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Analysis of Feedback-Resistant Anthranilate Synthases from *Saccharomyces cerevisiae*

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The initial step of tryptophan biosynthesis is catalyzed by the enzyme anthranilate synthase, which in most microorganisms is subject to feedback inhibition by the end product of the pathway. We have characterized the *TRP2* gene from a mutant *Saccharomyces cerevisiae* strain coding for an anthranilate synthase that is unresponsive to tryptophan. Sequence analysis of this *TRP2*(Fbr) (feedback-resistant) allele revealed numerous differences from a previously published *TRP2* sequence. However, *TRP2*(Fbr) was found to differ in only one single-point mutation from its own parent wild type, a C-to-T transition resulting in a serine 76-to-leucine 76 amino acid substitution. Therefore, serine 76 is a crucial amino acid for proper regulation of the yeast enzyme. We constructed additional feedback-resistant enzyme forms of the yeast anthranilate synthase by site-directed mutagenesis of the conserved LLES sequence in the *TRP2* gene. From analysis of these variants, we propose an extended sequence, $LLESX_{10}S$, as the regulatory element in tryptophan-responsive anthranilate synthases from prokaryotic and eukaryotic organisms.

The conversion of chorismic acid to anthranilic acid is the first step in the tryptophan branch of aromatic amino acid biosynthesis. This reaction is catalyzed by anthranilate synthase (EC 4.1.3.27), an enzyme that has been investigated in numerous species. Its substrate chorismate is a central compound in the biosynthesis of aromatic metabolites in microorganisms and plants. It serves as a precursor for the amino acids tryptophan, tyrosine, and phenylalanine as well as for other compounds such as *para*-aminobenzoate, ubiquinone, vitamin K, and phenazine pigments. Various regulatory mechanisms have therefore been developed by the cell to provide appropriate distribution of this important intermediate. The fact that tryptophan is essential for animals and humans has also resulted in considerable interest by the biotechnology industry, making the regulation of its synthesis in microorganisms an important area of research.

Anthranilate synthases have been characterized in many bacteria, in some species of archaea, and in some fungi, including the yeast *Saccharomyces cerevisiae* (for reviews, see references 7 and 35). The similarities observed among all of them suggest an evolution from a common ancestor enzyme, in most cases a complex consisting of two types of subunits that is necessary to convert chorismate (3-enolpyruvoyl-4-hydroxybenzoate) to anthranilate (2-aminobenzoate), using glutamine as the amino donor. Anthranilate synthase component I (AAS-I) is responsible for chorismate binding and allosteric regulation; in the presence of high concentrations of ammonia, this subunit is capable of performing an ammonia-dependent synthesis of anthranilate. AAS-II is a glutamine amidotransferase and catalyzes the transfer of the required amino group in the absence of ammonia. In protein sequence alignments, a high degree of conservation of primary structure is observed in AAS-II and in the C-terminal domain of AAS-I, whereas the N terminus of AAS-I is variable. The composition of the complex has been determined as either $(AAS\text{-}I)_1(AAS\text{-}II)_1$ or $(AAS\text{-}I)_2(AAS\text{-}II)_2$ in most species. An exceptional configuration has been found in *Rhizobium meliloti*, in which AAS-I and AAS-II are encoded by a *trpE(G)* fusion gene and form a single polypeptide (2).

In the yeast *S. cerevisiae*, the anthranilate synthase complex is heterodimeric, consisting of one molecule of each component encoded by the genes *TRP2* (AAS-I) and *TRP3* (AAS-II). Both genes have been cloned (1) and sequenced (36), and the complex has been purified from an overproducing yeast strain (23). The *TRP2* gene is the analog of the prokaryotic *trpE* gene. The deduced AAS-I peptide sequence shows the typical bipartite structure consisting of a conserved C-terminal half and an N-terminal part which displays a much higher variability, with only a limited number of conserved residues. *TRP3* encodes not only the glutamine amidotransferase, the analog of the bacterial *trpG* product, but also an indole-3-glycerol phosphate synthase domain (EC 4.1.1.48) catalyzing the fourth step in the tryptophan-specific pathway.

In *S. cerevisiae*, two levels of regulation are involved in the synthesis of anthranilate (for a review, see reference 4). The *TRP2* and *TRP3* genes are both subject to the general control of amino acid biosynthesis, which via the GCN4 protein induces the transcriptional levels of more than 30 genes two- to fivefold upon amino acid starvation. Tryptophan-specific regulation of enzyme activity level occurs through feedback regulation by the end product of the pathway. Although this phenomenon is common to almost all studied anthranilate synthases, information about the mode of action of the inhibitor is limited. The isolation of mutants deficient in feedback response to tryptophan has given preliminary insight into the structure of AAS-I. Detailed analysis, performed mostly in *Salmonella typhimurium*, suggests that the site of tryptophan binding resides in the variable N-terminal part (5), whereas the conserved C terminus contains the region of chorismate binding, catalytic activity, and subunit interaction.

