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Construction and Complementation of In-Frame Deletions of the Essential *Escherichia coli* Thymidylate Kinase Gene

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This work reports the construction of *Escherichia coli* in-frame deletion strains of *tmk*, which encodes thymidylate kinase, Tmk. The *tmk* gene is located at the third position of a putative five-gene operon at 24.9 min on the *E. coli* chromosome, which comprises the genes *pabC*, *yceG*, *tmk*, *holB*, and *ycfH*. To avoid potential polar effects on downstream genes of the operon, as well as recombination with plasmid-encoded *tmk*, the *tmk* gene was replaced by the kanamycin resistance gene *kka1*, encoding amino glycoside 3′-phosphotransferase kanamycin kinase. The kanamycin resistance gene is expressed under the control of the natural promoter(s) of the putative operon. The *E. coli tmk* gene is essential under any conditions tested. To show functional complementation in bacteria, the *E. coli tmk* gene was replaced by thymidylate kinases of bacteriophage T4 gp*1*, *E. coli tmk*, *Saccharomyces cerevisiae cdc8*, or the *Homo sapiens* homologue, *dTYMK*. Growth of these transgenic *E. coli* strains is completely dependent on thymidylate kinase activities of various origin expressed from plasmids. The substitution constructs show no polar effects on the downstream genes *holB* and *ycfH* with respect to cell viability. The presented transgenic bacteria could be of interest for testing of thymidylate kinase-specific phosphorylation of nucleoside analogues that are used in therapies against cancer and infectious diseases.

Metabolic pathways for the synthesis of the deoxynucleotide precursors required for DNA replication in *Escherichia coli* are well characterized (26, 40). The deoxynucleoside monophosphate kinases are universally conserved key catalysts involved in the turnover of natural nucleotides and are important in the phosphorylation of nucleoside analogues used in therapies against cancer and infectious diseases (35). Thymidylate kinases (EC 2.7.4.9) catalyze the phosphorylation of deoxythymidylate (dTMP) to the corresponding deoxythymidine diphosphate. This kinase is required after the merging of the de novo and salvage pathways of deoxythymidine triphosphate biosynthesis and is the last essential enzyme in the deoxythymidine triphosphate biosynthetic pathway of most organisms.

The *E. coli* Tmk (Swiss-Prot no. P37345) is expressed at low levels during cell growth; it acts as a dimer (25) and is known to represent ca. 0.01% of the soluble protein in *E. coli*. This is 10 to 20 times less than the most abundant nucleoside monophosphate kinase, adenylate kinase, Amk (34). The eukaryotic *Saccharomyces cerevisiae* thymidylate kinase TmpK (Swiss-Prot no. P00572) and *H. sapiens* thymidylate kinase dTYMK (Swiss-Prot no. P23919) are predominantly cytoplasmic or cell membrane bound, and their activity is cell cycle controlled (1, 16, 38). In view of the importance of dTMP kinase in the phosphorylation of nucleoside analogues (2, 15, 35), the crystal structures of *E. coli*, yeast, human, and other related monophosphate kinases were solved.

The *E. coli tmk* gene is located at 24.9 min in a putative five-gene operon on the chromosome (3, 28). The existence of a conditional-lethal, temperature-sensitive (Ts) mutant strain of *tmk* (9, 34) underlines the importance of Tmk in cellular metabolism and control of DNA replication and is estimated to be essential (9, 13, 34). It has been shown that a Ts strain of *S. cerevisiae* carrying a mutation in the thymidylate kinase gene *cdc8* can be complemented by the *E. coli tmk* gene (28). However, due to the lack of a suitable genetic system, the reverse situation could not be tested. Interestingly, the human dTYMK is able to complement the loss of dTMP kinase activity of a *S. cerevisiae cdc8* Ts strain, but the activity of the human kinase could not be purified from yeast (16). The complementation was explained by assuming a very low, yet sufficient level of human dTMP kinase activity (33). Several attempts to produce wild-type human dTMP kinase activity in *E. coli* failed, and dTYMK activity could only be efficiently detected by using a baculovirus system (16) or be purified as a fusion protein overproduced in *E. coli* (5).

In the present study, a replacement of the *E. coli tmk* gene by the kanamycin resistance (Km^r) gene *kka1*, which encodes the amino glycoside 3′-phosphotransferase kanamycin kinase (Swiss-Prot no. P00551) (EC 2.7.1.95), is presented. The Km^r gene *kka1* is expressed under the natural promoter(s) of the putative operon. The in-frame substitution construct of *tmk* shows no polar effects on the downstream genes *holB* and *ycfH* with respect to cell viability. The essential *E. coli tmk* gene was complemented by plasmids expressing either the bacteriophage T4 gene *1*, encoding a less-specific monophosphate kinase (Swiss-Prot no. P04531) (4, 6), the *S. cerevisiae* dTMP kinase gene *cdc8* (17, 30), or the human *dTYMK* homologue (20, 33). The last enzyme is of special interest, since this is the first time that the human thymidylate kinase protein dTYMK has been functionally produced as wild-type protein in bacteria in the absence of its bacterial counterpart. The presented

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TABLE 1. Strains, plasmids, and bacteriophages used or constructed in this study

