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Contribution of vesicle-protected extracellular DNA to horizontal gene transfer in *Thermus* spp.

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Summary. Highly efficient apparatus for natural competence and conjugation have been shown as the major contributors to horizontal gene transfer (HGT) in *Thermus thermophilus*. In practical terms, both mechanisms can be distinguished by the sensitivity of the former to the presence of DNAse, and the requirement for cell to cell contacts in the second. Here we demonstrate that culture supernatants of different strains of *Thermus* spp. produce DNAse-resistant extracellular DNA (eDNA) in a growth-rate dependent manner. This eDNA was double stranded, similar in size to isolated genomic DNA (around 20 kbp), and represented the whole genome of the producer strain. Protection against DNAse was the consequence of association of the eDNA to membrane vesicles which composition was shown to include a great diversity of cell envelope proteins with minor content of cytoplasmic proteins. Access of the recipient cell to the protected eDNA depended on the natural competence apparatus and elicited the DNA–DNA interference defence mediated by the Argonaute protein. We hypothesize on the lytic origin of the eDNA carrying vesicles and discuss the relevance of this alternative mechanism for HGT in natural thermal environments. [**Int Microbiol** 18(3):177-187 (2015)]

Keywords: *Thermus* · horizontal gene transfer · extracellular vesicles

Introduction

Horizontal gene transfer (HGT) is a common trait among bacteria, playing a main role in sudden acquisition of complex properties for adaptation to changing environments. In this sense, HGT seems to have played a major role in the adaptation of hyperthermophilic bacteria to high temperatures given the high percentage of genes from Archaeal origin in phylogenetic groups such as *Thermotogales* and *Thermales* [2,54].

Despite the description in the last decade of alternative mechanisms for HGT such as nanopods [49], gene transfer agents (GTAs) [30] or nanotubes [17], transformation, transduction and conjugation still remain as the major mechanisms for HGT in bacteria [20, 24]. From these classical pathways, transformation requires either the secretion of DNA to the environment or the lysis of the donor cells to produce the extracellular DNA (eDNA) taken up by natural competent cells. The bulk of this eDNA is linked to cell death and lysis, including fratricide processes [25]. In particular, eDNA release is often triggered by the lysis of a subpopulation of cells, sometimes as part of the end stage of the bacterial life cycle or as a consequence of unbalanced growth prompted by environmental variations such as nutrient availability or predation [43]. In other cases, eDNA release is related to apoptotic cell lysis, induced by autolysins such as LytM and AtlE in *Staphylococcus aureus* [4], GelE an SprE in *Enterococcus faecalis* [53] or LytF and AtlS in *Streptococcus gordonii* [57]. Also bacteriophage-mediated lysis contribute to the production of eDNA,

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in different bacteria [40]. In addition, antibiotic treatments, especially with beta-lactams produce cell lysis and eDNA accumulation.

In contrast to cell-lysis mediated eDNA production, many bacteria actively secrete extracellular eDNA in a lysis-independent way, leaded by specific mechanisms under the control of quorum-sensing systems [14,42]. Several studies in both Gram-positive and Gram-negative bacteria have pointed out variations in eDNA production during the growth cycle [28], either to battle host defence responses [38], or associated to high cell density populations [7]. Likewise, presence of antibiotics and detergents in the environment and changes in the salinity or pH of the extracellular milieu, for instance, may alter eDNA production as a reaction to environmental stress [13,37].

Whichever the mechanism involved, eDNA can play different roles in the environment. On the one hand, eDNA can be used by competent cells as the HGT source of new genes, including antibiotic resistances, virulence factors or enzymes. Also, eDNA is used by several bacteria as a stabilizing component of the biofilm matrix [14], being vital in the early stages of biofilm development [19, 46], as it facilitates adhesion, aggregation and maintenance of the integrity biofilm structures [4,14,44]. This is of particular interest from a clinical point of view, where biofilms play major roles in colonization and infection [9]. Studies on pathogenic multidrug-tolerant *Staphylococci*, *Pneumococci* or *Streptococci* have emphasized the high yields of eDNA present in their biofilms, being up to 50 % more than in *P. aeruginosa* biofilms [51], where degradation by DNase I treatment significantly reduced biofilm pathogenicity [31,33]. Therefore, eDNA production within the biofilm not only contributes to the spread of toxins but also promotes biofilm persistence, enduring the resistance of pathogens against antibiotics, ultimately broadening the prevalence of its virulence [9,31,33,38,51].

However, in the environment, eDNA coexists with DNases and nucleases secreted by the surrounding microbial community, which will rapidly degrade it as a way to gain nutrients for growth. An apparently widespread mechanism to protect eDNA from degradation involves the production of the so-called extracellular vesicles (EVs), that shelter the eDNA from the action of nucleases either by effective encapsulation, shielding the DNA, or by adsorption to their surface, blocking the access of DNAses by steric interactions. This way, the half-life of the eDNA in the environment increases, expanding the time window for HGT [5]. Such protective mechanisms could be especially relevant in thermal environments, where protective strategies could be required to prevent eDNA also from denaturation [50].

Thermus thermophilus is one of the best known thermophilic bacteria because its easy growth under laboratory conditions, sequence availability [23] and genetic accessibility [10]. Many strains of *T. thermophilus* can acquire linear or circular DNA at exceptionally high rates through a constitutively-expressed transformation apparatus [48]. Most of the DNA taken up by the cell is destroyed through a yet poorly understood DNA-DNA interference mechanism dependent on a thermophilic homologue (ttAgo) to the human Argonaute protein [52], supporting that natural competence more likely evolved to get nutrients from the environment than to acquire new genetic information. On the other hand, *T. thermophilus* is also able to transfer DNA by means of a conjugation-like mechanism poorly characterized, but that allows the transferred DNA to escape from the ttAgo's surveillance, suggesting a major relevance in HGT within the genus [6].

We describe here the existence of an alternative route of HGT in *T. thermophilus* based on the production of DNase-protected eDNA. Long-term protection of *T. thermophilus* eDNA is due to its association to extracellular membrane vesicles generated by cell lysis. We show that this vesicle associated eDNA can be incorporated into the genome of recipient *T. thermophilus* cells through its natural competence apparatus.

Materials and methods

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* DH5α was used for plasmid construction. *E. coli* was routinely grown at 37 ºC in LB broth or plates [32]. *T. thermophilus* was grown aerobically under rotational shaking (150 rpm) at 60°C or 70ºC in TB [41] or in mineral M162 medium [15]. Kanamycin (Km, 30 μg/l), Ampicillin (Am, 100 μg/l) and/or Hygromycin B (Hyg, 100 μg/l) were added when needed for selection.

Plasmids and insertional mutants. pMKpnqosYFP is a bi-functional plasmid selectable with Km both in *E. coli* and *T. thermophilus.* It encodes a thermostable yellow fluorescent protein (sYFP) expressed in *T. thermophilus* from the nqo_p promoter [1]. Transformation of this plasmid into *Thermus* spp. was done either by natural competence or by electroporation, depending on the strain. Insertion mutants in the *pyrE* or *gdh* genes was done by transformation with plasmid pK18*::pyrE* and genomic DNA from a *gdh::kat* derivative of *T. thermophilus* HB27 [11], respectively.

