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Intercellular communication and conjugation are mediated by ESX secretion systems in mycobacteria

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Abstract

Communal bacterial processes require intercellular communication mediated by secretion systems to coordinate appropriate molecular responses. Intercellular communication has not been described previously in mycobacteria. Here we show that the ESX secretion-system family member ESX-4 is essential for conjugal recipient activity in *Mycobacterium smegmatis*. Transcription of *esx4* genes in the recipient requires coculture with a donor strain and a functional ESX-1 apparatus in the recipient. Conversely, mutation of the donor ESX-1 apparatus amplifies the *esx4* transcriptional response in the recipient. The effect of ESX-1 on *esx4* transcription correlates with conjugal DNA transfer efficiencies. Our data show that intercellular communication via ESX-1 controls the expression of its evolutionary progenitor, ESX-4, to promote conjugation between mycobacteria.

Mycobacteria have elaborate cell envelopes and use ESX secretion systems to transport substrates across their diderm cell structure (1). Mycobacteria encode as many as five paralogous *esx* loci (2). Each *esx* locus encodes components for its own membrane transporter, secretion substrates, powering adenosine triphosphatase (ATPase), and other proteins that contribute structural or regulatory functions. Although they are homologous, the ESX conserved components (*ecc*) encoded by each locus are specific to their individual secretory apparatus (3) and are not functionally redundant, as phenotypes arise from mutations in individual paralogs. ESX secretion activity is required for virulence in pathogenic mycobacteria (4–6). However, the full range of functions of the various *esx* loci remains unknown.

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SUPPLEMENTARY MATERIALS

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Material and Methods

Figs. S1 to S4

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Mycobacterium smegmatis is a fast-growing saprophytic and nonpathogenic species that has been used as a model for slow-growing pathogenic species (7). *M. smegmatis* has three *esx* loci that encode the ESX-1, ESX-3, and ESX-4 secretion apparatuses. In *M. smegmatis*, the ESX-1 apparatus is required for distributive conjugal transfer (DCT), a distinct gene transfer process that occurs between independent and genetically distinct donor and recipient strains and results in progeny with mosaic genomes (fig. S1) (8–11). Mutations that inactivate ESX-1 in either the donor or recipient strains of *M. smegmatis* alter conjugal DNA transfer efficiencies. A direct model, in which ESX-1 is proposed to serve as the conduit for DNA traversing from the donor strain into the recipient, is ruled out by the finding that *esx1* mutations in the donor strain increase DNA transfer efficiencies up to 100-fold (9). Conversely, ESX-1 mutations in the recipient reduce DNA transfer to undetectable levels (8). Recent findings further show that genes determining donor or recipient mating identity in DCT have been mapped to a cluster of six of the 25 *esx1* genes (11). The disparate roles for the various ESX secretion apparatuses, in both slow- and fast-growing mycobacteria, indicate that they mediate secretion of substrates that function in diverse pathways.

The *esx4* loci appear to encode only the essential core components of a functional ESX apparatus and lack *eccA* and *espG* genes that are functionally important for substrate secretion and processing in other ESX systems (12, 13). This observation and the absence of any identified activity for ESX-4 led to the speculation that it is a vestigial locus (5). Loci encoding ESX-4 secretion systems are also found in other Actinobacteria and Firmicutes, which suggests that ESX-4 is the progenitor ESX (2, 3). In spite of its ancestral status, conserved composition, and broad distribution, a functional role for ESX-4 has not been identified.

Here we report mapping of a transposon insertion in the recipient *esx4* locus that abolished conjugation (Fig. 1). The transposon inserted into the recipient ortholog of *Msmeg_1536*, encoding a dedicated Ftsk-SpoIIIE-ATPase, $EccC_4$ (the subscript “4” indicates the associated *esx* locus), whose paralogs in other *esx* loci are required for function of their respective ESX apparatus (1, 14). To ensure that the transposon insertion resulted in a null phenotype, we created a precise deletion of *eccC*$_4$ in the recipient, and it too was defective for DCT (Fig. 1). To formally rule out any possibility of ESX-4 secretion activity, we created a precise deletion of *Msmeg_1535*, encoding the ESX-4 transporter, $EccD_4$. The recipient $EccD_4$ mutant was also transfer-defective (Fig. 1). Deletion of *eccC*$_4$ or *eccD*$_4$ from the donor strain, however, did not abrogate conjugation (Fig. 1). This recipient-specific requirement for ESX-4 is also seen with *esx1* mutants in DCT, although the increase in transfer efficiency seen with ESX-1 donor mutants was not evident with the loss of ESX-4 in the donor.

