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Substitutions in an Active Site Loop of *Escherichia coli* IscS Result in Specific Defects in Fe-S Cluster and Thionucleoside Biosynthesis *in Vivo**

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IscS catalyzes the fragmentation of L-cysteine to L-alanine and sulfane sulfur in the form of a cysteine persulfide in the active site of the enzyme. In *Escherichia coli* IscS, the active site cysteine Cys^{328} resides in a flexible loop that potentially influences both the formation and stability of the cysteine persulfide as well as the specificity of sulfur transfer to protein substrates. Alanine-scanning substitution of this 14 amino acid region surrounding Cys^{328} identified additional residues important for IscS function *in vivo*. Two mutations, S326A and L333A, resulted in strains that were severely impaired in Fe-S cluster synthesis *in vivo*. The mutant strains were deficient in Fe-S cluster-dependent tRNA thionucleosides (s^2C and ms^2i^6A) yet showed wild type levels of Fe-S-independent thionucleosides (s^4U and mnm^5s^2U) that require persulfide formation and transfer. *In vitro*, the mutant proteins were similar to wild type in both cysteine desulfurase activity and sulfur transfer to IscU. These results indicate that residues in the active site loop can selectively affect Fe-S cluster biosynthesis *in vivo* without detectably affecting persulfide delivery and suggest that additional assays may be necessary to fully represent the functions of IscS in Fe-S cluster formation.

The cysteine desulfurase IscS is the major cellular catalyst for the mobilization and distribution of sulfur from cysteine for a number of different biosynthetic pathways (Fig. 1) (1–4). It is a member of the NifS family of cysteine desulfurases whose first discovered member, NifS, was characterized as a necessary component for activity of the metalloenzyme nitrogenase (5). It was later shown in both *Azotobacter vinelandii* and *Escherichia coli* that the homolog *iscS* is part of a multicistronic operon (*isc*) required for the biosynthesis of protein Fe-S clusters (6). IscS is highly conserved among bacteria and eukarya and is required for viability in organisms such as *Saccharomyces cerevisiae* (7) *A. vinelandii* (6), and *Helicobacter pylori* (8). In *E. coli* (9–11) and *Salmonella enterica* serovar Typhimurium (12), elimination of *iscS* results in a viable organism that displays complex nutritional requirements and severely reduced activity of Fe-S cluster enzymes.

Most bacteria harbor multiple *nifS* homologs. *E. coli* contains *iscS* and two other paralogs, *csdA* and *sufS* (*csdB*). These enzymes can be placed into two groups, based on sequence of an active site loop that contains an essential cysteine and other criteria (13). By these criteria, *E. coli* IscS is a group I enzyme, whereas SufS and CSD are members of group II. All use cysteine and selenocysteine as a substrate *in vitro* with varying efficiency (14). IscS is required for selenium mobilization for the synthesis of the modified nucleoside mnm^5se^2U[1] in tRNA (15), even though SufS utilizes selenocysteine more efficiently *in vitro* (14). In *E. coli*, the *sufS* gene is part of the *suf* operon that is thought to contribute to Fe-S cluster formation under conditions of cellular stress (16). Deletion of *suf* genes cause no observable growth defects in *E. coli*. However, in combination with mutations in *iscSUA*, all but one *suf* gene is required for viability (17), suggesting that *suf* and *isc* genes have overlapping function.

The mechanism for cysteine desulfuration by NifS was shown by Dean and co-workers to be a novel utilization of pyridoxal phosphate for transferring the sulfur atom of substrate L-cysteine (or selenocysteine) to a cysteine residue in the active site of the enzyme to give a persulfide or hydrodisulfide (18). IscS was isolated based on its ability to provide sulfur for the Fe-S cluster of dihydroxy acid dehydratase and found to have a mechanism similar to NifS (19). Although the exact mechanism of sulfur incorporation in Fe-S clusters remains unclear, several groups have obtained evidence of complexation and persulfide transfer between IscS and IscU (20–22). The amino acid residues in IscS important for interaction with IscU and other potential target proteins (23) have not been defined, although a deletion of the C terminus of IscS reduces sulfur transfer and binding to IscU (21).

A primary role for IscS may be to provide sulfur for Fe-S clusters (24). However, as shown in Fig. 1, a number of cellular processes that do not involve Fe-S enzymes also require *iscS*. For instance, IscS is required for the biosynthesis of normal levels of all thionucleosides in *E. coli* (25) and *Salmonella typhimurium* (26), although two of them are not expected to involve Fe-S cluster enzymes in their synthesis. The biosynthesis of 4-thiouri-

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[1] The abbreviations used are: mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; s^4U, 4-thiouridine; s^2C, 2-thiocytidine; ms^2i^6A, 6-dimethallyl-2-methylthioadenosine; SDH, succinate dehydrogenase; WT, wild type.

