A method for the construction of in frame substitutions in operons: Deletion of the essential *Escherichia coli holB* gene coding for a subunit of the DNA polymerase III holoenzyme

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Abstract

To investigate the putative five-gene operon at 24.9 min on the *Escherichia coli* genome, which comprises the genes *pabC*, *yceG*, *tmk*, *holB* and *ycfH*, a method for the construction of an in frame deletion strain of the essential *E. coli holB* gene was developed. HolB, also referred to as delta prime or δ', is a subunit of the DNA polymerase III (Pol III) holoenzyme. The *holB* gene was replaced by the kanamycin resistance gene *kka1*, coding for amino glycoside 3′-phosphotransferase kanamycin kinase. The kanamycin resistance gene was expressed under the control of the promoter(s) of the putative five-gene operon. The *holB* gene is essential for bacterial growth and the deletion of *holB* exhibits no polar effects on the adjacent genes *tmk* or *ycfH* in terms of cell viability. The method of the *holB* null construction presented in this work allows for a simplified studying of interactions between the different subunits of DNA polymerase III.
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1. Introduction

The *E. coli holB* gene product designated HolB, delta prime or δ', is a subunit of the *E. coli* DNA polymerase III. The DNA polymerase III (Pol III) holoenzyme is essential for bacterial growth and is highly processive. It is responsible for the major part of DNA synthesis during the replication of the *E. coli* chromosome and is composed of at least 10 subunits (α, ε, θ, τ, γ, δ, δ', χ, ψ, and β) (Kornberg and Baker, 1991; McHenry, 2003). HolB is thought to stimulate the DNA-dependent ATPase activity in the so-called clamp loading complex (Carter et al., 1993; O'Donnell et al., 2001; Onrust and O'Donnell, 1993). However, in vitro studies showed that replication activity can be reconstructed without HolB. HolB is supposed to stimulate the assembly of the subunits of the DNA polymerase and to increase the ATPase activity of the γ complex (Onrust et al., 1991). Recent results indi-

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cate an essential role of HolB in DNA synthesis (Song et al., 2001).

The *holB* gene is located downstream of the *tmk* gene, coding for thymidylate kinase at 24.9 min on the *E. coli* chromosome. The *holB* gene was first identified using a reverse genetic approach (Carter et al., 1993) and found to be 1002 nucleotides long. Sequencing data showed a ribosomal binding site (RBS), a putative promoter site, and overlap of the *holB* structural gene and the *tmk* gene. The *holB* gene codes for a 36.9 kDa protein and its mRNA shows a rare codon usage, indicating a low HolB overexpression level (Konigsberg and Godson, 1983). Two HolB variants of similar protein mass appear on SDS-PAGE when detected with antibodies against the holoenzyme of DNA polymerase III (Dong et al., 1993). HolB has sequence similarity to other prokaryotic HolB proteins, to the *dnaX* gene products of *E. coli* (Dong et al., 1993; Flower and McHenry, 1986), to the replication factor C of HeLa cells (Chen et al., 1992), and to the gene *44* product of bacteriophage T4 (Spicer et al., 1984). Although the homology to the last two proteins is rather low, they are all involved in DNA replication.

In this work, a method for the construction of a *holB* deletion strain was established in which the *holB* gene has been replaced by the kanamycin resistance gene *kka1*. Co-transduction experiments indicate that *holB* is essential for growth of *E. coli*. No polarity effects on viability due to the deletion construct were observed under the conditions tested. The *holB* deletion construct presented in this report allows a simplified studying of interactions of the components of the clamp loading complex, or of *holB* homologues from other organisms. This article describes a method of gene substitutions in operons in the presence of a plasmid-encoded gene, facilitating the introduction of selectable markers in essential genes for complementation experiments.

