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Noncanonical Cell-to-Cell DNA Transfer in *Thermus* spp. Is Insensitive to Argonaute-Mediated Interference

Alba Blesa,[a] Carolina Elvira César,[a] Beate Averhoff,[b] José Berenguer[a]

Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid-Consejo Superior de Investigaciones Científicas, Madrid, Spain[a]; Molecular Microbiology and Bioenergetics, Institute of Molecular Biosciences, Goethe University Frankfurt, Frankfurt am Main, Germany[b]

Horizontal gene transfer drives the rapid evolution of bacterial populations. Classical processes that promote the lateral flow of genetic information are conserved throughout the prokaryotic world. However, some species have nonconserved transfer mechanisms that are not well known. This is the case for the ancient extreme thermophile *Thermus thermophilus*. In this work, we show that *T. thermophilus* strains are capable of exchanging large DNA fragments by a novel mechanism that requires cell-to-cell contacts and employs components of the natural transformation machinery. This process facilitates the bidirectional transfer of virtually any DNA locus but favors by 10-fold loci found in the megaplasmid over those in the chromosome. In contrast to naked DNA acquisition by transformation, the system does not activate the recently described DNA-DNA interference mechanism mediated by the prokaryotic Argonaute protein, thus allowing the organism to distinguish between DNA transferred from a mate and exogenous DNA acquired from unknown hosts. This Argonaute-mediated discrimination may be tentatively viewed as a strategy for safe sharing of potentially "useful" traits by the components of a given population of *Thermus* spp. without increasing the genome sizes of its individuals.

Lateral gene flow is responsible for the enormous plasticity observed in prokaryotic genomes, which confers on these organisms the ability to adapt rapidly to environmental changes (1–5). Three conserved mechanisms are traditionally associated with lateral gene transfer (LGT) in prokaryotes: phage-mediated transduction, natural transformation, and conjugation. Besides, alternative DNA transfer systems based on nanotubes (6), membrane vesicles, and nanopods in *Archaea* (7–9), or on the so-called "gene transfer agents" (10), have been described as contributors to genetic exchange among prokaryotes. Among these processes, transformation and conjugation depend only on functions encoded by the bacterial cell. Transformation involves the uptake of naked DNA from the environment and relies entirely on the ability of a competent recipient cell to incorporate DNA. Conjugation, on the other hand, is traditionally seen as the unidirectional transfer of a plasmid DNA molecule from a donor to a recipient cell, where the proteins responsible for DNA transfer are provided exclusively by the donor cell (11).

Conjugation differs from transformation not only in the imperative need for a mate for the transfer of DNA but also in the nature of the transferred DNA. While transformation can be envisioned as a mechanism that might have originally evolved in ancestral bacteria, such as *Thermus* spp., for the acquisition of DNA of any sort and origin as a nutrient, conjugation is more frequently associated with a gain of new capabilities from related species and commonly involves plasmids or megaplasmids that encode all the functions required for conjugation. In fact, transfer of chromosomal genes is apparently less common and in many cases requires the integration of a conjugative plasmid to generate a high frequency of recombination (Hfr) strain capable of transferring extensive regions of chromosomal DNA into recipient cells in a polar fashion (12). Transfer of chromosomal DNA can also be mediated by integrative and conjugative elements (ICEs). ICEs have recently received growing attention due to their ubiquitous presence among prokaryotes (13–15).

Albeit with variations, the general mechanism of conjugation is shared by *Archaea* and *Bacteria* and involves cell-to-cell contacts through a type 4-like secretion system (T4SS) (16). In addition, unconventional conjugative transfers, such as those using the single polar transfer protein TraB from *Streptomyces* spp. (17), or the chromosomally encoded multiple *cis*-acting sequences in *Mycobacterium smegmatis* (18), have been described as devoid of T4SS elements.

The ancient *Thermus-Deinococcus* clade (19) involves a group of extremophilic bacteria, including extreme thermophiles and radiation-resistant isolates. In addition to their thermophilic character, one of the most remarkable properties of *Thermus* spp. is the ability to take up and incorporate foreign DNA through a highly efficient natural competence system (20–22). The *Thermus thermophilus* laboratory model strains HB8 and HB27 were the first to be sequenced. Both harbor a highly syntenic ~1.8-Mbp chromosome and a more divergent and plastic megaplasmid (>200 kbp) that harbors most of the strain-specific genes (23). Actually, it has been suggested that this megaplasmid constitutes the main landing site for genes acquired laterally through natural competence (24), including loci that favor adaptation to a thermophilic lifestyle (22). Among the genes hypothetically acquired through LGT, several strains of *T. thermophilus* harbor a gene

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Address correspondence to José Berenguer, jose.berenguer@uam.es.

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encoding a homologue of the Argonaute protein, which is involved in RNA silencing in eukaryotes (25). It has been reported recently that the Argonaute homologue of *T. thermophilus* (ttAgo) constitutes a barrier to DNA acquired by transformation through a mechanism involving DNA-DNA interference, resulting in a ~10-fold decrease in the incorporation of genes acquired by natural competence. Also, the presence of ttAgo decreases the copy numbers of resident plasmids (26).