Isolation of extracellular membrane vesicles for TEM analysis. Isolation of EVs was performed following described methods [29]. Briefly, cultures were filtered through 0.45 μm nitrocellulose filters (Whatman PROTRAN BA85) and the cell-free fraction was further centrifuged at 6000 × *g* for 20 min at room temperature to eliminate large cell fragments. Supernatants were further filtered through 0.22 μm filters before ultracentrifugation (150.000 × *g* for 2.5 h at 4ºC) to collect the EVs. The EVs were washed twice in HEPES (50 mM, pH: 7.5) buffer by cen-

Table 1. Strains used in this work

Strain	Genotype	Phenotype/use	Reference
Escherichia coli DH5α	*F- endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA) U 169 Φ80dlacΔM15*	Cloning	[22]
Thermus aquaticus YT1	Wild type		DSZM 625
T. aquaticus syfp	*T. aquaticus* YT1 [pMKPnqos*yfp*]	KmR	This work
T. scotoductus SA01	Wild type		[21]
T. scotoductus pyrE	Δ*pyrE:kat*	KmR	This work
T. scotoductus syfp	*T. scotoductus* SA01 [pMKPnqos*yfp*]	KmR	This work
T. thermophilus HB27	ATCC BAA-163 / DSM7039	Wt	Y. Koyama
T. thermophilus HB27 *gdh*	*gdh::kat*	KmR	[11]
T. thermophilus HB27^{EC}	*ago::ISTth7*	Enhanced competence	[52]
T. thermophilus HB27*ago*$^{-}$	Δ*ago*	Enhanced competence	[52]
T. thermophilus HB27*ago-gdh*	Δ*ago, gdh::kat*	Enhanced competence. KmR	[6]
T. thermophilus HB27Δ*pilA4*	HB27^{EC} Δ*pilA4*	Non competent	[6]
T. thermophilus HB27Δ*pilQ*	HB27^{EC} Δ*pilQ*	Non competent	[6]
T. thermophilus HB8	Wild type		Y. Koyama
T. thermophilus HB8 *syfp*	*T. thermophilus* HB8 [pMKPnqos*yfp*]	KmR	This work
T. thermophilus SG0.5JP17-16	Wild type		J. Gladden
T. thermophilus SG0.5 *pyrE*	Δ*pyrE:kat*	KmR	This work
T. thermophilus NAR I *pyrE*	Δ*pyrE:kat*	KmR	This work
T. thermophilus VG7 *pyrE*	Δ*pyrE:kat*	KmR	This work
PK1	*T. thermophilus* HB27^{EC}[ttp0211::*kat*]	KmR	This work
CK1	*T. thermophilus* HB27^{EC} [*gdh*::*kat*]	KmR	[11]
CK2	*T. thermophilus* HB27^{EC} [ttc1844::*kat*]	KmR	[6]
CK3	*T. thermophilus* HB27^{EC} Δ*pilA4*::*kat,* Δ*ago*	KmR; non competent	[6]

trifugation (60.000 × *g* for 30 min), and re-suspended in 50 µl phosphate buffer (50 mM, pH 7.5). Control samples from each of the supernatants in the process were analysed for the presence of living cells and eDNA. For TEM analysis, EVs were adsorbed onto ionized Collodion-coated copper grids (300 mesh) and negatively stained with 2% (w/v) aqueous uranyl acetate. Grids were examined in a JEM 1010 transmission electron microscope (JEL, Japan) equipped with a TVIPS Tem Cam F416 (CCD SystemB) digital camera (Gauting, Germany).

Quantification and analysis of eDNA. Total DNase resistant eDNA was precipitated from 1 mL of filtered growth medium treated with DNase I (5 units, Roche) for 1 h at 37ºC. EVs-associated eDNA was extracted from EV fractions by phenol-chloroform and precipitation method [45]. Concentration of eDNA was measured by spectrophotometry and its integrity was analysed by agarose gel electrophoresis. Digestion with *HindIII* (Thermo Scientific) was carried out following manufacturers' indications. Susceptibility assays to DNase I (10 units, Roche) and nuclease SI enzymes (100 units, Thermo Scientific) were carried out according to manufacturers' indications. PCR assays of different regions of the genome were carried out with 9 oligonucleotide pairs (Table 2) that were further analysed by agarose gel electrophoresis.

Protein analysis. EVs samples from *T. thermophilus* HB27 were treated at 90ºC for 10 min with Laemli´s sample buffer [29] and incubated with trypsin in gel (Promega, Madison, WI) following manufacturer's indications. Gel extracted peptides were desalted and concentrated by OMIX C18 (Agilent

Table 2. Oligonucleotides used in this work. Sequence shown in low case indicates restriction site (underlined) and additional bases required for cloning

Name	Use	Oligonucleotides sequence (5′ to 3′)
AB225	Construction of pk18:: *pyrE*	aaagaattcCTAGACCTCCTCCAAGGGCAC
AB226	Construction of pk18:: *pyrE*	atcaagcttATGGACGTCCTGGAGCTTTAC
AB92	Check *pilA4* mutation	AAATGCTGAAGCTTGGCGGCAAC
AB93	Check *pilA4* mutation	AAAAGAATTCGGGAGTTAGGCTTGGGATTGTG
AB115	Check *pilQ* mutation	CTTCCCAAGAGGAGGCCCAG
AB116	Check *pilQ* mutation	CACGTCCCTCAGGTCCTTGTG
AB171	Check Δ*ago* mutation	GTCTTCCTTCTCCCTCCGGAC
AB172	Check Δ*ago* mutation	CTTCGGGCTTTCCCTGGAG
AB63	PCR analysis for *ttp0084* amplification	ttcgaattcCTTCATCCCCACCAAGTTTGAC
AB64	PCR analysis for *ttp0084* amplification	aacaagcttGTCCTTCACCTTCTTGAGCTCCAG
AB67	PCR analysis for *ttp00208* amplification	ttcgaattcTCCTCAAGGAGGCCCTCTG
AB68	PCR analysis for *ttp00208* amplification	atcaagcttGAAGTCCGCGAACTCGGTAAG
AB90	PCR analysis for *ttp00145-146* amplification	GATGCTGCTCGGATGGTTTG
AB91	PCR analysis for *ttp00145-146* amplification	CCTCCAGGGAACATCCAGTAGAG
CEC78	PCR analysis for *ttp00167* amplification	ttcgaattcGTCGCTGGTCATGGCGTC
CEC79	PCR analysis for *ttp00167* amplification	atcaagcttCACCGCTACCTGGTGGACTC
CEC82	PCR analysis for *ttc0893* amplification	ttcgaattcTACGTGGTGCTGGACGAGCTC
CEC83	PCR analysis for *ttc0893* amplification	atcaagcttGTTCCGCACCAGGTAGCTCTC
CEC84	PCR analysis for *ttc1415* amplification	ttcgaattcGTGGCGATGAGGATCTCCAG
CEC85	PCR analysis for *ttc1415* amplification	atcaagcttGTCCGGATAGACGGCAAGCTC
AB109	PCR analysis for *ttc1017-1018* amplification	atcagatctCAATGTCCCCATGCGGTTTC
AB110	PCR analysis for *ttc1017-1018* amplification	ttcaagcttGTAGATGGCGTCGTGGACCTC
CEC94	PCR analysis for *ttc638* amplification	ttcgaattcGCCAAAAGCCGCTCCTTCTC
CEC95	PCR analysis for *ttc638* amplification	atcaagcttCGGGACGAGGTCTTTCTTTC
AB169	PCR analysis for *ttc1879* amplification	atcaagcttGAGTTATTGGCCGCGCTTC
AB170	PCR analysis for *ttc1879* amplification	aaaccatggCATGCGGGTGCTCAGGTG

Technologies). Results from the LC-MS/MS analysis were surveyed with SEQUEST search algorithm from Proteome Discoverer software (*v.* 1.4; ThermoScientific) employing Uniprot's *T. thermophilus* HB27 database. Subcellular compartment allocation was performed according to PSORTb software results (v.3.0.2; Brinkman Laboratory). The frequency of peptides detected in relation to the molecular masses of the corresponding proteins was used as a proxy for the quantification of the proteins in the sample.

