Co-opting sulphur–carrier proteins from primary metabolic pathways for 2-thiosugar biosynthesis

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Sulphur is an essential element for life and is ubiquitous in living systems. Yet how the sulphur atom is incorporated into many sulphur-containing secondary metabolites is poorly understood. For bond formation between carbon and sulphur in primary metabolites, the major ionic sulphur sources are the persulphide and thio-carboxylate groups on sulphur-carrier (donor) proteins. Each group is post-translationally generated through the action of a specific activating enzyme. In all reported bacterial cases, the gene encoding the enzyme that catalyses the carbon–sulphur bond formation reaction and that encoding the cognate sulphur–carrier protein exist in the same gene cluster. To study the production of the 2-thiosugar moiety in BE-7585A, an antibiotic from Amycolatopsis orientalis, we identified a putative 2-thioglucose synthase, BexX, whose protein sequence mode of action seem similar to those of ThiG, the enzyme that catalyses thiazole formation in thiamine biosynthesis. However, no gene encoding a sulphur-carrier protein could be located in the BE-7585A cluster. Subsequent genome sequencing uncovered a few genes encoding sulphur-carrier proteins that are probably involved in the biosynthesis of primary metabolites but only one activating enzyme gene in the A. orientalis genome. Further experiments showed that this activating enzyme can adenylate each of these sulphur-carrier proteins and probably also catalyses the subsequent thiolation, through its rhodanese domain. A proper combination of these sulphur–delivery systems is effective for BexX-catalysed 2-thioglucose production. The ability of BexX to selectively distinguish sulphur-carrier proteins is given a structural basis using X-ray crystallography. This study is, to our knowledge, the first complete characterization of BexX and near the BE-7585A biosynthetic gene cluster impeded further functional characterization of BexX.

To search for the sulphur–carrier protein required for the BexX reaction, the entire genome of A. orientalis was sequenced. A total of 9,210 coding open reading frames were identified in approximately 9.8 megabases of genomic DNA, including genes encoding five cysteine desulphurase homologues, five rhodanese homologues and four sulphur–carrier protein homologues (ThiS, MoaD, CysO and MoaD2). Because it is unique in the genome and because it is not part of the molybdopterin or cysteine biosynthetic gene clusters in A. orientalis, the gene cluster in A. orientalis does not contain thiF, the gene that encodes the ThiS-activating enzyme, which is essential for converting ThiS to its thiocarboxylate form. The corresponding activating enzymes for MoaD and CysO are also missing from the respective molybdopterin and cysteine biosynthetic gene clusters. To test the proposed function of MoeZ, the A. orientalis thiF-like domain at the amino terminus and a rhodanese homology domain at the carboxy terminus (Extended Data Fig. 2 and Supplementary Table 1). Because it is unique in the genome and because it is not part of the molybdopterin or cysteine biosynthetic gene clusters, this protein, MoeZ, may be the universal activating catalyst for thiocarboxylate production in A. orientalis (Fig. 2a).

To test the proposed function of MoeZ, the ThiS and MoeZ proteins of A. orientalis were heterologously expressed in E. coli, each with an N-terminal His-tag. When ThiS was incubated with MoeZ and ATP, an electrospray ionization–mass spectrometry (ESI–MS) signal corresponding to adenylated ThiS (9) was detected, together with a few peaks probably derived from the reaction of the labile adenylated ThiS with buffer components (Extended Data Fig. 3c). On addition of excess bisulphide, complete conversion of 9 to its thiocarboxylate form (10) was observed (Extended Data Fig. 3d). Control experiments using bisulphide derived from G6P. The crystal structure of the BexX-substrate complex has now been determined to 2.3 Å resolution (Extended Data Fig. 1), confirming that G6P is covalently attached to the lysine at position 110 (Lys 110) of BexX (Fig. 1c). However, the absence of genes encoding potential sulphur–transfer enzymes, including common sulphur–carrier proteins, cysteine desulphurases, and rhodanese-like proteins, suggest that the BE-7585A biosynthetic gene cluster impeded further functional characterization of BexX.
in the absence of MoeZ showed no change in the original ThiS signals. These results demonstrated that MoeZ can charge ThiS to its ready-to-use thiocarboxylate form (Fig. 2a). The activated ThiS-COS\(^{-}\) was next incubated with the BexX-G6P complex (2). If sulphur transfer occurs and the resultant 2-thiosugar product (3) is released from the enzyme, a shift in the mass signal corresponding to the BexX-G6P complex to that of the free enzyme is anticipated (Fig. 2b). However, no increase in free BexX was observed in the presence of ThiS-COS\(^{-}\) (Fig. 2c–e), hence ruling out ThiS (and bisulphide) as the sulphur donor for BexX in 2-thiosugar formation.

To assess the competence of MoaD, CysO and MoaD2 of A. orientalis as sulphur-carrier proteins in BexX-catalysed reactions, N-terminally His\(_{10}\)-tagged MoaD, CysO and MoaD2 were prepared. Similar to the case for ThiS, thiocarboxylation of each protein in the presence of MoeZ, ATP and sodium sulphide (NaSH) was confirmed by mass spectrometric analysis (Extended Data Fig. 3f–k). Because activated MoaD was generated in small quantities at low purity, only CysO and MoaD2 were incubated with the BexX-G6P complex. The relative intensities of the mass signals corresponding to BexX-G6P (2) and the free enzyme were monitored before and after the addition of the activated sulphur-carrier proteins. Only signal ascribed to free BexX was discernible after treatment with CysO or MoaD2 (Fig. 2f, g). To gain further evidence, the thiosugar product (3) was derivatized with monobromobimane (mBBr)

**Figure 1** | Proposed mechanism for 2-thiosugar formation in BE-7585A biosynthesis. a. The active site lysine residue (Lys110)\(^{\text{C1}}\) of BexX initially forms an imine bond with G6P (1) at the C1 position, which is isomerized first to a C1–C2 enamine and then a C2-ketone intermediate (2) (the carbon atoms are numbered). Subsequent nucleophilic attack by a sulphur donor (red) occurs at the C2 position of 2, resulting in the incorporation of a sulphur atom in the 2-thio-G6P (2SG6P) (3) product. One arrow indicates one step, and two arrows suggest multiple steps between the transformations. b. ThiG-catalysed thiazole phosphate biosynthetic pathway. c. Stereo view of BexX active site.

**Figure 2** | Activation of sulphur-carrier proteins and sulphur transfer to the BexX-G6P complex. a. MoeZ-catalysed acyl adenylate of a sulphur-carrier protein (SCP)—for example, ThiS, MoaD, CysO or MoaD2—followed by nucleophilic attack of bisulphide, yielding the corresponding SCP-thiocarboxylate to produce 2SG6P (3), which was further derivatized with mBBr to give 2SG6P-bimane (11). c–g, Deconvoluted ESI–MS analyses of as-isolated C-His\(_{6}\)-BexX (where C denotes carboxy terminal) (calculated mass (calcd), 28,488), showing that the major species is 2 (28,730 calcd) (c), and the sulphur-transfer reactions using ThiS (d)—and a lower mass range of the same reaction showing N-His\(_{6}\)-ThiS-COSH (8,663 calcd) and its N-glucosamine derivatives (8,841 calcd) (where N denotes amino terminal) (e) (see also Extended Data Fig. 3)—CysO (f) or MoaD2 (g). h, HPLC traces (left) for BexX-catalysed reactions (right). In the reaction with ThiS, the amount of AMP, probably derived from partial decomposition of ATP during incubation, was comparable to that in the control with no added SCP.
before high-performance liquid chromatography (HPLC) analysis to yield 11, thereby facilitating detection (Fig. 2b). Indeed, when CysO or MoaD2 was used, a new peak (product peak) clearly appeared, together with an increase in AMP production (Fig. 2h, traces 3 and 4). The product peak was isolated and characterized as 2-thioglucose-6-phosphate-bimane (11) by ESI–MS and NMR spectroscopy (Supplementary Methods). Each assay sample was also treated with alkaline phosphatase, and the dephosphorylated product matched well with the synthetic standard (Extended Data Fig. 4). As expected, no thiosugar product was detected in the sample containing ThiS. These results firmly established that BexX-catalysed 2-thiosugar formation was able to proceed in the presence of either CysO-COS or MoaD2-COS but not ThiS-COS. These two examples clearly reveal the capability of some sulphur-delivery enzymes to ‘moonlight’ in natural product biosynthesis, bridging the biosynthetic pathways of primary and secondary metabolites.

