Investigation of *Escherichia coli* Dimethyl Sulfoxide Reductase Assembly and Processing in Strains Defective for the sec-Independent Protein Translocation System Membrane Targeting and Translocation*

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Dimethyl sulfoxide reductase is a heterotrimeric enzyme (DmsABC) localized to the cytoplasmic surface of the inner membrane. Targeting of the DmsA and DmsB catalytic subunits to the membrane requires the membrane targeting and translocation (Mtt) system. The DmsAB dimer is a member of a family of extrinsic, cytoplasmic facing membrane subunits that require Mtt in order to assemble on the membrane. We show that the MttA2, MttB, and presumably MttA, but not the MttC proteins are required for targeting DmsAB to the membrane. Unlike other Mtt substrates such as trimethylamine N-oxide reductase, the soluble cytoplasmic DmsAB dimer that accumulates in the mtt deletions is very labile. Deletion of the mttA2 or mttB genes also prevents anaerobic growth on fumarate even though fumarate reductase does not require Mtt for assembly. This was due to the lethality of membrane insertion of DmsC in the absence of the DmsAB subunits. In the absence of DmsC, DmsAB accumulates in the cytoplasm. A 45-amino acid leader on DmsA is removed during assembly. Processing does not require DmsC but does require Mtt. Translocation of DmsAB to the periplasm is not required for processing. The leader may be cleaved by a novel leader peptidase, or the long DmsA leader may traverse the membrane through the Mtt system resulting in cleavage by the periplasmic leader peptidase I followed by release of DmsA into the cytoplasm.

Until recently, protein translocation in bacteria was thought to take place either by the sec pathway or by specialized translocation systems (1). The translocation of proteins by the sec pathway requires the protein to remain in an open conformation during the process of translocation and extensive information exits for sec-mediated translocation (2, 3). The sec system has also been implicated in the translocation of selected periplasmic membrane-extrinsic and membrane-intrinsic proteins. For example, leader peptidase is targeted by the sec pathway (4), and a newly described protein, YidC (a component of sec-apparatus), was shown to target specifically membrane-intrinsic proteins (5–7). We and others (8–11) have reported an alternative protein targeting and translocation system termed membrane targeting and translocation (Mtt), also termed twin arginine translocase. The Mtt system is comprised of at least three proteins, MttA1A2B (also called TatABC) and has been shown to transport fully folded proteins to or across the membrane (8–10, 12). Unlike the sec system, no known integral membrane protein is targeted by the Mtt pathway (13). The discovery of the Mtt system explained the translocation into the periplasm of cofactor-containing proteins that were assembled in the cytoplasmic compartment. These proteins have a long N-terminal leader with a conserved twin arginine motif ((S/T)RXF(L/K)) (11). They have diversity in subunit composition, molecular weight, the nature of the redox cofactors, and cellular localization (13–15). Bacterial proteins utilizing the Mtt pathway include some proteins without cofactors such as Suf1, a member of the copper oxidase family, fusion proteins containing a reporter gene (e.g. β-lactamase), and even maltodextrin proteins (9, 16–19). Components of the Mtt protein translocation machinery are found in at least half of the complete bacterial genomes available to date as well as the genomes of chloroplasts and plant mitochondria. However, Mtt is totally absent from animal genomes (8, 11, 13).

Dimethyl sulfoxide (Me2SO) reductase is encoded by the dms operon and is expressed under anaerobic growth conditions in the absence of nitrate (20, 21). Extensive investigation has shown that the DmsAB subunits form a membrane extrinsic dimer facing the cytoplasm, and DmsC, an integral membrane protein, serves as a membrane anchor (22). Recently, we have confirmed that DmsA has an N-terminal twin arginine leader that functions as a membrane-targeting signal and is also essential for the stability of the holoenzyme (15).