In addition to DNA acquisition by natural competence, *T. thermophilus* is able to transfer DNA by a mechanism that involves cell-to-cell contacts (21, 27). However, bioinformatic analyses conducted on the conjugation-proficient strains HB27 and HB8 have failed to detect the presence of conjugative T4SS homologues (21), supporting the existence of a distinct mechanism for conjugation in these isolates. In this work, we provide insights into the genetic basis of this mechanism of cell-to-cell DNA transfer. We show that this mechanism requires the presence of an active competence system in the recipient mate, that the transfer system has a strong preference for megaplasmid-associated genes over chromosomal genes, and that the DNA transferred by this mechanism does not elicit the DNA-DNA interference control mediated by the ttAgo protein. Taken together, these data suggest that this novel conjugation-like mechanism may play a major role in lateral gene transfer in this species, allowing the genus to distinguish between potentially malicious DNA acquired from the environment and reliable DNA transferred from a mate.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this work are listed in Table 1. *Escherichia coli* strain DH5α, used for the construction of plasmids, was grown at 37°C in liquid or solid LB (28) selective medium. *T. thermophilus* strains were grown aerobically under rotational shaking (150 rpm) at 60°C in liquid or solid TB (*Thermus* broth [29]) medium, unless otherwise indicated. Kanamycin (Km; 30 μg/liter), ampicillin (Ap; 100 μg/liter), and/or hygromycin (Hyg; 100 μg/liter) was added when needed for selection.

Plasmids and construction of mutant strains. The plasmids used in this work are listed in Table 2. The oligonucleotides used for PCR amplification are listed in Table S1 in the supplemental material. DNA manipulation and cloning were performed using standard laboratory techniques (30). All constructs were checked by restriction analysis and sequencing, and mutants were confirmed by PCR analysis.

T. thermophilus integration mutants containing appropriate selective markers were constructed by transformation with the suicide plasmid pK18 or pH118, which confers thermostable resistance to Km or Hyg, respectively. An internal fragment of the target gene was used for single recombination to obtain the corresponding insertion mutant. Deletion mutants were constructed by double recombination with a linearized DNA construct containing upstream and downstream flanking regions around the target gene separated by the *hyg* or *kat* gene cassette, encoding thermostable resistance to Hyg or Km, respectively (31). In all cases, the cassette was inserted in the same transcription sense as the target gene to allow the expression of downstream genes. Transformation and selection were performed as described previously (32).

Transformation and conjugation assays. *T. thermophilus* strains were transformed by adding 10 to 200 ng of DNA to exponential-phase cultures of the strains as described previously (20, 32). Transformation frequencies were measured as the number of CFU on selective plates per viable cells of the transformed strain. For mating assays, *T. thermophilus* cultures, labeled with gene cassettes conferring thermostable resistance to Km or Hyg, were grown overnight to saturation. One hundred microliters of each mating pair was mixed in the presence of DNase I (5 units; Roche), and the mixture was centrifuged at 5,000 rpm for 4 min. Pellets were resuspended in 10 μl TB medium with DNase I and were laid on 0.22-μm sterile nitrocellulose filters (Protran BA85; Whatman) on prewarmed TB agar plates. After 5 h at 60°C, filters were introduced into Eppendorf tubes containing 1 ml liquid TB medium and were vortexed vigorously to wash off the cells. Appropriate serial dilutions were plated onto selective TB agar dishes. Conjugation frequencies were measured as the ratio of the number of CFU resistant to both antibiotics to the number of CFU corresponding to the chromosome-labeled mate.

For qualitative conjugation and transformation assays, 10^7 *T. thermophilus* cells were laid on top of selective agar medium and were topped either with 10^7 cells of the second member of the mating pair (1:1 ratio) or with 200 ng of naked DNA for transformation experiments.

Membrane pattern analyses. Protein profiles of the cell envelope were obtained by scraping cells from spots of transconjugant cells grown on selective agar plates. The cells were resuspended in 500 μl of water and were broken by sonication, and the envelope fraction was concentrated by centrifugation (14,000 × *g*, 15 min). Proteins were solubilized by boiling in Laemmli sample buffer, separated by SDS-PAGE (33), and revealed by Coomassie blue staining.

Statistical methods. Quantitative data were analyzed using SPSS Statistics, version 21.0 (2008; SPSS Inc., Chicago, IL). Results were considered significant if the *P* value was <0.05. To examine differences among conjugation frequencies (calculated as described above), Student's *t* test and the nonparametric Mann-Whitney U test and Kruskal-Wallis test were used. In studying differences in transfer frequencies among and within various loci, variance was addressed by one-way analysis of variance (ANOVA) and the Kruskal-Wallis test. *Post hoc* Tukey and Bonferroni tests were carried out when convenient.

