebacterium glutamicum* DS activity is synergistically inhibited by L-Phe and L-Tyr and the inhibition is enhanced by L-Trp (Hagino and Nakayama, 1974b; Shiio et al., 1974). The gene for *C. glutamicum* DS has been cloned and the deduced protein was 53% identical with the *E. coli* DS-phe protein and 48% and 47% with DS-trp and DS-tyr proteins, respectively (Chen et al., 1993).

DS proteins have been characterized from a few actinomycetes only. In *Streptomyces rimosus* (Stuart and Hunter, 1993), *Streptomyces coelicolor* (Walker, 1991), *Streptomyces antibioticus* (Murphy and Katz, 1980) and *Streptomyces aureofaciens* (Görisch and Lingens, 1971) a single DS is present that is inhibited by L-Trp only. No evidence for additional DS activities in these bacteria was found. The DS activity of *Amycolatopsis (Nocardia) mediterranei* was weakly inhibited by chorismate and by a combination of L-Phe, L-Tyr, and L-Trp (main effector) (Gygax et al., 1982). In *Amycolatopsis methanolica* (*Nocardia* sp. 239) a single DS protein was reported, cumulatively inhibited by L-Phe, L-Tyr, and L-Trp (main effector) (De Boer et al., 1989).

### 3-Dehydroquinate synthase

Conversion of DAHP into 3-dehydroquinate by 3-dehydroquinate synthase (DHQS) requires specific oxidation, β-elimination, reduction, and an intramolecular aldol condensation. All these steps are catalysed by a single enzyme, 3-dehydroquinate dehydratase. It requires the presence of catalytic amounts of NAD⁺, and Co²⁺ or Zn²⁺ ions for activity (Bender et al., 1989). The impressive number of reactions mentioned above appears to be the result of several kinetically feasible and thermodynamically favourable processes. In reality, the enzyme may be no more than a simple dehydrogenase with an enzyme bound NAD⁺ and a divalent metal ion required for activity (Haslam, 1993).

Only very little information is available about this protein in actinomycetes and other Gram-positive bacteria. The gene coding for 3-dehydroquinate synthase has been cloned from *Mycobacterium tuberculosis* revealing high similarity with 3-dehydroquinate synthase proteins from *E. coli*, *A. nidulans*, and *S. cerevisiae*. The Mr of the *M. tuberculosis* 3-dehydroquinate synthase is 38,000 (Garbe et al., 1991). It was found immediately upstream of the gene coding for 3-dehydroquinate dehydratase, the next enzymatic step in the shikimate pathway (Garbe et al., 1991). Also in *S. coelicolor* an open reading frame, immediately upstream of the 3-dehydroquinate dehydratase gene,
encoded a protein showing high homology with 3-dehydroquininate synthase (Hunter, 1992).

In *B. subtilis*, 3-dehydroquininate synthase was associated with chorismate synthase and NADPH-dependent flavin reductase. Activity of 3-dehydroquininate synthase was completely dependent on the presence of chorismate synthase (Hasan and Nester, 1978). The gene, *aroF*, encoding chorismate synthase was found immediately upstream of *aroB*, the gene encoding 3-dehydroquininate synthase. Both genes are part of the supra operon *aroFBH-trpEDCFBA-hisH-tyrA-aroE* (Babitzke *et al.*, 1992).

### 3-Dehydroquininate dehydratase

3-Dehydroquininate dehydratase (DHQD) catalyses the formation of 3-dehydroshikimate from 3-dehydroquininate. Two forms of 3-dehydroquininate dehydratase are known: type I, a biosynthetic enzyme present in for instance *E. coli*, and type II, a catabolic enzyme usually present in organisms growing on quinate as a carbon source. Quinate catabolism involves the enzymes quinate dehydrogenase, 3-dehydroquininate dehydratase, and 3-dehydroshikimate dehydratase (Fig. 3). The catabolic type II enzyme can easily be distinguished from the biosynthetic type I variant. Type II enzymes are thermostable and insensitive towards inactivation by NaBH₄ in the presence of 3-dehydroquininate (Kleanthous *et al.*, 1992).

The *B. subtilis* 3-dehydroquininate dehydratase is probably a type I enzyme, as judged from high sequence similarity with the *E. coli* type I enzyme (Yamamoto *et al.*, 1991).

The type II 3-dehydroquininate dehydratase enzymes of for instance *A. nidulans* (Kleanthous *et al.*, 1992) and *N. crassa* (Hawkins *et al.*, 1982) are dodecameric proteins. They are induced in the presence of quinate and required for quinate catabolism. An additional type I 3-dehydroquininate dehydratase in these organisms is present as a part of the pentafunctional *arom* protein. Some genes encoding type II 3-dehydroquininate dehydratase proteins have been cloned and the deduced proteins have no homology with type I enzymes. The subunits of type II enzymes have a Mr of about 16,000, smaller than the subunits of type I enzymes (Table 2).