Membrane targeting via the Mtt system is not limited to Me2SO reductase. Formate dehydrogenase-O is targeted to the membrane (but not translocated across) by a twin arginine leader (23). More recently, chlorophenol reductive dehalogenase (encoded by *cprAB* genes) of *Desulfitobacterium dehalogenase* was shown to be a membrane-bound enzyme, apparently targeted to the membrane by the twin arginine leader. CprA undergoes processing resulting in cleavage of the leader, and the mature form of the enzyme was localized to the cytoplasmic face of the membrane with the CprB subunit presumably serving a membrane anchor function (24). Thus certain membrane-
bound enzymes whose active sites face the cytoplasm appear to be targeted to the membrane by the Mtt system (15, 23, 24).

To better understand the mechanism of the membrane targeting of Me₃SO reductase, we have constructed several mutant strains defective in the mtt genes. We provide experimental evidence to show that Mtt catalyzes the targeting of DmsA to the cytoplasmic face of the membrane, but not its translocation, and that a functional Mtt system is required for processing of DmsA to the mature form and for the stability of the DmsAB dimer.

EXPERIMENTAL PROCEDURES

Materials—Molecular biology reagents and ECL Western blotting detection kit were purchased from Life Technologies, Inc., and American Pharmacia Biotech, respectively. Oligonucleotides were obtained from the Department of Biochemistry DNA core facility, University of Alberta, Edmonton, Canada. DNA polymerases (Taq and Elongase enzyme mix) were purchased from Life Technologies, Inc. Taq DNA polymerase was purchased from Stratagene. All other reagents used were of the highest purity available commercially.

Methods—Media, growth conditions, anaerobic growth profile on minimal media composed of glycerol as a carbon source and Me₂SO, Me₂SO reductase activity was monitored by the oxidation of reduced benzyl viologen by the substrate TMAO, or fumarate as the terminal electron acceptor. Me₂SO reductase activity was monitored by the oxidation of reduced benzyl viologen by the substrate TMAO or fumarate as the terminal electron acceptor. Me₂SO reductase activity was monitored by the oxidation of reduced benzyl viologen by the substrate TMAO or fumarate as the terminal electron acceptor. Me₂SO reductase activity was monitored by the oxidation of reduced benzyl viologen by the substrate TMAO or fumarate as the terminal electron acceptor.

The entire mtt operon was amplified using the oligonucleotide pairs RAT20/GZ-01 (see Table II for oligonucleotide sequences) with flanking EcoRI and SalI restriction enzyme sites at the 5' and 3' ends, respectively. The PCR product (2544 bp) was cloned into the EcoRI and SalI sites of plasmids pMS119EH or pTZ19R to yield the plasmids pMTT/Amp(B) or pMTT/Amp(3). The plasmid also differs from the mttA1A2BC sequences under control of the native mtt promoter amplified by PCR using the oligonucleotide pairs GZ-08/GZ-07 with an engineered EcoRI site at the 5' end. The PCR DNA (3596 bp) was digested with EcoRI and PstI enzymes, respectively, to yield 3375 bp of the mtt gene sequence. The PstI site is present within the 3' end of the amplified DNA, outside of the mtt operon. The EcoRI/PstI fragment was cloned into EcoRI/PstI-cleaved pBR322 resulting in deletion of the bulk of the amplicin coding region of the vector to yield the plasmid pMTT-322 (3). The plasmid also differs from the mtt operon. Plasmid pMTT/B was amplified by PCR using the oligonucleotides GZ-04/RAT22. The PCR DNA (827 bp) was cut with EcoRI and SalI prior to cloning into pMS119EH to generate the plasmid pMTT/B.

Plasmid pMDS193 encodes the DmsAB dimer under control of the dms promoter and was constructed using PCR with primers 23D-23/DS-24 and pMS160 as template DNA to amplify a 188-bp DNA fragment that covers the 3' end of dmsB with an engineered stop codon and flanking SalI and SalI/DhaI restriction enzyme sites at the 5' and 3' ends, respectively. The plasmid pMDS190 was digested with SalI and SalI to remove part of 3' end of dmsB and the entire dmsC (1073 bp). The DNA was gel-eluted, and the larger fragment (7494 bp) containing the entire dmsA and most of dmsB was mixed with the PCR DNA and ligated to yield the plasmid, pMDS193.

