# Recruitment and Spreading of the *C. elegans* Dosage Compensation Complex Along X Chromosomes

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**To achieve X-chromosome dosage compensation, organisms must distinguish X chromosomes from autosomes. We identified multiple, cis-acting regions that recruit the *Caenorhabditis elegans* dosage compensation complex (DCC) through a search for regions of X that bind the complex when detached from X. The DCC normally assembles along the entire X chromosome, but not all detached regions recruit the complex, despite having genes known to be dosage compensated on the native X. Thus, the DCC binds first to recruitment sites, then spreads to neighboring X regions to accomplish chromosome-wide gene repression. From a large chromosomal domain, we defined a 793–base pair fragment that functions in vivo as an X-recognition element to recruit the DCC.**

An essential, X-chromosome regulatory process called dosage compensation ensures that males (XO or XY) and females (XX) express equal levels of X-linked gene products in mammals, flies, and nematodes, despite their difference in X-chromosome dose (*1*–*3*). The strategies used to achieve dosage compensation are remarkably diverse, but in all cases, specialized complexes are targeted specifically to the X chromosome(s) of only one sex to regulate transcript levels (*1*–*3*). Defining the cis-acting sites on X that distinguish it from autosomes to recruit the DCC is fundamental for understanding mechanisms underlying dosage compensation. We tackled this problem in the nematode *Caenorhabditis elegans*, where the DCC binds to both X chromosomes of hermaphrodites to repress gene expression by half (*1*). This sex-specific, chromosome-wide regulation is superimposed on the temporal and spatial regulation of individual X-linked genes that occurs in both sexes. The DCC resembles the conserved 13*S* condensin complex required for mitotic and meiotic chromosome resolution and compaction (*4*–*7*). Targeting of the DCC to X is accomplished by the novel, trans-acting factor SDC-2, which is pivotal for X-chromosome recognition. SDC-2 collaborates with SDC-3 to recruit the complex (*8*).

If *C. elegans* X chromosomes contain discrete X-recognition elements that recruit the DCC, we envisage four possibilities for targeting this complex (Fig. 1A). In model I, a single site on X recruits the complex and nucleates long-range DCC spreading across the entire chromosome. This model resembles mammalian X-inactivation, in which random silencing of one female X chromosome initiates from the gene that produces *Xist*, a noncoding RNA (*3*). *Xist* RNA coats the inactive X and catalyzes formation of specialized chromatin. In model II, a limited number of recognition sites recruits the complex, and some or all sites nucleate short-range spreading. This model resembles fruit fly dosage compensation, in which up-regulation of X-chromosome gene expression occurs in males through binding of the MSL complex (*2*). In flies, X-linked genes that produce the noncoding RNAs *roX1* and *roX2* recruit the complex (*9*–*11*). The complex can spread along a chromosome in a *roX*-RNA–dependent process, but the requirement for spreading in dosage compensation is not known (*9*, *12*, *13*). In model III, a limited number of recognition sites recruit the DCC, but the DCC does not spread. It only occupies sites that autonomously recruit it, influencing gene expression from a long distance, perhaps by altering chromosome structure. In model IV, a high density of X-recognition sites recruit the complex, but no spreading occurs, implying direct, short-range regulation by the DCC.

We performed a systematic, chromosome-wide search for X-recognition elements in *C. elegans* by assaying regions of X (Fig. 1C) for their ability to recruit the DCC when detached from X. Regions were analyzed in 32-ploid intestinal cell nuclei (*14*) of XX hermaphrodite strains carrying either free or autosome-attached X-chromosome duplications. X-chromosome territories were marked by fluorescent in situ hybridization (FISH) probes; one identified the duplicated region (red), and a second identified the rest of X (blue) (Fig. 1B) (*15*). DCC localization (green) was visual-

