



Discovery and investigation of a new, second triose phosphate isomerase in *Klebsiella pneumoniae*

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Abstract

In this study, a *tpi1* gene encoding for the enzyme triose phosphate isomerase in *Klebsiella pneumoniae* DSM2026 was knocked out in an effort to metabolically engineer this strain as a model system for the production of 1,3-propanediol. Investigations of the *tpi1* knockout mutant led to the discovery of a second *tpi* gene (*tpi2*) in this organism. The new *tpi2* gene was cloned and sequenced. The coding region of the *tpi2* gene contains 795 bp (base pairs) and the deduced protein consists of 265 amino acids. Sequence comparison of TPI2 proteins in different organisms revealed the presence of a highly conserved signature A-Y-E-P-V-W-A-I-G-[EDVS]-[GKNASH], which is nearly the same as the reported TPI consensus signature. The *tpi1* gene of *K. pneumoniae* DSM2026 shows a high sequence similarity to that of *E. coli*, whereas, the *tpi2* gene resembles more its relatives in the alpha-proteobacteria, suggesting that they evolve from different ancestors. The overexpression of the *tpi2* gene restores the growth deficiency of *tpi1* knockout mutant on the minimal medium containing glucose or glycerol. Furthermore, the catalytic activity of this new triose phosphate isomerase was confirmed in both *tpi1* knockout mutant and *tpi2* over-expressing strain by enzyme assays. For the first time, the co-existence of two *tpi* genes in an enteric bacterium is experimentally confirmed.

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1. Introduction

Klebsiella pneumoniae, a Gram-negative enteric bacterium, is an organism with significance in both biomedicine and biotechnology. Some of the strains of *K. pneumoniae* are opportunistic pathogens, which

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can cause suppurative infection, pneumoniae and urinary tract infection in humans (Bouza and Cercenado, 2002). On the other hand, it is also a widely used model organism for elucidating biochemical and genetic mechanisms with respect to citrate metabolism (Bott et al., 1995), nitrogen fixation (Jack et al., 1999) and microbial production of useful chemicals such as 1,3-propanediol (Nakamura and Whited, 2003; Zeng and Biebl, 2002).

1,3-Propanediol (1,3-PD) can be used as a monomer for the synthesis of novel polyesters and biodegradable plastics. Different strategies for the microbial production of 1,3-PD have been investigated in recent years by using microorganisms such as *K. pneumoniae*, *Citrobacter freundii*, *Enterobacter agglomerans* and *Clostridium* strains from substrates such as glycerol and more abundant renewable resources like glucose (Biebl et al., 1998; Hartlep et al., 2002; Nakamura and Whited, 2003; Papanikolaou et al., 2000; Zeng and Biebl, 2002; Gonzalez-Pajuelo et al., 2005). For the production of 1,3-PD from glucose in *K. pneumoniae* an extensive metabolic engineering of the metabolism is necessary. Since *Klebsiella* cannot directly convert dihydroxyacetone phosphate (DHAP) to glycerol, heterogeneous genes encoding enzymes such as glycerol 3-phosphate dehydrogenase (GPD) and glycerol 3-phosphate phosphatase (GPP2) from *Saccharomyces cerevisiae* need to be introduced to this end. Another key strategy is to knock-out the *tpi* gene encoding the triose phosphate isomerase (TPI, EC. 5.3.1.1) to direct the carbon flux towards the formation of glycerol and further to 1,3-propanediol after the split of fructose biphosphate (Capitanio et al., 2002; Compagno et al., 2001). Triosephosphate isomerase (TPI) is the glycolytic enzyme that catalyzes the reversible inter-conversion of glyceraldehyde 3-phosphate (GAP) and DHAP (Knowles, 1991). TPI plays an important role in several metabolic pathways such as glycolysis and gluconeogenesis and is essential for efficient energy production. It is a dimer of identical subunits, each of which is made up of about 250 amino-acid residues. A glutamic acid residue is involved in the catalytic mechanism. The sequence around the active site residue is well conserved in all known TPI's. Deficiencies of TPI in human being are associated with haemolytic anaemia coupled with a progressive, severe neurological disorder (Olah et al., 2002). Structure study has led to the renewed interest in this enzyme because it could

serve as a drug target against many parasitic protozoans (Maithal et al., 2002).

In order to increase the yield of glycerol from glucose in *K. pneumoniae* we set out to knockout the *tpi1* gene. Surprisingly, the knockout of *tpi1* gene did not result in the expected phenotype but led to the discovery of a new, additional *tpi* gene (*tpi2*) in this organism. The gene *tpi2* was cloned and its functionality was demonstrated by enzyme activity assay. The phylogenetic relationship of this new gene was surveyed and its putative physiological function was discussed.

2. Materials and methods

2.1. Strains and plasmids

Bacterial strains and plasmids used in this study are given in Table 1. *E. coli* strains were routinely grown with LB medium at 37 °C in shaking flasks. For the cultivation of *K. pneumoniae* strain DSM2026 and its derivatives, LB medium was used as a rich medium and the medium as described by Hartlep et al. (2002) was used as a minimal medium. *K. pneumoniae* was grown at 37 °C. The concentrations of antibiotics used in medium are: ampicillin (Ap) 100 µg ml⁻¹, tetracycline (Tc) 10 µg ml⁻¹, and gentamicin (Gm) 10 µg ml⁻¹ for *E. coli*; carbenicillin (Cb) 100 µg ml⁻¹ plus Ap 150 µg ml⁻¹, Tc 20 µg ml⁻¹, and Gm 10 µg ml⁻¹ for *K. pneumoniae*.