An *S. cerevisiae* strain expressing a feedback-resistant anthranilate synthase has been previously isolated upon selection of mutants growing on 5-methyltryptophan (20,

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31). This amino acid analog acts as a false feedback inhibitor of anthranilate synthase, thereby causing tryptophan starvation of the cell. Wild-type yeast strains can partially compensate for this effect by activating the general control system to elevate enzyme synthesis and maintain slow growth. Mutants lacking the transcriptional regulatory response (*gcn* mutants) fail to grow on 5-methyltryptophan unless they are rescued by a second mutation, e.g., in the *TRP2* gene. In this work, we report the cloning and analysis of both the wild-type *TRP2* and the *TRP2*(Fbr) mutant gene encoding a feedback-resistant anthranilate synthase. The identification of the mutation, along with an analysis of homologous prokaryotic and eukaryotic sequences, suggests a relationship of the mutated residue to a conserved element. By construction of specific mutants, we tested the importance of this sequence in the yeast enzyme and gained evidence for an extended tryptophan regulatory element in anthranilate synthases.

MATERIALS AND METHODS

Strains and plasmids. All yeast strains are derivatives of *S. cerevisiae* S288C (*gal2 SUC2 mal CUP1*). The 5-methyltryptophan-resistant strain RH511 [TRP2(Fbr)] was constructed by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment of the general control nonderepressible strain RH380 (*gcn*), selection on 5-methyltryptophan, and backcrossing against the wild type (31). RH1682 (*MAT***a** *trp2 trp3A leu2-2 gcd2-1*) was isolated by selecting random spores after crossing RH1060 (*MAT*α *trp2 trp3A leu2-2*) and RH1378 (*MAT***a** Δ*ura3 gcd2-1*). The *trp2 trp3* genotype was verified by backcrossing against single *trp* mutants; the *gcd* mutation was verified by its characteristic slow-growth phenotype.

Escherichia coli MC1061 [(Δ*lacIPOZYA*)*X74 galU galK* StrA^r *hsdR* Δ(*ara*, *leu*)] (6) was used for cloning work, and the M13 host JM101 (Δ*lac pro thi supE* F' *traD36 proAB lacI*^{q}*Z*Δ*M15*) (18) was used for isolation of single-stranded DNA.

Yeast plasmids pME552 (*TRP1 TRP2 TRP3 LEU2*) (22) and pME557 [*TRP1 TRP2*(Fbr) *TRP3 LEU2*] (24) have been described elsewhere. pME824 is identical to pME552 except that a 360-bp *Cla*I-*Acc*I fragment within the *TRP2* gene has been exchanged for the corresponding fragment from the *TRP2*(Fbr) mutant.

pME825 was designed as a small shuttle vector providing a polylinker and blue/white selection in *E. coli* as well as high-copy-number selection by the *LEU2*^{d} (9) marker in *S. cerevisiae*. We cloned a 2.8-kb *Xba*I-*Eco*RI (partial) fragment of the 2μm-derived part of pJDB207 (3) into the single *Nae*I site of pGEM7Zf+ (Promega Biotec, Madison, Wis.), thereby obtaining a plasmid 5.8 kb in length (Fig. 1).

Expression plasmids pME826, pME827, pME828, and pME829, carrying mutant *TRP2* alleles, were assembled in the polylinker of pME825 as follows. A 452-bp fragment containing the *TRP2* promoter was prepared by polymerase chain reaction (PCR) (26) with specific primers (CT21 [22-mer; 5'-AGTTTGAATTCGCTCTGTCAGA-3'; positions 335 to 314] and MT21 [21-mer; 5'-GTCTTAGCTCTTTC CAACAGA-3'; positions 203 to 183]), introducing an artificial *Eco*RI site at the 5' end (position −325 relative to the ATG). It was isolated as a 286-bp *Eco*RI-*Cla*I fragment, and two independent isolates were verified by sequencing. The *TRP2* coding region, engineered in vitro as described below, was added as a 1,645-bp *Cla*I-*Bam*HI fragment. The *TRP3* gene was introduced in the opposite orientation as a 2,569-bp *Sac*I-*Bam*HI fragment to provide AAS-II for overexpression

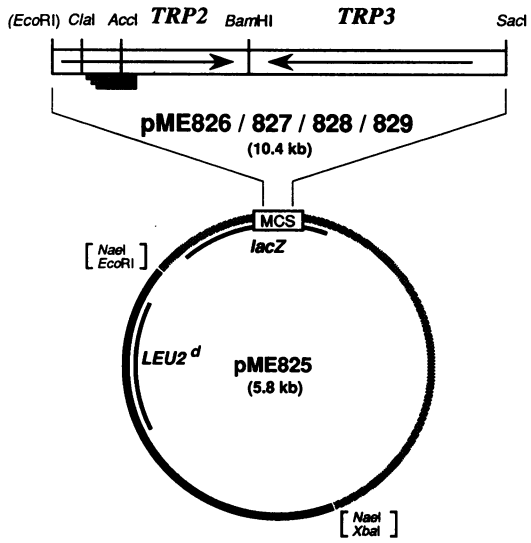


FIG. 1. Construction of the yeast shuttle vector pME825 and the *TRP2 TRP3* expression plasmids pME826, pME827, pME828, and pME829. Plasmid pME825 consists of a 2.8-kb *Eco*RI-*Xba*I fragment (black sector) from pJDB207 (3) cloned into the single *Nae*I site of pGEM7Zf+ (gray sector). Insertion of *TRP2* and *TRP3* into the multiple cloning site (MCS) yielded pME826 (wild type). Plasmids pME827 (mutant L_{76}), pME828 (mutant R_{65}), and pME829 (mutant $R_{65}L_{76}$) differ from pME826 in the *TRP2 Cla*I-*Acc*I fragment (shaded boxes) which has been modified by in vitro mutagenesis. Restriction sites in brackets are lost; the *Eco*RI site in parentheses has been introduced artificially.

