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Molecular Analyses of the Natural Transformation Machinery and Identification of Pilus Structures in the Extremely Thermophilic Bacterium *Thermus thermophilus* Strain HB27

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***Thermus thermophilus* HB27, an extremely thermophilic bacterium, exhibits high competence for natural transformation. To identify genes of the natural transformation machinery of *T. thermophilus* HB27, we performed homology searches in the partially completed *T. thermophilus* genomic sequence for conserved competence genes. These analyses resulted in the detection of 28 open reading frames (ORFs) exhibiting significant similarities to known competence proteins of gram-negative and gram-positive bacteria. Disruption of 15 selected potential competence genes led to the identification of 8 noncompetent mutants and one transformation-deficient mutant with a 100-fold reduced transformation frequency. One competence protein is similar to DprA of *Haemophilus influenzae*, seven are similar to type IV pilus proteins of *Pseudomonas aeruginosa* or *Neisseria gonorrhoeae* (PilM, PilN, PilO, PilQ, PilF, PilC, PilD), and another deduced protein (PilW) is similar to a protein of unknown function in *Deinococcus radiodurans* R1. Analysis of the piliation phenotype of *T. thermophilus* HB27 revealed the presence of single pilus structures on the surface of the wild-type cells, whereas the noncompetent *pil* mutants of *Thermus*, with the exception of the *pilF* mutant, were devoid of pilus structures. These results suggest that pili and natural transformation in *T. thermophilus* HB27 are functionally linked.**

Thermus thermophilus HB27 is a gram-negative, yellow pigmented bacterium which exhibits high competence for natural transformation (27, 36). All species of the genus *Thermus* are extremely thermophilic. Members of the genus *Thermus* grow at temperatures ranging from 50 to 85°C with a temperature optimum of 70°C. In contrast to most of the extremely thermophilic bacteria, *Thermus* spp. grow under aerobic conditions (5). The genus *Thermus* is closely related to the genus *Deinococcus*, and phylogenetic studies of conserved genes suggest that these two lineages form a eubacterial phylum (5, 56).

The process of natural transformation can be divided into four discrete steps: competence induction, DNA binding, DNA uptake, and the heritable integration of incoming DNA or reconstitution of plasmid DNA. Proteins involved in uptake of DNA via natural transformation have been studied in several gram-negative bacteria, such as *Neisseria gonorrhoeae* (15, 51, 57), *Acinetobacter* sp. strain BD413 (14, 41), *Haemophilus influenzae* (9), *Pseudomonas stutzeri* (16, 17), *Helicobacter pylori* (1, 21, 48), and *Synechocystis* sp. strain PCC6803 (58), and some gram-positive bacteria, such as *Bacillus subtilis* and *Streptococcus pneumoniae* (6, 12, 39). A common feature of the transformation machineries is the implication of proteins exhibiting significant similarity to components of type IV pilus systems (12, 20). The only exception, to our knowledge, is the transformation system in *H. pylori,* whose known competence proteins (HP0333, ComH, and ComB) do not share any similarity with components of type IV pilus systems.

The significant similarities of competence proteins to proteins of type IV pilus systems lead to the fundamental question of whether type IV pili are involved in DNA uptake. This question has not been settled yet, but it seems to emerge that different bacteria might have different mechanisms.

Very little is known with respect to natural transformation systems in thermophiles and hyperthermophiles, although this means of lateral gene transfer probably had a very important impact on the evolution of life. In some scenarios, the universal tree of life is not rooted to an ancestral organism but to a pool or a net of ancestors. These ancestors are assumed to be hyperthermophiles with readily exchangeable genetic material. Moreover, there is substantial evidence for massive gene exchange between archaeal and bacterial hyperthermophiles resulting from genome-scale comparisons of these organisms (2, 8).

We chose *T. thermophilus* HB27 as a model organism to get insights into the transformation machinery of extremely thermophilic bacteria. In this study, we identified 28 putative competence genes in the partially complete genome sequence of *T. thermophilus* HB27. We report the identification of nine competence genes using gene disruption and transformation studies, and we present evidence for a link between systems for pilus synthesis and natural transformation in this organism.

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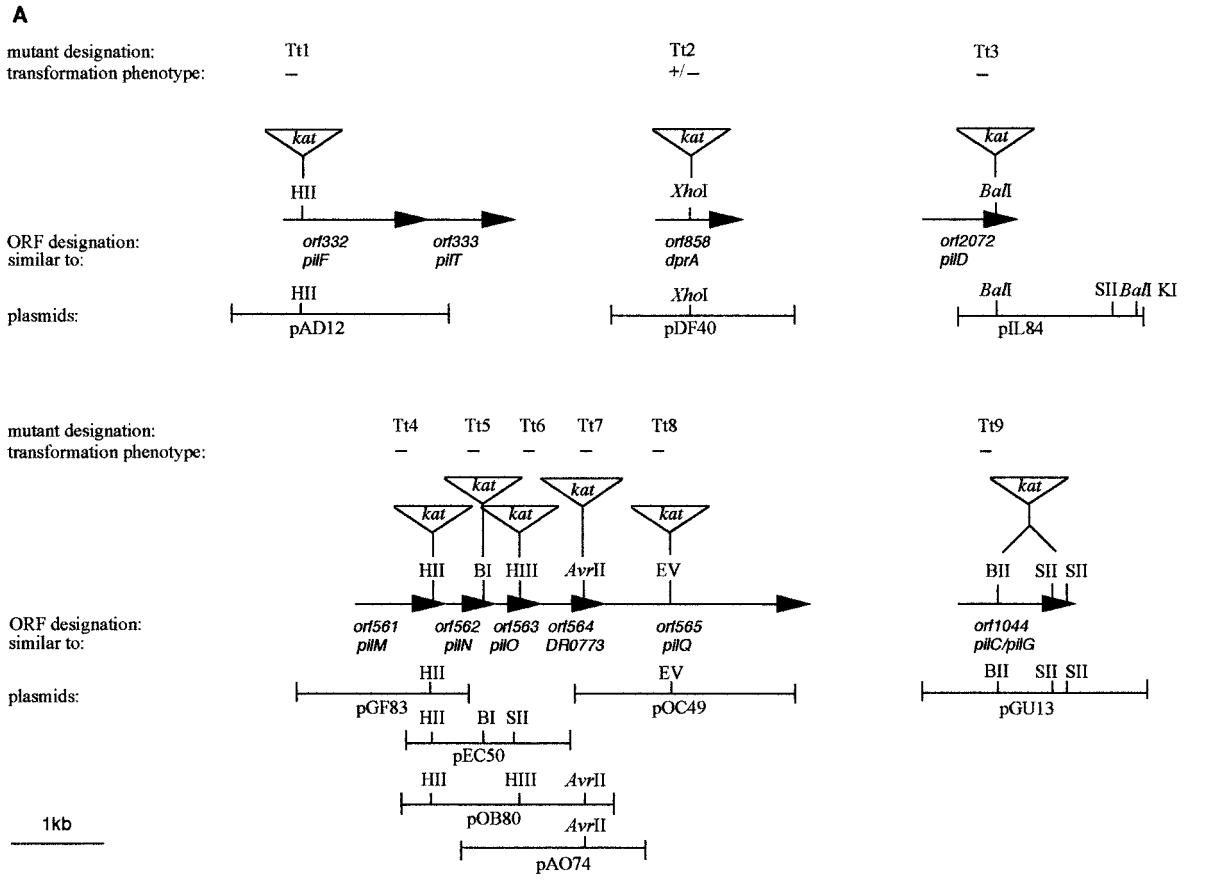
MATERIALS AND METHODS