Only one gene is exclusive to *esx4* (Fig. 1B). *Msmeg_1537* is of unknown function and is conserved in position in all *esx4* loci, but homologs are not found in other paralogous *esx* loci. The conserved presence of this gene within the *esx4* locus led us to hypothesize that it is necessary for ESX-4 function. We created a deletion mutant of *Msmeg_1537* in the recipient strain and found that this mutant strain was also defective for DCT, producing no transconjugants. Complementation by ectopic expression of *Msmeg_1537* restored conjugation (Fig. 1).

Together, these data show that ESX-4 is essential for DCT in the recipient but not the donor and that DCT is a sensitive and reliable assay for ESX-4 function. Thus, ESX-1 and ESX-4 have non-redundant roles in the same biological pathway. ESX-4 function cannot compensate for ESX-1 mutations and vice versa. Although the traditional *oriT*-based conjugation systems have evolved as a donor function encoded by a specific mobile element for self-propagation, all of the genes that have thus far been identified as necessary for mycobacterial DCT are recipient-specific.

Conjugation is a tightly regulated biological process that requires coordinated gene expression (15–17). We hypothesized that a subset of genes involved in DCT would respond to the presence of the opposite mating type. We used RNA profiling to detect key transcriptional programs that were activated or silenced upon coculture of donor and recipient strains. The many single-nucleotide polymorphisms (SNPs) between donor and recipient genomes act as strain-specific identifiers for the mRNAs and allowed us to perform strain-specific expression profiling (Fig. 2A). cDNA libraries were prepared from mRNA isolated from donor and recipient cells grown under mating conditions in either monoculture or coculture (fig. S2A). After library sequencing, reads were mapped back to each reference genome, and the embedded SNPs were used to identify which strain produced the mapped read. All genes were evaluated for their transcript levels under mating conditions relative to their levels from monoculture to identify the transcripts that responded to the presence of the opposite mating type (fig. S3).

During coculture of wild-type (WT) *M. smegmatis* strains, one of the most highly induced transcripts was from the *esx4* locus (Fig. 2B, fig. S2B, and table S1). *esx4* transcripts were elevated only in the recipient strain, as can be observed in the heat map of *esxUT* (Fig. 2B), the tandem gene pair encoding the primary WXG100 secretion substrates of ESX-4. Notably, the paralogous *esx1* and *esx3* WXG100 genes—*esxBA* and *esxGH*, respectively—did not transcriptionally respond in either strain to coculture with their mating partner (Fig. 2B). Therefore, coculture of donor and recipient strains specifically increased transcript levels of a recipient locus required for conjugation. The specific conditions required for the expression of *esx4* locus genes (in this case, coculture of a mating pair) may explain why identification of ESX-4 expression and function has been elusive. Overexpression of the sigma factor, SigM, has been associated with increased transcription of *esxUT* in *M. tuberculosis* (18, 19), but our *M. smegmatis* DCT RNA sequencing (RNA-seq) analyses detected no transcriptional change in *sigM* or conserved genes of its regulon.

We then tested whether ESX-1 function affected transcriptional profiles in DCT mating conditions. The *esx1*-encoded Ftsk-SpoIIIE-ATPase ortholog of $eccC_4$ (1), $eccC_{b1}$, was deleted in the donor and recipient strains for use as mating partners with WT strains. Coculture of the Δ$eccC_{b1}$ donor with a WT recipient induced transcription of the recipient WT *esx4* locus (Fig. 2B), as expected for mutated ESX-1 donors, which are known to perform DCT (9). In contrast, the pairing of the WT donor with the Δ$eccC_{b1}$ recipient failed to induce *esx4* transcription in this recipient strain (Fig. 2B). ESX-1 function in the recipient strain is required for DCT (8). Therefore, the recipient *esx4* locus transcriptionally responds only in conjugation-proficient mating pairs. Our RNA-seq analyses show that recipient *esx4*

gene transcripts are induced only upon coculture with the donor strain and that the induction requires a functional recipient ESX-1 secretion system.