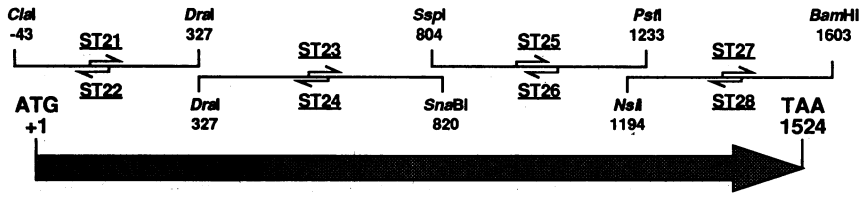
of the anthranilate synthase complex (Fig. 1). The vectors M13mp18 and M13mp19 (34) have been described elsewhere.

Media. YEPD complete and MV minimal media were used for the cultivation of yeast cells (25). *E. coli* strains were grown on LB broth and M9 minimal plates (19).

Molecular techniques. DNA manipulations (27), transformation of *E. coli* (32), and transformation of *S. cerevisiae* (14) were performed by standard procedures.

DNA sequencing and computer analysis. Nucleotide sequences were determined by the chain termination method (28), using Sequenase (U.S. Biochemical, Cleveland, Ohio), either from an M13-derived single-stranded template or from a double-stranded template. Oligonucleotide primers were synthesized by Microsynth (Windisch, Switzerland). Alignments of DNA and protein sequences were performed with the Genetics Computer Group (Madison, Wis.), software package, using the programs GAP, DISTANCES, and PILEUP for pairwise and multiple comparisons.

Site-directed mutagenesis by PCR. Specific mutations in the *TRP2* gene were introduced as described previously (12). We cloned a *Cla*I-*Bam*HI fragment containing the *TRP2* or *TRP2*(Fbr) open reading frame, respectively, into the pGEM7Zf+ vector to serve as a template for the amplification reactions. The specific mutagenic primer MT21 (21-mer; 5'-GTCTTAGCTCTTTCCAACAGA-3'; positions 183 to 203) was designed to change the serine 65 codon to an arginine 65 codon. Using an M13 universal primer and the MT21 oligonucleotide (100 pmol of each in a 100-μl reaction under standard conditions, with *Taq* polymerase [Boehringer, Mannheim, Germany]), we amplified a mutated 371-bp fragment. Thirty cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C were performed in a Biometra Trioblock



ST21	5'- GATCTGACTCCTCAC -3'	15-mer	115 > 129	ST25	5'- GTGGCTCGTTAAAAG-3'	15-mer	1004 > 1018
ST22	5'- GATCCAATGATGGCA-3'	15-mer	118 > 104	ST26	5'- GCACGGTCTTTTAA-3'	14-mer	1025 > 1012
ST23	5'- GTTACCAAGCTGCAG-3'	15-mer	563 > 577	ST27	5'- TGGCGGTGGTATTGT-3'	15-mer	1395 > 1409
ST24	5'- GTAACCTTCCTCCAA-3'	15-mer	567 > 553	ST28	5'- CGCCAGCTTGCAAGT-3'	15-mer	1399 > 1385

FIG. 2. Subcloning strategy for the sequencing of *TRP2* and *TRP2*(Fbr). Four fragments were cloned into both M13mp18 and M13mp19. Universal primers and sequence-specific oligonucleotides (ST21 through ST28; listed at the bottom) were used for sequencing. The positions of restriction sites and oligonucleotides are given relative to the *TRP2* translation start site. The open reading frame is symbolized by an arrow.

thermocycler (Biometra, Göttingen, Germany). This double-stranded product was isolated from an agarose gel, and 5 pmol was used as the primer for another amplification reaction, along with 10 pmol of the specific primer ST24 (15-mer; 5'-GTAACCTTCCTCCAA-3'; positions 553 to 667). The annealing temperature in the second PCR was elevated to 50°C. The product of the second PCR was a 735-bp fragment carrying the mutation approximately in the center and spanning the two unique *Cla*I and *Acc*I restriction sites. It could be isolated from agarose, cut, and recloned into its original position in the *TRP2* coding region. Use of the wild-type *TRP2* as the template for both amplification steps yielded the R_{65} mutant coding for arginine in codon 65. The analogous procedure using the *TRP2*(Fbr) mutant (L_{76}) as the template produced the double mutant $R_{65}L_{76}$. All amplified sequences were verified for the presence of the desired mutations and absence of possible second-site mutations due to the limited fidelity of *Taq* polymerase.