Strains, plasmids, and DNA manipulation. *T. thermophilus* HB27 wild-type (DSM 7039) and mutant strains were grown in a 1:1 mixture of TM broth (27)


A
mutant designation:
transformation phenotype:
Tt1
–
Tt2
+/–
Tt3
–
kat
HII
XhoI
BalI
ORF designation:
similar to:
orf332
pilF
orf333
pilT
orf858
dprA
orf2072
pilD
plasmids:
HII
pAD12
XhoI
pDF40
BalI
SII BalI KI
pIL84
mutant designation:
transformation phenotype:
Tt4
Tt5
Tt6
Tt7
Tt8
Tt9
HII
BI
HIII
AvrII
EV
BII
SII
SII
ORF designation:
similar to:
orf561
pilM
orf562
pilN
orf563
pilO
orf564
DR0773
orf565
pilQ
orf1044
pilC/pilG
plasmids:
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pOC50
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pEC50
pOB80
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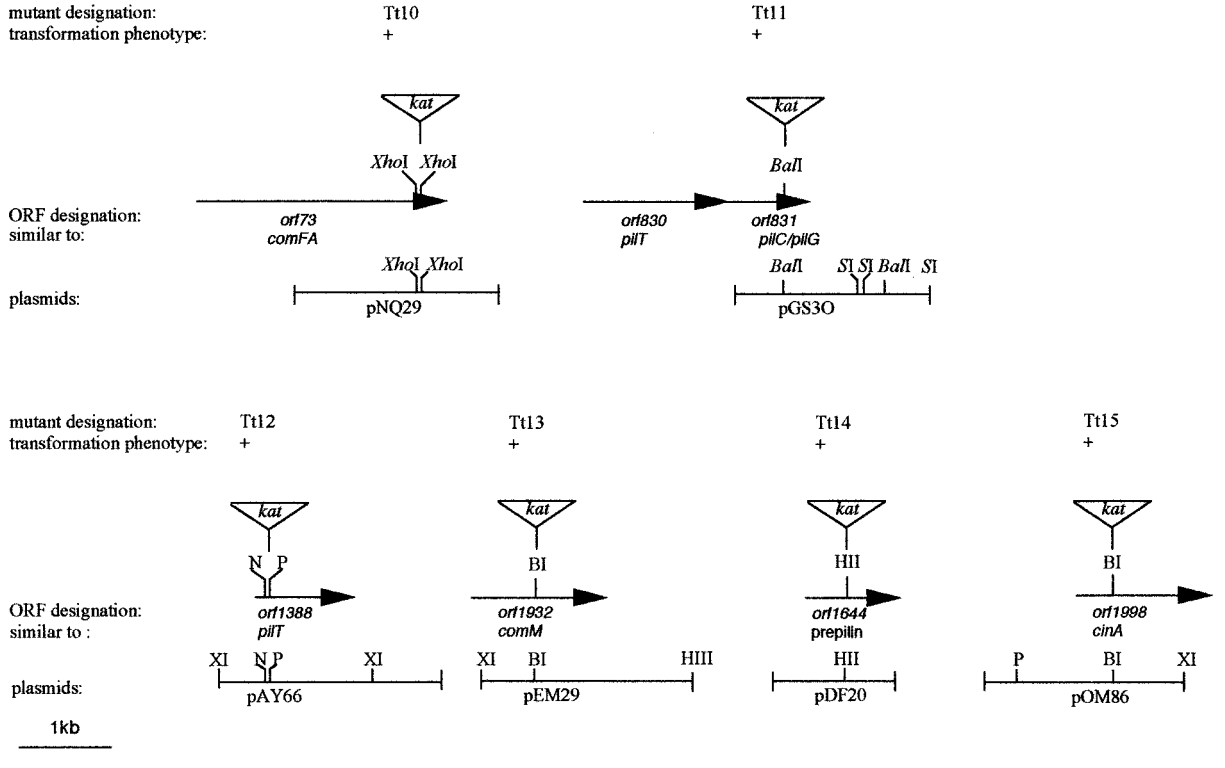
B
mutant designation:
transformation phenotype:
Tt10
+
Tt11
+
kat
XhoI
BalI
ORF designation:
similar to:
orf73
comFA
orf830
pilT
orf831
pilC/pilG
plasmids:
pNQ29
pGS3O
mutant designation:
transformation phenotype:
Tt12
+
Tt13
+
Tt14
+
Tt15
+
N P
BI
HII
ORF designation:
similar to :
orf1388
pilT
orf1932
comM
orf1644
prepilin
orf1998
cinA
XI
HIII
P
plasmids:
pAY66
pEM29
pDF20
pOM86
1kb

and Luria-Bertani (LB) medium at 70°C. Antibiotics were added when appropriate (kanamycin, 20 to 40 μg/ml; ampicillin, 100 μg/ml; and streptomycin, 100 to 500 μg/ml). *Escherichia coli* strains were cultured at 37°C in LB medium. The molecular and genetic procedures were standard techniques. Southern hybridization experiments were performed as described previously (40).

DNA sequence analysis. The nearly complete genomic sequence of *T. thermophilus* HB27 was determined by a whole-genome shotgun approach by the Göttingen Genomics Laboratory (G_2L). Clones carrying HB27 genomic DNA of approximately 2.0 kb in length from small insert libraries representative of the whole genome were sequenced from both ends using LICOR IL-4200 and ABI PRISM 377 DNA sequencers. The generated sequence readings were assembled into contigs with the Prap software implemented in the STADEN software package. Sequence data were analyzed with BLAST programs of the National Center for Biotechnology Information database, the software package (version 10.0) of the Genetics Computer Group (University of Wisconsin Biotechnology Center), and the WIT platform (Integrated Genomics).

