APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2001, p. 3140–3148 Vol. 67, No. 7
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.7.3140–3148.2001

Natural Transformation in Mesophilic and Thermophilic Bacteria: Identification and Characterization of Novel, Closely Related Competence Genes in *Acinetobacter* sp. Strain BD413 and *Thermus thermophilus* HB27

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Received 8 February 2001/Accepted 24 April 2001

The mesophile *Acinetobacter* sp. strain BD413 and the extreme thermophile *Thermus thermophilus* HB27 display high frequencies of natural transformation. In this study we identified and characterized a novel competence gene in *Acinetobacter* sp. strain BD413, *comA*, whose product displays significant similarities to the competence proteins ComA and ComEC in *Neisseria* and *Bacillus* species. Transcription of *comA* correlated with growth phase-dependent transcriptional regulation of the recently identified pilin-like factors of the transformation machinery. This finding strongly suggests that *comA* is part of a competence regulon. Examination of the genome sequence of *T. thermophilus* HB27 led to detection of a *comA*/*comEC*-like open reading frame (ORF) which is flanked by an ORF whose product shows significant similarities to the *Bacillus subtilis* competence protein ComEA. To examine whether these two ORFs, designated *comEC* and *comEA*, are implicated in natural transformation of *T. thermophilus* HB27, both were disrupted by using a thermostable kanamycin resistance marker. Natural transformation in *comEC* mutants was reduced 1,000-fold, whereas in *comEA* mutants the natural transformation phenotype was completely eliminated. These results strongly suggest that both genes, *comEC* and *comEA*, are required for natural transformation in *T. thermophilus* HB27. Several transmembrane α-helices are predicted based on the amino acid sequences of ComA in *Acinetobacter* sp. strain BD413 and ComEC in *T. thermophilus* HB27, which suggests that ComA and ComEC are located in the inner membrane and function in DNA transport through the cytoplasmic membrane.

Increasingly, data obtained in studies of molecular microbial ecology and genome analyses indicate that horizontal gene flow between different species and genera plays a major role in acquisition of novel metabolic capabilities, of the different mechanisms of gene transfer, natural transformation is perhaps the most versatile and provides an important mechanism for increasing the genetic variability of microorganisms. The ability to take up free DNA via natural transformation is widely distributed among representatives of different phylogenetic and trophic groups inhabiting distinct natural ecosystems (35). To gain insight into the structure of the DNA uptake machinery and the mechanism of natural transformation of gram-negative soil bacteria, we used *Acinetobacter* sp. strain BD413 as a mesophilic model organism. This strain is known for its high competence for natural transformation (25). *Acinetobacter* sp. strain BD413 has been shown to induce maximal competence immediately after the transition from the lag phase to the exponential growth phase. During prolonged exponential growth competence is gradually lost (43, 48). Prerequisites for induction of competence for natural transformation in *Acinetobacter* sp. strain BD413 have been investigated thoroughly and are well understood (44). Since *Acinetobacter* sp. strain BD413 does not discriminate between heterologous and homologous DNA, this strain has been broadly used as a model strain to elucidate the significance of intra- and interspecies gene transfer in natural soil habitats (15, 29, 35, 38, 39).

Although many thermophilic bacteria are known to exhibit high levels of competence for natural transformation, information on natural transformation systems of extremely thermophilic microorganisms is very scarce, in contrast to the abundant data on the transformation mechanisms of and the potential for DNA transfer between mesophilic bacteria (31). One extreme thermophile, *Thermus thermophilus* HB27, an aerobic, rod-shaped, gram-negative bacterium that grows at temperatures between 50 and 82°C, is known to exhibit high frequencies of natural transformation (22, 31). Transformation frequencies ranging from 10^{-2} to 10^{-1} were found when proliferating cells incubated at pH 6 to 9 and 70°C were exposed to chromosomal DNA (31). As observed in *Acinetobacter* sp. strain BD413, the highest transformation frequencies were obtained in the presence of divalent cations (Ca^{2+} or Mg^{2+}).

We recently identified five genes, *comC, comP, comB, comE*, and *comF*, that are required for DNA uptake in *Acinetobacter* sp. strain BD413 (1, 21, 34, 47, 48). Primary sequence comparisons, mutant studies, and biochemical analyses have suggested that ComP is part of a DNA binding and/or transport structure that is anchored in the cytoplasmic membrane and spans the periplasm (48). In contrast to our increasing knowledge concerning the structure and function of transformation mechanisms in mesophilic bacteria (8), nothing is known about the components of transformation systems of inhabitants of ex-

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treme environments, such as extremely thermophilic bacteria. The differences in physiology and environmental conditions between the genera *Acinetobacter* and *Thermus* led us to ask whether the transformation mechanisms of these phylogenetically distantly related bacteria are completely different or include conserved components.

In this study we identified a novel competence gene essential for natural transformation in *Acinetobacter* sp. strain BD413. The deduced protein encoded by this competence gene shows significant similarities to the competence proteins ComA of *Neisseria gonorrhoeae*, ComEC of *Bacillus subtilis*, Rec-2 of *Haemophilus influenzae*, and CelB of *Streptococcus pneumoniae* (2, 4, 10, 17). Due to the similarities, the novel competence gene in *Acinetobacter* sp. strain BD413 was designated *comA*. Examination of the genomic DNA sequence of *T. thermophilus* HB27 resulted in identification of a *comA*/*comEC*-like open reading frame (ORF) in *Thermus*, which is flanked by an ORF encoding a putative protein with significant similarities to the competence proteins ComEA of *B. subtilis* and CelA of *S. pneumoniae*. We obtained clear evidence that the *comEA-comEC* competence locus is essential for natural transformation in *T. thermophilus* HB27. Primary sequence comparisons and secondary structure predictions suggested that ComEC of *Thermus* and ComA of *Acinetobacter* are transmembrane proteins which may be involved in DNA transport through the cytoplasmic membrane. Our data strongly suggested that ComA- and ComEC-like proteins are ubiquitous, essential components of bacterial transformation mechanisms independent of the phylogenetic relationships and natural environments of the transformable bacteria.