We used the highly responsive bicistronic *esxUT* transcript in quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays to independently validate and quantify our global SNP-specific RNA-seq profiles (fig. S2B). DCT mating assays were repeated, with duplicate samples being processed for conjugal DNA transfer efficiency (Fig. 3A) and qRT-PCR (Fig. 3B). We used a polymorphic BstUI cleavage site to show that the induced *esxUT* transcript is exclusively from the recipient strain (Fig. 3C). The WT mating pair showed a 30-fold increase of the *esxUT* transcript relative to parental monocultures (Fig. 3B). The ESX-1 dependence in the recipient was corroborated, as *esxUT* was increased less than sixfold in the Δ$eccC_{b1}$ mutant. This level was slightly elevated relative to our RNA-seq data (Fig. 2B), although it is unclear whether this is normal variation in the assay, the *rpoB* qRT-PCR internal control, or an unknown factor. The accentuated recipient *esxUT* transcriptional response to the ESX-1 mutant donor strain indicated by RNA-seq data (Fig. 2B) was corroborated by qRT-PCR, which showed a 221-fold induction (Fig. 3B). Thus, coculture induction of ESX-4 corresponds to the reported effects of ESX-1 on conjugation: Recipient ESX-1 mutants do not induce recipient ESX-4 and do not produce transconjugants (8), whereas donor ESX-1 mutants hyperinduced recipient ESX-4 and are hyperconjugative (9). These results indicate that ESX-1 acts upstream of ESX-4 in DCT, directing the induction of *esx4* gene expression that results from mating-pair interactions.

The transfer of DNA between participating cells during DCT clearly requires physical contact, yet the communication might occur by diffusible signals. We performed coculture under typical DCT mating conditions. However, we separated the donor and recipient strains with a 0.45-μm filter membrane intended to allow transit of soluble signaling molecules but prevent cell-cell contact. qRT-PCR analysis revealed that recipient cells cultured with this porous separation did not show an *esxUT* transcriptional response to the underlying donor strain (Fig. 3B). These data indicated that direct cell-cell contact is needed to initiate the *esx4* transcriptional response. We speculate that ESX-1 secretes cell-surface mating identifiers, receptor proteins, and/or tethering scaffolds. One possibility consistent with our data is that ESX-1 secretes a cell surface receptor in both strains: In the recipient, a yet unknown ligand binding to this receptor initiates a signal cascade that induces ESX-4 necessary for DCT (fig. S4). Thus, disabling ESX-1 function in the donor strain would prevent its receptor secretion from competing for ambient ligand, resulting in hyperactivation of the recipient *esx4* locus. Candidates for potential involvement are encoded by the subset of *esx1* genes that constitute the mating identity locus (*mid*) (11).

Strain-specific RNA-seq during coculture also identified expression changes in genes that are not involved in DCT. Thus, coculture responses between these mycobacterial strains may not be limited to conjugation. Some of the largest transcriptional changes were dependent on ESX-1. The concept that ESX systems function in intercellular communication among mycobacteria has implications for other mycobacterial species and for intercellular communication in infection.

Supplementary Material

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ACKNOWLEDGMENTS

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REFERENCES AND NOTES

1. Abdallah AM et al., Nat. Rev. Microbiol 5, 883–891 (2007). [PubMed: 17922044]
2. Gey van Pittius NC et al., Genome Biol 2, research0044.1 (2001).
3. Bitter W et al., PLOS Pathog 5, e1000507 (2009). [PubMed: 19876390]
4. Simeone R, Bottai D, Brosch R, Curr. Opin. Microbiol 12, 4–10 (2009). [PubMed: 19155186]
5. Stoop EJ, Bitter W, van der Sar AM, Trends Microbiol 20, 477–484 (2012). [PubMed: 22858229]
6. Tan T, Lee WL, Alexander DC, Grinstein S, Liu J, Cell. Microbiol 8, 1417–1429 (2006). [PubMed: 16922861]
7. Shiloh MU, DiGiuseppe Champion PA, Curr. Opin. Microbiol 13, 86–92 (2010). [PubMed: 20036184]
8. Coros A, Callahan B, Battaglioli E, Derbyshire KM, Mol. Microbiol 69, 794–808 (2008). [PubMed: 18554329]
9. Flint JL, Kowalski JC, Karnati PK, Derbyshire KM, Proc. Natl. Acad. Sci. U.S.A 101, 12598–12603(2004). [PubMed: 15314236]
10. Parsons LM, Jankowski CS, Derbyshire KM, Mol. Microbiol 28, 571–582 (1998). [PubMed: 9632259]
11. Gray TA, Krywy JA, Harold J, Palumbo MJ, Derbyshire KM, PLOS Biol 11, e1001602 (2013). [PubMed: 23874149]
12. DiGiuseppe Champion PA, Champion MM, Manzanillo P, Cox JS, Mol. Microbiol 73, 950–962 (2009). [PubMed: 19682254]
13. Houben EN, Korotkov KV, Bitter W, Biochim. Biophys. Acta 1843, 1707–1716 (2014). [PubMed: 24263244]
14. Converse SE, Cox JS, J. Bacteriol 187, 1238–1245 (2005). [PubMed: 15687187]
15. Koraimann G, Wagner MA, Front. Cell. Infect. Microbiol 4, 54 (2014). [PubMed: 24809026]
16. Singh PK, Meijer WJ, FEMS Microbiol. Lett 358, 119–128 (2014). [PubMed: 24995588]
17. Dunny GM, Berntsson RP, J. Bacteriol 198, 1556–1562 (2016). [PubMed: 27021562]
18. Agarwal N, Woolwine SC, Tyagi S, Bishai WR, Infect. Immun 75, 452–461 (2007). [PubMed: 17088352]
19. Raman S et al., J. Bacteriol 188, 8460–8468 (2006). [PubMed: 17028284]