2.2. DNA manipulations

General DNA manipulations were performed as described by Sambrook and Russel (2001). Plasmids from *E. coli* and *K. pneumoniae* strains were isolated with the QIAprep spin Miniprep kit (Qiagen, Germany). Genomic DNA from *K. pneumoniae* was extracted with the blood and cell culture DNA kit (Qiagen, Germany). Electroporation of *K. pneumoniae* was done with the gene pulser II (BioRad, Germany) at 1.8 kV, 25 µF, and 200 Ω by using a 1 mm cuvette. Primers used in this study (Table 2) were designed by using the software VectorNTI and the genomic sequence of *K. pneumoniae* MGH78578 was used as a template to amplify specific genes from *K. pneumoniae* DSM2026. All the primers were synthesized by

Table 1
Bacterial strains and plasmids used in this work

Strains or plasmids	Genotype/phenotype	Source/reference
<i>Klebsiella pneumoniae</i> strains		
DSM2026	Wild type	DSMZ, Germany
DSM2026-1	Gm ^R , <i>sacB</i> ⁺ , DSM2026 strain with a plasmid pZH6 integrated into the chromosome	This study
DSM2026-2	Gm ^R , <i>sacB</i> ⁻ , DSM2026 strain with the <i>tpi1</i> gene replaced with the Gm ^R and GFP cassette on the chromosome,	This study
DSM2026-3	DSM2026 strain with a <i>tpi1</i> deletion on the chromosome	This study
<i>E. coli</i> MG1655 Δ <i>tpiA::FRT</i>	MG1655 strain with a <i>tpiA</i> deletion on the chromosome	Soucaille (personal communication)
Plasmids		
pUC18	Ap ^R , cloning vector	Yanisch-Perron et al. (1985)
pPS858	Ap ^R , Gm ^R , source of Gm ^R and GFP cassette	Hoang et al. (1998)
pEX18Tc	Tc ^R , <i>oriT</i> ⁺ , <i>sacB</i> ⁺ , gene replacement vector	Hoang et al. (1998)
pFLP2	Ap ^R , Flp recombinase-producing plasmid	Hoang et al. (1998)
pGEM-T	Ap ^R , T vector	Promega, Germany
pKD4	Km ^R , source of <i>oriR</i> _{R6K}	Datsenko and Wanner (2000)
pGPS5	Tc ^R , Km ^R , source of <i>oriR</i> _{R6K}	New England Biolabs, Germany
pZH5	Tc ^R , pEX18Tc derivative with the origin of pEX18Tc replaced with a R6K type of origin	This study
pZH1	Ap ^R , pUC18 with a 2.55 kb <i>Bam</i> HI– <i>Hind</i> III fragment containing <i>tpi1</i> gene and flanking region from <i>K. pneumoniae</i> DSM2026 genome	This study
pZH2	Ap ^R , Gm ^R , pZH1 with a 1.88 kb <i>Pst</i> I fragment replacing <i>tpi1</i> gene with Gm ^R and GFP cassette	This study
pZH4	Ap ^R , Gm ^R , pZH2 with a 3.6 kb <i>Aat</i> II– <i>Hind</i> III fragment from pEX18Tc	This study
pZH6	Gm ^R , pZH4 with a 0.39 kb <i>Aat</i> II– <i>Ase</i> I <i>oriR</i> _{R6K} fragment from pGEMT- <i>oriR</i> _{R6K} -1	This study
pGEMT- <i>oriR</i> _{R6K} -1	Ap ^R , pGEMT with a 0.52 kb <i>oriR</i> _{R6K} fragment from pKD4	This study
pGEMT- <i>oriR</i> _{R6K} -2	Ap ^R , pGEMT with 0.53 kb <i>oriR</i> _{R6K} fragment from pGPS5	This study
pGEMT-TPI2	Ap ^R , pGEMT with a 2.4 kb fragment containing <i>tpi2</i> gene and its flanking region from DSM2026 genome	This study
pZH8	Tc ^R , Gm ^R , pZH5 with a 3.6 kb <i>Bam</i> HI– <i>Spe</i> I PCR fragment containing Gm ^R and GFP cassette replaced the <i>tpi2</i> mutant allele	This study

Invitrogen, Germany. A high fidelity Pfu Turbo polymerase (Stratagene, The Netherlands) was used in PCR reactions except that a Taq polymerase (Qiagen, Germany) was used in colony PCR. PCR reactions were

generally carried out as follows: an initial denaturation cycle of 95 °C for 5 min, 30 cycles of 30 s at 95 °C, 30 s at 55–57.5 °C specific to the individual primers, 1 min kb⁻¹ at 72 °C and a final extension step

Table 2
Sequences of primers used in this study

Primer	Sequence 5'–3'
TPI1–1	ACGCGGATCCATGCGCCCTTTAATTGAGAA
TPI1–4	ACCGAAGCTTGCCGAATCTGGTGATGAGTG
TPI2–1	CTGACTGTTTGTGACTAGTGCATAGTCACAGGCTGCTTT
TPI2–2	TTTTGAAGCTAATTCGAGCTCAGCGGCTTGTTTCATTTTCC
TPI2–3	GGAAAATGAACAGCCGCTGAGCTCGAATTAGCTTCAAAA
TPI2–4	AAGATCCCCAATTCGAGCTCTCAATGGGTTGTTTATCGGC
TPI2–5	GCCGATAAAACAACCCATTGAGAGCTCGAATTGGGGATCTT
TPI2–6	TTACTCTTACTGGTGGATCCGGTCATTTCACCCGTATCG
TPI2–F1	GCGGACTAGTTATGACGGCTCACGAAGA
TPI2–F2	GACGGGATCCACGGGTTTGCCTGAATA

of 72 °C for 10 min. Colony PCR was done as described elsewhere (Datsenko and Wanner, 2000). Restriction enzymes (New England Biolabs, Germany) were used as recommended by the suppliers. DNA sequencing was performed by using the DYEnamic ET Terminator cycle sequencing kit (Amersham Biosciences, Germany) and the ABI PRISM 377 DNA Sequencer (Applied Biosystems, Germany).