MATERIALS AND METHODS

Strains, plasmids, and DNA manipulation. *Acinetobacter* wild-type and mutant strains were grown in Luria-Bertani (LB) medium (50) or mineral medium (41). Growth conditions described previously were used (47). A *p*-hydroxybenzoate hydroxylase (*pobA*) mutant strain of BD413, designated *Acinetobacter* sp. strain ADP239 (19), was used in all studies. *Escherichia coli* DH5α was used as a host strain for cloning of genomic DNA (18). *E. coli* S17-1(λpir) was used as a donor strain for conjugative transposition of mini-Tn*10*pLOF/Km (Ap^r, Tn*10* delivery plasmid with Km^r) (20). The *E. coli* strains were cultured at 37°C in LB medium. *T. thermophilus* HB27 wild-type and mutant strains were grown in a 1:1 mixture of TM broth (31) and LB medium at 60 to 70°C. When appropriate, antibiotics were added at the following concentrations: kanamycin 20 to 40 μg/ml; ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; and streptomycin, 100 to 500 μg/ml. The molecular and genetic procedures used were standard techniques (50, 51). Transformation, conjugation, and complementation studies of *Acinetobacter* mutants and Southern hybridization experiments were performed as described previously (47). For triparental matings helper plasmid pRK2013 (13) was used. *T. thermophilus* HB27 was transformed by using a modified protocol described by Koyama et al. (31).

Transformation studies. Transformation studies with *Acinetobacter* strains were performed as described previously (47). For transformation studies with *T. thermophilus* HB27 spontaneous streptomycin-resistant mutants were selected by plating 10^8 cells on TM medium plates containing streptomycin (500 μg/ml). The genomic DNA of the spontaneous streptomycin-resistant mutants were used as donor DNA for transformation studies as described previously (31).

Transposon mutagenesis in *Acinetobacter*. To identify competence loci in *Acinetobacter* sp. strain BD413, a genomic library of this strain was subjected to transposon mutagenesis by using a genetically engineered derivative of Tn*10*, mini-Tn*10*pLOF/Km, as described previously (20, 21). The BD413 library, which contained 6,000 clones harboring 2.5- to 6-kb *Xba*I DNA fragments, was generated in *E. coli* DH5α by using the mobilizable broad-host-range vector pRK415. Matings between the donor strain and pooled recipients were performed by using the filter mating technique as described recently (47). Transconjugants were selected on LB medium plates containing kanamycin and tetracycline and subjected to plasmid preparation. Plasmids were purified and digested with *Xba*I, and the *Xba*I fragments spanning the kanamycin resistance gene plus flanking BD413 DNA were transformed into *Acinetobacter* sp. strain ADP239. Selection of *Acinetobacter* sp. strain ADP239 transformants on LB medium plates containing kanamycin resulted in detection of 1,000 transformants that had acquired the kanamycin marker gene via homologous recombination of the marker-flanking DNA with ADP239 genomic DNA. Analysis of the transformants led to identification of one noncompetent mutant, T701. The proficiency of homologous recombination of this mutant was tested as described previously (16). The gene disrupted by mini-Tn*10*pLOF/Km was identified as *comA*. The mutant locus of T701 was recovered on a 10.5-kb *Xba*I fragment (pAF701-1) and an overlapping 3.9-kb *Eco*RI-*Eco*RV fragment (pAF701-1.2), which were cloned by using the vectors pGEM-7Zf(+) and pBluescriptII KS, respectively (Fig. 1A).

Generation of the wild-type *comA* gene. To clearly confirm the essential role of *comA* in natural transformation, complementation studies with the wild-type *comA* gene were performed. Isolation or generation of the *comA* wild-type gene was a prerequisite for these studies. Mutant T701 could not be used to generate the *comA* wild-type allele. Therefore, a second mutant had to be generated. To generate the second *comA* mutant, a 1.9-kb *Bam*HI-*Eco*RV DNA fragment that was located downstream of the mini-Tn*10* insertion in pAF701-1.2 and included 5′-terminal truncated *comA* was subcloned from pAF701-1.2. The *comA* allele in the resulting plasmid, pAF701-1.3 (Table 1), was disrupted by a kanamycin marker (*nptII*), which gave rise to plasmid pAF701-1.4 (Fig. 1A). This construct containing part of *comA*, disrupted by the *nptII* marker digested with *Bam*HI plus *Eco*RV, and the 3.2-kb *Bam*HI-*Eco*RV fragment carrying the disrupted *comA* gene was introduced into *Acinetobacter* sp. strain ADP239 by natural transformation. Transformants were selected on LB medium containing kanamycin. Several of the kanamycin-resistant transformants were analyzed to determine their transformation phenotypes and, as expected, were found to be noncompetent. One such mutant, designated T702, was used for further studies. Correct allelic replacement of chromosomal wild-type *comA* in mutant T702 by the disrupted ORF was verified by Southern hybridization.

The T702 mutant locus was recovered as a 12.5-kb *Hin*cII fragment (pAF702-2) (Table 1); this fragment contained the entire chromosomal copy of *comA*. To generate the *comA* wild-type gene, a 5.3-kb *Hin*cII-*Bam*HI DNA fragment was subcloned from pAF702-2 into pBluescriptII SK, resulting in plasmid pAF702-2.1 (Table 1). The *nptII* marker was eliminated by digestion with *Pst*I and subsequent religation, resulting in plasmid pAF702-2.2 (Table 1). The sequence of the analogous *comA* region in plasmid pAF702-2.2 (spanning the *Pst*I site) was found to be identical to the corresponding region in the 5′- and 3′-terminally truncated *comA* gene in pAF701-1.2 (Fig. 1A). This result confirmed that deletion of the *nptII* marker gene from pAF702-2.1 resulted in generation of the *comA* wild-type allele.

Complementation studies. To perform complementation studies, the insert of pAF702-2.2 was cloned into pRK415 (20). This was done by cloning the 4-kb insert of pAF702-2.2 as a *Kpn*I-*Bam*HI fragment into linearized pRK415, which resulted in pRK702-2.2 (Fig. 1A). This fragment contained the 3′ end of *orf1, orf2, comA*, and the 3′ end of *orf4*. For further complementation studies a second plasmid containing only the complete *comA* gene was constructed. To delete the *comA*-flanking *orf1* and *orf2* regions, plasmid pAF702-2.2 was digested with *Hin*cII plus *Cla*I, incubated in the presence of deoxynucleoside triphosphates (dNTPs) and the Klenow enzyme, and ligated. The 3.3-kb insert in the resulting plasmid, pAF702-2.3 (Table 1), included the 3′ end of *orf2, comA*, and the 3′ end of *orf4*. The insert of pAF702-2.3 was cloned as a 3.3-kb *Kpn*I-*Bam*HI DNA fragment into pRK415, which resulted in pRK702-2.3 (Fig. 1A). Recombinant plasmids pRK702-2.2 and pRK702-2.3 were conjugatively transferred into *comA* mutants T701 and T702, respectively. Transconjugants were selected for growth on LB medium plates containing tetracycline and analyzed for the presence of recombinant plasmids and transformation phenotype.

