

Pilin-Like Proteins in the Extremely Thermophilic Bacterium *Thermus thermophilus* HB27: Implication in Competence for Natural Transformation and Links to Type IV Pilus Biogenesis

Alexandra Friedrich,¹ Judit Rumszauer,¹ Anke Henne,^{2,3} and Beate Averhoff^{1,3*}

Bereich Genetik und Mikrobiologie, Department Biologie I, Ludwig-Maximilians-Universität München, D-80638 Munich,¹
and Laboratorium für Genomanalyse² and Institut für Mikrobiologie und Genetik,³
Georg-August-Universität, D-37077 Göttingen, Germany

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The extreme thermophile *Thermus thermophilus* HB27 exhibits high frequencies of natural transformation. Although we recently reported identification of the first competence genes in *Thermus*, the molecular basis of DNA uptake is unknown. A pilus-like structure is assumed to be involved. Twelve genes encoding prepilin-like proteins were identified in three loci in the genome of *T. thermophilus*. Mutational analyses, described in this paper, revealed that one locus, which contains four genes that encode prepilin-like proteins (*pilA1* to *pilA4*), is essential for natural transformation. Additionally, *comZ*, a new competence gene with no similarity to known genes, was identified. Analysis of the piliation phenotype revealed wild-type piliation of a *pilA1-pilA3Δkat* mutant and a *comZ* mutant, whereas a *pilA4* mutant was found to be completely devoid of pilus structures. These findings, together with the significant similarity of PilA4 to prepilins, led to the conclusion that the *T. thermophilus* pilus structures are type IV pili. Furthermore, the loss of the transformation and piliation phenotype in the *pilA4* mutant suggests that type IV pili are implicated in natural transformation of *T. thermophilus* HB27.

Analyses of complete genome sequences have suggested that large portions of bacterial genomes were acquired from archaea via lateral gene transfer; for example, it has been suggested that 16 and 24% of the genomes of the hyperthermophilic bacteria *Aquifex aeolicus* and *Thermotoga maritima*, respectively, are of archaeal origin (2, 6, 20, 21). Despite the substantial evidence that there has been massive DNA transfer between archaea and hyperthermophilic bacteria and the assumption that life originated in hot ecosystems, information on DNA transfer in hot environments and on the structure and function of transformation systems in extreme thermophiles is scarce.

Thermus thermophilus HB27 is an extremely thermophilic bacterium (23) which exhibits high frequencies of natural transformation (14, 17). The ability to take up free DNA has also been described for other representatives of the genus *Thermus*, such as *T. thermophilus* HB8, *Thermus flavus* AT62, *Thermus caldophilus*, and *Thermus aquaticus* YT1 (17). To gain insight into the mechanism of natural transformation in extremely thermophilic bacteria, we performed searches for homology in the genome sequence of *T. thermophilus* HB27 with sequences encoding known competence proteins of mesophilic model bacteria, such as *Neisseria gonorrhoeae*, *Acinetobacter* sp. strain BD413, *Bacillus subtilis*, and *Streptococcus pneumoniae* (4, 8, 18, 25, 26, 29), followed by gene disruption studies. These analyses led to identification of 11 competence genes in *T.*

thermophilus HB27 (9, 10). Seven of these genes encode proteins that are similar to type IV pilus biogenesis proteins of *Pseudomonas aeruginosa* or *N. gonorrhoeae*, one of which exhibits significant similarity to prepilin peptidases (10). The latter finding suggests that prepilin-like components, which were not identified, are implicated in the transformation machinery of *T. thermophilus* HB27. However, the *Thermus* genome contains 12 distinct genes that encode prepilin-like proteins, distributed over three chromosomal loci. To analyze the implication of these genes in transformation, genes encoding prepilin-like proteins from each of the three chromosomal loci were subjected to mutational analyses.

Here we describe the results of these analyses, which revealed that genes that encode pilin-like competence proteins (PilA proteins) and a novel *Thermus*-specific competence gene are implicated in natural transformation of *T. thermophilus*. We provide evidence that one of the pilin-like competence proteins represents a major pilus subunit. Taken together, our results suggest that the type IV pili are implicated in transformation of *T. thermophilus* HB27.

