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RESEARCH PAPER

Nonreplicating Intracellular Bacterial Vector for Conjugative DNA Transfer into Mitochondria

Young Geol Yoon • Michael D. Koob

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ABSTRACT

Purpose We have previously shown that DNA constructs can be introduced into isolated mitochondria through the process of conjugative transfer from an E. coli host. We set out to generate a conjugative E. coli strain that would be able to introduce itself into the cytoplasm of a mammalian cell for the purpose of transferring DNA into the mitochondria in the cell.

Methods We have now developed a method for making *E. coli* strains from which nonreplicating populations of daughter cells can be generated. We used this approach to modify a facultative intracellular enteroinvasive E. coli (EIEC) and introduced conjugative functions to this new strain.

Results We demonstrate that this new strain can generate large populations of nonreplicating cells that are capable of conjugative transfer to other cells and can readily invade mammalian tissue culture cells, live in the cytoplasm of the cell for several days, and that do not kill the invaded mammalian cell.

Conclusions We successfully constructed an E. coli host suitable for intracellular conjugative transfer but, due to the lack of suitable mitochondrial screening or selectable markers, we have not yet been able to determine if these bacterial vectors can in fact transfer DNA into intracelluar mitochondria.

M. D. Koob (✉)
Laboratory Medicine and Pathology, University of Minnesota
3-140 WMBB, 2101 6th St SE
Minneapolis, Minnesota 55455, USA
e-mail: koobx001@umn.edu

Present Address:
Y. G. Yoon
Department of Biomedical Sciences, Jungwon University
5 Dongbu-ri, Goesan-eup, Goesan-gun
Chungcheongbuk-do, Korea 367-805

KEY WORDS conjugative DNA transfer · facultative intracellular enteroinvasive *Escherichia coli* · mitochondria · mitochondrial DNA · nonreplicating bacterial cell

ABBREVIATIONS

eGFP	enhanced green fluorescent protein
EIEC	enteroinvasive *Escherichia coli*
PCR	Polymerase Chain Reaction

INTRODUCTION

Bacterial conjugation, which is sometimes referred to as bacterial mating, is an extremely efficient system that has evolved to transfer large broad-host-range DNA plasmids from one bacterial host to another (1). Because all of the molecular components of the DNA transfer machinery as well as those of the mating bridge that connects the DNA donor cell to the recipient cell are both encoded and synthesized in the donor cell, conjugation is also a very versatile process that is capable of transferring DNA from a bacterial host into a wide array of nonbacterial recipient cells (2,3). We have taken advantage of the flexibility of bacterial conjugation to develop a method for transferring DNA constructs directly from *E. coli* into isolated mammalian mitochondria (4). The biological activity of isolated mitochondria is much more limited than mitochondria within the cell, however, and we have been working to develop an intracellular mitochondrial conjugation system. In this paper we describe a novel approach for modifying *E. coli* strains to make them conditionally nonreplicating and for obtaining nonreplicating populations of daughter cells from these strains. We used this method to modify a strain of enteroinvasive *E. coli* (EIEC). We generated large numbers

of nonreplicating but metabolically active daughter cells from this novel strain that are capable of efficient conjugative DNA transfer and can actively enter into the cytoplasm of mammalian tissue culture cells without adversely affecting the growth of the invaded cells. To our knowledge, this is the first report in which terminally nonreplicating cells have been shown to serve as conjugative plasmid donors.

MATERIALS AND METHODS

Manipulation of Plasmid and *E. coli* Genomic DNA

Plasmid DNA was manipulated using standard molecular cloning techniques, as previously described (4). The *dnaA* gene, which encodes a protein that is required to initiate replication of the *E. coli* genome, was eliminated from the genomic DNA of the EIEC strain by recombining linear PCR fragments into the genome, using a described recombination system (5) as outlined in Fig. 1. Details are provided below. The complete sequence of previously unpublished