Disruption of *comA* flanking ORFs. To determine whether *orf4* has a potential function in natural transformation, *orf4* was disrupted by the kanamycin resistance gene, *nptII*. A 3.4-kb *Pst*I-*Hin*dIII fragment of plasmid pAF702-2 spanning the 3′ end of *comA, orf4, orf5*, and *orf6* was first subcloned into pBluescriptII KS (pAF702-4.4)(Table 1) and then was cloned as a 3.4-kb *Pst*I-*Kpn*I fragment into pRK415, which resulted in plasmid pRK702-4.2 (Table 1). The *nptII* marker was cloned into the unique *Bam*HI site of *orf4*, which resulted in plasmid pRK702-4.3. Plasmid pRK702-4.3 was digested with *Eco*RV, and the resulting 3.3-kb *Eco*RV fragment carrying the disrupted *orf4* DNA plus flanking DNA was introduced into *Acinetobacter* sp. strain ADP239 by natural transformation in order to disrupt chromosomal *orf4* by homologous recombination. Transformants were selected for growth on LB medium plates containing kanamycin. Correct allelic replacement of chromosomal wild-type *orf4* was verified by Southern hybridization. Several attempts were made to disrupt or to delete *orf1* and *orf2*. For these experiments different subclones of pAF701-1 carrying *orf1* and

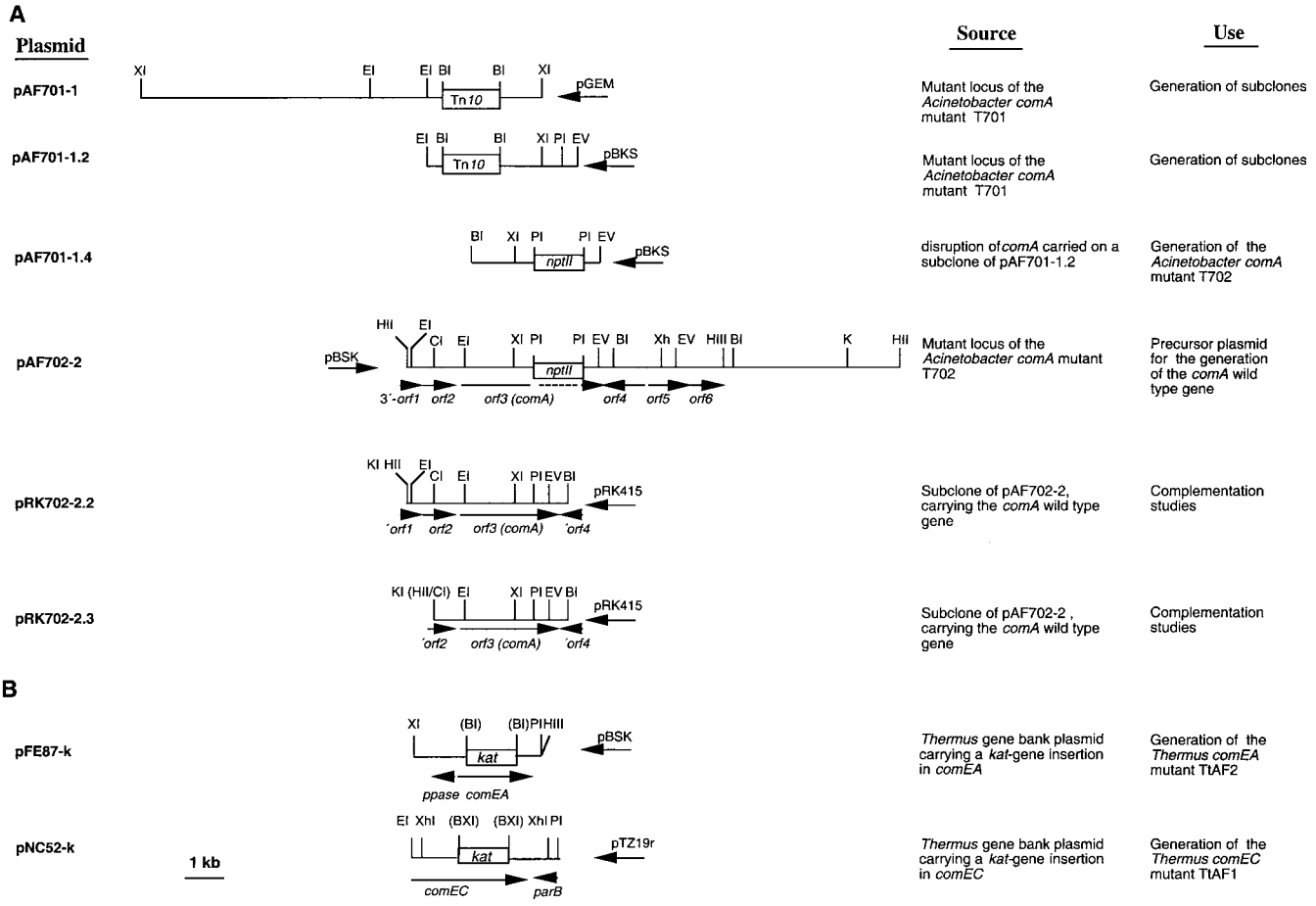

FIG. 1. Physical maps of plasmids used in this study for *Acinetobacter* (A) and *Thermus* (B) gene disruption and complementation studies. pBKS, pBluescriptII KS; pBSK, pBluescriptII SK; pGEM, pGEM-7Zf(+). The arrows indicate directions of transcription. B, *Bam*HI; BX, *Bst*XI; CI, *Cla*I; EI, *Eco*RI; EV, *Eco*RV; HII, *Hin*cII; HIII, *Hin*dIII; KI, *Kpn*I; XI, *Xba*I; Xh, *Xho*I; PI, *Pst*I.

orf2 were generated and used to insert the *nptII* marker into *orf1* and *orf2*, respectively. The resulting disrupted *orf1* and *orf2* DNA plus flanking wild-type DNA were transformed into *Acinetobacter* sp. strain ADP239, but despite several attempts with different constructs no replacement of wild-type *orf1* and *orf2* by the disrupted ORFs was observed.