Transformation frequency assays. *T. thermophilus* HB27 and its *ago*- derivatives strains were transformed by natural competence with either DNase-treated filtered supernatants of cultures, purified EVs, or purified DNA. DNase treatment of the DNA templates involved 1 ml of each filtered supernatant incubated in presence of 5 units of DNase I (Roche) for 1 h, amended with extra 5 units DNase I upon induction of transformation. In all cases, the donor strain was labelled with the *kat* gene cassette, and transformation frequencies were calculated as the number of transformants grown on Km-containing selective plates per recipient cell in triplicate assays.

Results

Production of DNase-resistant e-DNA by different *Thermus* spp. Occurrence of DNase-resistant eDNA was detected while performing conjugation assays with *T. thermophilus* strains, as we observed low levels of transfer of an antibiotic resistance marker in cultures of *T. thermophilus* HB27 derivatives separated by 0.22 μm nitro-

cellulose filters. Moreover, the control experiment showed that this DNA transfer was also detectable in the presence of DNase I, supporting that the transferred eDNA was protected from the enzyme activity. To confirm this, we used DNase-treated filtered growth medium of cultures from three *T. thermophilus* HB27 derivatives harbouring the *kat* gene cassette in the pTT27 megaplasmid (PK1) or in the chromosome (CK1, CK3), in transformation assays. As shown in Fig. 1A, we detected the transfer of the kanamycin resistance to the high transformation-efficiency *T. thermophilus HB27 Δago* strain in the three cases ($1.7 \pm 0.68 \times 10^{-6}$). In addition, we found no differences between the frequencies found for the transfer of megaplasmid genes (PK1) compared to that of genes located in the chromosome (CK1 and CK3).

Transformation assays mediating the single recombination of pK18*pyrE::kat* plasmid into the highly conserved *pyrE* gene were performed using a variety of *Thermus* strains. As shown in Fig. 1B, all the strains assayed (*T. thermophilus* NARI, *T. thermophilus* SG0.5JP17-16, *T. thermophilus* VG7, and *T. scotoductus* SA1) produced enough DNase-protected eDNA as to allow the detection of *pyrE::kat* transformants in *T. thermophilus ago-* derivatives. In addition, the low sized plasmid pMKpnqosYFP could also be transferred as DNase-protected eDNA (Fig. 1C), alike the observed transfer of genes from the chromosome and the megaplasmid.

Analysis of eDNA produced by *Thermus* spp. The genomic content of the DNase-resistant eDNA present in the growth media was analysed by gel electrophoresis. As shown in Fig. 2A, the eDNA from the HB27 strain and its CK2 and HB27^{EC} derivatives have a size (around 20 kbp) similar to that of whole genomic DNA isolated by conventional methods. Furthermore, confirmation of eDNA whole genome representation was attempted by two experimental strategies. On the one hand, the restriction pattern obtained from digesting eDNA samples with a restriction enzyme that has a relatively small number of cutting sites in the genome (*HindIII*), showed no defined products (Fig. 2A). In addition, PCR reactions on eDNA extracts provided positive products for the amplifications of several genes distributed along the chromosome (*ttc* genes) and the pTT27 megaplasmid (*ttp* genes) in all cases (Fig. 2B).

