Discovery of a sesamin-metabolizing microorganism and a new enzyme

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Sesamin is one of the major lignans found in sesame oil. Although some microbial metabolites of sesamin have been identified, sesamin-metabolic pathways remain uncharacterized at both the enzyme and gene levels. Here, we isolated microorganisms growing on sesamin as a sole-carbon source. One microorganism showing significant sesamin-degrading activity was identified as Sinomonas sp. no. 22. A sesamin-metabolizing enzyme named SesA was purified from this strain and characterized. SesA catalyzed methylene group transfer from sesamin or sesamin monochetol to tetrahydrofolate (THF) with ring cleavage, yielding sesamin mono- or di-catechol and 5,10-methylene tetrahydrofolate. The kinetic parameters of SesA were determined to be as follows: $K_m$ for sesamin = 0.032 ± 0.005 mM, $V_{max}$ = 9.3 ± 0.4 (μmol·min$^{-1}$·mg$^{-1}$), and $K_{cat}$ = 7.9 ± 0.3 s$^{-1}$. Next, we investigated the substrate specificity. SesA also showed enzymatic activity toward (+)-episesamin, (-)-asarinin, sesaminol, (+)-sesamolin, and piperine. Growth studies with strain no. 22, and Western blot analysis revealed that SesA formation is inducible by sesamin. The deduced amino acid sequence of sesA exhibited weak overall sequence similarity to that of the protein family of glycine cleavage T-proteins (GcvTs), which catalyze glycine degradation in most bacteria, archaea, and all eukaryotes. Only SesA catalyzes C1 transfer to THF with ring cleavage reaction among GcvT family proteins. Moreover, SesA homolog genes are found in both Gram-positive and Gram-negative bacteria. Our findings provide new insights into microbial sesamin metabolism and the function of GcvT family proteins.

sesamin | metabolism | lignan | tetrahydrofolate

We have been involved in studies of not only microbial metabolism of man-made compounds (1–3) but also biologically active natural compounds, such as curcumin (4). In this study, we characterize the microbial metabolism of the lignan sesamin.

Lignans (5) are plant-derived compounds consisting of dimers of phenylpropane units (6). They are found in a wide variety of plant-based foods. Whole-grain products, vegetables, fruits, nuts, seeds, and beverages such as tea, coffee, and wine are dietary sources of lignans. In Asian countries, sesame, which contains lignans, is used traditionally as a food. A major lignan is sesamin, which is a biologically active compound with antioxidative (7), cholesterol-lowering (8), lipid-lowering (9), antihypertensive (10), and antiinflammatory (11) properties.

In humans, sesamin is metabolized by CYP450 enzymes into sesamin mono- and di-catechol in liver microsomes (12). Sesamin monochetol is metabolized further by UDP-glucuronosyltransferase (UGT) and O-methyl transferase (COMT) (13), and the resulting glucuronides of sesamin metabolites are excreted in the bile and urine (14).

In microorganisms, on the other hand, metabolism of sesamin has been reported in few species. Aspergillus oryzae converts sesamin to sesamin mono- and di-catechol (15) whereas intestinal bacteria convert sesamin to the so-called “mammalian lignans” enterodiol and enterolactone (16). The activities of these metabolites make them useful dietary substances. Sesamin mono- and di-catechol show stronger antioxidant activity than sesamin (17). Also, mammalian lignans show other properties: higher antioxidant activity than vitamin E (18); reduction of cardiovascular disease risk (19); reduction of the risk of atherosclerosis by decreasing the total cholesterol level (20); and reduction of inflammation markers (21). However, neither sesamin-metabolizing enzymes nor their genes have been identified in microorganisms, including intestinal bacteria and A. oryzae.

Here, we describe the isolation and identification of a sesamin-catabolizing soil microorganism, Sinomonas sp. no. 22, together with purification and characterization of a previously unknown sesamin-modifying enzyme. This enzyme has the unique catalytic ability to transfer the methylene group from sesamin to tetrahydrofolate (THF) through ring cleavage (Fig. 1). In addition, we clarify the biochemical properties of this enzyme and propose a possible reaction mechanism. Our findings provide novel insights into the catabolism of natural compounds and THF-dependent metabolic pathways.