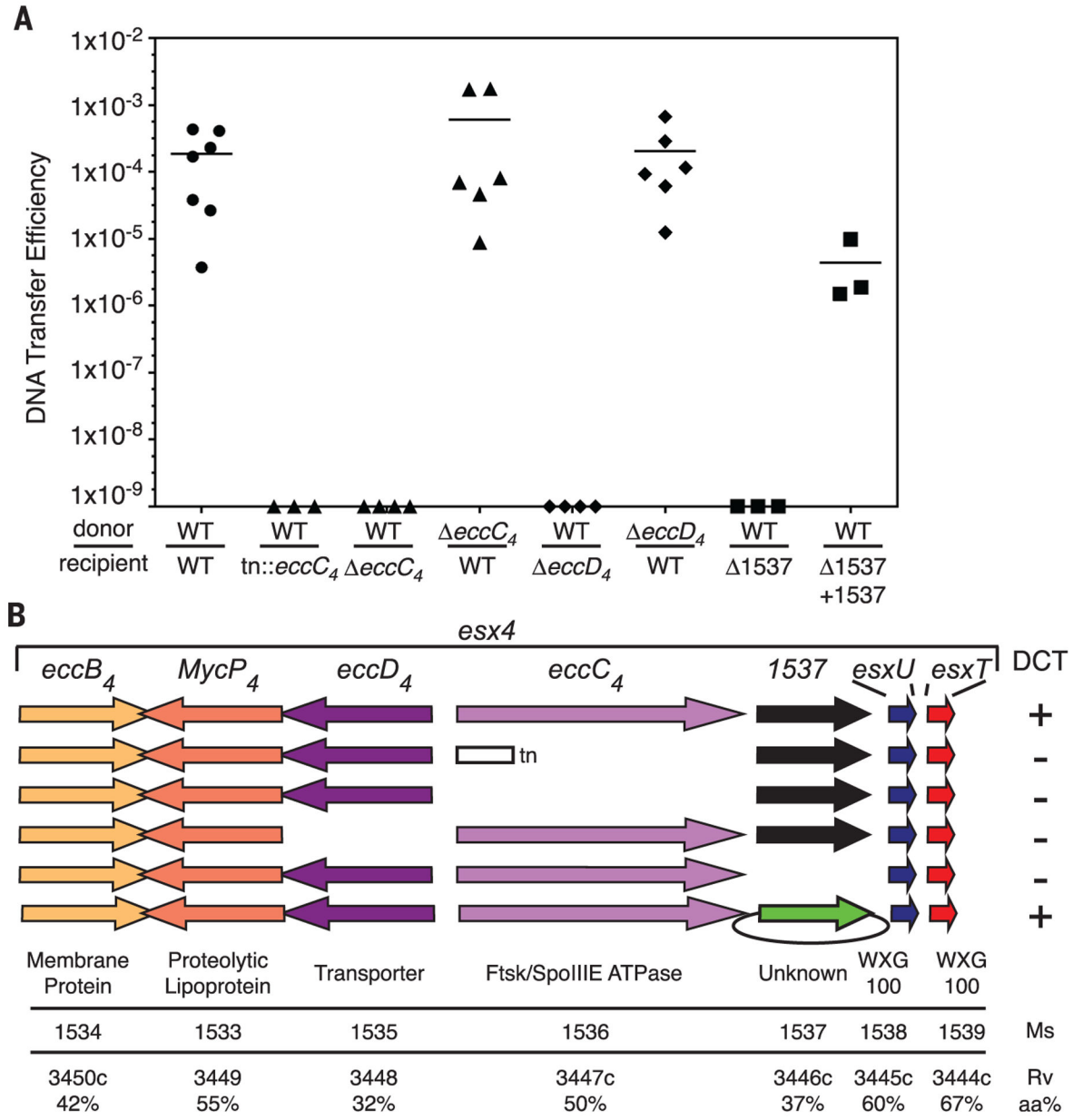

Fig. 1. Recipient ESX-4 is required for mycobacterial conjugation.