2.3. Construction of a suicide vector pZH6 and disruption of the *tpi1* gene

The *tpi1* gene with its flanking fragments was PCR amplified from *K. pneumoniae* DSM2026 genomic DNA by using primers TPI1–1 and TPI1–4 (Table 2). The PCR product was inserted into the plasmid pUC18, generating the plasmid pZH1. The insert was sequenced and the sequence has been deposited in the EMBL sequence database under accession number AJ567469. A suicide vector pZH6 (Fig. 1) was constructed as follows. First, the intact *tpi1* gene on the plasmid pZH1 was replaced with a Gm^R and GFP cassette, resulting in the plasmid pZH2 (Table 1). The constructed $\Delta tpi1$ mutant allele was further cut off and inserted into the pEX18Tc (Hoang et al., 1998), generating the plasmid pZH4 (Table 1). The ColE type origin of pZH4, which is replicative in *Klebsiella*, was then replaced by a non-replicative origin, oriR_{6K} γ from the plasmid pGEMT-oriR_{6K}-1 (Table 1). The resultant plasmid was designated as pZH6.

The disruption of the *tpi1* gene on the chromosome of *K. pneumoniae* DSM2026 (Fig. 2) was carried out by following a modified protocol described by Hoang et al. (1998). Briefly, the plasmid pZH6 was first electro-transformed into *K. pneumoniae* DSM2026; the merodiploid DSM2026-1 was isolated on LB medium containing Gm and then cultivated in LB liquid medium without Gm at 37 °C for several hours to allow homologous recombination. The diluted culture was then spread to LB medium containing Gm and 5% sucrose to select $\Delta tpi1::FRT$ mutant DSM2026-2. The unmarked $\Delta tpi1::FRT$ mutant *K. pneumoniae* strain DSM2026-3 was obtained after the excision of the Gm^R and GFP cassette with the aid of the helping plasmid pFLP2 and the subsequent curation of pFLP2. Colony PCR was performed to verify the structure of mutants by using primers TPI1–1 and TPI1–4.

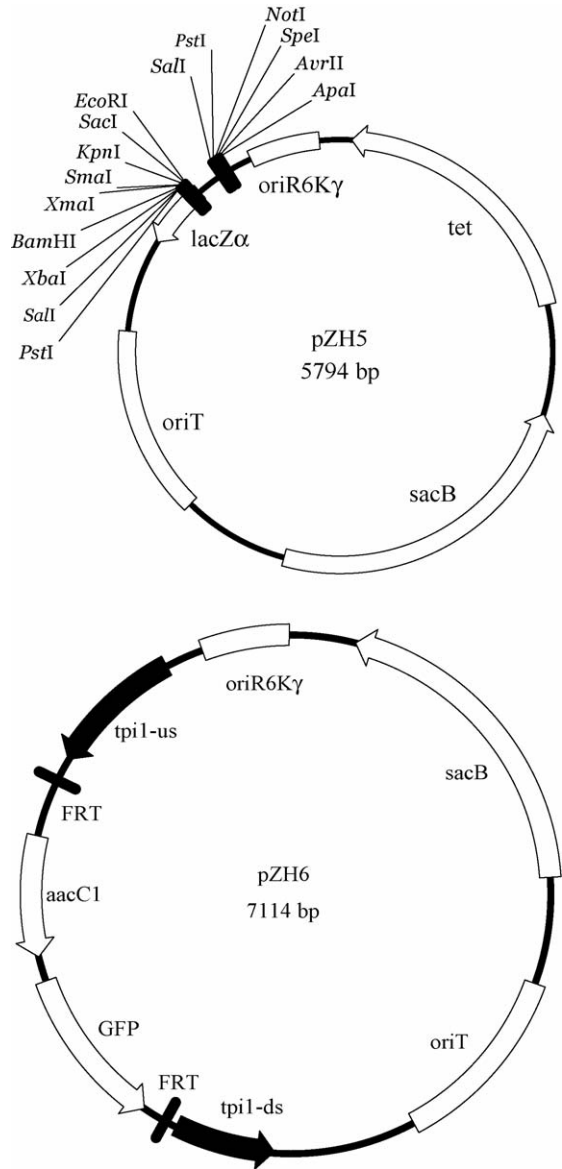


Fig. 1. Plasmids pZH6, pZH5 and pZH8 (the plasmids are not shown to the appropriate size): *tet*, tetracycline resistance gene; *aacC1*, gentamicin resistance gene; *GFP*, green fluorescent protein encoding gene; *lacZα*, gene encoding for beta-galactosidase alpha peptide; *sacB*, *B. subtilis* levansucrase-encoding gene; *FRT*, Flp recombinase target; *oriR6Kγ*, replication origin R6Kγ; *oriT*, origin of transfer; *tpi1-us*, the upstream sequence of *tpi1* gene; *tpi1-ds*, the downstream sequence of *tpi1* gene; *tpi2-us*, the upstream sequence of *tpi2* gene; *tpi2-ds*, the downstream sequence of *tpi2* gene.

