Perturbation of Cytochrome c Maturation Reveals Adaptability of the Respiratory Chain in *Mycobacterium tuberculosis*

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**ABSTRACT** *Mycobacterium tuberculosis* depends on aerobic respiration for growth and utilizes an *aa*3-type cytochrome *c* oxidase for terminal electron transfer. Cytochrome *c* maturation in bacteria requires covalent attachment of heme to apocytochrome *c*, which occurs outside the cytoplasmic membrane. We demonstrate that in *M. tuberculosis* the thioredoxin-like protein Rv3673c, which we named CcsX, is required for heme insertion in cytochrome *c*. Inactivation of CcsX resulted in loss of *c*-type heme absorbance, impaired growth and virulence of *M. tuberculosis*, and induced cytochrome *bd* oxidase. This suggests that the bioenergetically less efficient *bd* oxidase can compensate for deficient cytochrome *c* oxidase activity, highlighting the flexibility of the *M. tuberculosis* respiratory chain. A spontaneous mutation in the active site of vitamin K epoxide reductase (VKOR) suppressed phenotypes of the CcsX mutant and abrogated the activity of the disulfide bond-dependent alkaline phosphatase, which shows that VKOR is the major disulfide bond catalyzing protein in the periplasm of *M. tuberculosis*.

**IMPORTANCE** *Mycobacterium tuberculosis* requires oxygen for growth; however, the biogenesis of respiratory chain components in mycobacteria has not been explored. Here, we identified a periplasmic thioredoxin, CcsX, necessary for heme insertion into cytochrome *c*. We investigated the consequences of disrupting cytochrome *c* maturation (CCM) for growth and survival of *M. tuberculosis in vitro* and for its pathogenesis. Appearance of a second-site suppressor mutation in the periplasmic disulfide bond catalyzing protein VKOR indicates the strong selective pressure for a functional cytochrome *c* oxidase. The observation that *M. tuberculosis* is able to partially compensate for defective CCM by upregulation of the cytochrome *bd* oxidase exposes a functional role of this alternative terminal oxidase under normal aerobic conditions and during pathogenesis. This suggests that targeting both oxidases simultaneously might be required to effectively disrupt respiration in *M. tuberculosis*. 

*Mycobacterium tuberculosis* has evolved to survive for decades within host granulomas. An infected host often develops different types of granulomas, including cavitating, fibrotic, and cavitating lesions, each providing a different environment (1, 2). Even bacteria within the same lesion may experience different microenvironments depending on their location within the granuloma (3), and they can exist as multiple subpopulations (4). The availability of oxygen and nutrients can also differ vastly between various *in vivo* niches. It has been shown that *M. tuberculosis*, while residing in cavities, experiences the same oxygen tension as in the bronchi in the lung (5). However, *M. tuberculosis* further away from the cavity surface or localized in noncavitating lesions is exposed to microaerobic or anaerobic conditions (6). Thus, throughout the course of infection, even within the same lesion, *M. tuberculosis* must adapt its metabolism to survive and persist. The ability of *M. tuberculosis* to adapt to multiple environments can in part be attributed to the modulatory nature of its respiratory chain (7, 8). Electrons flow from NADH dehydrogenase and succinate dehydrogenase complexes into the menaquinone- *aa*3-type oxidoreductase supercomplex or the *bd*-type menaquinol oxidase (9–12). Both oxidases use O2 as the terminal electron acceptor. The cytochrome *bc*1-*aa*3 oxidoreductase (CcO) supercomplex consists of a *bc*1 di-heme-*c*-type cytochrome reductase, encoded by *gcrCB*, that transfers electrons to the terminal *aa*3-type Cu-heme oxidase, encoded by *ctaB*, *ctaC*, *ctaD*, *ctaE*, and *ctaF* (8). Cytochromes of the *c* type are characterized by covalently bound heme, which is attached via two thioether bonds to the two cysteine residues in the heme binding motif Cys-Xxx-Xxx-Cys-His (13, 14). This cytochrome *c* maturation (CCM) occurs on the outside the cytoplasmic membrane (15). *M. tuberculosis* is predicted to use a type II CCM system, consisting of four proteins, that is best characterized in *Bacillus subtilis* (ResA, ResB, ResC, CcdA) and *Bordetella pertussis* (CcsA, CcsB, CcsX, DipZ) (16–19) (see Fig. S1 in the supplemental material). The integral membrane proteins ResB/CcsA bind heme in the cytoplasm and export it to the extra-cellular domains of ResC/CcsB, priming it for covalent attachment to apocytochrome *c*. Thiol reduction is catalyzed by CcdA/DipZ and the membrane-anchored thioredoxin-like protein, ResA/CcsX, which reduces the disulfide bond of the Cys-Xxx-
Xxx-Cys-His motif of apocytochrome c (18–21). In this study, we identified the ResA/CcsX homolog in *M. tuberculosis*, encoded by *rv3673c*, and investigated the consequences of disrupting cytochrome c biogenesis. We provide evidence that the bioenergetically less efficient cytochrome bd oxidase substitutes for impaired CcO activity and that loss of CcsX can be compensated for by a mutation in the disulfide bond-forming protein vitamin K epoxide reductase (VKOR).