Anthranilate synthase enzyme assay. Glutamine-dependent anthranilate synthase activities were determined either in cells permeabilized by Triton X-100 (21) or in crude extracts desalted over disposable Sephadex G-25 columns (PD-10; Pharmacia, Uppsala, Sweden) (15, 30). We used the stop-assay method (8) in the presence of various concentrations of tryptophan.

Nucleotide sequence accession number. The nucleotide sequence of the wild-type *TRP2* has been deposited in the EMBL data library under accession number X68327.

RESULTS

Isolation and sequencing of *TRP2* and *TRP2*(Fbr). We have determined the nucleotide sequences of both the wild-type and the feedback-resistant anthranilate synthase-encoding genes. The wild type has been cloned by using a library from wild-type strain S288C as described previously (1). The *TRP2*(Fbr) allele was isolated analogously from strain RH511, an *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-mutagenized derivative of the same wild type. A 1.6-kb *Cla*I-*Bam*HI fragment containing the entire coding region of *TRP2* or *TRP2*(Fbr) was sequenced by using four subclones of this fragment and a series of specific oligonucleotides (Fig. 2). We determined the nucleotide sequences of both strands of both alleles (Fig. 3).

The DNA sequence of our wild type, compared with that of a *TRP2* wild type which was independently cloned and sequenced previously (36), showed a considerable number of discrepancies. In summary, we found in the previously published sequence 15 base exchanges, 3 base insertions, 6 base deletions, and a 10-base duplication within the open reading frame. In addition, we identified two base exchanges and another four-base duplication in the 282 bp upstream of the *Cla*I site during sequencing of a PCR-cloned promoter fragment (see Materials and Methods for construction of plasmids pME826 to pME829). The insertions and deletions cause several stretches of frameshift in the central part and at the end of the gene, leading to a number of differences in the amino acid sequence and a shorter open reading frame of 507 instead of 527 codons. A computer alignment of the translations of the two wild-type sequences yields a similarity of 89% and an identity of 85% on the amino acid level. In addition, the shorter open reading frame in our sequence leaves a longer 3' noncoding part and reveals a consensus element for yeast termination and polyadenylation, a TAG ...TAGTTTT motif (37) (Fig. 3).

We compared the amino acid sequences of the two yeast wild types with a large set of anthranilate synthase-related sequences. For computer alignments, we used 19 bacterial *trpE* genes, two isogenes coding for anthranilate synthase in *Arabidopsis thaliana*, the *phnA* gene from *Pseudomonas aeruginosa* encoding an alternative anthranilate synthase, and six paralogous *pabB* genes encoding 4-amino-4-deoxychorismate synthases. All of them yielded 2 to 4% higher similarity and identity values to the *S. cerevisiae* sequence determined in this work than to the sequence published previously (36). The highest values were found for the *trpE* gene from *Pseudomonas putida*, which shows 63.2% similarity and 40.6% identity to our sequence but only 60.3% similarity and 36.8% identity to the sequence reported previously.

Identification of the *TRP2*(Fbr) mutation. Comparison of the *TRP2*(Fbr) sequence with our wild-type sequence revealed a single-point mutation, a C-to-T transition in residue 227, relative to the initiator codon ATG. On the amino acid level, this change leads to a serine-to-leucine substitution at position 76. To verify that the site responsible for the feedback resistance of the *TRP2*(Fbr)-encoded enzyme resides in the N-terminal part of the protein, we replaced a small portion of the 5' terminus of *TRP2* (*Cla*I-*Acc*I; positions −42 to 319; 360 bp) by the corresponding fragment of *TRP2*(Fbr). Anthranilate synthase activities were assayed in yeast cells carrying either plasmid pME552 (wild-type *TRP2*), pME557 [TRP2(Fbr)], or pME824 [*TRP2*(Fbr)-*TRP2* hybrid]. The assays were performed in permeabilized cells with and without tryptophan. In terms of tryptophan inhibition, the hybrid construct was indistinguishable from *TRP2*(Fbr). The wild type was inhibited to 9% of its activity by 0.5 mM tryptophan concentration, whereas *TRP2*(Fbr)