Name	Genetic characteristics[a]	Reference or source
Strains		
293T	Human embryonic kidney 293T cells	A. Melotti
B178	W3110 *galE sup*$^+$	12
DY378	W3110 λ*cI-857* Δ(*cro-bioA*)	39
DC1	B178 miniTn*10* (Tc^r) linked to *tmk*	8
DC3	DY378 miniTn*10* (Tc^r) linked to *tmk*	8
DC11	B178 Δ*tmk*::*kka1* + pDC13; Km^r Ap^r	This work
DC12	B178 Δ*tmk*::*kka1* (RBS) + pDC3; miniTn*10* (Tc^r) linked to *tmk*; Km^r Ap^r	This work
DC13	DC11 miniTn*10* (Tc^r) linked to *tmk*; Km^r Tc^r Ap^r	This work
DC14	B178 Δ*tmk*::*kka1*; miniTn*10* (Tc^r) + pDC15	This work
Plasmids		
pACYC177	Ap^r Km^r; low-copy p*15A* origin	7
pFYZ1	Ap^r; low-copy *oriF* origin plasmid	18
pGP189	pSE380; 39-bp deletion	11
pHsTmpK	pGexRB; *dTYMK-1*	5
pKO3	Cm^r; low-copy pSC101 Ts origin, *sacB*	21
pMPM-A4Ω	Ap^r; high-copy pColE1 origin	23
pMPM-A6Ω	Ap^r; low-copy p15A origin	23
pScTmpK	pJC20HisC; *cdc8*	19
pSE380	Ap^r; high-copy pColE1 origin	Invitrogen
pSR1613	pWSK29; 7.1-kb DNA fragment at 24.9 min on the *E. coli* genome	S. Raina
pWSK29	Ap^r; low-copy pSC101 origin	36
pDC1	pWSK29; *pabC yceG tmk holB ycfH*	8
pDC2	pMPM-A6Ω; *holB*	8
pDC3	pMPM-A6Ω; *tmk*	8
pDC4	pMPM-A6Ω; *tmk holB*	8
pDC5	pMPM-A6Ω; *tmk holB ycfH*	8
pDC6	pDC1; *tmk* in-frame deletion	8
pDC7	pKO3; *pabC yceG holB ycfH*	8
pDC11	pDC7; Δ*tmk*::*kka1*	This work
pDC12	pDC6; Δ*tmk*::*kka1* (RBS)	This work
pDC13	pFYZ1; *pabC yceG tmk holB ycfH*	This work
pDC14	pMPM-A6Ω; T4 gene *1*	This work
pDC15	pMPM-A6Ω; *cdc8*	This work
pDC16	pMPM-A6Ω; *dTYMK-1*	This work
pDC17	pMPM-A4Ω; *dTYMK-1*	This work
pDC18	pGP189; *dTYMK-1*	This work
pDC19	pGP189; *tmk*	This work
pDC20	pGP189; *dTYMK-2*	This work
pDC21	pGP189; *tmk*, C-*myc* tag	This work
pDC22	pGP189; *dTYMK-2*, C-*myc* tag	This work
Bacteriophages		
P1*vir*	Bacteriophage P1	24
T4 Do	Bacteriophage T4 Do	D. Ang

[a] Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline.

transgenic bacteria, expressing various thymidylate kinases in a simplified cellular system, could be of interest for testing the phosphorylation of thymidine-like nucleoside analogues that are used in therapies against cancer and infectious diseases.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The various bacterial strains, plasmids, and phages used and constructed in the present study are listed in Table 1.

Bacterial media and culture conditions. Luria-Bertani (LB) medium (10 g of tryptone [Difco], 5 g of yeast extract [Difco], and 5 g of NaCl per liter; pH 7.0) was used for bacterial growth. For LB agar plates, 10 g of agar (Difco) were added per liter. For selection of antibiotic resistance, 100 μg of ampicillin, 50 μg of kanamycin, 20 μg of chloramphenicol, and 10 μg of tetracycline ml^{-1} were used as required. For the *tmk* replacement strains and plasmid constructs, 25 μg of kanamycin ml^{-1} was added. Growth was always performed at 30°C unless otherwise indicated.

Cultivation of human embryonic kidney 293T cells and mRNA preparation. Human embryonic kidney 293T cells (gift of A. Melotti) were cultivated by standard laboratory techniques using Dulbecco modified Eagle medium, fetal calf medium, and antibiotics. About 10^8 cells were centrifuged for 3 min at room temperature at 900 rpm and then kept frozen at −20°C. Using QIAGEN RNeasy mRNA mini-prep columns, mRNA of 5×10^7 293T cells was purified and diluted in 200 μl of distilled H_2O.

PCR, RT-PCR, and oligonucleotides. Standard PCR was performed with *Pfu* DNA polymerase according to the manufacturer's instructions (Promega) using a MiniCycler (MJ Research). PCR amplifications were cycled 30 times for 1 min at 94°C, 1 min at 53°C, and between 1 and 4 min at 72°C, depending on the template and the primer pair used. The human dTMP kinase gene dTYMK (*dTYMK-2*) was amplified and cloned from mRNA of human embryonic kidney 293T cells by using reverse transcription-PCR (RT-PCR) DNA amplification techniques (SuperScript one-step RT-PCR, Platinum Taq; Gibco-BRL/Life Technologies). The reaction mixture was incubated for 30 min at 50°C to allow the RT to produce cDNA from the human mRNA, followed by a 40-cycle standard PCR amplification at an annealing temperature of 53°C and an elongation time of 1 min at 72°C using primers 9 and 10.

