Quantitative real-time PCR

Total RNA extraction using Trizol Reagent

1. Bacterial cells (3 mL) were harvested and resuspended in 0.5 mL culture medium to which 0.5 mL of TRIzol was added and thoroughly mixed by pipetting.
2. The mixture was incubated at 15 – 30^{o}C for 5 min and thoroughly mixed with 0.2 mL chloroform.
3. After incubating at room temperature for 2 – 3 minutes, sample was centrifuged at 4^{o}C for 15 min at 12,000 x g. RNA in the aqueous phase was precipitated with 0.5 mL isopropyl alcohol.
4. Precipitated RNA was washed with 1 mL 75% alcohol and resuspended in RNase-free water.
5. Residual genomic DNA was removed by digestion with 1 unit of DNase I (Invitrogen) at room temperature for 15 minutes. DNase I digestion was terminated with 1 µL of 25 mM EDTA solution and further incubation at 65^{o}C for 10 min.

Synthesis of first-strand cDNA

1. Random primers (0.5 µL; Promega) was added to 1 µg of DNase I-treated RNA and incubated at 70^{o}C for 5 min, and then cooled on ice.
2. Reverse Transcription master mix (13.5 µL) which consisted of 5x reaction buffer, 10 mM each of dNTP, RNaseOUT (40 units/µL; Invitrogen) and MMLV reverse transcriptase (200 U; Promega) was added and incubated at 37^{o}C for 1 hour. The cDNA was stored at -20^{o}C until ready to use.

Quantitative real-time PCR

Real-time PCR was performed using the StepOnePlus Real-time PCR system (Applied Biosystems) with SYBR green (KAPA Biosystems) as the fluorescent reporter dye.

1. RT-PCR mixture in 10 µL consisted of 2 µL of first-strand cDNAs, 0.18 µL of each primer (10 µM), 5 µL of 2x KAPA SYBR FAST qPCR Master Mix and 0.2 µL of 50x KAPA SYBR FAST ROX High.
2. Results were normalized to the 16S rRNA to determine the expression levels of various target genes. The gene-specific primers used are shown in Table 1 below. Gene expression levels in different samples were compared by

calculating the relative expression levels of mRNA using the comparative delta C_T (threshold cycle number) method. The following formula: $2^{-\Delta\Delta CT}$, where ΔCT is the difference in CT between the gene of interest and 16S rRNA, and ΔΔCT for the sample = (ΔCT for the treated sample - ΔCT of the control sample), was used for the calculation.

Table 1. Gene-specific primers for qRT-PCR analysis

Gene Target	Forward Primer Sequence 5'→3'	Reverse Primer Sequence 5'→3'
16S rRNA	CAGCCACACTGGAACTGAGA	GTTAGCCGGTGCTTCTTCTG
Δ15desaturase	CAATTCCGGCAGATTGTTTT	TTCAGGGTTTTGCTTTTGCT
FadD	GGCACGGTAGTCAATTTCGT	TATTGCGGTGAGTCAGCATC
FadL	CCTTTGATGACAGCCCAGTT	CAGCCAGGCTTTACCTTCAG
TesA	TTCCTGTTCCTGGTCCTGTT	TGATGCTGTTTCAGCAGAGC