SUPPORTING MATERIAL FOR

The Subunit Structure of Benzylsuccinate Synthase†

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Construction of expression plasmids used in this study.

Materials.
*D*NA-modifying enzymes and reagents were purchased from New England Biolabs (Beverly, MA). Oligonucleotide primers were obtained either from IDT Integrated DNA Technologies (Coralville, IA) or from Invitrogen (Carlsbad, CA). *E. coli* BL21(λDE3) and expression vectors pET-28b, pACYC-Duet, pET-Duet, pRSF-Duet were purchased from Novagen (Madison, WI); Pfu turbo DNA polymerase and *E. coli* XL1-Blue were from Stratagene (Cedar Creak, TX)..

Subcloning of tut genes from PWC-301
The genes were subcloned from PWC-301, a pBluescript plasmid carrying a cosmid clone which contains the *tutE* gene and the *tutFDGH* that has been described previously (1). The *tutFDGH* gene cluster was amplified using by polymerase chain reaction (PCR) technology. The forward primer (5′-GAG ACC CAT ATG AAC GAC ATC GTA AGC-3′) included an *Nde*I restriction site (underlined) flanked by a 6 bp GAG ACC clamp. The reverse primer (5′-GAA GCT TGG CGG CGA TGT ATT GGA CG-3′) contained a *Hind*III restriction site (underlined) flanked by a 1 bp G clamp. The PCR was performed with a Biometra (Horsham, PA) TRIO-thermoblock according to a procedure recommended by the manufacture. The product was directly ligated into the PCR-BluntII-TOPO vector (Invitrogen^{TM}) by following the given protocol. The resultant vector was designated pTopoTutD.

The Plasmid pTopoTutD was digested with *Nde*I/*Hind*III and the *tutFDGH* gene cluster was ligated into pET 28b vector previously digested with the same enzymes and the resultant vector is named pET 28_FDGH.

Construction of tutF expression vector
To insert *tutF* into the pET 28 vector, a forward primer (5'-GGA GAC TCA CAT ATG GGA ACC ACC ACA TGC AAG CAG-3') and a reverse primer (5'-CTG CTT GCA TGT GGT GGT TCC CAT ATG TGA GTC TCC-3') were used to introduce an *Nde*I restriction site (underlined) at the start codon of the *tutFttttttt* gene in pET 28_FDGH through a typical QuikChange II PCR procedure. The resulting plasmid was digested with *Nde*I/*EcoR*I and the restriction fragment isolated by gel electrophoresis and ligated into pET 28 vector digested with the same enzymes. The resultant vector was named pET 28_F.

Construction of tutG expression vector
With primers (5'-CGG TTG CAC GAA AAC ATA TGG AGG GCA GCA ACA TGG -3') and (5'-CCA TGT TGC TGC CCT CCA TAT GTT TTC GTG CAA CCG-3'), an *Nde*I restriction site (underlined) was introduced at the start codon of the *tutG* gene in pET 28_FDGH using a typical QuikChange II PCR procedure. The resulting plasmid was digested with *Nde*I/*Hind*III and the restriction fragment isolated by gel electrophoresis and ligated into pET 28 vector digested with the same enzymes. The resultant vector was named pET 28_G (which contains both *tutG* and *tutH* genes; the later is not expressed).

Construction of tutD expression vector

An *Nde*I restriction site (underlined) was introduced at the start codon of the *tutD* gene in pET 28_FDGH using a typical QuikChange II PCR procedure with primers (5'-AAA CTA CAG ATC AAG GAG ACC CAT ATG AAC GAC ATC GTA AGC GCC-3') and (5'-GGC GCT TAC GAT GTC GTT CAT ATG GGT CTC CTT GAT CTG TAG TTT-3'). The resultant plasmid was digested with *Nde*I/*Hind*III and the restriction fragment isolated by gel electrophoresis and ligated into the pET 28 vector digested with the same enzymes. The resultant plasmid was named pET 28_D (which contains *tutD*, *tutG* and *tutH*).