The following PCR primers were used: primer 1 (5′-*kka1* 5′→3′; 5′-ATCTGC ATGCTAAGTTATGAGCCATATCAAC-3′), primer 2 (3′-*kka1* 3′→5′; 5′-ATC TGCATGCCATTTAGAAAAACTCATCGAGCA-3′), primer 3 (5′-*kka1* RBS 5′→3′; 5′-ATCTGCATGCTAAGGAGAATTCATATGAGCCATATTCAAC-3′), primer 4 (3′-*kka1* RBS 3′→5′; 5′-ATCTGCATGCCATATGAATTCTCCTTA GAAAAACTCATCGAGCA-3′), primer 5 (5′-T4 gene *1* 5′→3′; 5′-GGAGGA ATTCATATGAAACTAATCTTTTTAAGCG-3′), primer 6 (3′-T4 gene *1* 3′→5′; 5′-ACTATCTAGATTATAGTACCTTTAGTGTATTTT-3′), primer 7 (5′ of the operon; 5′-GTAGTGGCGGGCGAGG-3′), primer 8 (SalI *ycfH* 3′→5′; 5′-GCC AGAACGTCATCCACGTC-3′), primer 9 (5′-*dTYMK* 5′→3′; 5′-GGAGGCATG CTAAGGAGAATTCATATGGCGGCCCGGCGC-3′), primer 10 (3′-*dTYMK* 3′→5′; 5′-ACTATCTAGAGGCCGGCCTCACTTCCATAGCTCCCC-3′), primer 11 (5′-*tmk* 5′→3′; 5′-GGAGGAATTCACCATGCGCAGTAAGTATATCGT-3′), primer 12 (3′-*tmk* 3′→5′; 5′-ACGCGCATGCTCATGCGTCCAACTCCTTC-3′), primer 13 (3′-*myc dTYMK* 3′→5′; 5′-ACTATCTAGATCACAGATCCTCTTCTG AGATGAGTTTTTGTTCCTTCCATAGCTCCCCA-3′), and primer 14 (3′-*myc tmk* 3′→5′; 5′-ACTATCTAGATCACAGATCCTCTTCTGAGATGAGTTTTT GTTCTGCGTCCAACTCCTTCAC-3′). Lyophilized primers were dissolved in H_2O and stored at −20°C.

Plasmid construction. General techniques for plasmid DNA preparation, restriction enzyme manipulation, molecular cloning, and agarose gel electrophoresis were carried out as described in Sambrook and Russell (29).

The plasmids pDC11 and pDC12, used for the genomic replacement constructs to generate the Δ*tmk*::*kka1* null strains, were prepared by inserting the SphI-digested *kka1* PCR products of pACYC177 (7) (primer pair 1 and 2 for pDC11 and 3 and 4 for pDC12, respectively) into the unique SphI site of plasmids pDC7 and pDC6, respectively. Plasmid pSR1613 (a gift from S. Raina) was digested with BglII and HindIII, and a fragment of approximately 4.8-kb harboring the five-gene operon was cloned into the BamHI- and HindIII-digested vector pFYZ1 (18), resulting in pDC13.

Plasmid pDC14 was constructed by inserting the EcoRI/XbaI-digested PCR amplification product (primer pair 5 and 6) of genomic bacteriophage T4 Do DNA (gift of D. Ang) into the EcoRI/XbaI-digested vector pMPM-A6Ω (23). Plasmid pDC15 was constructed by ligating a fragment containing the *S. cerevisiae cdc8* gene from the plasmid pScTmpK (19) into pMPM-A6Ω. The above-mentioned two vectors were linearized with NdeI and EcoRI, respectively, followed by filling the ends by using T4 DNA polymerase. Plasmid pScTmpK was digested with BamHI, and pMPM-A6Ω with BglII, the appropriate fragments were gel purified and ligated together. The PCR product, using the template pHsTmpK (5) and the primers 9 and 10, was digested with EcoRI/XbaI and inserted into the EcoRI/XbaI-digested vectors pMPM-A6Ω, pMPM-A4Ω (23), or pGP189 (11), resulting in plasmids pDC16, pDC17, and pDC18, respectively. Plasmid pDC19 was constructed by ligating the EcoRI/XbaI-digested PCR product (primer pair 11 and 12, template pDC3) into the EcoRI/XbaI-digested vector pGP189. Plasmid pDC20 (primers 9 and 10) was constructed by inserting the EcoRI/XbaI-digested PCR fragments into the EcoRI/XbaI-digested vector pGP189. The cDNA product of the RT-PCR amplification of human embryonic kidney 293T mRNA served as a template for the PCRs (primer pair 9 and 10), resulting in pDC20. The plasmids overproducing C-terminal *myc* tag proteins were constructed in a similar way and are all derivatives of pGP189. Vector and PCR products were digested with EcoRI/XbaI prior to ligation. The *tmk* insertion in plasmid pDC21 was amplified from the template pDC19 using primers 11 and 14. The DNA insertion in plasmid pDC22 was amplified from pDC20 by using the primers 9 and 13. The PCR amplified thymidylate kinase genes were sequenced and proved to be identical to database sequences of *E. coli* (gb U00096.2), bacteriophage T4 (gb AF158101.6), and *H. sapiens* (*dTYMK-2*) (gb BT020055.1), respectively.