Generation of *Thermus* mutants. To analyze the role of potential competence genes, mutants were disrupted by a kanamycin resistance marker (*kat*) derived from the *E. coli*/*T. thermophilus* shuttle vector pMK18 (7). For gene disruption, recombinant plasmids of the *Thermus* gene library were used. Based on the sequence information, 15 gene library plasmids covering 11 different loci were selected (Fig. 1). The *kat* gene was inserted into unique sites of *orf332*, *orf858*, *orf561*, *orf564*, and *orf565* or by substitution of distinct DNA fragments within *orf73* and *orf1044* by the *kat* gene (Fig. 1). To allow insertion of the *kat* gene into unique cleavage sites within the putative competence genes *orf562*, *orf563*, *orf1388, orf1932*, and *orf1998*, cleavage sites within the multiple cloning site had to be eliminated by subcloning into another vector. Therefore, the inserts of pEC50, pOB80, pAY66, pEM29, and pOM86 (Fig. 1) used for disruption of *orf562*, *orf563*, *orf1388*, *orf1932,* and *orf1998*, respectively, were subcloned into pBluescriptII KS/SK (Stratagene).

To allow insertion of the *kat* gene into the *Bal*I site of *orf2072* or *orf831*, the *Bal*I sites present in the flanking DNA regions had to be eliminated. Therefore, pIL84 and pGS30 (Fig. 1) were digested with *Sac*II/*Kpn*I and *Sac*I, respectively, which was followed by treatment with Klenow enzyme and religation. This cloning strategy resulted in deletion of *Bal*I restriction sites in the flanking DNA regions and allowed marker insertion into unique *Bal*I sites in *orf831* and *orf2072*, respectively.

The plasmids carrying the disrupted conserved open reading frames (ORFs) were transformed into *E. coli* DH5α, and transformants were selected on LB medium containing 100 μg of ampicillin/ml and 20 μg of kanamycin/ml. Plasmids were prepared, and the inserts were purified and transformed into *T. thermophilus* HB27 by natural transformation. *Thermus* transformants were selected on TM medium containing 40 μg of kanamycin/ml. The correct allelic replacement of chromosomal wild-type DNA by disrupted ORFs was verified by Southern hybridization.

Transformation studies. For transformation of *T. thermophilus* HB27, a modified protocol of Koyama et al. (27) was used. For transformation studies with *T. thermophilus* HB27, spontaneous streptomycin-resistant mutants were selected by plating 10^8 cells on TM medium containing streptomycin (500 μg/ml). The genomic DNA of one selected streptomycin-resistant mutant was isolated and used as donor DNA for transformation studies. To enable parallel analyses of several *Thermus* mutants, a rapid transformation test system was established. One colony of a *Thermus* mutant, disrupted in one of the potential competence genes, was mixed with 20 μl of chromosomal DNA (100 ng/μl) of the streptomycin-resistant *Thermus* strain, plated on TM agar, and incubated for at least 8 h at 70°C to allow expression of the streptomycin marker. To select for transformants that had acquired the genes mediating the streptomycin resistance phenotype, the cells were plated on TM agar containing 100 μg of streptomycin/ml and subsequently incubated at 70°C overnight. The *T. thermophilus* HB27 wild-type strain was used as a transformation control.

Electron microscopy. *Thermus* wild-type and mutant strains grown overnight on freshly prepared TM plates were negatively stained with 4% (wt/vol) uranylacetate. After drying on Formvar-coated copper grids, the cells were viewed with a Philips model EM301 transmission electron microscope at 80 kV.

Nucleotide sequence accession number. The sequence data have been submitted to the GenBank database and the accession numbers are listed below in Table 2.

RESULTS

Identification of competence genes by gene disruption. Analysis of the transformation machineries of gram-positive and gram-negative bacteria have shown that DNA transfer systems often comprise homologues of type IV pilus biogenesis factors or homologues of proteins implicated in protein secretion machineries. Similarity searches within the *Thermus* genomic database with known proteins of type IV pilus biogenesis led to the identification of 24 homologues of type IV pilus biogenesis systems (Table 1). The deduced proteins of two other potential competence genes are similar to proteins of DNA translocation machineries, such as DprA and ComFA of *H. influenzae* and *B. subtilis*, respectively (Table 1). The deduced products of two more potential competence genes show similarities to proteins implicated in recombination of the incoming DNA with genomic DNA in *H. influenzae* and *S. pneumoniae* (Table 1).

To determine whether the conserved potential competence genes are essential for transformation, 15 ORFs (Fig. 1), distributed over 11 conserved loci, were selected for gene disruption studies by use of a kanamycin resistance gene, *kat*. As indicated in Fig. 1A, the potential competence locus comprising *orf561, orf562, orf563*, *orf564,* and *orf565* was subjected to single gene disruptions. The resulting mutants, Tt4 (*orf561*::*kat*), Tt5 (*orf562*::*kat*), Tt6 (*orf563*::*kat*), Tt7 (*orf564*::*kat*), and Tt8 (*orf565*::*kat*) were found to be completely noncompetent (Fig. 1A and Table 2). Due to the similarities of the deduced *Thermus* proteins to *Neisseria* type IV pilus proteins with the noncompetent mutant phenotypes, the *Thermus* ORFs *orf561, orf562, orf563,* and *orf565* will be referred to hereafter as *pilM*, *pilN*, *pilO,* and *pilQ* (Table 1). Since *orf564* is also a member of this competence gene cluster, although its deduced protein does not show any similarities to type IV pilus proteins, it was designated *pilW*. Due to the tandem arrangement of the *pil* genes within this *Thermus* competence locus and the analogous orientation, it cannot be excluded that a disruption of upstream-located genes results in a polar effect on downstream-located genes. However, since the ORFs located immediately downstream of *pilQ* encode key enzymes of tryptophan biosynthesis, a highly conserved chorismate synthase, and a shikimate kinase, a role for these genes in natural transformation can be excluded. This finding shows that at least *pilQ*, the last gene of this competence locus (Fig. 1A), is essential for natural transformation.

Three potential competence loci are comprised of genes whose deduced proteins show similarities to the type IV pilus factor PilT. To address the question of a role for the three *pilT* loci in natural transformation, single ORFs for each of these

FIG. 1. Structural organization and gene disruption strategy of conserved ORFs within potential competence loci in the genome of *T. thermophilus* HB27. Physical map of mutant loci that are involved (A) and not involved (B) in natural transformation. In the restriction maps of gene bank plasmids covering different conserved ORFs of the potential competence loci, only selected restriction sites are shown. The triangle indicating the *kat* gene denotes the insertion site of the Km^r marker gene. The arrows denote direction of transcription. +, wild-type transformation frequencies; ±, 100-fold-reduced transformation frequencies; −, not transformable. BI, *Bam*HI; BII, *Bgl*II; EV, *Eco*RV; HII, *Hinc*II; HIII, *Hin*dIII; N, *Nco*I; P, *Pst*I; XI, *Xba*I.