Results

Isolation and Identification of Sesamin-Metabolizing Bacteria. The soil samples used for microbial screening were collected from sesame gardens at the University of Tsukuba. At ~1 moi from the start of this study, by using the enrichment culture method described in Materials and Methods, we isolated 40 microorganisms that were able to grow on culture medium containing sesamin as the sole carbon source. We selected one isolate, strain no. 22, for further study. This strain showed 99% 16S rRNA gene sequence similarity to Sinomonas atrocyanea DSM20127T. Morphological and biochemical properties of strain no. 22 are shown in SI Appendix, Supplementary Data.

Significance

Lignans, including sesamin, are produced by a wide variety of plants, but the microbial degradation of lignan has not been identified biochemically. Here, we show that Sinomonas sp. no. 22 can catabolize sesamin as a sole-carbon source. We identified the sesamin-converting enzyme, SesA, from strain Sinomonas sp. no. 22. SesA catalyzed methylene group transfer from sesamin to tetrahydrofolate (THF). The resulting 5,10-CH2-THF might find use as a C1-donor for bioprocesses. SesA gene homologs were found in the genomes of both Gram-positive and Gram-negative bacteria, suggesting that sesamin (lignan) utilization is a widespread, but still unrecognized, function in environments where lignans are produced and degraded.

Author contributions: T.K., E.F., and M.K. designed research; T.K. and E.F. performed research; T.K., E.F., Y.H., and M.K. analyzed data; and T.K. and M.K. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The nucleotide sequence data reported in this paper have been deposited in the DNA Data Bank of Japan (DDBJ) database [accession nos. LC101493 (sesA) and thr2], LC101494 (thr1), and LC101495 (16S rRNA gene)].

1T.K. and E.F. contributed equally to this work.

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Two products (compounds A and B) were isolated by ethyl acetate extraction and preparative HPLC (Fig. 2).

High-resolution mass spectrometry (HRMS) analysis in the negative mode revealed the molecular ion of compound A at \( m/z \) 341.1029 [M-H]⁻, which was in agreement with the calculated mass of \( C_{18}H_{17}O_6 \). From the sequence information, the mass of compound B at \( m/z \) 329.1022 [M-H]⁻, in agreement with the calculated mass of \( C_{15}H_{17}O_6 \).

The gene for the sesamin-metabolizing enzyme, named SesA, was purified as a single band on SDS/PAGE (Table 1 and SI Appendix, Table S3). Considering the relationship between the protein amount and the activity of each fraction obtained on gel-filtration chromatography, the target enzyme was the ~50-kDa protein band observed on SDS/PAGE (SI Appendix, Figs. S1 and S4). An N-terminal amino acid sequence of the protein was determined to be TAEQAIN.

The 1.4-kb region of the SesA-coding gene was inserted into an expression vector, and recombinant SesA was produced in Escherichia coli and purified. The specific activity of the recombinant SesA was approximately the same (8.8 units/mg) as that of SesA purified from strain no. 22.

We determined the effects of temperature and pH on SesA activity. The optimal reaction temperature and pH were below 40 °C and pH 7.5–8.5, respectively (SI Appendix, Figs. S8 A and B). SesA was most stable under 30 °C and in the pH range of 5.5–10.0 (SI Appendix, Fig. S8 C and D).

The absorption spectrum of the purified SesA showed an absorbance maximum near 280 nm. No other absorption peak or shoulder was observed at higher wavelengths (SI Appendix, Fig. S9). These results suggest that no cofactor is bound to the purified enzyme. The CD spectrum is shown in SI Appendix, Fig. S10. Qualitative analysis of metal content was performed by inductively coupled plasma atomic emission spectroscopy analysis. The enzyme contained 1.70 mol of phosphorus and 0.70 mol of sulfur per mole of subunit. No other metal was detected within the assay limits (SI Appendix, Supplementary Data 3).

The stoichiometry of the SesA reaction was examined. The amounts of sesamin, sesamin monocatechol, sesamin di-catechol, and folates were determined by HPLC and liquid chromatography (LC)/MS/MS. After 30 min incubation, sesamin mono- and di-catechol increased to 77 and 9.3 μM, respectively. Coincidentally, sesamin decreased by 80 μM (SI Appendix, Fig. S11 A).