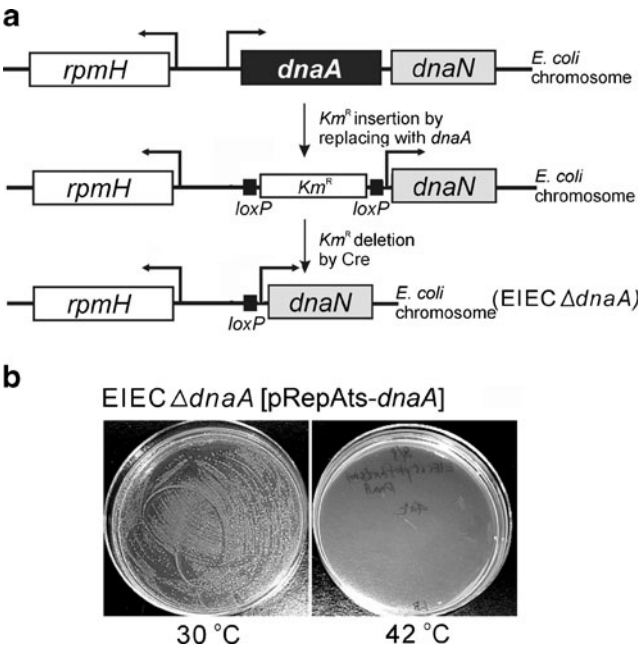

Fig. 1 Generating nonreplicating daughter cells from a facultative intracellular strain of *E. coli*. (**a**) Modification of an EIEC strain to remove the *dnaA* gene, which is required for *E. coli* chromosomal replication initiation. A plasmid containing a copy of the *dnaA* gene was transformed into the EIEC strain and the kanamycin resistance Km^R gene flanked by *loxP* sites was integrated into the chromosome in place of the genomic *dnaA* gene. Cre recombinase was subsequently used to remove the Km^R gene from the genome. (**b**) Temperature sensitive chromosomal bacterial genomic replication. The *dnaA* gene was cloned into a vector containing temperature sensitive origin of pSC101 and maintained in the EIEC*ΔdnaA* strain. This EIEC strain can grow at low temperature (30°C) but is not able to replicate its genomic DNA after the *dnaA* gene on the temperature-sensitive plasmid is lost at higher temperatures (42°C).

vectors and constructs used for this work are available from GenBank with the following accession numbers: pANTS-AraλRED (JQ301470); pANTS-AraλRED *dnaA dnaN* (JQ301471); pK^{Flox}ANTS-Asc (JQ301472); pK^{Flox}ANTS-Asc tsOri-pBR (JQ301473); and $pRepA^{ts}$-*dnaA* (JQ301474).

Cloning of the dnaA *Operon into a λRED Expression Plasmid*

The *dnaA* operon containing the *dnaA* and *dnaN* genes and their promoter sequences was amplified by PCR using following primers: 5′-AATACTAGTCTATGGTCATTAAATTTTCCAAT-3′ and 5′-CGACCATGGCCGAGCGTATAG-3′ (*Spe*I and *Nco*I sites underlined). The 3-kb PCR product was digested with SpeI and NcoI and cloned into the *Spe*I and *Nco*I sites of pANTS-AraλRED, a novel Amp-resistant construct that expresses the phage lambda recombination proteins (5) after induction by arabinose. The resulting plasmid was transformed into the EIEC strain and provided both the dnaA DNA replication-initiation protein and the phage lambda recombination proteins that allowed us to further manipulate the *E. coli* genome.

Deletion of the dnaA *Gene from the Chromosome of EIEC Strain*

To remove the *dnaA* gene from the *E. coli* genome, a recombinant PCR technique was used to prepare a linear DNA fragment in which the *dnaA* is deleted from the *dnaA-N* operon and a kanamycin (Km^R) selection marker flanked by *loxP* sequences is inserted upstream of the *dnaA-N* operon promoter. The *dnaA* operon and the Km^R gene fragments were amplified using following primers: (a) a floxed Km^R gene with *dnaA* insertion homology (loxPKmloxP 3′, 5′-GATCGATTAAGCCAATTTTT*GTTGAACTGCGGATCTTGCG*-3′ and loxPkmloxP 5′, 5′-GGAAAATTTAATGACCATAGAC*GGGCCATCGATTCGAATAAC*-3′; bases that correspond to sequences 5′ of the of *dnaA* promoter are underlined and sequences for floxed Km^R fragment amplification are italicized), (b) *dnaA-N* operon promoter region (DnaAOp promoter5′, 5′-GTCTATGGTCATTAAATTTTCCAATATG-3′ and DnaAOp promoter3′, 5′-TTCATAGGTTTACGATG(*delta dnaA*)*ACAAAAGTCGATAATGACTAAGGC*-3′; sequences homologous to the flanking PCR products are underlined, and the *dnaA* deletion junction is noted) and (c) *dnaN* gene fragment (DnaN5′, 5′-GACTTTTGTCATCGTAAACCTATGAAATT-3′ and DnaN-OutsideHomology3′, 5′-TAAGCCAATCGCTGTCTCGC-3′; sequences homologous to the promoter PCR product are underlined). These three PCR products from (a), (b) and (c) above were combined and recombinant PCR was carried out using primers DnaN-OutsideHomology3′ and RpmH-outsideHomol (5′-