Replacement of *tmk* on the *E. coli* chromosome. Plasmid pDC11 was used for the replacement of the *tmk* gene by the *kka1* gene using the Ts-plasmid integration method as described by Link et al. (21). Strain B178 (12) was transformed with the plasmid pDC11 and selected at 43°C in the presence of chloramphenicol and kanamycin on LB agar plates. Colonies isolated at 43°C were then grown at 37°C overnight. The next day, they were diluted and grown at 37°C for the preparation of competent cells. Cultures were transformed with pFYZ1 as a control, or with the plasmid pDC13 carrying the *tmk* operon, and selected on plates containing ampicillin, chloramphenicol, and kanamycin at 37°C overnight. From overnight cultures grown at 37°C, dilutions of equal numbers of cells were spread on LB agar plates at 30°C containing 5% sucrose, kanamycin, and ampicillin. DC11 shows Km^r, ampicillin resistance (Ap^r), and sucrose resistance (Suc^r) but chloramphenicol sensitivity (Cm^s) (Table 1).

Alternatively, using the plasmid pDC12 (Δ*tmk*::*kka1* [RBS]), a second Δ*tmk*::*kka1* deletion strain containing additional RBS before and after *kka1* was constructed by linear transformation as described by Yu et al. (39) (Fig. 2b and data not shown). DC3 transformed with pDC3 in the presence of 0.05% L-arabinose was transformed with a PCR amplification product of plasmid pDC12 using the primer pair 7 and 8. P1 lysates were grown on candidates with the Δ*tmk*::*kka1* (RBS) deletion at 30°C. Cotransduction experiments using B178 previously transformed with pDC3 as recipient strain resulted in strain DC12.

Bacteriophage P1 lysates, transduction, and cotransduction. Lysate preparations and transduction were performed as described by Miller (24). A lysate grown on strain DC1 was used for the transduction of DC11 to yield strain DC13 (Table 1). The frequency of cotransduction between Δ*tmk*::*kka1* and the linked tetracycline resistance (Tc^r) marker miniTn*10* (Tc^r) was determined with lysates grown on the strains DC13 or DC14. These P1 lysates were used to infect B178 carrying various plasmids. Transductants were first selected on LB agar plates containing sodium citrate, tetracycline, ampicillin, and various concentrations of L-arabinose or IPTG (isopropyl-β-D-thiogalactopyranoside). To determine the linkage frequency, candidates were then screened on the same plates additionally containing kanamycin. A transductant of B178 previously transformed with pDC15 in the presence of 0.05% L-arabinose indicating Tc^r, Km^r, and Ap^r resulted in DC14.

Protein expression. For protein expression, overnight cultures were diluted to an optical density of 0.1 at 600 nm and cultivated at 37°C. When the cultures reached an optical density of 0.5, they were induced with 1 mM IPTG and grown for an additional 1.5 h at 37°C. Matching uninduced cultures were also grown for 1.5 h at 37°C. The cultures were centrifuged, and the pellets boiled for 10 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Standard SDS-PAGE was performed and gels were stained with Coomassie blue (29).

Western blot analysis. Western blots were prepared as described by Harlow and Lane (14). Blots were first treated with a mouse anti-*myc* tag antibody, followed by treatment with a goat anti-mouse antibody linked to horseradish peroxidase. For imaging, the SuperSignal West Pico Trail-Kit (Pierce) was used with X-ray films (Kodak).

RESULTS

Construction of Δ*tmk*::*kka1* *E. coli* strains by the plasmid-based gene replacement method. The *tmk* gene was replaced in strain B178 by the Km^r gene *kka1*, whose expression is under the control of the promoter(s) of the putative five-gene operon (Fig. 1a). Since the structure in this operon is relatively complex (Fig. 1b and c), two different replacement strains were constructed by using plasmids pDC11 and pDC12, respectively (Fig. 2). The basic strategy for the replacement of *tmk* was to construct deletion strains in which the genomic organization of the operon stays intact. In plasmid pDC11 (Fig. 2a), the Km^r gene *kka1* was flanked by short 5′ and 3′ sequences of *tmk*, which overlap with the *yceG* and *holB* genes, but has no clear RBS for the translation initiation of the Km^r gene *kka1*. Plasmid pDC12 (Fig. 2b) was identical to pDC11 except for the addition of RBS 5′ upstream and 3′ downstream of *kka1*. However, no difference in results with either of the two *tmk* replacement constructs was observed.

Plasmid pDC11 was used to replace *tmk* on the genome

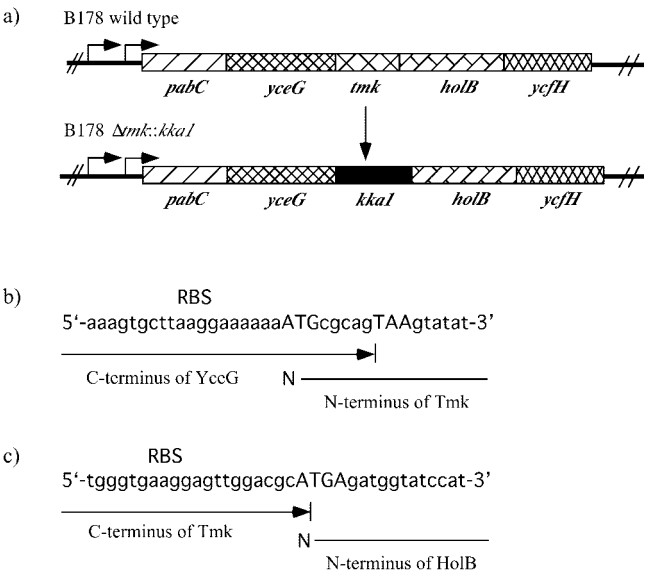

FIG. 1. (a) Construction of Δ*tmk*::*kka1* replacement strains: *pabC*, 4-amino-4-deoxy-chorismate lyase; *yceG*, gene of unknown function; *tmk*, dTMP kinase; *holB*, DNA polymerase III subunit; *ycfH*, conserved gene of uncertain function; *kka1*, Km^r gene. (b and c) Overlapping genes at 24.9 min on the *E. coli* genome and translation products of the mRNA(s) at the 5′ (b) and 3′ (c) termini of the *tmk* gene; the RBS location is based on sequence analysis (28, 31). Lines and arrows indicate translation products; capital triplets indicate start and stop codons.