2. Materials and methods

2.1. Bacterial strains, plasmids and phages, media and culture conditions

The bacterial strains, plasmids, and phages used and constructed during the course of this study are listed in Table 1. LB medium containing 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl per liter, pH 7.0, was used for bacterial growth. For LB agar plates, 10 g of agar was added per liter of medium. For selection of antibiotic resistance in liquid and solid medium, 100 μg/ml ampicillin, 25 μg/ml kanamycin, 10 μg/ml chloramphenicol and 10 μg/ml tetracycline were used. Cells were grown at 37 °C with the exception of strains harboring the insertion *yceG*::miniTn*10* (Kan^r) or when otherwise indicated.

2.2. PCR, oligonucleotides and plasmid construction

Polymerase chain reactions were performed with *Pfu* DNA polymerase according to the manufacturer's protocol (Promega, Madison, WI, USA). The plasmid pSR1613 (Table 1) served as template in the PCR reactions and for the amplification of the kanamycin resistance gene *kka1*, the plasmid pACYC177 was used. The PCR fragment used in linear DNA transformation was obtained using plasmid pDC10 as template.

DNA sequences of PCR primers used in this work: Primer 1 (N-*tmk* 5′→3′): 5′-GGAGGAATTCAC-CATGCGCAGTAAGTATATCGT-3′, primer 2 (C-*tmk* 3′→5′): 5′-ACGCGCATGCTCATGCGTCCAAC-TCCTTC-3′, primer 3 (N-*holB* 5′→3′): 5′-GGA-GGAATTCACCATGAGATGGTATCCATGGTTA-3′, primer 4 (C-*holB* 3′→5′): 5′-ACGCGCATGCTTAA-AGATGAGGAACCG GTA-3′, primer 5 (5′ of the operon): 5′-GTAGTGGCGGGCGAGG-3′, primer 6 (N-*tmk* 3′→5′): 5′-ATCTGCATGCTTCCAGCCCCT-CAATG-3′, primer 7 (C-*tmk* 5′→3′): 5′-ATCTG-CATGCTGGGTGAAGGAGTTGG-3′, primer 8 (*SalI* *ycfH* 3′→5′): 5′-GCCAGAACGTCATCCACGTC-3′, primer 9 (N-*holB* 3′→5′): 5′-ATCTGCATGCT-CTCATGCGTCCAACTC-3′, primer 10 (C-*holB* 5′→3′): 5′-ATCTGCATGCCTTTAAGAGAGACAT-CATGTTTT-3′, primer 11 (universal primer): 5′-TAA-TACGCTCACTATAGGG-3′, primer 12 (N-Kan 5′→3′): 5′-ATCTGCATGCTAAGTTATGAGCCA-TATTCAAC-3′, primer 13 (C-Kan 3′→5′): 5′-ATC-TGCATGCCATTTAGAAAAACTCATCGAGCA-3′, primer 14 (C-*yceG* 5′→3′): 5′-ATCTGCATGCCCA-GGTGCGATAGCGA-3′. Lyophilized primers synthesized by Invitrogen Ltd. (Paisley, UK) were dissolved in H$_2$O and stored at −20 °C.