**RESULTS AND DISCUSSION**

**Identification of a membrane-bound, periplasmic thioredoxin.**

We sought to identify the gene encoding *M. tuberculosis*’s ResA/CcsX and searched for genes encoding proteins predicted to contain a thioredoxin fold and a single transmembrane helix or to be localized to the membrane, using the Tuberculist database (22) and the transmembrane prediction server, TMHMM version 2.0 (23). Of the proteins that matched our criteria, *Rv0526* and *Rv3673c* were the most likely candidates, with 24% and 33% sequence identity to *B. subtilis* ResA, respectively. *Rv3673c* is predicted to contain a single transmembrane helix, and *Rv0526* is predicted to be a secreted lipoprotein. We focused on *Rv3673c* because of its membrane-anchoring transmembrane helix and sequence similarity to ResA even though *rv0526* is located in the genomic region that contains the other predicted components of the CCM system. Based on the results of the work presented here, *Rv3673c* was named CcsX.

To experimentally confirm that the catalytic domain of CcsX is located in the periplasm, we created fusions with alkaline phosphatase (PhoA) at residues leucine 41, serine 47, and proline 65 and expressed them in *Mycobacterium smegmatis* (see Fig. S2 in the supplemental material). PhoA is active only when located in the oxidizing extracytoplasmic environment and has been used to determine the topology of membrane proteins (24), including those in mycobacteria (17, 25). All CcsX-PhoA fusions demonstrated alkaline phosphatase activity (see Fig. S2B in the supplemental material). A plasmid containing PhoA lacking a signal sequence served as a negative control, and a plasmid expressing PhoA fused to the signal sequence of the secreted antigen 85B served as our positive control (25). Immunoblot analysis of *M. tuberculosis* expressing Flag-tagged CcsX confirmed its association with the cell membrane/wall fraction (see Fig. S2C). Taken together, these data suggest that CcsX is a membrane-associated protein with an extracellular thioredoxin domain.