following the procedure of Link et al. (21). First, pDC11 was introduced into the chromosome of B178 by selecting for both Cm^r and Km^r at 43°C. Next, the vector pFYZ1 (control) or the plasmid pDC13 (five-gene operon of *tmk*) were transformed into candidates that already had integrated the plasmid pDC11 into the genome due to its Ts *pSC101* origin of replication. Excision and loss of pDC11 was selected in the presence of sucrose, since sucrose was toxic to the cell due to the pDC11-encoded levansucrase *sacB* gene product (10). Dilutions of an equal number of transformants were plated at 30°C on LB agar plates containing 5% sucrose, ampicillin, and kanamycin. The efficiency of plating was approximately 1,000-fold lower for cells carrying the pFYZ1 control plasmid than for transformants harboring a second copy of *tmk* (pDC13). This was the first indication that the Tmk protein was essential for *E. coli*. Colonies showing Suc^r, Ap^r, and Km^r phenotypes, harboring either the control plasmid pFYZ1 or the plasmid pDC13, were tested for Cm^s. Of the pFYZ1-transformant cells, all colonies (15 of 15) indicated Cm^r and Km^r, whereas all of the pDC13-transformants (104 of 104) had efficiently lost the pDC11 plasmid and therefore showed Cm^s and Km^r. This was a further indication for *tmk* being an essential gene. Replacement of *tmk* by the *kka1* gene (Δ*tmk*::*kka1*) using the above method and the plasmid pDC11 resulted in strain DC11.

Alternatively, plasmid pDC12 was used to construct a Δ*tmk*::*kka1* strain with 5′- and 3′-RBS flanking *kka1* (Fig. 2b). Using the method of linear DNA fragment transformation as described by Yu et al. (39), the construction of the Δ*tmk*::*kka1* (RBS) was only possible in the presence of a second, plasmid-encoded *tmk* (pDC3). The second procedure resulted in strain DC12.

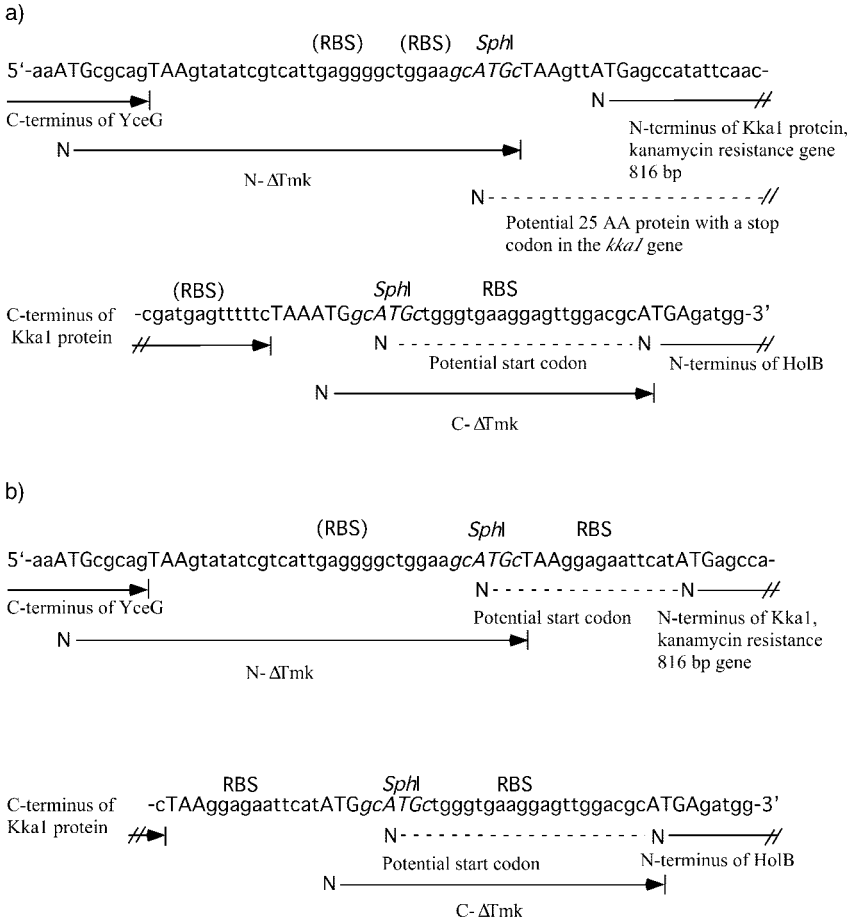

FIG. 2. DNA sequences present on plasmid pDC11 (a) and plasmid pDC12 (b), as well as their translational products: sequence in italics, SphI site; (RBS) or RBS, potential RBS or RBS based on sequence analysis (31). Lines and arrows indicate translation products. Capital triplets represent start and stop codons. Dashed lines represent potential translational products.