Table 1
Strains, plasmids and phages used and constructed in this study

Name	Description	Source/reference
Strains		
B178	W3110*galEsup$^+$*	Georgopoulos, 1971
DC1	B178/λ	Gift of D. Ang
DH5α	F′ / *endA1 hsdR17* ($r_K^-m_K^-$) *glnV44 thi-1 recA1 gyrA* (NalR) *relA1* Δ(*lacIZYA-argF*) *U169 deoR* (Φ*80dlac*Δ(*lacZ*)*M15*)	Woodcock et al., 1989
DY378	W3110 *λcI857* Δ(*cro-bioA*)	Yu et al., 2000
SR749	*E. coli yceG*::miniTn*10* (Kan^r)	Gift of S. Raina
DC2	B178 miniTn*10* (Tet^r) linked to *holB*	This work
DC3	DY378, miniTn*10* (Tet^r) linked to *holB*	This work
DC4	DC3 with pMPM-A6Ω	This work
DC5	DC3 with pDC2	This work
DC6	DC5, Δ*holB::kka1*	This work
DC7	B178, Δ*holB*, with pDC2, Tet^r, Kan^r	This work
Plasmids		
pACYC177	Amp^r, Kan^r, low copy *p15A* origin	Chang and Cohen, 1978
pKO3	Cm^r, low copy *pSC101* Ts origin, *sacB*	Link et al., 1997
pMPM-A6Ω	Amp^r, low copy *p15A* origin	Mayer, 1995
pSR1613	pWSK29 with a 7.1 kb DNA fragment at 24.9 min on the *E. coli* genome	Gift of S. Raina
pWSK29	Amp^r, low copy *pSC101* origin	Wang and Kushner, 1991
pDC1	pWSK29, *pabC, yceG, tmk, holB, ycfH*	This work
pDC2	pMPM-A6Ω, *holB*	This work
pDC3	pMPM-A6Ω, *tmk*	This work
pDC4	pMPM-A6Ω, *tmk* and *holB*	This work
pDC5	pMPM-A6Ω, *tmk, holB* and *ycfH*	This work
pDC6	pDC1, *tmk* in frame deletion	This work
pDC7	pKO3, *pabC, yceG, holB, ycfH*	This work
pDC8	pWSK29, *pabC, yceG, tmk* and *ycfH*	This work
pDC9	pKO3, *pabC, yceG, tmk* and *ycfH*	This work
pDC10	pDC9, *kka1* in *Sph*I	This work
Phages		
P1*vir*	Bacteriophage P1 for transductions	Our collection
λ1098	λ hop miniTn*10* (Tet^r)	Way et al., 1984

General techniques for plasmid DNA preparation, restriction enzyme manipulation, molecular cloning, and agarose gel electrophoresis were carried out by standard protocols. Restriction endonucleases and enzymes for DNA modification were purchased from New England Biolabs (Beverly, MA, USA) and Boehringer Mannheim (Roche Diagnostics, Germany). Transformations and plasmid preparations were done in DH5α (Woodcock et al., 1989). The plasmid pSR1613, a pWSK29-based low copy number vector (Wang and Kushner, 1991), contains a 7.1 kb fragment spanning the region at 24.9 min on the *E. coli* genome (gift of S. Raina). Cloning of the *Bgl*II/*Hin*dIII fragment of pSR1613 into the unique *Bam*HI/*Hin*dIII sites of pWSK29 created plasmid pDC1. The plasmids pDC2, pDC3 and pDC4 were generated by insertion of the *Eco*RI- and *Sph*I-digested PCR products resulting from amplification using the primer pairs 1 and 2, 3 and 4, and 1 and 4, respectively, into the *Eco*RI/*Sph*I-digested, arabinose-inducible vector pMPM-A6Ω (Mayer, 1995). Plasmid pDC5 was engineered by the insertion of the *Nhe*I/*Hin*dIII fragment harboring *ycfH* of the plasmid pDC1 into the *Nhe*I/*Hin*dIII-digested plasmid pDC4, expressing *tmk*, *holB* and *ycfH* under the arabinose-inducible *pBAD* promoter. Plasmid pDC6 was constructed by ligation of the PCR fragments obtained with the primers 5 and 6 (digested with *Aat*II/*Sph*I) and primers 7 and 8 (digested with *Sph*I/ *Nhe*I) into the *Aat*II/*Nhe*I-digested plasmid pDC1. The *Bgl*II/*Xho*I fragment containing the *tmk* deletion construct of pDC6 was inserted into the *Bam*HI/ *Sal*I-digested vector pKO3 (Link et al., 1997) resulting in plasmid pDC7. Plasmid pDC8 was constructed by ligation of the PCR products of primers 5 and 9 (*Xho*I/*Sph*I) and primers 10 and 11 (universal primer) (*Kpn*I/*Sph*I) into pDC1, digested with *Kpn*I and *Xho*I. Plasmid pDC9 resulted from the insertion of the *Rsr*II/*Ase*I fragment of pDC8 into the *Rsr*II/*Ase*I-digested plasmid pDC7. Plasmid pDC9 contains a 2100 bp homology region at the 5′ end of the *holB* gene and 700 bp at the 3′ end around the *holB* deletion. Plasmid pDC10 was constructed by introducing the *Sph*I digested PCR product (kanamycin resistance gene *kka1*) of pACYC177 (primers 12 and 13) into the unique *Sph*I site of plasmid pDC9, followed by selection for Kan^r.