Not only in fungi but also in bacteria, type I and type II enzymes may be present simultaneously. In *Acinetobacter calcoaceticus* (Ingledew *et al.*, 1971), *Pseudomonas aeruginosa* (Ingledew and Tai, 1972), and in various actinomycetes (Cain, 1981), both type I and type II 3-dehydroquininate dehydratases are present in quinate grown cells.

Type II enzymes (but not type I enzymes) are present in *M. tuberculosis* (Garbe *et al.*, 1991), *S. coelicolor* (White *et al.*, 1990) and most likely also in various other actinomycetes (Cain, 1981). Although these organisms possess a type II enzyme, they are unable to grow on quinate. The enzyme, therefore, functions in aromatic amino acid biosynthesis only. The *M. tuberculosis* 3-dehydroquininate dehydratase has been
overexpressed and subsequently purified. Like other type II enzymes it is a dodecameric thermostable protein (Moore et al., 1992). Crystals of the enzyme have been obtained and attempts to determine its 3-dimensional structure are in progress (Gourley et al., 1994).

Type I enzymes are inhibited by NaBH₄ in the presence of 3-dehydroquinate. An imine intermediate is formed between the active-site lysine and 3-dehydroquinate, resulting in a Schiff’s base that can be reduced in a stable substrate-protein complex by NaBH₄ (Shneier et al., 1991). Type II enzymes cannot be inactivated in this way because a different mechanism is used for the conversion of 3-dehydroquinate in which lysine does not play a role (Garbe et al., 1991; Harris et al., 1993; Kleanthous et al., 1992; Shneier et al., 1993). Inhibition studies with the arginine modifier phenylglyoxal identified an arginine residue in the N-terminus of the type II 3-dehydroquinate dehydratase protein that is essential for its activity (Krell et al., 1995). A preliminary reaction mechanism based on the involvement of this arginine has been proposed (Krell et al., 1995). An interesting aspect of type I and type II 3-dehydroquinate dehydratases thus is that two different enzymes use different mechanisms (opposite stereo chemistry) for the same conversion (Shneier et al., 1993).

**Shikimate dehydrogenase**

Shikimate dehydrogenase (SAdeh) catalyses the reduction of 3-dehydroshikimate. Three types of shikimate dehydrogenase enzymes can be distinguished depending on the involvement of either NAD⁺, NADP⁺, or pyrroloquinoline quinone (PQQ).

In all organisms studied the biosynthetic shikimate dehydrogenase enzyme is specific for NADPH and 3-dehydroshikimate.

The NAD⁺-specific shikimate dehydrogenase is the first enzyme in shikimate catabolism; it is also referred to as quinate dehydrogenase in view of its ability to convert quinate into 3-dehydroquinate (Cain, 1972a). NAD⁺-dependent shikimate dehydrogenases from *Rhodococcus rhodochrous* (Bruce and Cain, 1990), *N. crassa* (Barea and Giles, 1978), and *A. nidulans* (Hawkins et al., 1988) are all monomeric proteins of approximately Mᵋ 40,000 and able to function as quinate dehydrogenases. Significant sequence homology exists between the NAD⁺- and the NADP⁺-dependent shikimate dehydrogenases, including the shikimate dehydrogenase domain from the *arom* protein (Anton and Coggins, 1988).

A catabolic PQQ-dependent shikimate/quinate dehydrogenase has been characterized (Van Kleef and Duine, 1988) and cloned from *A. calcoaceticus* (Elsemore and Ornston, 1994). No homology was found between the PQQ- and the NAD(P)⁺-dependent shikimate dehydrogenase proteins. Other PQQ-dependent quinate/shikimate dehydrogenases have not been described thus far.
Shikimate kinase

Shikimate kinase (SK) catalyses the formation of shikimate-3-phosphate from shikimate and ATP. In *E. coli* two shikimate kinases are present (Table 2). The higher affinity of shikimate kinase II for shikimate and ATP suggested that it normally functions in aromatic amino acid biosynthesis (DeFeyter and Pittard, 1986). Both shikimate kinase I and II activity of *E. coli* are not feedback inhibited. Recent studies with *E. coli aroK* showed that it is transcribed differently than originally reported. The new predicted M_r of shikimate kinase I is now in accordance with the M_r of purified shikimate kinase I and the protein is more homologous to other shikimate kinase enzymes (Griffin and Gasson, 1995; Whipp and Pittard, 1995).