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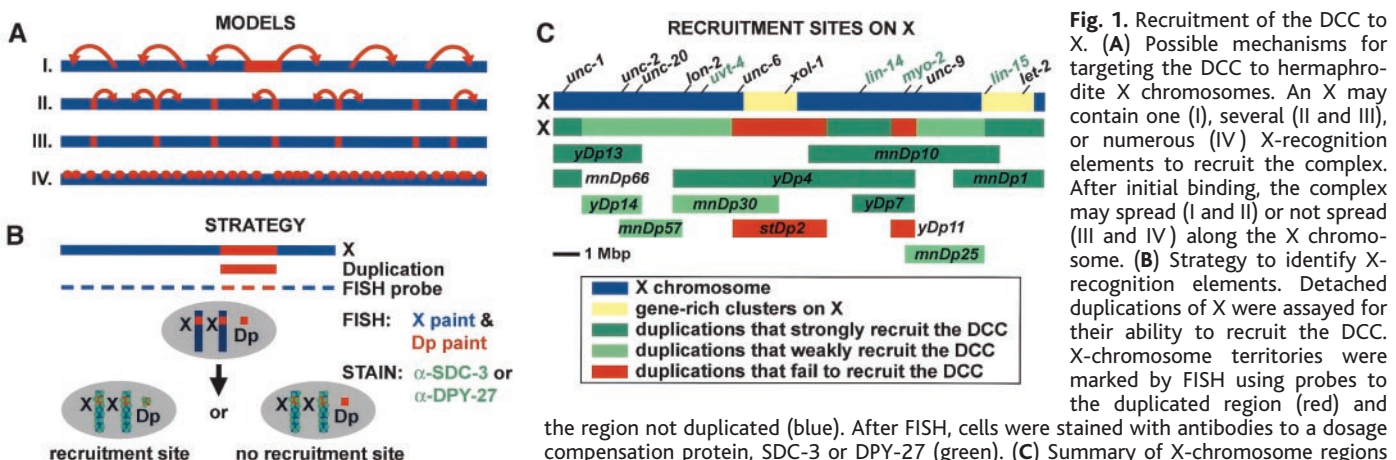


**Fig. 1.** Recruitment of the DCC to X. (**A**) Possible mechanisms for targeting the DCC to hermaphrodite X chromosomes. An X may contain one (I), several (II and III), or numerous (IV) X-recognition elements to recruit the complex. After initial binding, the complex may spread (I and II) or not spread (III and IV) along the X chromosome. (**B**) Strategy to identify X-recognition elements. Detached duplications of X were assayed for their ability to recruit the DCC. X-chromosome territories were marked by FISH using probes to the duplicated region (red) and the region not duplicated (blue). After FISH, cells were stained with antibodies to a dosage compensation protein, SDC-3 or DPY-27 (green). (**C**) Summary of X-chromosome regions that recruit the DCC. Shown is a genetic map of the *C. elegans* X chromosome (blue), with its two gene-rich clusters (yellow), known dosage-compensated genes (green), and duplications (below) analyzed in this study. Duplications were scored as strongly recruiting (dark green), weakly recruiting (light green), or failing to recruit (red). A summary of X-recruitment regions defined by duplication assays is below the genetic map.

ized by antibodies to either DPY-27 or SDC-3. Previously, only the large duplication *mnDp10* was shown to recruit the DCC, but with a different approach (*16*).

Model I, the presence of a single recruitment site, was eliminated by analysis of hermaphrodites carrying one copy of the right-end X duplication *mnDp1* and two X chromosomes deleted for the corresponding region (*mnDf1*) (Fig. 2A) (*15*). Antibodies to the DCC colocalized with both the duplication and the truncated X chromosomes, which indicated that *mnDp1* harbors at least one X-recognition element, as does the rest of X (*15*).

Multiple, independent X-recruitment sites were found through analysis of other X duplications (Fig. 1C). The X chromosomes accompanying these duplications were either wild type or had a small deletion removing only part of the duplicated region (*15*), making X chromosomes appear both red and blue, and duplications appear only red. Because chromosomes are only roughly aligned in polyploid intestinal cells, we analyzed duplications only if they occupied spatial domains distinct from X chromosomes. The left end of X (*yDp13* and *mnDp66*) recruited the complex robustly (Fig. 2A) (*17*). Duplications from the middle of X (*yDp4* and its smaller derivative *yDp7*) also recruited the complex strongly (Fig. 2A) (*15*, *17*). Limited, but reproducible, recruitment of the DCC was observed with four other duplications [*mnDp57* (Fig. 2B), *mnDp30*, *mnDp25*, and *yDp14*] (*15*, *17*). Discovery of multiple X-recognition elements is consistent with models II to IV.