2.3. MiniTn10 tetracycline resistance (Tet^r) marker linked to holB

Preparations of bacteriophage P1-lysates and transductions were performed as described (Miller, 1972). A Tet^r marker linked to *tmk* was isolated using the λ1098 bacteriophage (Way et al., 1984) to create a library of miniTn*10* (Tet^r) insertions in strain SR749 (*yceG*::miniTn*10* (Kan^r), gift of S. Raina), selecting for both Tet^r and Kan^r colonies. A P1-lysate was grown on the pooled library of Tet^r and Kan^r candidates of previously transduced SR749 and used to simultaneously transduce the two markers into DC1, strain B178 harboring a λ−prophage (B178/λ, gift of D. Ang). Seven Tet^r and Kan^r transductants of DC1 were used for the preparation of P1-lysates and tested further to determine their co-transduction frequency. B178 was transduced with the lysates of the candidates, selecting first for Kan^r, followed by screening for Tet^r. This procedure resulted in strains with co-transduction frequencies between 6% and 72% (Tet^r and Kan^r), which have both markers, a miniTn*10* (Tet^r) insertion linked to *holB* and the *yceG*::miniTn*10* (Kan^r) marker. DC2 with a co-transduction frequency of about 40% was used for further experiments (Table 1, Fig. 1, data not shown).

2.4. Construction of holB replacement deletion in the E. coli genome

The deletion was done by linear transformation as described (Yu et al., 2000). As a first step, the strain DY378 was transduced at 30 °C with a P1-lysate grown on DC2 to introduce the miniTn*10* (Tet^r) marker next to *holB*. The resulting strain DC3 was further transformed at 30 °C with the plasmid pMPM-A6Ω

Table 2
Linear transformation for the construction of the ΔholB::kka1 strain and co-transduction experiments

a)

Plasmids	Control (no DNA)	Control (Kan^r) non-essential	PCR ΔholB::kka1
pMPM-A6Ω, control, DC4	−	+	−
pDC2, *holB*, DC5	−	+	+

b)

Plasmids	P1-lysate grown on DC6 (ΔholB::kka1)
pMPM-A6Ω (control)	0/140=0%
pDC2 (*holB*)	44/148=30%
pDC3 (*tmk*)	0/36=0%
pDC4 (*tmk*, *holB*)	14/34=41%
pDC5 (*tmk*, *holB*, *ycfH*)	12/34=35%

a) Transformation of strains DC4 and DC5 was performed without DNA, with PCR amplified DNA from a non-essential kanamycin resistance construct, or with the 1600 bp PCR product from pDC10 (ΔholB::kka1). DC4 is DC3 (DY378, miniTn*10* (Tet^r) linked to *holB*) transformed with pMPM-A6Ω (control); DC5 is DC3 (DY378, miniTn*10* (Tet^r) linked to *holB*) transformed with pDC2 (arabinose-inducible second copy of *holB*). (−)= no transformants, (+) transformants. b) Co-transduction frequencies between ΔholB::kka1 and the nearby miniTn*10* (Tet^r) marker: B178 carrying various arabinose-inducible plasmids was co-transduced with a P1-lysate grown on DC6 (ΔholB::kka1) in the presence of 0.05% L-arabinose at 30 °C. In general, about 40 colonies were tested in each experiment; higher numbers are total numbers of different experiments. Transductants were first selected for Tet^r, followed by screening for both Kan^r and Tet^r. Fractions indicate number of Tet^r and Kan^r/number of Tet^r transductants, followed by the calculated co-transduction frequency in %.