Thiocarboxylated sulphur-carrier proteins recognize their partners through specific protein–protein interactions20–22. Because BexX has 37% sequence similarity to ThiG22 and because the two enzymes are structurally homologous, it was surprising to find that ThiS is not a sulphur-carrier protein for BexX. To understand the sulphur-carrier protein specificity of BexX, the BexX–CysO structure was determined to 2.6 Å resolution (Fig. 3a and Extended Data Fig. 1f). We were unable to crystallize BexX–MoaD2; however, because of the compact ubiquitin-like fold and similar sizes of CysO (90 residues) and MoaD2 (96 residues), we were able to construct a reliable homology model for BexX–MoaD2 using the BexX–CysO structure as a template. We also constructed a hypothetical model of BexX–ThiS using ThiS from Thermus thermophila (Protein Data Bank (PDB) ID, 2HTM) as a guide.

CysO and MoaD2 superimpose well, with a root mean squared deviation (r.m.s.d.) of 0.1 Å for 80 Cα atoms. By contrast, CysO and ThiS show significant differences, especially in the loop regions, with an r.m.s.d. of 2.7 Å for 43 Cα carbon atoms (Fig. 3b and Extended Data Figs 1d, e and 5). The most significant difference between ThiS (66 residues) and either CysO or MoaD2 is the insertion of two additional α-helices, which are located at the BexX–sulphur-carrier protein interface (Extended Data Fig. 5). As a result, the amount of accessible surface area buried on complex formation is ~1,000 Å² for BexX–CysO and BexX–MoaD2 but only ~600 Å² for BexX–ThiS (Extended Data Figs 5 and 6a). CysO contributes 19 residues and BexX contributes 26 residues to the interface of BexX–CysO, similarly to the 16 residues contributed by MoaD2 and the 23 by BexX in BexX–MoaD2. By contrast, only eight ThiS residues contribute to the interface in the BexX–ThiS model. Ten of the interface residues are conserved between CysO and MoaD2, but only four of these are conserved in ThiS (Extended Data Fig. 5a). The hydrogen-bonding scheme is also conserved between BexX–CysO and BexX–MoaD2 (Extended Data Fig. 6b–d). A comparison of the BexX–CysO complex and the Bacillus subtilis ThiG–ThiS complex (PDB ID, 1TYG)23 complex provides further insight. Superposition of BexX–CysO and ThiG–ThiS results in an r.m.s.d. of 1.7 Å for the BexX–ThiG core (Fig. 3c); however, CysO and ThiS do not overlay well (r.m.s.d. ~40 Å). Thus, even though the overall sulphur-carrier protein folds are similar, and even though each sulphur-carrier protein is positioned to insert its C-terminal tail into the active site of its partner (Extended Data Fig. 6e, f), the selection of CysO or MoaD2 by BexX is clearly determined by the interface interactions.

Finally, we also examined whether the C-terminal rhodanese domain of MoeZ has a role in sulphur transfer. In a typical rhodanese reaction, the conserved cysteine residue in rhodanese (for example, Cys 360 in MoeZ) is converted to a persulphide group in the presence of thiolsulphate or through the action of a cysteine desulphurase using l-cysteine as the sulphur source27. Because the resultant persulphide is a known sulphur donor28, it can be used to charge the adenylation sulphur-carrier proteins to the thiocarboxylate forms (Fig. 4). To test this potential second role of MoeZ as a sulphur donor, MoeZ was incubated with CysO or MoaD2, first in the presence of ATP and thiolsulphate (with no addition of reducing agent to prevent bisulphide formation). MoeZ was observed to catalyse the thiolation of both CysO and MoaD2 but not when replaced with a MoeZ(Cys360Ala) mutant (Extended Data Fig. 7), which retained a similar level of adenylation activity to wild-type MoeZ (Extended Data Fig. 8). These observations are consistent with the C-terminal rhodanese domain of MoeZ being involved in sulphur transfer. Next, we assessed BexX-catalysed 2-thiosugar formation with MoeZ and MoaD2 in the presence of ATP, using either thiosulphate (Fig. 4a) or l-cysteine and a cysteine desulphurase (CD4, Extended Data Table 1) from A. orientalis (Fig. 4b) as the primary sulphur sources. As expected, the 2-thioglucose product was detected in both cases in the absence of reducing agents (Extended Data Fig. 9). Taken together, these results support the probable dual role of MoeZ in catalysing both the adenylation and thiolation of sulphur-carrier proteins in A. orientalis.

In summary, we carried out whole-genome sequencing of A. orientalis and demonstrated that the sulphur delivery for 2-thiosugar production in the biosynthesis of BE-7585A is achieved by hijacking the sulphur-transfer systems from primary metabolism. Although the overall reaction mechanism of 2-thiosugar formation resembles that of thiamine.
bio-synthesis, BexX cannot utilize the corresponding sulphur-carrier protein, ThiS, from the thiamine pathway. Instead, the sulphur-carrier proteins that are probably involved in cysteine (CysO) and molybdothionine (MoaD2) biosynthesis are recruited to transfer their C-terminal thio-carboxylate sulphur to the BexX-G6P complex (2). Two structural snapshots, of the BexX-G6P ketone intermediate (2) and the BexX-CysO heterotetramer, provide significant insight into the proposed sulphur incorporation mechanism, as well as the structural basis by which sulphur-carrier proteins are selected. These results indicate that a functional alliance between a sulphur-carrier protein and its acceptor protein is not specific but is entirely random. The assembly of operational sulphur-transfer machinery from components of the sulphur-carrier systems of primary metabolism, to deliver a sulphur atom to produce 2-thiosugars, is an efficient strategy for the biosynthesis of a relatively rare metabolite. Such an ad hoc approach to sulphur transfer may be a paradigm for yet undiscovered pathways of sulphur-containing natural product biosynthesis. The revelation that MoeZ is the universal activating enzyme for all known sulphur-carrier proteins in A. orientalis is another significant finding. The presence of only a single ThiF-type enzyme in the entire genome has also been noted in several other microorganisms (Extended Data Table 2). The charging of multiple sulphur-carrier proteins in different biosynthetic pathways by a single activating enzyme may be a common phenomenon in nature (at least in the Actinomycetales).

In addition, the finding that functional pairs of sulphur-carrier proteins and their acceptor proteins are not necessarily located in the same gene cluster raises the possibility that some cryptic gene clusters in various genomes may encode pathways for the biosynthesis of sulphur-containing natural products. Such a possibility has generally been overlooked in recent efforts to deconstruct genomic information.