We detected 5,10-CH₂-THF in SesA reaction mixtures. After 30-min incubation, 5,10-CH₂-THF increased to 85 μM (SI Appendix, Fig. S11 B). The specific activity of the enzyme increased after 8 h cultivation, followed by a decrease in sesamin in the medium and an increase in the protein compared with cell growth. On the contrary, in medium containing glucose as sole-carbon source, cells grew exponentially for 4 h, but sesamin-converting activity was not observed during cultivation. Furthermore, in 2× YT liquid medium, sesamin-converting activity was detected only in the presence of added sesamin (SI Appendix, Fig. S7B).

Cloning and Heterologous Expression of sesA and Biochemical Properties of SesA. The 1.4-kb region of the SesA-coding gene was inserted into an expression vector, and recombinant SesA was produced in Escherichia coli and purified. The specific activity of the recombinant SesA was approximately the same (8.8 units/mg) as that of SesA purified from strain no. 22.

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Appendix, Fig. S11B); there was no change in the 5-CH$_3$-THF
amount. THF could not be determined because of its instability
under our assay conditions. These results demonstrate that

sesamin mono catechol, di catechol, and 5,10-CH$_2$-THF were
formed stoichiometrically with the consumption of sesamin dur-
ing the enzymic reaction (Fig. 4).  

Kinetic Analysis. Using varying concentrations of sesamin in the
presence of 1 mM THF, a typical hyperbolic curve of product
formation over substrate concentration was obtained (SI Appen-
dix, Fig. S11C), indicating that the reaction followed Michaelis–
Menten kinetics. Apparent steady state kinetic constants were
estimated for sesamin by quantitative measurements of sesamin
monocatechol by HPLC analysis. Nonlinear regression analysis
revealed the following: $K_m = 0.032 \pm 0.005$ mM, $V_{max} = 9.3 \pm
0.4$ (μmol·min$^{-1}$·mg$^{-1}$), and $K_{cat} = 7.9 \pm 0.3$ s$^{-1}$.

Substrate Specificity. To examine the substrate specificity of SesA,
we investigated the compounds listed in Fig. 3. The structures of
the products were determined by LC/MS/MS and NMR analyses.
SesA catalyzed the demethylation of (+)-episesamin, (-)-asarinin,
and (+)-sesaminol to yield monocatechols 3 (SI Appendix,
Figs. S12–S16), 4 (SI Appendix, Figs. S17–S22), 6 (SI Appendix,
Figs. S23–S25), and 8 and 9 (SI Appendix, Figs. S26–S28), and
di catechols 5 (SI Appendix, Figs. S17 and S29–S32), 7 (SI Appendix,
Figs. S23), and 10 (SI Appendix, Fig. S26). Moreover, piperine (derived
from black pepper) was also demethylated (SI Appendix, Figs.
S33–S35). SesA activity was specific for the methylenedioxy-
phenyl and not the methoxyphenyl group.

Mutational Analysis of SesA. To investigate the reaction mecha-
nism of SesA, we constructed a set of mutants with single amino
acid substitutions. SesA was found to belong to a diverse family
of enzymes that include specific domains of dimethylglycine
oxidase (DMGO) (23), sarsosine oxidase (24), dimethylsulfo-
niopropionate demethylase (DmdA) (25), and demethylase. The
enzyme acts on lignin degradation products such as syringate
and vanillin [DesA (26, 27) and LigM (28), respectively], as well as
GcvT (29).

Based on the amino acid sequence alignment of these enzymes,
we prepared three mutants of SesA: D95A, E189A, and Y221A.
Each of the mutant enzymes was expressed in E. coli and purified
by the method described in Materials and Methods (SI Appendix,
Fig. S36). Enzymatic activity was measured using the method
as that for wild type SesA. E189A and Y221A exhibited no activity
at all. The activity of the D95A derivative was 40% compared
with that of the wild type enzyme.

Discussion

In nature, many physiologically active compounds, such as fla-
vonoids, terpenoids, alkaloids, steroids, coumarins, glycosides,
and nucleosides, are produced by plants and microorganisms.
Unique pathways of microbial metabolism of these compounds
have been reported (30–34). Research on the microbial metab-
olism of a diversity of natural compounds can be expected to
reveal novel enzymes (4) and catalytic functions (34). We recently
reported a curcumin metabolic pathway in E. coli and identified
a novel curcumin/dihydrocurcumin reductase (4). Here, we isolated
sesamin catabolizing microorganisms and determined the initial
steps of the sesamin metabolic pathway at both protein and
gene levels.