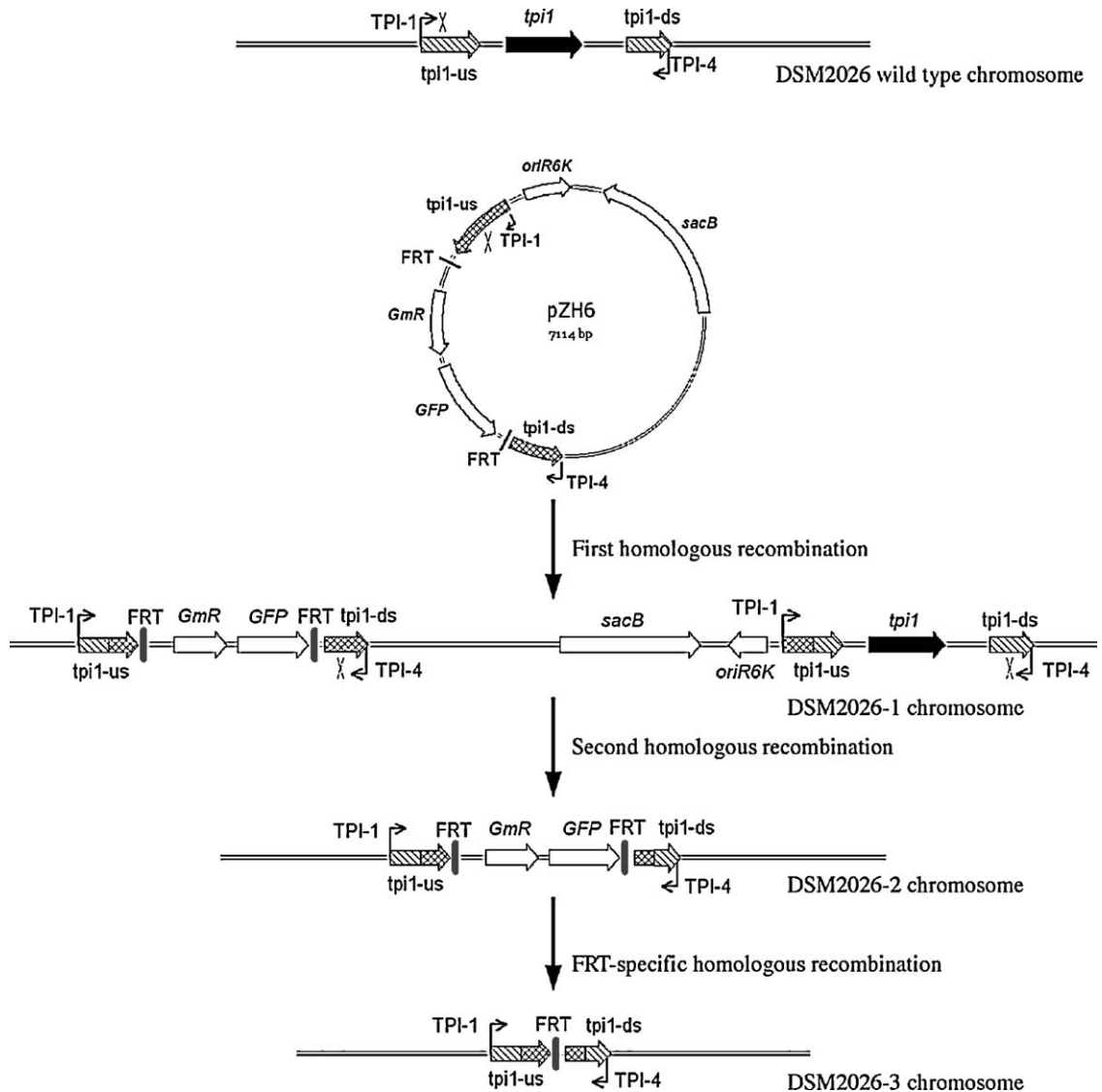


Fig. 2. Plasmid-based gene disruption strategy using *tpi1* as an example. Black solid arrows marked with *tpi1* indicate the wild type gene encoding for the triose phosphate isomerase. Arrows with dash line indicate the chromosomal flanking sequences of *tpi1*. *tpi1-us*, the upstream sequence of the *tpi1* gene; *tpi1-ds*, the downstream sequence of the *tpi1* gene. Arrows with cross line indicate the flanking sequences of *tpi1* on the plasmid pZH6. Empty arrows represent genes on the plasmid pZH6. TPI1-1 and TPI1-4 represent the primers, which were used to confirm the structure of each cell line in colony PCR. First, a homologous recombination happened between the chromosomal *tpi1-us* and that on plasmid, which resulted in a merodiploid. Then, a second recombination happened between the chromosomal *tpi1-ds* and the homologous plasmid-borne *tpi1-ds* and the wild type *tpi1* gene was knocked out. Finally, with the aid of plasmid pFLP2-carried Flp recombinase, which recognizes the specific FRT site and eliminates the sequences between two FRT sites, the unmarked $\Delta tpi1::FRT$ mutant DSM2026-3 was generated.

2.4. Cloning and sequencing of a new gene *tpi2* and its flanking regions

The *tpi2* gene with its flanking regions was amplified by using primers TPI2–1 and TPI2–6 (Table 2) and the template *K. pneumoniae* DSM2026 genomic DNA. PCR product was ligated with pGEM-T. The generated plasmid was named as pGEMT-TPI2. The insert was sequenced. The sequence has been deposited in the EMBL sequence database and the accession number is AJ567470.

2.5. Construction of a suicide vector pZH5

The plasmid pZH5 was constructed on the basis of pEX18Tc. A 549 bp *NdeI*–*PflMI* fragment from pGEMT-oriR_{6K}-2 (Table 1), which contains an oriR_{6K} replication origin (non-replicative in *Klebsiella*) insert from pGPS5, was introduced into pEX18Tc to replace the original ColE1 type replication origin (replicative in *Klebsiella*). Meanwhile, several rare restriction sites such as *ApaI*, *AvrII*, *SpeI* and *NotI* were introduced during the plasmid improvement. The created plasmid is named as pZH5 and shown in Fig. 1.

2.6. Construction of a suicide vector pZH8 and disruption of the *tpi2* gene

To construct a *tpi2* mutant allele, a three-fragment-fusion strategy was used (Kuwayama et al., 2002). First, three fragments including the upstream DNA fragment flanking the *tpi2* gene, the Gm^R and GFP cassette from the plasmid pPS858, and the downstream flanking fragment of the *tpi2* gene were amplified by PCR with respective primers TPI2–1 and TPI2–2, TPI2–3 and TPI2–4, TPI2–5 and TPI2–6 (Table 2). The Gm^R and GFP cassette contains a 40 bp fragment at each end, which is complementary to the 3'-end of the upstream flanking fragment of the *tpi2* gene and the 5'-end of the downstream flanking fragment of the *tpi2* gene, respectively. Then, three fragments were fused by PCR by using primers TPI2–F1 and TPI2–F2 (Table 2), resulting in an in-frame replacement of 651 bp of the *tpi2* gene (795 bp). The *Bam*HI and *SpeI* digested fusion-PCR product, which contains the *tpi2* mutant allele was then introduced into the *Bam*HI and *SpeI* digested vector pZH5. The generated plasmid is named as pZH8. To knockout the *tpi2*

gene, the plasmid pZH8 was transformed into the wild type *K. pneumoniae* DSM2026 or the Δ *tpi1* mutant DSM2026-3 by following the procedure as described above.