Deletion or Insertional Inactivation of the mtt Genes—The mttA gene was inactivated by insertionional mutagenesis. The plasmid pMTT/B was cut with SacII at the unique site within mttA. The recessed ends were filled in to create blunt ends. A blunt-ended chloramphenicol acetyltransferase (CmR) gene cartridge, CAT, generated using PCR (see

### Table I

| E. coli strains | Genotype | Source or Ref. |
|----------------|----------|----------------|
| T3G1           | Δlac-pro supE thi hsdD5/F' traD36proAB lacY lacZ DM15 | Laboratory collection |
| DSS301         | as T3G1; Km³ΔmmsABC | Laboratory collection |
| K38            | HfrC, λ | Laboratory collection |
| HB101          | supE44 hsdS20 (r-Mp) recA13 ara-14 proA2 lacY1 galK2 rpsL20 syl-5 nth-1 thr-1 ara-1 leuB6 Δ (proA2) lacY1 sbeC201 tex-33 supE44 galiK2 A sbeC15 | Laboratory collection |
| JC7623         | hisG4 recB1 recC22 rpl31 syl-5 nth-1 argE3 thi-1 | Laboratory collection |
| D308           | RP497 (chc¹ lac Y eda¹ argA¹ Tn10 recD109 | Laboratory collection |
| DSS640⁴        | as T3G1; Cm⁺ΔmttA₁A₂BC | This study |
| DSS641⁴        | as T3G1; Cm⁺ΔmttA₂ | This study |
| DSS642⁴        | as T3G1; Cm⁺ΔmttA₃ | This study |
| DSS643⁴        | as T3G1; Cm⁺ΔmttB | This study |
| DSS644⁴        | as T3G1; Cm⁺ΔmttC | This study |
| DSS740⁴        | as DSS301; Cm⁺ΔmttA₁A₂BC | This study |
| DSS743⁴        | as DSS301; Cm⁺ΔmttB | This study |
| DSS744⁴        | as DSS301; Cm⁺ΔmttC | This study |

* DSS640 represents a total deletion of mtt operon; DSS641, DSS642, DSS643, and DSS644 represent deletion in the first (mttA₁), second (mttA₂), third (mttB), and fourth (mttC) genes of the mtt operon, respectively.
* Double deletions are designated as DSS740 (total deletion of mtt and dms operons). DSS743 and DSS744 indicate the deletion of the dms operon plus the third or the fourth genes of mtt operon (mttB or mttC), respectively.
below) was inserted at the filled in SacII site of the pMTT/19R plasmid to generate pMTT/CAT. The CmR gene sequence (CAT) was amplified using pACYC184 DNA as template and the oligonucleotides CAT5/CAT3 (Table II). The CAT cartridge has BamHI-flanking ends and was also used to generate various mtt gene deletions as summarized below and in Table II.

### TABLE II

| Oligonucleotide pairs   | Sequencea | Derived plasmid/DNAb |
|-------------------------|-----------|----------------------|
| RAT20/GZ-01             | gctaagaattcGGTGGTCTGATCGCC          | pMTT or pMTT/19R     |
| GZ-08/GZ-07             | tatagtcgacAGTGTGAAGAATACC           | pMTT-322             |
| GZ-04/RAT22             | tatagtcgacATGGTGATCGC               | pMTTB                |
| DS-23/DS-24             | GATTTCGCCCTATACGCA                  | pDMS193              |
| CAT5/CAT3               | gacggatccCTGTTGATACC                |                      |
| GZ-09’                  | tatagtcgacGGTATAACGCAAAC            |                      |
| GZ-20’                  | gacggatccCTGTTGATACC                |                      |
| GZ-07’                  | tatagtcgacGGTATAACGCAAAC            |                      |
| GZ-14’                  | tatagtcgacGGTATAACGCAAAC            |                      |
| GZ-11’                  | tgcggatccTAAAGGTCAGCT              |                      |
| GZ-12’                  | tgcggatccTAAAGGTCAGCT              |                      |
| GZ-13’                  | tgcggatccAAGAGGAGCCG              |                      |

a Non-homologous sequences are shown in lowercase, and the restriction enzyme sites are underlined.
b CAT5/CAT3-amplified DNA was used in combination with the PCR-amplified sequences to generate the plasmids deleted in the individual mtt genes as described.