Regions of native X chromosomes corresponding to strongly recruiting duplications (*mnDp1*, *yDp7*, and *yDp13*) showed equally robust DCC binding (Fig. 2C) (*15*, *17*). In fact, the DCC appears to assemble along the entire native X chromosome (Fig. 2C), which raises the possibility that X-recognition sites are densely distributed throughout X, as in model IV. However, two duplications (*stDp2* and *yDp11*), representing a large portion of X, appear unable to recruit the DCC (Fig. 3A) (*15*), which makes model IV very unlikely. These negative results were unexpected, because the *stDp2* region overlaps a gene-rich cluster that presumably contains dosage-compensated genes, and the *yDp11* region contains the known dosage-compensated gene *myo-2* (*18*). For *yDp11*, lack of recruitment cannot be attributed to an inhibitory position effect caused by attachment to an autosome, because duplications with the same attachment site (*yDp4* and *yDp7*) (*19*) do recruit.

These results suggest that the DCC regulates gene expression in regions lacking DCC recruitment sites (*stDp2* and *yDp11*) either by spreading into these regions from neighboring X-recognition elements (model II) or by acting over a distance (model III). Model II predicts the DCC will bind to the *stDp2* and *yDp11* regions of native X chromosomes; model III predicts it will not. We found robust localization of the DCC to both regions (Fig. 3B). Moreover, the DCC localizes robustly to regions of X corresponding to duplications with limited, but reproducible, binding (Fig. 3B) (*15*). We conclude, therefore, that *C. elegans* X chro-

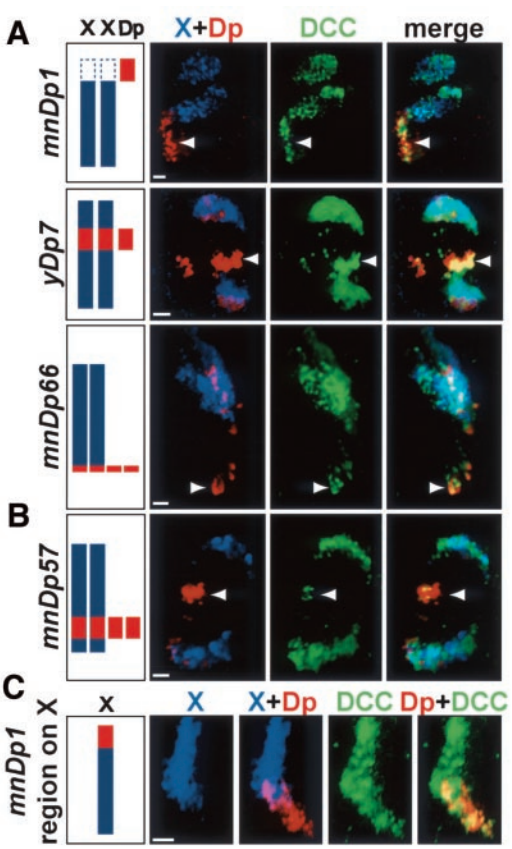


**Fig. 2 (left).** Multiple regions of the X chromosome recruit the DCC when detached from X. (**A** to **C**) Confocal images of individual, 32-ploid XX intestinal cell nuclei with (A and B) or without (C) X-chromosome duplications. Diagrams (left) represent the genotype of each nucleus, showing the copy number of each X duplication (red) and the location on X (blue) of the region duplicated (red). (A) X duplications that strongly recruit. (B) X duplication that weakly recruits. Images show DCC staining (green) and FISH paint of the duplication (red), corresponding X region (red), and all other X regions (blue). Colocalization of a duplication (arrowhead) and DCC appears yellow in the merged image. (C) Single wild-type X chromosome showing colocalization (yellow) of DCC (green) with the *mnDp1* region (red) on the native X. Scale bar, 2 μm.