Cotransduction experiments with the Δ*tmk*::*kka1* construct. To independently confirm the essential role of *tmk*, the miniTn*10* (Tc^r) marker in DC1 (8) was transduced into DC11 by bacteriophage P1 transduction, selecting for Km^r, Ap^r, and Tc^r. This resulted in strain DC13. Phage P1 lysates grown on DC13 or DC14 were used to infect *E. coli* B178 strains, previously transformed with various L-arabinose-inducible plasmids harboring parts of the genomic region of *tmk*. This was done in the presence of 0.05% L-arabinose and at 30°C (Table 2). For the Δ*tmk*::*kka1* replacement construct (Fig. 2a), cotransductants were isolated only in the presence of a second copy of *tmk* at an average frequency of 25% (96 of 390 candidates). In the absence of a second *tmk* gene, no cotransduction was observed (0 of 322 candidates). The cotransduction experiments confirmed that the Tmk protein was absolutely essential and indicated no severe polarity effects of the replacement construct on viability due to altered expression of the up- or downstream genes *yceG*, *holB*, and *ycfH*. The variable level of gene expression of the complementing plasmid constructs may explain the difference in the frequency of cotransduction (22 to 43%) compared to the expected 40% cotransduction between the linked miniTn*10* (Tc^r) and a *yceG*::miniTn*10* (Km^r) marker insertion in the gene *yceG* upstream of *tmk* in the operon (Fig. 1) (8, 27). Using two independent Δ*tmk*::*kka1* replacement constructs (Fig. 2a and b), no difference in the cotransduction frequency was observed (data not shown). The deletions of *tmk* and its replacement by *kka1* were confirmed by PCR amplification, as well as by Southern blot analysis (data not shown).

Complementation of the genomic *E. coli tmk* gene by plasmid-encoded thymidylate kinases of bacteriophage T4, *E. coli*,

TABLE 2. Frequencies of cotransduction between the miniTn*10* (Tc^r) marker and the Δ*tmk*::*kka1* (Km^r) marker[a]

Plasmid	Gene(s)	Frequency of cotransduction
pMPM-A6Ω	Control	0 (277)
pDC2	*holB*	0 (45)
pDC3	*tmk*	28 (39)
pDC4	*tmk*, *holB*	22 (311)
pDC5	*tmk*, *holB*, *ycfH*	43 (40)
tmk on complementing plasmids		25 (390)
Control, without *tmk*		0 (322)

[a] Strain B178 transformed with various L-arabinose-inducible plasmids was transduced in the presence of 0.05% L-arabinose at 30°C with phage P1 lysates grown on DC13. In general, ca. 40 colonies were tested, and higher numbers are total numbers of different experiments. Numbers indicate calculated frequency of cotransduction in percent, followed in parentheses by the numbers of candidates tested.

TABLE 3. Complementation of the *E. coli tmk* gene by homologues of various origins[a]

Plasmid	Gene	Frequency of cotransduction (0.05% arabinose at 30°C)
pMPM-A6Ω	Control	0 (26)
pDC3	*E. coli tmk*	29 (28)
pDC14	T4 gene *1*	29 (28)
pDC15	*S. cerevisiae cdc8*	16 (38)

[a] Complementation of the Δ*tmk*::*kka1* allele by plasmid-encoded homologues of dTMP kinases from bacteriophage T4, *E. coli*, and *S. cerevisiae.* B178 strain transformed with various L-arabinose-inducible plasmids was transduced in the presence of 0.05% L-arabinose at 30°C with a phage P1 lysate grown on DC13. Numbers indicate the calculated percent frequency of cotransduction, followed in parentheses by the numbers of candidates tested.

TABLE 4. Complementation of the *E. coli tmk* gene by human thymidylate kinase gene *dTYMK*[a]

Plasmid	Gene	Frequency of cotransduction (IPTG, 37°C) at:		
		0 mM	0.1 mM	1 mM
pGP189	Control	0 (40)	0 (28)	0 (140)
pDC19	*tmk*	15 (41)	27 (37)	23 (70)
pDC18/pDC20	*dTYMK*	0 (44)	27 (70)	25 (276)

[a] Complementation of *E. coli tmk* by the human thymidylate kinase gene *dTYMK.* B178 transformed with IPTG-inducible plasmids was transduced in the presence of various concentrations of IPTG at 37°C with phage P1 lysates grown on DC14. Numbers indicate the calculated percent frequency of cotransduction, followed in parentheses by the numbers of candidates tested.

and *S. cerevisiae*. Several expression systems and vectors of various copy numbers were tested for the expression of genes presumed to complement the *E. coli tmk* gene. Since *tmk* is expressed at low levels (34), these genes were generally first cloned into the low-copy-number plasmid pMPM-A6Ω (*p15A* origin of replication) and tightly regulated by the L-arabinose-inducible promoter (23). If the complementation of genomic *E. coli tmk* was not successful, they were cloned into the L-arabinose-inducible high-copy-number vector pMPM-A4Ω (*pUC*-like origin of replication) (23). Later, the genes of highest interest were cloned into a derivative of the IPTG-inducible vector pSE380 (Invitrogen) using the restriction enzymes EcoRI and XbaI. The derivative of pSE380, called pGP189 (11), has a 39-bp deletion near the *lac* promoter site of pSE380. This deletion replaces the NcoI site of pSE380 by an EcoRI site, so that genes originally cloned into pMPM-A6Ω have an optimal translation initiation in pGP189.