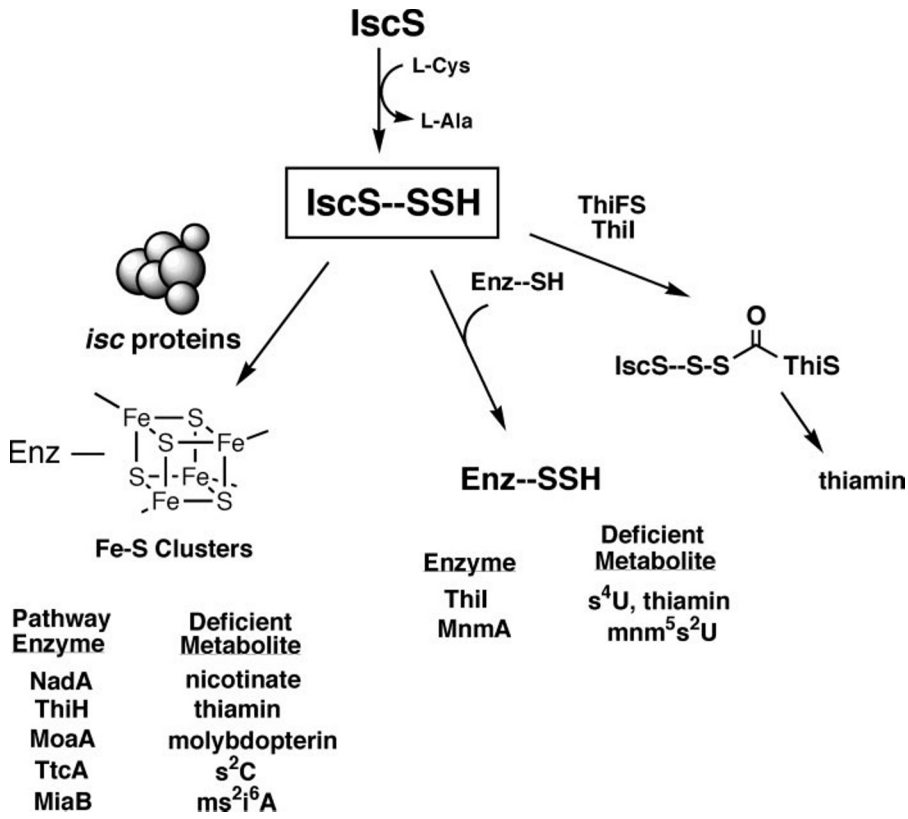

FIG. 1. **Biosynthetic pathways requiring IscS persulfide (*boxed*) in *E. coli*.** The persulfide transfer role of IscS is schematically illustrated as having two main branches. This includes Fe-S cluster biosynthesis via sulfur transfer to IscU and interaction with other *isc* cluster proteins (*left*) and persulfide transfer to tRNA binding proteins for the synthesis of thiouridines mnm^5s^2U and s^4U in tRNA (*middle*). In addition, a novel role for IscS persulfide has been proposed for sulfur incorporation into the thiazole ring of thiamin (*right*) (45). *E. coli* Δ*iscS* mutants are defective in the biosynthesis of all of the pathway metabolites shown, most probably because of defects in the enzymes listed.

dine (Fig. 2, *s^4U*) involves the transfer of sulfane sulfur from Cys^{328} of IscS to a cysteine of the ThiI protein (27–29). ThiI, in turn, binds tRNA, activates a specific uridine using Mg-ATP, and transfers the sulfur, during which it is oxidized to a disulfide (30). The synthesis of 2-thiouridine requires IscS *in vitro* (31) and *in vivo* (15, 25, 26) and may be similar to s^4U synthesis with the MnmA protein as the recipient of the IscS persulfide (31). Both *mnmA* and *iscS* mutants contain mnm^5U instead of the thiolated derivative, indicating that the modifications at the 2 and 5 positions are formed independently.

The remaining two thionucleosides, dimethallyl-2-methylthioadenosine (ms^2i^6A) and 2-thiocytidine (s^2C) are both considered to require the action of Fe-S proteins for their synthesis. The *miaB* gene is required for ms^2 incorporation into i^6A37 in certain tRNAs (32). MiaB has been shown by sequence homology (33) and *in vitro* reconstitution (34) to be a 4Fe-4S protein similar in some respects to biotin and lipoate synthases. *E. coli* mutants lacking either *miaB* or *iscS* produce i^6A in place of the fully modified base (Fig. 2). Jager *et al.* (35) report that the *ttcA* gene required for s^2C synthesis contains two required Cys-*X*-*X*-Cys motifs (35) that may be indicative of an Fe-S cluster protein. Recently, Leipuviene *et al.* (36) reported additional evidence consistent with our results that supports the dual role of IscS in thionucleoside biosynthesis as depicted in Fig. 1.

It has been suggested from sequence comparisons and structural data that the active site cysteine in all NifS proteins resides in a loop (37). In the crystal structures of IscS from *Thermotoga maritima* (37) and *E. coli* IscS (38), portions of the loop are unstructured. Models of the unresolved region of the loop in *E. coli* IscS both place Cys^{328} at least 17 Å from the active site pyridoxal phosphate (38) and support the suggestion that a flexible loop would allow transfer of the persulfide sulfur at a greater distance to protein substrates (37). Because the role of *iscS* in *E. coli* requires distribution of sulfur for a variety of pathways, a major question is how specificity is manifested in sulfur transfer from IscS to each of its protein substrates. This study was initiated to probe the active site loop region of *E. coli* IscS by alanine-scanning mutagenesis. In this work, we report the characterization of two mutants that are defective in Fe-S biosynthesis *in vivo* but functional in persulfide formation and transfer *in vitro*.

MATERIALS AND METHODS

Strains—The parent strain for the *iscS* mutant (CL100) used in this study is *E. coli* MC1061. Preparation and characterization of an in-frame *iscS* deletion in this strain has been described previously (8). *E. coli* strain OD1190, which has a polar disruption of the *iscU* gene, was a generous gift of Dr. James Imlay. *S. enterica* strain DM5420 has been described previously (12) and has a polar MudJ insertion in *iscA*.

Construction of iscS Alanine Mutants—Alanine mutants were prepared using the QuikChange mutagenesis protocol (Stratagene) and plasmid pCL010, a pET21c-derived plasmid containing wild type *iscS* sequence (27). Mutant plasmids were isolated, sequenced, and used to transform strain CL100(DE3) (a DE3 lysogen containing a nonpolar *iscS* deletion) (9). This strain exhibited basal expression of T7 RNA polymerase in the absence of isopropyl-1-thio-β-D-galactopyranoside and hyper-expression in its presence. Mutations of interest were also prepared in the low copy plasmid pCL199, a derivative of pKO3 (39). This plasmid contains the *iscS* gene and adjacent 500-bp flanking regions from the *E. coli* genome. These mutant plasmids were used to observe low copy effects when expressed in *trans* as well as to reintegrate the mutant *iscS* genes back into the chromosome of strain CL100 using previously described techniques (8). Chromosomal mutations were confirmed by amplification of *iscS* using IscS.No and IscS.Co primers (9) followed by sequence analysis.