MATERIALS AND METHODS

Strains, plasmids, DNA manipulation, and sequencing. *T. thermophilus* HB27 wild-type and mutant strains were grown in a 1:1 mixture of *Thermus* medium and Luria-Bertani medium at 60 to 70°C (10). For transformation of *T. thermophilus* HB27 a modified protocol of Koyama et al. (17) was used. *Escherichia coli* strains were cultured at 37°C in Luria-Bertani medium. Antibiotics were added when appropriate (20 to 40 µg of kanamycin per ml, 100 µg of ampicillin per ml, and 100 to 500 µg of streptomycin per ml). The molecular and genetic procedures used were standard procedures. The complete genomic sequence of *T. thermophilus* HB27 was determined at the Göttingen Genomics Laboratory (G₂L) as described recently (10). Generation of the *Thermus* gene bank by using the vector pTZ19r (MBI, Fermentas) has been described previously (9).

* Corresponding author. Mailing address: Department Biologie I, Bereich Genetik und Mikrobiologie, Ludwig-Maximilians-Universität München, Maria-Ward-Strasse 1a, D-80638 Munich, Germany. Phone: 49-89-21806186. Fax: 49-89-21806160. E-mail: B.Averhoff@lrz.uni-muenchen.de.

Generation of *Thermus* mutants. To analyze the role of the open reading frames (ORFs) encoding prepilin-like proteins in natural transformation, gene disruption or allelic replacement of selected DNA regions within distinct ORFs was performed by using a thermostable kanamycin resistance gene (*kat*), which was derived from the *E. coli*-*Thermus* shuttle vector pMK18 (5). For gene disruption either gene bank plasmids, such as pGL54 and pEY61, were used (Fig. 1) or the ORFs were amplified by PCR and subsequently inserted into pBlue-script II KS or pGEM-7Zf(+). The latter approach resulted in recombinant plasmids pAF1699, pAF1698, pJR1, and pJR2 (Fig. 1). PCR primers used for amplification of these DNA fragments are listed in Table 1. To allow insertion of the *kat* gene into the intergenic region between *orf1698* and *orf1699*, two adjacent *HincII* restriction sites upstream of *orf1699* (Fig. 1) were used. To allow use of these *HincII* sites, an additional *HincII* site present in *orf1698* had to be eliminated. Therefore, pAF1698 was digested with *EcoRI* and *NcoI*, treated with the Klenow enzyme, and religated. The *kat* gene was inserted into selected ORFs by using restriction sites within the ORFs in the recombinant plasmids, as indicated in Fig. 1. Mutant generation by allelic replacement of the wild-type loci by mutant loci was performed as described recently (10). Correct allelic replacement of chromosomal wild-type DNA by disrupted ORFs was verified by Southern hybridization and PCR. Plasmids that were generated for *Thermus* gene disruption studies were sequenced by using the standard primers or primers generated from the genomic sequence information (Table 1).

Electron microscopy. *Thermus* wild-type and mutant strains grown overnight on freshly prepared *Thermus* medium plates were negatively stained with 4% (wt/vol) uranyl acetate or 3% (wt/vol) phosphotungstic acid. After the cells were dried on Formvar-coated copper grids, they were viewed with a Philips model EM301 transmission electron microscope at 80 kV.

Nucleotide sequence accession number. The nucleotide sequence data have been deposited in the GenBank database under accession no. AY116643.

RESULTS AND DISCUSSION

Identification and analyses of genes that encode prepilin-like competence proteins. Similarity searches with the genomic database of *T. thermophilus* HB27 led to detection of 12 conserved ORFs which encode prepilin-like proteins and are distributed over three distinct loci (Fig. 1). One of these loci (locus I) was found to be located on the *Thermus* megaplasmid pTT27, whereas locus II and locus III are located on the *Thermus* chromosome. To determine whether the ORFs that encode prepilin-like proteins are implicated in natural transformation, gene disruption and deletion studies were performed as indicated in Fig. 1.