(**A**) DNA transfer efficiencies (transconjugants per donor cell) show that DCT requires an intact *esx4* locus in the recipient strain. Transconjugants were not recovered in matings that lacked a recipient ESX-4 component. Mating-pair genotypes are indicated for donor and recipient, above and below the line, respectively. (**B**) Schematic showing the conserved gene content and order of *esx4* loci. ESX nomenclature of the encoded proteins is shown above the arrows (*ecc* designates an ESX essential core protein). The gene specific to *esx4* is indicated by its *M. smegmatis* gene name, *Msmeg_1537*. Recipient locus mutations are summarized by a transposon insertion (tn), a deletion (the absence of an arrow), or a complementing plasmid (green arrow with oval). *M. smegmatis* (Ms) and *M. tuberculosis*

(Rv) gene numbers and amino acid identities (aa%) and the putative function of the encoded proteins are shown below each gene.

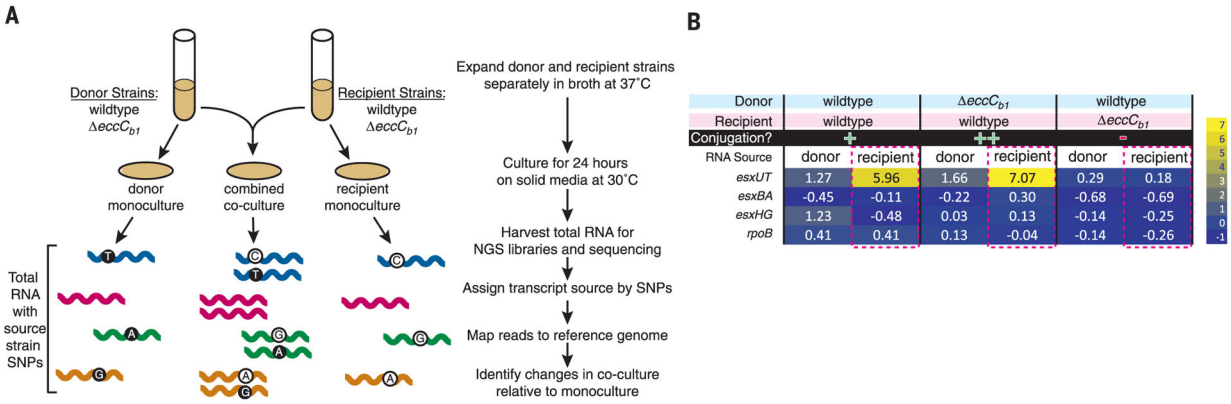

Fig. 2. Coculture with donor induces recipient *esx4* transcript levels and requires recipient ESX-1 activity.

(**A**) Experimental design for SNP-guided RNA-seq of DCT. The ESX-1_{mut} strains have targeted deletions of *eccC*$_{b1}$, encoding the ATPase required for ESX-1 secretion activity and required in the recipient for conjugation. The four strains were grown individually and in mixed cultures of WT × WT, WT × ESX-1_{mut}, or ESX-1_{mut} × WT. Conditions, processing, and analysis are as indicated at right. (**B**) Heat-map cells of WXG100 genes from *esx4* (*esxUT*), *esx1* (*esxBA*), and *esx3* (*esxHG*), with changes upon coculture shown as $\log_2$ insets. For each mating, the donor and recipient genotypes are shown, and the conjugal mating proficiencies are indicated. A housekeeping gene (*rpoB*) is included as an independent expression control.