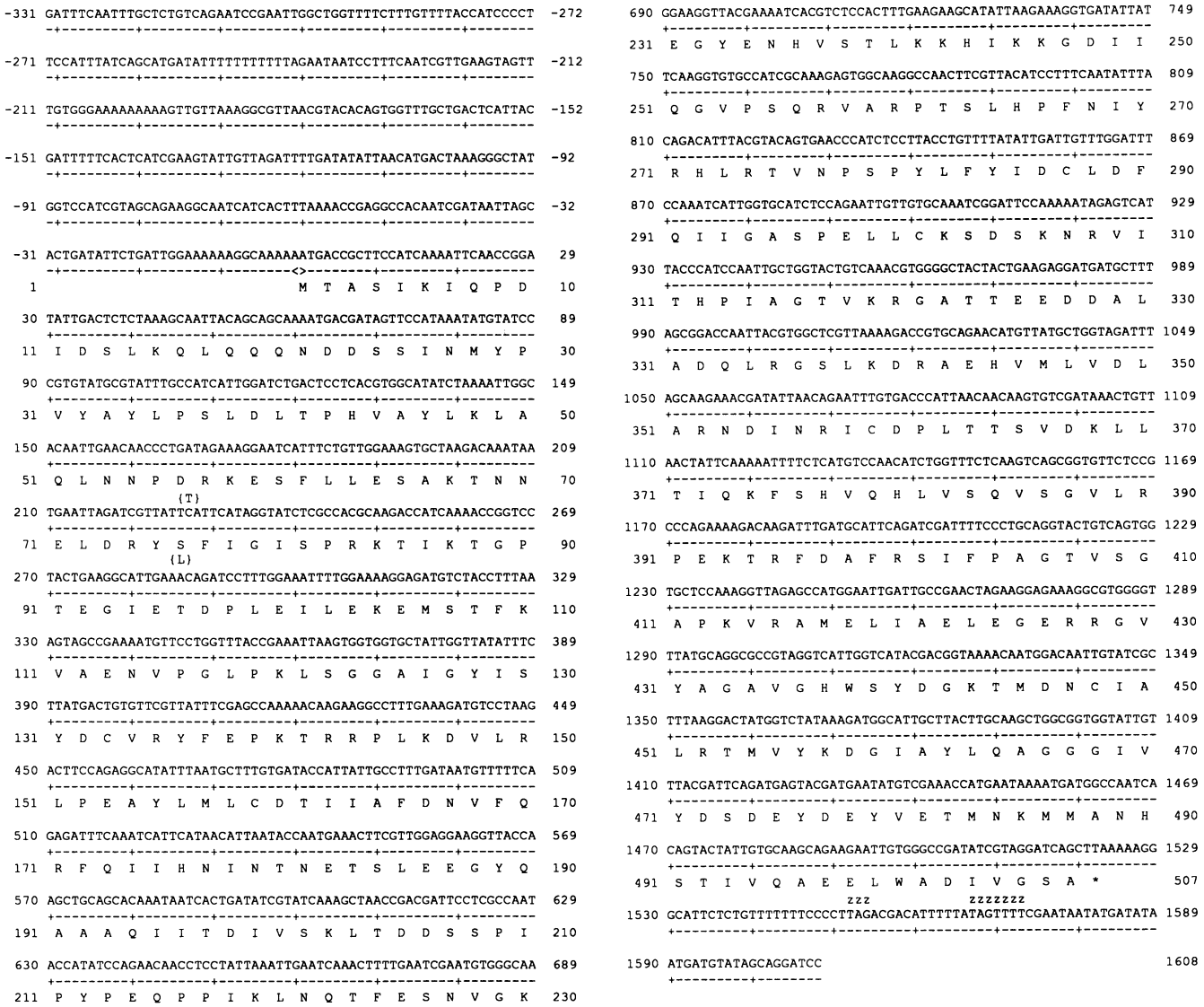


FIG. 3. Nucleotide and amino acid sequences of the *S. cerevisiae TRP2* gene encoding AAS-I. In the feedback-resistant TRP2(Fbr) allele, a C-to-T transition in nucleotide 227 leads to a serine 76-to-leucine 76 change. The transcription termination consensus sequence according to Zaret and Sherman (37) is indicated by z's.

and the hybrid construct showed at the same conditions 86 and 84%, respectively, of their uninhibited activities. The regulatory defect could be transferred from the mutant to the wild-type enzyme by the 107 N-terminal amino acids.

Mutations in the N-terminal part of anthranilate synthase causing feedback resistance have been characterized in *Brevibacterium lactofermentum*, *E. coli*, and *Salmonella typhimurium* (5, 16, 17). In those studies, several mutations revealed a particular element, a Leu-Leu-Glu-Ser (LLES) stretch, which is one of the few highly conserved sites in the upstream part of the large subunit of anthranilate synthase. In the *TRP2*(Fbr) product of *S. cerevisiae*, however, it is not this LLES element (positions 62 to 65) that is mutated but rather a serine located 11 residues further downstream. The sequence of this region in a multiple sequence alignment of anthranilate synthase-related proteins (Fig. 4) reveals that the serine, separated from the LLES by a spacer of 10 amino acids, is conserved in all tryptophan-regulated anthranilate synthases analyzed so far. The *TRP2*(Fbr) mutant of *S. cerevisiae* provides the first evidence for this residue's involvement in the regulation of the enzyme.

Construction of regulatory mutants of yeast *TRP2*. The highly conserved LLES element has been shown to be involved in tryptophan regulation of anthranilate synthases of some bacteria. To test its role in the yeast enzyme and to determine a possible relationship with the *TRP2*(Fbr) mutation that we have located nearby, we used a PCR-based method for site-directed mutagenesis of this element in the yeast anthranilate synthase sequence. By changing the serine 65 codon to an arginine 65 codon, we created the constellation LLER, which has been shown to cause feedback resistance of anthranilate synthase in *B. lactofermentum* (17) and *E. coli* (16). Analogous mutagenesis using *TRP2*(Fbr) as the template yielded a double mutant carrying an arginine for serine 65 and a leucine for serine 76. Constructs carrying *TRP2*(Fbr) are referred to as L_{76}, the