Three of the resulting mutants, Tt17 (*orf822::kat*), Tt18 (*orf824::kat*), and Tt21 (*orf1646-orf1644Δkat*), exhibited wild-type transformation frequencies, which led to the conclusion that the ORFs are not implicated in natural transformation (Fig. 1 and Table 2). Simultaneous deletion of the 3' end of *orf1695*, *orf1696*, and the 5' end of *orf1697* resulted in mutant Tt22 (*orf1695-orf1697Δkat*), which was found to be not competent (Fig. 1 and Table 2). From this finding we concluded that locus III is a competence-specific locus. For conclusions concerning functions of distinct ORFs that encode pilin-like proteins within this competence locus, the possibility of a potential polar effect of the *kat* marker had to be excluded. Northern studies have shown (33; J. Berenguer, personal communication) that analogous orientation of the *kat* marker gene and the gene disrupted by the *kat* marker prevents polar effects on the transcription of genes located downstream. Because the *kat* marker is in an orientation which does not result in any polar effects, we concluded that at least one of the ORFs examined (*orf1695*, *orf1696*, or *orf1697*) is essential for natural transformation. Disruption of *orf1699*, the last ORF that encodes a prepilin-like protein of locus III, resulted in the non-transformable mutant Tt20 (Fig. 1 and Table 2). Although the

marker is inserted in the orientation opposite that of *orf1699*, which may cause polar effects, the possibility that there is a polar effect on genes located downstream can be excluded since *orf2532*, which is located immediately downstream of *orf1699*, is transcribed in the opposite direction. This clearly shows that *orf1699* is essential for natural transformation. Based on the similarities of Orf1695, Orf1696, Orf1697, and Orf1699 to pilins (PilA of *P. aeruginosa* and PilE of *N. gonorrhoeae*) and their function in natural transformation, the ORFs encoding these proteins were designated *pilA1*, *pilA2*, *pilA3*, and *pilA4*.

The overall levels of amino acid identity of the deduced *Thermus* prepilin-like proteins to prepilins are rather low, ranging from 22 to 35%. There are significant similarities within the N-terminal domains. Particularly within the first 30 amino acids the levels of amino acid identity of *Thermus* prepilin-like proteins and prepilins are high, ranging from 44 to 73% with PilA of *P. aeruginosa*. All prepilins and prepilin-like proteins have a short leader peptide, a cleavage motif for the prepilin peptidase, a conserved glutamate residue at position 5 (E₅) in the mature protein, and a characteristic size (145 to 160 amino acids) (15). For the E₅ residue a critical role in polymerization of the pilin subunits has been demonstrated (24, 28). Additionally, most prepilins are characterized by a pair of cysteine residues near the C terminus, which form a disulfide loop (15). All these conserved features are also present in the *pilA1* to *pilA4* gene products, suggesting that these pilin-like competence proteins are assembled in a pilus-like structure.

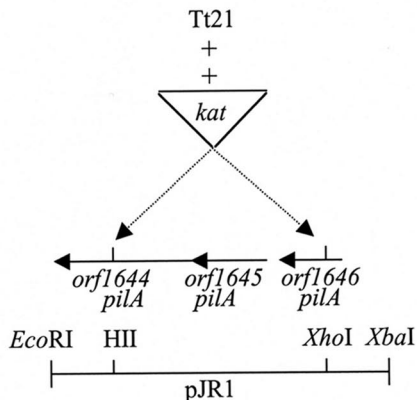
***comZ*, a novel competence gene.** The protein product of one ORF in the novel competence locus, *orf1698*, did not show any similarity to proteins in databases. Disruption of *orf1698* resulted in the noncompetent mutant Tt24 (Fig. 1 and Table 2). Since the marker was found to be inserted into *orf1698* in the opposite orientation, the possibility that the marker insertion had a polar effect on the downstream competence gene, *pilA4*, could not be excluded. To analyze the role of *orf1698* in natural transformation, disruption of the intergenic space spanning 254 bp between *orf1698* and *pilA4* was performed by inserting the *kat* gene 171 bp upstream of the *pilA4* start codon (Fig. 1). In contrast to marker insertion into *orf1698*, marker insertion into the intergenic region resulted in a wild-type transformation phenotype of mutant Tt25 (Fig. 1). This clearly shows that the noncompetent phenotype of *orf1698* mutant Tt24 does not result from a polar effect and that *orf1698* is essential for natural transformation. The novel competence gene, designated *comZ*, encodes a 59-kDa protein. The presence of one hydrophobic N-terminal domain suggests a membrane location for ComZ.