Analysis of Growth Phenotype—Rich medium was LB and minimal medium was M9-supplemented with 0.2% glucose, 1 mM $MgSO_4$, and leucine (40 μg/ml) unless otherwise stated (MC1061 is a leucine auxotroph). The following nutrients were added at the given concentrations when indicated: nicotinic acid (12.5 μg/ml), thiamin (1 μg/ml), isoleucine (40 μg/ml), and valine (40 μg/ml). Ampicillin was used at a final concentration of 100 μg/ml, and chloramphenicol was used at 20 μg/ml. To determine doubling times, cells from overnight cultures in LB were pelleted, washed once with minimal medium, resuspended, and inoculated (1:500) into LB. Cultures were incubated with shaking at 37 °C, and growth was measured by monitoring the absorbance at 600 nm. For assessment of nutritional requirements, cells were grown in nutrient broth, washed, and inoculated into minimal medium containing the

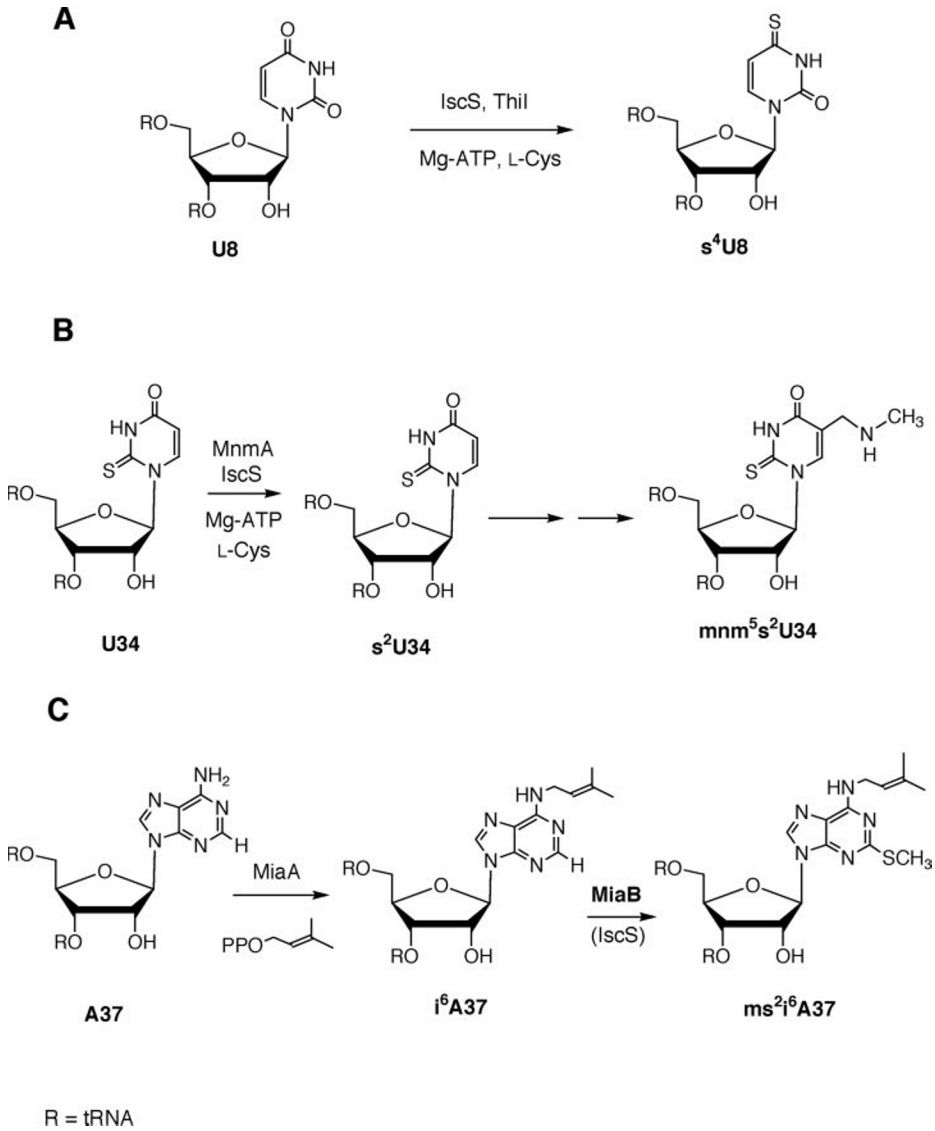

FIG. 2. **Biosynthesis of three naturally occurring tRNA thionucleosides in *E. coli*.** Relevant steps in the biosynthetic pathways for s^4U8, mnm^5s^2U34, and ms^2i6A37 are shown as understood. The gene products known to be involved are indicated near the relevant step. The synthesis of a fourth thionucleoside, s^2C, is largely uncharacterized but is known to require *iscS* and *ttcA* (35).

relevant supplements prior to monitoring growth.

Isolation and Analysis of Unfractionated tRNA—Bulk tRNA was isolated as described previously (9) from 100 ml of uninduced cultures in LB supplemented with ampicillin (100 μg/ml). All of the cultures for tRNA analysis were harvested at mid-log phase (A_{600} = 0.5). Bulk gel-purified tRNA was quantitated by UV absorption at 260 nm, digested (100 μg) to nucleosides using nuclease P1 and alkaline phosphatase, and analyzed by high pressure liquid chromatography by the method of Gherke *et al.* (40) as described by us (9, 25). All of the thionucleosides were analyzed at 260 nm with the exception of 4-thiouridine, which was also analyzed at 330 nm (data not shown). To account for variation in sample loading, the thiouridines and s^2C were quantitated by taking the ratio of each peak area to that of pseudouridine in the same chromatogram (41). The levels of ms^2i^6A were taken as a relative percentage of the sum of ms^2i^6A and i^6A, which should be independent of the amount of tRNA loaded onto the column. The values were then reported relative to the wild type, which is set to 1.0 (Table I).

Western Analysis of iscS Expression—Antiserum to IscS was prepared from a single rabbit by Bethyl Laboratories (Montgomery, TX). Affinity-purified serum was obtained by passing the serum in 0.5 M NaCl through a column of consisting of 50 mg of IscS immobilized on CNBr-activated Sepharose 4B beads (Amersham Biosciences). Antiserum was eluted from the column using 50 mM glycine (pH 3.0) followed by immediate neutralization with 0.5 M Tris-HCl (pH 8.0). For Western analysis, cultures were grown to mid-log phase (A_{600} 0.5) in LB medium, lysed by French Press, and separated into soluble and insoluble fractions by centrifugation at 30,000 × *g* for 15 min. Samples were subjected to SDS-PAGE analysis (42) using 12% gels, and immunoblotting was carried out using enhanced chemiluminescence detection (Amersham Biosciences). A 1:500 dilution of rabbit anti-IscS affinity-purified antiserum was followed by a 1:10,000 dilution of an anti-rabbit horseradish peroxidase conjugate.