TABLE 1. Putative competence genes identified via homology searches in the genome sequence of *T. thermophilus* HB27

Group and *Thermus* ORF(s)	Similar competence protein	Reference	% Amino acid similarity[a] and conserved motifs[b]
Group I (DNA translocation)			
orf858	DprA (H.i.)[c]	25	43
orf73	ComFA (B.s.)	29	ATP binding motif
Group II (type IV pili)			
orf561	PilM (P.a)	32	42
orf562	PilN (P.a)	32	—[b]
orf563	PilO (P.a.)	32	—[b]
orf564	Hypothetical protein DR0773 (D.r.)	56	32
orf565	PilQ (P.a)	31	35
orf332	PilF (N.g.)	13	49
orf333	PilT (P.a.)	55	59
orf830	PilB (P.a.)	35	47
orf1388	PilT (P.a.)	55	53
orf2072	PilD (P.a.)	35	44
orf1044	PilC (P.a.)	35	52
orf831	PilC (P.a)	35	48
orf822, orf824–825, orf827–828, orf1644–1646, orf1695–1697, orf1699	Prepilins	12	Prepilin cleavage motif
Group III (recombination)			
orf1932	ComM (H.i.)	19	57
orf1998	CinA (S.p.)	38	46

[a] The amino acid sequence similarities were calculated by comparing the deduced protein of the conserved ORFs in *Thermus* with the protein products of the prominent similar genes listed in Table 2.
[b] Due to direct sequence comparison.
[c] Strain designation: B.s., *B. subtilis*; D.r., *D. radiodurans* R1; H.i., *H. influenzae*; N.g., *N. gonorrhoeae*; P.a., *P. aeruginosa*; S.p., *S. pneumoniae*.

loci, *orf332*, *orf1388*, and *orf831* (Fig. 1), were subjected to gene disruption. (Fig. 1). Analyses of the transformation phenotypes of the resulting mutants revealed that the natural transformation phenotype is completely abolished in mutant Tt1 (*orf332*::*kat*), whereas Tt11 (*orf831*::*kat*) and Tt12 (*orf1388*::*kat*) exhibit wild-type transformation frequencies (Table 2; Fig. 1). From these results, we conclude that *orf831* and *orf1388* are not implicated in natural transformation. Downstream of *orf332*, an ORF designated *orf333* is present whose deduced protein shows similarities to PilT in *N. gonorrhoeae* and *P. aeruginosa*. Thus, it cannot be excluded that the noncompetent phenotype of the *orf332* mutant is due to a polar effect on *orf333* (Table 2; Fig. 1). However, since *orf333* is flanked by the known *T. thermophilus* gene encoding a glutamyl-tRNA-amidotransferase (3), a polar effect on potential competence genes located downstream of *orf332* and *orf333* can be excluded.

The deduced protein of *orf1044* is similar to the *Pseudomonas* PilC and the *Neisseria* PilG proteins (35, 50). Disruption of this ORF, designated *pilC,* resulted in the noncompetent mutant Tt9 (Fig. 1; Table 2). Since the ORF located immediately downstream of *orf1044* is transcribed in the opposite direction, this finding clearly shows that *orf1044* is essential for natural transformation.

Disruption of the *pilD*-like *orf2072* (Table 1) resulted in mutant Tt3 (Fig. 1A), which was found to be noncompetent (Table 2). It is interesting that the *Thermus pilD*-like gene, in contrast to *pilD* genes in other proteobacteria, such as *P. aeruginosa*, *P. stutzeri*, *H. influenzae*, and *N. gonorrhoea*, is not clustered with other *pil* genes (Fig. 2A). PilD homologues are

TABLE 2. Identified competence genes in *T. thermophilus* HB27

Annotation (accession no.)	bp	Molecular mass (kDa)	Mutant phenotype	Proposed function
dprA (*orf858*) (AF439555)	1,005	36	Transformation deficient	DNA translocation or recombination
pilM (*orf561*) (AF436067)	1,134	41	Noncompetent, nonpiliated	DNA transformation and assembly of pili
pilN (*orf562*) (AF436067)	624	23	Noncompetent, nonpiliated	DNA transformation and assembly of pili
pilO (*orf563*) (AF436067)	582	21	Noncompetent, nonpiliated	DNA transformation and assembly of pili
pilW (*orf564*) (AF436067)	879	30	Noncompetent, nonpiliated	DNA transformation and assembly of pili
pilQ (*orf565*) (AF436067)	2,274	83	Noncompetent, nonpiliated	Secretin-like competence protein
pilF (*orf332*) (AF436070)	1,695	62	Noncompetent, piliated	ATP-dependent function in assembly of the transformation apparatus
pilC (*orf1044*) (AF436068)	1,326	48	Noncompetent, nonpiliated	IM[a] protein involved in assembly of pili and the transformation apparatus
pilD (*orf2072*) (AF436069)	1,065	38	Noncompetent, nonpiliated	Prepilin peptidase involved in processing of prepilins

[a] IM, inner membrane.