GCGCAAGGATCGTCCTGGATCTTTATTAGATC GATTAAGCCAATTTTT-3′, sequences homologous to the floxed Km^R PCR product are underlined).The recombinant PCR product containing the Km^R flanked by *loxP* sequences and the genomic homology was transformed into the EIEC [pANTS-AraλRED *dnaA dnaN*] strain. The PCR product was integrated into the *dnaA* locus by homologous recombination mediated by λ recombinase (5), thereby removing the *dnaA* gene and integrating the floxed Km^R marker in the genome 5′ of the promoter that now expressed only *dnaN*. The Km^R gene was subsequently eliminated by transient expression of Cre recombinase to complete the markerless removal of *dnaA*, generating the Δ*dnaA* EIEC strain (see Fig. 1). This deletion junction (noted in the DnaAOp promoter3′primer sequence above) was confirmed by PCR and sequencing of the PCR product.

Construction of dnaA-Containing Plasmid with Temperature Sensitive Origin

The temperature sensitive origin (tsORI) sequence was amplified by PCR from pEL3 (mini pSC101 plasmid) (6) with the following primers: tsOri-AscI, 5′-ACGGGCGCGCC TGTTGATGATACCGCTGCCT-3′ (*Asc*I site underlined) and tsOri-NheNco, 5′-CCGCTAGCCCATGG TAACGGTGAACAGTTGTT-3′ (*Nhe*I site underlined). The 1.8-kb PCR amplified tsORI sequence was cloned into the *Nhe*I and *Asc*I sites of the pK^{Flox}ANTS-Asc vector, a novel construct derived from a *Not*I-flanked pBR-ori series of "pANTS" vectors (7,8). The *dnaA* gene was then amplified by PCR using these primers: 5′-GCCAAGCTTG TCTATGGTCATTAAATTTTCCAAT-3′ and 5′-CGGGGATCCTTAGCTGCTTAATGTTCTGAT TAAATTTGAAAAATC-3′ (*Hind*III and *Bam*HI cloning sites underlined). Note that 7 nt near the 3′ end of the dnaA reading frame were changed without altering the protein sequence in order to eliminate the minimal overlap that would otherwise exist between the gene in the plasmid construct and the Δ*dnaA* genomic allele. The PCR-amplified *dnaA* gene fragment was digested with HindIII and BamHI and cloned into the pK^{Flox}ANTS-Asc tsOri-pBR plasmid (above). After removing the pBR-ori sequence by NotI digestion and ligation, the resulting recombinant plasmid ($pRepA^{ts}$-*dnaA*) was transformed into the Δ*dnaA* EIEC strain, and we screened the Km^R transformants at 30°C to find clones from which the Amp^R pANTS-AraλRED *dnaA* construct had been spontaneously lost in the absence of ampicillin selection.

Preparing Populations of Nonreplicating EIEC

After growing liquid cultures of our temperature-sensitive Δ*dnaA* EIEC [$pRepA^{ts}$-*dnaA*] strains at 30°C until mid-log phase, we grew the cultures at 42°C for 3 h to stop the replication of the *dnaA*-containing plasmid and to allow nonreplicating daughter cells that no longer contained this plasmid to be generated. We then added 50 mg/ml of ampicillin to the culture and incubated the cultures to 37°C overnight to lyse any remaining dividing cells.

Conjugation and Cytoplasm Invasion Assays

Conjugation experiments were performed as previously described(4). To evaluate cytoplasmic invasion, populations of nonreplicating EIEC cells (above) were overlayed onto the top of HeLa229 cells. After culturing for 4 h culture at 37°C in a 5% CO_2 incubator, the tissue culture cells were washed with PBS three times to remove the remaining *E. coli* in the culture medium. In some experiments, gentamicin was added to the culture media to eliminate only extracellular bacteria (9).