The detection of nonconserved genes in the *Thermus* transformation machinery, such as *comZ* in this study and *pilW* in previous studies (10), underlines the presence of distinct features in the transformation apparatus of *Thermus*, which might have evolved due to the extreme environment of *Thermus* and the specific outermost layer of *Thermus* cells, which consists of S-layer and lipid components (J. Berenguer, personal communication).

Piliation and transformation are linked in *T. thermophilus* HB27. The significant similarities of the proteins encoded by 12 ORFs to prepilin-like proteins, together with the recent detection of pilus structures on the surface of *Thermus* cells

Locus I

mutant designation:
transformation phenotype:
piliation phenotype:

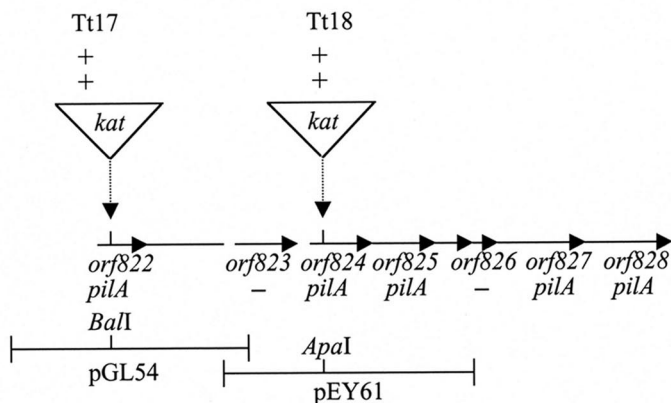


ORF designation:
similar to:

plasmids:

Locus II

mutant designation:
transformation phenotype:
piliation phenotype:



ORF designation:
similar to:

plasmids:

Locus III

mutant designation:
transformation phenotype:
piliation phenotype:

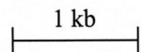
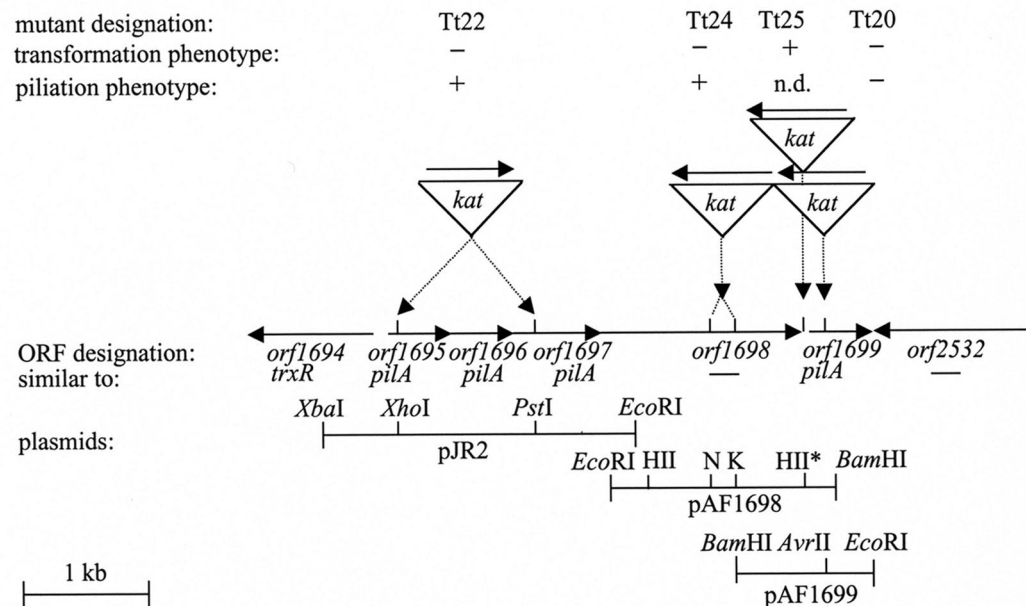


FIG. 1. Organization of the ORFs detected in the *Thermus* genome sequence that encode 12 prepilin-like proteins and gene disruption of conserved ORFs within potential competence loci in the genome of *T. thermophilus* HB27. In the restriction maps of plasmids covering different conserved ORFs of the potential competence loci only selected restriction sites are shown. The triangle indicating the *kat* gene indicates the insertion site of the Km^r marker gene. The arrows indicate the directions of transcription. *pilA*, prepilin gene; *trxR*, putative thioredoxin reductase gene; HII, *HincII*; N, *NcoI*; K, *KpnI*; n.d., not determined. The asterisk indicates two closely associated *HincII* sites.