Gene disruption in *T. thermophilus* HB27. *comEC* and *comEA* of *T. thermophilus* HB27 were disrupted by a kanamycin resistance gene (*kat*) encoding a thermostable kanamycin nucleotidyltransferase (32, 36), which was derived from the shuttle vector pMK18. pMK18 confers kanamycin resistance in *E. coli* (37°C) and in *Thermus* (70°C) (6). Two plasmids from an HB27 gene bank were used for these disruption experiments. The gene bank was generated by cloning physically sheared, blunt-ended chromosomal DNA from *T. thermophilus* HB27 in *Hin*cII-digested vector pTZ19r. Based on the sequence information, two of the gene bank plasmids, designated pNC52 and pFE87, were determined to carry homologs of the *B. subtilis comEC* and *comEA* competence genes. These two plasmids were used for disruption of the HB27 *comEC*- and *comEA*-like genes via insertion of a thermostable kanamycin resistance marker gene (*kat*).

To insert the kanamycin resistance gene (*kat*) into the *comEC*-like ORF, plasmid pNC52 (Table 1) was digested with *Bst*XI and incubated in the presence of dNTPs and the Klenow enzyme. pMK18 was digested with *Hin*dIII plus *Bam*HI, which yielded a 1.2-kb fragment containing the *kat* gene. After incubation with the Klenow enzyme, the *kat* gene was ligated to *Bst*XI-linearized pNC52, and the ligation mixture was transformed into *E. coli* DH5α. Transformants were selected on LB medium containing 100 μg of ampicillin per ml and 20 μg of kanamycin per ml. The resulting plasmid, pNC52-k (Fig. 1B), was digested with *Xho*I, and the resulting 3.2-kb fragment containing the disrupted *comEC*-like gene plus flanking DNA was introduced into *T. thermophilus* HB27 by natural transformation. Transformants were selected on TM medium containing 40 μg of kanamycin per ml.

To disrupt the *comEA*-like gene, the insert of pFE87 was cloned as an *Hin*dIII-*Xba*I fragment into pBluescriptII KS in order to delete restriction sites from the multiple cloning site. The resulting plasmid, pFE87-HX (Table 1), was digested with *Bam*HI and incubated in the presence of dNTPs and the Klenow enzyme. The blunt-ended *kat* gene was ligated to blunt-ended, linearized plasmid pFE87-HX, and the ligation mixture was transformed into *E. coli* DH5α. Transformants were selected on LB medium containing 20 μg of kanamycin per ml and 100 μg of ampicillin per ml. The resulting recombinant plasmid, designated pFE87-k (Fig. 1B), was digested with *Pst*I plus *Xba*I, and the 3.3-kb fragment containing the disrupted ORF plus flanking wild-type DNA was introduced into *T. thermophilus* HB27 via natural transformation. Transformants were selected on TM medium containing 40 μg of kanamycin per ml. Correct allelic replacement of chromosomal wild-type *comEA*- and *comEC*-like genes by the disrupted ORFs was verified by Southern hybridization.

Construction of *comA*::*lacZ* transcriptional fusions and assay for β-galactosidase activities. To generate *comA*::*lacZ* transcriptional fusions, vector pKOK 6.1 (30) containing a promoter-free *lacZ* gene was digested with *Pst*I. The 4.7-kb *Pst*I fragment, carrying the promoter-free *lacZ* gene, was inserted into the *Pst*I site in pAF702-2.3, which resulted in pAF702-3.2 (Table 1). Correct orientation of the insert with respect to the *lacZ* gene was confirmed by restriction analysis. The insert of pAF702-3.2 was subcloned as a 8-kb *Kpn*I-*Bam*HI fragment in the opposite orientation with respect to the *lac* promoter into the broad-host-range vector pRK415, resulting in plasmid pRK702-3.2 (Table 1). pRK702-3.2 was transferred into *Acinetobacter* sp. strain ADP239 via triparental mating. As a control *Acinetobacter* sp. strain ADP239 carrying the *lacZ* gene of pKOK6.1 cloned in the opposite direction with respect to the *lac* promoter in vector pRK415 was used. The β-galactosidase activities were determined as described by Miller (37) and were expressed in Miller units, which are proportional to the increase in absorbance of free *o*-nitrophenol per minute and cell density.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
Acinetobacter strains		
Acinetobacter sp. strain BD413	Wild type	25
Acinetobacter sp. strain ADP239	Spontaneous *pobA* mutant of BD413	19
Acinetobacter sp. strain ADP197	*recA* mutant (*rec100*::Tn*5*) of BD413	16
T701	*pobA comA*::Tn*10*, noncompetent	This study
T702	*pobA comA*::*nptII*, noncompetent	This study
T703	*pobA orf4*::*nptII*	This study
Thermus strains		
T. thermophilus HB27	Wild type	42
TtAF1	*comEC*::*kat*, noncompetent mutant	This study
TtAF2	*comEA*::*kat*, noncompetent mutant	This study
E. coli strains		
DH5α	F *lacZ*Δ*M15 recA1* π^- *hsdR17 supE44* Δ(*lacZYA argF*)	18
S17-1(λpir)	*trp* Sm^r *recA thi pro hsdM*$^+$ RP4-Tc::Mu::Km Tn7 λpir, *hsdR* mutant	54
Plasmids		
pBluescriptII KS/SK	Ap^r	Stratagene
pRK415	Tc^r *lacPOZ′*	28
pTZ19r	Ap^r	MBI, Fermentas
pGEM-7Zf(+)	Ap^r	Promega, Serva GmbH
pUC4K	Ap^r Km^r	Pharmacia
pLOF/Km	Ap^r, Tn*10*-based delivery plasmid with Km^r	20
pMK18	Km^r	6
pKOK6.1	Ap^r Km^r Cm^r	30
pAF701-1	10.5-kb *Xba*I fragment of mutant T701 in pGEM-7Zf(+) (*comA*::Tn*10*)	This study
pAF701-1.2	3.9-kb *Eco*RI-*Eco*RV fragment of mutant T701 in pBIIKS (*comA*::Tn*10*)	This study
pAF701-1.3	1.9-kb *Bam*HI-*Eco*RV fragment of pAF701-1.2 in pBIIKS (*′comA′*)	This study
pAF701-1.4	*nptII* gene inserted into *Pst*I site of pAF701-1.3 (*comA*::*nptII*)	This study
pAF702-2	12.5-kb *Hin*cII fragment of mutant T702 in pBIISK (*comA*::*nptII*)	This study
pAF702-2.1	5.3-kb *Hin*cII-*Bam*HI fragment of pAF702-2 in pBIISK (*comA*::*nptII*)	This study
pAF702-2.2	4-kb *Hin*cII-*Bam*II fragment (*′orf1 orf2 comA ′orf4*)	This study
pAF702-2.3	3.3-kb insert of pAF702-2.2 in pBIISK (*′orf2 comA ′orf4*)	This study
pRK702-2.2	4-kb *Bam*HI-*Kpn*I insert of pAF702-2.2 in pRK415	This study
pRK702-2.3	3.3-kb *Bam*HI-*Kpn*I insert of pAF702-2.3 in pRK415	This study
pAF702-3.2	*lacZ* gene inserted into *Pst*I site of pAF702-2.3 (*comA*::*lacZ*)	This study
pRK702-3.2	8-kb *Bam*HI-*Kpn*I insert of pAF702-3.2 in pRK415 (*comA*::*lacZ*)	This study
pAF702-4.4	3.4-kb *Pst*I-*Hin*dIII insert of pAF702-2 in pBIIKS	This study
pRK702-4.2	3.4-kb insert of pAF702-4.4 in pRK415	This study
pRK702-4.3	*nptII* gene inserted into *Bam*HI site of pRK702-4.2 (*orf4*::*nptII*)	This study
pNC52	*Thermus* gene bank plasmid carrying a 2.6-kb fragment in pTZ19r (*comEC*)	This study
pFE87-HX	*Thermus* gene bank plasmid cloned as 2.1-kb *Hin*dIII-*Xba*I fragment in pBIIKS (*comEA*)	This study
pNC52-k	*kat* gene inserted into *Bst*XI site of pNC52 (*comEC*::*kat*)	This study
pFE87-k	*kat* gene inserted into *Bam*HI site of pFE87-HX (*comEA*::*kat*)	This study