**METHODS SUMMARY**

Whole-genome sequencing of Amycolatopsis orientalis was carried out at the High Throughput Sequencing Core Facility at Academia Sinica, Taiwan, using a 454 GS FLX Titanium analyser (Roche) and a Genome Analyzer IIx (Illumina). Contig extension and genome annotation were carried out using Glimmer (Gene Locator and Interpolated Markov ModelIDER) version 3.0 (ref. 25), TRNAscan-SE\(^*\) and RNAmer\(^*\). The thiS, moaD, cysO, moaD2 and moeZ genes were PCR-amplified from A. orientalis genomic DNA\(^*\) and ligated into a pET22b(+) vector (Novagen). The resultant plasmids were overexpressed in the Escherichia coli BL21 Star (DE3) strain (Invitrogen) and purified under conditions similar to those previously described for preparing BexX.\(^*\) Each of the purified sulphur-carrier proteins (50–90 μM) was subjected to ESI–MS analysis before and after incubation with 80 μM MoeZ and 5 mM ATP in 100 mM Tris–HCl buffer (pH 8.0) containing 5 mM MgCl\(_2\) in the presence or absence of 10 mM NaSH. The corresponding sulphur-carrier protein thiocarboxylates generated in situ were incubated with 100 μM BexX and 2 mM G6P (4) in 50 mM NH\(_4\)HCO\(_3\) buffer, pH 8.0, at 25 °C for 8 h to yield the 2-thio-D-glucose-6-phosphate product (3). The resultant reaction mixture was then added to a solution of 5 mM mBr in methanol to give 11 and subjected to HPLC analysis. Crystals of BexX-G6P were grown from 40% (v/v) polyethylene glycol (PEG) 300, 0.2 M calcium acetate and 0.1 M sodium cacodylate–HCl, pH 6.5, and crystals of BexX-CysO complexes were grown from 28% PEG 4000, 0.2 M LiSO\(_4\) and 0.1 M Tris, pH 8.0. Data were collected at the Cornell High Energy Synchrotron Source (CHESS) and the Advanced Photon Source (APS). The structures were determined by molecular replacement.

**Online Content**

Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Parry, R. J. in Comprehensive Natural Products Chemistry Vol. 1 (eds Meth-Cohn, O., Barton, D. & Nakanishi, K.) 825–863 (Elsevier Science, 1999).
2. Fontecave, M., Ollagnier-de-Choudens, S. & Mulliez, E. Biological radical sulfur insertion reactions. Chem. Rev. 103, 2149–2166 (2003).
3. Mueller, E. G. Trafficking in persulfides: delivering sulfur in biosynthetic pathways. Nature Chem. Biol. 2, 185–194 (2006).
4. Kessler, D. Enzymatic activation of sulfur for incorporation into biomolecules in prokaryotes. FEMS Microbiol. Rev. 30, 825–840 (2006).
5. Burroughs, A. M., Iyer, L. M. & Aravind, L. Natural history of the E1-like superfamily: implication for adenylation, sulfa and transfer, and ubiquitin conjugation. Proteins 75, 895–910 (2009).
6. Sasaki, E., Ogasa-wara, Y. & Liu, H.-W. A biosynthetic pathway for BE-7585A, a 2-thiosugar-containing angucycline-type natural product. J. Am. Chem. Soc. 132, 7405–7417 (2010).
7. Sasaki, E. & Liu, H.-W. Mechanistic studies of the biosynthesis of 2-thiosugars: evidence for the formation of an enzyme-bound 2-ketosulfide intermediate in BexX-catalyzed reaction. J. Am. Chem. Soc. 132, 15544–15546 (2010).
8. Thibodeaux, C. J., Melanço, C. E. & Liu, H.-W. Unusual sugar biosynthesis and natural product glycodiversification. Nature 446, 1008–1011 (2007).
9. Thibodeaux, C. J., Melanço, C. E. & Liu, H.-W. Natural product sugar biosynthesis and enzymatic glycodiversification. Angew. Chem. Int. Edn Engl. 47, 9814–9859 (2008).
10. Liu, C.-L., McCarty, R. M. & Liu, H.-W. The biosynthesis of nitrogen-, sulfur- and high-carbon chain-containing sugars. Chem. Rev. 42, 4377–4407 (2013).
11. Braunschweiler, A. & Seebeck, F. P. Identification and characterization of the first ovotitloid biosynthetic enzyme. J. Am. Chem. Soc. 133, 1757–1759 (2011).
12. Stark, J. H. in The biosynthesis of the thiazole moiety of thiamin pyrophosphate (Edwin B.), Biochem. J. 42, 12430–12438 (2003).
13. Begley, T. P. Cofactor biosynthesis: an organic chemist’s treasure trove. Nat. Prod. Rep. 23, 15–25 (2006).
14. Jurgenson, C. T., Begley, T. P. & Ellick, S. E. The structural and biochemical foundations of thiamin biosynthesis. Annu. Rev. Biochem. 78, 569–603 (2009).
15. Iyer, L. M., Burroughs, A. M. & Aravind, L. The pyrrocatenolic prevention of the ubiquitin-signaling system and the early evolution of ubiquitin-like β-grasp domains. Genome Biol. 7, R60 (2006).
16. Mihara, H. & Ezaki, N. Bacterial cysteine desulfurases: their function and mechanisms. Appl. Microbiol. Biotechnol. 60, 12–23 (2002).
17. Cipollone, R., Ascenzi, P. & Visca, P. Common themes and variations in the thiamine biosynthesis. J. Bacteriol. 51, 51–59 (2007).
18. Schwarz, G., Mendel, R. R. & Robe, M. W. Molybdenum cofactors, enzymes and pathways. Nature 460, 839–847 (2009).
19. Burns, K. E. et al. Reconstitution of a new cysteine biosynthetic pathway in Mycobacterium tuberculosis. J. Am. Chem. Soc. 127, 11602–11603 (2005).
20. Jurgenson, C. T., Burns, K. E., Begley, T. P. & Ellick, S. E. Crystal structure of a sulfur-carrier protein complex found in the cysteine biosynthetic pathway of Mycobacterium tuberculosis. Biochemistry 47, 10354–10364 (2008).
21. Rudolph, M. J., Wuebbrens, M. M., Rajagopal, K. V. & Schindelin, H. Crystal structures of molybdothionine synthase and its evolutionary relationship to ubiquitin activation. Nature Struct. Biol. 8, 42–46 (2001).
22. Settembre, E. C. et al. Thiamin biosynthesis in Bacillus subtilis: structure of the thiazole synthase/sulfur carrier protein complex. Biochemistry 43, 11647–11657 (2004).
23. Shigii, N., Sakaguchi, Y., Asai, S., Suzuki, T. & Watanabe, K. Common thiolation mechanism in the biosynthesis of RNA thio-uridine and sulfur-containing cofactors. EMBO J. 27, 3267–3278 (2008).
24. Voss, M., Nintz, M. & Leimkühler, S. Elucidation of the dual role of mycobacterial MoaZ in molybdenum cofactor biosynthesis and cysteine biosynthesis. PLoS ONE 6, e28170 (2011).

**Figure 4** | Possible involvement of the rhodanese domain of MoeZ in thiolation of sulphur-carrier proteins. The C-terminal rhodanese domain (RHOD) of MoeZ catalyses thiolation of the adenylated SCP. The sulphur source for charging the rhodanese domain can be from thiosphate (a) or from l-cysteine mediated by a cysteine desulphurase (CD) (b). Nucleophilic attack (red dashed line) on the adenylated sulphur-carrier protein followed by intramolecular disulphide bond formation (with another cysteine residue in MoeZ) allows sulphur transfer from the persulphide group to SCP. The protein persulphide intermediate can be reduced to release sulphur, which can also attack adenylated sulphur-carrier proteins (blue dashed line). To prevent such complications, the experiments were carried out in the absence of reducing agents. [Red], reduction; S\(_{hit}\), the wild-type Cys 360 residue.
Supplementary Information is available in the online version of the paper.

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Author Contributions H.-W.L. provided the scientific direction and the overall experimental design for the studies. E.S. and H.G.S. designed and performed the biochemical experiments. X.Z. and S.E.E. were responsible for the crystal structure studies. M.-Y.J.L., T.-L.L., A.O., J.-Y.L. and Y.-H.C. carried out the whole-genome sequencing and gene annotation. E.S., X.Z., H.G.S., S.E.E. and H.-W.L. wrote the manuscript.