c The PCR-amplified DNA from GZ-08/GZ-09 (5’ region of mttA1 gene, 768 bp) and GZ-20/RAT22 (3’ region of mttA1 plus the mttB gene, 1352 bp) was used to generate pMTT/19R in pTZ19R vector background. A CAT cartridge with BamHI-flanking ends was ligated into the BamHI-opened pMTT/19R to generate pMTT/CAT with a 127-bp deletion in the mttA1 gene.

d The PCR-amplified DNA from GZ-08/GZ-09 (3’ region of mttA1 gene, 768 bp) and a CAT cartridge to generate pMTT/CAT plasmid in a pTZ19R vector background that has a 1912-bp deletion of the mtt operon, missing in the 3’ mttA1, complete mttA2, and the bulk of the mttC genes, respectively.

e The PCR-amplified DNA from GZ-08/GZ-11 (1650-bp DNA with the mtt promoter region, mttA1A2, and the downstream sequences) were cut with appropriate restriction enzymes, ligated to a CAT cartridge, and cloned into pTZ19R to generate a plasmid, pMTT/CAT, with a 274-bp deletion in the mttB gene.

f The PCR-amplified DNA from GZ-08/GZ-13 (2405 bp, is composed of the promoter region along with the mttA1A2B and the 5’ sequence of the mttC gene) was cloned with a CAT cartridge into the gel-purified pMTT/CAT vector (deleted for the EcoRI and BamHI-intervening sequences from the previous cloning step) to generate a plasmid, pMTT/CAT, with a 274-bp deletion in mttC.

**Fig. 1. Growth of the mtt deletions on glycerol/Me₂SO minimal medium.**

Bacterial growth was monitored at 37 °C in a Klett spectrophotometer. Klett units are plotted as a function of time (hours) for the various strains tested. For a description of the strains see Table I. A, TG1 versus DSS641 (ΔmttA1) and DSS642 (ΔmttA2), B, TG1 versus DSS644 (Δmtt C1), C, TG1 versus DSS649 (ΔmttA1A2BC) and DSS640/pMTT, D, TG1 versus DSS643 (ΔmttB) and complementation of DSS643/pmtTB.
RESULTS

Characterization of mtt Deletion Strains—Deletions in mttA₂, mttB, and mttA₁₂BC failed to support anaerobic growth on GD (15, 25) (Fig. 1, A, C, D, and G) or GT (15, 25) minimal medium (data not shown). Deletion of mttA₁ showed moderate inhibition of growth compared with the wild-type strain, TG1 (Fig. 1A). This was shown to be due to a compensating effect of a functional homologue of MttA₁, TatE, present on Escherichia coli chromosome (9). Deletion of mttC alone had very little effect on the growth profiles measured under experimental conditions (Fig. 1B). Growth on glycerol/Me₂SO medium was restored when the Mtt polypeptides were expressed from a multicopy plasmid pMTT carrying the entire mtt operon in the DmsA strain (Fig. 1C). Similarly, growth of the DmsB strain was corrected by a plasmid (pMTTB) expressing the MttB polypeptide (Fig. 1D). The defective growth phenotype observed for the strain deleted in mttA₂ was also corrected by a plasmid expressing the Mtt polypeptides (data not shown).

These results clearly demonstrate that the mttA₂ and mttB genes are critical for anaerobic respiration on Me₂SO.