Plasmids pMPM-A6Ω (control), pDC3 (*tmk*), pDC14 (T4 gene *1*) and pDC15 (*cdc8*) were transformed into B178, and the resulting strains were used as recipients in cotransduction experiments in the presence of 0.05% L-arabinose. Table 3 indicates that thymidylate kinase activity of the bacteriophage T4 gene *1*, *E. coli tmk*, and *S. cerevisiae cdc8* can complement the absence of the genomic *E. coli tmk* gene. The control plasmid pMPM-A6Ω gave no cotransduction. The absence of genomic *E. coli tmk* was confirmed by PCR analysis. A B178 Δ*tmk*::*kka1* candidate complemented with the *S. cerevisiae cdc8* gene (pDC15) resulted in DC14 and was further used for the preparation of lysates for cotransduction experiments. In contrast to DC11, which harbors the *tmk* operon of plasmid pDC13, a recombination of the genomic Δ*tmk*::*kka1* construct with the plasmid-encoded yeast *cdc8* of pDC15, can be excluded in DC14. All following work was therefore done with the Δ*tmk*::*kka1* construct present in DC14.

Complementation by the human dTMP kinase gene *dTYMK*. The human *dTYMK* gene was subcloned from pHsTmpK (*dTYMK-1*) (5) to create plasmids pDC16, pDC17, and pDC18, existing at various copy numbers per cell. To prove that the complementation activity of the human dTMP kinase is not allele specific, a second allele of the human *dTYMK* gene was amplified from mRNA of human embryonic kidney 293T cells and cloned into pGP189 resulting in pDC20 (*dTYMK*-2). Indeed, the two dTYMK proteins proved to be identical in their amino acid sequence.

To test whether the human *dTYMK-1* gene can complement the Δ*tmk*::*kka1* allele, *E. coli* B178 transformed with various plasmids was cotransduced by using a phage P1 lysate grown on DC14 in the presence of either 0.05 or 0.5% L-arabinose. The presence of plasmid pDC3 (containing *E. coli tmk*) allows deletion of *tmk* at a concentration of 0.05% L-arabinose, whereas cotransduction was not observed in the presence of pDC16 (human *dTYMK-1*) (data not shown). Therefore, the human dTMP kinase gene was cloned into the high-copy-number vector pMPM-A4Ω, resulting in plasmid pDC17 (*dTYMK-1*). This plasmid was transformed into B178, and cotransduction experiments were carried out as before. Plasmid pDC17 (*dTYMK-1*) allowed deletion of *tmk* at 0.5% L-arabinose. Since this concentration of L-arabinose is rather high, the two human *dTYMK* alleles (expressed from pDC18 and pDC20) and the *E. coli tmk* gene (pDC19) were cloned into the IPTG-inducible high-copy-number vector pGP189. These plasmids were then transformed into B178, which was again cotransduced. The plasmids pDC18 (*dTYMK-1*) and pDC20 (*dTYMK-2*) were able to complement the Δ*tmk* allele in the presence of 0.1 mM IPTG (Table 4). Plasmid pDC19 (*tmk*) complemented even in the absence of any inducer since its promoter is leaky. The replacement of *tmk* on the chromosome was again confirmed by PCR analysis (data not shown). The data resulting from these experiments confirm the basic proposition that *E. coli* may grow with dTMP kinase activity of various origins.

Overexpression of *E. coli* and human dTMP kinases and Western blot analysis. Strain B178 was transformed with plasmids pGP189 (control), pDC19 (*tmk*), pDC18 (*dTYMK-1*), and pDC20 (*dTYMK-2*). Cultures were induced with IPTG, and total cellular protein extracts were analyzed by SDS-PAGE. Whereas *E. coli* Tmk was visible as a band of approximately 28 kDa (28, 34), the human kinases could not be detected by Coomassie blue staining (data not shown).

After induction by IPTG, B178 carrying plasmids pGP189 (control), pDC21 (*tmk*-C-*myc*) and pDC22 (*dTYMK-2*-C-*myc*) were prepared for Western blot analysis, detecting expressed protein with an anti-*myc*-tag antibody. The bacterial Tmk could be visualized even without IPTG induction, whereas the human protein appeared only as a weak band upon induction with IPTG (Fig. 3).

DISCUSSION

Construction of a *tmk* deletion strain. Up to now, no *E. coli tmk* deletion strain has been constructed. Such a strain is

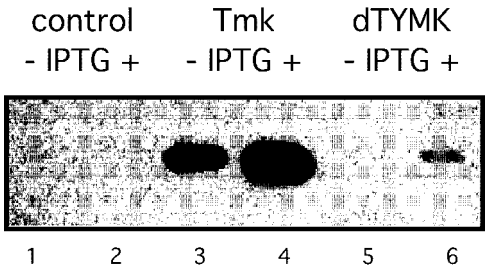

FIG. 3. Western blot analysis of *E. coli* Tmk and human dTYMK kinases modified with a C-terminal *myc* tag. B178 transformed with various high-copy-number vectors was grown in the presence of 1 mM IPTG as described in Materials and Methods. The proteins were detected by a primary antibody specific for the C-terminal *myc*-tag followed by a secondary horseradish peroxidase-linked antibody for imaging. Lanes: 1, pGP189 (control) without IPTG; 2, pGP189 (control) with 1 mM IPTG; 3, pDC21 (*tmk*-C-*myc*) without IPTG; 4, pDC21 (*tmk*-C-*myc*) with 1 mM IPTG; 5, pDC22 (*dTYMK-2*-C-*myc*) without IPTG; 6, pDC22 (*dTYMK-2*-C-*myc*) with 1 mM IPTG.