RESULTS

Generating Nonreplicating Daughter Cells from a Cytoplasmic Invasive Strain of Enteroinvasive *E. coli*

Enteroinvasive *E. coli* (EIEC) are capable of entering into and replicating within the cytoplasm of epithelial cells (10). We confirmed that a minimally characterized EIEC strain (EIEC65) (11) was able to invade the cytoplasm of both human and mouse cells tissue culture cells. However, we found that after entering these tissue culture cells, the EIEC rapidly replicated and killed the mammalian cell host. We wanted to eliminate the ability of these bacterial cells to replicate after entering into the mammalian cytoplasm without adversely affecting their ability to synthesize protein and replicate DNA, which are both needed for conjugative DNA transfer. To achieve this, we inserted the *E. coli dnaA* gene into a plasmid with a temperature sensitive origin of replication (6), introduced this construct into the EIEC cells, and then deleted the genomic copy of the *dnaA* gene from these cells (Fig. 1). Because the *dnaA* gene is required for initiating genomic DNA replication in *E. coli* (12), cells deleted for this gene will not be capable of replicating their own genome but will be otherwise metabolically normal. At 30°C, which is a temperature at which the $pRepA^{ts}$-*dnaA* plasmid can replicate, the Δ*dnaA* EIEC cells replicate in a normal manner and can form colonies (Fig. 1b). At 42°C, however, the *dnaA* plasmid does not replicate and so the Δ*dnaA* EIEC cells also stop replicating and cannot form colonies at this temperature (Fig. 1b).

At the cellular level, the cells which have a nonreplicating $pRepA^{ts}$-*dnaA* plasmid will continue to replicate their

genomic DNA and divide, but the daughter cells will have lost the pRepA^{ts}-*dnaA* plasmid and so will have a permanent Δ*dnaA* genotype and can no longer replicate at any temperature. Experimentally, we found that we could isolate large populations of irreversibly nonreplicating Δ*dnaA* EIEC cells by growing our strain at 30°C, shifting the culture to 42°C for several hours, and then adding ampicillin, which will lyse dividing cells but will not harm the non-dividing cells (13).

Conjugative DNA Transfer from Nonreplicating Populations of Invasive *E. coli*

To make our replication deficient Δ*dnaA* EIEC cells capable of conjugative DNA transfer, we introduced the RK2-derived plasmid pRK2013 (14) to generate Δ*dnaA* EIEC [pRepA^{ts}-*dnaA*, pRK2013]. The pRK2013 conjugative "helper" plasmid encodes the conjugative functions of RK2 but has the replication origin from ColE1. We then transformed into this strain pT7hpGFP+oriT(4), a plasmid designed to be mobilized into other strains by conjugation when RP4 functions are provided *in trans*, and then tested the conjugation functions of both replicating and nonreplicating populations of these EIEC cells by mating them with recipient *E. coli* DH5α (Fig. 2). As expected, we found that

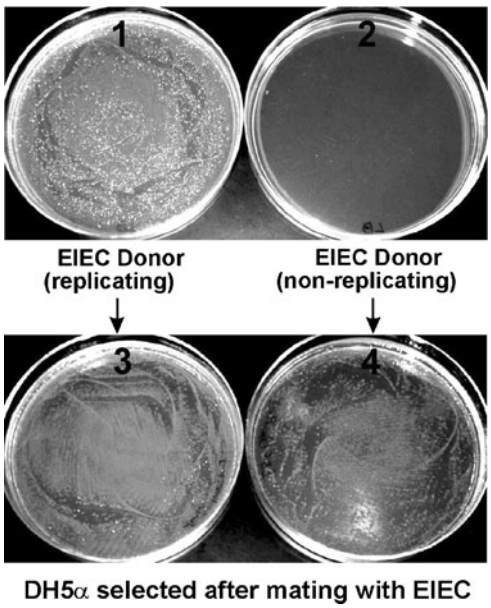

Fig. 2 Efficient conjugative DNA transfer from nonreplicating *E. coli* into recipient bacterial cells. Populations of nonreplicating EIEC daughter cells are generated by growing cultures at elevated temperatures and then treating them with ampicillin (Amp) to lyse any cells that can still divide, as described. The replicating parent cells can form colonies on culture plates at 30°C (-Amp, plate 1), but the nonreplicating population has irreversibly lost its ability to divide and form colonies (Amp, plate 2). We found that a similar number of recipient *E. coli* can be selected after mating with the ampicillin-treated EIEC strains (plate 4) as can be selected after conjugation with the replicating parent culture (plate 3), demonstrating that the nonreplicating population of modified EIEC is still able to transfer DNA by conjugation.