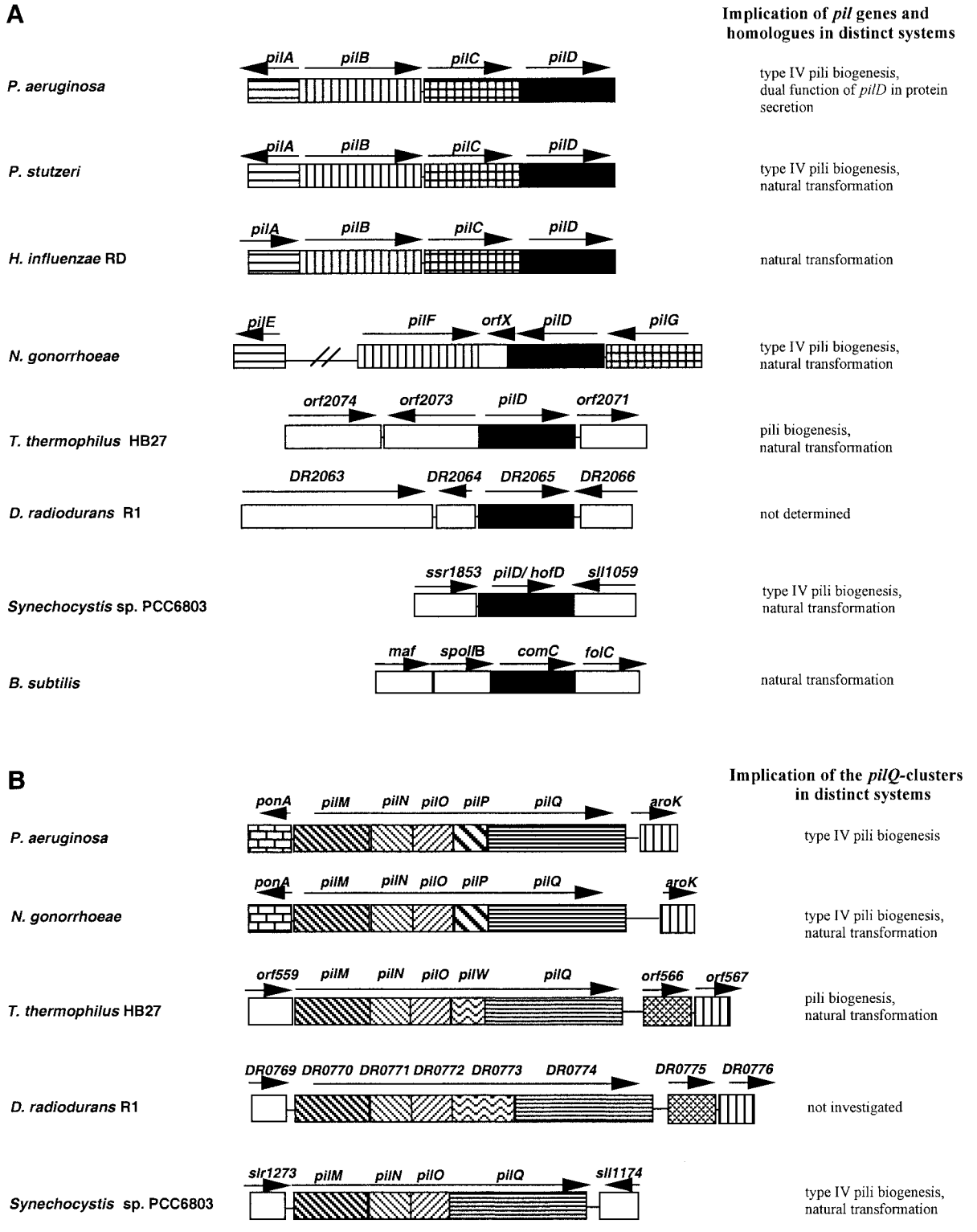

FIG. 2. (A) Comparison of the *pilD* locus in different gram-negative proteobacteria and nonproteobacteria. *orf2073* encodes a thermostable carboxypeptidase and *orf2071* encodes a hypothetical protein in *Thermus. ssr1853* encodes a hypothetical *Synechocystis* protein, and *sll1059* encodes an adenylate cyclase. *DR2065* is a *pilD* homologue in *Deinococcus*. The *Deinococcus* ORFs *DR2063*, *DR2064*, and *DR2066* encode a polynucleotide phosphorylase, a hypothetical 17.3-kDa protein, and a conserved hypothetical protein, respectively. *comC* is a *pilD* homologue in *B. subtilis. spoIIB* and *folC* encode a sporulation factor and the enzyme folypoly-γ-glutamate synthetase-dihydrofolate synthetase, respectively. (B) Comparison of the genetic organization of the *pilQ* cluster in different gram-negative proteobacteria and nonproteobacteria. The *Thermus* ORFs *orf559*, *orf566,* and *orf567* encode isopropylmalate dehydrogenase, chorismate synthase, and shikimate kinase, respectively. *DR0775* is an *aroC* homologue and *DR0776* is an *aroK* homologue in *Deinococcus. slr1273* and *sll1174* are predicted to encode hypothetical proteins in *Synechocystis*.

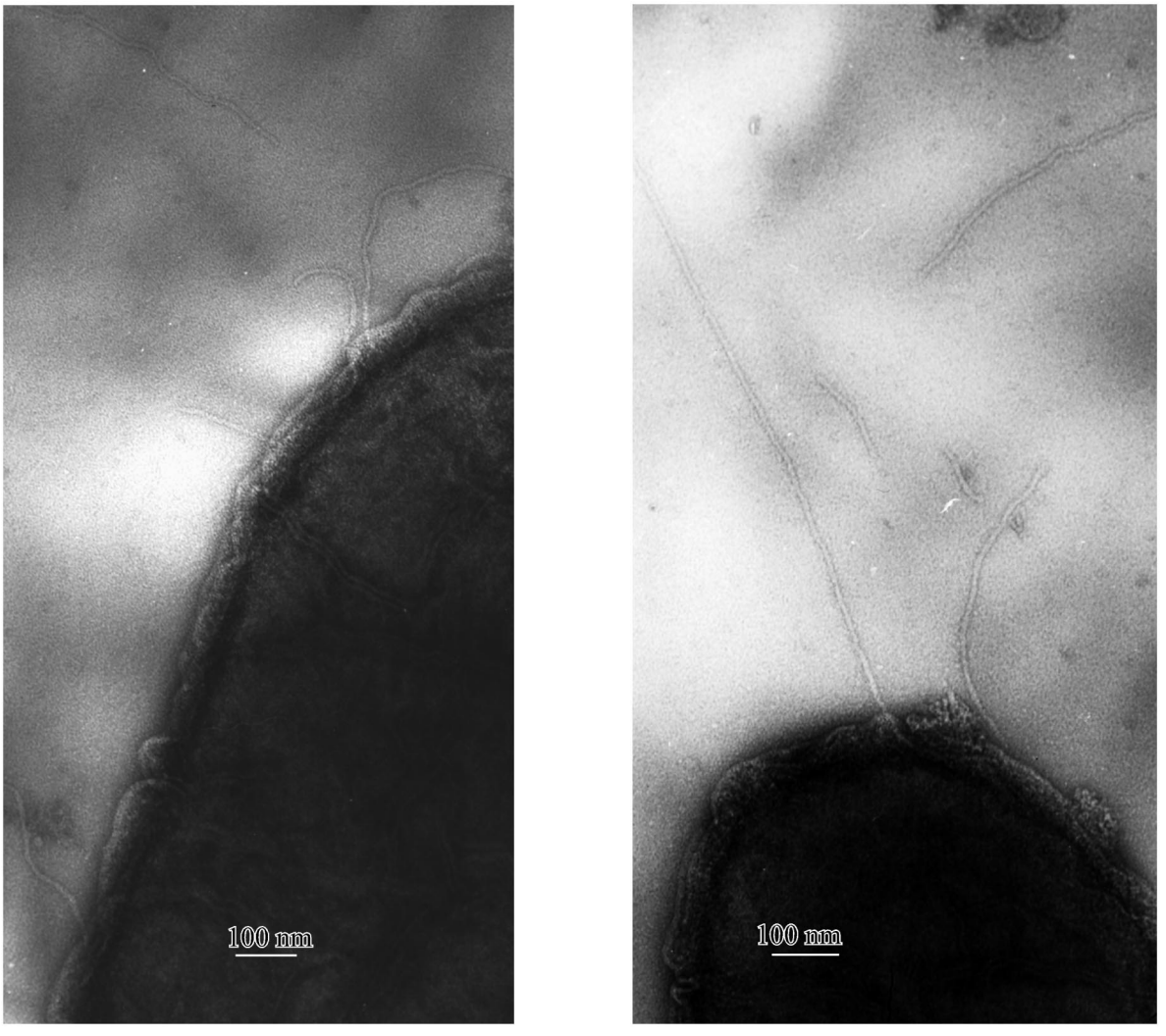

FIG. 3. Representative sample showing the pilus structures on the surface of *T. thermophilus* HB27 wild-type cells. Electron microscopic investigations were conducted with uranylacetate-stained cells.