Author Information The nucleotide sequences of the thiamine biosynthetic gene cluster, moeZ and the surrounding genes, the molybdopterin biosynthetic gene cluster, the cysteine biosynthetic gene cluster, and moaD2 and the surrounding genes have been deposited in the GenBank database under the accession numbers JN602207, JN602208, JN602209, JN602210 and JN602211, respectively. The atomic coordinates and structure factors for BexX–G6P and BexX–CysO have been deposited in the Protein Data Bank under the accession numbers 4N6F and 4N6E, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to H.-W.L. (h.w.liu@mail.utexas.edu).
METHODS
Whole-genome sequencing and analysis. Soybean-casein digest (TSB) medium (10 ml) was inoculated with spores of Amylomucor orientalis and incubated in a rotary incubator at 30 °C and 250 r.p.m. for 2 days. The resultant seed culture (4 ml) was transferred to 100 ml TSB medium and grown under the same conditions for 2 days. The growth culture (25 ml) was centrifuged at 5,000 g for 20 min at 4 °C, and the cells were washed with 25 ml 10 mM EDTA. After another centrifugation, the cells were stored at −80 °C until use. The cells were resuspended in 5 ml 1 mM EDTA, and the suspension was divided into 1.5 ml tubes (0.4 ml each). The genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer’s instructions. The resultant DNA solution (0.5 μg ml−1, 50 μl from each tube) was subjected to massively parallel sequencing using a 454 FLX Titanium analyser (Roche) and a Genome Analyzer IIx (Illumina) at the High Throughput Sequencing Core Facility at Academia Sinica, Taiwan. Primary assembly was carried out using 454 Newbler software (Roche). Contig extension and the closing of short gaps was achieved by scripts built in-house at the core. Genome annotation was carried out using Glimmer (Gene Locators and Interpolated Markov ModelERK) version 3.0 (ref. 25), iRNAscan-SE26 and RNAmmer27. Homologous protein sequences were identified in the NCBI database using the Basic Local Alignment Search Tool (BLAST).

Preparation of proteins. C-His6-BeX (where C denotes carboxy terminal) was prepared as described previously. The thi (orf13794), moaD (orf13839), CysO (orf06461), moaD2 (orf10102), moeZ (orf02110) and cd4 (orf04673) genes were PCR-amplified from A. orientalis genomic DNA using primers with engineered NdeI and HindIII restriction sites. The sequences of the primers are described in the Supplementary Methods. The PCR-amplified gene fragments were purified, digested with NdeI and HindIII and ligated into a PET28b (+) vector (Novagen) that had been digested with the same enzymes. For crystallization studies, the beX gene was also sub-cloned into PET28b (+) and produced as an N-terminally His6-tagged protein. In addition, the cysO gene was cloned into an IMPACT pTYB1 vector (New England Biolabs) that had been digested with NdeI and Sapi for the production of CysO thiocarboxylate28. The resultant plasmids were used to transform the Escherichia coli BL21 Star (DE3) strain (Invitrogen) for protein overexpression. An overnight culture of E. coli transformants grown in 10 ml LB medium containing 50 μg ml−1 kanamycin at 37 °C was used to inoculate 1 l of the same growth medium. The culture was incubated at 37 °C with shaking (230 r.p.m.) until the optical density at 600 nm (OD600) reached ~0.5. Protein expression was then induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM, and the cells were allowed to grow at 18 °C with shaking at 125 r.p.m. for an additional 24 h. The cells were collected after centrifugation at 4,500 g for 15 min and stored at −80 °C until lysis. All purification steps were carried out at 4 °C using nickel (Ni-NTA) resin according to the manufacturer’s protocol. The proteins were eluted using 250 mM imidazole buffer containing 10% glycerol, except those for crystallization studies. The collected protein solution was dialysed three times against 1 l 50 mM Tris-HCl buffer, pH 8, containing 300 mM NaCl and 15% glycerol. The protein solution was then flash-frozen in liquid nitrogen and stored at −80 °C for analysis. To the reaction mixture (10 ml) prepared above was added for colour development. Formation of SCN− was detected spectrophotometrically using a CarboPac PA1 analytical column (4 × 250 mm; Dionex). The sample was eluted with a gradient of water (solvent A) and 1 M ammonium acetate (solvent B). The gradient was run from 5% to 15% B over 5 min, from 15% to 30% B over 15 min and from 30% to 100% B over 7 min, with a 5-min wash at 100% B, and from 100% to 5% B over 3 min, followed by equilibration at 5% B for 5 min. The flow rate was 1 ml min−1, and the detector was set at 260 nm (Fig. 2h). The peak corresponding to the enzymatic reaction product was isolated and subjected to ESI–MS analysis (see Supplementary Methods for structural characterization). Alternatively, the reaction mixture stored at −20 °C was thawed and treated with 0.2 μl calf intestinal alkaline phosphatase (CIP) (2 units) and incubated at 37 °C for 1 h. The precipitant that appeared during the incubation was removed by centrifugation at 16,000g for 2 min, and 2 μl 100 mM mBBr in methanol was added to the reaction solution (the final concentration of mBBr was 5 mM). The resultant mixture was incubated at 25 °C for 5 min, and the supernatant (5 μl) was diluted with deionized water (95 μl) before HPLC analysis using an analytical C18 column (4 × 250 mm). The sample (20 μl) was eluted with a gradient of water (solvent A) and 80% acetonitrile (solvent B). The gradient was run from 5% to 30% B over 15 min, from 30% to 80% B over 5 min and from 80% to 5% B over 5 min, followed by re-equilibration at 5% B for 10 min. The flow rate was 1 ml min−1, and the detector was set at 260 nm. The 2-thio-i-glucose-bimane standard (0.1 mM) was prepared from the chemically synthesized 2-thio-i-glucose29 incubated with mBBr at room temperature for 5 min. The peak corresponding to the enzymatic reaction product was also isolated and subjected to ESI–MS analysis (Extended Data Fig. 4).

Determination of rhodanese activity of MoeZ. The site-specific Cys360Ala mutant of MoeZ was constructed according to the manufacturer’s site-directed mutagenesis protocol (Stratagene) using moezPET28b (+) as a DNA template. The forward primer (5′-GATGTTCTGCAGCAAGCTATCGG-3′) and the reverse primer (5′-GGGCGGCGGCGCGACTGGGGTGGAGG-3′) were used in the PCR amplification to introduce the site of mutagenesis (CDM-moaD/Cys360Ala/PET28bH+) was used to transform the E. coli BL21 Star (DE3) strain for protein overexpression. The rhodanese activity of MoeZ was determined using a previously described assay30. A typical assay mixture contained 50 mM Tris-HCl, pH 8.0, 50 mM potassium cyanide, approximately 2 μM MoeZ or MoeZ(Cys360Ala) and a variable amount of sodium thiosulphate (0–35 mM) in 100 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 15% glycerol. The reaction was initiated by the addition of MoeZ and was quenched after 10–15 s incubation at 25 °C by the addition of 50 μl acetic acid (15% formic acid). Then, 150 μl reagent B (1 Fe(Nb)3·9H2O and 2.65% HNO3 in 13 ml H2O) was added for colour development. Formation of SCN− in the reaction was quantified using the extinction coefficient of SCN− (ε(400 nm) = 22900)31. The steady-state kinetic parameters were determined in triplicate by fitting the experimental data using the Michaelis–Menten equation (Extended Data Fig. 7e). The assay for protein thiocarboxylate formation was performed in an anaerobic chamber to minimize the oxidation of MoeZ or MoeZ(Cys360Ala). A typical reaction mixture contained 80 μM MoeZ or MoeZ(Cys360Ala), 4 mM ATP, 5 mM MgCl2, 5 mM Na2SO3 in 50 mM HEPES, pH 8.0, with 500 mM glycerol (from enzyme stock solution) and 100 μM of one of the sulphur-carrier proteins, N-His6-MoaD2 or N-His6-CysO. The reaction was incubated in the glove box at −30 °C for 40 min and quenched by flash-frying in liquid nitrogen. Samples were then analysed by ESI–MS (Extended Data Fig. 7d).