				LLES xxxxxxx...xxx S
Escherichia coli	*TrpE*		+	..PATL**LLES** ADIDSKD...DLK **S**LLLVDSALR
Salmonella typhimurium	*TrpE*		+	..PATL**LLES** ADIDSKD...DLK **S**LLLVDSALR
Vibrio parehaemolyticus	*TrpE*		nd	..TDSL**LLES** AEIDSKQ...NLK **S**LLIVDSAVR
Brevibacterium lactofermentum	*TrpE*		+	TADDAA**LLES** ADITTKN...GIS **S**LAVLKSSVR
Corynebacterium glutamicum	*TrpE*		nd	TADDAA**LLES** ADITTKN...GIS **S**LAVLKSSVR
Pseudomonas aeruginosa	*TrpE*		+	A.PNSY**LLES** VQGGEKW...GRY **S**IIGLPCRTV
Pseudomonas putida	*TrpE*		+	Q.PNSY**LLES** VQGGEKW...GRY **S**MIGLPSRTV
Pseudomonas syringae	*TrpE*		+	Q.PNSY**LLES** VQGGEKW...GRY **S**IIGLPCRTV
Chlostridium thermocellum	*TrpE*		nd	S.SCCF**LLES** VEGGEKW...ARY **S**IIGKNPFLV
Bacillus pumilus	*TrpE*		nd	KQDIVY**LLES** KDESSSW...SRY **S**FIGLHPFLT
Bacillus subtilis	*TrpE*		nd	DREITY**LLES** KDDTSTW...SRY **S**FIGLNPFLT
Bacillus caldotenax	*TrpE*		nd	REEAVF**LLES** KDDESPW...ARY **S**FIGVAPFLT
Leptospira biflexa	*TrpE*		+	KYENCF**LLES** AGDNQYD...SRY **S**VIGFQPSHL
Spirochaeta aurantia	*TrpE*	(*)	nd	.....V**VLES** SSSKKGR...DRY **S**LLLLQEAFR
Acinetobacter calcoaceticus	*TrpE*	(*)	+	Y.TQAY**LFES** VEGGENW...ARY **S**IIGLGESTV
Lactococcus lactis	*TrpE*	(*)	nd	KGKNKV**ILES** IPRENDQ...SRF **S**IIALNPVKH
Methanobacterium thermoautotrophicum	*TrpE*		nd	EYESSF**LLES** MESDTGL...ARY **S**FIGFEPEMI
Haloferax volcanii	*TrpE*	*	nd	RSDYGF**LLES** AE -*(22)*- RF **S**FVGYDPEAV
Thermus thermophilus	*TrpE*	*	nd	A.PVSF**LLES** VERGRQ....SRF **S**IVGVGARRT
Saccharomyces cerevisiae	***TRP2***		+	DRKESF**LLES** AKTNNEL...DRY **S**FIGISPRKT
Arabidopsis thaliana	*ASA1*	*	nd	E.APSF**LFES** VEPGSQMSSVGRY **S**VVGAQPAME
Arabidopsis thaliana	*ASA2*	*	nd	D.APSF**LFES** VEPGSQSSNIGRY **S**VVGAQPTIE
Pseudomonas aeruginosa	*PhnA*	*	-	AGANRM**LFDC** FDVDSKA...ARR **S**VAILSSCLR
Salmonella typhimurium	*PabB*	*	-	HLPWAM**LLHS** GDAIHPY...NRF **D**ILVADPVTT
Klebsiella aerogenes	*PabB*	*	-	SQPWAM**LLHS** GFAEHAH...NRF **D**IIVAQPRAT
Escherichia coli	*PabB*	*	-	HLPWAM**LLHS** GYADHPY...SRF **D**IVVAEPICT
Bacillus subtilis	*PabB*	*	-	R.KHHV**LLES** ARGG.....RY **S**IAGLDPIAT
Lactococcus lactis	*PabB*	*	-	AMNNGI**LLES** VEGN..K...PRY **S**IGGAEPIGT
Streptomyces lividans	*PabB*		-	PRCHAE**LLES** VTGASRM...SRY **S**IIVLDPIGT
				LLES xxxxxxx...xxx S

FIG. 4. Alignment of the $LLESX_{10}S$ regions of anthranilate synthases and *para*-aminobenzoate synthases from various organisms. The conserved motif is indicated on the top and bottom lines. The sequence of the X_{10}-spacer of *H. volcanii* is simplified because of its unusual length of 26 amino acids. *, sequence with aberrations from the consensus; (*), sequence with conservative mutations; −, enzymes not responsive to tryptophan; +, feedback-regulated enzymes; nd, regulation of the enzyme has not yet been determined.

arginine 65 mutant is called R_{65}, and the double mutant is referred to as $R_{65}L_{76}$ (Fig. 5B).

All four variations of *TRP2* (the wild type, L_{76}, and the newly generated R_{65} and $R_{65}L_{76}$) were expressed in yeast cells. To achieve a high specific activity in the analyzed cell extracts, the in vitro-modified *TRP2* genes were overexpressed from high-copy-number plasmids. On the basis of the well-established yeast plasmid pJDB207, we constructed the smaller shuttle vector pME825. The mutant *TRP2* genes were cloned into its multiple cloning site, along with a copy of *TRP3* to provide AAS-II for a functional anthranilate synthase complex. The resulting expression plasmids pME826, pME827, pME828, and pME829 (Fig. 1) were transformed into strain RH1682 (*trp2 trp3 leu2 gcd2*), which has a constitutively activated general amino acid control system for enhanced expression of GCN4-regulated genes.