^{35}S Transfer Assays—Persulfide transfer activity was determined as described previously (21) with the following modifications. Assays were carried out at 23 °C for 30 s and contained 1 μM IscS, 18.5 μM IscU, 50 mM Hepes (pH 7.3), 150 mM KCl, and 10 mM $MgCl_2$ in a final volume of 35 μl. Reactions were initiated by the addition of L-[^{35}S]cysteine (0.19 Ci/mmol; Amersham Biosciences) to a final concentration of 57 μM and were terminated by centrifugation at 1000 × *g* through a size-exclusion column, Bio-Gel P-6 column (Bio-Rad). The spin column eluant was mixed with SDS-PAGE sample loading buffer to give final concentrations of 1% glycerol, 5 mM Tris (pH 8.0), and 0.001% bromphenol blue. Samples were immediately analyzed by gel electrophoresis using a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate in the absence of added reducing agent. ^{35}S was visualized by exposure on a phosphorimaging screen and analyzed using a Personal Molecular Imager FX (Bio-Rad) and analyzed with Quantity One quantitation software supplied with the instrument.

Assembly of Fe-S Clusters on IscU—IscS-mediated assembly of iron-sulfur clusters on IscU was assayed as described previously (21). Reaction mixtures contained 2 μM IscS, 50 μM IscU, 2 mM ferric citrate, and 10 μM pyridoxal phosphate in 50 mM Hepes (pH 7.3), 150 mM KCl, and 10 mM $MgCl_2$. Cluster assembly was initiated by the addition of 2.5 mM L-cysteine and 5 mM dithiothreitol and was monitored by circular dichroism on a Jasco J-720 spectropolarimeter.

Enzyme Assays

Cysteine Desulfurase—Cysteine desulfurase activity of IscS proteins was performed by monitoring sulfide release from L-cysteine as de-

scribed previously (19, 21) and quantifying the sulfide in the reaction mixture by the methylene blue procedure of Segel (43). Specific activity of the purified proteins was determined at a cysteine concentration of 2.5 mM. Protein concentration was 100 nM.

Succinate Dehydrogenase—In replicates of six, cultures (5 ml) were grown in LB medium to mid-log phase (A_{650} = 0.6). Cell pellets from 3 ml of culture were frozen at −20 °C and assayed within 1 week. Pellets were resuspended and washed in 300 μl of 20 mM Tris citrate buffer (pH 8) and assayed as described previously (12).

6-Phosphogluconate Dehydratase—Cultures were grown in minimal A salts supplemented with 0.2% sodium gluconate and 0.2% casamino acids as well as with leucine, isoleucine, nicotinate, and thiamin at the levels described above. Cells were harvested at A_{600} = 0.8 by centrifugation at 8000 × *g* for 10 min. After storage at −80 °C, cells were resuspended in 50 mM Tris (pH 7.5), incubated with lysozyme for 20 min on ice, sonicated (3 × 20-s bursts), and then centrifuged in a microcentrifuge for 5 min. The supernatant was removed and immediately frozen on dry ice and stored until assayed (<24 h). Dehydratase was assayed in two steps for the formation of pyruvate according to the procedure described by Gardner and Fridovich (44). Activity is expressed in units/milligram protein where 1 unit = 1 μmol/min pyruvate formed.