Spectrophotometric analysis of the adenylation reaction catalysed by MoeZ and its Cys360Ala mutant. The adenylation of sulphur-carrier proteins catalysed by MoeZ and its Cys360Ala mutant was monitored using a coupled enzyme assay in the presence of an excess of NaSH (Extended Data Fig. 8a). The coupled enzyme

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reaction was monitored by detecting the consumption of NADH (ε_{340} = 6.220 M⁻¹ cm⁻¹) at 340 nm. A typical reaction mixture (120 μl) contained 3 μM MoEZ or its Cys606Ala mutant, 10 μM MoaD2, 80 μM ATP, 3 mM NADH, 2 mM phosphoenolpyruvate, 0.16 mM NAD and ~1.5 unit each of adenylate kinase, pyruvate kinase and lactate dehydrogenase (LDH) in 50 mM NH₄HCO₃ buffer, pH 8.0, containing 2.5 mM MgCl₂. The reaction was initiated on addition of ATP at time 0, and the absorbance at 340 nm was monitored for 30 s (Extended Data Fig. 8b).

2-Thiosugar formation using other sulphur sources. A typical reaction mixture (40 μl) contained 15 μM C-His, BexX, 18 μM N-His, MoaD2, 90 or 90 μM N-His, MoEZ or its Cys606Ala mutant, 2.5 mM ATP, 2.5 mM G6P and different sulphur sources ((i) 0.2 mM Na₂S₂O₃, or (ii) 0.15 mM 1-cysteine plus 25 μM cysteine desulphurase CD4 (in 0.25 mM pyridoxyl 5'-phosphate) in 50 mM NH₄HCO₃ buffer, pH8.0, containing 5 mM MgCl₂. The resultant reaction mixture was incubated at 30 °C for 30 or 70 min for (i) and 0, 10, 20 or 50 min for (ii). The reaction was quenched by adding an equal volume of acetonitrile, and mBBr was added to the collected supernatant (to a final concentration of ~2 mM). After incubation at 25 °C for 30 min, the reaction mixture was dried by vacuum concentration. The residue was re-dissolved in 50 mM NH₄HCO₃, pH 8.0, and treated with 3 units CIP and 0.16 mM NADH and ~1.5 unit each of adenylate kinase, pyruvate kinase and lactate dehydrogenase (LDH) in 50 mM NH₄HCO₃ buffer, pH 8.0, containing 2.5 mM MgCl₂. The reaction mixture was incubated at 30 °C for 30 or 70 min for (i) and 0, 10, 20 or 50 min for (ii). The reaction was quenched by adding an equal volume of acetonitrile, and mBBr was added to the collected supernatant (to a final concentration of ~2 mM). After incubation at 25 °C for 30 min, the reaction mixture was dried by vacuum concentration. The residue was re-dissolved in 50 mM NH₄HCO₃, pH 8.0, and treated with 3 units CIP.

Crystallization of BexX–G6P. Crystals of BexX–G6P were grown using the vapour diffusion hanging drop method. A solution containing 10 mg ml⁻¹ BexX in 10 mM Tris, pH 8.0, and 50 mM NaCl was pre-incubated on ice with G6P (at a final concentration of 2 mM) for about 1 h. Hanging drops were formed by mixing 1:5 μl protein solution and 1:5 μl well solution containing 40% (v/v) PEG 300, 0.1 M HEPES, pH 7.0, and 0.2 M magnesium acetate. The crystals belonged to space group P2₁ 2₁ 2₁ with unit cell dimensions of a = 251.7 Å, b = 181.7 Å, c = 99.0 Å. The Mathews coefficient is 3.5 Å³ Da⁻¹, corresponding to a solvent content of 56.1%.

Crystallization of the BexX–CysO complex. Crystals of BexX–CysO were grown using the vapour diffusion hanging drop method. Both CysO and CysO-thioribosecarboxylate were used for crystallization trials; however, CysO consistently yielded better crystals and was used for the structures reported here. The BexX–CysO complex was formed by pre-incubating 0.55 mg ml⁻¹ BexX and 1.0 mg ml⁻¹ CysO in 10 mM Tris, pH 8.0, containing 50 mM NaCl for 1 h. Hanging drops were formed by mixing 1:5 μl protein solution and 1:5 μl well solution containing 28% PEG 4000, 0.1 M Tris, pH 8.0, and 0.2 M LiSO₄. Plate-shaped crystals appeared within 5 days and grew to a maximum size of 0.5 mm × 0.2 mm in about 2 weeks. The crystals belonged to space group F422 with unit cell dimensions of a = 168.9 Å and c = 42.4 Å. The Matthews coefficient assuming two monomers of BexX per asymmetric unit was 2.8 Å³ Da⁻¹, corresponding to a solvent content of 56.1%.

X-ray data collection and processing. X-ray diffraction data for BexX–G6P were collected at beamline A1 at the Cornell High Energy Synchrotron Source (CHESS) using a Quantum 210 charge-coupled display (CCD) detector (Area Detector Systems Corporation, ADSC) with a crystal-to-detector distance of 200 mm and a wavelength of 0.9767 Å. The data collection temperature was 100 K. A total of 180° of data were collected with an oscillation range of 0.5° per frame and an exposure time of 3 s per frame. Data for BexX–G6P–CysO were collected at the Northeastern Collaborative Access Team (NE-CAT) beamline 24-ID-C at the Advanced Photon Source (APS) using a Q315 CCD detector (ADSC). The wavelength was 0.9791 Å; the data collection temperature was 100 K; and the detector distance was 400 mm. Individual frames were collected over a range of 180° using 1 s for each 1°. X-ray diffraction data were indexed, integrated, scaled and merged using the program HKL-2000 (ref. 31).

Structure determination and refinement. The structure of BexX was determined by molecular replacement using the program Phaser as implemented in the PHENIX program package. A monomer of ThiG from Bacillus subtilis (PDB ID, 1ITY) was used as the search model. The initial molecular replacement solution was refined to an R_sync of 40.0% and R_free of 44.0%. All side chains were added, and the model was manually adjusted using COOT. After several cycles of refinement using PHENIX and REFMAC5 (ref. 36), G6P and water molecules were added. The final model was refined to an R_sync of 19.1% and R_free of 22.2%. The Ramachandran plot shows 94.7% of residues in the most favourable regions and 5.3% in the allowed regions. No residues were in generously allowed regions or disallowed regions. The structure of BexX–CysO was determined by molecular replacement using a monomer of BexX from the BexX-G6P complex and a monomer of CysO from Mycobacterium tuberculosis (PDB ID, 3DWM) as the search models. An initial model, corresponding to one monomer of BexX and one monomer of CysO, was generated by Phaser as implemented in PHENIX. Packing analysis showed that a BexX–CysO dimer is formed by crystallographic twofold symmetry. The initial refinement resulted in an R_sync of 33.8% and R_free of 39.5%. Subsequent cycles of model building in COOT and refinement in PHENIX and REFMAC5 (ref. 36) resulted in a final R_sync of 19.6% and R_free of 24.0%. The Ramachandran plot shows that 90.0% of residues are located in the most favourable regions, 9.7% in the allowed regions and 0.3% in the generously allowed regions. No residues were in the disallowed regions.
Extended Data Figure 1 | Structures of BexX and CysO from *Amycolatopsis orientalis*. **a**, A stereo ribbon diagram of the \((\beta\alpha)_8\)-barrel fold of BexX is shown from the top view. The α-helices, β-strands and loops are marked in blue, green and yellow, respectively. The ketone-intermediate (2) formed by Lys 110 and G6P is shown as sticks and coloured in purple. **b**, The typical secondary structure composition of the classical \((\beta\alpha)_8\)-barrel is shown as a topology model; the conserved Lys 110 is highlighted in red. **c**, The quaternary structure of BexX is shown as a ribbon diagram with two monomers coloured by chain. **d**, A ribbon diagram of CysO from the BexX–CysO structure. Secondary structural elements are coloured blue for α-helices, green for β-strands and yellow for loops. **e**, A topology diagram of CysO. **f**, Data collection and refinement statistics. One crystal was used for each of the two data sets. *The values in parentheses are for the highest-resolution shell.
Extended Data Figure 2 | Putative thiamine, molybdenum cofactor and cysteine biosynthetic genes found in *A. orientalis* and their proposed functions. 