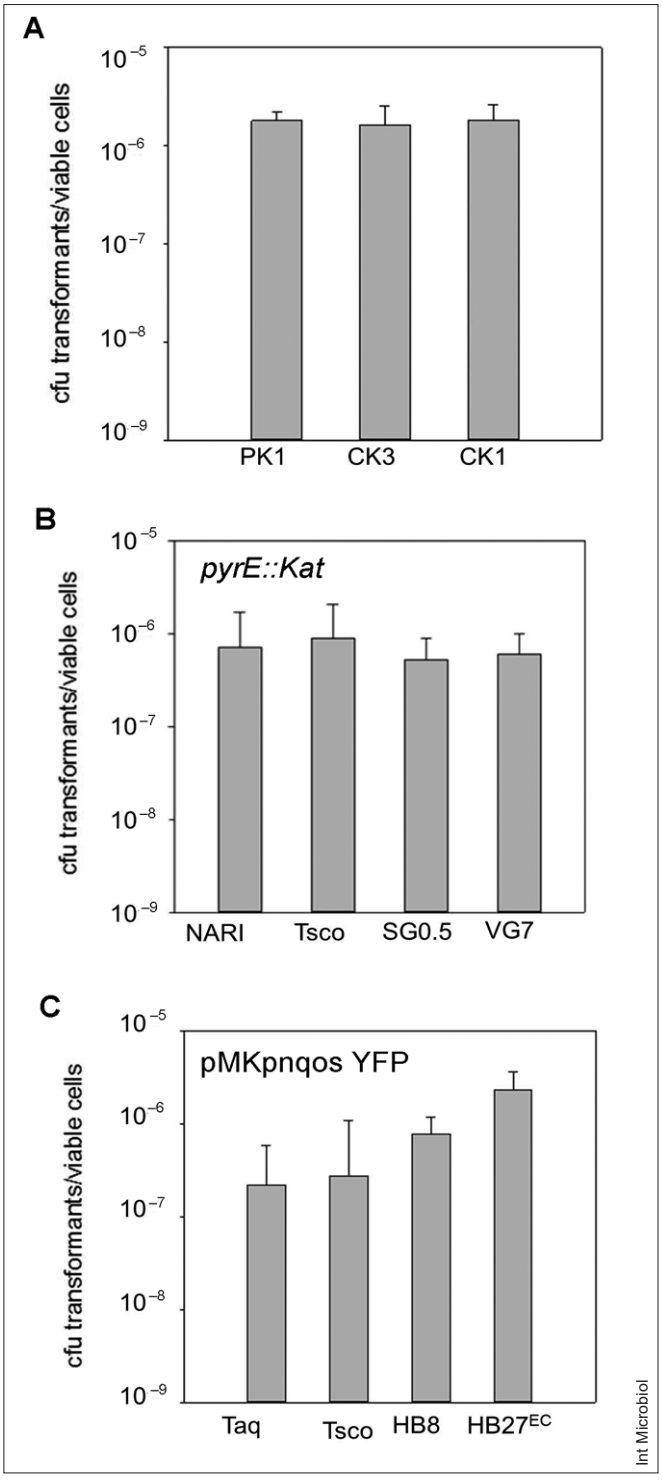

Fig. 1. Production of DNase-resistant e-DNA by *Thermus spp*. (**A**) Production of eDNA by *T. thermophilus* HB27. Transfer frequencies of two chromosome (CK1, CK3) and one megaplasmid (PK1) gene labelled with the *kat* cassette to the *T. thermophilus Δago* strains were measured. (**B**) Production of eDNA by different *Thermus* spp. Transformation efficiencies to *T. thermophilus Δago* were measured with eDNA from cultures of the indicated *Thermus* spp. *pyrE::kat mutants*. Donor *pyrE::kat* strains: *T. thermophilus* NARI (NARI), *T. scotoductus* SA01 (Tsco), *T. thermophilus* SG0.5JP17-16 (SG0.5), *T. thermophilus* VG7 (VG7). (**C**) eDNA mediated transference of replicative plasmid**.** Transformation efficiencies were obtained as above for different *Thermus* spp. strains harboring plasmid pMKPnqosYFP. Donor strains: *T. aquaticus* YT1 (Taq), *T. scotoductus* SA01 (Tsco), *T. thermophilus* HB8 (HB8), *T. thermophilus* HB27^{EC} (HB27^{EC}). Error bars correspond to the mean standard deviation (n = 3)

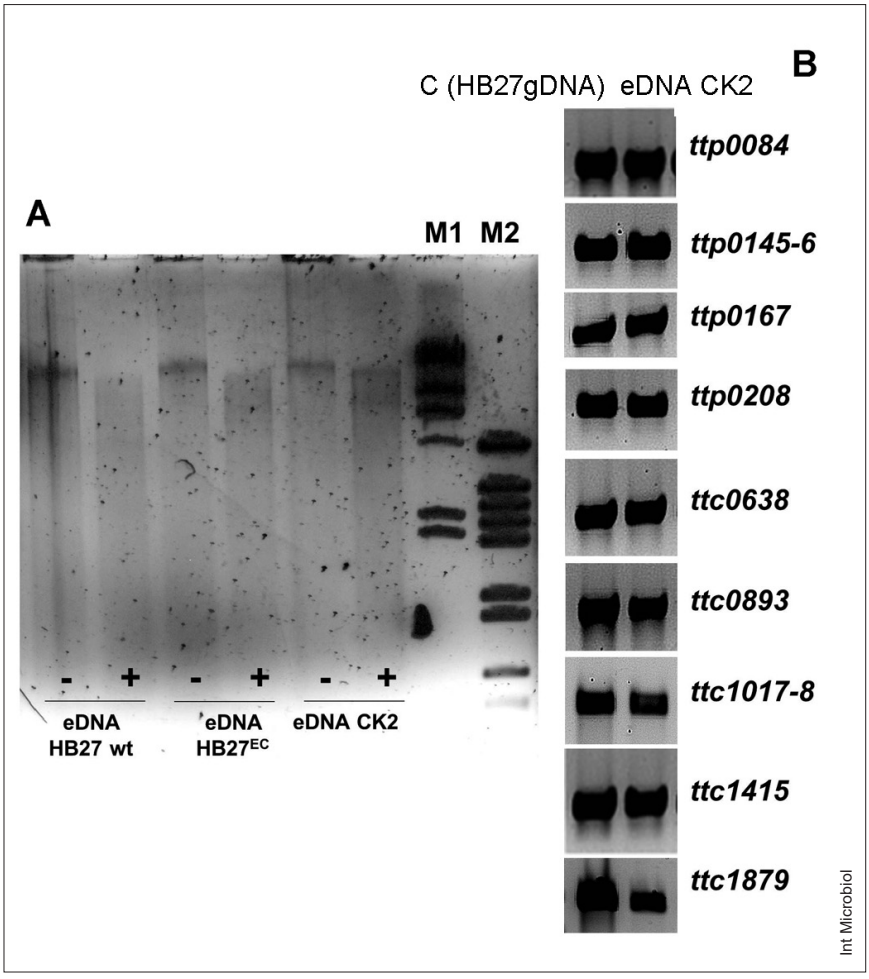

Fig. 2. DNase-protected eDNA is representative of the whole genome of *T. thermophilus*. (**A**) Samples of DNase resistant eDNA, isolated from cultures of the *T. thermophilus* strain strains HB27, HB27^{EC} and CK2, were treated (+) or not (-) with *HindIII.* Size markers (bp): M1 (23130-9420-6560-4360-2320-2020); M2 (4370-2899-2498-2201-1933-1331-1150-759-611). (**B**) PCR products of the indicated genes were obtained from eDNA isolated from the CK2 strain. Genomic DNA from the HB27 strain was used as positive control. Oligonucleotides employed for PCR are described in Table 2.

Production of eDNA along cell growth. The production of eDNA along the growth cycle was assayed for the wild type HB27 strain, its *Δago* derivative, and the insertional mutants CK1 and CK2 (Table 1). As shown in Fig. 3, production of eDNA in the four strains was high in early exponential phase and decreased with the growth rate. Actually, production of eDNA was exacerbated during growth in rich medium at 70ºC compared to 60ºC. In contrast, in mineral medium, no eDNA was detected at 60 or 70ºC (data not shown).

Protection of eDNA within extracellular membrane vesicles. All the *Thermus* spp. strains assayed in Fig. 1, were shown to produce extracellular vesicles. Figure 4A shows a representative image of such structures. In all cases, the sizes of such vesicles were rather heterogeneous.

The composition of such EVs was explored through a proteomic analysis. In addition to the identification of trypsin-generated peptides, we deduced a semi-quantitative analysis that gives an approximate idea of the protein content of the vesicles. Our data supported the abundance of ABC transporters and cell wall associated proteins. Moreover, bioinformatic prediction of the subcellular allocation of the identified proteins showed that most of the proteins for which a localization could be predicted belonged to cell envelope components (inner/outer membranes and periplasm) (Fig. 4B). However, a lower but significant fraction of proteins in the external vesicles were identified as cytoplasmic components (14.2%). In summary, there was a great diversity of both the EVs morphology as well as the protein components extracted from such EVs.

Analysis of the DNA within EVs. As shown in Fig. 5, eDNA purified from supernatants or chemically extracted from EVs was sensitive to DNase (lanes 3 and 5) but not to S1 nuclease (lane 6). Control experiments with whole EVs