(control) and with pDC2 (*holB*) to give strains DC4 and DC5. PCR amplification of the kanamycin resistance construct ΔholB::kka1 was performed on plas-
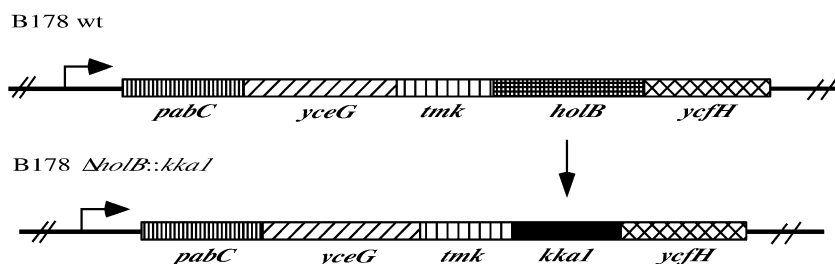

Fig. 1. Construction of the *holB* replacement strains: *pabC*=4-amino-4-deoxy-chorismate lyase, *yceG*=gene of unknown function, *tmk*=thymidylate kinase, *holB*=subunit of DNA polymerase III, *ycfH*=conserved gene of uncertain function, *kka1*=kanamycin resistance gene.

mid pDC10 (primers 8 and 14). Besides the kanamycin resistance gene, the PCR product contains 600 bp of DNA located upstream of *holB* and 100 bp of DNA located downstream. After digestion with *Dpn*I, the purified PCR product was used for transformation of strains DC4 and DC5. As a control, no DNA or PCR-amplified DNA from a non-essential kanamycin insertion strain was used (gift of P. Genevaux). Kanamycin resistant colonies were selected at 30 °C on LB agar plates containing ampicillin, kanamycin, tetracycline and 0.05% L-arabinose. Table 2a shows data of the linear transformations of DC4 and DC5 depending on the DNA fragment used. This resulted in strain DC6.

2.5. Co-transduction experiments

A bacteriophage P1-lysate was grown on DC6 and was used to infect B178 harboring various plasmids. Transductants were first selected on LB agar plates containing 10 mM sodium citrate, tetracycline, ampicillin and 0.05% L-arabinose. In a second step, transductants were screened on LB-agar plates containing 10 mM sodium citrate, kanamycin, tetracycline, ampicillin and 0.05% L-arabinose to determine co-transduction frequency between the nearby marker (Tet^r) and the deleted *holB* gene (Kan^r). Co-transduction was only observed in the presence of a second plasmid-encoded *holB* gene (Table 2b). A Tet^r and Kan^r candidate of B178 transformed with pDC2 resulted in strain DC7.

3. Results

To generate a Δ*holB*::*kka1 E. coli* strain, a set of plasmids was constructed as described in Table 1. The *E. coli holB* gene was replaced by the kanamycin resistance gene *kka1*, which is expressed under the control of the promoter(s) of the putative five-gene operon (Fig. 1). The gene *kka1* was cloned into the unique *Sph*I site of pDC9, the ligation transformed into DH5α and selected for Kan^r. The resulting plasmid pDC10 expresses the kanamycin resistance from the promoter(s) of the five-gene operon. Thus, in the plasmid pDC10 and later in the *E. coli* chromosome, no additional ribosomal binding sites (RBS) (Shine and Dalgarno, 1974) for *kka1* and *ycfH* were introduced (Fig. 2c and d).

The starting strain DY378 was transduced with the miniTn*10* (Tet^r) marker of strain DC2, resulting in strain DC3. The gene deletion experiment was performed with DC3 by linear transformation (Yu et al., 2000), using either the control plasmid pMPM-A6Ω (DC4) or the arabinose-inducible plasmid pDC2 (DC5), carrying a second copy of the *holB* gene. The strains containing the nearby Tet^r marker and a plasmid were then transformed with a linear DNA fragment that was obtained by PCR amplification of the kanamycin resistance gene insertion in *holB* present on plasmid pDC10. Additionally, negative (without DNA) and positive controls (non-essential Kan^r insertion, gift of P. Genevaux) were performed. In the transformations with the positive control (non-essential Kan^r insertion) (Table 2a), both strains, DC4 (pMPM-A6Ω, control) and DC5 (pDC2, arabinose-inducible *holB* gene), showed Kan^r linear transformants (+). For the transformation of the PCR amplified insertion construct in *holB*, only Kan^r linear transformants were observed for DC5 (pDC2, arabinose-inducible *holB* gene),(+), but not for DC4 (pMPM-A6Ω, control), (−). Without DNA, no transformants were observed (−). The number of positive (+) transformants varied from a few to several thousand colonies depending on the quality and amount of DNA, the electro-competent strains as well as the electroporation chambers used. However, no transformants were observed in cases indicated by (−) in Table 2a.