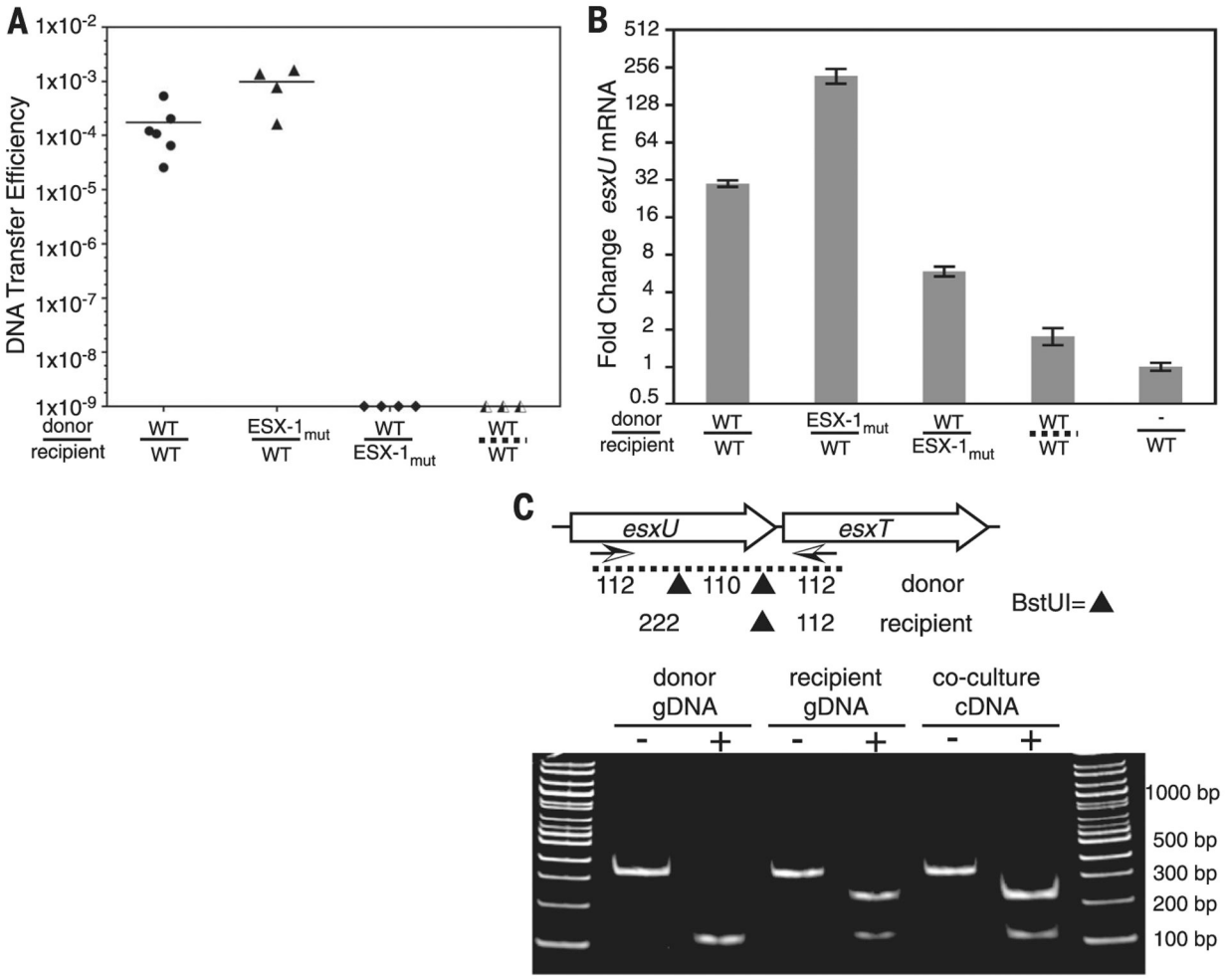

Fig. 3. ESX-1 and contact dependence of conjugal communication.

(**A**) DCT mating efficiencies for conjugal pairs used for RT-PCR. Strains are identified as WT or ESX-1_{mut} (Δ*eccC*$_{b1}$) donors (above the line) or recipients (below). The dashed line at bottom right indicates separation of conjugal strains by a porous membrane. (**B**) qRT-PCR analysis for *esxU* from cocultures of ESX-1 mutants or physically separated mycobacteria. *esxU* signals were normalized to *rpoB* expression. Error bars indicate SD (n = 3). (**C**) RT-PCR and restriction fragment length polymorphism analysis of BstUI fragments identifies recipient origin of the elevated *esxUT* transcripts. Arrows below the genetic map indicate the primers used for amplification, and the sites of BstUI cleavage are indicated by triangles. Monoculture genomic DNA (gDNA) controls show the expected parental patterns upon digestion (+) with BstUI. Digestion of coculture-derived cDNA shows a recipient pattern. bp, base pairs.