Construction of the TutF_TutD co-expression vector

The *tutF* gene was amplified from the pET 28_F plasmid described above using PCR. The forward amplification primer (5′-ATC CAT GGG AAC CAC CAC ATG CAA GCA G-3′) included an *Nco*I restriction site (underlined) flanked by a 2 bp AT clamp and the first 21 bases of the *tutF* gene. The reverse primer (5′-TT AAG CTT TTA GCG CTT CGT TTG AAA GGC TTC-3′) contained a *Hind*III restriction site (underlined) flanked by a 2 bp TT clamp and the last 24 bases of the *tutF* gene, including the stop codon. The PCR product was isolated and digested with *Nco*I and *Hind*III and ligated into multiple-cloning site I of a similarly digested pET-Duet vector by standard procedures. The resultant vector was designated pET-Duet_F.

The *tutD* gene was amplified from the pET 28_D vector. The forward amplification primer (5′-AAT TAA CAT ATG AAC GAC ATC GTA AGC GCC AAG-3′) included an *Nde*I restriction site (underlined) flanked by a 6 bp AAT TAA clamp and the first 21 bases of the *tutD* gene. The reverse primer (5′-TT GGT ACC TTA GAT TTC GAC GTT TAG GAA CTC-3′) contained a *Kpn*I restriction site (underlined) flanked by a 2 bp TT clamp and the last 24 bases of the *tutD* gene, including the stop codon. The PCR product was digested with *Nde*I and *Kpn*I and ligated into the multiple-cloning site II of a similarly digested pET_F vector. The resultant vector is called pET_D_F_1.

Construction of a His_6-tag at the 3'-terminus of tutD

To add a His-Tag encoding sequence at the 3'-terminus of *tutD*, an *XbaI* restriction site on the pET_D_F_1 vector was mutated away (underlined) using primers (5′-GGG GAA TTG TGA GCG GAT AAC AAT TCC CCT TTA GAA ATA ATT TTG-3′) and (5′-CAA AAT TAT TTC TAA AGG GGA ATT GTT ATC CGC TCA CAA TTC CCC-3′) by PCR. These primers were added to a typical QuikChange II reaction mixture together with the pET_D_F_1 vector as template and the PCR reaction was performed following the procedure recommended by the manufacturer. The resultant vector was called pET_D_F_2.

Next, a new *XbaI* restriction site (underlined) was introduced into the pET_D_F_2 vector immediately after the S-tag-encoding sequence using primers (5′-GCC AGC ACA TGG ACT CGT CTA GAA GCG CAG CTT A-3′) and (5′-TAA GCT GCG CTT CTA GAC GAG TCC ATG TGC TGG C-3′) under the same PCR procedure described above. The resultant construct was named pET_D_F_3.

To introduce the 6-His-tag and following stop codon into pET_D_F_3, two short oligonucleotides (5′-CGG ATC CGG CAG CAG CCA TCA CCA TCA TCA CCA C**TA A**T-3′) and (5′-CTA GAT TAG TGG TGA TGA TGG TGA TGG CTG CTG CCG GAT CCG GTA C-3′) including sequence coding for 6 His (underlined) and a TAA stop codon

(Shown in bold) were used. They had 38 bases complementary to each other as well as flanked bases corresponding to the overhangs after *Kpn*I and *Xba*I digestion. These oligos were treated with T4 polynucleotide kinase (NEB, Beverly, MA) to phosphorylate the 5' termini, allowed to anneal by heating to 95 ºC followed by slowly cooling to ambient temperature, and ligated into the pET_D_F_3 vector digested previously with *Kpn*I and *Xba*I and purified by a QIAquick PCR purification Kit from Qiagen. The resulting construct was named pET_D_F_4.
Finally, the original stop codon in the *tutD* gene was mutated to TCA (underlined) by PCR using primers (5′-CCT AAA CGT CGA AAT CTC AGG TAC CGG ATC CGG CAG C-3′) and (5′-GCT GCC GGA TCC GGT ACC TGA GAT TTC GAC GTT TAG G-3′) and vector pET_D_F_4 as template. The resultant construct was designated pET-Duet_D_F, in which *tutD* contains a sequence encoding a C-terminus His_6 tag to facilitate protein purification through Ni-NTA affinity column.