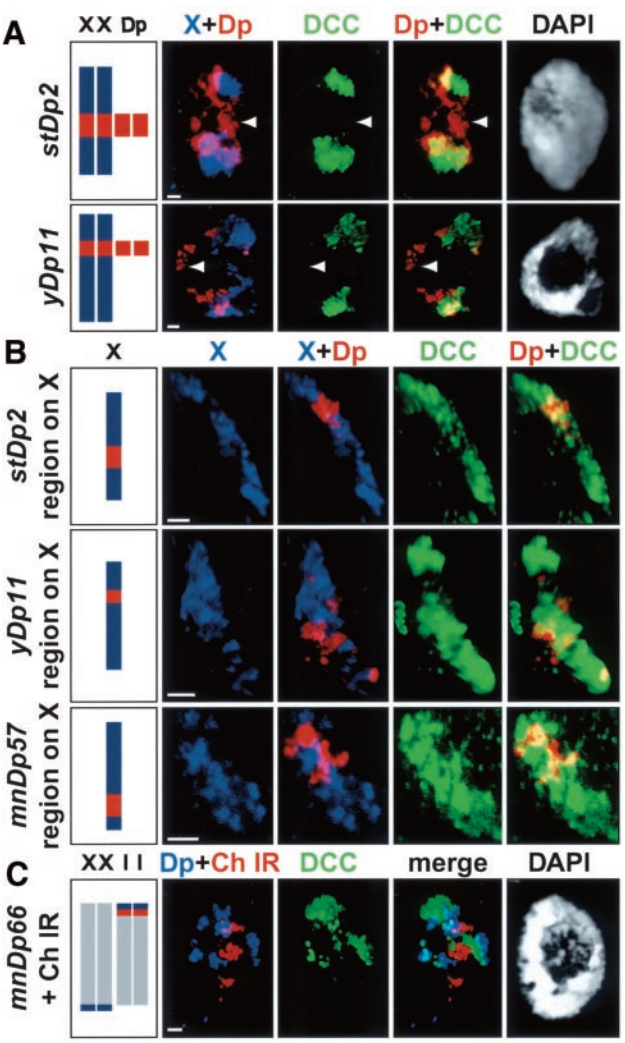


**Fig. 3 (right).** The DCC spreads along the X chromosome once bound to initial sites of recruitment. (**A**) The X duplications *stDp2* and *yDp11* do not recruit the DCC and are unlikely to contain X-recognition elements. Nuclei were stained as in Fig. 2, DNA (gray). (**B**) Robust localization of the DCC to regions of native X chromosomes corresponding to *stDp2*, *yDp11*, or mnDp57 indicates spreading of DCCs from neighboring recruitment sites. (**C**) The DCC (green) does not spread into autosomal DNA [right end of chromosome I (Ch IR, red)] located 500 kb internal to the attachment site of the strongly recruiting X duplication *mnDp66* (blue). Scale bar, 2 μm.

mosomes have discrete X-recognition elements that recruit the DCC and nucleate DCC spreading along X chromosomes (model II), over short or long distances (Fig. 4A).

We also asked whether the DCC spreads into autosomal territories adjacent to strongly recruiting X duplications. We found that the DCC does not spread into the right end of chromosome I, from either *mnDp10* (*17*) or *mnDp66* (Fig. 3C) (*15*). For both duplications, the complex bound neither to the 28*S* ribosomal DNA (rDNA) cluster at the attachment site nor to the adjacent 500-kb region (*15*), precluding the possibility that the 28*S* rDNA chromatin structure prevents binding to the rDNA cluster but permits spreading and binding beyond it. DCC spreading was also not detected from *mnDp1* into its attachment site at the left end of chromosome V (*15*). The lack of obvious DCC spreading into autosomal DNA indicates that either X chromosomes contain elements required for spreading or the analyzed autosomal regions contain elements that block spreading.