Nucleotide sequence accession number. The sequence data have been deposited in the GenBank database under accession no. AF320001 and AF319938.

RESULTS

Isolation and characterization of a noncompetent *Acinetobacter* mutant. Transposon mutagenesis of an *Acinetobacter* sp. strain BD413 genomic library resulted in 200 tetracycline- and kanamycin-resistant *E. coli* DH5α clones. Retransfer of the mutant loci into *Acinetobacter* sp. strain ADP239 led to 1,000 kanamycin-resistant transformants which were analyzed to determine their transformation phenotypes. These studies led to detection of one completely noncompetent mutant, designated T701 (Fig. 2A).

During previous transformation studies of mutants of *Acinetobacter* sp. strain ADP239, a deletion the *pca* operon, which encodes the key enzymes used for further breakdown of the *p*-hydroxybenzoate hydroxylase (PobA) reaction product (protocatechuate), was observed. A spontaneous large deletion in the *pca* operon would interfere with the screening system used for transformation-deficient mutants, which is based on the failure of transformation-deficient ADP239 mutants to grow on *p*-hydroxybenzoate in the presence of wild-type DNA since they cannot acquire the wild-type *pobA* allele. To exclude the possibility that there was a deletion in the *pca* operon, which could have resulted from integration of two noncontiguous DNA fragments into the ADP239 genome, mutant T701 was analyzed to determine its ability to use protocatechuate as a sole carbon and energy source. These studies revealed that mutant T701 was able to grow on protocatechuate, and so the possibility that there was a deletion in the *pca* operon was eliminated. Furthermore, mutant T701 showed a high proficiency for DNA repair during growth in the presence of the DNA-alkylating agent methyl methanesulfonate, which eliminated the possibility that the transformation defect was a result of an impaired RecA function. Complementation studies with recombinant plasmids carrying *comP, comB, comC, comE*, and/or *comF* revealed that the noncompetent phenotype of mutant T701 was not due to a defect in these recently identified competence genes. This was confirmed by hybridization studies (data not shown).

Cloning and nucleotide sequence analysis of the novel *Acinetobacter* competence locus. The mutant locus of T701 was re-

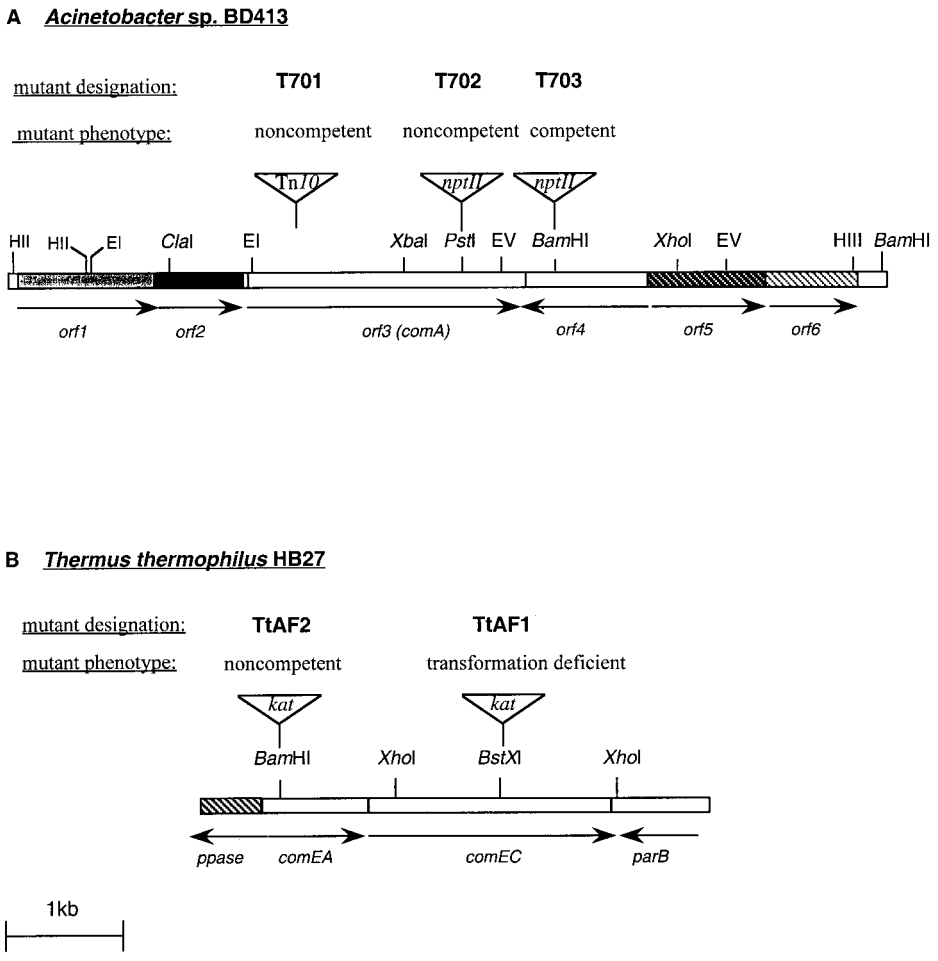

FIG. 2. (A) Chromosomal region of *Acinetobacter* sp. strain BD413 containing *orf1, orf2, orf3* (*comA*), *orf4, orf5*, and *orf6* and physical map of the mutant loci. (B) Chromosomal region of *T. thermophilus* HB27 containing *ppase, comEA, comEC*, and *parB*. The arrows indicate directions of transcription. *nptII*, insertion site of the Km^r gene; Tn*10*, insertion site of mini-Tn*10*; *kat*, insertion site of the *kat* gene; EI, *Eco*RI; EV, *Eco*RV; HII, *Hin*cII; HIII, *Hin*dIII.

