EXPERIMENTAL PROCEDURES

Purification of proteins—For the purification of CsdA, the cells from the 11.5 L culture were suspended in 150 ml of buffer A (20 mM Tris, 1 mM DTT, pH 7.5) and disrupted by sonication on ice. After removing the cell debris by centrifugation, the crude extract was treated by addition of ammonium sulfate at a concentration of 1.5 M. The mixture was kept on ice for 1 hour and centrifuged. The supernatant solution was filtrated and applied into 50 ml of a Butyl-Toyopearl (TOSOH, Japan) column equilibrated with buffer A containing 1.5 M ammonium sulfate. After that, the column was washed with the same buffer. The fractions containing CsdA confirmed by SDS-PAGE were pooled. Ammonium sulfate powder was added to the collected solution at 1.5 M. Then, the enzyme solution was loaded onto 30 ml of a Butyl-Toyopearl column equilibrated with buffer A containing 1.5 M ammonium sulfate. After the column was washed with 500 ml of the equilibration buffer, the enzyme was eluted with a linear gradient of 1.5-0.5 M ammonium sulfate in buffer A (1 L). The enzyme fractions containing CsdA confirmed by SDS-PAGE were collected and dialyzed against buffer A. The enzyme solution was then applied into a DEAE-Toyopearl (TOSOH, Japan) column (30 ml) equilibrated with buffer A. After the column was washed with 600 ml of buffer A, the adsorbed protein was eluted with 4 L of buffer A containing 150 mM NaCl. The fractions containing CsdA confirmed by SDS-PAGE were collected. Ammonium sulfate powder was added to the enzyme solution at 1.5 M. Then, the enzyme solution was applied to 30 ml of a Butyl-Toyopearl column equilibrated with buffer A containing 1.5 M ammonium sulfate. Without washing, the enzyme was directly eluted with 500 ml of buffer A. The fractions containing CsdA were pooled, dialyzed against buffer A containing 10 µM PLP, and concentrated using the AmiconUltra-15 centrifugal filter device (10K MWCO) (MILLIPORE, Japan). The final preparation of the enzyme was stored at -80 °C until use.

The cell pellet of E. coli expressing SufS from a 12 L culture was suspended in 150 ml of buffer A. After sonication on ice, the cell debris was removed by centrifugation. The supernatant was treated with 1 M ammonium sulfate and centrifuged. Then, SufS in the secondary supernatant was precipitated with 1.75 M ammonium sulfate. After another round of centrifugation, the pellet was resolubilized by buffer A containing 1 M ammonium sulfate. The enzyme solution was filtrated and applied into 50 ml of a Butyl-Toyopearl column equilibrated with buffer A containing 1 M ammonium sulfate. After washing with the same buffer, the column was eluted with 1 L of 0.9 M ammonium sulfate in buffer A and 1 L of buffer A. The fractions containing SufS were collected and dialyzed against buffer A. Then, the sample was loaded onto 100 ml of a DEAE-Toyopearl column and washed with buffer A containing 100 mM NaCl (500 ml). A linear gradient of 100-150 mM NaCl in buffer A (1 L) and 3 L of 150 mM NaCl in buffer A was used to elute the protein. The fractions containing SufS were pooled. Ammonium sulfate powder was added to the collected solution at 1 M. Then, the enzyme solution was loaded onto 50 ml of a Butyl-Toyopearl column equilibrated with buffer A containing 1 M ammonium sulfate. Without washing, the protein was eluted with 1 L of buffer A containing 0.8 M ammonium sulfate and a linear gradient of 0.7-0 M ammonium sulfate in buffer A (500 ml). The collected solution containing SufS was dialyzed against buffer A with 10 µM PLP and then purified by gel filtration with a Hiload 16/60 Superdex 200 column and AKTA explorer 10S (GE Healthcare Biosciences, Japan). The protein was eluted with 1 L of buffer A containing 200 mM NaCl. The pooled enzyme solution of SufS was dialyzed against buffer A with 10 µM PLP and concentrated. The final preparation of the enzyme was stored at -80 °C until use.