also present in nonproteobacteria such as *D. radiodurans*, *Synechocystis* strain PCC6803, and *B. subtilis* (4, 34, 56, 58). Interestingly, the *pilD*-like genes in these transformable phylogenetically distant nonproteobacteria are, analogously to the *Thermus pilD*-like gene, not associated with other *pil* genes (Fig. 2A). The differences in the organization of the *pilD*-like gene in *Thermus* and *pilD* homologues in the genomes of transformable proteobacteria (Fig. 2A) might reflect potential distinct horizontal gene transfer events or internal recombination events in *Thermus*.

To address the question of a role of prepilin-like proteins in natural transformation, *orf1644* was disrupted (Table 1; Fig. 1B). The resulting mutant Tt14 shows wild-type transformation frequencies (Fig. 1B), which leads to the conclusion that the prepilin-like ORF, *orf1644*, is not implicated in transformation.

Disruption of the *H. influenzae dprA* homologue *orf858* resulted in mutant Tt2, which was found to exhibit a 100-fold reduced transformation frequency of 5×10^{-5} transformants/viable count (Fig. 1A; Table 2), whereas the wild type exhibited transformation frequencies of 6×10^{-3}. Since the flanking ORF downstream of the *dprA*-like gene is orientated in the opposite direction, this result provides clear evidence that *orf858* is essential for natural transformation of *T. thermophilus* HB27.

The disruption of the three additional selected ORFs, *orf73, orf1932,* and *orf1998* (Fig. 1B), resulted in mutants exhibiting wild-type transformation frequencies, which leads to the conclusion that these ORFs are not implicated in the natural transformation of *T. thermophilus*.

Piliation phenotypes of *Thermus* wild-type cells and noncompetent mutants. Electron microscopic studies of *T. thermophilus* HB27 cells led to the identification of individual pilus structures on the cell surface that were 6 nm in diameter and 1 to 3 μm in length (Fig. 3).

The significant similarities of the identified *Thermus* competence proteins to components of the type IV pilus systems, together with the presence of pilus structures on the cell surface of *T. thermophilus* HB27, led to the question of whether the transformation system is linked to the pilus biogenesis

system in *Thermus*. To answer this question, noncompetent *Thermus* mutants disrupted in the competence genes *pilD, pilC,* and *pilF* or in individual genes of the *pilM-pilQ* cluster were analyzed with respect to their piliation phenotype. The *pilC* and *pilD* mutants and the *pilM-pilQ* mutants were found to be devoid of pilus structures (Table 2). This finding suggests either that transformation and pili are linked systems or that pili are implicated in transformation. The *pilF* mutant was found to exhibit pilus structures indistinguishable from those of the HB27 wild type. From these findings, we conclude that PilD, PilC, PilM, PilN, PilO, PilW, and PilQ but not PilF have a dual function in transformation and pilus biogenesis.

DISCUSSION

Detection of potential competence genes in the *T. thermophilus* genome database. Homology searches in the genome sequence database of *T. thermophilus* HB27 led to the identification of 28 conserved ORFs whose protein products are similar to proteins involved in natural transformation of different gram-negative and gram-positive bacteria and to components of type IV pilus systems (Table 1). The deduced protein products of the potential competence genes show similarities to components of three major functional systems: (i) DNA translocation, (ii) type IV pilus biogenesis, and (iii) DNA recombination (Table 1).

Genetic organization of competence genes. The genes of the *pil* competence locus in *Thermus*, comprising *pilM, pilN, pilO, pilW* (DR0773), and *pilQ* (Fig. 1A), are analogously orientated and tightly clustered, such that all adjacent genes have overlapping stop and start codons. This *Thermus* locus is related to *pilMNOPQ* loci implicated in type IV pilus biogenesis in *P. aeruginosa* and in pilus biogenesis and natural transformation in *N. gonorrhoeae* (10, 31, 32). In *N. gonorrhoeae* and *P. aeruginosa*, the *pilMNOPQ* cluster is preceded by *ponA*, which encodes a penicillin-binding protein (33, 46). Downstream of the *Neisseria* and *Pseudomonas pilMNOPQ* cluster is *aroK*, a shikimate kinase gene (31, 37). In contrast to this organization, there is no *ponA* homologue in the close vicinity of the conserved *pilM-Q* cluster of *T. thermophilus*, *Synechocystis* strain PCC6803, and *D. radiodurans* (Fig. 2B). *aroK* homologues are present downstream of the conserved *pilM-Q* cluster in the *Thermus-Deinococcus* group and are separated from the conserved *pilM-Q* cluster by a chorismate synthase gene. No *aroK* homologue was found in the close vicinity of the *pil* cluster in *Synechocystis*. The analogous organization of the genes within the conserved *pilM-Q* cluster in *Thermus, Synechocystis, Pseudomonas,* and *Neisseria* suggests that this conserved *pil* module in these distantly related bacteria has been acquired via horizontal gene transfer; and the differences in the flanking DNA regions might be due to an integration of the complete *pil* module in different genomic loci of the host genomes. The protein product of the fourth gene within the *Thermus pilM-Q* cluster, designated *pilW*, is similar to the hypothetical protein of *D. radiodurans* R1, encoded by the fourth ORF, DR0773, of the putative *pilM-Q* cluster in *D. radiodurans.* In *P. aeruginosa* and *Neisseria*, the fourth gene of the conserved gene cluster, *pilP*, encodes a lipoprotein which is predicted to be implicated in stabilization of PilQ multimers (11). ORFs encoding PilP homologues are missing in the genome sequence of *T. thermophilus* HB27.