**CcsX is required for heme insertion in cytochrome c, and cytochrome bd oxidase can compensate for loss of CcsX.**

We created an *M. tuberculosis* mutant strain, the /H9004CcsX mutant, in the background of H37Rv, in which the entire *ccsX* gene was replaced with a hygromycin cassette. Deletion of *ccsX* was confirmed by Southern blotting (see Fig. S3 in the supplemental material). To determine the impact of loss of CcsX on CCM, we stained for proteins with covalently bound heme in membrane fractions of *M. tuberculosis* H37Rv wild type (WT), the /H9004CcsX and /H9004CcsX-comp mutants. Heme staining of WT membranes revealed five bands at ~110, ~80, ~60, ~25, and ~15 kDa (Fig. 1A), and we attempted to identify the proteins containing covalently bound heme. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed multiple proteins in each band, at least one of which had a predicted heme-binding motif. Using the LC-MS/MS data, heme-binding motif predictions, and molecular masses, we assigned a
heme-bound protein to each band. The band at ~80 kDa corresponds to the catalase-peroxidase KatG (26); the band at ~60 kDa corresponds to SirA, a sulfite reductase with a covalently bound siroheme moiety (27); and the band at ~25 kDa stems from QcrC, the cytochrome c subunit of the bc₁ complex (8). Attempts to identify the ~15-kDa protein were unsuccessful, and the 150-kDa band appeared to be an aggregate containing more than one of the heme-containing proteins found in the lower-molecular-mass bands. Heme staining of membranes from the ΔCcsX mutant did not reveal bands for SirA or the ~15-kDa protein, and the band corresponding to QcrC was drastically reduced in intensity (Fig. 1A). This strongly suggested that CcsX is important for cytochrome c assembly. Heme binding to KatG was unaffected by the lack of CcsX, which is not surprising, because KatG is cytosolic and should thus not depend on the extracytoplasmic thioredoxin CcsX for heme attachment. More surprising was the heme deficiency in SirA, formerly known as NirA, a sulfite reductase with covalently bound siroheme (27, 28). SirA is a predicted cytosolic protein and thus unlikely to rely on the extracytoplasmic CcsX for heme insertion. Siroheme can, however, be hijacked and processed into heme in some bacteria and archaea (29). The defective heme incorporation into QcrC may have resulted in excessive heme export and depletion of intracellular heme stores, which in turn caused conversion of siroheme to heme, thereby depleting siroheme in SirA. Alternatively, SirA might be an extracytoplasmic protein and directly rely on CcsX for heme acquisition, as proteomics identified SirA in the membrane/lipid fractions of M. tuberculosis (30, 31).

To confirm the loss of c-type heme incorporation into QcrC, reduced minus oxidized difference spectroscopy was used to analyze the cytochrome content of the aerobically grown WT and ΔCcsX strains. In WT membranes, major absorbance peaks were observed at 552, 563, and 600 nm, characteristic of c-, b-, and a-type cytochromes, respectively (9, 32) (Fig. 1B). In membranes from the ΔCcsX mutant, the major peak at 552 nm was no longer visible, indicating a lack of c-type heme. The peak at 563 nm was unchanged; however, a prominent peak was visible at 631 nm, which we did not observe in WT membranes. This peak is characteristic of a d-type heme and has been demonstrated to indicate the presence of the bd oxidase in M. smegmatis following exposure to microaerobic conditions (O₂ < 1%) but was undetectable when the bacteria were grown aerobically (9). The bd terminal oxidase is also induced in M. smegmatis lacking the cytochrome bc₁ oxidase (12) and in M. tuberculosis treated with the cytochrome c-specific inhibitors cyanide and azide or agents affecting CCM, such as ZnSO₄ and dithiothreitol (DTT) (33). Consistent with the hypothesis that bd oxidase is upregulated in the ΔCcsX mutant to compensate for the perturbation in CCM, mRNA levels of genes of the bd oxidase encoding the cyd operon (cydABDC) were 2-3-fold higher in the mutant than in the WT, while expression levels of qcrC and ctaD were unchanged in the ΔCcsX mutant (Fig. 1C). Furthermore, the ΔCcsX mutant was hypersusceptible to cyanide and to the proton-translocating F₁,Fₒ-ATP synthase inhibitor N,N’-dicyclohexylcarbodiimide (DCCD), suggesting impaired cytochrome c oxidase activity and reduced respiratory energy generation (Fig. 1D). In contrast, the minimal inhibitory concentration of thioreidazine (TRZ), a phenothiazine derivative thought to inhibit the type II NADH dehydrogenase (11, 33, 34), was not different from that of the WT and the ΔCcsX-comp mutant, suggesting that type II NADH dehydrogenase activity is not decreased in the mutant.

Together, these data demonstrate that (i) CcsX is important for CCM in M. tuberculosis, (ii) Rv0526 is not functionally redundant with CcsX, and (iii) deletion of CcsX is accompanied by increased expression of bd oxidase, suggesting that defective CCM resulted in reduced electron flow through the bc₁-aa₃ oxidase, which can at least partially be compensated for by increased bd oxidase activity.