RESULTS

Cell-to-cell DNA transfer among *T. thermophilus* strains shows preference for the pTT27 megaplasmid. DNA transfer between *T. thermophilus* strains was monitored in mating experiments involving the transfer of genes conferring thermostable resistance to Km or Hyg, inserted at different positions in the chromosome (CK or CH clones, respectively) or the megaplasmid (PK or PH clones, respectively) (Table 1). Mating experiments were carried out with equal amounts of the two mates in the presence of thermostable DNase I in order to avoid transfer by natural competence after putative cell lysis or DNA secretion. Figure 1 shows that cell-to-cell transfer between strains CK1 and PH1 (referred to below as the CK1 × PH1 cross) or between strains CK1 and CH1 was insensitive to the presence of DNase I, whereas this enzyme prevented the transfer of plasmid pMH::Pnqo::s*gfp* by natural competence in the control experiment. Besides, experiments in which a nitrocellulose membrane was used to separate the mates did not produce recombinants harboring both resistances (not shown). Therefore, a DNA exchange system that, like classical conjugation processes, is not sensitive to DNase and requires direct cell-to-cell contacts for transfer exists in *T. thermophilus*. We will thus refer to the cells carrying both markers as transconjugants. However, in contrast to classical conjugation conditions, the strains used for these experiments are isogenic, which implies the absence of an active surface exclusion system and, at the same time, hinders discrimination between "donor" and "recipient" strains.

As can be observed in Fig. 1, a higher number of transconjugants was routinely detected when one of the mates was labeled in the megaplasmid (PH1) than when both mating strains were labeled in the chromosome. Thus, we quantified the frequency of transfer as the number of transconjugants (with both antibiotic resistances) per chromosomally labeled Km-resistant cell (CK1). For this experiment, either the chromosomally labeled strain CH1

TABLE 1 Bacterial strains used in this work

Strain	Genotype	Phenotype/use	Reference and/or source
E. coli DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* ϕ80d*lac*ΔM15	Cloning	47
Thermus thermophilus			
HB27	ATCC BAA-163/DSM7039	Wild type	Y. Koyama
HB27^{EC}	HB27 *ago*::*ago*IS*Tth7*	Enhanced competence	Lab strain; 26
HB27Δ*ago*	Δ*ago*	Lacking Argonaute	26
NAR1	Wild type	Wild type; partially denitrifying	48
PH1	HB27^{EC}(pTT27::*ttp0146*::*hyg*)	Hyg^r	This work
PH2	HB27^{EC}(pMHPnqos*gfp*)	Hyg^r	This work
CK1	HB27 *gdh*::*kat*	Km^r	48
CK3	NAR1 *gdh*::*kat*	Km^r; partially denitrifying	This work
CH1	HB27^{EC}::Δ*ttc0313*::*hyg*	Hyg^r	49
PK1	HB27^{EC}(pTT27::*ttp0046*::*kat*)	Km^r	This work
PK2	HB27^{EC}(pTT27::*ttp0085*::*kat*)	Km^r	This work
PK3	HB27^{EC}(pTT27::*ttp0140*::*kat*)	Km^r	This work
PK4	HB27^{EC}(pTT27::*ttp0167*::*kat*)	Km^r	This work
PK5	HB27^{EC}(pTT27::*ttp0191*::*kat*)	Km^r	This work
PK6	HB27^{EC}(pTT27::*ttp0219*::*kat*)	Km^r	This work
CK5	HB27^{EC} *ttc0638*::*kat*	Km^r	This work
CK6	HB27^{EC} *ttc0893*::*kat*	Km^r	This work
CK7	HB27^{EC} *ttc1415*::*kat*	Km^r	This work
CK8	HB27^{EC} *ttc1844*::*kat*	Km^r	This work
CK11	HB27 Δ*pilA4*::*kat* Δ*ago*	Km^r; noncompetent	This work
CK12	HB27 Δ*pilQ*::*kat* Δ*ago*	Km^r; noncompetent	This work
CK13	HB27 *pilF*::*kat*	Km^r; noncompetent	35
CK14[a]	HB27 *pilA1-3*::*kat*	Km^r; noncompetent	50
CH2	HB27 *pilQ*::*hyg*	Hyg^r; noncompetent	This work
CH3	NAR1 *pilQ*::*hyg*	Hyg^r; noncompetent	This work
CH4	HB27 Δ*pilA4*::*hyg*	Hyg^r; noncompetent	This work
CK15	HB27 *comEA*::*kat*	Km^r; noncompetent	35
CK16	HB27 *pilT*::*kat*	Km^r	35
CK17	HB27 *comZ*::*kat*	Km^r; noncompetent	50
CK18	HB27 *pilD*::*kat*	Km^r; noncompetent	50
CK19	HB27 *pilA3*::*kat*	Km^r; noncompetent	50
CK20	HB27 *comEC*::*kat*	Km^r; 0.001-fold competent	35
CK21	HB27 *pilT2*::*kat*	Km^r	This work
CK22	HB27 *gdh*::*kat* Δ*ago*	Km^r	This work
CK23	HB27 *gdh*::*kat*	Km^r	This work
CH5	HB27 Δ*ttc0313*::*hyg* Δ*ago*	Hyg^r	This work
CH6	HB27 Δ*ttc0313*::*hyg*	Hyg^r	This work
CK24	HB27 *ttc0638*::*kat* Δ*ago*	Km^r	This work
CK25	HB27 *ttc0638*::*kat*	Km^r	This work
CK26	HB27 *ttc0858*::*kat*	Km^r	This work
CK27	HB27 *ttc1621*::*kat* Δ*ago*	Km^r	This work
CK28	HB27 *ttc1844*::*kat* Δ*ago*	Km^r	This work
CK29	HB27 *ttc1844*::*kat*	Km^r	This work

[a] Insertion mutant lacking the genes *pilA1*, *pilA2*, and *pilA3*.