Sesamin is a major lignan in sesame oil with characteristic
methylenedioxyphenyl groups. A methylenedioxyphenyl group
is present in some plant metabolites, such as berberin (isolated
from Berberis), piperonal (isolated from dill, vanilla, violet
flowers, and black pepper), and piperine (isolated from black pepper),
as well as sesamin. Also, drugs such as tadalafil and 3,4-methyl-
enedioxyamphetamine (MDMA) have a methylenedioxyphenyl
group. In humans, the methylenedioxy bridges (O C O) of
methylenedioxyphenyl compounds are oxidized to catechols by
CYP450s (12, 35).

<table>
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<tr>
<th>Substrate</th>
<th>Product</th>
<th>Specific activity (units/mg)</th>
<th>Relative activity</th>
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<tbody>
<tr>
<td>sesamin</td>
<td>1</td>
<td>8.8</td>
<td>100%</td>
</tr>
<tr>
<td>(+)-episesamin</td>
<td>3</td>
<td>2.7</td>
<td>31%</td>
</tr>
<tr>
<td>(-)-asarinin</td>
<td>4</td>
<td>7.7</td>
<td>88%</td>
</tr>
<tr>
<td>diasesamin</td>
<td>no products</td>
<td>9.8</td>
<td>112%</td>
</tr>
<tr>
<td>sesaminol</td>
<td>6</td>
<td>8.5</td>
<td>97%</td>
</tr>
<tr>
<td>sesamolin</td>
<td>8</td>
<td>10</td>
<td>100%</td>
</tr>
<tr>
<td>pinoresinol</td>
<td>11</td>
<td>0.95</td>
<td>11%</td>
</tr>
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Fig. 3. SesA activities for plant derived methylenedioxyphenyl compounds and their derivatives. The specific activities for each substance and structures of reaction products are indicated.
On the other hand, microbial enzymes that metabolize methylenedioxyphenyl bridges have not been reported although metabolites have been identified in a few cases. For example, sesamin is converted into sesamin monocatechol and sesamin di-catechol by A. oryzae (15). In addition, cell-free extracts of Pseudomonas fluorescens strain PM3 oxidize piperyonid acid into protocatechuic and formic acid, requiring NADH or NADPH as a cofactor (36). Recently, it was reported that intestinal bacteria convert sesamin into mammalian lignans (16).

In this study, we identified SesA, which converts sesamin into sesamin mono- and di-catechol. The cleaved methylene group of sesamin is transferred to THF, 5,10-CH2-THF being formed. Thus, SesA is a THF-dependent sesamin/sesamin-monocatechol methyltransferase. In this reaction, the methylene group from the methylenedioxy bridge is transferred without an apparent change in redox state to THF. This mechanism is distinct from that of CYP450 (12), which oxidatively removes the methylene group of sesamin. Compared with the enzymatic activity of SesA and CYP2C9, which contributes significantly to the metabolism of sesamin in the liver, the K_m values of SesA and CYP2C9 were 32 μM and 5.4 μM, respectively. On the other hand, the k_cat value of SesA for sesamin was 220 times higher than that of CYP2C9 (12).

Homology searches of the protein database demonstrated that SesA exhibits low similarity (~20%) to GeVT, which is involved in the glucose cleavage system (GCS) together with P-protein, H-protein, and L-protein. In general, GCS is found in most bacteria, archaea, and the mitochondria of all eukaryotes and plays a role in the 5,10-CH2-THF.

The folate-binding domain of GeVT (pfam01571) is conserved in SesA and also in DMGO, sarcosine oxidase, DmdA, DesA, and LigM. These GeVT family proteins exhibit weak sequence identities and are classified into separate clades from one another (SI Appendix, Fig. S37).

SesA is distinct as follows: (i) SesA is a homo-trimer that forms no complex with any other proteins; (ii) the substrates of SesA are aromatic compounds; and (iii) SesA catalyzes a ring cleavage reaction to transfer the methylene group to THF (Fig. 1).