covered on a 10.5-kb *Xba*I fragment (pAF701-1) and an overlapping 3.9-kb *Eco*RI-*Eco*RV fragment (pAF701-1.2) (Fig. 1A). Sequence analysis of the mini-Tn*10*-flanking genomic DNA of the mutant loci recovered led to identification of a 3′-end-truncated ORF, *′orf3*, that was disrupted by transposon insertion. This finding suggests that *orf3* plays an essential role in natural transformation of *Acinetobacter* sp. strain BD413. To allow cloning of the complete *orf3* wild-type allele, an additional *Acinetobacter orf3* mutant, designated T702, was generated by inserting a kanamycin marker gene (*nptII*) into the *Pst*I site of *orf3*. Analysis of the transformation phenotype of the resulting mutant, T702 (Fig. 2A), revealed a completely noncompetent phenotype. The mutant locus of T702 was recovered on a 12.5-kb *Hin*cII fragment (pAF702-2) (Fig. 1A). To characterize the mutant loci of mutant T701 and T702, the inserts of plasmids pAF701-1 and pAF702-2 were subcloned into a series of overlapping clones, and a sequence analysis was performed. These studies led to identification of six ORFs, designated *orf1, orf2, orf3, orf4, orf5*, and *orf6* (Fig. 2A).

Characterization of *orf1, orf2, orf3, orf4, orf5*, and *orf6*. Each ORF starts with an ATG start codon and is preceded by a well-conserved and well-placed ribosome binding site (53). Two potential ATG start codons were found for *orf3*. Due to the well-placed conserved ribosome binding site, the second ATG is thought to represent the start codon. A conserved site for a presumptive σ^{70}-dependent promoter was detected 68 bp upstream of this start codon. Additional conserved sites for presumptive σ^{70} -dependent promoters were found 25 bp upstream of *orf1* and 31 bp upstream of *orf5*. A sequence with dyad symmetry that could form a stem-loop structure and that may act as a rho-independent terminator (46) was found 18 bp downstream of the stop codon of *orf2* and 13 bp downstream of the stop codon of *orf6*. The ORFs are tightly clustered, and the stop codon of *orf1* overlaps the start codon of *orf2*. This suggests that *orf1* and *orf2* may form an operon.

Similarities of the deduced proteins with proteins in databases. The predicted proteins encoded by *orf1, orf2, orf3, orf4, orf5*, and *orf6* comprise 411, 228, 792, 337, 338, and 268 amino acids, respectively, and have deduced molecular masses of 45, 25, 91, 39, 37, and 31 kDa, respectively. Orf1 and Orf2 are similar to components of ABC transporters. The protein product of *orf1* is 22% identical to membrane protein HI1548 of *H. influenzae* RdKW20 (14) and 20% identical to DevC of *Anabaena* sp. strain PCC 7120, the integral membrane com-

ponent of the DevBCA exporter (12). Hydropathy plots indicated that Orf1 is strongly hydrophobic and has at least four transmembrane helices. Orf2 is similar to ATP binding proteins of ABC transporters, exhibiting 41, 41, and 35% identity with UgpC (52) and GlnQ (40) of *E. coli* and DevA of *Anabaena* (12), respectively. ATP binding proteins of ABC transporters are characterized by an ATP binding motif (Walker sites A and B) which is essential for ATP binding and is highly conserved in Orf2 (11). The deduced protein encoded by *orf3* is similar to proteins required for genetic transformation of different gram-negative and gram-positive bacteria; for example, Orf3 is 40% similar to gonococcal ComA (10), *B. subtilis* ComEC (18) and *H. influenzae* Rec-2 (5) and 37% similar to CelB of *S. pneumoniae* (45). Based on the significant similarities and the suggested function of Orf3 in natural transformation, Orf3 was designated ComA. *Acinetobacter* ComA also exhibits structural similarties with its homologs, including several hydrophobic domains in the central part of the protein. At least six hydrophobic potentially membrane-spanning domains were found in *Acinetobacter* ComA between residues 250 and 530, and two additional hydrophobic domains are present close to the N terminus.

The deduced protein encoded by *orf4* is similar to HtrB proteins, exhibiting 35 and 33% similarity to HtrB of *H. influenzae* (33) and HtrB of *E. coli* (26), respectively. HtrB proteins are membrane-associated proteins that are involved in lipid A biosynthesis and are required for rapid growth of *E. coli* at temperatures above 33°C (3, 27).

The protein product encoded by *orf5* is similar to proteases. The deduced protein is 36 and 41% similar to proteinase IV of *E. coli* (23) and PspA of *Campylobacter jejuni* (7), respectively. Proteinase IV of *E. coli* is a nonessential protease which digests cleaved signal peptides (23), and PspA of *C. jejuni* is thought to be involved in induction and/or processing of pilus subunits (7). Database searches with the deduced protein encoded by *orf6* revealed no significant similarities to known proteins.

***comA* complementation studies and disruption of *comA*-flanking ORFs.** pRK415 derivatives pRK702-2.2 and pRK702-2.3 (Fig. 1A), both carrying the *comA* wild-type gene, were mobilized into noncompetent mutants T701 and T702, respectively, and were found to fully restore transformation competence. These results provide clear evidence that *comA* is essential for natural transformation in *Acinetobacter* sp. strain BD413. Complementation of the noncompetent mutants was independent of the orientation of the insert with respect to the *lac* promoter, indicating that expression of *comA* is under control of its native promoter.

To address the question whether *orf1, orf2*, and *orf4* are also implicated in natural transformation, the ORFs were subjected to mutagenesis individually, and the resulting mutants were analyzed to determine their natural transformation phenotypes. Despite many different attempts, mutagenesis of *orf1* and *orf2* was not possible. This suggests that *orf1* and *orf2* have functions that are essential for cell viability. The *nptII* marker gene was successfully inserted into *orf4* (Fig. 2A). The resulting mutant, T703, exhibited normal growth rates and was not affected in terms of natural transformation.