TABLE 1. Primers designed for cloning of putative *Thermus* competence genes

Plasmid generated	Primer designation	Primer sequence (5'-3') ^a
pAF1699	orf1699-BI	CCT CTT CGG ATC CGT CAT TAG
	orf1699-EI	CGG CTG GAA TTC CCA CAA TGA G
pAF1698	orf1698-EI	GTT GCA GAA TTC CAA GGC TGC
	orf1698-BI	GAT ACA TCC GGA TCC CGT TTG TC
pJR1	orf1644-EI	CGT GGC GGA ATT CGC GGA TGG
	orf1646-XI	GAC GAG CTC TAG ACC CGC GTC
pJR2	orf1695-XI	CGG ATG CTC TAG ACC TCG GTG
	orf1697-EI	GCA GCC TTG GAA TTC TGC AAC CC

^a Modified base pairs are indicated in bold.

(10), raised the question of whether the ORFs that encode prepilin-like proteins are implicated in pilus biogenesis. To answer this question, mutants having mutations in genes encoding prepilin-like proteins and a mutant having a mutation in *comZ* were analyzed to determine their piliation phenotypes. Tt17 (*orf822::kat*), Tt18 (*orf824::kat*), and Tt21 (*orf1644-orf1646Δkat*) were found to have a wild-type piliation phenotype (Fig. 1). These findings led to the conclusion that the ORFs are implicated in neither pilus biogenesis nor transformation. The transformation-defective mutants Tt22 (*pilA1-3Δkat*) and Tt24 (*comZ::kat*) were also found to have a wild-type piliation phenotype, suggesting that the pilin-like proteins encoded by the ORFs and ComZ play a role in natural transformation but are not essential for the pilus structures. Despite the wild-type piliation phenotype of these mutants, the data do not rule out the possibility that the *orf822*, *orf824*, *orf1644* to *orf1646*, and *pilA1* to *pilA3* genes have a function in pilus biogenesis. In contrast, the transformation-defective mutant Tt20 having a mutation in *pilA4* was found to be devoid of pilus structures (Fig. 1 and Table 2). From these findings we concluded that PilA4 has a dual function in transformation and pilus biogenesis. Since so far no other prepilin-like proteins have been found to be involved in pilus biogenesis in *Thermus*, we concluded that the prepilin-like competence protein PilA4 represents a pilus subunit and that the pilus structures are type IV pili. Although the finding that mutations of distinct genes in loci I and II that encode prepilin-like proteins (Fig. 1) had no

effect on piliation suggests that these loci are not implicated in piliation, the possibility that other genes within these loci that encode prepilin-like proteins might be implicated in piliation cannot be completely excluded.

A connection between type IV pilus expression and competence for natural transformation has been reported for several competent bacteria, such as *N. gonorrhoeae*, *Pseudomonas stutzeri*, *Dichelobacter nodosus*, *Legionella pneumophila*, and *Synechocystis* sp. strain PCC6803 (11, 16, 27, 29, 32), but despite these findings it cannot be concluded in general that the expression of type IV pili is a prerequisite for natural transformation. Gram-positive competent bacteria, such as *B. subtilis* and *S. pneumoniae*, as well as the gram-negative organism *Haemophilus influenzae*, do not exhibit type IV pili, although their transformation apparatus consists of components that are similar to the pilus subunit (7, 8).

The central question that arises from the observation that a loss of pilus structures is accompanied by a loss of transformability is whether the pili themselves are involved in DNA uptake. The piliation and transformation defect of the *Thermus pilA4* mutant, together with identification of other competence proteins with similarities to type IV pilus biogenesis proteins, suggests that transformation and the piliation apparatus are closely linked. However, this suggestion does not allow any conclusion concerning pilus-mediated DNA transfer. The possibility that the PilA4 protein plays a role in two distinct systems, the type IV pilus and the DNA uptake apparatus, cannot be excluded. The essential roles of several additional prepilin-like PilA proteins (PilA1 to PilA3) in transformation, together with the finding that these prepilin-like components are not essential for piliation, support the hypothesis that there is a distinct transformation apparatus consisting of several prepilin-like components plus the structural subunit of the pilus structures. It is tempting to speculate that such an apparatus resembles a rudimentary pilus structure. On the other hand, it is possible that a functional pilus is required for DNA binding followed by DNA transport via pilus retraction. The latter possibility is supported by the finding that pili retract in a PilT-dependent process (19) and the finding that the PilT protein, which is dispensable for pilus biogenesis, is essential for pilus-mediated twitching motility (pilus retraction) and natural transformation in *N. gonorrhoeae*, *P. stutzeri*, and *Synecho-*