In particular, point iii is unique to SesA among the GeVT family proteins.

SesA, LigM, and DesA all metabolize aromatic compounds although the sequence similarities of SesA to LigM and DesA are only 22% and 20%, respectively. On the other hand, SesA showed distinct enzymatic activities compared with LigM and DesA. LigM and DesA transfer the “methyl” group to THF, yielding “5,10-CH2-THF.” On the contrary, SesA transferred the “methylene” group of sesamin to THF, giving “5,10-CH2-THF.” Although we investigated each of the compounds listed in Fig. 3, we found that SesA does not transfer the methyl group to THF.

The enzymatic activity of SesA was influenced by the stereochemistry of the methylenedioxyphenyl group (Fig. 3). Dialsesamin and (++)-episesamin monocatechol are inert substrates (Fig. 3), suggesting that SesA catalyzes only the demethylation of an equatorial methylenedioxyphenyl group, when the stereochemistry of the tetrahydrofurans ring is 8R, 8'S. On the other hand, (−)-asarinin is converted into (−)-asarinin di-catechol, which suggests that SesA is able to catalyze the demethylation of methylenedioxyphenyl groups that are in both axial and equatorial positions, when the stereochemistry of the tetrahydrofuran ring is 8S, 8’S.

Also, the activity of SesA was affected by substrate size. Pipérine was an active substrate whereas small methylene dioxyphenyl compounds such as samin were inert substrates (Fig. 3).

The crystal structure of GeVT of Thermotoga maritima shows that aspartic acid (D96), glutamic acid (E195), and tyrosine (Y100) are hydrogen-bonded to THF (29). These hydrogen bonds are also observed in the structures of DMGO, sarcosine oxidase, and DmdA; sequence alignment of SesA, LigM, DesA, GeVT, DMDA, and DMGO demonstrated that D95 and E189 of SesA corresponded to D96 and E195 of GeVT (SI Appendix, Fig. S38).

Tyrosine is not conserved at the corresponding position in the protein sequences of GeVT family proteins. Strictly speaking, Y100 of GeVT, Y660 of DMGO, and Y206 of DmdA are hydrogen-bonded to THF. In this alignment, Y221 of SesA corresponded to Y247 and Y242 of LigM and DesA, respectively. The E189A and Y221A mutants of SesA were inactive. Considering the reported crystal structures of DmdA (25) and GeVT (29), this

<table>
<thead>
<tr>
<th>Table 1. Purification of SesA</th>
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<tr>
<td>Step</td>
</tr>
<tr>
<td>Cell-free extract</td>
</tr>
<tr>
<td>(NH4)2SO4</td>
</tr>
<tr>
<td>Hiprep Butyl FF</td>
</tr>
<tr>
<td>Mimetic Orange 1 A6XL</td>
</tr>
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</table>

Fig. 4. Sesamin metabolic pathway and proposed reaction mechanism. (A) Proposed sesamin metabolic pathway in strain no. 22. (B) DNA sequence of region encoding Sesa and its flanking region. The thf gene exhibits 60% amino acid sequence identity with that of Mycobacterium bovis (UniProtKB accession code P0A5T7), and the th72 gene exhibits 68% amino acid sequence identity with that of Clavibacter michiganensis (UniProtKB accession code B0RD2). (C) Proposed reaction mechanism for Sesa. In C, “B” represents a base.

methyl transfer is suggested to be coupled with proton transfer mechanism, in which proton donors (indicated by BH-family enzymes. Therefore, we propose a possible reaction of THF attacks the CH3 group on the sulfonium ion of the substrate, followed by the cleavage of the C–S bond of the arm after migration of a proton from the protonated R223 to the substrate, yielding a reactive iminium intermediate (38). The iminium intermediate reacts with the N5 atom of THF to form ammonia and an iminium ion including N5, to form 5,10-CH2-THF. (ii) In DmdA, methyl transfer is suggested to be coupled with proton transfer that is initiated by a base and mediated by a water molecule in the active site. In this reaction, as a nucleophile, Sp3-hybridized-N5 of THF attacks the CH3 group on the sulfonium ion of the substrate, to yield 3-(methylthio)propionic acid (25). Therefore, the proton donor is not required in this reaction. (iii) In DMOG, THF attacks the iminium ion of the substrate via the nucleophile N10 atom, with concomitant deprotonation by D552, followed by the formation of sarcosine and 5,10-CH2-THF through intramolecular rearrangement of the covalent intermediate formed between THF and the iminium intermediate (i.e., N5 of THF attacks on the covalent intermediate with concomitant deprotonation of N5 of THF by the nascent sarcosine) (23).