*a*, Organization of the putative thiamine biosynthetic gene cluster and the proposed thiamine biosynthetic pathway in *A. orientalis*. The genes encoding MoeZ and ThiL (one of the genes involved in thiazole biosynthesis) are not found in the gene cluster. The gene encoding the ThiS-activating enzyme, ThiF, is also absent from the genome. 

*b*, Organization of the putative molybdopterin biosynthetic gene cluster and the proposed molybdenum cofactor biosynthetic pathway in *A. orientalis*. The genes encoding MoeZ and MoeA are not found in the gene cluster. The gene encoding the MoaD-activating enzyme, MoeB, is also absent from the genome. 

*c*, Organization of the putative cysteine biosynthetic gene cluster and the proposed cysteine biosynthetic pathway in *A. orientalis*. The gene encoding MoeZ is not found in the gene cluster. 

*d*, Organization near the *moaD* homologue, *moaD2*, found in the *A. orientalis* genome. 

*e*, Organization near *moeZ* in the *A. orientalis* genome and the conserved domains of MoeZ predicted by BLAST analysis.
Extended Data Figure 3 | ESI–MS analyses of the MoeZ-catalysed activation of sulphur-carrier proteins and SDS–PAGE separation of the purified proteins. a, Reaction scheme of the MoeZ-catalysed activation of ThiS. b–e, Deconvoluted ESI–MS analyses of as-isolated ThiS (b), ThiS in the presence of MoeZ and ATP (c), ThiS in the presence of MoeZ, ATP and bisulphide (d) and ThiS in the presence of bisulphide (control) (e). The calculated molecular masses are shown as the neutral form in the upper right corner. Analysis of purified N-His6-ThiS (where N denotes amino terminal) shows two mass signals (observed (obsd), 8,646 and 8,824 Da) consistent with the calculated molecular mass of the recombinant enzyme in its native and N-gluconoylated form (where N denotes amino terminal) (calcd, 8,647 and 8,825 Da). Gluconoylation of the N-terminal His6 tag is a known post-translational modification when expressing recombinant proteins in E. coli. Such a modification should not affect ThiS activity, because the predicted active site for ThiS is at the C terminus. Indeed, when N-His6-ThiS was incubated with N-His6-MoeZ and ATP, a mass spectrometric signal corresponding to adenylated N-His6-ThiS (9) was detected together with a few peaks that were probably derived from a reaction of the labile adenylated ThiS with buffer components (see c). f–k, Deconvoluted ESI–MS analyses of as-isolated MoaD (N-His6-MoaD, 105 amino acids; calcd, 11,022 Da) (f), as-isolated CysO (N-His6-CysO, 109 amino acids, and its N-gluconoylated derivative; calcd, 11,688 and 11,866 Da, respectively) (g), as-isolated MoaD2 (N-His6-MoaD2, 115 amino acids, and its N-gluconoylated derivative; calcd, 12,473 and 12,651 Da, respectively) (h), MoaD incubated with MoeZ, ATP and NaSH (N-His6-MoaD-COSH; calcd, 11,038 Da) (i), CysO incubated with MoeZ, ATP and NaSH (N-His6-CysO-COSH and its N-gluconoylated derivative; calcd, 11,704 and 11,882 Da, respectively) (j) and MoaD2 incubated with MoeZ, ATP and NaSH (N-His6-MoaD-COSH and its N-gluconoylated derivative; calcd, 12,489 and 12,667 Da, respectively) (k). l, SDS–PAGE gel of purified sulphur-carrier proteins, MoeZ and CD4: N-His6-ThiS (85 amino acids, 8.7 kDa, lane 2), N-His6-MoaD2 (115 amino acids, 12.5 kDa, lane 3), N-His6-CysO (109 amino acids, 11.7 kDa, lane 4), N-His6-MoeZ (421 amino acids, 43.3 kDa, lane 9). The molecular weight markers are 220, 160, 120, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa (top to bottom, lanes 1, 6 and 8). The protein MoaD did not express well, and the partially purified protein solution contained significant amounts of endogenous proteins from the E. coli host.
Extended Data Figure 4 | BexX-catalysed 2-thio-D-glucose-6-phosphate formation followed by alkaline phosphatase treatment. a, Reaction scheme to synthesize the expected bimane derivative. b, HPLC traces of the C-His$_6$-BexX-catalysed reactions (where C denotes carboxy terminal) using N-His$_6$-ThiS, N-His$_6$-CysO or N-His$_6$-MoaD2, and the control reactions. The thiosugar product was treated with alkaline phosphatase (CIP) and then derivatized with mBBr. HPLC analysis of the synthetic standard of 2-thio-D-glucose-bimane is shown in the bottom trace (trace 7). c, High-resolution ESI–MS (positive) of the isolated product peak (2-thio-D-glucose-bimane C$_{16}$H$_{22}$N$_2$NaO$_7$S$^+$ [M + Na]$^+$, calcd, 409.1040 Da; obsd, 409.1038 Da).
Extended Data Figure 5 | Sequence alignment of *A. orientalis* CysO, MoaD2 and ThiS and hydrophobic interactions for BexX complexes. a, Sequence alignment was based on structural supersession using the programs 3D-Coffee\(^{38}\), MultAlin\(^{39}\) and ESPript\(^{40}\). The main differences between CysO (or MoaD2) and ThiS result from an insertion of ten residues between \(\beta_1\) and \(\beta_2\) of CysO (or MoaD2) and an insertion of 14 (or 15) residues between \(\alpha_1\) and \(\beta_3\) of CysO (or MoaD2). The first insertion includes the short helix 3_10, and the second includes helix \(\alpha_2\). Both of these insertions are involved in the BexX–CysO (or BexX–MoaD2) interface. Ten interface residues (red stars and red triangles) are conserved between CysO and MoaD2; however, only four of these residues are conserved in ThiS (red triangles). Two differences between CysO and MoaD2 represent conservative substitutions; while Thr 9 and Ala 86 in CysO are replaced by Gly 11 and Ser 92 in MoaD2, the interface interaction is contributed by hydrogen bonds that are formed by the backbone atoms. b–d, Hydrophobic interactions of BexX–CysO (b), BexX–MoaD2 (c) and BexX–ThiS (d). BexX monomers are shown as grey ribbon diagrams with hydrophobic interaction regions coloured in cyan. CysO, MoaD2 and ThiS are shown as cartoons and coloured in green, blue and yellow, respectively. Hydrophobic interaction regions in sulphur-carrier proteins are coloured in red. The \(\alpha\)-helices and \(\beta\)-strands in BexX and the sulphur-carrier proteins are labelled in black and red, respectively.
Extended Data Figure 6 | The A. orientalis BexX–CysO interface, predicted hydrogen bonds between BexX with sulphur-carrier proteins, and a comparison of the BexX–CysO interface with the Bacillus subtilis ThiG–ThiS interface. 

**a**, Interacting surfaces of BexX (left) and CysO (right). The surface is colour coded by atom type (oxygen, red; nitrogen, blue; carbon, green). Non-interacting surfaces are shown in grey.

**b**, Hydrogen bonds on the surface of BexX with CysO are shown as black dashes.

**c**, Hydrogen bonds formed by the C-terminal tail of CysO and the surrounding residues from BexX are shown as black dashes. The Fo – Fc simulated annealing omit map of the C-terminal residues (Ala-Val-Ala-Gly-Gly) is rendered in grey and contoured at 3.0 σ. Residues are shown as sticks with the carbon atoms in grey for BexX and green for CysO. CysO residues are labelled in red; BexX residues are labelled in black.

**d**, Predicted hydrogen bonds between BexX and other sulphur-carrier proteins. The hydrogen-bonding scheme for the BexX–CysO complex (9 of 12 involve the C-terminal tail) is conserved in the model of the BexX–MoaD2 complex. 

**e**, The interface between BexX (blue) and CysO (pink). Secondary structural elements of CysO are labelled in black, the β2 and α2 elements in BexX are labelled in red. 