or the megaplasmid-labeled strain PH1 or PH2 was mated in a 1:1 ratio with strain CK1. As shown in Fig. 2A, the numbers of transconjugants with megaplasmid-associated markers (PH1 and PH2) were around 15-fold higher ($93.3 \times 10^{-5} \pm 6.7 \times 10^{-5}$) than the number of transconjugants that had incorporated chromosomal genes (CH1) ($6.1 \times 10^{-5} \pm 1.1 \times 10^{-5}$). To determine if these differences were affected by the position of the transferred locus or the type of antibiotic marker used, we repeated a series of mating experiments with a common chromosomally labeled mating strain (CH1) and several kanamycin-tagged strains, labeled at different positions in the megaplasmid (PK1, PK2, PK3, PK4, PK5, and PK6) or in the chromosome (CK1, CK5, CK6, CK7, and CK8). As shown in Fig. 2B and in Fig. S2 in the supplemental material, the frequencies of transconjugants with megaplasmid-associated markers relative to strain CH1 were at least an order of magnitude higher than those involving only chromosome markers. Bearing in mind that the copy numbers of the chromosome and the megaplasmid are similar (4 to 5 according to reference 34), these figures support the existence of a strong bias toward genes located in the megaplasmid rather than the chromosome.

In contrast, no significant differences were detected among the transfer frequencies of megaplasmid-labeled strains (Fig. 2B). Therefore, it was not possible to infer an order of transfer of loci in

TABLE 2 Plasmids used in this work

Plasmid	Description	Use[a]	Reference
pAB6	pK18::*ttp0191*	Insertion; Km^r; generation of PK5 mutant	This work
pAB8	pK18::*ttp0085*	Insertion; Km^r; generation of PK2 mutant	This work
pAB15	pK18::*ttp0140*	Insertion; Km^r; generation of PK3 mutant	This work
pAB22	pUC19::Δ*pilA4*::*kat*	*pilA4* deletion; Km^r; generation of CK11 and CK26 mutants	This work
pAB52	pUC19::Δ*pilA4*::*hyg*	*pilA4* deletion; Hyg^r; generation of CH4 mutant	This work
pAB36	pUC19::Δ*pilQ*::*kat*	*pilQ* deletion; Km^r; generation of CK12 mutant	This work
pAB54	pUC19::Δ*ttc1844*::*kat*	*ttc1844* deletion; Km^r; generation of CK28 and CK29 mutants	This work
pCBG2	pK18::*ttp0046*	Insertion; Km^r; generation of PK1 mutant	This work
pCBG3	pUC119::*ttp0146*::*hyg*	Insertion; Hyg^r; generation of PH1 mutant	This work
pCEC30	pK18::*ttp0167*	Insertion; Km^r; generation of PK4 mutant	This work
pCEC31	pK18::*ttp0219*	Insertion; Km^r; generation of PK6 mutant	This work
pCEC33	pK18::*ttc1415*	Insertion; Km^r; generation of CK7 and CK21 mutants	This work
pCEC34	pK18::*ttc1844*	Insertion; Km^r; generation of CK8 mutant	This work
pCEC35	pK18::*ttc0638*	Insertion; Km^r; generation of CK5, CK24, and CK25 mutants	This work
pCEC36	pK18::*ttc0893*	Insertion; Km^r; generation of CK6 mutant	This work
pCEC40	pH118::*pilQ*	*pilQ* disruption; Hyg^r; generation of CH2 and CH3 mutants	This work
P#36	p*orf333*::*kat*	*pilT* disruption; Km^r; generation of CK16 and CK27 mutants	35
pH118	Hyg resistant	Suicide vector	49
pK18	Km resistant	Suicide vector	51
pMH::Pnqo::*sgfp*	*Tth* replicative	Hyg^r; episome transfer, generation of PH2 mutant	52
pMK184	*Tth* replicative	Km^r; episome transfer	48
pUC119	Ap resistant	Cloning vector	31
pUC119::Δ*0313H*	pUC119::Δ*ttc0313*::*hyg*	*ttc0313* deletion; Hyg^r; generation of CH1 mutant	49

[a] Km^r or Hyg^r refers to a phenotype of resistance to kanamycin or hygromycin, respectively. Insertional plasmids contain a ~1-kb sequence of the target gene in a suicide vector. Deletion plasmids include a ~1-kb sequence upstream of the target gene and a ~1-kb sequence downstream of the target gene, interrupted by the appropriate antibiotic marker.

the megaplasmid. On the other hand, larger differences were found among the transfer frequencies of chromosomally labeled strains, but different tests failed to reveal enough significance to allow us to infer an order of transfer of the markers.

The natural competence system is involved in cell-to-cell DNA transfer. In order to evaluate a putative relationship between cell-to-cell DNA transfer and natural competence, we employed a series of mutants (CK11 to CK21) known to be affected in natural competence (22) and carried out mating experiments with a transformation-proficient mate (CH1). We observed strong differences in the transfer frequencies among the mutants without any deducible pattern with regard to piliation (see Fig. S3 in the supplemental material). However, when we tried to mate these competence-deficient strains (CK11 to CK21) with another competence-deficient strain (CH4), no transconjugants were obtained (Fig. 3). Therefore, all the components of the natural competence system of *T. thermophilus* in at least one of the mates are required for cell-to-cell transfer.