As a control for the growth experiments, we investigated the growth of our mtt mutants on GF (15, 25) minimal medium. None of the fumarate reductase polypeptides bear a twin arginine leader, and this enzyme is not targeted to the membrane by the Mtt system, thus growth on GF medium should be unaffected in these mutants. Surprisingly, DmsC₁ and DmsC₂ showed limited growth, whereas DmsA and a total deletion of the mtt operon (Δmtt) nearly abolished growth on GF medium (Fig. 2, A and B). Anaerobic growth on GF medium was restored by in vivo complementation of the Δmtt strain by pMTTB (Fig. 2B); similarly the Δmtt and ΔmttA₂ strains were complemented by expression plasmids carrying the entire mtt operon (data not shown). The mtt deletions grew normally under aerobic conditions and anaerobically with nitrate (data not shown).

We hypothesized that the lack of growth on GF medium resulted from the insertion of DmsC into the membrane without the DmsAB subunits. Previously, we showed that expression of DmsC in the absence of DmsAB is lethal (34). In confirmation of this hypothesis, growth was observed on GF medium for the double mutants DSS740 and DSS743 (Δmtt ΔdmsABC) and DSS740/pDMS160 strain (Fig. 2C). Predictably, introduction of the DmsABC expression plasmid (pDMS160) into the double mutant, Δmtt ΔdmsABC (DSS740), suppressed growth on fumarate (Fig. 2C), whereas the expression of the DmsAB subunits without DmsC (pDMS193) did not suppress growth (data not shown).

Comparison of Me₂SO Reductase Activities in the Control and the mtt Deletion Strains—To correlate growth to the mem-


**TABLE III**

**Comparison of the Me$_2$SO reductase activity in control and mtt deletion strains**

Bacterial cultures were grown anaerobically in peptone-fumarate medium for 24 h. Preparation of membrane (M) and soluble (S, periplasm plus cytoplasm) fractions and the assay of the Me$_2$SO reductase using benzyl viologen (BV) and TMAO as the electron donor-acceptor species were as described under "Experimental Procedures." Total activity units represent the combined activities of membrane and soluble fractions. The percent distribution and the specific activity of the individual fractions for each bacterial strain tested were as indicated. TG1, DSS640 ($\Delta$mttA$_1$A$_2$BC), DSS641 ($\Delta$mttA$_1$), DSS642 ($\Delta$mttA$_2$), and DSS643 deletions strains are described under "Experimental Procedures."

| Strain/plasmid | Total activity (BV/TMAO) | Distribution | Specific activity |
|---------------|--------------------------|--------------|------------------|
|               | M | S | M | S | % | |
| TG1           | 137 | 94 | 6 | 4.00 | 0.38 |
| DSS640        | 23.2 | 0.2 | 99 | 0.01 | 0.13 |
| DSS640/pMTT322 | 148 | 83 | 17 | 2.80 | 0.20 |
| DSS641        | 156 | 83 | 17 | 5.10 | 0.23 |
| DSS642        | 42 | 16 | 84 | 0.20 | 0.40 |
| DSS642/pMTT   | 140 | 73 | 27 | 2.20 | 0.30 |
| DSS643        | 19 | 18 | 82 | 0.20 | 0.70 |
| DSS643/pMTTB  | 153 | 79 | 21 | 4.40 | 1.40 |
| DSS644        | 119 | 93 | 7 | 3.50 | 0.28 |
| DSS644/pMTTC  | 129 | 80 | 20 | 4.30 | 1.10 |

**FIG. 3.** **Comparison of the stabilities of TMAO and Me$_2$SO reductases in the mtt deletions.** A, bacteria were grown in peptone/fumarate medium (TG1, DSS640, DSS641, DSS642, DSS643, and DSS644) or B, peptone/fumarate/TMAO medium (DSS301, DSS740, and DSS744). Membrane and supernatant (periplasm + cytoplasm) fractions were prepared and assayed for enzyme activity as described under "Experimental Procedures," using TMAO as substrate for both TMAO and Me$_2$SO reductases. The combined total activity units in the membrane and supernatant fractions were used for the histogram.