pT7hpGFP+oriT (Tc^{R}) is transferred at a high frequency into the recipient DH5α *E. coli* after mating with the either replicating (Fig. 2, plates 1 and 3) or the nonreplicating ampicillin-treated EIEC strains (Fig. 2, plates 2 and 4). This result demonstrates that the nonreplicating (*i.e.*, heat and ampicillin treated) populations of Δ*dnaA* EIEC [pRK2013] retain the ability to transfer DNA into recipient cells, even though they have lost the ability to replicate their own genomic DNA and can no longer form colonies (Fig. 2, plate 2).

Nonreplicating *E. coli* Can Actively Invade Mammalian Tissue Culture Cells without Decreasing the Viability of the Mammalian Host Cell

We next confirmed that the nonreplicating forms of Δ*dnaA* EIEC [pRK2013] are still able to actively invade the cytoplasm of human tissue culture cells (Fig. 3). We assayed this invasion by labeling the *E. coli* with enhance Green Fluorescent Protein (eGFP), introduced the bacteria into a culture of HeLa cells, and visually confirmed that the fluorescently-labeled bacteria were present in the cytoplasm of the HeLa cells. After washing out the bulk of the *E. coli*, any extracellular bacteria that may have remained attached to the tissue culture cells were eliminated by adding gentamicin to the media, which kills all of the bacteria not protected within the tissue culture cells(9). Although the nonreplicating *E. coli* are more elongated than the replicating forms of these bacteria, presumably due to continued cell growth in the absence of genomic DNA replication, these bacteria are still capable of efficient invasion of mammalian cells (Fig. 3).

Unlike replication proficient EIEC, which will continue to grow and divide within the cytoplasm of mammalian tissue culture cells and thus kill them, the nonreplicating EIEC enter the cytoplasm of mammalian tissue culture cells but will neither kill them nor even significantly inhibit their growth (Fig. 4). We continued to observe invaded HeLa cells for 6 days and found that, although the labeled bacteria were clearly visible within the cytoplasm of cells for several days, these cells continued to divide at essentially the same rate as untreated and non-invasion control populations of HeLa cells (Fig. 4).

In order to determine if the intracellular bacteria were capable of conjugative transfer of DNA into intracelluar mitochondria, we hoped to take advantage of the same RNA assay we used to measure conjugative transfer into isolated mitochondria(4). To do this, we treated cultures of modified HeLa cells in which the T7 RNA polymerase was targeted into the mitochondrial matrix with nonreplicating populations of Δ*dnaA* EIEC [pRK2013] carrying either the conjugative competent pT7hpGFP+oriT or a nearly identical control plasmid that cannot be transferred by pRK2013 (*i.e.*, -oriT)(4). After isolating and assaying total

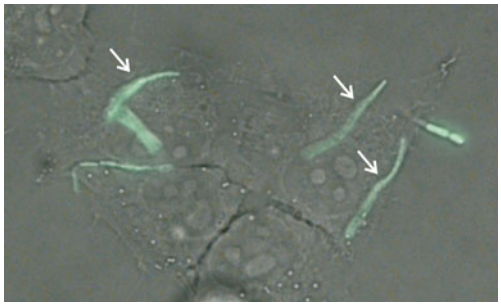

Fig. 3 Invasion of nonreplicating EIEC bacteria into tissue culture cells. Fluorescently-labeled, nonreplicating EIEC can actively invade into the cytoplasm of tissue culture cells (*arrows*).

RNA from the experimental and the control cells, however, we found that both samples contained RNA that had been transcribed from the assay plasmids (not shown). We subsequently found that basal levels of RNA transcript are generated from these plasmids when they are present in the *E. coli* cells. Because the plasmids are already in the bacteria inside the cells, we are not able to use our established RNA transcription assay to distinguish between intra-bacterial and intra-mitochondrial DNA and so were not able to measure intracellular conjugation into mitochondria.