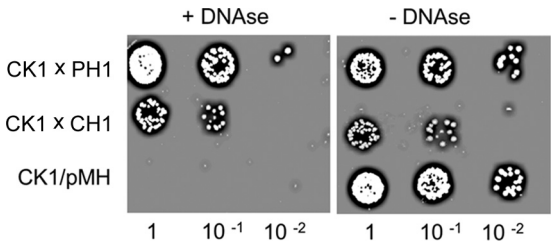

FIG 1 Cell-to-cell DNA transfer in *T. thermophilus*. Shown is the growth on Hyg-Km double-selective TB plates of dilutions of strain CK1 (Km resistant) mated in a 1:1 ratio with an isogenic Hyg-resistant strain labeled in the megaplasmid (PH1) or the chromosome (CH1). As a control, CK1 was transformed with 200 ng of plasmid pMH::Pnqo::*sgfp*, and the growth of serially diluted transformants was assessed. The media for matings and transformation experiments were either supplemented with DNase I before mixing (left) or left unsupplemented (right).

To determine in which of the mates, donor or recipient, the competence system was required, we performed mating experiments between competence mutants of two different *T. thermophilus* strains, NAR1 and HB27, which can be phenotypically distinguished by differences in their respective patterns of membrane proteins (i.e., several traits encoded at different regions are followed simultaneously) (29). Thus, a competence-deficient HB27 (CH2) or NAR1 (CH3) strain was mated with an HB27 or NAR1 derivative proficient (CK1 or CK3, respectively) or deficient (CK11) in competence. As shown in Fig. 4A, plasmid transformation of strains CK11 (HB27 Δ*pilA4*::*kat*) (spot 4a), CH2 (HB27 Δ*pilQ*::*hyg*) (spot 3d), and CH3 (NAR1 Δ*pilQ*::*hyg*) (spot 2d) confirmed their deficiency in competence, whereas transformation was positive for strains CK3 (NAR1 *gdh*::*kat*) (spot 4b) and CK1 (HB27 *gdh*::*kat*) (spot 4c). When competence-deficient strain CH2 or CH3 was mated with the competence-proficient partner CK1 or CK3, we obtained transconjugants (Fig. 4A, spots 2b, 3b, 2c, and 3c). Thus, to analyze the parenthood of these transconjugant derivatives, we compared their whole membrane protein patterns. As shown in Fig. 4B, the membrane protein pattern of the transconjugants obtained after mating between a competent NAR1 strain (CK3) and a noncompetent HB27 mutant (CH2) is identical to the pattern obtained after mating between two NAR1 strains (the CK3 × CH3 cross). Conversely, the protein pattern of transconjugants after mating between a proficient HB27 strain (CK1) and a NAR1 competence mutant (CH3) is identical to the pattern observed for the mating between two HB27 derivatives (the CK1 × CH2 mating pair). Data for the complete experiment and controls are shown in Fig. S4 in the supplemental material.

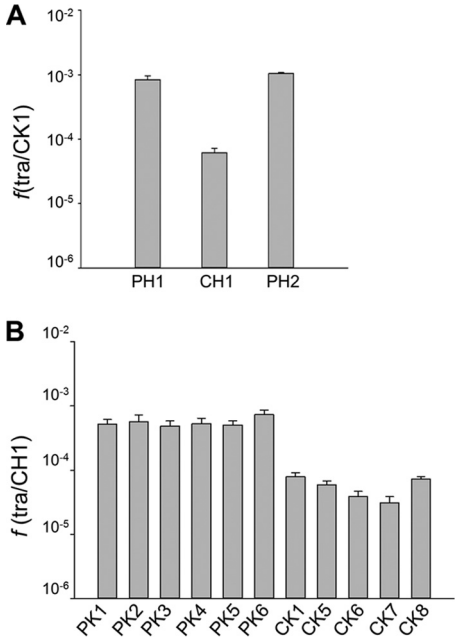

FIG 2 Preference for megaplasmid genes. (A) Transfer frequencies were obtained after equal amounts of a strain labeled with the Hyg cassette in the chromosome (CH1) or in the megaplasmid (PH1, PH2) and strain CK1, labeled with the Km marker in the chromosome, were mixed. Frequencies (*f*) are averages of ratios between colonies grown on selective medium containing Km and Hyg and colonies grown with Km (tra/CK1) in 9 independent experiments. Error bars correspond to the standard deviations of the means. Differences in transfer frequencies between chromosomal (CH1) and plasmid (PH1, PH2) markers were statistically significant, as assessed by *t* tests ($n = 9$) for the CK1 × PH1 (*P*, <0.001) and CK1 × PH2 (*P*, 0.001) mating pairs versus the CK1 × CH1 mating pair. No statistical difference could be observed between megaplasmid-linked frequencies (PH1 versus PH2 [*P*, 0.134]). (B) Transfer frequencies were obtained as described above for matings between the Hyg-resistant strain labeled in the chromosome (CH1) and Km-resistant strains labeled either in the chromosome (CK1, CK5 to CK8) or in the megaplasmid (PK1 to PK6). Frequencies are averages of ratios between colonies grown on selective medium containing Km and Hyg and colonies grown with Hyg (tra/CH1) in 9 independent experiments. Error bars correspond to the standard deviations of the means. Note that the transfer frequencies for megaplasmid-linked genes were approximately an order of magnitude higher than those for chromosomal genes.