brane association of the reductase activity, we measured enzyme activities with the artificial electron donor, benzyl viologen with TMAO as a substrate. As expected, the control strain TG1 showed up to 94% of the activity in the membrane fraction (Table III). The reductase activity in the mutants DSS640 ($\Delta$mtt), DSS642 ($\Delta$mttA$_2$), and DSS643 ($\Delta$mttB) was predominantly soluble. Complementation of the deletions with appropriate plasmids resulted in membrane-bound reductase activity. $\Delta$mttA$_1$ (DSS641) and $\Delta$mttC (DSS644) exhibited profiles close to the control strain. The distribution and the specific activity of fumarate reductase remained unaffected indicating that this anaerobic enzyme was not influenced by the Mtt pathway (data not shown).

**Stability of Me$_2$SO and TMAO Reductases in the mtt Deletions—**The total Me$_2$SO reductase activity in $\Delta$mttA$_1$ and $\Delta$mttB was greatly reduced, compared with the control, DSS641 and DSS644 (Fig. 3A). By comparison the periplasmic enzyme TMAO reductase was stable in these mutants even though it accumulated in the cytoplasm (Fig. 3B). We used DSS301 as the strain for comparison of TMAO reductase activity because this strain has a deletion in the dms operon and thus Me$_2$SO reductase does not interfere with the benzyl viologen/TMAO assay. Upon in vivo complementation, the specific activity as well as the total activity of Me$_2$SO reductase approached the control values.

**Localization of DmsAB—**As a precursor to examining the processing of the DmsA leader, we felt that it was essential to re-examine the cytoplasmic localization of DmsAB. Eliminating the anchor DmsC should simplify interpretation as expression of DmsAB closely mirrors a typical dual subunit Mtt substrate such as the E. coli hydrogenase-2. Such an experiment was reported earlier, with a construct that encoded DmsAB and the first two transmembrane loops of DmsC (pDMS59X) (8). Expression from this construct showed some accumulation of soluble DmsAB dimer in the periplasmic compartment (8) leading us to propose that DmsC served a "stop-transfer" role. However, as the experiment lacked adequate controls for lysis and the quality of the periplasmic fraction and to eliminate potential problems arising from expression of a partial DmsC subunit, we constructed a plasmid encoding only DmsAB (pDMS193). Localization was studied in the control strain (DSS301) and the mtt deletions by immunoblotting of appropriate fractions (Fig. 4). As an internal control, the plasmid encoded $\beta$-lactamase was also monitored in these same fractions loaded identically on a separate gel and transferred onto a blot for probing with $\beta$-lactamase-specific antibody (Fig. 4). $\beta$-Lactamase is a known sec-dependent and periplasmically localized enzyme. The DmsAB subunits encoded by the plasmid pDMS193 in the control strain (DSS301) were predominantly cytoplasmic. The membrane fraction had a very small amount of DmsAB, presumably due to the contamination from occluded soluble reductase in the membrane pellet. The periplasmic fraction was almost devoid of DmsAB. In these samples $\beta$-lactamase was predominantly in the periplasmic fraction. A small amount of the $\beta$-lactamase was noted in the cytoplasmic fraction and none in the membrane fraction. These findings clearly indicate that the DmsAB subunits are present only in the cytoplasm. A soluble cytoplasmic enzyme marker (glucose-6-phosphate dehydrogenase) was also monitored in these fractions (35). Glucose-6-phosphate dehydrogenase was present only in the cytoplasmic fraction, also validating the quality of the extracts prepared (data not shown). As expected, the DmsAB subunits were localized to the cytoplasmic fraction in the $\Delta$mtt strain. The $\beta$-lactamase (Fig. 4) and the glucose-6-phosphate dehydrogenase (data not shown) were present in the expected cell compartments. $\beta$-Lactamase export was not affected by deletion of the mtt genes. These experiments were carried out in a strain totally lacking DmsC (DSS301/pDMS193) and a strain with sub-stoichiometric levels of DmsC relative to DmsAB (DSS640/pDMS193). However, it is expected that in strain DSS640/pDMS193 the DmsAB dimer would accumulate in the cytoplasm, regardless of the level of...
Considerable information has accumulated on the role of Mtt in the translocation of periplasmic proteins. The necessity for MttA1, MttA2, and MttB but not MttC has been documented for TorA, FdhF, Hya1, SulII, and YaeK proteins (9, 19, 36). Me2SO reductase is a membrane-bound protein with the extrinsic DmsAB subunits facing the cytoplasm (22). Assembly of Me2SO reductase requires Mtt and DmsA has a typical twin arginine leader that is essential (15). It was of interest to see if Me2SO reductase had the same requirements for Mtt proteins. We constructed a series of mtt chromosomal deletions and confirmed that Me2SO reductase requires MttA2 and MttB using anaerobic growth measurements (Fig. 1). Deletion of MttA1 had only a marginal effect on growth. This is due to the redundancy of MttA1 resulting from the presence of the homologous gene, ybec (tatE), on the E. coli chromosome. A strain deleted for tatA (mttA1) and tatE failed to direct the Me2SO reductase to the membrane implying a role for these proteins (9). MttC was not required, and MttC has been shown to be a protein exhibiting nuclease activity and is apparently unrelated to the Mtt system (37). We have noted that amplification of Me2SO reductase in an mttC deletion strain showed only 50% of the membrane-bound reductase compared with the wild type. We assume that in the mttC deletion, the mtt mRNA may be less stable resulting in the lower activity measurements. Complementation of the mutants with appropriate mtt genes on plasmids corrected the phenotype. However, we have observed a significant lag in the complemented strains (Fig. 1). Overexpression of the Mtt proteins from multicopy plasmids hinders growth and in some instances may totally inhibit cell growth (36). These studies confirm and extend the observations of others (9, 36) who have shown that the growth defects on Me2SO and TMAO are due to a generalized defect in the Mtt system.