DISCUSSION AND CONCLUSION

We have previously demonstrated that DNA constructs can be introduced into isolated mitochondria by conjugative DNA transfer. Although mitochondria that have been removed from a cell continue to have some limited metabolically activity, isolated mitochondria have a fairly limited utility in comparison to mitochondria in living cells. In particular, using a conjugative DNA transfer approach to stably transform mitochondria with DNA constructs or complete mitochondrial genomes that would be capable of replicating in the mitochondrial matrix would require us to adapt these techniques for use with intracellular mitochondria. Although it may be possible to perform conjugative DNA transfer into isolated mitochondria and to then return these mitochondria to the cytoplasm of a cell, a much more attractive alternative is to generate bacterial strains that would be capable of invading the cytoplasm of a cell and then conjugating with mitochondria in their native environment.

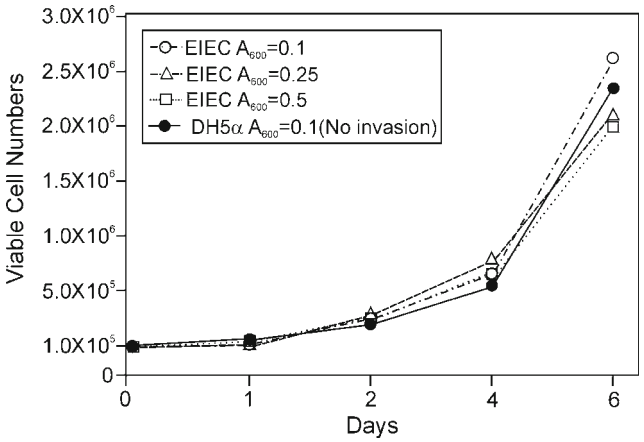

Fig. 4 Viability of tissue culture cells after invasion of nonreplicating EIEC cells. Populations (OD_{600}=0.1, 0.25 and 0.5) of nonreplicating EIEC cells were overlaid on top of a culture of HeLa229 cells. After a 4 h culture at 37°C in a 5% CO_2 incubator, the tissue culture cells were washed with PBS three times to remove the remaining *E. coli* in the culture medium. The HeLa229 cells were then incubated further for 6 days to measure the viable cell numbers. The EIEC strain that was invaded into cytoplasm did not inhibit the growth of tissue culture cells. The *E. coli* strain DH5α was used as a non-invasion control.

In this paper we describe an approach that allows us to generate large numbers of nonreplicating *E. coli* cells by transferring the *dnaA* gene from the genome to a plasmid with a temperature-sensitive replication origin. The *dnaA* gene is required to initiate replication of the *E. coli* genome (12) as well as of some plasmids (15), but loss of this protein does not affect the replication of other plasmids (*e.g.*, ColE1- or R100-derived plasmids) nor is DNA synthesis initiated through other mechanisms affected, such as the nick-extension DNA synthesis involved in conjugative transfer (1). When replication of the plasmid pRepA^{ts}-*dnaA* is stopped by culturing the modified *E. coli* at the non-permissive temperature, daughter cells are generated that no longer contain a *dnaA* gene and so are now terminally nonreplicating but otherwise have all cellular functions. Note that the pRK2013 conjugative "helper plasmid" has a ColE1-derived replication origin that is not dependent on DnaA to replicate and is still present in all daughter cells (14). We found that we could eliminate all cells that could still replicate and divide in these cultures by adding ampicillin to the culture, which selectively lyses dividing cells but does not affect non-dividing cells (13).

We have used this approach to construct an *E. coli* host suitable for intracellular conjugative transfer and have demonstrated that these bacterial hosts have three essential traits: 1) they can conjugate with and transfer DNA into a recipient cell, 2) they can efficiently invade and are metabolically active within the cytoplasm of mammalian cells, and 3) they do not significantly damage the mammalian host cell.

Unfortunately, due to the lack of a suitable mitochondrial assay, we have not yet been able to determine if these bacterial vectors can in fact transfer DNA into intracelluar mitochondria. Because bacterial conjugative DNA transfer introduces a single-stranded DNA molecule into the recipient, we do not know if DNA introduced through this route into mitochondria would be a suitable substrate for either the native

mitochondrial DNA replication or RNA transcription system. For this reason, we have devised a system for measuring transcription from a synthetic promoter sequence that is functional in either single-stranded or double stranded DNA and have shown that this assay can detect DNA transferred into isolated mitochondria(4). In this previous work, however, we were able to completely eliminate the bacterial donor prior to performing this RNA transcript assay and so could eliminate the background RNA that made from the assay plasmid inside the bacteria. We are therefore continuing to develop mitochondrial screening assays, potential selective markers (16), and mitochondrial genomic plasmid constructs for use with our intracellular conjugative bacterial host.

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