s^4U Assays—DEAE filter disc assays were performed as previously described for the formation of s^2U (30) and s^4U (27). For s^4U, the assay mixture contained 50 mM Tris (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 4 mM ATP, 20 μM unmodified $tRNA^{Phe}$, 20 μM L-[^{35}S]cysteine, 300 nM ThiI, and 1 μM His_6-tagged mutant or WT IscS. Reaction time was 8 min at 37 °C. Assays for s^2U formation were performed similarly using unmodified $tRNA^{Glu}$ as substrate (30).

```
          315         323         333
   ECiscS malk.d.lav SsgSaCtsas LepSyvlral
   TMNifS nllsgygiyv stSSACTSKD ERLShvldam
   BSnifZ hmleeqdifv sttsaCsake hkpskvllem
   BSnifS lecnrsnici stgsaCsagy hgpsetmkal
   MTiscS lllnakgiya stgsaCnsss lepshvltac
 AFnifS-1 lsldmagiqa stgsaCsskt lqpshvlmac
   BSyrvO vnldmagvav ssgsaCtags vlpshvlta.
 ANalr2505 lglq.pvvav ssgsaCsstk tapshvltal
   YPO2138 lels.d.val ssgsaCtsdq qipshvlqal
   HSNFS1 malk.d.val ssgssCslhp wsplmcleql
 HSLOC96810 malk.d.val ssgsaCtsas lepsyvlrai
   SCNFS1 malr.d.ial ssgsaCtsas lepsyvlhal
 CEB0205.6 malk.s.ial ssgsaCtsas lepsyvlrai
   TViscS tkip.e.faf ssgsaCihnd depsyvlkai
   PA2062 pqalesslav sstsaCnsar papshvllal
  AAnifS2 lrldlmgiet asgsaCvsla lkqshvltai
   ML1708 mlldangiec stgsaCtsgv pqpshvliam
Consensus ---------- S-GSAC---- --PS-VL---
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FIG. 3. **Sequence alignment of the putative active site loop region of selected IscS homologs.** IscS homologs from a wide taxonomic range of organisms are represented. The *top sequence* is from *E. coli* IscS and is the basis for the numbering. The region of *E. coli* IscS mutated in this study (Ser^{323}-Ser^{336}) is shown in *boldface*, and mutated residues chosen for further study are *capitalized* as is the active site cysteine, Cys^{328}. The regions of the active site loops of *E. coli* IscS (37) and *T. maritima* (36) that were disordered in their crystal structure are *underlined*. Abbreviations used are *EC*, *E. coli*; *TM*, *T. maritima*; *BS*, *Bacillus subtilis*; *HS*, *Homo sapiens*; *SC*, *S. cerevisiae*; *CE*, *Caenorhabditis elegans*; *AF*, *Archaeoglobus fulgidus*; *AA*, *Aquifex aeolicus*; *YP*, *Yersinia pestis*; *MT*, *Methanococcus thermoautotropicus*; *AN*, *Anabena* sp.; *PA*, *Pseudomonas aerogenosa*; *TV*, *Trichomonas vaginalis*; *ML*, *Mycobacterium leprae*.

RESULTS

Generation of IscS Mutants—Alanine-scanning mutagenesis was performed on a 14 amino acid region (Ser^{323}-Ser^{336}) that encompasses the active site loop region of *E. coli* IscS. Fourteen plasmids containing the relevant alanine substitutions in *IscS* were generated (see "Materials and Methods"). The plasmids were confirmed by sequencing and transformed into CL100(DE3) where the plasmid-encoded gene provided the only source of IscS in the cell. Alignment of this region from a selection of group I IscS homologs from taxonomically diverse organisms is shown in Fig. 3.

Thionucleoside Profile of iscS Mutants—As an initial screen for mutant IscS function, thionucleoside levels in each of the 14 plasmid-containing strains were determined by high pressure liquid chromatography analysis of tRNA enzymatic digests. The strain containing the C328A mutant had a thionucleoside profile identical to the iscSΔ strain, consistent with previous reports that Cys^{328} is a critical residue in IscS. Two of the 13 remaining mutations (S326A and L333A) resulted in altered thionucleoside levels. These alleles and two additional mutations as controls (S323A and S336A) were integrated into the chromosome for further analysis. The levels of each of the four tRNA thionucleosides isolated from the four resulting mutants are shown in Table I. The two control mutants (S323A and S336A) had thionucleoside levels indistinguishable from those of a wild-type (MC1061) strain. In contrast, the S326A and L333A mutants had significantly decreased levels of s^2C and ms^2i^6A while maintaining wild-type levels of the thiouridines mnm^5s^2U and s^4U. The spectrum of thionucleosides of the S326A chromosomal mutant is shown in Fig. 4. A similar spectrum was observed with the L333A mutant. The mutant spectra are different than either the parental or Δ*iscS* strains being instead similar to that produced by a block in *miaB* (Fig. 1) (32), consistent with the MiaB-catalyzed step being defective in the *iscS* mutants. MiaB is a member of the SAM radical superfamily of proteins that contain an oxygen labile Fe-S cluster (33). Thus, for this thionucleoside in particular, the data support the conclusion that an Fe-S cluster protein is defective.