As a control for the growth studies, we examined the ability of the mtt deletions to grow on TMAO, fumarate, and nitrate. TMAO reductase is a soluble periplasmic reductase, with a twin arginine leader, that utilizes Mtt for translocation. Growth and activity results with this enzyme reflected the Me2SO reductase data (data not shown). Fumarate and nitrate reductase are membrane-bound enzymes that lack twin arginine leaders and do not utilize Mtt. We were surprised to find that the mtt deletions (Δmtt and ΔmttB) failed to grow on fumarate (Fig. 2) but grew normally on nitrate (data not shown). By use of a double deletion for both dms and mtt, we traced the phenotype to the expression of incorrectly assembled Me2SO reductase in the mtt deletions. Expression of DmsC, the Me2SO reductase anchor subunit in the absence of correctly assembled DmsAB catalytic dimer is lethal (34). As expected, deletion of both the dms operon and the mtt operon restored growth on fumarate. As nitrate represses the expression of dmsABC, growth on nitrate was unaffected.
The enzyme activity distribution of Me₂SO reductase (Table III) and TMAO reductase (9) also confirms that the proteins are mislocalized in strains with deletions of the mtt operon. Me₂SO reductase was very labile compared with TMAO reductase in these mutants (Fig. 3) suggesting rapid proteolytic degradation of mistargeted DmsAB.

A large amount of experimental evidence utilizing immunoblotting of periplasmic fractions obtained by osmotic shock and chloroform washing, protease susceptibility, lactoperoxidase-catalyzed iodination, immunogold electron microscopy of etched membrane preparations, TaphoA fusions, and electron paramagnetic resonance monitoring the effects of the probe dysprosium(III) on an engineered 3Fe4S cluster in DmsB indicated that DmsAB faces the cytoplasm (22, 38). Others (11) have suggested that DmsAB faces the periplasm based solely on the presence of a twin arginine leader sequence in DmsA. In this study we have re-examined the question of the localization of DmsAB in both wild-type and mtt deletions (Fig. 4). Our results once again confirm that DmsAB is cytoplasmic.