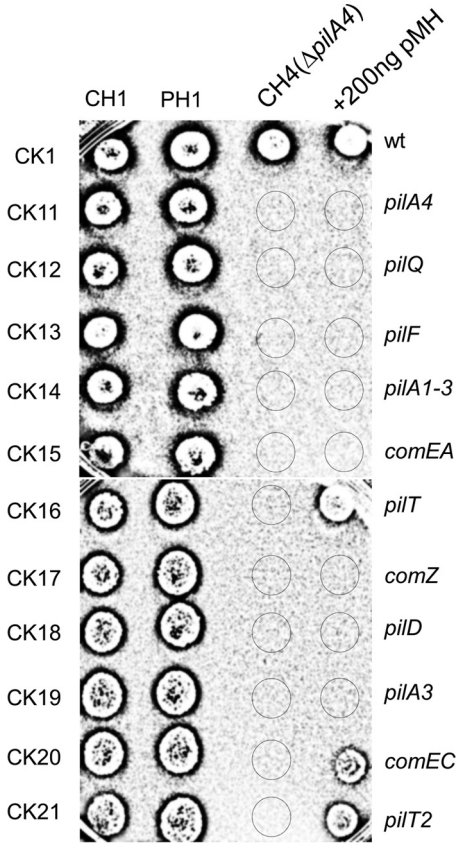

FIG 3 Competence genes are required for cell-to-cell DNA transfer. Selection plates with Hyg and Km were spotted in mating assays (1:1) between Km-resistant mutants in which the indicated components of the natural competence apparatus were affected (CK11 to CK21) and either of two competence-proficient strains, labeled in the chromosome (CH1) or the megaplasmid (PH1), or between CK11 to CK21 and another competence-deficient mutant (CH4). The results of transformation assays with a plasmid conferring Hyg resistance (pMH) are shown on the right. Note that most mutants are completely unable to take up DNA, whereas in others (e.g., CK16, CK20, and CK21), competence is still detectable. Other competence-defective mutants for which data are not shown here (*pilO*, *pilW*, *pilM*, *pilN*, and *dprA* mutants) yielded results similar to those for CK11.

These data show that only competence-proficient strains can function as recipient strains in mating experiments.

The Argonaute protein distinguishes between DNA acquired by competence and DNA acquired by cell-to-cell transfer. The presence of a homologue of the eukaryotic Argonaute protein in *T. thermophilus* (ttAgo) has been shown to protect the bacteria against plasmid DNA acquired by natural competence, leading to a decrease in the transformation efficiency by an order of magnitude (26). In order to know if ttAgo also protects the bacteria against DNA acquired by the cell-to-cell transfer process described here, we constructed a Δ*ago* (ΔTTP0026) mutant and labeled its chromosome at different positions with the Km (CK22) or Hyg (CH5) marker in two derivative strains that were used for mating. As shown in Fig. 5A, no significant differences in transfer efficiency were found in matings involving Δ*ago* or *ago*$^+$ strains. In contrast, transformation of these strains with genomic DNA of the respective mating partners (high-G+C isogenic lineal DNA) revealed a decrease of an order of magnitude in transformation efficiency associated with the presence of the ttAgo protein (*P*, <0.001). Similar results were obtained when other pairs of *ago*$^+$ and Δ*ago* counterpart mates were used (Fig. 5B). Therefore, despite the requirement for a functional competence system in the recipient cell, the way in which the DNA enters the cell by conjugation does not induce the ttAgo-mediated DNA-DNA interference mechanism that protects the cells from DNA acquired by natural competence.

DISCUSSION

Most *T. thermophilus* strains contain a highly conserved chromosome (around 1.8 Mbp) and a very dynamic megaplasmid (230 to 400 kbp), which harbors most of the interstrain differences (23), including genes likely of archaeal or eukaryotic origin (24). It is believed that the extreme plasticity observed in these species is due to the presence of a highly efficient natural competence system (21, 22, 35) and that the concentration of such plasticity in the megaplasmid could be the result of counterselection of insertion in essential genes, which are mostly encoded in the chromosome. In