Growth Is Compromised in iscS Mutants—*E. coli* strains lacking *iscS* have well documented growth defects, including slow growth in rich medium. Growth properties of the four *iscS* mutants were explored. The doubling time of these mutant strains in rich medium are listed in Table I. The two mutants, L333A and S336A, that were defective in thionucleoside production had a longer doubling time than the other two point mutants or the wild-type strain. However, in both cases, the doubling time was significantly shorter than that of an Δ*iscS* mutant. In M9 minimal media supplemented with glucose and

TABLE I
Characterization of chromosomal iscS mutants

Levels of the indicated thionucleosides are presented as the ratio of peak area to that of pseudouridine with the level in MC1061 set to 1.0. Errors were based on duplicate runs and are ≤15%. Doubling time (t_2) is for growth in LB at 37 °C. SDH and 6-phosphogluconate dehydratase (PGD) activities each represent the mean ± S.D. of duplicate experiments. SDH activity is reported relative to WT. PGD activity is expressed as units/milligram protein in extracts where 1 U = 1 μmol/min. ND, not determined.

Strain	s^2C	mnm^5s^2U	s^4U	ms^2i^6A	t_2	SDH	PGD
					min		
MC1061 (WT)	1.0	1.0	1.0	1.0	40 ± 5	1.0 ± 0.1	0.64 ± 0.03
CL100 (Δ*iscS*)	0.05	<0.01	<0.01	<0.01	136 ± 6	0.17 ± 0.04	0.25 ± 0.01
S323A	1.0	1.0	0.9	1.0	46 ± 2	0.94 ± 0.07	ND
S326A	0.1	0.9	0.9	<0.01	105 ± 11	0.19 ± 0.08	0.30 ± 0.01
L333A	0.2	1.1	1.0	<0.01	64 ± 4	0.17 ± 0.04	0.39 ± 0.01
S336A	1.0	1.0	0.9	1.0	47 ± 4	0.81 ± 0.06	0.50 ± 0.03

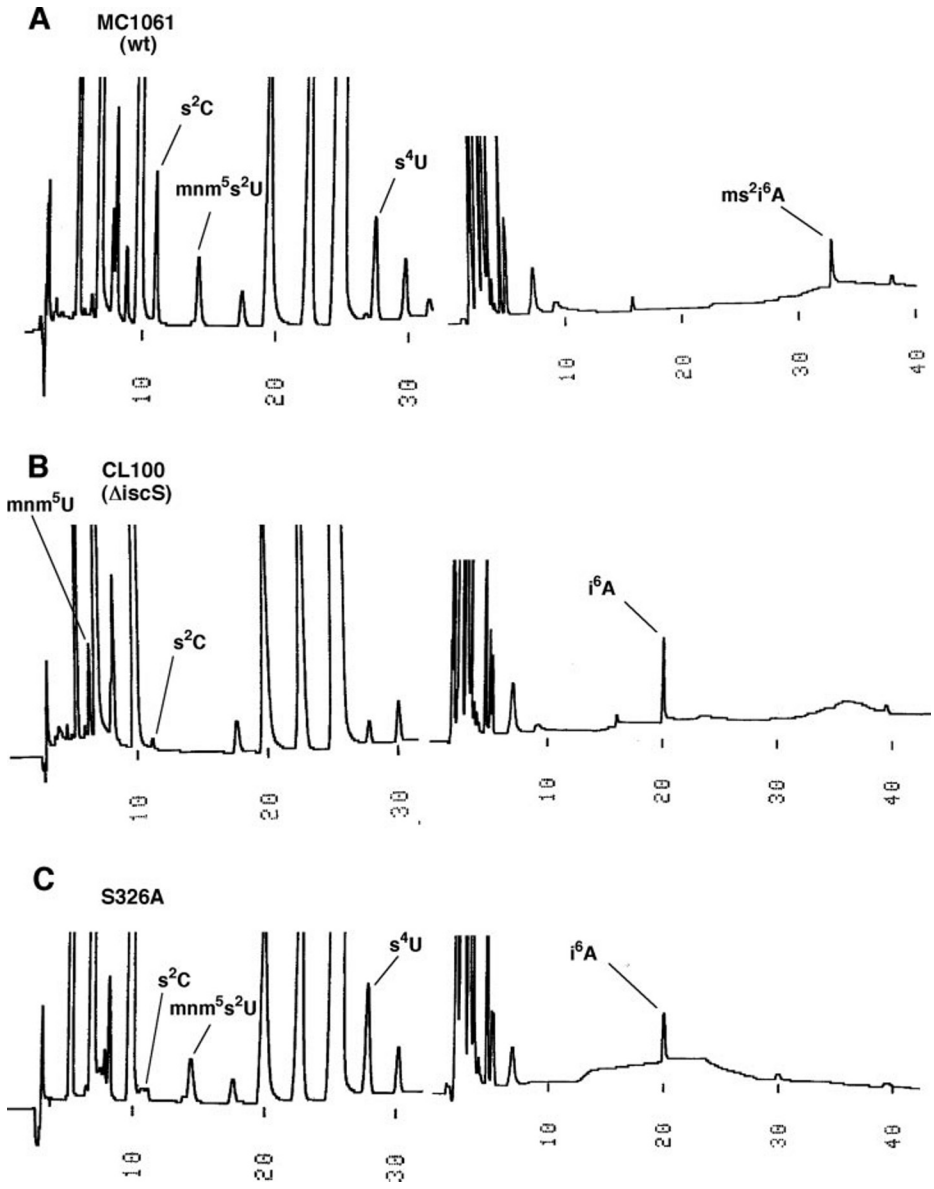

FIG. 4. **High pressure liquid chromatography thionucleoside profile of parent strain MC1061 (*A*), Δ*iscS* strain CL100 (*B*), and *iscS* S326A chromosomal mutant (*C*).** Mutants lacking ms^2i^6A display an increased peak that represents the precursor i^6A. Similarly, mutants lacking mnm^5s^2U contain mnm^5U.

leucine (to satisfy parental auxotrophy), strain CL100 (Δ*iscS*) required nicotinic acid, thiamine, and isoleucine for growth (9). Under the same conditions, the S326A and L333A mutants grew in the absence of nicotinic acid, thiamine, or isoleucine after a significant lag. The lag time could be shortened by isoleucine or thiamine. The control S323A and S336A mutants exhibited growth behavior similar to the parent strain. In summary, the S326A and L333A mutants differ from the remaining alanine mutants and from the *iscS* null mutant in growth properties as they did in the thionucleoside analysis. To more directly test the status of in Fe-S clusters in the S326A and L333A mutants, succinate dehydrogenase and phosphogluconate dehydratase levels were measured in cell-free extracts. These data, presented in Table I, showed that SDH activity in the S326A and L333A point mutants was impaired to a similar extent as in the *iscS* deletion strain, whereas the dehydratase level is also consistent with defects in these two mutants.

iscS (L333A and S326A) Mutant Phenotypes Are Similar to Those of Null Mutants in iscAU—Of the genes in the *isc* operon, *iscS* is uniquely required for sulfur transfer to other proteins (*e.g.* ThiI) in addition to its role in Fe-S cluster synthesis. The data above were consistent with mutants L333A and S326A being defective solely in the function of IscS required for Fe-S biosynthesis, predicting that mutants defective in the downstream *isc* genes would have a similar phenotype. Results from the analysis of two additional *isc* mutant strains were consistent with this hypothesis. The first strain, DM5420, is a *S. enterica* mutant described previously (12) carrying a polar insertion in *iscA*. The second, OD1190, is an *E. coli* strain that carries a polar mutation in *iscU*. Data in Fig. 5 show that tRNA isolated from the *iscU* mutant contained no ms^2i^6A or s^2C (<0.01) and thiouridines mnm^5s^2U and s^4U were unaffected (1.0). Similar results were found for strain DM5420 (data not shown). In addition, as reported previously (12) and confirmed herein, the SDH activity in DM5420 was ~35% that of the wild-type strain. In summary, by every assay used (growth, SDH activity, thionucleoside accumulation), the L333A,S326A mutants were more similar to the *iscA* and *iscU* mutants than the Δ*iscS* mutant.

The iscS Mutations Do Not Reduce Protein Levels—To eliminate the possibility that the phenotype of the two *iscS* mutants was a result of decreased amounts of the mutant proteins, levels of IscS in the parent and chromosomal mutant strains were measured by immunoblotting. Fig. 6 shows a comparison of the levels of IscS in cells grown to mid-log phase ($A_{600} = 0.6$) in rich medium (LB). Mutant protein was found in the insoluble fraction of cell lysates, but each of the mutant strains accumu-

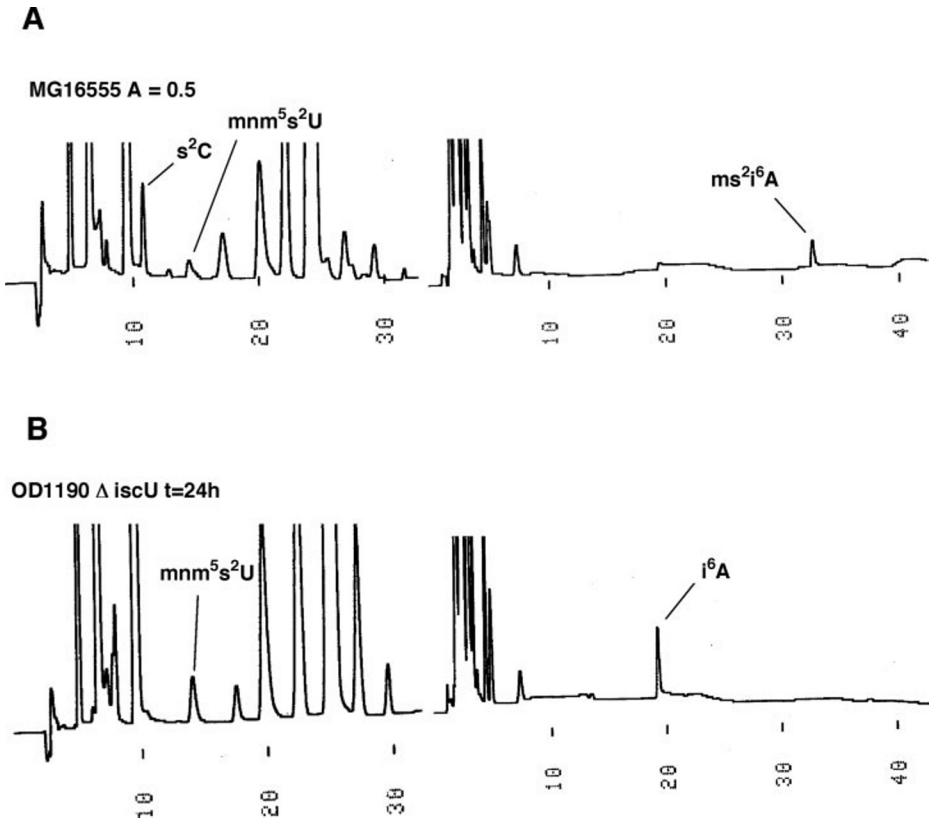