In *B. subtilis* 168 shikimate kinase is active in a trifunctional complex with the bifunctional DS-CM protein and is inhibited by chorismate and prephenate (Nakatsukasa and Nester, 1972; Nasser et al., 1969). Such a complex was not present in *B. licheniformis* indicating that this is not a common feature in *Bacillus* spp. (Nasser et al., 1969). The activity of shikimate kinase in *B. licheniformis* was feedback inhibited by chorismate only (Nasser et al., 1969). The gene, *aroI*, encoding shikimate kinase has been cloned from *B. subtilis*; the deduced protein has 59% similarity with *E. coli* shikimate kinase II (Nakane et al., 1994).

The shikimate kinase gene has also been cloned from *Brevibacterium lactofermentum* and has been found in a cluster with the genes encoding 3-dehydroquinate synthase and shikimate dehydrogenase but no sequence information is available yet (Matsui et al., 1988). Activity of shikimate kinase was found to be limiting in *B. lactofermentum* strains overproducing L-Tyr because an increase in its gene dosage stimulated/enhanced L-Tyr production (Ito et al., 1990).

5-Enolpyruvyl-shikimate-3-phosphate synthase

5-Enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) catalyses the formation of EPSP from shikimate-3-phosphate and PEP. Only a few reports have been published describing EPSP synthase of Gram-positive bacteria. The *aroA* gene encoding EPSP synthase has been cloned from *M. tuberculosis* and the predicted protein was shown to be structurally related with EPSP synthases from other sources (Garbe et al., 1990). The *B. subtilis* gene, *aroE*, encoding EPSP synthase has been cloned, sequenced and overexpressed. Kinetic studies together with site-directed mutagenesis showed that the enzyme has allosteric behaviour towards both substrates, a feature not observed with other known EPSP synthases. Another characteristic was its ability to form several multimers resulting in several bands upon native gel electrophoresis and activity staining (Majumder et al., 1995). The activity of EPSP synthase from *B. subtilis* was inhibited by glyphosate
(N-(phosphonomethyl)glycine, a broad spectrum herbicide) *in vivo* and *in vitro* (Fischer *et al.*, 1986). Other bacterial and plant EPSP synthases are also inhibited by glyphosate (Amrhein *et al.*, 1983) but the enzyme of most *Pseudomonas* species was remarkably glyphosate resistant (Schulz *et al.*, 1985). Resistance against glyphosate has been found to correlate with increased synthesis of a sensitive EPSP synthase enzyme or the synthesis of a mutated, insensitive EPSP synthase (Reinbothe *et al.*, 1991). Cloning of a gene conferring increased glyphosate resistance in *Pseudomonas* sp. did not complement an *E. coli* strain with an inactivated EPSP synthase. Apparently a desensitized mutated EPSP synthase is not the only mechanism for glyphosate resistance. Other mechanism may involve increased export or even degradation of glyphosate (Fitzgibbon and Braymer, 1990).

Genetically defined *aroA* mutants of (mycobacterial) pathogens may be constructed via gene replacement. These weakened strains may be used for raising vaccines (Garbe *et al.*, 1990).

**Chorismate synthase**

Chorismate synthase (CS) catalyses the conversion of EPSP into chorismate, the end product of the shikimate pathway. The enzyme requires reduced FMN or FAD but their role in catalysis is currently unknown (Izumi *et al.*, 1993; Ramjee *et al.*, 1991, 1992).

The *N. crassa* chorismate synthase is a bifunctional enzyme and is a tetramer with a subunit Mr of 50,000, larger than the tetrameric *E. coli* enzyme (Table 2). It has been proposed that the difference in Mr was due to the presence of a diaphorase domain on the *N. crassa* chorismate synthase. In another eukaryote, *S. cerevisiae*, chorismate synthase is a monofunctional enzyme similar to the *E. coli* enzyme (Jones *et al.*, 1991).

Chorismate synthase has been purified from *B. subtilis* and was present in a complex with NADPH-dependent flavin reductase and 3-dehydroquinate synthase (Hasan and Nester, 1978). The role of the flavin reductase may be in providing reduced FAD or FMN for the chorismate synthase reaction. The presence of 3-dehydroquinate synthase in the complex was not required for chorismate synthase activity (Hasan and Nester, 1978; Haslam, 1993). 3-Dehydroquinate synthase activity, however, was dependent on the presence of chorismate synthase (Hasan and Nester, 1978). The genes *aroB* and *aroF*, encoding 3-dehydroquinate synthase and chorismate synthase respectively, were both present in the supra operon *aroFBH-trpEDCFBA-hisH-tyrA-aroE*. The NADPH-dependent flavin reductase was not present in the same operon (Babitzke *et al.*, 1992).
L-PHE AND L-TYR BIOSYNTHETIC PATHWAYS