Characterization and possible functions of the identified competence genes. The deduced protein of the first gene in this *Thermus* cluster is similar to PilM in *P. aeruginosa* and *N. gonorrhoeae* (Table 1). The PilM proteins in *P. aeruginosa*, *N. gonorrhoeae*, and *T. thermophilus* HB27 share a highly conserved C-terminal domain characteristic for FtsA cell division proteins. The presence of a total of three hydrophobic domains distributed over the whole *Thermus* PilM suggests that PilM is a cytoplasmic membrane protein. No clues to the function of PilN and PilO in *Thermus* can be derived from their homologues in *P. aeruginosa* and *N. gonorrhoeae* since their function in type IV pilus biogenesis is still unknown.

PilQ is similar to members of the secretin family, such as PilQ in *Myxococcus xanthus* (54), ExeD in *Aeromonas salmonicida* (24), PilQ in *P. aeruginosa* (31), and PilQ in *N. gonorrhoeae* (10). Secretins are conserved within a 250-amino acid (aa) C-terminal stretch, whereas the N-terminal and central parts are variable. As shown by Guilvout et al. (18), the C terminus of the *Klebsiella* secretin PulD is required for multimer formation, and it is concluded from their experiments that the β-domain of the C-terminal part is the major determinant of multimer stability. For the gonococcal PilQ, a dependence of multimerization on conserved C-terminal residues was also demonstrated (10). Since the C terminus in *Thermus* PilQ is well conserved, we conclude that the *Thermus* PilQ also forms ring-like structures which mediate DNA transport into the periplasm.

PilF (*orf332*) and PilT (*orf333*) are similar to pilus assembly proteins and to proteins of the general secretion pathway (Table 1). *pilF* (*orf332*) and *pilT* (*orf333*) are tightly clustered and analogously orientated (Fig. 1A). Both proteins contain a Walker A motif (53) and a conserved aspartate box (Fig. 4), and they are very similar to each other (49% similarity). Both motifs, the Walker A motif and the aspartate box (Fig. 4), are highly conserved in proteins of the PilT family (43). The two short motifs (TXEDPXE and RXXPDXXXGEI/MRD), containing at least one aspartate residue and therefore referred to as aspartate boxes, are typical for PulE-PilF-PilB and PilT homologues and are not found in other proteins with ATP-binding sites, such as ABC transporters (23, 43). The Walker A motif GXXXXGK(S/T)T of the *Thermus* PilF contains a phenylalanine in place of the second conserved threonine (Fig. 4). It has to be noted that this threonine residue is generally less conserved in PulE homologues, such as PilB in *P. aeruginosa* and PilF in *Neisseria*, which contain a valine instead. It has been shown by Possot and Pugsley (43) that replacement of key amino acids within the Walker A box of PulE, such as exchange of the conserved last glycine residue by alanine or of the conserved lysine residue by arginine, abolishes protein secretion in *K. oxytoca*. Analogously, Turner et al. (52) demonstrated that the highly conserved glycine residues within the Walker A box of the *Pseudomonas* proteins XcpR and PilB are essential for protein secretion and type IV pilus biogenesis, respectively, in *P. aeruginosa*. Likewise, the replacement of aspartate residues within each of the two aspartate boxes in PulE led to reduction of secretion efficiency in *K. oxytoca* (43). Although PilF and PilT share conserved regions, such as the Walker A motif and the conserved aspartate boxes, they are different in size (Fig.

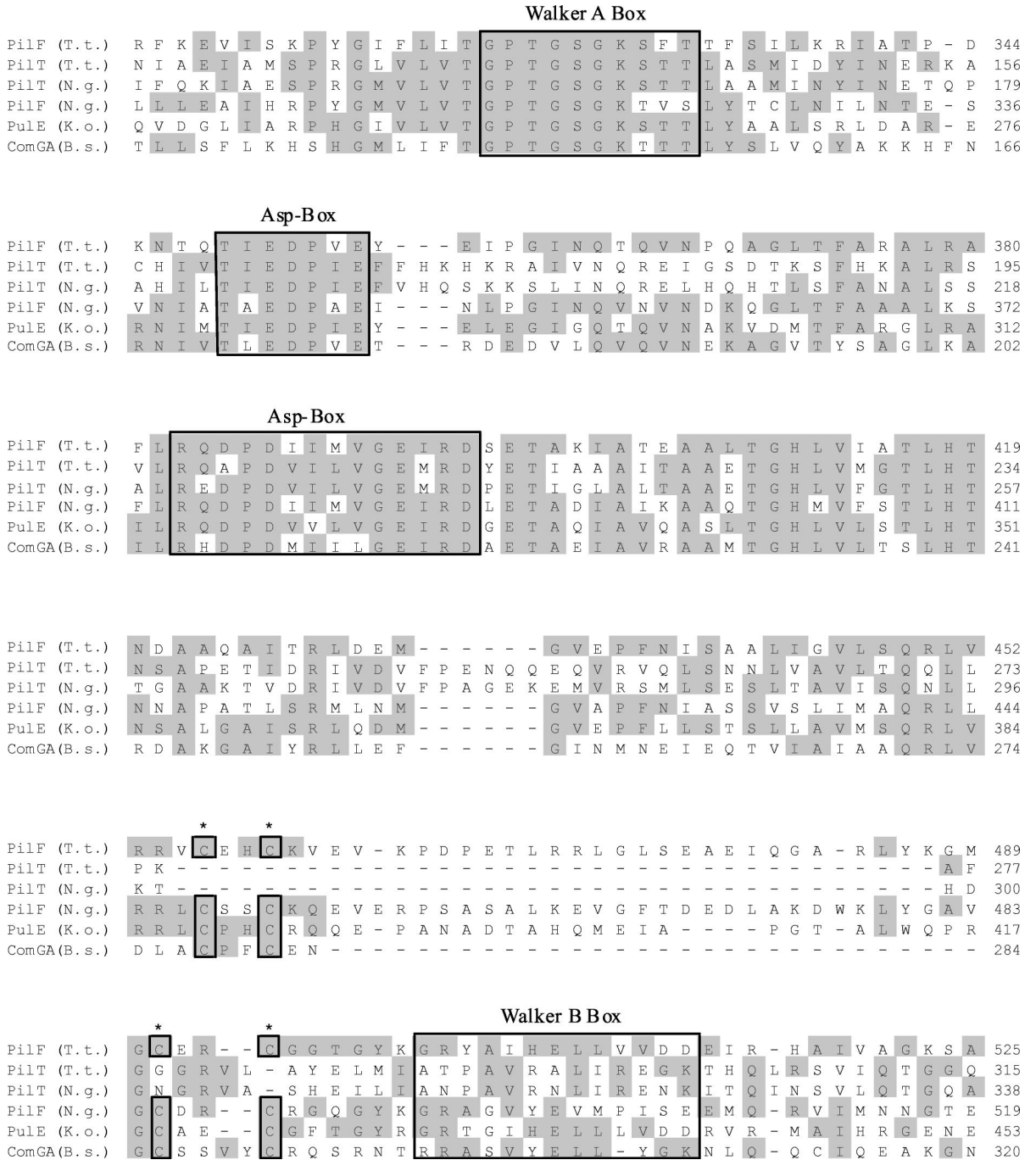

FIG. 4. Alignment of the conserved central and C-terminal part of PilT-like proteins. Identical residues are indicated by grey shadows. The conserved cysteine residues found in PulE homologues are marked by a star (*) and are boxed. B.s., *B. subtilis*; K.o., *K. oxytoca*; N.g. *N. gonorrhoeae*; T.t., *T. thermophilus* HB27.