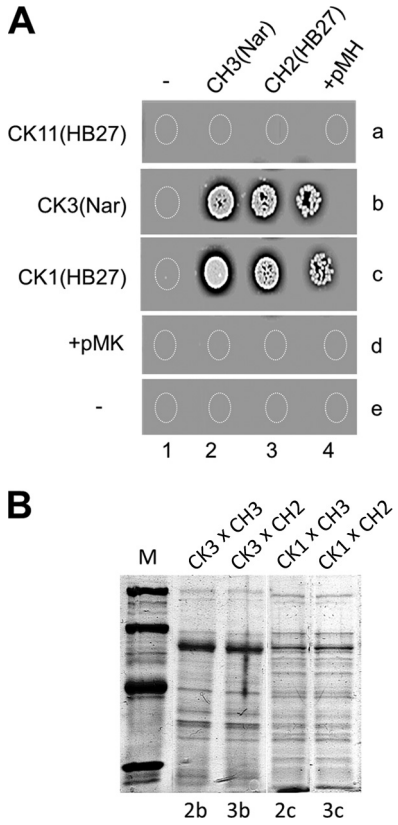

FIG 4 The competence system is required in the recipient but not in the donor cells. (A) A transformation-deficient derivative of strain HB27 (CK11, CH2) or NAR1 (CH3) was mated with the transformation-proficient derivative CK1 (HB27) or CK3 (NAR1). A total of 10^7 cells were spotted onto selective (Km and Hyg) plates either alone (−) or topped with the indicated mating counterpart or 200 ng of plasmid DNA conferring resistance to Km (+pMK) or Hyg (+pMH). (B) SDS-PAGE of whole membrane proteins from spots 2b (the CK3 × CH3 cross), 3b (the CK3 × CH2 cross), 2c (the CK1 × CH3 cross), and 3c (the CK1 × CH2 cross) of panel A. Note that the protein pattern always corresponds to the competence-proficient strain used in the mate: NAR1 for 2b and 3b, HB27 for 2c and 3c. Lane M, molecular size markers of 97.4, 66.2, 45, and 31 kDa.

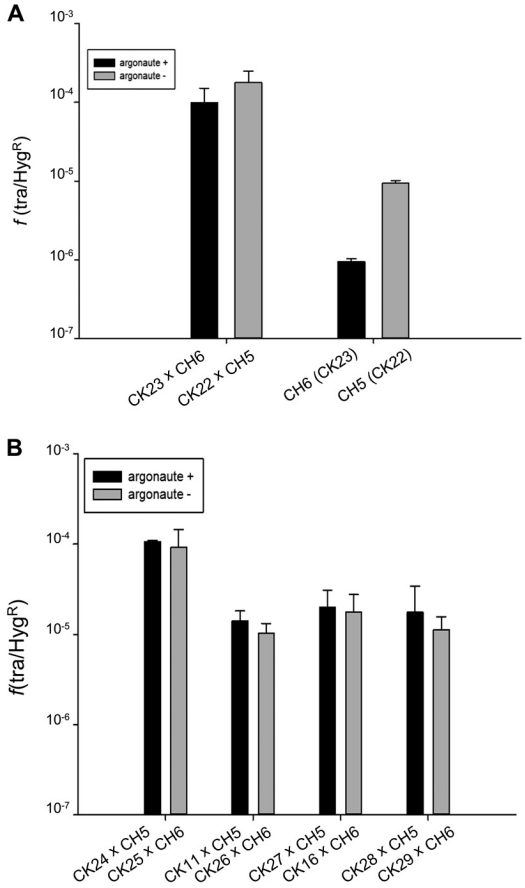

FIG 5 The ttAgo interference pathway is not activated by cell-to-cell DNA transfer. (A) Transfer frequencies were obtained after mating of equal cell amounts of Δ*ago* strains (the CK22 × CH5 mating pair) as well as *ago*$^+$ strains labeled in the same genes (the CK23 × CH6 mating pair). Parallel transformation of strains CH5 and CH6 with 10 ng of genomic DNA isolated from the respective counterparts (CK22 and CK23) was carried out to show the Ago-mediated interference against high-G+C isogenic lineal DNA acquired by natural competence. Frequencies {ratios of Km- and Hyg-resistant transconjugants to the Hyg-resistant partner [f(tra/Hyg^R)]} are averages of results from 9 independent experiments. Error bars correspond to the standard deviations of the means. Differences in transfer frequencies between Δ*ago* and *ago*$^+$ matings were statistically nonsignificant (P, 0.501; n = 9). However, there were significant statistical differences between transformation frequencies (P, <0.001). (B) Transfer frequencies obtained after mating of equal cell amounts of CH5 (Δ*ago*) or CH6 (*ago*$^+$) with Km-resistant mutants labeled in different chromosomal loci (CK11, CK16, CK24 to CK29) that were either Δ*ago* (shaded bars) or *ago*$^+$ (filled bars). Frequencies are averages of results of 5 independent experiments. Error bars correspond to the standard deviations of the means. Differences in transfer frequencies between *ago*$^+$ and Δ*ago* strains were not significant (P, 0.968). Likewise, no significant differences among locus groups or within each locus group could be detected (P, 0.339 and 0.105, respectively).

contrast, our results suggest that concentration of foreign genes in the pTT27 megaplasmid could actually be associated with a preference for active cell-to-cell transfer of megaplasmid genes. In addition, we show that cell-to-cell DNA transfer does not elicit ttAgo-mediated DNA-DNA interference, which constitutes a major barrier against DNA acquired by natural competence.

Nature of the mating partners. In several conjugation systems, DNA transfer takes place in an unidirectional way between a donor and a recipient mate (12, 15, 16). In this process, a mechanism of exclusion exists that prevents transfer between organisms that carry the same type of conjugative plasmids (36). However, we show here that in *T. thermophilus*, transfer takes place between completely isogenic strains. For this reason, the directionality of DNA transfer is difficult to assess, and simultaneous analysis of various phenotypes, such as membrane protein patterns, has to be used to confirm the parenthood of the transconjugants (Fig. 4B). This bidirectionality phenomenon is somehow similar to conjugative retrotransfer processes, where recipient cells can occasionally act as donor cells (37–40). However, in contrast to classical conjugative retrotransfer, where gene flow from recipient to donor is the consequence of two sequential rounds of DNA transfer in which the recipient becomes a secondary donor after obtaining DNA from the primary donor (39), the cell-to-cell DNA transfer system of *T. thermophilus* could *a priori* permit simultaneous bidirectional DNA transfer, resembling a hermaphroditism-like trait.