Deficient CCM is associated with increased resistance to H₂O₂. Consistent with transposon mutagenesis studies, which predicted ccsX to be required for optimal growth (35, 36), the ΔCcsX mutant grew slower than the WT on agar plates and in liquid medium (Fig. 2A and B). Introducing a copy of ccsX back onto the mutant chromosome complemented these phenotypes. Deletion of ResA/CcsX homologs caused increased resistance to H₂O₂ treatment in Agrobacterium tumefaciens (37) but hypersusceptibility to H₂O₂ in Neisseria gonorrhoeae (38). Consistent with the phenotype in A. tumefaciens, the ΔCcsX mutant was hypersusceptible to H₂O₂ (Fig. 2C), and H₂O₂ hyperresistance was returned to WT levels in the complemented mutant, confirming that this phenotype was due to the loss of CcsX. This response was specific to H₂O₂ stress. Exposure to superoxide-generating plumbagin, acidified sodium nitrite resulting in the generation of reactive nitrogen species, and acidification (pH 4.5) did not affect the mutant differently than the WT (see Fig. S4 in the supplemental material). To explore the mechanism responsible for H₂O₂ hyperresistance, we measured expression of genes important for antioxidant defense in M. tuberculosis (39–42). There was no difference in katG mRNA or protein abundance between the ΔCcsX mutant and the WT (Fig. 2D and E), similar to the finding that H₂O₂ hyperresistance of the TlpA (CcsX homolog) mutant in A. tumefaciens was catalase independent (37). mRNA levels of the thioredoxin genes, trxB1, thix, and the thioredoxin reductase-encoding trxB2, were also similar in all strains. In contrast, mRNA levels of alkyl hydroperoxide reductase (AhpC) were 2.5-fold increased in the ΔCcsX mutant and returned to WT levels in the complemented strain (Fig. 2D). Consistent with the increased mRNA levels, protein amounts of AhpC and AhpD, a thioredoxin-like adaptor protein (43), were also increased in the ΔCcsX mutant (Fig. 2E). AhpC and AhpD are subunits of M. tuberculosis’s peroxidase and peroxynitrite reductase complex and have been implicated in oxidative and nitrosative stress responses (39, 42, 43). However, deletion of ahpC in M. tuberculosis resulted in hypersusceptibility to cumene hydroperoxide but not H₂O₂ (44). The increased resistance to H₂O₂ of the ΔCcsX mutant may also be a consequence of overexpression of the cytochrome bd oxidase, as E. coli cyd mutants are hypersusceptible to H₂O₂ stress (45, 46). The cytochrome bd oxidase retains a high affinity for oxygen (47), and increased expression would result in efficient scavenging of oxygen radicals produced from the breakdown of H₂O₂, leading to increased resistance. Consistent with this is the observation that cytochrome bd oxidase plays a respiratory protective role for nitrogen-fixing enzymes which are sensitive to oxidative damage (48). It is noteworthy that unlike other terminal cytochrome oxidases, the cytochrome bd oxidase does not generate superoxide radicals during catalytic reduction of oxygen, which serves as a barrier for the formation of excessive endogenous oxidative species (49).

The role of CcsX in pathogenesis. Gene expression analysis revealed that genes encoding subunits of the cytochrome oxidase...
and bd oxidase were differentially expressed at different phases of mouse infection (50). This suggested that cytochrome c oxidase is most important for M. tuberculosis’s ability to replicate during the acute phase, while the less-energy-efficient bd oxidase is preferentially required during transition from acute to chronic infection. The ΔCcsX mutant reached 100-fold-lower CFU than the WT in mouse lungs at day 21 following low-dose aerosol infections, after which it maintained a constant titer (Fig. 3A). A similar trend was observed in spleens, to which the ΔCcsX mutant disseminated more slowly than the WT and never reached the same bacterial load (Fig. 3A, right). Growth in lungs and spleens was restored to WT levels in the complemented mutant (Fig. 3B). Thus, perturbed CCM affected replication of M. tuberculosis in vivo but had no impact on persistence during chronic mouse infection. These data suggest that bd oxidase can sufficiently substitute for impaired cytochrome c oxidase activity during the chronic phase of the infection when the bacilli are only slowly or not replicating but are exposed to the host-adaptive immune response. In addition to fulfilling a respiratory role in the electron transport chain, cytochrome bd oxidase may protect tubercle bacilli against reactive oxygen species generated through the adaptive immune response. In contrast, normal replication early in infection depends on intact CCM and cytochrome c oxidase activity.