In our study, on the other hand, the activity of D95A was found to be 40% less compared with that of the wild-type enzyme. Considering this finding and the proposed reaction mechanisms of other GcvT family enzymes, direct nucleophilic attack on sesamin by N5 of THF would initiate the reaction, as seen in the case of DmdA. However, the reaction mechanism of SesA is not the same as other members of the GcvT family. In the SesA reaction, the methylenedioxy groups (O=C–O) are cleaved to yield OH groups of catechol moieties; proton donation is required to cleave the O=C–O bond, which is not present in substrates of other GcvT family enzymes. Therefore, we propose a possible reaction mechanism, in which proton donors (indicated by BH′ and B′H′ in Fig. 4C) are involved. In previously reported reaction mechanisms of GcvT family enzymes, proton donation does not occur except in GevT. In GevT, R223 is predicted to donate a proton to the substrate for cleavage of the C–S bond. According to the amino acid alignment of these proteins, an arginine residue, which corresponds to R223 in GevT, is not conserved in SesA, LigM, and DesA. We predict that the amino acid residues act as proton donors that provide the methylenedioxy bridges of sesamin with a proton. Considering the crystal structures of GevT, DmdA, and DMOG in the GevT family enzymes, candidates of proton donor residues in the predicted active site of SesA are as follows: R81, H82, R100, R179, and H225 (SI Appendix, Fig. S38). BH′ and B′H′ provide protons and become :B and :B′, respectively, in sesamin. In the proposed reaction (Fig. 4C), the ring closure to yield 5,10-CH2-THF is predicted to be initiated by a base (B′). Then, the :B′ should accept the proton on the N10 atom of an iminium ion, including the N5 atom of 5,10-CH2-THF in the last step (step iii). The SesA reaction is different from that of other GevT family enzymes in that SesA requires proton donors for the reaction. To identify the amino acid residues B and B′ studies on the crystal structure and site-directed mutagenesis studies of SesA are required.

At the beginning of this study, we isolated strain no. 22 by enrichment culture using sesamin as a sole-carbon source. In the growth experiment, the enzymatic activity of SesA was found to increase just before the onset of cell growth (SI Appendix, Fig. S7). These experiments and Western-blot analysis revealed that SesA formation was induced by sesamin in both media. Moreover, 5,10-CH2-THF produced by the SesA reaction would be metabolized as follows. Analysis of the gene annotation of strain no. 22 indicated that strain no. 22 has a putative formyltetrahydrofolate deformylase gene (thf1), and a putative bifunctional 5,10-CH2-THF dehydrogenase/cyclohydrolase gene (thf2) upstream and downstream of the sesA gene, respectively (Fig. 4B). Thf2 could convert 5,10-CH2-THF into 10-formyltetrahydrofolate (10-CHO-THF), which is a substrate for the biosynthesis of purine. Also, Thf1 could convert 10-CHO-THF into THF. These findings suggest that strain in sesamin no. 22 is important physiologically.

Some SesA homologs, which we found by Blast searches, form a gene cluster with folate-metabolizing enzyme genes (SI Appendix, Fig. S39). Most were derived from Actinobacteria, but this gene set was also observed in the Gram-negative bacterium, Bradyrhizobium japonicum WSM2793. These findings suggest that THF-dependent C1 transfers are distributed in various microorganisms.

Materials and Methods

Bacterial Strains, Plasmids, Primers, and Additional Methods. For bacterial strains, plasmids, and primers, see Supplementary Tables S1 and S2.

For chemicals, HPLC and LC/MS/MS analyses, structure determination, purification of the sesamin-metabolizing enzyme from Sinomonas sp. no. 22, the draft genome sequence of Sinomonas sp. no. 22, cloning and heterologous expression of sesA, determination of the molecular mass of SesA, time courses of cell growth and enzymatic activity, Western blot analysis, measurement of folate, temperature dependency and stability, pH dependency and stability, substrate specificity, circular dichroism analysis, and site-directed mutagenesis, see SI Appendix, Supplementary Methods.