We devised a general strategy to identify discrete X-recognition elements responsible for recruitment activity. Transgenic nematode lines carrying extrachromosomal DNA arrays with tandem copies of 4 to 8 cosmids from a recruitment region were made and then assayed in intestinal cells for DCC binding (*15*). Arrays with six X cosmids near *lon-2* were the first to show recruitment (Fig. 4B and fig. S1B). All recruitment activity was subsequently attributed to cosmid R160; 94% of R160 arrays were positive compared with 2% of control arrays (Fig. 4B and fig. S1B). This activity was further mapped to a 4.5-kb fragment of R160 that strongly recruits the DCC in both polyploid intestinal cell nuclei and diploid nuclei of XX embryos (Fig. 4C and fig. S1, A and B) (*15*).

Multiple copies of a bona fide X-recognition element may titrate complexes away from X chromosomes and thereby impair dosage compensation. Indeed, a genetic test revealed compromised dosage compensation in animals carrying arrays with X-recognition elements. Mutations in the master regulatory gene *xol-1* inappropriately activate dosage compensation in XO males, causing assembly of the DCC on the single X and complete XO-specific lethality from reduced X-linked gene expression (*20*). Arrays with X-recognition elements suppressed the XO-specific lethality, which showed that arrays compete for DCCs and prevent them from binding to X (*15*). As further indication of titration, X chromosomes are easily detected by DCC antibodies only in XX cells that lost arrays because of mitotic instability (Fig. 4, C and D). Strong recruitment of the DCC by this X-recognition element, which comes from a region of X (*mnDp57*) with weak recruitment activity when detached, suggests that X duplications showing limited recruitment have high-affinity recruitment sites. These sites likely occur at lower frequency than in duplications showing strong recruitment.

The 4.5-kb fragment carrying the X-recognition element has several noteworthy features but appears not to contain any open reading frames (ORFs) (Fig. 4B). It includes an X-specific repeated element (EL-1), a 196–base pair (bp) region with >85% identity to the syntenic *C. briggsae* region, a G-rich region (Fig. 4B and fig. S1D) atypical for *C. elegans* intercistronic regions, a cluster of putative transcription factor–binding motifs that makes the region resemble an enhancer, and a 32-nucleotide repeated sequence enriched on X (Fig. 4B, fig. S1D). Array analysis of ~1.37-kb subfragments (A to C) and a 793-bp subfragment (D) of the positive recruitment fragment B (Fig. 4, B and D; fig. S1, A to D) showed that neither EL-1, the *C. briggsae* region, nor the 32-nucleotide oligomer are critical for recruitment. Further dissection will reveal features of the X-recognition element essential for recruitment.

We have demonstrated that a discrete X-recognition element can distinguish the X chromosome from autosomes to recruit the DCC. We predict that additional, molecularly tractable recognition elements reside in other regions of X that autonomously recruit the complex. Our work suggests the strategy by which the *C. elegans* X chromosome attracts the condensin-like DCC to repress X chromosome–wide gene expression. Discrete X-recognition elements serve as entry sites both to recruit the DCC and to nucleate spreading of the complex to X regions that lack recruitment sites. Through this process, a