2.7. Enzyme activity assay

Cell extracts from the different strains cultivated in the minimal medium containing glucose were used to test the triose phosphate isomerase activity. Three independent experiments were performed. The enzyme activity was determined by coupling the conversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate and the reduction of DHAP (Plaut and Knowles, 1972). The decrease of absorbance at 340 nm caused by the oxidation of NADH was monitored. One activity unit (U) is defined as the conversion of 1 μ mol substrate per minute at 25 °C. The reaction mixture was composed of 100 mM triethanolamine buffer, pH 7.6, 5 mM EDTA, 0.2 mM NADH, 20 μ g ml⁻¹ α -glycerolphosphate dehydrogenase, and 1 mM glyceraldehyde 3-phosphate, 20 μ l cell extract. The substrate glyceraldehyde 3-phosphate was prepared from diethylacetal monobarium according to the manufacturer's instruction. The concentration of GAP was estimated by using glyceraldehyde-3-phosphate dehydrogenase. Chemicals used in the enzymatic assay were bought from Sigma.

2.8. Bioinformatic analysis

The genome sequences of *K. pneumoniae* MGH78578 were downloaded from the Genome Sequencing Center in the Medical School of Washington University (<http://genome.wustl.edu/projects/bacterial/>, April 2005) and were reformatted as a local database of BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997).

Similarity comparisons of sequences were done through the BLAST server of NCBI. Domain structure analysis was performed through the server of Interpro (Apweiler et al., 2001). The software package Vector NTI Suite version 8.0 (InforMax, USA) was used for multiple alignment, phylogenetic analysis and other routine analysis.

To analyze the evolution of TPI2, TPI2 was used as a query to search against the NCBI protein database "nr" (all non-redundant GenBank CDS trans-

lations + RefSeq Proteins + PDB + SwissProt + PIR + PRF). An E-value of $1E-5$ was set as cutoff. All acquired homologs were aligned using the Vector NIT suite and a phylogenetic tree was generated. A subset of 48 homologs was selected to represent different phylogenetic branches with emphasis on organisms locating in the same branch as TPI2 and the ones having more than one TPI2 homologs and aligned again using the Vector NTI suite. A phylogenetic tree was built based on the multiple sequence alignment and its stability was statistically validated using the software package “Phylip” version 3.64 (<http://evolution.genetics.washington.edu/phylip.html>) (Felsenstein, 1996). At first the multiple-sequence alignment from the Vector NTI suite was resampled 1000 times via the program “seqboot” of the phylip package (bootstrap method). Then for each sample a distance matrix was calculated by the program “protdist” applying the Jones–Taylor–Thornton (JTT) model of amino acid replacement. Subsequently, the distance file was used to compute phylogenetic trees using the program “neighbor” (Neighbor-Joining method of clustering) which generated 1000 trees from the 1000 samples. Finally, the “consense” tree program (extended majority rule) was used to find the conserved tree.

3. Results and discussion

3.1. Sequencing of the *tpi1* gene and its flanking regions from *K. pneumoniae* DSM2026

A 2.5 kb DNA fragment containing *tpi1* was amplified from *K. pneumoniae* DSM2026 by PCR and sequenced as described in Section 2. It also contains an intact ORF for the putative *yiiQ* gene (600 bp) at the upstream of *tpi1* and an ORF (1302 bp) for a hypothetical protein at the downstream of *tpi1*. It was found by sequence analysis that the *tpi1* gene in *K. pneumoniae* DSM2026 strain is 765 bp long and encodes a deduced protein of 255 amino acid (aa) with a predicted molecular weight of 26,900 and an isoelectric point (pI) of 5.78, which is consistent to the proteomic analysis reported by Wang et al. (2003). The protein showed a 94% identity to the TPI of *Salmonella typhimurium* and *Enterobacter cloacae* and a 93% identity to the TPI of *E. coli* by BLAST searching against the NCBI protein database “nr” (09/03).

3.2. Disruption of the *tpi1* gene

The *tpi1* gene in *K. pneumoniae* DSM2026 was knocked out as described in Section 2. Meanwhile, a 3'-end fragment (159 bp) of the upstream putative *yiiQ* gene and a 3'-end fragment (358 bp) of the downstream-unknown gene were also excised. The gene-organization structure of each strain was verified by Colony PCR. The result is shown in Fig. 3. Two bands (2.5 and 2.93 kb) are detected when the merodiploid DSM2026-1 is used as the template. It clearly shows the presence of both the wild type *tpi1* gene and the *tpi1*-replaced heterogeneous allele. The absence of the 2.5 kb small band in the case of the mutant DSM2026-2 as the template means the successful knockout of the wild type *tpi1* gene. A 1.27 kb band is found when the unmarked $\Delta tpi1::FRT$ mutant

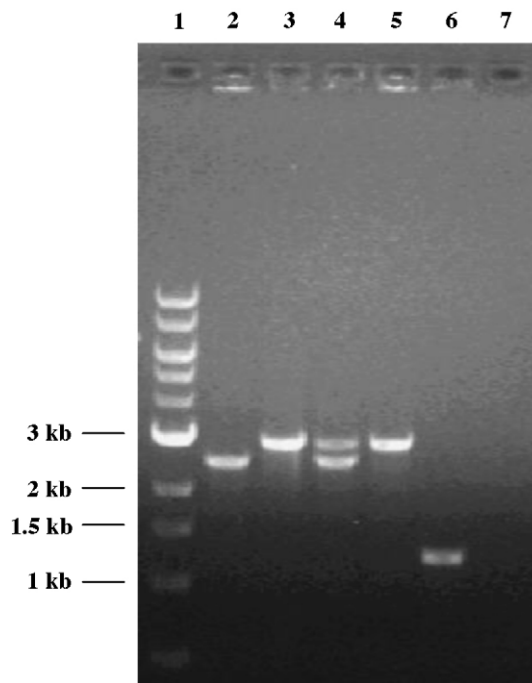


Fig. 3. Colony PCR verification of the gene structure of strains by using primers TPI1–1 and TPI1–4. Lane 1 represents the 1 kb ladder (New England Biolabs). Lanes from nos. 2 to 7 indicate the product patterns in colony PCR using different templates. Templates and the length of the expected products are as follows: Lane 2, wild type DSM2026, 2.5 kb; lane 3, vector pZH6, 2.93 kb; lane 4, merodiploid DSM2026-1, 2.5 kb and 2.93 kb; lane 5, DSM2026-2, 2.93 kb; lane 6, DSM2026-3, 1.27 kb; lane 7, negative control, no template, no product.

is used as the template, suggesting the elimination of the Gm^R and GFP cassette.