IscS was purified from the cells harboring pEF1. The cells from the 4 L culture were suspended in 40 ml of 50 mM potassium phosphate (KPB), 5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4, and sonicated on ice. Then, the mixture was centrifuged to remove the cell debris. The supernatant was filtrated and loaded onto 70 ml of a DEAE-Toyopearl column equilibrated with 10 mM KPB (pH 7.5). After washing with 400 ml

of an equilibration buffer, the proteins were eluted with a linear gradient of 0-0.15 M NaCl (1 L) and 0.15 M NaCl (200 ml) in 10 mM KPB (pH 7.5). The fractions containing IscS were collected. Ammonium sulfate powder was added to the collected solution at 0.9 M. Then, the enzyme solution was applied into 25 ml of a Phenyl-Toyopearl column equilibrated with 10 mM KPB and 0.9 M ammonium sulfate, pH 7.5. The IscS-containing solution passed through the column was concentrated and dialyzed against 10 mM KPB and 1 M ammonium sulfate, pH 7.5. Then, the sample was applied to a Phenyl-Toyopearl column equilibrated with 10 mM KPB and 1 M ammonium sulfate, pH 7.5. After washing with the same buffer, IscS was eluted with a linear gradient of 1-0 M ammonium sulfate in 10 mM KPB (pH 7.5). The fractions containing IscS were pooled and concentrated. During the concentration process, the solvent was exchanged with 10 mM KPB, 1 mM DTT, and 10 μ M PLP, pH 7.5. The final preparation of the enzyme was stored at -80 °C until use.

The N-terminal His₆ tag-containing MoaD and MoeB were purified using His•Bind Kits (Novagen, Japan). The cells were suspended in 30 ml of a binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) and disrupted by sonication. After the cell debris was removed by centrifugation, the supernatant solution was filtrated and applied to a His-Bind Resin Chromatography column (17 ml) pretreated with 100 ml of a strip buffer (0.5 M NaCl, 20 mM Tris-HCl, 100 mM EDTA, pH 7.9), 100 ml of sterile deionized water, 100 ml of a charge buffer (50 mM NiSO₄), 100 ml of sterile deionized water, and 100 ml of the binding buffer. Then, the column was washed with 100 ml of the binding buffer and 200 ml of the wash buffer (0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole, pH 7.9), and the absorbed enzyme was eluted with a linear gradient of 1× wash buffer to 0.5× elute buffer (0.5 M NaCl, 20 mM Tris-HCl, 1.0 M imidazole, pH 7.9) (500 ml). For the purification of MoeB, the fractions containing enzyme were collected and dialyzed against 20 mM Tris-HCl and 1 mM DTT, pH 8.0. Then, the sample was concentrated by using an AmiconUltra-15 centrifugal filter device (10K MWCO). The final preparation of the enzyme was stored at -80 °C until use. For the purification of MoaD, the collected sample (90 ml) was concentrated to 3 ml using an AmiconUltra-15 centrifugal filter device (3K MWCO) and diluted with 100 ml of the binding buffer. The protein solution was then loaded onto 5 ml of a His-Bind Resin Chromatography column. After the same washing described before, MoaD was eluted with a linear gradient of 1× wash buffer to 0.5× elute buffer (250 ml). The fractions were pooled, dialyzed against 20 mM Tris-HCl, 1 mM DTT, pH 8.0, and concentrated using an AmiconUltra-15 centrifugal filter device (3K MWCO). The final preparation of the enzyme was stored at -80 °C until use.

All of the purified proteins were analyzed by SDS-PAGE (Fig. S1).

HPLC/ESI-MS analysis—HPLC/electrospray ionization mass spectrometry (ESI-MS) was performed with an API 3000 triple quadrupole instrument equipped with a nebulizer-assisted electrospray ionization interface (PE Sciex, Canada) under the same chromatographic conditions as described in the above-mentioned HPLC experiment. The effluent was split in a ratio of 1:19, resulting in 50 μ l/min of it being admitted into the mass spectrometer. The ion spray, orifice, and focusing ring voltages were set to 5.0 kV, 40 V, and 200 V, respectively. Positive ion mass spectrometry (MS) spectra were acquired at 3 seconds per scan over a range of m/z 200-500.