TABLE 2. ORFs analyzed in this study^a

ORF	Protein			Mutant	Mutant phenotype	Proposed function
	No. of amino acids	Mol wt (10 ³)	C-terminal Cys residues			
<i>orf822</i>	116	12	C ₆₅ -(27 aa)-C ₉₃ ^b	Tt17	Competent, piliated	Unknown
<i>orf824</i>	142	16	C ₈₃ -(17 aa)-C ₁₀₀	Tt18	Competent, piliated	Unknown
<i>orf1644</i>	305	33	None	Tt21	Competent, piliated	Unknown
<i>orf1645</i>	159	17	C ₁₂₆ -(8 aa)-C ₁₅₉	Tt21	Competent, piliated	Unknown
<i>orf1646</i>	145	16	None	Tt21	Competent, piliated	Unknown
<i>orf1695 (pilA1)</i>	156	17	None	Tt22	Noncompetent, piliated	Prepilin-like protein of DNA transformation machinery
<i>orf1696 (pilA2)</i>	193	21	C ₁₇₀ -(14 aa)-C ₁₈₅	Tt22	Noncompetent, piliated	Prepilin-like protein of DNA transformation machinery
<i>orf1697 (pilA3)</i>	233	25	C ₁₃₆ -(22 aa)-C ₁₅₉ ^a C ₁₇₀ -(38 aa)-C ₂₀₉	Tt22	Noncompetent, piliated	Prepilin-like protein of DNA transformation machinery
<i>orf1698 (comZ)</i>	554	59	None	Tt24	Noncompetent, piliated	Unknown function in DNA transformation machinery
<i>orf1699 (pilA4)</i>	131	14	C ₉₅ -(34 aa)-C ₁₃₀	Tt20	Noncompetent, nonpiliated	Pilin-like subunit of pili and DNA uptake apparatus

^a All deduced proteins except ComZ have the conserved cleavage motif for the prepilin endonuclease.

^b aa, amino acids.