3.3. Behaviour of the $\Delta tpi1$ mutant DSM2026-3 and evidence for the existence of a second tpi gene in *K. pneumoniae*

Minimal medium with different carbon sources (glucose, glycerol and gluconate) was used to examine the phenotype of $\Delta tpi1::FRT$ mutant (Fig. 4). The experimental results showed a normal growth of the *K. pneumoniae* $\Delta tpi1::FRT$ mutant DSM2026-3 on gluconate as expected since gluconate can be metabolized through the Entner-Doudoroff pathway without the involvement of TPI (Nelson and Cox, 2004). The $\Delta tpi1$ mutant was not expected to grow on the medium using glucose or glycerol as sole carbon source due to the accumulation of DHAP. To our surprise, *K. pneumoniae* $\Delta tpi1::FRT$ mutant DSM2026-3 grew on glucose and glycerol although its growth was very weak. This is quite unusual as the intact ORF of the tpi gene was

already knocked out. The close relative *E. coli* MG1655 does not grow on glycerol at all after its tpi gene was deleted (Soucaille, personal communication). This phenomenon may be caused by unknown genes or non-specific function(s) of enzymes that catalyze the same reaction as TPI. To examine this possibility, enzyme assays for the activity of triose phosphate isomerase were carried out with the wild type *K. pneumoniae* DSM2026 and the $\Delta tpi1::FRT$ mutant DSM2026-3. Compared to the high TPI activity in the wild type strain ($83.6 \pm 1.11 \text{ U mg}^{-1} \text{ protein}$), only 1% of the original activity ($0.89 \pm 0.12 \text{ U mg}^{-1} \text{ protein}$) was detected in the mutant *K. pneumoniae* DSM2026-3, indicating a successful knockout of the tpi gene. Nevertheless, the remaining TPI activity detected in DSM2026-3 seems to be still quite significant, suggesting the existence of other enzyme(s) with similar TPI function. For an indirect comparison, we also measured the remaining TPI activity in an *E. coli* MG1655 $\Delta tpiA::FRT$ mutant (a gift from Soucaille). It showed an activity as low as $0.10 \pm 0.08 \text{ U mg}^{-1} \text{ protein}$.

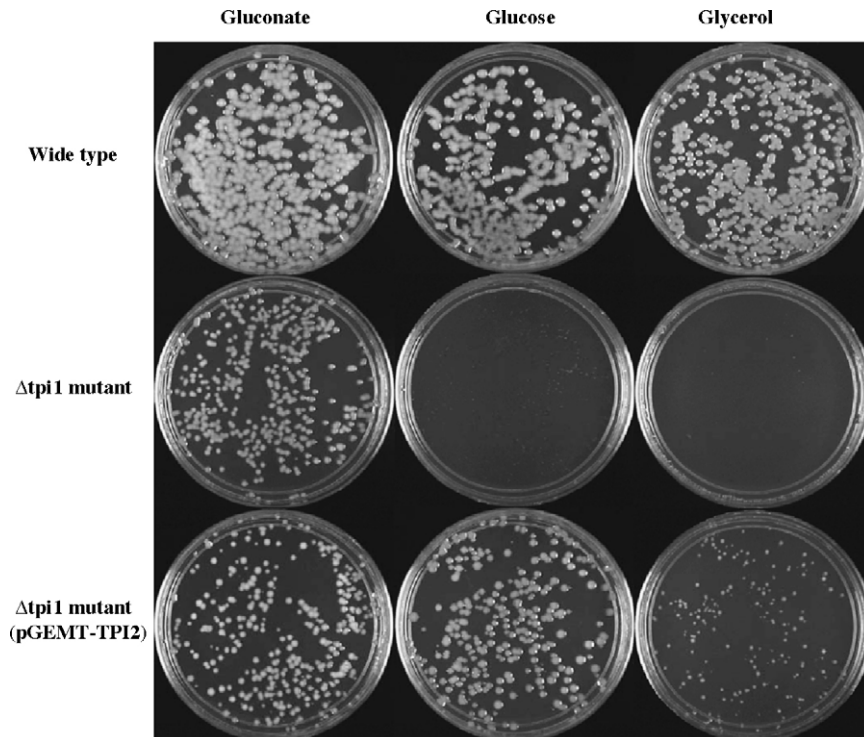


Fig. 4. Comparison of the growth of variant strains on the minimal media supplied with different sole carbon source.

The above finding prompted us to query the *K. pneumoniae* MGH78578 genomic sequences database with the *tpi1* gene of *K. pneumoniae* DSM2026. Besides the known *tpi1* gene, a 588 bp ORF was found when the 7.9-fold unfinished genome sequences were originally queried (a 795 bp ORF was later confirmed upon the availability of the 10-fold genome sequences). This ORF exhibits a 40% and 29% amino acid identity to the *tpiA* of *Pasteurella multocida* and the disrupted *tpi1* gene in *K. pneumoniae* DSM2026, respectively. It implies the existence of a putative second *tpi* gene (*tpi2*) in *K. pneumoniae* DSM2026 that may contribute to the rest TPI activity and support the growth of the mutant on glucose and glycerol (Fig. 4).