ESI-MS/MS analysis—The peaks of 300-nm absorbing material eluting approximately between 5 and 7 min in the above-described HPLC experiment were collected and analyzed using ESI-MS/MS with the same mass spectrometer as in the above-mentioned HPLC/ESI-MS experiment. The channel electron multiplier voltage was set to 2.8 kV; the MS/MS spectra were acquired over a range of m/z 30-360.

UV-Vis spectroscopy—For ultraviolet-visible (UV-Vis) spectroscopy, the fractions of accumulated compound from the Capcell Pak C₁₈ SG120 column were further purified using ion-exchange HPLC with an IEC QA-825 column (Showa Denko, Japan) equilibrated with water. Compound Z was eluted with a linear gradient of 0-1 M NaCl. The fractions of 300-nm absorbing material eluting between 20 and 22 min were collected and dispensed in 200-µl aliquots in 1.5-ml tubes. Next, 0.01 N HCl or 0.01 N NaOH was added to the aliquots in order to adjust the pH to 1, 5, or 12. The absorption spectrum of the sample was measured from 300

nm to 450 nm with a UV2450 spectrophotometer (Shimadzu, Japan).

RESULTS AND DISCUSSION

Identification of the compound accumulated in the $\Delta iscS$ mutant. To ascertain whether the 300-nm absorbing material accumulated in the $\Delta iscS$ and $\Delta moaD$ strains is compound Z, we performed HPLC/ESI-MS analysis of the acid extracts of these strains. The positive ion mass spectra obtained for the 300-nm absorbing material accumulated in the $\Delta iscS$ strain were identical to those accumulated in the $\Delta moaD$ strain (Fig. S4). Since the theoretical mass for compound Z with the formula $C_{1q}H_{10}N_5O_7P$ is 343, the ions at m/z 344 and m/z 366 in the spectrum were attributed to be [M + H] and [M + Na] ions, respectively.

Furthermore, fractions of the compounds eluted at 5.75 min from the $\Delta iscS$ and $\Delta moaD$ mutants were collected and analyzed using ESI-MS/MS spectrometry. Two ions at m/z 246 and m/z 344 were detected in the samples of both the $\Delta iscS$ and $\Delta moaD$ mutants (Fig. S5A, B). The ion at m/z 344 corresponds to the parent $[M + H]^{-1}$ ion of compound Z and that at m/z 246 has properties that are in accordance with those of a predicted daughter ion that is probably formed via cleavage of the 1,3,2-dioxaphosphorinane ring of compound Z (Fig. S5C).

Compound Z is known to exhibit characteristic UV-Vis absorption properties at different pH values (1). Therefore, we analyzed the 300-nm absorbing material accumulated in the acid extracts of the $\Delta iscS$ strain by UV-Vis spectroscopy. Fractions eluting between 5 and 7 min from a Capcell Pak C₁₈ column were collected and further purified using an ion exchange column. The main peak detected at 21.15 min was collected, and the absorption spectra of this fraction were measured at pH 1, 5, and 12. At pH 1, the purified sample exhibited an absorption maximum at 315 nm and a shoulder at 370 nm (Fig. S6A). When the pH was adjusted to 5, it displayed an absorption peak at 310 nm and a broad absorption band centered at 350 nm (Fig. S6B). At pH 12, the sample exhibited an absorption maximum at 370 nm (Fig. S6C). These characteristic spectral features of the material accumulated in the $\Delta iscS$ mutant are similar to those of compound Z (1).

These data indicate that the 300-nm absorbing material eluted at 5.75 min in the HPLC analysis of acid extracts of the $\Delta iscS$ and $\Delta moaD$ mutants is compound Z. These results confirm the accumulation of compound Z in the *moaD*-deficient strain and provide the first demonstration of compound Z accumulation in an *iscS*-deficient bacterial strain.

Genetic complementation of the $\Delta iscS$ mutant. To further confirm the involvement of *iscS* in the biosynthesis of MPT, we performed a genetic complementation experiment. The cells containing the plasmid were grown in the presence of 100 µg/ml ampicillin and 1 mM IPTG at 37°C. As expected, the introduction of the pIscS plasmid expressing IscS into the $\Delta iscS$ strain resulted in absolutely no accumulation of compound Z, differently from that in the parent $\Delta iscS$ strain (Fig. S7).