thought to be a useful tool for complementation studies using thymidylate kinases that might be of medical or biotechnological interest. The *tmk* gene is located in the third position of a putative five-gene operon, and therefore a simple insertion inactivation by a cassette encoding an antibiotic resistance is not possible. The *ycfH* gene was proposed to be nonessential (37), but *holB*, the gene next to *tmk* downstream in the operon, is essential (8, 32). Therefore, the goal of the present study was to replace the *tmk* gene by a genetic marker without influencing the expression of up- or downstream genes. Although a conditional lethal mutant of *tmk* already existed, this Ts *tmk* strain has a point mutation (G146A) in the *tmk* gene and loses its essential activity only at 42°C (9, 34). The fact that thymidylate kinase is a key enzyme for phosphorylation of thymidine nucleotides underlines the importance of constructing a stable null mutant of *tmk*, excluding the possibility of true revertants.

***E. coli tmk* is an essential gene and can be complemented with thymidylate kinases of various origins.** I obtained evidence for an essential function of the *E. coli tmk* gene product by various experiments: the first Δ*tmk*::*kka1* replacement strain was constructed according to the method of Link et al. (21). This method tests the frequency of excision and loss of the integrated plasmid harboring the Δ*tmk*::*kka1* construct or the *tmk* gene. The frequency was approximately 1,000-fold higher for the strains carrying pDC13 (five-gene *tmk* operon) compared to the one with the control vector pFYZ1. In addition, the colonies thus obtained were tested for loss of chromosome-released plasmid by checking for Cm^s. All of the pFYZ1 (control), but none of the pDC13 (five-gene *tmk* operon) colonies were Cm^r, Km^r, and Suc^r. This also indicates that *tmk* is essential, since without a plasmid-encoded copy, *tmk* cannot be deleted from the chromosome. In a second independent approach, using the linear transformation system of Yu et al. (39), a replacement of *tmk* could only be achieved in the presence of a second copy of *tmk*. Finally, cotransduction of a nearby miniTn*10* (Tc^r) marker with the Δ*tmk*::*kka1* (Km^r) marker solely occurred in the presence of a second copy of *tmk*, underlining again the essential role of the Tmk protein in *E. coli*. Furthermore, cotransduction experiments were repeated in various other genetic backgrounds with similar results (data not shown).

In addition, plasmids overproducing thymidylate kinase activity of bacteriophage T4, *S. cerevisiae* or human origin could complement the *tmk* deletion; however, under different conditions. In the case of the human dTMP kinase, various explanations can be given for the low overproduction of human dTYMK protein. Since *dTYMK* does not have an unusually rare codon usage, this may not be inferred as a reason for poor overproduction. The human protein may not be correctly folded in *E. coli* and gets degraded or, for proper functioning, the kinase may have to undergo posttranslational modifications. Also, the *dTYMK* mRNA could be unstable in bacteria, or the human protein contains signals for its degradation or export out of *E. coli*. Furthermore, the human dTYMK protein may only be stable and active in supra-molecular complexes, such as the proposed "metabolon" multienzyme complex (22).

Advantages of a *tmk* deletion construct. The *E. coli* Δ*tmk*::*kka1* replacement strains presented in this study are clearly advantageous compared to conditional-lethal mutants. For example, the Ts strain of Daws and Fuchs (9) can only be used for complementation studies at high temperature such as 42°C. Although the *tmk* Ts allele of this strain does not support *E. coli* growth at high temperature, this does not exclude that there could be residual, yet insufficient Tmk activity to allow *E. coli* to grow at high temperature (9). Most likely, if this strain was used to test complementation by homologues from other species, it would not be clear whether the residual *E. coli tmk* does, or does not, play a role in providing the cell with mutant dTMP phosphorylation activity. It is noteworthy that the Tmk(G146A) mutant enzyme present in this *E. coli* Ts strain (9) is not characterized by lower thermal stability but by significantly lower catalytic efficiency that is thought to be insufficient to support growth at high temperature (34). Furthermore, complementation studies with bacterial Tmks could lead to the formation of heterodimers of the endogenous Ts *E. coli* kinase and the complementing kinase, restoring the function of the otherwise inactive Tmk(G146A) mutant. Moreover, since the human thymidylate kinase is able to complement the genomic *tmk* deletion at 37°C, but not at 42°C (data not shown), the *E. coli* Ts strain is not suitable for complementation studies based on the human enzyme. Therefore, the *tmk* replacement strain (B178 Δ*tmk*::*kka1*) has significant advantages in any in vivo selection strategies, since there is no residual *E. coli* Tmk activity present and revertants can be excluded. Indeed, this is the first time that the wild-type human thymidylate kinase *dTYMK* has been functionally expressed in *E. coli* in the complete absence of the *E. coli tmk* gene. The described recombinant bacteria, overproducing bacteriophage, bacterial, fungal or mammalian thymidylate kinases from plasmids, will be of interest for testing in a simplified cellular system effects of thymidine-like nucleoside analogues that are used in therapies against cancer and infectious diseases.

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