In a recent study, the localization of DmsAB was compared in a strain expressing a partial DmsC subunit (pDMSc59X) with full-length DmsC originating from the chromosomal copy of the dms operon. In those experiments we reported (8) some DmsAB in the periplasm in the absence of full-length DmsC, leading us to suggest that DmsC might serve a stop-transfer role. In the current experiments, with a complete DmsC deletion, DmsAB subunits were only observed in the cytoplasm. It is possible that the absence of DmsC, or sub-stoichiometric levels of this polypeptide, could alter the localization of the DmsAB dimer. However, the large body of experimental evidence gathered to date supports the cytoplasmic localization of DmsAB in the wild-type and in the reductase-amplified strains (with stoichiometric amounts of DmsC) (22, 38) as well as DmsC-deficient strains (Fig. 5). The earlier studies lacked compartment-specific marker enzyme controls for the fractionation protocols, and as a result a small amount of cell lysis and/or an increase in membrane leakiness due to expression of the partial DmsC subunit could have contributed to the observed DmsAB in the periplasm (8).

In the absence of the DmsC anchor subunit, one might expect that DmsAB should be translocated to the periplasm directed by the twin arginine leader. Why is DmsAB not translocated even in the absence of DmsC? It could be that the DmsA leader contains some stop-transfer information. This seems unlikely because in a previous study we showed that replacement of the dms leader with the tor leader did not affect the topology of DmsAB (15). It appears that DmsAB contains information in the mature polypeptides that prevents translocation to the periplasm. This conundrum is not limited to DmsAB. The localizations of FdoGH in E. coli (23) and CprAB in D. dehalogenasase (24) were also shown to be cytoplasmic despite the presence of twin arginine leader sequences. Furthermore, N-acetylumarmyl-l-alanine amidase (AmiA), a twin arginine leader sequence containing protein, was shown to be targeted and translocated by an Mtt-independent mechanism (39). These studies imply that information in the total polypeptide and not solely the signal sequence determines the localization of a given protein. Elegant information on the importance of information in the mature protein for membrane targeting came from studies of a TorA leader peptide (TorA-Lep) fusion protein. The full-length TorA-Lep fusion protein was directed to the membrane via the Sec pathway and not by the Mtt system, even though a twin arginine leader was present (4). Conversely, the DmsA twin arginine leader was shown to mediate the export of a fusion protein (yeast cytochrome c) via the Mtt system (40). Taken together, these results favor the view that both the twin arginine leader and the mature polypeptide contribute to the final localization of a specific protein.

The results of whole cell immunoblotting (Fig. 5) clearly indicate that the MttA₁, MttA₂, and MttB proteins are required for processing of the DmsA leader. This was seen with both the catalytic DmsAB dimer and the holoenzyme indicating that the DmsC anchor does not play a role in processing of pre-DmsA to mature DmsA. It is not clear how the DmsA leader is processed. There is no evidence to date that any of the known Mtt subunits are directly involved in the processing of the leader sequence. It has been assumed that the leader peptide is cleaved by leader peptidase I which faces the periplasm (4). Attempts to study the processing of the DmsA leader using a conditional lethal leader peptidase mutant were inconclusive, due to the poor growth of this strain and the difficulty in obtaining total peptidase deficiency under our experimental conditions (data not shown). Since none of the substrates of the Mtt system studied to date are true soluble cytoplasmic proteins, the observation of apparent DmsA processing in the cytoplasm should be interpreted with caution. The DmsAB dimer attached to the membrane-bound Mtt system possesses a leader sequence of 45 amino acids that is sufficient to traverse the bilayer and mediate processing on the periplasmic side, without the bulk of the DmsAB ever crossing the bilayer. The processed DmsAB would then lack affinity for the Mtt system and could be released into the cytoplasm. Alternatively, the DmsA leader could be cleaved by a novel cytoplasmic protease. Interestingly, mutation of the two conserved alanines to asparagines at the DmsA leader cleavage site did not affect the membrane targeting or the processing (15).

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