FIG. 5. **High pressure liquid chromatography thionucleoside profile of *E. coli* WT strain MG16555 (*A*) and Δ*iscU* mutant strain OD1190 (*B*) after extended growth (24 h).**

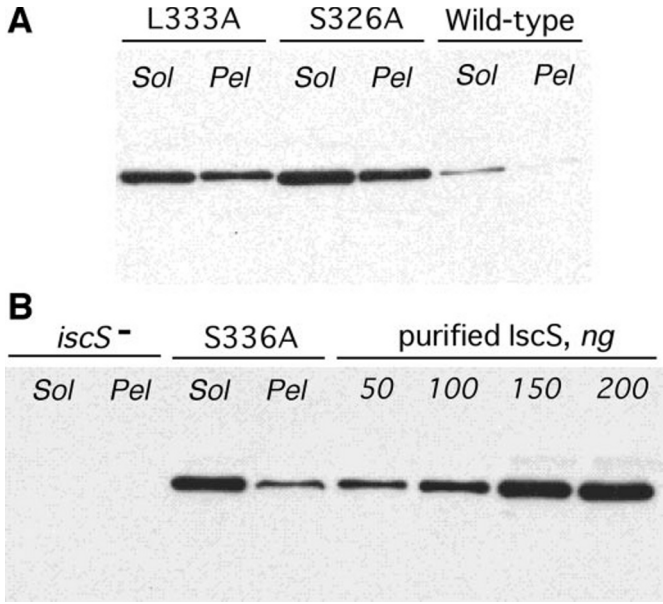

FIG. 6. **Western analysis of expression levels of IscS in wild type and mutant strains harvested at mid-log phase.** *Panel A* shows L333A and S326A mutants and parent strain MC1061. *Panel B* shows Δ*iscS* strain CL100, S336A mutant, and IscS standard. Each lane contained 5 μg of protein. *Sol* and *Pel* refer to soluble fraction and cell pellet, respectively.

lated levels of soluble IscS greater than the parent strain. This finding, together with the cysteine desulfurase activity studies described below, suggest that the phenotypes observed did not result from reduced levels of the mutant proteins.

In Vitro Activity of Mutant IscS Proteins—The above results suggest that the S326A and L333A IscS mutants have a defect in Fe-S synthesis *in vivo*. To correlate this defect with a specific biochemical function of IscS, *in vitro* assays were used to measure the activity of IscS. IscS from the four mutants described in Table I was purified as the N-terminal His_6 fusion protein. Table II shows the results of three enzymatic activity assays with these forms as well as that of a C328A mutant. As anticipated, the C328A mutant is essentially devoid of cysteine desulfurase activity and activity in s^4U and s^2U synthesis. Each of the other active site loop mutant proteins exhibited cysteine desulfurase activity that was equal to or greater than that of the wild-type enzyme. Furthermore, an *in vitro* assay of s^4U biosynthesis using ^{35}S-labeled cysteine showed that each mutant protein (≥1 μM) was able to transfer the sulfur of cysteine to the appropriate tRNA substrate with similar efficiency, although the S326A mutant was consistently ~30–40% lower at all of the concentrations tested (0.2–1 μM). All of the mutant proteins showed s^2U synthesis activity that was not significantly different from wild type at all of the concentrations tested. These data showed that the inability of the S326A and L333A mutants to build Fe-S clusters *in vivo* was not the result of lowered cysteine desulfurase activity.

IscS Mutants Are Proficient to Transfer Sulfur to IscU—The S326A and L333A mutant IscS proteins were assayed for their ability to transfer sulfur to IscU. The assay employed monitors IscS-catalyzed transfer of [^{35}S]sulfur from L-[^{35}S]cysteine to IscU using nonreducing SDS-PAGE (21). Fig. 7 shows that each of the IscS mutants was labeled to a similar extent to wild-type IscS and that each was able to transfer the labeled sulfur to IscU with efficiency similar to wild type IscS. These results indicate that replacement of Ser^{326} and Leu^{333} with alanine does not block sulfur transfer to IscU. The similar extent of labeling of the band corresponding to an IscS-IscU heterodimer further suggests that these substitutions do not have dramatic effects on complex formation between IscS and IscU.

The ability of the S326A mutant to participate in assembly of iron-sulfur clusters on IscU was also investigated. Samples containing IscS, IscU, cysteine, and ferric chloride were incubated anaerobically in the presence of dithiothreitol, and cluster assembly was monitored by circular dichroism (21). Fig. 8

TABLE II
In vitro assays of wild-type and mutant IscS proteins

All of the assays used purified proteins with the exception of the C328A form that was expressed in strain CL100(DE3) and was ~40% pure. Assay procedures are as described under "Materials and Methods."

Isc protein	Cys desulfurase	s^4U synthesis	s^2U synthesis
	min^{-1}	*nmol/min/mg*	
Wild type	3.6 ± 0.4	3.6 ± 0.5	+
C328A	0.2 ± 0.1	<0.1	−
S323A	4.8 ± 0.4	3.4 ± 0.2	+
S326A	4.2 ± 0.2	2.2 ± 0.2	+
L333A	5.4 ± 0.2	4.3 ± 0.5	+
S336A	5.9 ± 0.7	3.8 ± 0.3	+

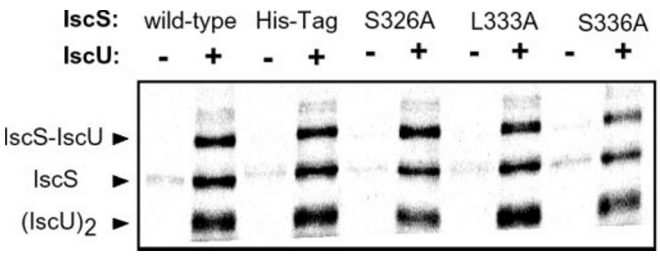

FIG. 7. **Sulfur transfer activities of IscS mutants.** L-[^{35}S]Cysteine was incubated for 30 s with wild-type or mutant forms of His-tagged IscS (1 μM) in the absence or presence of IscU (18.5 μM), separated on spin column, and subjected to non-reducing SDS-PAGE. ^{35}S label incorporation was analyzed with a PhosphorImager. Protein migration positions determined by Coomassie Blue staining are indicated on the *left*.

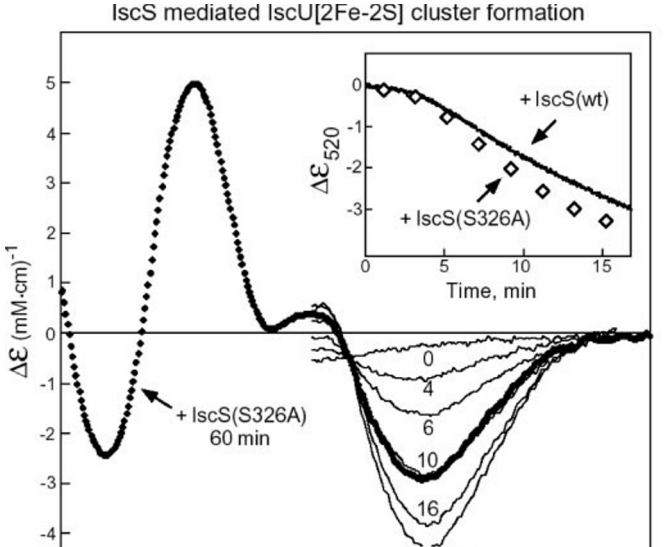