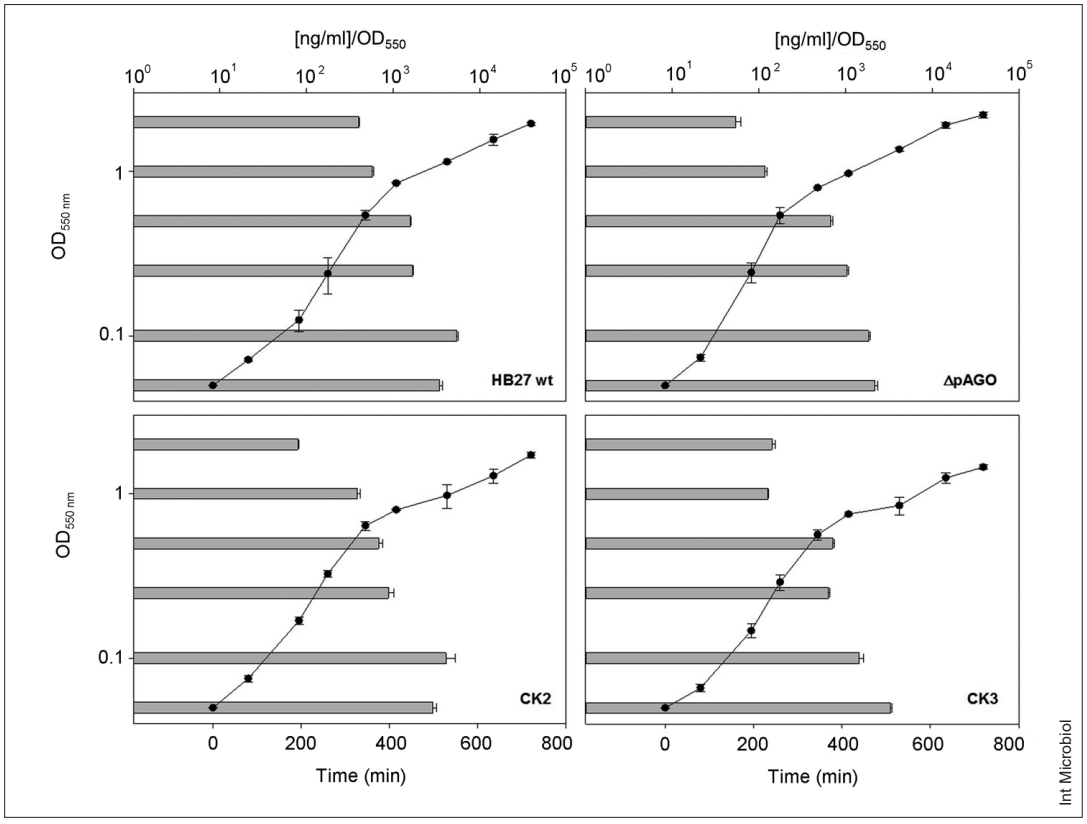

Fig. 3. Production of eDNA is linked to the growth rate. The production of DNAse-resistant eDNA by different strains of *T. thermophilus* along the growth ($OD_{550\ nm}$) in TB rich medium is represented for the HB27 wild type strain and its derivatives *Δago*, CK2 and CK3.

show the expected protection against both enzymes (lanes 2 and 4). Thus, EV-associated eDNA is double stranded.

Activation of Argonaute interference by EVs-linked eDNA. The relevance of the competence apparatus in the mechanisms by which the EV-associated eDNA gets access to the cell was analysed. For this, we used as host the HB27 wild type strain or mutant derivatives defective in transformation (*ΔpilQ, ΔpilA4*). The outcome of these assays (Fig. 6A) was that neither *ΔpilA4* nor *ΔpilQ* could be transformed, whereas transformation was detected in the wild type strain with both EVs and genomic DNA, but with much lesser efficiency in the first. We further checked if acquisition of eDNA was also subjected to DNA-DNA interference mediated by the Argonaute protein. For this, the wild type strain and its *Δago* derivative were transformed with EVs isolated from strains CK1 or PK1. As shown in Fig. 6B, the *Δago* strain was around 10 folds more efficient in transformation than its parental wild type strain, in a way similar to that found for genomic DNA.

Discussion

Transformation and conjugation have been described as the major contributors to HGT in *Thermus* spp. [3,6,12]. Whereas the success of HGT by conjugation depends on a donor cell to encode a selectable property beneficial for the recipient cell, the broad range transformation capability of the genus allows the cells to receive DNA from distant Phyla including Archaea, as described for many genes of *T. thermophilus* [3,8,48]. However, the success of a transformation event depends on the availability of large sized dsDNA in the natural environment in which *Thermus* spp. live.

In thermal environments, the integrity of the eDNA is challenged not only by the presence of DNases, secreted by several organisms to gain nutrients, but also by the melting of dsDNA at high temperatures. In this scenario, the protection of the extracellular DNA from both hydrolytic action of enzymes and melting could expand the possibility of HGT both in time and distance. Here, we provided evidence, on the one

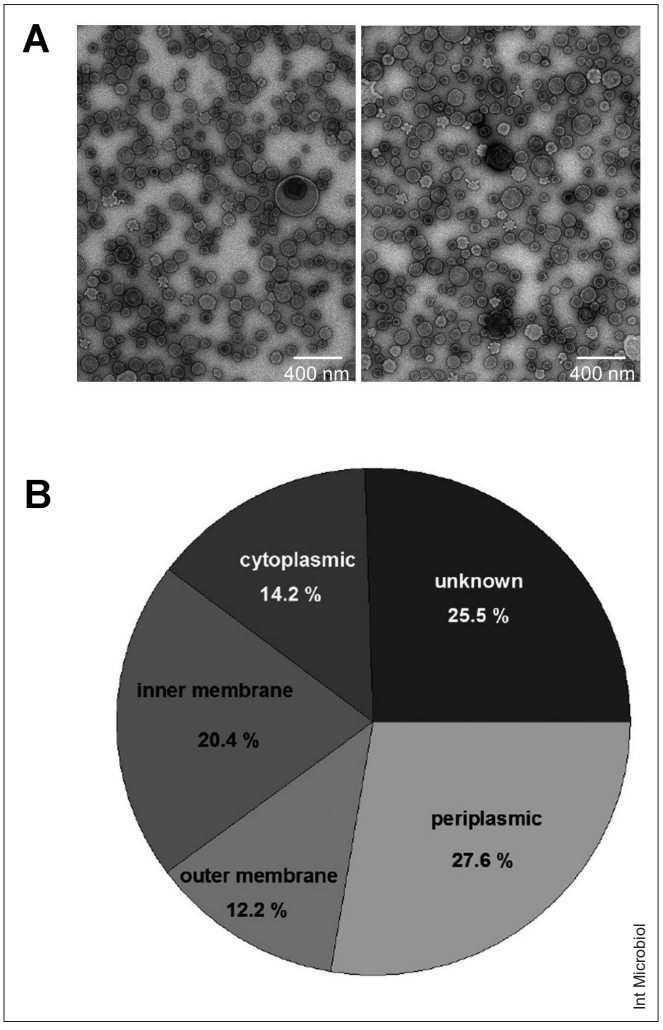

Fig. 4. eDNA is protected within membrane vesicles. (**A**) Representative TEM images of EV fractions of the HB27^{EC} and CK2 strains are presented. Samples adsorbed onto Collodion-coated copper grids were negatively stained with uranyl acetate. Samples were observed in a JEM 1010 transmission electron microscope. Bar represents 400 nm. **(B**) Pie chart displaying the relative abundance of the proteins associated to the EVs fraction, classified by their predicted subcellular localization, as predicted by P-SORT software after LC MS/MS analysis.