**f**, The interface between ThiG (grey) and ThiS (yellow) from *B. subtilis*. Secondary structural elements of ThiS are labelled in black, and the β2 and α2 elements in ThiG are labelled in red. The β2–α2 loop region in BexX and ThiG is highlighted in red. For CysO, 3_{10} and α2 form hydrophobic contacts with the β2–α2 loop and α2 of BexX. ThiG also uses its β2–α2 loop to interact with ThiS; however, ThiS uses two different loop regions to form the interface. In addition, the β2–α2 loop of BexX is closer to the (β2)_{8}-barrel than in ThiG, in which the β2–α2 loop extends outwards and covers the top of ThiS.

|        | BexX          | CysO          | MoaD2       | ThiS         |
|--------|---------------|---------------|-------------|--------------|
| Ile30  | Gly96 (O) (3.0 Å) | Gly95 (O) (3.0 Å) | Gly85 (O) (3.0 Å) | Gly90 (O) (3.0 Å) |
| Ile30  | Gly96 (O) (3.0 Å) | Gly95 (O) (3.0 Å) | Gly85 (O) (3.0 Å) | Gly90 (O) (3.0 Å) |
| Ile52  | Gly96 (O) (2.7 Å) | Gly95 (O) (2.7 Å) | Gly85 (O) (2.7 Å) | Gly90 (O) (2.7 Å) |
| Thr54  | Val87 (O) (2.9 Å) | Val83 (O) (2.9 Å) | Val63 (O) (2.9 Å) | Val90 (O) (2.9 Å) |
| Thr54  | Val87 (O) (2.9 Å) | Val83 (O) (2.9 Å) | Val63 (O) (2.9 Å) | Val90 (O) (2.9 Å) |
| Asn59  | Thr9 (N) (3.0 Å) | Val11 (N) (3.0 Å) | Thr9 (N) (3.0 Å) | Thr9 (N) (3.0 Å) |
| Ser63  | Ala68 (O) (2.9 Å) | Ala68 (O) (2.9 Å) | Ala68 (O) (2.9 Å) | Ala68 (O) (2.9 Å) |
| Leu64  | Ala68 (O) (2.9 Å) | Ala68 (O) (2.9 Å) | Ala68 (O) (2.9 Å) | Ala68 (O) (2.9 Å) |
| Leu66  | Ala68 (O) (2.9 Å) | Ala68 (O) (2.9 Å) | Ala68 (O) (2.9 Å) | Ala68 (O) (2.9 Å) |
| Asp68  | Arg45 (N) (2.8 Å) | Arg45 (N) (2.8 Å) | Arg45 (N) (2.8 Å) | Arg45 (N) (2.8 Å) |
| Ser65  | Gly90 (OXT) (3.1 Å) | Gly90 (OXT) (3.1 Å) | Gly90 (OXT) (3.1 Å) | Gly90 (OXT) (3.1 Å) |
| Asp95  | Asp62 (O) (3.2 Å) | Asp62 (O) (3.2 Å) | Asp62 (O) (3.2 Å) | Asp62 (O) (3.2 Å) |
| Lys580 | Gly90 (OXT) (3.2 Å) | Gly90 (OXT) (3.2 Å) | Gly90 (OXT) (3.2 Å) | Gly90 (OXT) (3.2 Å) |
Extended Data Figure 7 | MoeZ-dependent protein thiocarboxylate formation in sulphur-carrier proteins using thiosulphate as the sulphur source. a–d, Deconvoluted ESI–MS of MoaD2 incubated with MoeZ (the observed peaks are consistent with the calculated molecular masses of N-His6-MoaD2-COSH (12,489 Da), N-His6-MoaD2-glycerol (12,547 Da), and N-gluconoylated-His6-MoaD2-COSH (12,667 Da)) (a), MoaD2 incubated with the MoeZ(Cys360Ala) mutant (the observed peaks are consistent with the calculated molecular masses of N-His6-MoaD2 (12,473 Da) and N-His6-MoaD2-glycerol (12,547 Da)) (b), CysO incubated with MoeZ (the observed peaks are consistent with the calculated molecular masses of N-His6-CysO-COSH (11,704 Da), N-His6-CysO-glycerol (11,762 Da) and N-gluconoylated-His6-CysO-COSH (11,882 Da)) (c), and CysO incubated with the MoeZ(Cys360Ala) mutant (the observed peaks are consistent with the calculated molecular masses of N-His6-CysO (11,688 Da), N-His6-CysO-glycerol (11,762 Da), their N-gluconoylated derivatives (11,866 Da, and 11,940 Da, respectively) and N-His6-CysO-AMP (12,017 Da)) (d). Observed masses corresponding to protein thiocarboxylate are shown in red. Two peaks corresponding to the dehydration of N-His6-MoaD2 and N-His6-CysO were probably caused by in-source collision-induced dissociation (CID) during the ESI–MS analysis.

e, Kinetic parameters for the thiosulphate:cyanide sulphur transferase activity of MoeZ from *A. orientalis*. Bovine liver rhodanese is a typical rhodanese enzyme. Compared with bovine rhodanese, human molybdopterin synthase sulphurase (human MOCS3) displayed much lower thiosulphate:cyanide sulphur transferase activity. In the case of human MOCS3, L-cysteine and cysteine desulphurase are proposed as the physiological sulphur source over thiosulphate because of its lower rhodanese activity. In the case of human MOCS3, L-cysteine and cysteine desulphurase are proposed as the physiological sulphur source over thiosulphate because of its lower rhodanese activity. However, this may not be the case for MoeZ from *A. orientalis* because its rhodanese activity is comparable to bovine liver rhodanese.

| Proteins                  | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | Reference | Assay conditions |
|---------------------------|------------|----------------------|-----------|------------------|
| MoeZ                      | 7.6 ± 0.7  | 32 ± 1               | this study| 25 °C, pH 8.0    |
| MoeZ(C360A)               | N. D.      | N. D.                | this study| 25 °C, pH 8.0    |
| Bovine liver rhodanese    | 18.1 ± 1.0 | ~ 300                | ref. 41   | 40 °C, pH 8.7    |
| human MOCS3               | 80.8 ± 3.8 | 2.11 ± 0.20          | ref. 42   | 37 °C, pH 8.0    |

N. D. = not detected.
Extended Data Figure 8 | Relative adenylation activity of MoeZ and the MoeZ(Cys360Ala) mutant. a, Reaction scheme for the MoeZ-catalysed adenylation activity assay. The adenylation activities of MoeZ and its Cys360Ala mutant were inferred using a colorimetric assay to monitor the production of AMP (indicated by a decrease in NADH at 340 nm) when MoeZ or its Cys360Ala mutant was co-incubated with a sulphur-carrier protein (MoaD2) in the presence of ATP, NaSH, adenylate kinase (AK), pyruvate kinase (PK) and lactate dehydrogenase (LDH). b, The relative adenylation activity of MoeZ (open circles) and its Cys360Ala mutant (filled circles), as well as a no MoeZ/no Cys360Ala control (open squares), was measured by the coupled enzyme assay, as described in a. Little difference in the decrease in absorption at 340 nm was observed between MoeZ and its Cys360Ala mutant (compared with the control with no MoeZ), suggesting that the mutation at Cys360 had little effect on the adenylation activity of MoeZ.
Extended Data Figure 9 | BexX-catalysed 2-thiosugar formation using various sulphur sources. a, b, Reaction scheme for C-His_{6}-BexX-catalysed 2-thiosugar formation using N-His_{6}-MoeZ, N-His_{6}-MoaD2 and thiosulphate (a) or l-cysteine and the cysteine desulphurase (CD4) from A. orientalis (b). The reactions were carried out in the absence of reducing agent to avoid complications from the generation of bisulphide from protein persulphide (*see also below). Under these conditions, MoeZ cannot be regenerated after single turnover. The thiosugar product was derivatized with mBBr and then treated with alkaline phosphatase (CIP) to yield 2-thio-D-glucose-bimane (2SG-bimane). c, d, The 2SG-bimane product concentrations at different time points of incubation with thiosulphate (c) or l-cysteine and CD4 (d) as the sulphur source were estimated on the basis of the product peak area of each HPLC trace. The 2SG-bimane synthetic standard (10, 25, 50, 77, 100 and 200 μM) was used for calibration. The filled and open circles denote product formation from the incubation with N-His_{6}-MoeZ and the N-His_{6}-MoeZ(Cys360Ala) mutant, respectively. *The observed minor product formation with the MoeZ(Cys360Ala) mutant, l-cysteine and CD4 (see d, open circles) is probably caused by the formation of bisulphide, which could be generated on reduction of CD4-persulphide in the presence of free cysteine molecules. In fact, a small amount of bisulphide was detected under similar conditions with l-cysteine and CD4 (in the absence of other proteins and reducing agents) by the methylene blue assay within 15 min of incubation^{45}. 
### Extended Data Table 1 | Putative cysteine desulphurases, rhodaneses and sulphur-carrier proteins found in the *A. orientalis* genome