A VKOR mutation compensates for loss of CcsX. During the course of these experiments, especially after multiple passages, the ΔCcsX phenotypes became less robust. When plating the mutant

FIG 2 Deletion of CcsX results in impaired growth and increased resistance to H2O2. Growth of WT, ΔCcsX, and ΔCcsX-comp strains on agar plates (A) and in Sauton’s liquid medium (B). (C) Survival of M. tuberculosis strains after exposure to hydrogen peroxide. Bacteria were plated and CFU were determined after exposure to 4 and 16 mM H2O2 for 4 h. Data are means ± SD of triplicate cultures and representative of two independent experiments (*, P < 0.05, compared to the WT and compared to the complemented strain). (D) Relative mRNA amounts of potential antioxidant genes in the ΔCcsX and ΔCcsX-comp mutants. mRNAs were quantified by quantitative real-time PCR and normalized to sigA and are expressed as abundance relative to mRNA levels in the WT. Error bars represent SD from three biological replicates. Student’s t test P values are indicated (*, P < 0.05). (E) Immunoblot analysis of proteins extracts from the WT (lane 1), ΔCcsX (lane 2), and ΔCcsX-comp (lane 3) strains using antisera against AhpC, AhpD, KatG, and DlaT (loading control). Soluble (s) and insoluble (is) protein fractions were run for the KatG immunoblot.

FIG 3 The ΔCcsX mutant is attenuated in the mouse model of tuberculosis. (A) Growth and survival in mouse lungs (left) and spleens (right) of the WT and ΔCcsX strains. Data are from 4 mice per strain and time point and representative of 2 independent experiments. (B) The ΔCcsX-comp strain grows like the WT in mouse lungs and spleens. Data are from 4 mice per strain and time point.
on 7H10 agar, we noticed a heterogeneous population of colonies, with some significantly larger than others (see Fig. S5A in the supplemental material). Both small and big colonies lacked the ccsX gene, suggesting that the large colonies represented a suppressor mutant (see Fig. S5B and C in the supplemental material). This putative suppressor strain, the /H9004 CcsX-S mutant, also grew significantly faster than the /H9004 CcsX mutant in liquid medium (Fig. 4A) and was more susceptible to H2O2 than the /H9004 CcsX mutant (see Fig. S5D). Whole-genome sequencing revealed a point mutation in rv2968c/vkor, resulting in an amino acid change in vitamin K epoxide reductase (VKOR). M. tuberculosis’s VKOR is functionally similar to DsbB in E. coli (51, 52), which together with DsbA catalyzes disulfide bond formation in the periplasm (53). The ΔCcsX-S mutation caused the proline residue at position 140 to be replaced with a serine (Fig. 4B). This proline lies in the Cys-Xxx-Xxx-Cys thioredoxin active site and is conserved in most thioredoxin family members (54, 55). TMHMM predicts that this sequence in VKOR is located in the periplasmic space consistent with its function. To determine whether the substitution of proline with serine affected the function of VKOR, we expressed the antigen 85B secretion signal-PhoA fusion protein, whose activity depends on intramolecular disulfide bond formation (56), in the WT, the ΔCcsX mutant, and the ΔCcsX-S mutant and measured alkaline phosphatase activity. PhoA activity was drastically reduced in the ΔCcsX-S mutant compared to in the other strains, suggesting that the point mutation in VKOR-P140S impaired disulfide bond formation in the periplasm of the ΔCcsX-S mutant (Fig. 4C). Finally we complemented the ΔCcsX-S mutant with intact VKOR to prove that the suppressor phenotype was caused by the mutated VKOR. Expression of the native, intact vkor gene from a tetracycline repressor-controlled promoter in the ΔCcsX-S mutant resulted in small colonies, while expression of the mutated gene did not affect growth of the ΔCcsX-S mutant (Fig. 4D). Even in the absence of the inducer anhydrotetracycline (atc), the ΔCcsX-S mutant transformed with WT VKOR formed smaller colonies than when transformed with VKOR-P140S, likely because of leaky expression. The addition of atc exacerbated this phenotype. Similarly, growth of the ΔCcsX-S mutant expressing VKOR in atc-containing liquid medium was significantly reduced compared to that of the untransformed control and mimicked the slow growth of the ΔCcsX mutant (Fig. 4A). In contrast, the ΔCcsX-S mutant transformed with VKOR-P140S replicated like the untransformed ΔCcsX-S mutant, and the growth of the WT was not affected by either VKOR or VKOR-P140S. Together, these data demonstrate that the point mutation in VKOR caused the suppressor phenotype of the ΔCcsX-S mutant. Mutations in DsbA and DsbB in Rhodobacter capsulatus (57) and B. subtilis (58) have been demonstrated to compensate for the loss of CcdA and restore
cytochrome c biogenesis. CcdA and its redox partner ResA/CcsX are required for the periplasmic reduction of the disulfide bond in apocytochrome c, allowing heme attachment (19, 59). The redox pair DsbA and DsbB forms the disulfide; thus, mutation in either of these proteins or the addition of a reducing agent to the medium abrogates the need for CcdA and ResA/CcsX. Similarly, mutation of VKOR suppressed the defect caused by lack of CcsX. Together, these data not only establish that CcsX is part of M. tuberculosis’s CCM system but also support the hypothesis that VKOR is the major disulfide bond catalyzing protein in M. tuberculosis’s periplasm.