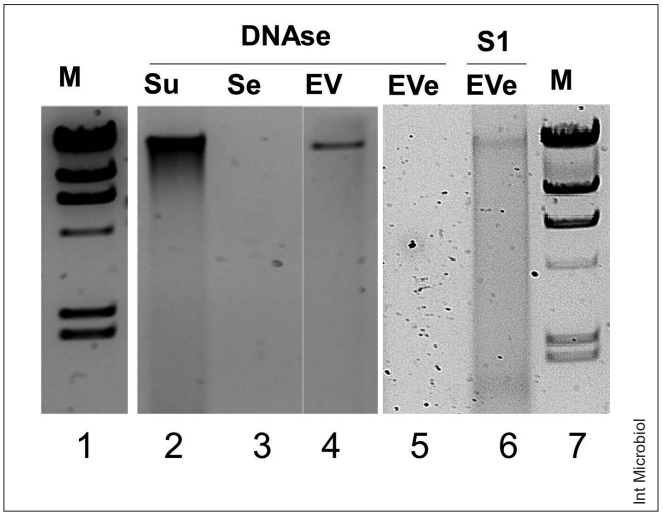

Fig. 5. eDNA is double stranded and barrier-protected from nucleases. Samples were treated with DNase I (10 units, 1 h at 37ºC) or Nuclease S1 (100 units, 1 h at 37ºC) as indicated. Su) Supernatant from HB27; Se) eDNA extracted from Su by phenol-chloroform treatment and precipitated with ethanol; EV) Evs purified from Su by ultracentrifugation and washing; Eve) eDNA extracted from EVs; M) Molecular size markers: 23130-9420-6560-4360-2320-2020 bp.

hand, of the ability of all the strains of *Thermus* spp. assayed to produce DNase-resistant eDNA, and on the other, of the capability of *T. thermophilus* to integrate this eDNA into its genome.

The production of DNase-resistant DNA was assayed in *T. aquaticus* YT1, *T. scotoductus* SA1, and different strains of *T. thermophilus,* for which Km resistant insertion mutants or Km-resistance plasmid transformants could be isolated. Thus, it is likely that production of eDNA is a common trait of the genus. Moreover, through a double experimental strategy involving restriction profiles and PCR amplification, we could demonstrate that the eDNA was double stranded, not fragmented, and included the whole genome of the producer, distributed in a random manner, with no overrepresentation of any specific genome regions (Figs. 2 and 5). Therefore, under the decribed growth conditions the genome of all the *Thermus* spp. strains assayed was available in the growth medium in a DNase protected form.

Then, we wondered what was the nature of such protection against DNases. As classical chemical treatments to purify eDNA makes it sensitive to these enzymes (Fig. 5), we deduced that protection was the consequence of physical barriers blocking the access of the enzymes to the eDNA, and related it with the production of membrane vesicles (EVs) that were easily isolated and visualized by electron microscopy in cultures of all the strains analysed (Fig. 4A). Therefore, protection against DNase would be the consequence of the association or integration of eDNA to or into the EVs

Production of EVs is a relatively common trait described in many bacteria and in a few Archaea, including extreme thermophiles [34,35,50]. In Gram-negative bacteria, EVs are produced in many species through the local destabilization of the outer membrane that produces unspecific spontaneous release of membrane blebs [34,35]. These EVs can play different functions, from secretion of proteins, to production of encapsulated eDNA. In the latter case, their function as vehicles for HGT has been shown for *Neisseria gonorrhoeae* [16],

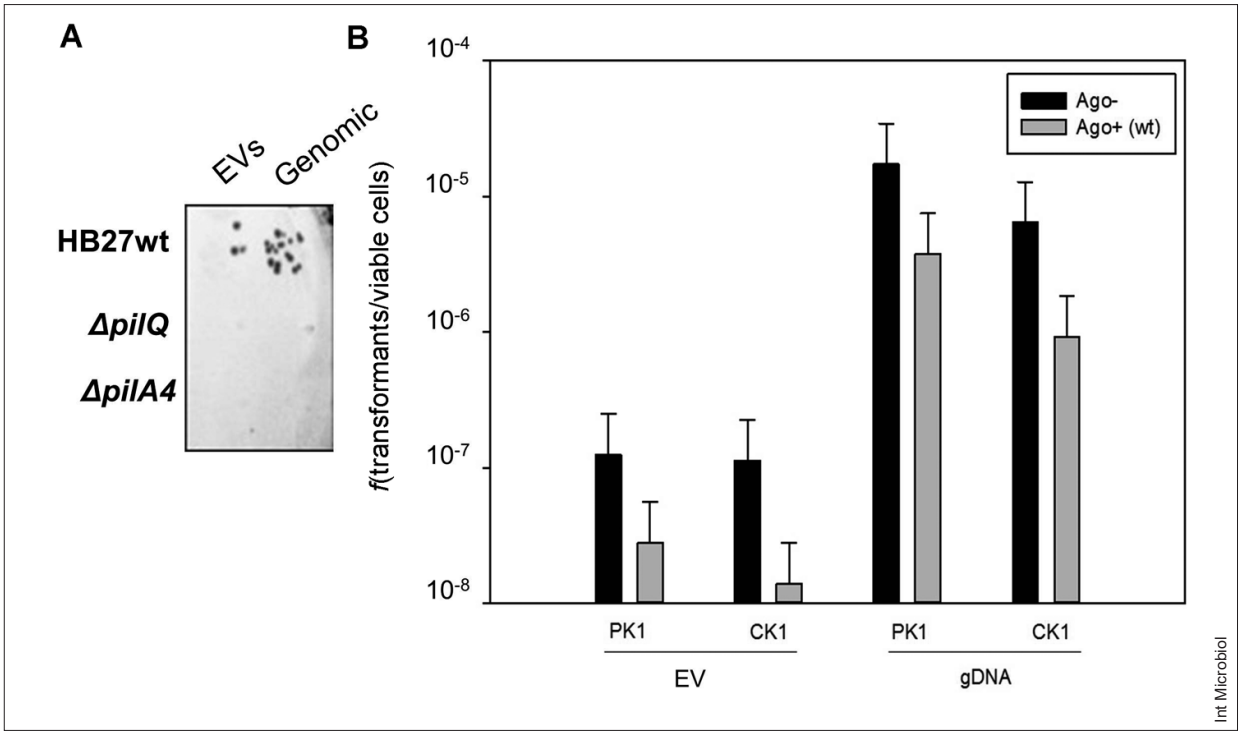

Fig. 6. Transformation with eDNA requires the competence apparatus and elicits Argonaute DNA-DNA interference. (**A**) Transformation of the HB27 strain and competence defective mutants *ΔpilQ* and *ΔpilA* with EVs fraction containing 300 ng of eDNA produced by cultures of the CK1 strain or with 20 ng of genomic DNA from the same strain. **(B)** Transformation frequencies of *T. thermophilus* HB27 (*ago*$^+$) (grey bars) and its Δ*ago* (*ago*$^-$) (black bars) derivative with EVs fraction (EV) from the CK1 and PK1 strains containing 500 ng of eDNA. Transformation controls with 20 ng of genomic DNA from same strains were carried out in parallel (gDNA). Transformation efficiency is expressed as the number of Km-resistant colonies per viable *Δago* cell. Error bars correspond to the mean standard deviation. (n = 3)

Haemophilus spp [27], *E. coli* [58], *Pseudomonas aeruginosa* [26], *Helicobacter pylori* [42] and *Salmonella* spp [55]. Among Gram-positives, production of EVs seems less frequent, or at least few reports have been published, including production of vesicles in *Streptomyces* [47] and *Thermoanaerobacterium thermosulfurogenes* EM1 [36].