3.4. Cloning of the gene *tpi2* and experimental confirmation of its function in *K. pneumoniae* DSM2026

The indications from genomic analysis and enzyme activity assay further prompted us to clone the putative *tpi2* gene from *K. pneumoniae* DSM2026 for more direct evidence and for further characterization. A 2.4 kb fragment containing the putative *tpi2* gene was amplified by PCR from the genomic DNA of *K. pneumoniae* DSM2026. The fragment also contains a partial 5'-end fragment of the gene *pdxA* (548 bp of 1008 bp) at the upstream of *tpi2* and a partial 5'-end fragment of an ORF for an unknown protein at the downstream. It was then ligated into the plasmid pGEM-T to generate a new plasmid pGEMT-TPI2 that was further introduced into *K. pneumoniae* $\Delta tpi1$ mutant DSM2026-3 for over-expression of *tpi2*. The *tpi2* over-expressing strain exhibits a recovered growth on the minimal medium containing glucose or glycerol comparing to the growth of the $\Delta tpi1$ mutant (Fig. 4). Moreover, enzyme assay of TPI activity shows that the TPI activity in this strain (109.91 ± 2.77 U mg^{-1} protein) is recovered to the same level as that in the wild type. These results give direct evidences for the existence and functionality of the discovered *tpi2* gene in *K. pneumoniae*. For the first time, the co-existence of two *tpi* genes in an enteric bacterium is confirmed experimentally. The functionality of *tpi2* explains the growth of the $\Delta tpi1::FRT$ mutant *K. pneumoniae* on glucose and glycerol (Fig. 4). To further experimentally confirm the function of *tpi2*, we attempted to knock it out. The suicide plasmid pZH8 (Fig. 1) was used to construct a

$\Delta tpi1 \Delta tpi2$ double mutant or a single $\Delta tpi2$ mutant as described in the methods. However, all attempts have failed so far.

3.5. Bioinformatic analysis of the new *tpi2* gene

The cloned DNA fragment containing *tpi2* was sequenced. The deduced protein TPI2 in *K. pneumoniae* DSM2026 is 265 aa in length and has a predicted molecular weight of 29,200 and a pI value of 6.27.

The translated TPI2 sequence was used to do a BLAST search (07/05) against the nr database with a cutoff E-value set at $1E-5$. Six hundred and seventy-eight TPI proteins from 400 organisms including prokaryotes and eukaryotes are found to be homologous to the query. TPI2 is found to very much resemble the second *tpi* gene of α -proteobacteria *Gluconobacter oxydans* 621H (with 49% identity), *Rhodospirillum rubrum* (45%), *Mesorhizobium loti* (46%), *Brucella abortus* (46%), *Brucella melitensis* 16M (46%), *Rhizobium* sp. NGR234 (45%), and *Sinorhizobium meliloti* (45%). Furthermore, the TPI2 of *K. pneumoniae* DSM2026 shows a 45% and 43% identity to the TPIA (349 aa) of *Pasteurella multocida* and TPIA (260 aa) of *Mannheimia succiniciproducens* MBEL55E strain (both belonging to the Pasteurellaceae of γ -proteobacteria), respectively. All strains mentioned above (except for *Rhizobium*) bear two *tpi* genes on their chromosome. The *tpi2* of *Rhizobium* sp. NGR234 is on a mega-plasmid. It should be mentioned that the functionality of *tpi2* in these organisms has not been experimentally studied so far.

Protein sequence alignment reveals the presence of a consensus pattern A-Y-E-P-V-W-A-I-G-[EDVS]-[GKNASH] among the *tpi* genes mentioned above (Fig. 5). The highly conserved motif is nearly the same as the reported *tpi* consensus signature [AVG]-[YLV]-E-P-[LIVMEPKST]-[WYEAS]-[SAL]-[IV]-[GN]-[TEKDVS]-[GKNAD] (E is the active site residue) (PROSITE access number: PS00171) except the slight difference in the last conserved position (Knowles, 1991). This finding again supports the functionality and annotation of the novel *tpi2*.

The genome context of *tpi2* of *K. pneumoniae* MGH78578 was also investigated (Fig. 6). An ORF (orf2) downstream of the *tpi2* gene was shown to have a 70% identity with a putative transcriptional regula-

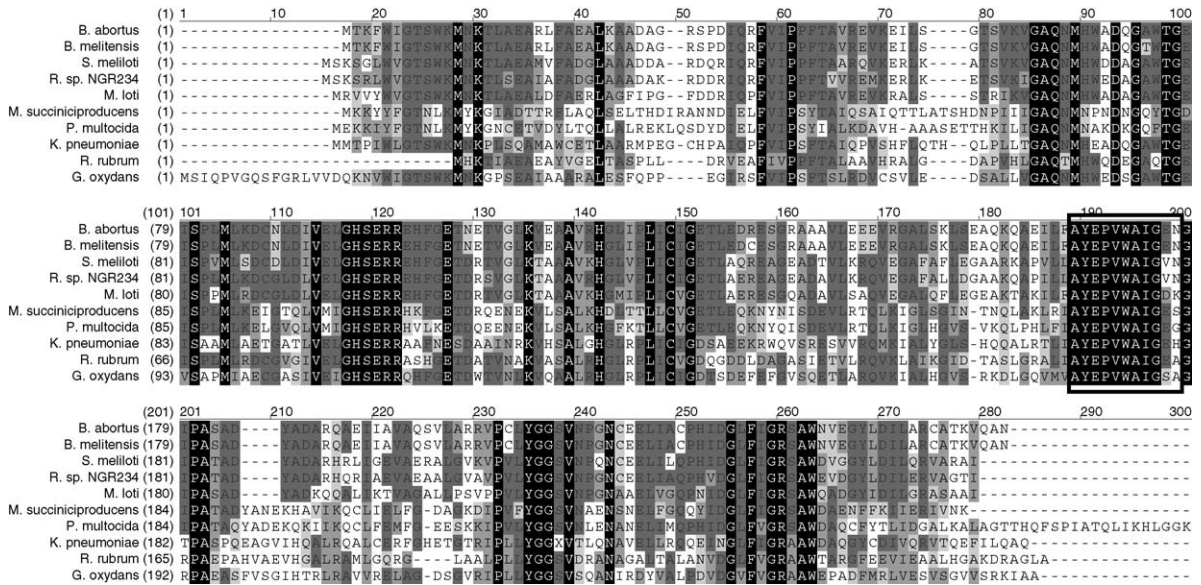


Fig. 5. Multiple sequence alignment of TPI2 homologues of *Brucella abortus biovar 1* str. 9-941 (YP.223155), *Brucella melitensis* 16M (NP.541403), *Sinorhizobium meliloti* (CAC46805), *Rhizobium* sp. NGR234 (AAQ87129), *Mesorhizobium loti* MAFF303099 (NP.107631), *Mannheimia succiniciproducens* MBEL55E (AAU36986), *Pasteurella multocida subsp. multocida* str. Pm70 (AAK03724), *K. pneumoniae* DSM2026 (CAD98882), *Rhodospirillum rubrum* (ZP.00269390) and *Glucobacter oxydans* 621H (YP.192608). Identical amino acids are shaded in black and conserved amino acids are shaded in gray. The sequence enclosed in box is conserved in all these proteins and exhibits a consensus pattern A-Y-E-P-V-W-A-I-G-[EDVS]-[GKNASH].