Growth phase-dependent transcription of *comA* in *Acinetobacter* sp. strain ADP239. In order to gain insight into regulation of *comA* expression and to address the question of potential coregulation of *comA* with the recently identified pilin-like genes required for genetic transformation of *Acinetobacter*, we constructed transcriptional fusions of *comA* and a promoter-free *lacZ* gene and monitored β-galactosidase activities during growth. Upon transfer of the cells into fresh medium, *comA* transcription decreased rapidly, but then it recovered steadily during growth, reaching a maximum in the late stationary phase (Fig. 3). The course of growth phase-dependent regulation is correlated with growth phase-dependent transcriptional regulation of the pilin-like competence genes *comB, comE, comF*, and *comP* (21, 48).

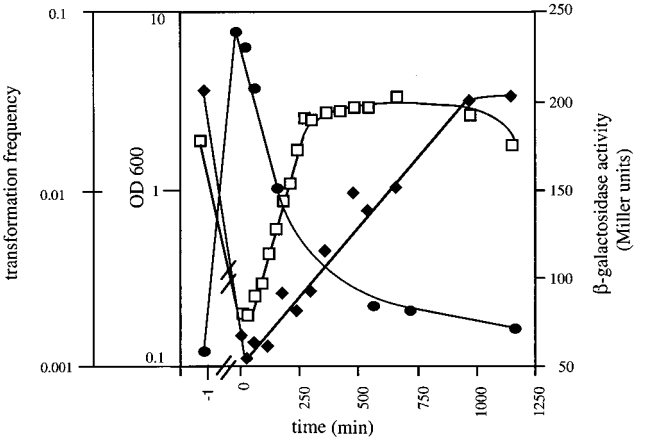

FIG. 3. Growth phase-dependent transcription of *comA* in *Acinetobacter* sp. strain ADP239. *comA* transcription was monitored by using *comA*::*lacZ* reporter fusions located on low-copy-number plasmid pRK415. β-Galactosidase activity was monitored as described by Miller (37). Optical densities at 600 nm (OD 600) (□), β-galactosidase activities (◆), and transformation frequencies (●) during growth were determined at the times indicated.

Identification of *comEC* and *comEA* competence genes in *T. thermophilus* HB27. Searches in the genome database for *T. thermophilus* HB27 with *Acinetobacter* sp. strain BD413 ComA led to identification of an ORF whose product shows significant similarities to the *Acinetobacter* sp. strain BD413 and *N. gonorrhoeae* ComA competence proteins and the *B. subtilis* ComEC competence protein. Analysis of the region upstream of this conserved ORF led to detection of an ORF encoding a putative protein with similarities to the *B. subtilis* protein ComEA, which is essential for genetic transformation of *B. subtilis* (17, 24). Based on the significant similarities and the organization of these two ORFs, which were analogous to the *B. subtilis comEA-comEC* cluster, they were designated *comEA* and *comEC*. The TGA stop codon of *comEA* in *T. thermophilus* HB27 overlaps the ATG start codon of *comEC*, indicating that putative cotranscription of the two ORFs occurs. Downstream of *comEC* an ORF nearly identical to the putative *parB* gene (accession no. AJ277593) of *T. thermophilus* HB8 was identified. The orientation of this ORF is opposite the orientation of *comEA* and *comEC* (Fig. 2B).

To examine the potential function of *comEA* and *comEC* in the transformation mechanism of the highly competent extreme thermophile *T. thermophilus* HB27, both ORFs were subjected to gene disruption by using the thermostable kanamycin gene *kat*. The resulting kanamycin-resistant *T. thermophilus* HB27 mutants, designated TtAF1 and TtAF2 (Table 1

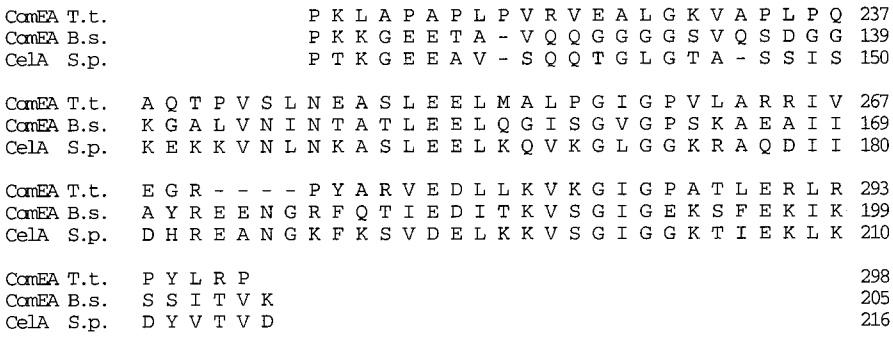

FIG. 4. Alignment of ComEA proteins. Identical residues are indicated by gray boxes. B.s., *B. subtilis*; S.p., *S. pneumoniae*; T.t., *T. thermophilus* HB27.

and Fig. 2B), were analyzed to determine their transformation phenotypes by using the genomic DNA of a spontaneous streptomycin-resistant *T. thermophilus* HB27 mutant. In the *comEA* mutant natural transformation was completely absent, whereas in the *comEC* mutant natural transformation was significantly impaired (the natural transformation frequencies were 1,000-fold lower). Since the orientation of the downstream putative *parB* gene is opposite the orientation of *comEA* and *comEC*, the transformation defects of the *comEA* and *comEC* mutants cannot be due to a polar effect on the *parB* gene. The gene organization results together with the results of the mutant studies provide clear evidence that the *Thermus comEA-comEC* locus is involved in natural transformation. Since *comEC* is located downstream of *comEA*, the possibility that marker insertion in *comEC* has a polar effect on the preceding *comEA* gene can be excluded. The organization of *comEA* and *comEC*, together with the results of the mutant studies leads to the conclusion that *comEC* is important for natural transformation. Although the possibility that marker insertion in *comEA* has a polar effect on *comEC* cannot be excluded, the phenotypic differences of the *comEA* and *comEC* mutants indicate that analogous to *B. subtilis comEA* and *comEC*, both genes, *comEA* and *comEC*, are essential for *T. thermophilus* transformation.