Specific anthranilate synthase activity was determined as 1.5 U/mg (nanomoles/minute per milligram of protein) in permeabilized cells of the *S. cerevisiae* wild type S288C. The various plasmid-bearing strains reached overexpression rates in a range of 20- to 50-fold, depending on individual transformants.

For feedback inhibition assays, we used crude extract preparations partially purified over Sephadex-G-25 to remove free tryptophan. Since the physiological tryptophan pool in *S. cerevisiae* has been determined to be as low as 0.02 to 0.03 mM in a wild-type strain, but levels elevated about 50-fold to 1 mM are detected in strains expressing a feedback-resistant anthranilate synthase from the chromosome (11), we investigated the feedback behavior of our *TRP2* mutants at inhibitor concentrations of between 0 and 2 mM.

The feedback responses of the four anthranilate synthases are presented in Fig. 5A. The difference between feedback resistance and wild-type behavior is evident from the comparison of the wild type and L_{76} [the original *TRP2*(Fbr)]; the relative activity of the wild-type enzyme dropped below 20% at a tryptophan concentration of 0.5 mM and reached a plateau at 2 to 12% beyond 1 mM, whereas the mutant retained more than half of its maximum activity (55%) even at the extreme inhibitor concentration of 2 mM. The curve of the second single mutant, R_{65}, shows that a destroyed LLES element in the yeast enzyme can cause a feedback resistance equivalent to that exerted by the L_{76} mutant; R_{65} kept 69% activity at the highest inhibitor concentration, its relative activity values being even slightly higher than those of L_{76}. This finding adds further evidence for the central role of the LLES sequence in the regulation of anthranilate synthases.

Although the effects of both single mutations on the feedback response were strong, they did not confer to the enzyme complete resistance to high tryptophan concentrations. It is interesting that a combination of both was not able to enhance the effect. In the double mutant, no additivity of the partial effects of the single mutations could be observed;

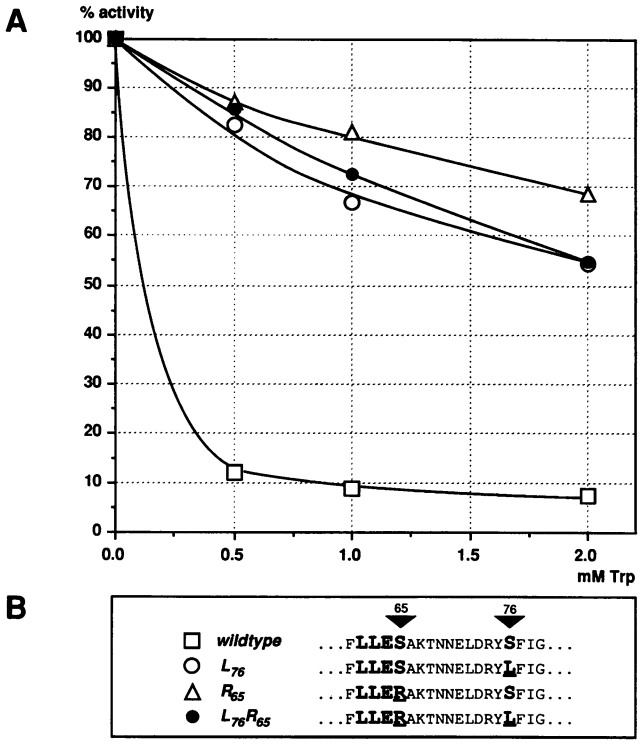


FIG. 5. Relative anthranilate synthase activities of the *TRP2* wild type and the mutants R_{65}, L_{76}, and $R_{65}L_{76}$, measured at tryptophan concentrations of 0.0, 0.5, 1.0, and 2.0 mM. The data are means of at least two measurements of two independent transformants of each mutant. The values shown are percentages of uninhibited activity of the respective strain. The amino acid sequences of the $LLESX_{10}S$ region in the investigated enzymes are shown at the bottom.

the $R_{65}L_{76}$ enzyme behaved essentially in the same way as did R_{65} and L_{76}.

DISCUSSION

We have analyzed the yeast *TRP2* gene, which encodes one polypeptide of the tryptophan-regulated anthranilate synthase complex, as well as three *TRP2* alleles encoding gene products which are unresponsive to tryptophan. Our wild type showed a number of discrepancies from the *TRP2* sequence published previously (36). This divergence is surprising, since both genes originated from derivatives of wild-type strain S288C. Our *TRP2* sequence gave higher similarity and identity values when aligned to any of 28 anthranilate synthases or 4-amino-4-deoxychorismate synthases found in the data bases. The frameshifts in the C-terminal part of our yeast sequence contributed significantly to this improvement, because they restored several conserved residues in the alignment. In theory, corrections of the *S. cerevisiae* sequence have already been proposed. In a detailed computer analysis of anthranilate synthases reported in reference 7, the author has postulated frameshifts in yeast *TRP2* to obtain better alignments of the gene product. On the basis of (i) the fact that we have sequenced two *TRP2* alleles that differ in only one nucleotide as a result of a chemical mutagenesis step and (ii) the higher similarity values of our sequence to related prokaryotic anthranilate synthases, we conclude that the differences to the original *TRP2* sequence are not artifacts.