FIG. 8. **Fe-S cluster formation mediated by IscS(C326A).** Reaction mixtures contained 2 μM IscS, 50 μM IscU, 2 mM ferric citrate, and 10 μM pyridoxal phosphate in 50 mM Hepes (pH 7.3), 150 mM KCl, and 10 mM $MgCl_2$. Cluster assembly was initiated by the addition of 2.5 mM L-cysteine and 5 mM dithiothreitol. CD spectra were recorded from 500 to 680 nm at different times, and spectra after 0, 4, 6, 10, 16, and 26 min are shown. The spectrum from 350 to 700 nm recorded after 1 h reveals some decay of the cluster. The *inset* shows a comparison of the rate of cluster formation for the S326A mutant in this experiment with that for wild-type IscS monitored continuously at 560 nm.

shows visible region CD spectra recorded at different times following cysteine addition for a reaction containing the S326A mutant. Maximal cluster formation was observed in ~30 min with some decay apparent at longer times. The CD spectrum is similar to that previously reported for wild-type IscS (21) with minima near 380 and 560 nm and a maximum near 430 nm. The *inset* shows that the initial rate of cluster formation for the S326A mutant is similar to or slightly greater than that of the wild-type parent. This result indicates that sulfur transfer to IscU mediated by the S326A mutant is effective in iron-sulfur cluster formation.

DISCUSSION

Based on the complex phenotype of *E. coli iscS* mutants and extensive *in vitro* work with purified protein, it is clear that IscS interacts with multiple protein sulfur acceptors. Here alanine scanning of the active site loop of *E. coli* IscS has been used to identify residues required for *in vivo* function of this protein. The data show that two substitutions, the highly conserved Ser^{326} and the nonconserved Leu^{333}, selectively impair the role of IscS in the synthesis of Fe-S clusters. Analysis of thionucleoside accumulation served as an initial screen for IscS function. IscS is required for the biosynthesis of normal levels of all of the thionucleosides in *E. coli* (25) and *S. typhimurium* (26), although only two of them are expected to require Fe-S cluster enzymes in their synthesis. The finding that of the four thionucleosides, substitution mutants (S326A and L333A) were defective in synthesis of only s^2C and ms^2i^6A indicates that the two roles of IscS have been separated. Decreased levels of the Fe-S cluster enzyme succinate dehydrogenase also support the conclusion that the S326A and L333A mutations result in a defect in Fe-S cluster biosynthesis *in vivo*.

Although these mutants were defective in Fe-S cluster biosynthesis *in vivo*, no *in vitro* defect was identified. The S326A and L333A mutant proteins were able to transfer sulfur to IscU *in vitro*. This experiment also shows that persulfide formation and stability are not detectably impaired in these mutants. Preliminary experiments that measure the rate of persulfide transfer to IscU for the S326A and L333A mutants show no significant differences with WT IscS.[2] These results suggest that the role of IscS in Fe-S biosynthesis *in vivo* may be more complex than previously appreciated in that persulfide transfer to IscU is a necessary but not sufficient step.

Structural data from a crystal form of *E. coli* IscS shows that the loop containing the C328 can be positioned near the protein surface, greater than 17 Å from the buried pyridoxal phosphate cofactor (38). In both the *T. maritima* (37) and *E. coli* (38) structures, portions of this loop are disordered, consistent with its proposed flexibility. Neither Leu^{333} of *E. coli* IscS nor the equivalent of Glu^{329} in *T. maritima* NifS was observable in the crystal structure. Since Leu^{333} is not conserved, it may be required for interactions that are organism-specific. Unlike Leu^{333}, Ser^{326} is resolved in the *E. coli* structure. The Ser^{326} serine hydroxyl is within hydrogen bonding distance to Arg^{39} of the other IscS monomer in the homodimer. However, Arg^{39} is not conserved (data not shown); therefore, this interaction may be of limited importance. Because Ser^{326} is highly conserved, the interactions in which it is required must also to some extent be conserved. Preliminary results from saturation mutagenesis of position 326 suggest that serine is the only acceptable amino acid at this position for normal growth.[3] Aside from the Arg^{39} interaction, there is no obvious indication of the role of the serine based on the crystal structure. It may be required for a subtle modulation of persulfide reactivity or for protein-protein interaction with Isc proteins other than IscU. Future work will focus on the isolation of specific mutants defective in non Fe-S functions of IscS in an attempt to understand the structural basis of its sulfur transfer specificity.

[2] H. Urbina, and L. Vickery, unpublished observation.
[3] E. Steenblock, J. S. Williams, and C. T. Lauhon, unpublished observation.

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