Among extreme thermophiles there are fewer reports on EVs. In *Sulfolobus* the formation of EVs seems to be the result of a specific process mediated by endosomal sorting complex similar to that of eukaryotic cells [18]. In *Thermococcus* and *Pyrococcus*, virus-like membrane vesicles are produced that contain genomic DNA making it highly resistant to DNase treatment and heat denaturation [50].

The EVs produced by *Thermus* spp. were quite heterogeneous in size (Fig. 4A) and contained such a large variety of proteins (including cytoplasmic ones) that we concluded that their generation was more likely the consequence of cell lysis rather than the products of a specific EV-generation apparatus, for which a much defined protein composition pattern could be expected. Actually, our data showing decreased production of eDNA in cells growing at lower rates (60ºC in rich medium, or mineral medium), support that exacerbation of lysis is the consequence of unbalanced growth under non natural conditions. In this sense, population stress increased by accelerated nutrient demand of fast growing cultures would drive a higher cell lysis which, in return, would feed the survivals [39,56]. In this scenario, protection of eDNA against DNases would be a lateral consequence of the production of lytic vesicles.

If, as supported by our data, the EVs produced are the consequence of lysis of fast growing cultures, the following question is how the EVs-protected eDNA gets access to a recipient cell. Fusion processes between the EVs and the outer envelope of *Thermus* spp. cannot be ruled out at present, but having in mind the presence of a regular surface layer acting as a scaffold of the outer membrane in these bacteria [10], the existence of such fusion processes seems unlikely. On the other hand, the likely lytic origin of these EVs suggests that the eDNA could be not just inside the EVs, but that it could be adsorbed to their surface tight or deep enough as to block the

access to DNase, but still allowing the competence apparatus of *Thermus* to take it up. In favour of this hypothesis we found >100-fold difference in transformation efficiencies between eDNA and genomic DNA (Fig. 6B). Moreover, the requirement for an active competence system, demonstrated by the lack of Km resistance colonies on transformation assays involving *ΔpilQ* and *ΔpilA4* strains (Fig. 6A), and the sensitivity of this eDNA to the Argonaute DNA-DNA mediated interference supports that transformation is the way by which EVs-associated eDNA enters the cell.

A final question is the biological significance of the EVs in HGT in natural environments. Under the laboratory conditions assayed, production of EVs is associated to rapid growth in rich medium, an unlikely circumstance in environmental context where the scarcity of nutrients makes the growth uneven and rarely fast. However, cell lysis is part of normal life in nature more related with cell stress than with fast growth, and the formation of lytic EVs from different origins with protected eDNA bound to their surface but still accessible to the competence apparatus of *Thermus* is likely to occur frequently. In this scenario, EVs could function as reservoir of genetic material in a protected but still accessible way, increasing its resistance to enzymes, heat and time, as shown for the EVs of *Thermococcus* and *Pyrococcus* [50]. Actually, EVs produced by *Thermus* and stored at 4ºC for 20 months in the presence of DNase are still active in transformation experiments, supporting the role of EVs as vehicles for HGT over long periods of time.

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References

1. Al-Ramahi, Y (2013) Ingeniería de proteínas fluorescentes y aplicaciones de localización celular en microorganismos termófilos. 2013. PhD Thesis. Universidad Autónoma de Madrid

1. Aravind L, Tatusov R, Wolf Y, Walker D, Koonin E (1998) Evidence for massive gene exchange between archaeal and bacterial hyperthermophiles. Trends Genet 14:442-444
2. Averhoff B (2009) Shuffling genes around in hot environments: the unique DNA transporter of *Thermus thermophilus*. FEMS Microbiol Rev 33:611-626
3. Bao Y, Zhang X, Jiang Q, Xue T, Sun B (2015) Pfs promotes autolysis-dependent release of eDNA and biofilm formation in *Staphylococcus aureus*. Med Microbiol Immun 204:215-226
4. Basse G, Lorenz MG, Wackernagel W (1994) A biological assay for the sensitive and quantifiable detection of extracellular microbial DNases. J Microbiol Methods 20:137-147
5. Blesa A, César CE, Averhoff B, Berenguer J (2015) Non canonical cell-to-cell DNA transfer in *Thermus* spp. is insensitive to Argonaute-mediated interference. J Bacteriol 197:138-146
6. Blokesch M, Schoolnik GK (2008) The extracellular nuclease Dns and its role in natural transformation of *Vibrio cholerae*. J Bacteriol 190:7232-7240
7. Brüggemann H, Chen C (2006) Comparative genomics of *Thermus thermophilus*: Plasticity of the megaplasmid and its contribution to a thermophilic lifestyle. J Biotechnol 124:654-661
8. Carrolo M, Frias MJ, Pinto FR, Melo-Cristino J, Ramirez M (2010) Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae*. PLoS One 5:e15678-e15678
9. Cava F, Hidalgo A, Berenguer J (2009) *Thermus thermophilus* as biological model. Extremophiles 13:213-231
10. Cava F, Zafra O, Magalon A, Blasco F, Berenguer J (2004) A new type of NADH dehydrogenase specific for nitrate respiration in the extreme thermophile *Thermus thermophilus.* J Biol Chem 279:45369-45378
11. César CE, Álvarez L, Bricio C, van Heerden E, Littauer D, Berenguer J (2012) Unconventional lateral gene transfer in extreme thermophilic bacteria. Int Microbiol 14:187-199
12. Chatterjee S, Chaudhuri K (2012) Outer membrane vesicles of bacteria. Springer Science & Business Media
13. Das T, Sehar S, Manefield M (2013) The roles of extracellular DNA in the structural integrity of EPS and bacterial biofilm development. Env Microbiol Rep 5:778-786
14. Degryse E, Glansdorff N, Pierard A (1978) A comparative analysis of extreme thermophilic bacteria belonging to the genus *Thermus*. Arch Microbiol 117:189-196
15. Dorward DW, Garon CF (1989) DNA-binding proteins in cells and membrane blebs of *Neisseria gonorrhoeae*. J Bacteriol 171:4196-4201
16. Dubey GP, Ben-Yehuda S (2011) Intercellular Nanotubes Mediate Bacterial Communication. Cell 144:590-600
17. Ellen A, Albers SV, Huibers W, Pitcher A, Hobel CV, Schwarz H, Folea M, Schouten S, Boekema E, Poolman B, Driessen AM (2009) Proteomic analysis of secreted membrane vesicles of archaeal *Sulfolobus* species reveals the presence of endosome sorting complex components. Extremophiles 13:67-79
18. Flemming H-C, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623-633
19. Gogarten JP, Townsend JP (2005) Horizontal gene transfer, genome innovation and evolution. Nat Rev Microbiol 3:679-687
20. Gounder K, Brzuszkiewicz E, Liesegang H, Wolherr A, Daniel R, Gottschalk G, Reva O, Kumwenda B, Srivastava M, Bricio C, Berenguer J, van Heerden E, Litthauer D (2011) Sequence of the hyperplastic genome of the naturally competent *Thermus scotoductus* SA-01. BMC Genomics 12:577
21. Grant SG, Jessee J, Bloom FR, Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc Natl Acad Sci USA 87:4645-4649
22. Henne A, Bruggemann H, Raasch C, Wiezer A, Hartsch T, Liesegang H, Johann A, Lienard T, Gohl O, Martinez-Arias R, Jacobi C, Starkuviene V, Schlenczeck S, Dencker S, Huber R, Klenk H, Kramer W, Merkl R, Gottschalk G, Fritz H (2004) The genome sequence of the extreme thermophile *Thermus thermophilus*. Nat Biotechnol 22:547-553
23. Jain R, Rivera MC, Moore JE, Lake JA (2003) Horizontal gene transfer accelerates genome innovation and evolution. Mol Biol Evol 20:1598-1602