Characterization of the *comEC* and *comEA* competence genes in *T. thermophilus* HB27. The protein encoded by *comEC* consists of 677 amino acids and has a calculated mass of 72 kDa. *Thermus* ComEC is 40, 40, 40, 37, and 37% similar to ComA of *Acinetobacter* sp. strain BD413, ComEC of *B. subtilis*, Rec-2 of *H. influenzae*, ComA of *N. gonorrhoeae*, and CelB of *S. pneumoniae*, respectively. Like ComA of *Acinetobacter* sp. strain BD413, ComEC of *T. thermophilus* HB27 is predicted to be an inner membrane protein with six potentially membrane-spanning domains in the central part.

The ComEA protein of *T. thermophilus* HB27 consists of 298 amino acids, has a calculated mass of 30 kDa, and is similar to ComEA of *B. subtilis* and CelA of *S. pneumoniae*, which comprise 205 and 216 amino acids, respectively (17, 45). In a C-terminal 60-amino-acid overlap ComEA of *T. thermophilus* is 60% similar to the C-terminal domain of *Bacillus* ComEA and *Streptococcus* CelA (Fig. 4). This domain includes a helix-hairpin-helix motif which is responsible for DNA binding, as demonstrated in *B. subtilis* (49).

DISCUSSION

Here we describe identification of novel competence genes in the mesophilic soil bacterium *Acinetobacter* sp. strain BD413 and the extremely thermophilic bacterium *T. thermophilus* HB27. The *T. thermophilus* HB27 competence genes are the first competence genes identified in an extremely thermophilic bacterium so far. Based on sequence similarities, the novel competence genes in *Acinetobacter* and *Thermus* were designated *comA* and *comEC*, respectively. Both *comA* of *Acinetobacter* sp. strain BD413 and *comEC* of *T. thermophilus* HB27 were found to be essential for natural transformation. Sequence analysis of the *comA* locus of *Acinetobacter* sp. strain BD413 revealed six tandemly arranged (ORFs), designated *orf1, orf2, comA, orf4, orf5*, and *orf6*. The *comEC* gene of *T. thermophilus* HB27 is preceded by an ORF that encodes a protein with significant similarities to the *B. subtilis* competence protein ComEA (17, 24). This ORF, designated *comEA*, was also found to be essential for natural transformation in *Thermus*. A *comEA*-like competence gene was not detected close to the *comA* gene in *Acinetobacter* sp. strain BD413.

Despite the fact that *comA* of *Acinetobacter* sp. strain BD413 is not closely associated with the recently identified *Acinetobacter* competence genes *comP, comB, comE*, and *comF*, growth phase-dependent transcriptional regulation this *comA* gene is similar to regulation of the pilin-like competence genes *comP, comB, comE,* and *comF*.

Although *T. thermophilus, B. subtilis*, and *S. pneumoniae* are not closely related bacteria, the genes of the conserved *comEC* locus of *T. thermophilus* and *B. subtilis* and the homologous *celB* locus of *S. pneumoniae* are organized analogously. In *B. subtilis, T. thermophilus*, and *S. pneumoniae* the competence gene *comEC* or *celB* is closely associated with a second competence gene that was first described in *B. subtilis* as *comEA*. However, in contrast to *B. subtilis*, a homolog of an additional gene, *comEB*, which is present in the *Bacillus* competence-specific operon, is not present *T. thermophilus* and *S. pneumoniae* (2, 17, 45).

Clues to the function of ComA in *Acinetobacter* sp. strain BD413 and the function of ComEC in *T. thermophilus* HB27 can be derived from the significant similarities of these proteins to ComA of *N. gonorrhoeae*, ComEC of *B. subtilis*, and Rec-2 of *H. influenzae* (8). *comA* mutants of *N. gonorrhoeae* have been shown to take up DNA in a DNase-resistant state

(9). This finding, together with the prediction that there are several transmembrane α-helices, led to the proposal that the gonococcal ComA protein may be an integral membrane protein involved in DNA transfer across the cytoplasmic membrane (9, 10). The *comEC* product in *B. subtilis* was also predicted to be a polytopic integral membrane protein with 8 to 10 transmembrane segments and was found to be essential for DNA uptake but not for DNA binding (17, 24). An analogous function has been suggested for Rec-2 of *H. influenzae* (4). Based on secondary structure predictions for ComA of *Acinetobacter* sp. strain BD413 and ComEC of *T. thermophilus* HB27, six central and two N-terminal hydrophobic regions are present in ComA and ComEC, respectively. Furthermore, ComA of *Acinetobacter* sp. strain BD413 and ComEC of *T. thermophilus* both have a large, hydrophilic, positively charged domain near the N terminus, as well as a large hydrophilic, positively charged C-terminal domain which may be localized in the cytoplasm, based on the rule that positive charges of inner membrane proteins are oriented towards the cytoplasm (5). The N terminus of ComA of *Acinetobacter* sp. strain BD413 has structural features characteristic of signal peptides (56) including (i) a positively charged N terminus (Arg-4), (ii) a hydrophobic core (Ile-15 to Ile-23), and (iii) a small neutral C-terminal residue. Positions −1 and −3 are known to be particularly important for specifying the cleavage site, which could be between Ala-25 (position −1) and Ile-26 (position 0) according to the proposal of von Heinje (55). In contrast to ComA of *Acinetobacter* sp. strain BD413, the N terminus of ComEC of *T. thermophilus* does not exhibit structural features characteristic of signal peptides. Based on the structural characteristics and topology data, we propose that ComA and ComEC of *Acinetobacter* and *Thermus*, repectively, are channel-forming polytopic membrane proteins implicated in transport of DNA through the cytoplasmic membrane.

The broad distribution of ComA- and ComEC-like factors among very different bacteria independent of their phylogenetic relationships and their natural environments indicates that these proteins may play a central role in the transformation mechanisms of very different bacteria and that these components are highly conserved.

It has to be noted that except for the *comEA* gene of the *comEC* locus in *T. thermophilus*, no *comEA*-like genes have been found close to conserved *comA*/*comEC*-like genes in gram-negative bacteria, but interestingly, multiple copies of *comEA*-like genes have been identified in the genome of *N. gonorrhoeae*, and at least one of them is involved in natural transformation (I. Chen and E. C. Gotschlich, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. D/B-10, p. 211, 1999). Analogously, the possibility that there are genes in *Acinetobacter* sp. strain BD413 that exhibit similarities to the *B. subtilis* competence gene *comEA* also cannot be excluded, and this question remains an interesting topic for the future.

ACKNOWLEDGMENTS

This work was supported by grant Av 9/4-4 from the Deutsche Forschungsgemeinschaft. A. Friedrich was supported by the Fond der Chemischen Industrie.

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