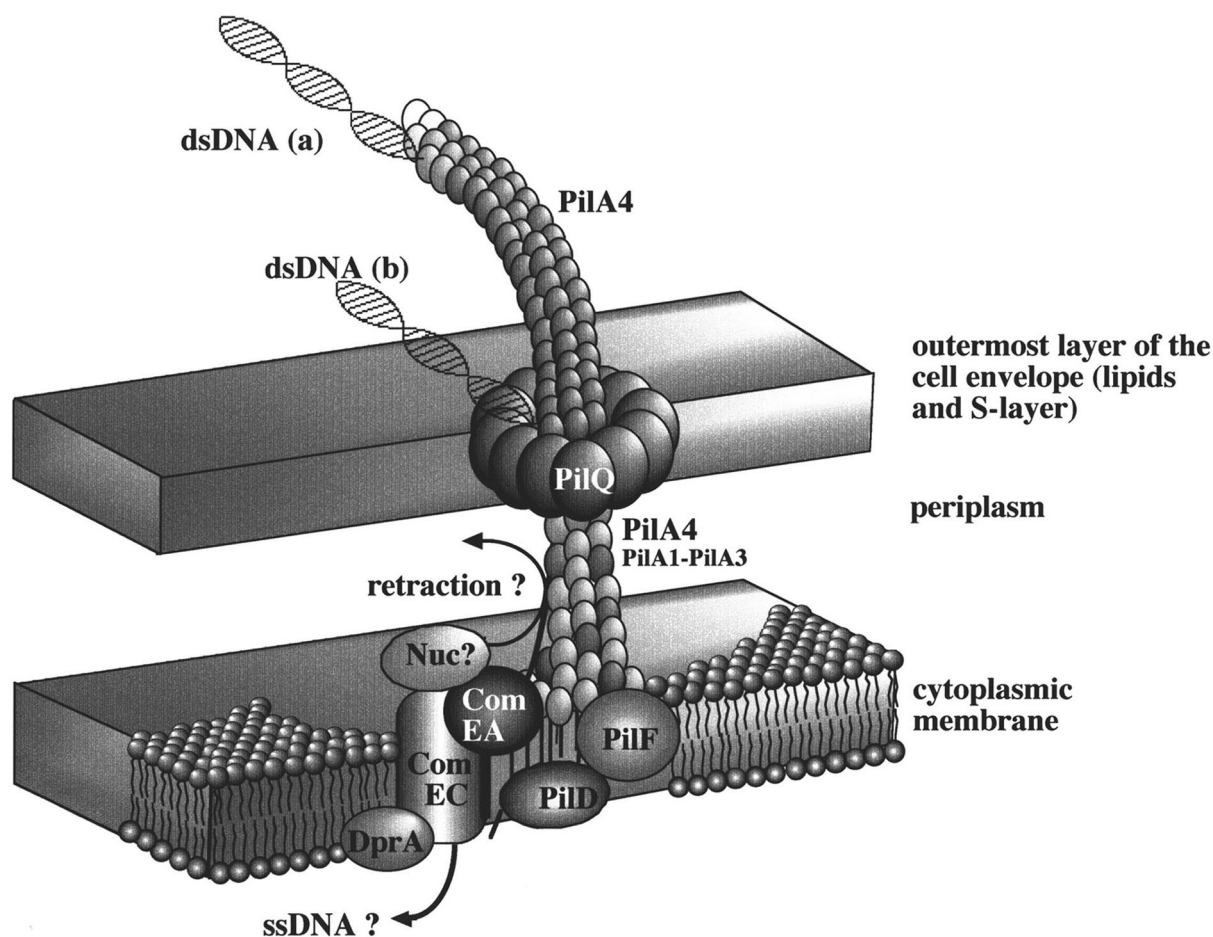


FIG. 2. Hypothetical model of the transformation system in *T. thermophilus* HB27. Two possible DNA binding sites are indicated; they are at the tip of the pilus structures (a) and close to the potential ring-like structure consisting of secretin-like subunits in the outermost layer (b). dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; Nuc, postulated but unidentified competence-specific endonuclease which cleaves double-stranded DNA upon transport through the inner membrane.

cystis sp. strain PCC6803 (13, 22, 30). The finding that a *Thermus* mutant defective in the *pilF* gene, which is similar to the gonococcal *pilT* gene, still has pilus structures but is defective in transformation (10) correlates with the described *pilT* mutant phenotypes. When these data are taken into account, it is tempting to speculate that retraction of *Thermus* pili might be important for DNA uptake in *Thermus*.

From the transformation-defective but pilus-positive phenotype of *Thermus* mutant Tt22 (*pilA1-3Δkat*), it is obvious that one of the proteins or even all of the proteins encoded by *pilA1* to *pilA3* are essential in natural transformation but are not essential in pilus biogenesis. From this finding we concluded that the *pilA* locus in *Thermus* consists of two classes of competence genes, one with a dual function in natural transformation and piliation (*pilA4*) and the other with a competence-specific function (*pilA1* to *pilA3* and *comZ*). A similar situation is encountered in *Synechocystis* sp. strain PCC6803; PilA1 is implicated in both pilus biogenesis and natural transformation, while PilA2 is a competence-specific protein (3, 32). In *N. gonorrhoeae* the structural subunit of the type IV pili (PilE) is required for transformation, a second pilin-like protein (ComP) is competence specific, and a third pilin-like protein

(PilV) functions as an inhibitor of transformation as it acts as an antagonist of ComP (1, 31). An antagonistic effect was also reported for PilAII in *P. stutzeri*, which inhibits PilAI, the structural subunit (12). From these results it was concluded that pilin-like proteins have different functions, such as being major and minor structural components of the transformation and/or pilus apparatus and as regulatory components. From the results obtained in this study we concluded that PilA4 is the major subunit of the pilus and/or the transformation apparatus and that PilA1 to PilA3 are components of an associated structure rather than proteins that are involved in regulatory processes. A hypothetical model of a pilus-like DNA translocator taking into account all *Thermus* competence genes identified so far is presented in Fig. 2.

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