The formation of L-Phe or L-Tyr from chorismate involves three enzymatic steps in both cases (Fig. 4). Chorismate is converted into prephenate via an intramolecular rearrangement of the pyruvyl sidechain at C-3 to C-1 via the formation of a new bond between C-9 and C-1. Prephenate can be converted into phenylpyruvate, 4-hydroxyphenylpyruvate or into L-arogenate. The formation of L-arogenate is a classical amino transfer of the amino group from L-glutamate to the keto group of prephenate. Conversion of prephenate into phenylpyruvate involves decarboxylation of the C-1 carboxyl group and dehydration of the C-4 hydroxyl resulting in aromatization of the cyclohexadiene. Formation of 4-hydroxyphenylpyruvate involves the same decarboxylation but oxidation results in aromatization, keeping the C-4 hydroxyl unchanged. L-Arogenate can undergo the same reactions as prephenate. Dehydration and decarboxylation of L-arogenate will yield L-Phe and oxidative decarboxylation L-Tyr. Transfer of the amino group from L-glutamate to the keto group of phenylpyruvate or 4-hydroxyphenylpyruvate will yield L-Phe or L-Tyr, respectively.

ENZYMEOLOGY OF L-PHE AND L-TYR BIOSYNTHESIS

Chorismate mutase

Chorismate mutase catalyses the conversion of chorismate into prephenate. Chorismate mutase enzymes occur either in a free form (CM-F) or associated with other proteins (Bentley, 1990). Chorismate mutase may be associated with prephenate dehydratase (P-protein), prephenate dehydrogenase (T-protein) or DS (DS-CM) (see above) (Bentley, 1990). The bifunctional *E. coli* P- and T-proteins possess separate domains for each activity (Baldwin and Davidson, 1981; Turnbull and Morrison, 1990). From the bifunctional proteins, active, monofunctional proteins can be constructed (Stewart *et al.*, 1990; Xia *et al.*, 1992a). The prephenate dehydrogenase domain of the T-protein can convert both prephenate and arogenate and is therefore referred to as a cyclohexadienyl dehydrogenase (Ahmad and Jensen, 1987). The P-protein is present in superfamilies A and B of the Gram-negative bacteria whereas the T-protein has been detected in the enteric lineage of superfamily A only (Jensen and Ahmad, 1991). CM-F is present in several Gram-negative bacteria in addition to P-and/or T-proteins (Ahmad *et al.*, 1990; Bonner *et al.*, 1990; Patel *et al.*, 1977; Xia and Jensen, 1992). This rules out the possibility that the T-protein originates from a gene fusion between the genes encoding CM-F and cyclohexadienyl dehydrogenase. Surprisingly, the *Erwinia herbicola aroQ* gene product, CM-F, was located in the periplasmic space with a yet unknown function;
its activity was not feedback inhibited by aromatic amino acids. The AroQ protein is a positively charged dimer with a subunit Mr of 18,000 and has low homology with other chorismate mutase enzymes (Xia et al., 1993).

A single, monofunctional chorismate mutase enzyme has been detected in virtually all tested yeast species and it is exclusively located in the cytosol. Activity of the chorismate mutase enzyme is regulated via activation by L-Trp and feedback inhibition by L-Tyr and/or L-Phe (Bode and Birnbaum, 1991). The best characterized enzyme from eukaryotes is the chorismate mutase from S. cerevisiae. The enzyme is activated by L-Trp and inhibited by L-Tyr and does not show homology with bacterial chorismate mutases (Ramilo et al., 1993; Schmidheini et al., 1989). A single amino acid substitution resulted in a permanently activated, feedback inhibition resistant, chorismate mutase (Graf et al., 1995; Schmidheini et al., 1989, 1990). The crystal structure of this mutated chorismate mutase was determined and no structural homology was found with the monofunctional

**Figure 4.** Chorismate and beyond- primary metabolic pathways. 1, chorismate mutase; 2, prephenate/cyclohexadienyl dehydratase; 3, prephenate/cyclohexadienyl dehydrogenase (NAD(P)⁺); 4, arogenate/cyclohexadienyl dehydrogenase (NAD(P)⁺); 5, prephenate/aromatic amino acid aminotransferase; 6, arogenate/cyclohexadienyl dehydratase.
chorismate mutase from *B. subtilis* (Xue and Lipscomb, 1994; Xue et al., 1994). In the yeast *Rhodotorula glutinis* two chorismate mutase enzymes were detected; one of these is feedback inhibited by L-Tyr and activated by L-Trp while the other one is insensitive towards feedback inhibition or activation (Fiske and Kane, 1984).