Construction of the tutG co-expression vector
The *tutG* gene was amplified from the pET 28_G vector. The forward amplification primer (5′-AAT TAA CAT ATG GAG GGC AGC AAC ATG GAA ACA-3′) included an *Nde*I restriction site (underlined) flanked by a 6 bp AAT TAA clamp and the first 21 bases of the *tutG* gene. The reverse primer (5′-ATT CTC GAG TCA GAC GTG GTC GCG GAA ACT -3′) contained an *Xho*I restriction site (underlined) flanked by a 3 bp ATT clamp and the last 21 bases of the *TutG* gene, including the stop codon. The PCR product for *tutG* was isolated and digested with *NdeH*I and *Xho*I and ligated into multiple-cloning site II of a similarly digested expression vector pRSF-Duet by standard procedures. The resultant vector was called pRSF-Duet_G.

Construction of the tutF_tutD co-expression vector with an early stop codon inserted into the tutF gene
To make a construct with only *tutD* gene expressed, an early stop codon was inserted into the *tutF* gene to yield a prematurely terminated *tutF* gene with only the first 16 amino acid expressed. The forward primer (5' - AAA CTT CTT TCC CGT CCA C**TA A**AG ACG CGG ATG AC - 3') contained an extra A (underlined) so that a new TAA stop codon (shown in bold type) was generated right after this insertion. The reverse primer (5' - GTC ATC CGC GTC TTT AGT GGA CGG GAA AGA AGT TT - 3') contained 34 bases and was complementary to the forward primer. These primers were added to a typical QuikChange II reaction mixture together with the pET_D_F vector as template and the PCR reaction was performed accordingly. The resulting plasmid was designated pET-Duet_D_F_stop.

Construction of the tutF C9S mutant_tutD co-expression vector
The *tutF* C9S mutant was conducted with a forward primer (5'-ACC ACC ACA TGC AAG CAG TCG GCA AAC TTC TTT CCC-3') and a reverse primer (5'-GGG AAA GAA GTT TGC CGA CTG CTT GCA TGT GGT GGT-3'). The primers were added to a typical QuikChange II reaction mixture together with the pET-Duet_D_F vector as template and the PCR reaction was performed accordingly. The resulting plasmid was designated pET-Duet_D_F_C9S.

Construction of the tutG C29S mutant co-expression vector
This *tutG* C29S mutant was constructed with a forward primer (5'-GCC GTG CCG GAG TTC GAA ATG GCA AAC CCC-3') and a reverse primer (5'-GGG GTT TGC CAT TTC GAA CTC CGG CAC GGC-3'). The primers were added to a typical QuikChange II reaction mixture together with the pRSF-Duet_G plasmid as template and the PCR reaction was performed accordingly. The resulting plasmid was designated pRSF-Duet_G_C29S.

Construction of the His-tagged tutF C9S mutant in pACYC-Duet vector
The *tutF C9S* gene was amplified by PCR using the pET-Duet_D_F_C9S vector as template. The forward amplification primer (5'-AAG CTT GGA TCC TGG AAC CAC CAC ATG CAA GCA GTC G-3') included a *BamH*I restriction site (underlined) flanked by a 6 bp AAG CTT clamp and the first 24 bases of the *TutF C9S* gene. In addition, a single T was inserted right after the *BamH*I cutting site to keep the gene in the correct reading frame. The reverse primer (5'-GAA TTC CTC GAG TTA GCG CTT CGT TTG AAA GGC TTC-3') contained a *Xho*I restriction site (underlined) flanked by a 6 bp GAA TTC clamp and the last 24 bases of the *tutF* gene, including the stop codon. The PCR product was isolated and digested with *BamH*I and *Xho*I and ligated into the multiple-cloning site I of a similarly digested expression vector pACYC-Duet by standard procedures. The resultant vector is called pACYC_F_C9S which contains a His_6-tag at the N-terminus.

Construction of the His-tagged tutG C29S mutant in pET 28 vector
This *tutG* C29S mutant was constructed with same primers used in pRSF-Duet_G_C29S preparation and a similar PCR procedure except using pET_28_G as template. The resulting plasmid was designated pET28_G_C29S which contains a His_6-tag at the N-terminus.

Reference:

(1) P.W. Coschigano, Transcriptional analysis of the tutE tutFDGH gene cluster from Thauera aromatica strain T1, Applied and Environmental Microbiology 66 (2000) 1147-1151.