The cell-to-cell transfer described here is not restricted to isogenic strains. Actually, natural isolates of *T. thermophilus* that carry genes encoding the ability to respire nitrogen oxides anaer-

obically (PRQ25 or NAR1) can transfer such a property to the aerobic HB27 strain by cell-to-cell contacts (21, 41). In such cases, differences among membrane protein patterns provide the easiest way to identify donor and recipient strains, as shown in Fig. 4. In fact, a collection of *T. thermophilus* isolates actually behave as donors in mating experiments with strain HB27 (not shown), supporting the notion that this conjugative-like mechanism is widely distributed, at least within this species.

Requirement for competence genes. The paradoxical aspect of the DNA transfer process described here is that while the DNA is transported from a donor to a recipient cell in a DNase I-resistant state, all the known components of the transformation machinery assayed are apparently required in the recipient cell (Fig. 3 and 4). A tentative explanation may be that the competence apparatus is required for pulling the DNA supplied by the donor cell, which, in turn, should have a DNA-pushing system apparently independent of competence. Therefore, we propose a two-step model for cell-to-cell conjugative transfer in *T. thermophilus*. The donor cell would actively transfer DNA to the recipient cell in a first, conjugation-like step, which does not require the transformation machinery. In the second, transformation-like step, the recipient cell would play an active role in pulling the transferred DNA to ease its passage into the cytoplasm, a process requiring the DNA transport machinery of the competence apparatus. This process is remarkably different from classical conjugation, where the recipient cell remains basically passive, waiting for DNA transfer (11).

An additional argument supporting the existence of a two-step push-pull mechanism is that it clearly favors the transfer of genes located in the megaplasmid over that of genes located in the chromosome (Fig. 2), despite their similar copy numbers. Actually, the genetic polymorphism observed in the megaplasmid could, to some extent, be related to this greater feasibility of transfer. The reasons underlying this preferential transfer of megaplasmid genes are not known, but it might be related to the presence of one or more yet unknown sequences acting as an origin of transfer, reminiscent of conjugative *oriT* sequences. In this scenario, the transfer of chromosomal genes could be the consequence of sporadic integration of the megaplasmid by recombination between insertion sequences (ISs), which are abundant in both elements (http://www-is.biotoul.fr/), as has been described for Hfr formation by the *E. coli* F factor (42). In this regard, previous data from our group suggest that some strains of *T. thermophilus* behave like Hfr strains (27), likely because such integration has become stable.

Similar generalized genomic transfer mechanisms have been described for *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Streptococcus agalactiae* (18, 43, 44). These transfer models differ from the classical Hfr model in that initiation of transfer is predicted to occur at multiple defined sites located throughout the genome. For *S. aureus* and *S. agalactiae*, conventional conjugation involves conjugative proteins (T4SS, a coupling protein, and a relaxase), which are responsible for the processive transfer of hundreds of kilobases of the bacterial chromosome (43, 44). In contrast, in *M. smegmatis*, DNA transfer is mediated by the virulence-associated locus *esx-1* (45). In another example of unconventional conjugation models, *Streptomyces* conjugative transfer seems to depend exclusively on a single polar protein (17), which resembles the FtsK/SpoIIIE system (46). In the genus *Thermus*, the presence of a conspicuous conjugative T4SS is restricted to a few strains and species, including *T. thermophilus* strains SG0.5J (21) and JL-18 (NC_017590). However, neither strain HB27 nor any of its derivatives used in this work contain homologues of these classical conjugation-related genes. Likewise, no candidates for an *oriT* sequence, coupling protein (CP), or relaxase have been detected in their genomes by bioinformatic analysis. Hence, it is difficult to predict whether transfer initiation in the genus *Thermus* occurs at a single defined site or at multiple sites simultaneously. Nevertheless, the DNA to be transferred must first be secreted from the donor so that the recipient cell can pull it into the cytoplasm.

DNA discrimination by ttAgo. As described in a previous article, the ttAgo protein is involved in a DNA-DNA interference mechanism that limits the entrance of DNA by natural competence (26). In that work, the ttAgo protein was shown *in vitro* to have a preference for A+T-rich regions in supercoiled plasmids. However, we show here that ttAgo is also able to protect the bacterium against the entrance of lineal isogenic DNA with a high G+C content (Fig. 5A). Such discrimination is not active when the same DNA is transferred by the cell-to-cell mechanism described here. Therefore, two hypotheses could explain this discrimination. Either the entry of DNA by competence stimulates specifically a putative ttAgo interference pathway, or the form in which DNA enters the cell makes the difference (i.e., double-stranded DNA versus single-stranded DNA, or the presence or absence of pilot proteins). In both cases, this discrimination system might have evolved to permit this very promiscuous bacterium to distinguish between potentially hazardous DNA of unknown origin and trustworthy DNA acquired from a reliable mate.

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