24. Jakubovics NS, Shields RC, Rajarajan N, Burgess JG (2013) Life after death: the critical role of extracellular DNA in microbial biofilms. Lett Appl Microbiol 57:467-475
25. Kadurugamuwa JL, Beveridge TJ (1995) Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. J Bacteriol 177:3998-4008
26. Kahn ME, Barany F, Smith HO (1983) Transformasomes: specialized membranous structures that protect DNA during *Haemophilus* transformation. Proc Natl Acad Sci USA 80:6927-6931
27. Kulp A, Kuehn MJ (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol 64:163-184.
28. Laemmli UK, Paulson JR, Hitchins V (1974) Maturation of the head of bacteriophage T4. V. A possible DNA packaging mechanism: *In vitro* cleavage of the head proteins and the structure of the core of the polyhead. J Supramol Struct 2:276-301
29. Lang AS, Zhaxybayeva O, Beatty JT (2012) Gene transfer agents: phage-like elements of genetic exchange. Nat Rev Microbiol 10:472-482
30. Lappann M, Claus H, Van Alen T, Harmsen M, Elias J, Molin S, Vogel U (2010) A dual role of extracellular DNA during biofilm formation of *Neisseria meningitidis*. Mol Microbiol 75:1355-1371
31. Lennox E (1955) Transduction of linked genetic characters of the host by bacteriophage P1. Virology 1:190-206
32. Martins M, Henriques M, Lopez-Ribot JL, Oliveira R (2012) Addition of DNase improves the *in vitro* activity of antifungal drugs against *Candida albicans* biofilms. Mycoses 55:80-85
33. Mashburn LM, Whiteley M (2005) Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature 437:422-425
34. Mashburn-Warren LM, Whiteley M (2006) Special delivery: vesicle trafficking in prokaryotes. Mol Microbiol 61:839-846
35. Mayer F, Gottschalk G (2002) The bacterial cytoskeleton and its putative role in membrane vesicle formation observed in a Gram-positive bacterium producing starch-degrading enzymes. J Mol Microbiol Biotechnol 6:127-132
36. McBroom AJ, Kuehn MJ (2007) Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. Mol Microbiol 63:545-558
37. Olaya-Abril A, Prados-Rosales R, McConnell MJ, Martín-Peña R, González-Reyes JA, Jiménez-Munguía I, Gómez-Gascón L, Fernández J, Luque-García JL, García-Lidón C, Estévez H, Pachón J, Obando I, Casadevall A, Pirofski L, Rodríguez-Ortega MJ (2014) Characterization of protective extracellular membrane-derived vesicles produced by *Streptococcus pneumoniae*. J Prot 106:46-60
38. Pavlostathis SG, Marchant R, Banat IM, Ternan NG, McMullan G (2006) High growth rate and substrate exhaustion results in rapid cell death and lysis in the thermophilic bacterium *Geobacillus thermoleovorans.* Biotechnol Bioeng 95:84-95
39. Petrova OE, Schurr JR, Schurr MJ, Sauer K (2011) The novel *Pseudomonas aeruginosa* two-component regulator BfmR controls bacteriophage-mediated lysis and DNA release during biofilm development through PhdA. Mol Microbiol 81:767-783
40. Ramirez-Arcos S, Fernandez-Herrero LA, Marin I, Berenguer J (1998) Anaerobic growth, a property horizontally transferred by an Hfr-like mechanism among extreme thermophiles. J Bacteriol 180:3137-3143
41. Ricci V, Chiozzi V, Necchi V, Oldani A, Romano M, Solcia E, Ventura U (2005) Free-soluble and outer membrane vesicle-associated VacA from *Helicobacter pylori*: two forms of release, a different activity. Biochem Biophys Res Commun 337:173-178
42. Rice KC, Bayles KW (2008) Molecular control of bacterial death and lysis. Microbiol and Mol Biol Rev 72:85-109
43. Sahu PK, Iyer PS, Oak AM, Pardesi KR, Chopade BA (2012) Characterization of eDNA from the clinical strain *Acinetobacter baumannii* AI-IMS 7 and its role in biofilm formation. Scientific World J 2012
44. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press.
45. Schooling SR, Beveridge TJ (2006) Membrane vesicles: an overlooked component of the matrices of biofilms. J Bacteriol 188:5945-5957
46. Schrempf H, Merling P (2015) Extracellular *Streptomyces lividans* vesicles: composition, biogenesis and antimicrobial activity. Microb Biotech 8:644-658.
47. Schwarzenlander C, Averhoff B (2006) Characterization of DNA transport in the thermophilic bacterium *Thermus thermophilus* HB27. FEBS J 273:4210-4218
48. Shetty A, Chen S, Tocheva EI, Jensen GJ, Hickey WJ (2011) Nanopods: a new bacterial structure and mechanism for deployment of outer membrane vesicles. PLoS One 6:e20725
49. Soler N, Marguet E, Verbavatz J-M, Forterre P (2008) Virus-like vesicles and extracellular DNA produced by hyperthermophilic archaea of the order *Thermococcales*. Res Microbiol 159:390-399
50. Steinberger RE, Holden PA (2005) Extracellular DNA in single- and multiple-species unsaturated biofilms. Appl Env Microbiol 71:5404-5410
51. Swarts DCJ, Matthijs M, Westra ER, Zhu Y, Janssen JH, Snijders AP, Wang Y, Patel DJ, Berenguer J, Brouns ST, van der Oost J (2014) DNA- guided DNA interference by a prokaryotic Argonaute. Nature 507:258-261
52. Thomas VC, Thurlow LR, Boyle D, Hancock LE (2008) Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. J Bacteriol 190:5690-5698
53. van Wolferen M, Ajon M, Driessen AJ, Albers SV (2013) How hyperthermophiles adapt to change their lives: DNA exchange in extreme conditions. Extremophiles 17:545-563
54. Wang H, Huang Y, Wu S, Li Y, Ye Y, Zheng Y, Huang R (2014) Extracellular DNA inhibits *Salmonella enterica* serovar *Typhimurium* and *S. enterica* serovar *Typhi* biofilm development on abiotic surfaces. Curr Microbiol 68:262-268
55. Wells JE, Russell JB (1996) The effect of growth and starvation on the lysis of the ruminal cellulolytic bacterium *Fibrobacter succinogenes*. Appl Env Microbiol 62:1342-1346
56. Xu Y, Kreth J (2013) Role of LytF and AtlS in eDNA release by *Streptococcus gordonii.* PLoS One 8: e62339
57. Yaron S, Kolling GL, Simon L, Matthews KR (2000) Vesicle-mediated transfer of virulence genes from *Escherichia coli* O157: H7 to other enteric bacteria. Appl Env Microbiol 66:4414-4420