The members of the anthranilate synthase family have an N-terminal domain that shows high sequence variability, in contrast to a remarkably conserved C terminus. Only a small number of amino acid residues appear invariant in the proximal part of the sequence. Some of them, like the LLES element (corresponding to positions 62 to 65 in yeast AAS-I) and a second stretch located around a methionine (aligning with leucine 280 in *S. cerevisiae*), were shown to be involved in tryptophan regulation of the *Salmonella* and *Brevibacterium* enzymes (5, 17). We have identified an additional residue, serine 76 of *S. cerevisiae*, that elicits feedback resistance upon mutation. This amino acid is almost perfectly conserved in a characteristic but less stringent region that is separated from LLES by a spacer of 10 residues. This short distance suggests that LLES, the spacer, and the next conserved serine belong together to form an $LLESX_{10}S$ element. The multiple alignment of this region in Fig. 4 reveals how remarkably stable this motif has remained in evolution; all bacterial TrpE proteins except those of *Acinetobacter calcoaceticus* (LFES), *Spirochaeta aurantia* (VLES), and *Lactococcus lactis* (ILES) show a perfectly conserved $LLESX_{10}S$ sequence, suggesting that conservative substitutions of hydrophobic amino acids for either of the leucines are tolerated. In the archaea and in eukaryotes, the spacer length appears to be less stringent; the sequences of *Methanobacterium thermoautotrophicum* and *S. cerevisiae* match the $LLESX_{10}S$ consensus, but the TrpE proteins of *Thermus thermophilus* and *Haloferax volcanii* and the two recently published anthranilate synthase isoenzymes from the higher plant *A. thaliana* show some variability. However, the regulation of these particular anthranilate synthases has not yet been described in the literature. In summary, the $LLESX_{10}S$ element is commonly conserved within the variable domain of feedback-regulated anthranilate synthases.

One group of sequences in Fig. 4, i.e., the *phnA*-encoded anthranilate synthase from the phenazine pigment biosynthetic pathway of *P. aeruginosa* (10) and the various 4-amino-4-deoxychorismate synthases which catalyze a closely related chemical reaction (13), does not conform to the rest very well. These enzymes are not involved in tryptophan biosynthesis, are not regulated by this compound, and do not match the $LLESX_{10}S$ consensus in all cases but one. Some enzymes show variations in the LLES core itself (e.g., LFDC in PhnA of *P. aeruginosa*), some show variations in the length of the spacer (eight amino acids in *L. lactis* PabB and six amino acids in *Bacillus subtilis* PabB), and the three 4-amino-4-deoxychorismate synthases from *E. coli*, *Salmonella typhimurium*, and *Klebsiella aerogenes* contain an aspartate at the position of yeast serine 76. However, it is an interesting finding that the $LLESX_{10}S$ sequence is varied but not completely absent in the tryptophan-independent members of the anthranilate synthase family. It therefore appears to be an old structural element which in PhnA and PabB has not withstood the mutational pressure due to the lack of function and has diverged from the correct structure in various ways.

The deregulation of an allosteric enzyme by a point mutation can follow different mechanisms. In some instances, such as aspartate transcarbamoylase (33), the amino acid exchange was found to destroy the binding site for the effector. Consequently, the enzyme cannot sense the feedback signal and remains constitutively active. On the other hand, a point mutation in chorismate mutase of *S. cerevi-*

siae, an enzyme which is regulated by tryptophan in the wild type, was found to lock the enzyme in its active allosteric conformation without affecting the binding of the effector (29). In the case of anthranilate synthase, there is evidence for the first mechanism, since mutants in the LLES of *Salmonella typhimurium* were found to be unable to bind tryptophan (5).

We have tested the inhibition of the mutants L_{76}, R_{65}, and $R_{65}L_{76}$ by tryptophan at different effector concentrations. Comparison of the two single mutants revealed that an exchange of either of the two serines can elicit an equally strong resistance to the feedback inhibitor. Since in both cases a residual feedback response was observed, albeit at very high inhibitor concentrations, an increase of the effect could be expected in the double mutant. Interestingly, this was not the case; $R_{65}L_{76}$ was not feedback superresistant but rather behaved essentially in the same way as did the two single mutants. This phenomenon suggests that the two single mutations in the prominent serines destroy the same element and supports our idea that the entire $LLESX_{10}S$ sequence is a highly sensitive functional unit in the regulatory mechanism of anthranilate synthase.

In summary, an element consisting of an LLES stretch and a serine, separated by usually 10 variable amino acid residues, is conserved in tryptophan-regulated anthranilate synthases from bacteria, archaea, and eukaryotic microorganisms. Variations of this sequence are found preferentially in related but tryptophan-unresponsive enzymes. Artificial point mutations in the LLES sequence were known to cause feedback resistance in bacterial anthranilate synthases; we have now demonstrated the importance of both serines of the consensus in the enzyme of *S. cerevisiae*. The analysis of a double mutant favors the postulation that the LLES and the downstream serine are both part of the tryptophan binding site.

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