The *B. subtilis* monofunctional chorismate mutase is the best studied chorismate mutase protein from Gram-positive organisms thus far (Chook et al., 1994; Gray et al., 1990a; Gray and Knowles, 1994; Rajagopalan et al., 1993). Two chorismate mutase enzymes are present in *B. subtilis*; a bifunctional DS-CM protein that forms a trifunctional complex with shikimate kinase (see above) and a monofunctional protein that is believed to be the natural chorismate mutase (Kurahashi et al., 1987). The chorismate mutase activity of the DS-CM protein may be the result of a mutation in the chorismate regulatory site of DS (see above). The enzyme has been crystallized and its structure was determined (Chook et al., 1994). Both the monofunctional and the bifunctional chorismate mutase are not feedback inhibited by L-Phe or L-Tyr (Gray et al., 1990b; Lorence and Nester, 1967). In *B. licheniformis* two chorismate mutase enzymes were detected but no complex formation with DS and shikimate kinase as in *B. subtilis* was observed with either chorismate mutase (Nasser et al., 1969).

The *A. mediterranei* chorismate mutase is feedback inhibited by L-Phe and L-Tyr and slightly activated by L-Trp (Xia and Chiao, 1989). Also in *A. methanolica* inhibition by L-Phe and L-Tyr was observed (De Boer et al., 1989). In some other species of the *Actinomycetales* chorismate mutase activity is inhibited by L-Tyr and (partly) by L-Trp. L-Phe had an inhibitory effect on chorismate mutase activity in *Dactylosporangium thailandensis* only. In six out of thirteen actinomycete species tested, chorismate mutase activity was activated by L-Phe (Hund et al., 1987). In *Microtetraspora glauca* chorismate mutase was activated by L-Phe and feedback inhibited by L-Tyr and L-Trp (Speth et al., 1989). Chorismate mutase has been purified from *S. aureofaciens*; the protein is a trimer with a subunit Mr of 14,300. Activity was not inhibited by aromatic amino acids or by shikimate, anthranilate, phenylpyruvate, or 4-hydroxyphenylpyruvate. The protein was not associated with any other protein (Görisch, 1987; Görisch and Lingens, 1974). Chorismate mutase in the chloramphenicol producing strain *Streptomyces* sp. 3022a is a protein with a Mr of 75,000 and not feedback inhibited by the end products or intermediates of the shikimate pathway (Lowe and Westlake, 1972).

In *B. flavum* chorismate mutase is present in a complex with DS (Shiio and Sugimoto, 1979). Chorismate mutase activity of *B. flavum* (and also of *C. glutamicum*) is inhibited by L-Phe or L-Tyr and the inhibition is released by L-Trp (Hagino and Nakayama, 1975; Sugimoto and Shiio, 1980b). The chorismate mutase of *B. flavum* is a dimer with subunit Mr of 13,500 and active in the presence of DS only (Sugimoto and Shiio, 1980b).
Prephenate dehydratase

Prephenate dehydratase (PDT) catalyses the formation of phenylpyruvate from prephenate. In all members of superfamily A and B of the Gram-negative bacteria prephenate dehydratase is associated with chorismate mutase in a bifunctional P-protein (see above).

In *Ps. aeruginosa* (Fiske et al., 1983) and *E. herbicola* (Xia et al., 1991) a second monofunctional prephenate dehydratase was detected. The enzymes differed in their substrate specificity from the prephenate dehydratase component of the P-protein. Not only prephenate but also arogenate could be used as a substrate. Prephenate dehydratases that can use both prephenate and arogenate as substrates are referred to as cyclohexadienyl dehydratases. The *Ps. aeruginosa* gene *pheC* encoding cyclohexadienyl dehydratase has been cloned and sequenced. The calculated subunit Mr was 30,480 and the native protein is a dimer (Zhao et al., 1992). Localization studies showed that cyclohexadienyl dehydratase is present in the periplasmic space (Zhao et al., 1993). In other Gram-negative bacteria, e.g. *E. herbicola*, cyclohexadienyl dehydratase is also present in the periplasmic space (Zhao et al., 1993). Together with the periplasmic chorismate mutase (see above), a functional pathway from chorismate to phenylpyruvate exists in the periplasm (Xia et al., 1993; Zhao et al., 1993). A possible function may be to scavenge L-arogenate from plants since especially members of the plant pathogens, *Klebsiella*, *Erwinia* and *Serratia* possess the periplasmic cyclohexadienyl dehydratase (Zhao et al., 1993). Recently it was shown that this cyclohexadienyl dehydratase displays homology with periplasmic receptor proteins for polar amino acids. These receptor proteins may have a function in chemotaxis (Tam and Saier, 1993). The function for chorismate to phenylpyruvate conversion in the periplasmic space is currently not known (Xia et al., 1993).