FIGURE LEGENDS

<u>Fig. S1.</u> SDS-PAGE (15% polyacrylamide gel) analysis of the purified proteins. Lane 1, MoaD (10.9 kDa); lane 2, MoeB (28.9 kDa); lane 3, CsdA (43.2 kDa); lane 4, SufS (44.4 kDa); lane 5, IscS (45.1 kDa).

Fig. S2. Amino acid sequences of MoeB in various organisms were aligned with CLUSTALW. Two highly conserved cysteine residues were found (Cys142 and Cys187 in MoeB of *Escherichia coli*).

Fig. S3. Analysis of the sulfur transfer from cysteine desulfurases to MoaD by ³⁵S-labeled L-cysteine. Lane 1, CsdA and MoeB; lane 2, CsdA, MoeB, and MoaD; lane 3, CsdA and MoeB (C142A, C187A); lane 4, CsdA, MoeB (C142A, C187A), and MoaD; Lane 5, SufS and MoeB;

lane 6, SufS, MoeB, and MoaD; lane 7, SufS and MoeB (C142A, C187A); lane 8, SufS, MoeB (C142A, C187A), and MoaD; Lane 9, IscS and MoeB; lane 10, IscS, MoeB, and MoaD; lane 11, IscS and MoeB (C142A, C187A); lane 12, IscS, MoeB (C142A, C187A), and MoaD. The gel was stained with Coomassie Brilliant Blue (A), and the [³⁵S] radioactivity was visualized on a phosphor imaging plate (B).

<u>Fig. S4.</u> HPLC/ESI-MS of compound Z accumulated in the $\Delta iscS$ and $\Delta moaD$ strains. The positive ion mass spectra of the 300-nm absorbing material accumulated in the $\Delta iscS$ (A) and $\Delta moaD$ (B) strains are shown.

Fig. S5. ESI-MS/MS analysis of compound Z accumulated in the $\Delta iscS$ and $\Delta moaD$ strains. MS/MS spectra of the 300-nm absorbing material accumulated in the $\Delta iscS$ (A) and $\Delta moaD$ (B) strains are shown. A possible scheme for the cleavage of compound Z and the structural assignment of the major fragment ion under the ESI-MS/MS conditions are shown in C.

<u>Fig. S6.</u> UV-Vis absorption spectra of compound Z isolated from the $\Delta iscS$ strain. The absorption spectra of the $\Delta iscS$ -derived compound Z fraction eluting between 20 and 22 min from an IEC QA-825 column were measured at pH 1 (*A*), pH 5 (*B*), and pH 12 (*C*).

<u>Fig. S7.</u> Genetic complementation of the $\Delta iscS$ mutant by the expression of IscS. After oxidization with 1% I₂ and 2% KI, the acid extracts from the wild-type(pUC118), $\Delta iscS$ (pUC118), and $\Delta iscS$ (pIscS) were analyzed using HPLC as described in Experimental Procedures. The arrow indicates the accumulation of compound Z in the $\Delta iscS$ (pUC118) strain.

REFERENCE

 Johnson, J. L., Wuebbens, M. M., and Rajagopalan, K. V. (1989) J. Biol. Chem. 264, 13440-13447.