tory protein of *Yersinia pseudotuberculosis* (Chain et al., 2004). A further domain structure analysis showed that *orf2* was fully aligned to a transcriptional regulator DeoR. At the upstream of the *tpi2* gene, there are three successive genes *rpi*, *tal* and *tkt* encoding for a ribose 5-phosphate isomerase, a transaldolase and a transketolase involved in the pentose phosphate pathway, respectively. Moreover, genomic analysis reveals an ORF (in the upstream flank of the *tpi2* gene) that shows a high similarity to the gene *pdxA*, encoding the 4-hydroxythreonine-4-phosphate dehydrogenase that is essential in the synthesis of Vitamin B6 (Laber et al., 1999; Sivaraman et al., 2003). Two (*pthA* and *pthB*) of the three ORFs between the *pdxA* gene and *rpi* show

significant similarities to the components IIA and IIB of the phosphotransferase system (PTS), respectively. The other ORF (*kdgT*) is annotated as a permease, which is responsible to transport 2-keto-3-deoxy-gluconate, the metabolite in the Entner-Doudoroff pathway. Considering the genome context of *tpi2*, it seems that *tpi2* could play important roles in the central metabolism involving glycolysis, pentose phosphate pathway, Entner-Doudoroff pathway and Vitamin B6 synthesis.

On the basis of BLAST searching results a phylogenetic tree was built to investigate the evolution relationship of *tpi1* and *tpi2* (Supplementary Fig. 1). Seventy-eight organisms are found to harbor more than one *tpi* genes, suggesting that the multiple-*tpi* event is

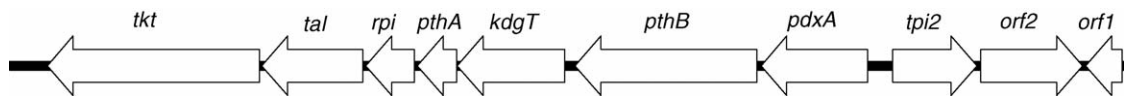


Fig. 6. The genomic context of the *tpi2* gene of *K. pneumoniae* MGH78578. *orf1*, hypothetical protein; *orf2*, putative transcriptional regulatory protein; *tpi2*, triose phosphate isomerase; *pdxA*, putative 4-hydroxythreonine-4-phosphate dehydrogenase; *pthB*, PTS system glucitol/sorbitol-specific EIIBC component; *kdgT*, 2-keto-3-deoxygluconate permease; *pthA*, PTS system glucitol/sorbitol-specific EIIA component; *rpi*, ribose 5-phosphate isomerase; *tal*, transaldolase; *tkt*, transketolase.

not rare. Among them, 36 bacteria have two or three *tpi* genes and the multiple *tpi* genes in one organism mostly fall into different subfamilies. The TPI1 of *K. pneumoniae* is classified into the same group as those TPis from gamma-proteobacteria such as *E. coli*. Whereas, the TPI2 of *K. pneumoniae* locates on the same branch of the seven TPI proteins from the alpha-proteobacteria mentioned above (*G. oxydans*, *R. rubrum*, *M. loti*, *B. abortus*, *B. melitensis*, *S. meliloti* and one megaplasmid of *Rhizobium* sp.) and two TPis from the gamma-proteobacteria (*P. multocida* and *M. succiniciproducens*). The close distance between *K. pneumoniae* TPI2 and the TPis of all the seven alpha-proteobacteria suggests that *K. pneumoniae* probably obtains the second *tpi* gene from an ancestor of alpha-proteobacteria by horizontal gene transfer. Multiple TPis in the Enterobacteriaceae strain *Erwinia carotovora* subsp. *Atroseptica* SCRI1043 and *Microbulbifer degradans* 2-40 (a marine bacterium belonging to the gamma-proteobacteria) show low similarity to the *Klebsiella* TPI2 and exhibit a different evolution pattern.

It is also noticed that all neighbor genes of the *tpi2* have (at least one) another corresponding paralog elsewhere in the chromosome of the *K. pneumoniae* MGH7858 strain (data not shown). These paralogs are more similar to the genes from *E. coli*, the close relative of *K. pneumoniae*, both at the sequence level and at the genomic context level. Therefore, these paralogs should function as those in *E. coli*. However, *E. coli* does not possess such an additional set of genes as *tpi2* and its neighbor genes in *Klebsiella*. This fact supports the conjecture that *tpi2* and its flanking region in *K. pneumoniae* originate from horizontal gene transfer.

4. Concluding remarks

The successful knockout of the *tpi1* gene in *K. pneumoniae* DSM2026 and the physiological study of mutants grown on different carbon sources led to the discovery of a novel *tpi2* gene in this bacterium. The direct enzyme assay and the finding of a highly conserved TPI signature in genome sequences of different organisms unambiguously demonstrate its enzymatic activity as triose phosphate isomerase. Moreover, the over-expression of *tpi2* gene in the $\Delta tpi1::FRT$ mutant almost restores the growth phenotype on the minimal

medium containing glucose or glycerol as the carbon source, further supporting its TPI activity. However, attempts to knock out this new, second *tpi* gene for further functional study failed although the method used worked well for the knockout of *tpi1*. To shed more light on the intrinsic function of the new discovered *tpi2* in *K. pneumoniae*, more comprehensive investigations including purification of the enzyme and biochemical assay of its function should be done in future work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [10.1016/j.jbiotec.2006.03.034](https://doi.org/10.1016/j.jbiotec.2006.03.034).

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