Cyclohexadienyl dehydratase or P-proteins have never been detected in Gram-positive bacteria; only monofunctional, prephenate specific dehydratase enzymes have been reported. In *B. subtilis* a monofunctional prephenate dehydratase exists which has some unusual regulatory properties. The enzyme is inhibited by L-Phe and L-Trp and activated by L-Met and L-Leu. Positive regulators force the protein in a hyperactive, octameric structure while the negative effectors promote the formation of a dimeric, less active, structure (Fischer and Jensen, 1987a; Pierson and Jensen, 1974; Riepl and Glover, 1979). Activation by L-Met and L-Leu may contribute to a balanced synthesis of these hydrophobic amino acids, thereby optimizing the process of protein synthesis (Pierson and Jensen, 1974).

The prephenate dehydratase encoding gene (*pheA*) of *B. subtilis* has been cloned and sequenced, revealing high homology with prephenate dehydratase from *C. glutamicum* and the C-terminus of the P-protein from *E. coli* (Trach and Hoch, 1989). Prephenate dehydratase from both *C. glutamicum* and *B. flavum* are feedback inhibited by L-Phe and
L-Trp and activated by L-Tyr. Inhibition or activation did not alter the Mₚ of these enzymes, differing from the situation in *B. subtilis* (Fazel and Jensen, 1980). Hydrophobic amino acids activated prephenate dehydratase of *C. glutamicum* only at relatively high concentrations (Hagino and Nakayama, 1974a). In *B. flavum* these amino acids did not affect activity (Sugimoto and Shiio, 1974). The *pheA* gene, of *C. glutamicum* has been cloned and sequenced. The predicted protein has a Mₚ of 33,740 (Follettie and Sinskey, 1986). Considering the Mₚ of 260,000, an octamer is the most likely quaternary structure (Fazel and Jensen, 1980).

Prephenate dehydratase in sporeforming members of the *Actinomycetales* is feedback inhibited by L-Phe and activated by L-Tyr. In some members, prephenate dehydratase activity is inhibited not only by L-Phe but also by L-Tyr (Hund et al., 1987). In *A. mediterranei* prephenate dehydratase activity was inhibited by L-Phe and activated by L-Tyr (Xia and Chiao, 1989). A similar situation was found in *A. methanolica* (De Boer et al., 1989). Prephenate dehydratase from *Micromonospora glauca* was partially purified as a protein with a Mₚ of 110,000, inhibited by L-Phe and by the remote effectors L-Tyr and L-Trp (Speth et al., 1989). The *Streptomyces 3022a* prephenate dehydratase has a Mₚ of 220,000 and its activity is inhibited by L-Phe only (Lowe and Westlake, 1972). In other *Streptomyces*, prephenate dehydratase is feedback inhibited by L-Phe only although some species did not show any inhibition or activation in the presence of L-Phe or L-Tyr (Keller et al., 1983).

### Prephenate dehydrogenase

Prephenate dehydrogenase (PDH) catalyses the formation of 4-hydroxyphenylpyruvate from prephenate. As is the case for prephenate dehydratase, prephenate dehydrogenase can exist in three different forms, namely together with chorismate mutase in a bifunctional T-protein (see above), as a monofunctional, prephenate specific dehydrogenase, or as cyclohexadienyl dehydrogenase.

The prephenate dehydrogenase enzymes from the yeasts *Pichia guilliermondii* (Koll et al., 1988), *C. maltosa* (Bode and Birnbaum, 1991), and *S. cerevisiae* (Jones and Fink, 1982) are inhibited by L-Tyr. In *C. maltosa* and in *Neurospora* prephenate dehydrogenase activity is activated by L-Trp (Bode et al., 1984) or by L-Phe, respectively (Catcheside, 1969).

Most cyclohexadienyl dehydrogenases are specific for NAD⁺ (Ahmad et al., 1990; Patel et al., 1977; Wood and Ganem, 1990; Zhao et al., 1993) and sensitive to feedback inhibition by L-Tyr (Byng et al., 1980; Patel et al., 1977; Turnbull et al., 1991). An allosterically insensitive, NAD⁺-specific, cyclohexadienyl dehydrogenase has been characterized from *Zymomonas mobilis* (Zhao et al., 1993) and from several *Pseudomonas* species (Subramaniam et al., 1994).
In Gram-positive bacteria either an arogenate dehydrogenase (see below) or prephenate dehydrogenase is present. Both enzymes generally are specific for L-arogenate or prephenate, respectively, which leads to the conclusion that cyclohexadienyl dehydrogenases are not present in Gram-positive bacteria. Thus far, the only exception may be found in *Microtetraspora glauca*, from which a prephenate dehydrogenase has been partially purified that coeluted with arogenate dehydrogenase activity. Both activities were specific for NAD+ and are feedback inhibited to the same extent by L-Tyr and 4-hydroxymercuribenzoate. These results suggest that this enzyme is a cyclohexadienyl dehydrogenase (Speth et al., 1989).