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MoeB_Thermosynechococcus	1	MLNPDLSTIQUTKDDYERYS
MoeB_Azobacteroides	1	MNFTSEQIERYS
MoeB_Homo	1	MASREEVLALQAEVAQREEELNSLKQKLASALLAEQEPQPERLVPVSPLPPKAAUSRDBIURVS
MoeB_Gluconacetobacter	1	MIPDFSEABIORY
MoeB_Methylobacterium	1	
MoeB_Escherichia	1	MAELSDQBMLRY
MoeB_Shigella	1	MAELSDQBMLRYN
MoeB_Yersinia	1	MPEDSDAQALRY
MoeB_Neisseria	1	MTTTEHDNDDAFL
MoeB_Xylella	1	STHLPDTNAEIVLICQSGQRSRHTGERLQAAGYRRLYSVAGGTDAWRKAGLPLLRPILSTDEQDFME
MoeB Thermosynechococcus	21	RHLIDPEVGVEGOKRUKAASVICIGTGGIGSPLLLVIAAAGVGRIGIVDFDVVDSSNLOROVIHGTSWVG
MoeB Azobacteroides	13	RHTILLODVGLEGOEKTGDSKVLVIGVGGLGAPVLFYLAAAGIGTLGUIDREVVDLSNLOROUTHFASDVG
MoeB_Homo	65	ROAVLEELGVHGOLRLGTACVLIVGEGGLGEPLAOYLAAAGVGRLGIVDVDVDVMSNLAROVLHGEALAG
MoeB Gluconacetobacter	15	RHILLPEIGGTGŐAALRAASVLIVGAGGLGSPLGLYLAAAGVGRIGLVDDDVVDLSNLORŐFAHTTGRIG
MoeB_Methylobacterium	16	RHEVLREVGGPGOARLKAGRVLVIGAGGLGAPLIQYLAAAGIGTIGIVDDDTVSLSNLOROVIHGTPDIG
MoeBEscherichia	14	ROILLRGFDFDGOEALKDSRVLIVGLGGLGCAASOYLASAGVGNLTLLDFDTVSLSNLOROTLHSDATVO
MoeB Shigella	14	ROIILRGFDFDGQEALKDSRVLIVGLGGLGCAASQYLASAGVGNLTLLDFDTVSLSNLQRQTLHSDATVG
MoeB Yersinia	14	ROIVLRGFDFDGQEKLNAAKVLIVGLGGLGCAAAQYLTVAGVGHLTLLDFDKVSLSNLQRQVLHRDNRIG
MoeB_Neisseria	18	RHILLDEIGIEGQQKLSAAHILVVGCGGLGAAALPYLAASGVGTLTIADSDTVELHNLQRQVAFDEGDVG
MoeB_Xylella	71	RHLRLPHIGPHGQQRLAEARVLLIGAGGLGSPAAFYLTAAGVGHLRIADHDTVERSNLQRQILHVDAELG
MoeB Thermosynechogogers	0 1	
MoeB Azobacteroides	83	K TKWOSAKEKI KOTNESWKWYY O ESEN WINAFD VI KUNDETWICTEN DEWENTNE WULL A CHEP SUC
MoeB Homo	135	OAKA FSAAA SIREDINS AVECUPYTO AITPATIL DIVERVOUND SINU PAPERANN DAVIA CODING SINU
MoeB Gluconacetobacter	85	ORKWESTARE AMRA WINDLING WE PHAT RIMDAGNAR ALANG GYDIAYG GSDNJR TRY I WAD AGA L PO D THI VSA
MoeB Methylobacterium	86	RPKVESAAETVARIN PHVRVETHACRITPENAPAINI AGVDI VADGSDAFATRVAVSDACE A GVDI VTA
MoeB Escherichia	84	OPKVESARDANTE IN PHIAIT PVNALMDDAELAAM TAEHDIAVIDCTDNWAVENOINAGCEAAEVPIAVSC
MoeB Shigella	84	OPKVESARDALTE INPHIAITPVNALMDDAELAAMTAEHDIAVLDCTDNVAVENOLNAGCFAARVDAVS
MoeB Yersinia	84	MSKVASAALTISEMNPSLIIKTIDAOMDDEOLAIATAEHOMVIDCTDNVASEEOLNBICHAOPKPIVSC
MoeB Neisseria	88	KLKTBALAGRIKRINH TVNVRAVNEKLDGCRLTGDVOA ADIVLDCCDNYATROAVNRACVOTKTPIAVSG
MoeB Xvlella	141	VEKAASAARRUSAIN PRVOVEAMOVRANSSNIETULOD VDVAIDGADNEPARVIJINDACVKMGTPLVVG