This experiment showed that a plasmid-encoded copy of *holB* was required in order to delete *holB*, demonstrating an essential role of the HolB protein in cell growth (Table 2a). These experiments resulted in strain DC6. Since the plasmid pDC10 can also be used for genomic recombinations using the plasmid pK03 system (Link et al., 1997), another recombination experiment under similar conditions was performed, confirming the result that *holB* is an essential gene (data not shown).

A P1-lysate grown on strain DC6 was used to transduce strain B178, previously transformed with arabinose-inducible plasmids harboring different genes of the *holB* operon, pDC2 to pDC5 (see Table 1). This procedure allowed delineating whether it is possible to delete the *holB* gene, or whether the *holB* is essential for *E. coli* growth. If the *holB* gene can be deleted from the *E. coli* genome, then a co-transduc-
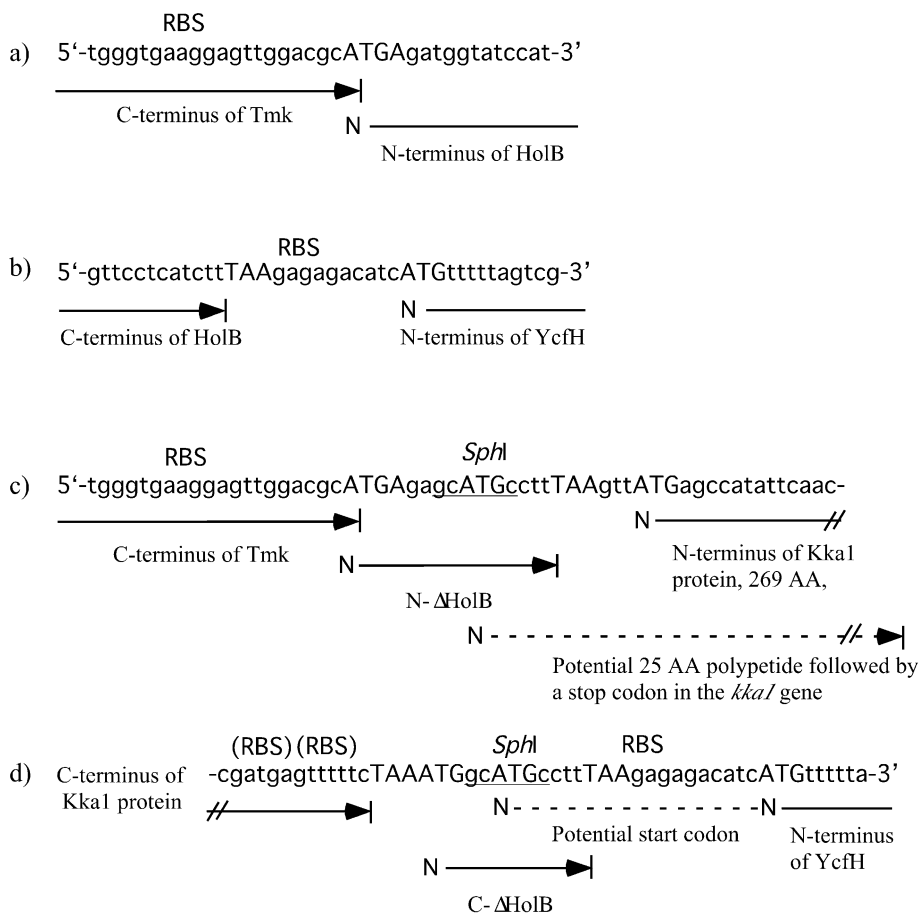
Fig. 2. Genomic sequence details at the 24.9 min region on the *E. coli* chromosome and translation products of the mRNA(s) at a) the N-terminus and b) the C-terminus of the HolB protein. DNA sequences of the Δ*holB*::*kka1* construct and its translational products at c) the N-terminus and d) the C-terminus of the original *holB* translational product. Lines and arrows indicate translation products. Capital triplets represent start and stop codons. Dashed lines represent potential translational products. *Sph*I and underlined sequences=restriction site of *Sph*I, (RBS)=potential ribosomal binding site, RBS=ribosomal binding site derived from sequence analysis.