Prephenate dehydrogenase activity is present in *B. subtilis* and activity is feedback inhibited by L-Tyr (Fischer and Jensen, 1987b). The gene encoding prephenate dehydrogenase (*tyrA*) has been cloned and was part of the supra operon *aroFBH-trpEDCFBA-hisH-tyrA-aroE* (Babitzke et al., 1992).

In *Streptomyces refuineus*, an L-Phe activated NAD+-dependent prephenate dehydrogenase activity was detected but no feedback inhibition by L-Tyr was found (Speedie and Park, 1980). In *A. mediterranei* prephenate dehydrogenase activity was not regulated (Xia and Chiao, 1989). In *Nocardia* sp. 239 prephenate dehydrogenase activity was feedback inhibited by both L-Phe and L-Tyr (De Boer et al., 1989). Although prephenate dehydrogenase was detected in *C. glutamicum*, as judged from prephenate dependent NAD(P)H formation (Hagino and Nakayama, 1974a). Fazel and Jensen (1979b) reported that this was based on an assay artefact. The combination of prephenate, glutamate (present in high concentrations in crude extracts), NAD(P)+, prephenate aminotransferase and arogenate dehydrogenase also results in prephenate dependent NAD(P)H formation.

**Arogenate dehydrogenase**

Arogenate dehydrogenase catalyses the formation of L-Tyr from arogenate. The enzyme has been detected in a variety of members of the *Actinomycetales*. NAD+ is the preferred coenzyme but sometimes NADP+ is also used but with lower efficiencies (Hund et al., 1987; Keller et al., 1983). In all but one *Streptomyces* species tested, L-Tyr acts as a feedback inhibitor and in a single case L-Phe is an activator of enzyme activity (Keller et al., 1983). In other actinomycetes tested, arogenate dehydrogenase activity was not feedback inhibited by L-Tyr (Hund et al., 1987).

Arogenate dehydrogenase enzymes have been purified from *Actinoplanes missouriensis* and *Streptomyces phaeochromogenes*. Both enzymes are dimers with a subunit Mr of 36,000 and 28,100, respectively, and specific for NAD+ and arogenate. Activity is not feedback inhibited by L-Tyr or any other intermediate in aromatic amino acid biosynthesis (Hund et al., 1989; Keller et al., 1985).
Aromatic amino acid aminotransferase

Aromatic aminotransferase catalyses the formation of L-Phe, L-Tyr or arogenate from respectively phenylpyruvate, 4-hydroxyphenylpyruvate, or prephenate in the presence of an appropriate amino donor. Pyridoxal 5'-phosphate is used as a cofactor and is frequently bound to the enzyme. Aromatic amino acid aminotransferases usually employ broad substrate specificities resulting in the presence of several isoenzymes.

In *E. coli* three enzymes capable of transaminating L-Phe, L-Tyr (Pittard, 1987), and prephenate were detected (Bonner et al., 1990). These isoenzymes (aromatic aminotransferase, aspartate aminotransferase, and branched-chain aminotransferase) are not only functional *in vitro* but also *in vivo*. Mutants blocked in aromatic aminotransferase still behaved as if they were prototrophs. Only mutants blocked in aspartate aminotransferase, aromatic aminotransferase plus branched-chain aminotransferase were auxotrophic for L-Phe and L-Tyr (Gelfand and Steinberg, 1977). In the nitrogen fixing plant symbionts *Rhizobium meliloti* and *Rhizobium leguminosarum* biovar *trifolii* multiple aromatic aminotransferases were present (Kittell et al., 1989; Perezgaldona et al., 1992). Inducible aminotransferases play a role in the catabolism of aromatic amino acids in *Pseudomonas putida* (Ziehr and Kula, 1985). In *B. subtilis* four aromatic amino acid aminotransferases were detected. Two enzymes were active with L-Phe and L-Tyr only, whereas the other two were also active with histidinol phosphate and L-Asp (Mavrides and Comerton, 1978). Histidinol phosphate aminotransferase, active with aromatic amino acids, was also present in *Z. mobilis* (Gu et al., 1995).