MoeB_Thermosynechococcus	161	IFRFEGQATVFNYEGGPNYRDLYPEPPPPGLVPSCAEGGVLGILPGIIGVIQATETIKIILG
MoeB_Azobacteroides	153	ILQFEQQTLTY-LPGTMCYRCVFHSPPPSNSIPTCSQAGVLGTIAGILGTIQATEVLKFLIGT
MoeB_Homo	205	ALRFEGQITVYHYDGGPCYRCIFPQPPPAETVTNCADGGVLGVVTGVLGCLQALEVLKIAAGI
MoeB_Gluconacetobacter	155	VLRFEGOISTERPHRGGPCYRCLYPAPPODGAVPSCAEAGVFGAVTGVMGTLOATEALKEILEI
MoeB_Methylobacterium	156	LGAEDCSLUTIRAHETGPTGEPNPTVRCLEPSPPPPGSVAPCSBAGVLGALAGVMCSLMAMEVIRALAD
MoeB_Escherichia	154	AIRMEGOITVFTYODGEPCYRCLSRLFGENALTCVEAGVMAPLIGVIGSLOAMEAIRMLAGY
MOEB_Shigella	154	AIRMEGOITVFTYDDGEPCYRCLSRL-FGENALTCVEAGVMAPLIGVIGSLOAMBAIRLLAG
MOEB_IEFSINIA	154	AIR AGOVSVATINGDOPOTICLS RL-FS DARLTOV MACHARPLY GILGANDARAINLIG
MoeB Xvlella	211	
MOED_AVIEIIA	211	
		-
MoeB_Thermosynechococcus	224	GTTISGRIJIFNAMEMKFRELKERPNPERPVIDKLIDYEEFCGIRQAKAQEAAQMAEIPEMTV
MoeB_Azobacteroides	215	GELITNRII TFNAKSMIFRTIKINKNRECLIGGSYPSITNLAESERIVCELENK
MoeB_Homo	268	GPSYSGSILLFDALRGHFRSIRLRSRRLDCAACGERETVTDLLDYEAFCGSSATDKCRSLQLLSPEERVS
MoeB_Gluconacetobacter	219	GESIDGRILVWDALAARFHTVRLAPDPDCALCGPHASIHDLSAHGAADVR
MoeB_Methylobacterium	226	GEPINGRIJMVDARSMRFETLAXAWDPDNPLNGVRTGPAAEPAGHPLPPR
MoeB_Escherichia	216	ekiaastekivwyDAMTCOBREM() MRNPGCEVCCO
MOEB_Shigella	216	ekijastek i vm xD/AMTCQ3/REM(YMRN) PGGEV/CGQ
Moes_Yersinia	216	GOVISGNIVMIDAMTAEBNSENJAKDANGEVIGKDELAKVADQPVL
MOCE_NEISSEFIA	221	
WOOD_VATETIG	2/0	GALENAGATEGET CATEGARY CAN BERNAR PORT PORT OF THE ARE CARELED TO TAKE CARELED TAKE TAKE TAKE TAKE TAKE TAKE TAKE TAKE
MoeB_Thermosynechococcus	287	QELKALMDSGAQDYVLVDVRNPNEYEIARIPGSVLVPLSEIENGPGVEKIRSLLNGHR
MoeB_Azobacteroides		· · · · · · · · · · · · · · · · · · ·
MoeB_Homo	338	VTDYKRLLDSGAFHLLLDVRPQVEVDICRLPHALHIPLKHLERRDAESLKLLKEAIWEEKQGTQEGAAVF
MoeB_Gluconacetobacter		
MoeB_Methylobacterium		
MoeB_Escherichia		
MoeB_Shigella		
MoeB_Yersinia		
MOCE_NEISSERIA		
WOOD_VATETIG		
MoeB_Thermosynechococcus	345	LLVHCKMGGRSAKALGILKEAGIEGINIKGGINAWSQEVDPSVPTY
MoeB_Azobacteroides		
MoeB_Homo	408	IYVICKLGNDSQKAVKILQSLSAAQELDPLTVRDVVGGLMAWAAKIDGTFPQY
MoeB_Gluconacetobacter		
MoeB_Methylobacterium		
MoeB_Escherichia		
MoeB_Shigella		
Moes_Yersinia		
MOCE_NEISSEFIA		
NOCD_ATTELLA		





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Fig. S5 Zhang et al.



Fig. S6 Zhang et al.



Fig. S7 Zhang et al.