| gene (orf #) | Name of the protein | protein with the highest sequence similarity and origin | identity / similarity (%) | protein accession number |
|-------------|---------------------|--------------------------------------------------------|---------------------------|-------------------------|
| 10706       | CD1                 | cysteine desulphurase/selenocysteine lyase [Amycolatopsis mediterranei U32] | 97 / 98                   | YP_003765029            |
| 11099       | CD2                 | cysteine desulphurase [Amycolatopsis mediterranei U32] | 87 / 91                   | YP_003765163            |
| 14916       | CD3                 | cysteine desulphurase [Amycolatopsis mediterranei U32] | 88 / 93                   | YP_003766845            |
| 04763       | CD4                 | cysteine desulphurase [Amycolatopsis mediterranei U32] | 92 / 96                   | YP_003763873            |
| 09299       | CD5                 | cysteine desulphurase [Amycolatopsis mediterranei U32] | 97 / 99                   | YP_003762467            |
| 04658       | RHO1                | rhodanese-like protein [Amycolatopsis mediterranei U32] | 89 / 93                   | YP_003763825            |
| 08287       | RHO2                | rhodanese-like protein [Amycolatopsis mediterranei U32] | 89 / 91                   | YP_003764815            |
| 09690       | RHO3                | rhodanese-like protein [Amycolatopsis mediterranei U32] | 95 / 96                   | YP_003762363            |
| 10524       | RHO4                | rhodanese-like protein [Amycolatopsis mediterranei U32] | 81 / 87                   | YP_003763440            |
| 12151       | RHO5                | rhodanese-like protein [Amycolatopsis mediterranei U32] | 97 / 99                   | YP_003771104            |
| 02110       | MoeZ                | molybdenopterin biosynthesis-like protein MoeZ [Amycolatopsis mediterranei U32] | 99 / 99                   | YP_003763336            |
| 13974       | ThiS                | thiamin biosynthesis protein ThiS [Amycolatopsis mediterranei U32] | 88 / 93                   | YP_003770674            |
| 13839       | MoaD                | ThiS/MoaD family protein [Amycolatopsis mediterranei U32] | 82 / 90                   | YP_003770615            |
| 06461       | CysO                | ThiS/MoaD family protein [Amycolatopsis mediterranei U32] | 97 / 100                  | YP_003769822            |
| 10102       | MoaD2               | ThiS/MoaD family protein [Amycolatopsis mediterranei U32] | 91 / 94                   | YP_003764220            |
Extended Data Table 2 | BLASTP (protein BLAST) analysis of E1-like proteins in genomes of selected strains of the Actinomycetales

| Family                  | # of E-1 like protein | Name of bacterial strain                          | protein accession number |
|-------------------------|-----------------------|---------------------------------------------------|--------------------------|
| Streptomycesaceae       | 1                     | Streptomyces coelicolor A3(2)                      | MoeZ (NP_629326)         |
| Streptomycesaceae       | 1                     | Streptomyces avermitilis MA-4680                   | MoeZ (NP_824258)         |
| Streptomycesaceae       | 1                     | Streptomyces griseus subsp. griseus NBRC 13350     | MoeZ (YP_001823859)      |
| Streptomycesaceae       | 1                     | Streptomyces cattleya NRRL 8057                    | MoeZ (YP_004913535)      |
| Streptomycesaceae       | 1                     | Streptomyces violaceusniger Tu 4113               | MoeZ (YP_004811516)      |
| Mycobacteriaceae        | 4                     | Mycobacterium tuberculosis H37Rv                   | MoeZ (YP_177842)         |
|                         |                       |                                                   | MoeB (YP_177929)         |
|                         |                       |                                                   | Rv2338c (NP_218554)      |
|                         |                       |                                                   | Rv1355c (NP_215871)      |
| Mycobacteriaceae        | 4                     | Mycobacterium bovis AF2122/97                      | MoeZ (NP_866876)         |
|                         |                       |                                                   | MoeB (NP_866788)         |
|                         |                       |                                                   | MB2366c (NP_866015)      |
|                         |                       |                                                   | Mb1390c (NP_855044)      |
| Mycobacteriaceae        | 2                     | Mycobacterium avium subsp. paratuberculosis K-10  | MoeZ (YP_962240)         |
|                         |                       |                                                   | MoeY? (YP_960282)        |
| Mycobacteriaceae        | 2                     | Mycobacterium abscessus ATCC 19977                 | MoeZ (YP_001704255)      |
|                         |                       |                                                   | E1 family (YP_001702828) |
| Corynebacteriaceae      | 2                     | Corynebacterium glutamicum ATCC 13032              | MoeZ (? (NP_599461)      |
|                         |                       |                                                   | MoeZ (? (NP_601246)      |
| Corynebacteriaceae      | 1                     | Corynebacterium jeikeium ATCC 43734                | MoeZ (ZP_05845904)       |
| Nocardioaceae           | 2                     | Nocardia farcinica IFM 10152                       | MoeZ (YP_120782)         |
|                         |                       |                                                   | nfa49170 (YP_121133)     |
| Nocardioaceae           | 2                     | Rhodococcus jostii RHA1                            | MoeZ (YP_706296)         |
|                         |                       |                                                   | RHA1_ro05762 (YP_705688) |
| Pseudonocardioaceae     | 1                     | Saccharopolyspora erythraea NRRL 2338              | MoeZ (YP_001103327)      |
|                         |                       |                                                   | MoeZ (YP_003763336)      |
|                         |                       |                                                   | MoeB7 (YP_003764340)     |
|                         |                       |                                                   | AMED-1241 (YP_00376458)  |
|                         |                       |                                                   | E1-family (YP_003766676) |
| Frankiaceae             | 5                     | Frankia alni ACN14a                               | MoeZ (YP_716188)         |
|                         |                       |                                                   | HesA? (YP_716927)        |
|                         |                       |                                                   | HesA2? (YP_716575)       |
| Micrococaceae           | 1                     | Anthrobacter chlorophenolicus A6                   | MoeZ (YP_002488550)      |
| Microbacteriaceae       | 1                     | Clavibacter michiganensis subsp. michiganensis NCBB 382 | MoeZ (YP_001223108)    |
| Micromonosporaceae      | 2                     | Micromonospora aurantiaca ATCC 27029               | MoeZ (YP_003838663)      |
|                         |                       |                                                   | E1-family (YP_003839126) |
| Micromonosporaceae      | 3                     | Saltinispora arenicola CNS-205                     | MoeZ (YP_001535374)      |
|                         |                       |                                                   | E1-family (YP_001538912) |
|                         |                       |                                                   | E1-family (YP_00153897)  |
| Nocardioaceae           | 1                     | Nocardioides sp. JS614                             | MoeZ (YP_922675)         |
| Propionibacteriaceae    | 1                     | Microbactenus phosphovorus NM-1                    | MoeZ (YP_004573609)      |