The thermophilic *Bacillus* IS1 contained a single, monomeric, thermostable aromatic aminotransferase, *M* of 45,000, active with L-Tyr and L-Phe but not with L-Asp (Schutten et al., 1987). Thermostable aromatic aminotransferases have also been characterized from the hyperthermophilic archaea *Thermococcus litoralis* (Andreotti et al., 1994) and *Pyrococcus furiosus* (Andreotti et al., 1995). Both organisms contain two aromatic aminotransferases that were specific for L-Phe, L-Tyr, and L-Trp. The aminotransferases from *T. litoralis* were active as dimers with a subunit *M* of 47,000 and 45,000, respectively. *P. furiosus* are ATI has been purified and characterized as a dimer with a subunit *M* of 44,000. N-Terminal amino acid sequence analysis revealed that the *P. furiosus* are ATI is similar to are ATI from *T. litoralis*. In the archaeon *Methanococcus aeolicus* two aromatic aminotransferases were detected; one of these aromatic aminotransferases coeluted with the branched-chain aminotransferase activity in gel filtration experiments (Xing and Whitman, 1992).

In the coryneform bacteria *C. glutamicum* and *B. flavum* two aromatic amino transferases also active with L-Asp and branched-chain amino acids were detected. Both enzymes were most active with prephenate and glutamate. Surprisingly, L-Phe biosynthesis seems to be dependent on the availability of L-Leu as donor in the
aminotransferase reaction whereas prephenate aminotransferase was most active with L-Glu as donor (Fazel and Jensen, 1979a; Shiio et al., 1982). From *Brevibacterium lines* an inducible aromatic aminotransferase was characterized as a key enzyme in nitrogen metabolism (Lee and Desmazeaud, 1985a, 1985b). Two other constitutive aromatic aminotransferases were also present, one of which was characterized as aspartate aminotransferase (Asano et al., 1987).

In *S. cerevisiae* L-Trp and other aromatic amino acids are degraded via an inducible aromatic aminotransferase. The single constitutive aromatic aminotransferase present in *S. cerevisiae* has most likely a role in L-Phe and L-Tyr biosynthesis (Kradolfer et al., 1982).

**Arogenate dehydratase**

Arogenate dehydratase catalyses the formation of L-Phe from arogenate. Arogenate dehydratases were reported only to be present in Gram-negative bacteria and in eukaryotes. The enzyme is specific for its substrate and is feedback inhibited by L-Phe (Zamir et al., 1985).

In the eukaryotic algae *Euglena gracilis* arogenate dehydratase is exclusively involved in the biosynthesis of L-Phe (Byng et al., 1981).

In the Gram-negative *Pseudomonas diminuta* the rare situation exists that the P-Protein is absent and L-Phe biosynthesis proceeds exclusively via arogenate and not via phenylpyruvate (Whitaker et al., 1981).

Arogenate dehydratase was reported to be present in the actinomycetes *Micromonospora echinospora* and *Dactylosporangium thailandesis*. Both organisms, however, also possessed prephenate dehydratase activity, indicating dual pathways towards L-Phe or the presence of a cyclohexadienyl dehydratase (Hund et al., 1987).

**L-TRP BIOSYNTHESIS AND OTHER METABOLITES FROM CHORISMATE**

L-Trp is synthesized from chorismate in five enzymatic steps that are invariant in those organisms that have been studied to date. Variation is found in the organisation and regulation of the genes and enzymes in the pathway. A discussion of L-Trp biosynthesis in various organisms is beyond the scope of this introduction and for a recent review the reader is referred to Haslam (1993) and references therein.

Chorismate is not only the precursor for aromatic amino acids but also for several other metabolites (Figs. 3, 4). 4-Aminobenzoate and 4-hydroxybenzoate, the precursors
for tetrahydrofolate and benzo- and naphtoquinones, respectively, are directly synthesized from chorismate. Menaquinone, 2,3-dihydroxybenzoate and salicylate biosynthesis proceeds via isochorismate. For a detailed description of the biosynthesis of these metabolites the reader is referred to Haslam (1993) and references therein.

**REGULATION OF AROMATIC AMINO ACID BIOSYNTHESIS**

Biosynthesis of aromatic amino acid in most organisms is regulated mainly via feedback inhibition/activation of enzymes converting the branchpoint intermediates; the aromatic amino acids themselves are generally involved as effectors. The only exception to date is the regulation of aromatic amino acid biosynthesis in *B. subtilis* via chorismate, prephenate, and non-aromatic amino acids.

In all organisms studied thus far, L-Trp controls its own synthesis mainly via feedback inhibition of anthranilate synthase (Haslam, 1993).

The regulation of L-Tyr and L-Phe is not that uniform and several mechanisms to control their levels have evolved (see above).

Attenuation of expression together with TyrR and TrpR controlled repression of the synthesis of the pathway enzymes also play a role in maintaining sufficient amounts of aromatic amino acids in *E. coli* (Gavini and Pulakat, 1991; Pittard, 1987; Pittard and Davidson, 1991).

**REFERENCES**

References are listed on pages 147-160.