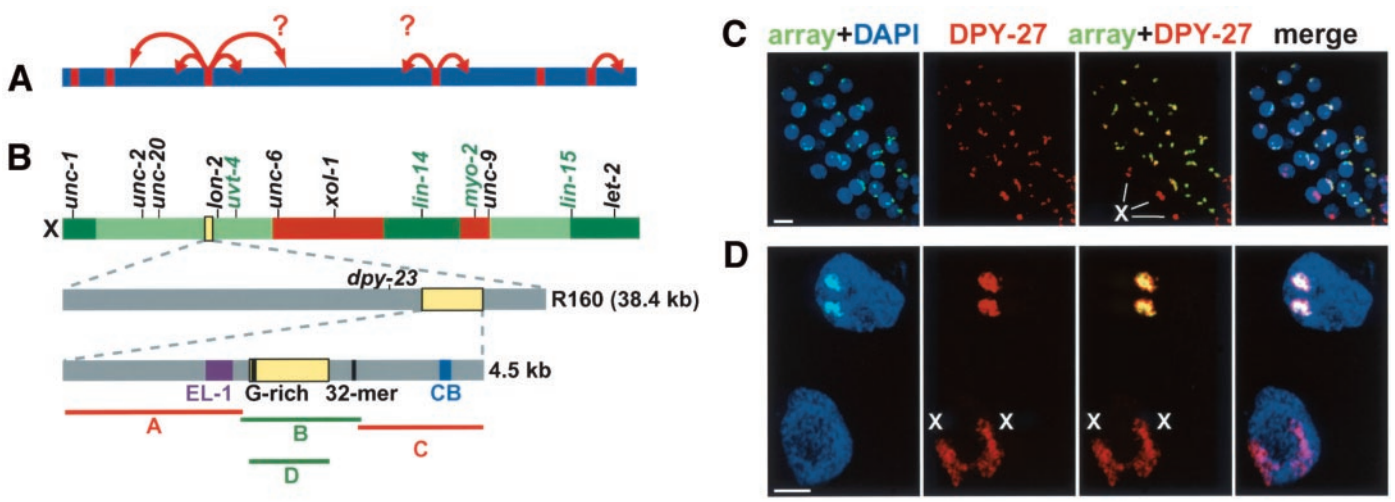


**Fig. 4.** Identification of an X-recognition element. (**A**) Current model of X-chromosome recognition and binding by the DCC. Multiple X-recognition elements on X recruit the DCC and nucleate spreading, potentially over short or long range, to repress chromosome-wide gene expression. (**B**) Cloning of an X-recognition element. DCC recruitment activity was detected in extrachromosomal transgenic arrays carrying cosmid R160 (yellow box on map of X). This map summarizes regions of strong DCC recruitment (dark green), weak recruitment (light green), and no recruitment (red). Recruitment activity (yellow box) within R160 was delimited to a 4.5-kb fragment that lacks ORFs. Array analysis of subfragments A to D further delimited recruitment activity to the 793-bp fragment D. (**C**) XX embryo showing recruitment of the DCC to an array carrying the 4.5-kb X-recognition element. Arrays also carried *lacO* repeats and encoded a LacI::GFP fusion protein, which allowed array detection by GFP-specific antibodies (green). DPY-27 antibodies (red) mark bound DCCs. DNA was visualized by DAPI (blue). (**D**) Adjacent XX intestinal cell nuclei with or without arrays that carry the X-recognition element on the 1.37-kb fragment B. In both (C) and (D), arrays bind the DCCs and titrate them from X. X chromosomes are detectable only in nuclei without arrays. Scale bar, 5 μm.

repressed chromatin state can be propagated in cis over short or long distances from initial sites of recruitment, to establish the global regulation of X chromosomes that is maintained throughout the lifetime of the animal.


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21. We thank D. Albertson for X chromosome yeast artificial chromosomes; C. Y. Loh for cosmid array *yEx708* found to carry an X-recognition element; T. Cline, A. Severson, and C. Tsai for critical comments on the manuscript; and A. Lee, D. Akey, and T. Blumenthal for discussions. Some nematode strains were provided by the *Caenorhabditis* Genetics Center, funded by NIH's National Center for Research Resources. This work was supported by NIH Grant R37-GM30702 (B.J.M.) and NIH Fellowship F32-GM065007 (G.C.). B.J.M. is an investigator of the Howard Hughes Medical Institute.