MATERIALS AND METHODS

Strains, media, and molecular biology techniques. M. tuberculosis (H37Rv) strains were grown in Middlebrook 7H9 or Sauton’s medium as described (60). Cultures were grown aerated in roller bottles rotating at 1 rpm or in stationary tissue culture flasks in small volumes (10 ml) and agitated regularly to ensure aeration. Bacteria were also grown on Middlebrook 7H10 or 7H11 agar plates containing 10% oleic acid-albumin-dextrose-catalase (OADC) supplement (Becton Dickinson) and 0.5% glycerol. Hygromycin B (50 μg/ml), kanamycin (20 μg/ml), and streptomycin (20 μg/ml) were included when selection was required. The ΔccsX mutant was constructed using a suicide plasmid (61). Gateway cloning technology (Invitrogen) was used for clonings (60). For complementation, ccsX was cloned downstream of the hsp60 promoter into a plasmid that integrates into the chromosomal attB site and electroporated into the ΔccsX mutant. Alkaline phosphatase fusions were created and analyzed as described (25, 62). RNA isolation and analysis by quantitative real-time PCR was performed as described (60).

Heme staining and cytochrome spectra. M. tuberculosis lysates were prepared from mid-log-phase cultures, and membranes were isolated as described (63). Membranes for heme staining were resuspended in ammonium bicarbonate (NH₄HCO₃, Fisher) by sonication until dissolved. A total of 80 μg of membrane fraction was separated on a 4 to 15% Tris-HCl gradient gel (Bio-Rad). Heme staining was performed as described (64). LC-MS/MS analysis of excised bands was performed by the Proteomics Resource Center, Rockefeller University. Membranes for cytochrome spectra were isolated as described above, except TC buffer (10 mM Tris-HCl [pH 7.4], 16 mM cholate) was used instead of phosphate-buffered saline (PBS) for lysis and resuspension of the membrane pellet (61). Reduced minus oxidized cytochrome spectra were recorded on a UV-Vis spectrophotometer (Uvikon XS/SL) at 24°C using a few grams of solid sodium dithionite as reductant and a few drops of 100 or 200 μM potassium ferricyanide as oxidant (9).

Mouse infections. C57BL/6 mice (Jackson Laboratories) were infected using an Inhalation Exposure System (Glas-Col) to deliver ~100 to 1000 CFU per animal. Procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

Whole genome sequencing and analysis. The ΔccsX strain and a large-colony suppressor mutant were sequenced using an Illumina Genome Analyzer Ixx. Approximately 5 μg of DNA was processed using the standard Illumina sample preparation protocol (Illumina, Inc.), and the samples were sequenced in paired-end mode with a read length of 54 bp. The genome was assembled by mapping reads to the parental H37Rv genome and calling single-nucleotide polymorphisms and insertions/deletions as described (65). The mean depths of coverage (number of reads covering each site) were 27.9X and 101.2X for the two strains.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00475-13/-/DCSupplemental.

Figure S1, TIF file, 0.9 MB. Figure S2, TIF file, 1.3 MB. Figure S3, TIF file, 0.3 MB. Figure S4, TIF file, 0.4 MB. Figure S5, TIF file, 1.4 MB.

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