tion efficiency of about 40% is expected, since the closely linked *yceG*::miniTn*10* (Kan^r) marker of SR749 exhibits a similar co-transduction frequency (data not shown). The results presented in Table 2b clearly show that *holB* cannot be removed from the *E. coli* chromosome unless another copy of this gene is provided *in trans*, implying that *holB* gene is indispensable for *E. coli* viability. These data also suggest that the *holB* deletion does not exert a significant polarity effect on the neighboring genes, such as *tmk* or *ycfH*.

Strains carrying the Δ*holB* construct, such as DC7, were verified by various PCR amplification reactions using primers specific for the genome or for plasmids used for the construction of strains (data not shown). These PCR experiments indicate the correct substitution of the *holB* gene by the insertion of the *kka1* gene. In addition, Southern blot analyses confirmed that the *kka1* gene correctly replaces the *holB* gene in the Δ*holB*::*kka1* strains (data not shown).

4. Discussion

The rationale behind the replacement of *holB* by the kanamycin resistance gene *kka1*, coding for the

amino glycoside 3′-phosphotransferase kanamycin kinase, was to almost completely eliminate the *holB* gene from the *E. coli* genome. This should be done in a way to introduce a selectable marker into *holB*, but to exclude polar effects on down-stream as well as on up-stream genes.

Two different approaches were tested for the introduction of the *holB* deletion and *kka1* replacement construct (Link et al., 1997; Yu et al., 2000). Both methods gave identical results, demonstrating an essential role of the HolB protein for *E. coli* viability. The genetic environment of the *holB* gene is relatively simple (Blattner et al., 1997), since there are no overlapping structural gene sequences, except for the start codon of the *holB* open reading frame (ORF) (Fig. 2a) and the stop codon of the *tmk* ORF (Fig. 2b). However, in the case of the up-stream gene *tmk* the genomic situation appears more complex (Chaperon, unpublished). The resulting genomic sequence regions of the Δ*holB*::*kka1* replacement construct are indicated in Fig. 2c and d.

A PCR fragment of 1600 bp amplified from plasmid pDC10 was used for linear transformation (Yu et al., 2000). Kan^r colonies could be obtained only in the presence of an arabinose-inducible second copy of *holB* (pDC2) (Table 2a). When the Δ*holB*::*kka1* strain was constructed by the method of the temperature-sensitive and negative-selectable plasmid pK03 (Link et al., 1997), an identical phenotype was observed. This finding was confirmed by co-transduction experiments, where double-resistant colonies were only seen in the presence of an arabinose-inducible second copy of *holB* (Table 2b). These results are in agreement with a *holB* conditional null strain (Song et al., 2001).

However, whereas the *holB* null construct of Song et al. (2001) contains a double-ochre mutation, the *holB* null mutant generated in the present work is nearly completely deleted. This has the advantage that recombination in-between the genomic substitution construct and plasmid-encoded *holB* genes can be excluded. Therefore, expressing *holB* from the arabinose-inducible plasmid pDC2, recombination with the genomic *holB* null allele can be excluded with high probability. Furthermore, using the idea of complete gene deletion/substitution, interactions of remaining protein fragments with complementing proteins or other proteins of the cell are not possible. The method described here allows experiments in any genetic background as long as HolB activity is provided from a plasmid *in trans*. Therefore, this method allows a simplified studying of essential genes of operons by complementation with a plasmid-encoded copy of a gene.

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