**Supporting Online Material**

www.sciencemag.org/cgi/content/full/303/5661/1182/DC1
Materials and Methods
Fig. S1
References

24 October 2003; accepted 31 December 2003

# An Engineered Pathway for the Formation of Protein Disulfide Bonds

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**We have engineered a pathway for the formation of disulfide bonds. By imposing evolutionary pressure, we isolated mutations that changed thioredoxin, which is a monomeric disulfide reductase, into a [2Fe-2S] bridged dimer capable of catalyzing $O_2$-dependent sulfhydryl oxidation in vitro. Expression of the mutant protein in *Escherichia coli* with oxidizing cytoplasm and secretion via the Tat pathway restored disulfide bond formation in strains that lacked the complete periplasmic oxidative machinery (DsbA and DsbB). The evolution of [2Fe-2S] thioredoxin illustrates how mutations within an existing scaffold can add a cofactor and markedly change protein function.**

The pathways for the formation of disulfide bonds in secreted proteins of eukaryotic cells and bacteria are mechanistically very similar: Electrons are transferred from the protein thiols to soluble catalysts of disulfide bond formation, then to membrane-associated enzymes, and finally to terminal electron acceptors such as oxygen (*1*, *2*). In *Escherichia coli*, catalysis of disulfide bond formation is mediated by the periplasmic protein DsbA. DsbA is recycled by the membrane enzyme DsbB with concomitant reduction of quinones (*2*) (Fig. 1A). Inactivation of either the *dsbA* or the *dsbB* gene abolishes the oxidation of secreted proteins.

We sought to design a pathway for the formation of disulfide bonds that would be independent of the action of this disulfide catalytic machinery. Our aim was to create a pathway consisting of a protein carrier that acquires a disulfide bond within the cytoplasm, is subsequently translocated across the membrane, and then donates its disulfide bond stoichiometrically to periplasmic proteins, enabling their proper folding. Thus, a single protein would substitute for the entire DsbA-DsbB catalytic system, including its connection to the electron transport chain.

The designed pathway hinges upon three steps: (i) the selection of an appropriate protein that can form disulfide bonds, (ii) a means for forming a disulfide bond in the carrier protein within the normally reducing cytoplasm, and (iii) a mechanism for the export of the protein carrier, including its disulfide, across the membrane. Inactivation of the cytoplasmic redox balancing systems by deletion of the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes allows the formation of disulfide bonds in the cytoplasm (*3*). For the membrane translocation of a protein containing a disulfide, we chose to employ the twin arginine transporter pathway (Tat). Tat is responsible for the membrane translocation of proteins that have acquired cofactors in the cytoplasm and also for folded proteins, including those that contain disulfides (*4*).

We elected to use thioredoxin as the disulfide exchange protein for the designed pathway. Many enzymes with disulfide oxidoreductase activity, including protein disulfide isomerase and DsbA, contain a thioredoxin fold (*1*, *2*). These enzymes contain the active-site CXXC motif, where the cysteines reversibly form a disulfide bond and can undergo rapid thiol-disulfide exchange reactions. Although thioredoxin is normally a reductant, it can serve as a catalyst of disulfide bond formation in the cytoplasm under oxidizing conditions and can also weakly substitute for DsbA when secreted via the Sec secretory pathway (*5*). This ability to serve as a general periplasmic oxidant is completely dependent on recycling by DsbB.

*dsbB*$^-$ strains are nonmotile because of the misfolding of the flagella component FlgI, which contains an essential disulfide (*6*). The engineering of a pathway capable of mediating disulfide bond formation in the absence of DsbB should lead to the restoration of cell motility. TrxA was fused to the prototypical Tat-specific leader peptide ssTorA of the *E. coli* trimethylamine N-oxide reductase (TorA) protein and then expressed in the *E. coli* strain DR473 *dsbB*::kan5. This strain has an oxidizing cytoplasm and also carries a deletion in *dsbB*, so that disulfide bond formation in the periplasm is abolished. However, *E. coli* DR473 *dsbB*::kan5 expressing ssTorA-TrxA were nonmotile, indicating that this fusion is unable to mediate the formation of the critical disulfide bond in FlgI.

The central XX residues of the CXXC motif in proteins with a thioredoxin fold are critical for determining their redox properties (*7*). Specifically, substitution of the active-site CGPC dipeptide in thioredoxin by the sequence CPHC found in DsbA generates a thioredoxin mutant with a considerably more oxidizing redox potential (*7*). This mutation was generated, but the ssTorA-TrxA(CPHC) fusion also failed to restore motility, suggest-

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