1). Furthermore, a tetracysteine motif found in PilF is missing in PilT. This tetracysteine motif present in the C terminus of PilF (Fig. 4) resembles a zinc-binding motif. Such tetracysteine motifs are generally present in members of the PulE-PilB-PilF subgroup and some kinases such as adenylate kinases, but they are absent in proteins of the PilT subgroup. Since it was demonstrated in *Klebsiella* that this Cys motif is required for proper PulE function (44), we propose that this motif is important for the function of the *Thermus* PilF.

orf1388 is the third ORF in the *Thermus* genome whose deduced protein exhibits conserved motifs characteristic for PilT proteins (Table 1) but, in contrast to *pilF* and *pilT*, an implication of *orf1388* in natural transformation can be excluded. This observation clearly demonstrates that, despite

their similarities, conserved proteins in *Thermus* do not necessarily belong to the same functional system, and it highlights the fact that sequence similarities might not (always) allow conclusions with respect to function, even in cases of highly conserved proteins.

The *Thermus* competence factor PilC (Table 2), which is closely related to the *P. aeruginosa* PilC type IV pilus protein (Table 1), is comprised of extended hydrophobic domains spanning the central region from aa 210 to 270 and spanning the last 40 C-terminal amino acids. It has been proposed that PilC-like proteins are polytopic integral membrane proteins, probably located in the cytoplasmic membrane (35, 42). The exact function of PilC-like proteins remains to be established, but it has been proposed that the *Pseudomonas* PilC and the *Klebsiella* homologue, PulF, are required for specific interactions with other type IV pilus assembly proteins (26, 43) and might be essential for their optimal localization or stabilization (26).

The product of *orf2072* (PilD) is similar to prepilin peptidases. It has at least eight hydrophobic domains, which indicates a localization in the inner membrane. The highest identities of the *Thermus* PilD and prepilin peptidases are present within the N and C termini of the proteins. The N-terminal domain includes two pairs of cysteines which are required for the leader peptidase and methyltransferase activities of the bifunctional enzyme PilD, as demonstrated in *P. aeruginosa* (49). This motif is highly conserved, with the exception of XspO in *Xanthomonas campestris* (22), in all PilD proteins (30), and it is required for the proper function of prepilin peptidase proteins (44). The significant similarities, together with the highly conserved cysteine cluster, lead to the conclusion that PilD in *T. thermophilus* HB27 represents a prepilin peptidase most likely involved in proteolytic processing and methylation of prepilin-like proteins. It should be mentioned that no other prepilin peptidase was detected in the *Thermus* genome. Therefore, PilD should be implicated in the processing of all prepilins and prepilin-like proteins of *T. thermophilus* HB27.

The *Thermus* DprA (*orf858*) is similar to Smf and DprA proteins, which are widely distributed in transformable and nontransformable prokaryotes. DprA plays an essential role in transformation of *H. influenzae*, *H. pylori,* and *S. pneumoniae* (1, 6, 25). For the competence proteins of this family, a function in DNA transport through the cytoplasmic membrane and/or in recombination is postulated. The *Thermus* DprA and its homologues in *H. influenzae* have 57% similarity within a central 205-aa overlap (positions 60 to 265). This central region contains highly conserved amino acid stretches [VGXSR, positions 100 to 105; TSGLALGID(X_3)H, positions 127 to 139; VLGS(X_5)YP, positions 152 to 163; PRRNR, positions 190 to 194; and SGSLITA, positions 212 to 218]. The high degree of conservation indicates a critical function of these amino acid stretches.

Distinctive features of the *Thermus* transformation system. The results presented here suggest that the DNA translocating machinery of *T. thermophilus* HB27 is related to type IV pili. In analogy to *N. gonorrhoeae* and *P. stutzeri* (15, 16), but in contrast to *Acinetobacter* sp. strain BD413 (28), the pili, as visible in electron micrographs, either are involved in natural transformation or pili and transformation are closely linked systems. This is concluded from the finding that the noncompetent mutants, with the exception of the *pilF* mutant, are devoid of pili structures. Despite the similarities of the components of the natural transformation machineries in *Thermus* and other transformable bacteria, the *Thermus* DNA translocation machinery is different from corresponding systems in other bacteria. The absence of a *pilP* homologue, the presence of a nonconserved *pilW,* and the low similarities of the *Thermus* PilN and PilO to homologues in other microorganisms might reflect the potential structural distinctiveness of the DNA transformation machinery in *Thermus.*

The suggestion that the *Thermus* transformation system differs from known transformation systems is also supported by the results obtained from characterization of the secretin-like PilQ protein in *Thermus*. Members of the secretin family form ring-like structures in the outer membrane which are implicated in type IV pilus biogenesis, protein export, and natural transformation of gram-negative bacteria, and also in phage assembly. It is suggested that the N terminus of secretins, which is implicated in protein export, folds back into the cavity of the channel that is formed by the C-terminal domain of the native complex. The N terminus of the secretin-like phage assembly protein pIV, which shows no homologies to the N termini of other secretins, is suggested to consist of a periplasmic substrate-binding domain that confers specificity to phage assembly (47). The N terminus of the *Thermus* PilQ shows no similarities to members of the secretin family, neither to secretins implicated in protein export or DNA import in gram-negative bacteria nor to proteins implicated in phage assembly. This finding indicates that the *Thermus* PilQ might interact with very distinct components.

The potential structural differences of the DNA transformation machinery in *Thermus* might be due to the structural distinctiveness of the cell envelope and the peptidoglycan of *T. thermophilus.* The murein from *Thermus* shows significant differences in complexity compared to the murein of other gram-negative bacteria; for example, the composition of murein and peptide cross bridges of *T. thermophilus* are typical for gram-positive bacteria, whereas the murein content, degree of cross-bridging, and glycan chain length are more similar to those from gram-negative bacteria. The outermost layer of the *Thermus* cell envelope is built by an S-layer covered by amorphous material (45). The distinct features of the *Thermus* cell envelope and the murein layer might have triggered the evolution of the *pilMNOWQ* cluster in *Thermus.*

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