Isolation of Sesamin-Metabolizing Microorganisms. Sesamin-metabolizing microorganisms were isolated from soil in the University of Tsukuba and sesame gardens by the following enrichment method. Step 1 was as follows: 1 g of collected soil was added to 10 mL of sesamin medium, which consisted of 0.1% (wt/vol) sesamin, 1% (wt/vol) aminopterin, 0.5% (wt/vol) thymine, 0.025% (wt/vol) K3HPO4, 0.0045% (wt/vol) MgSO4·7H2O, 0.0005% (wt/vol) FeSO4·7H2O, and 10% (vol/vol) tap water, adjusted to pH 7.0 with NaOH, followed by incubation at 28 °C or 37 °C for 3 d. Step 2 was as follows: 2% (vol/vol) of the cultivated medium was added to the same fresh medium, followed by incubation at 28 °C or 37 °C for 3 d. Step 2 was repeated three times.

After enrichment, the culture broth was spread on sesamin sole-carbon agar plates, which contained 1.5% (wt/vol) agar in addition to the above sesamin sole-carbon medium, and colonies that grew on these plates on 1 wk incubation at 28 °C were isolated. Each of the isolated strains was inoculated into a test tube containing 10 mL of sesamin sole-carbon medium, followed by incubation at 28 °C for 2 d. Cells were harvested by centrifugation (4,000 × g, 10 min, 4 °C) and, after washing twice with 10 mM potassium phosphate buffer (pH 7.0), were resuspended in 200 μL of buffer. Then, 250 μL of 0.5% (wt/vol) FeSO4·7H2O, and the cell debris was removed by centrifugation (27,000 × g, 10 min, 4 °C) to prepare a cell-free extract. Two hundred microliters of the reaction mixture comprised 10 μL of 100 mM KPB (pH 7.0), 10 μL of 10 mM sesamin (in DMSO), 100 μL of the cell-free extract, and milliQ water. After incubation at 28 °C for 16 h, the reaction was stopped by adding 100 μL of acetonitrile. The reaction samples were analyzed by HPLC and LC/MS.

Enzyme Assay. Measurement of enzyme activity was performed as follows. One hundred microliters of the reaction mixture [1 μL of 0.73 mg/mL SesA, 5 μL of 1 M Tris-HCl (pH 8.0), 3 μL of 10 mM substrate (in DMSO), 10 μL of 10 mM THF, and 2 μL of TWEEN 80 were used]. THF was dissolved in 50 mM Tris-HCl (pH 9.0), 1% 2-mercaptoethanol, and 2% (wt/vol) ascorbate. One unit of sesamin-converting activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol of sesamin monooctetehol per minute. Specific activity is expressed as units per milligram of protein.

The reaction was initiated by adding the enzyme, followed by incubation at 28 °C for an appropriate time. After incubation, the reaction was stopped by adding 100 μL of acetonitrile.

For determination of the kinetic parameters for the demethylenation of sesamin, 100 μL of the reaction mixture consists of 1 μL of 0.073 mg/mL SesA, 5 μL of 1 M Tris-HCl (pH 8.0), 10 μL of 10 mM THF, 2 μL of TWEEN 80, and from 0.05 mM to 0.3 mM sesamin. The reactions were initiated by the addition of SesA, followed by incubation at 28 °C, and then termination at 1, 3, 5, 7, and 10 min by the addition of 50 μL of acetonitrile. The experiments

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were carried out in duplicate independently. The \( k_{\text{cat}} \) values were calculated using a M, of 50,385 for SesA.

**Nucleotide Sequence Accession Numbers.** The nucleotide sequence data reported in this paper appear in the DDBJ/GenBank database under accession numbers LC101493 for sesA and thf2, LC101494 for thf7, and LC101495 for the 16S rRNA gene.

**ACKNOWLEDGMENTS.** We thank Dr. Kentaro Shiraki (University of Tsukuba) for help with circular dichroism spectra analysis.

Supplementary Information

Discovery of a sesamin-metabolizing microorganism and a new enzyme

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Figure S39. SesA homologs and the flanking genes.

Supplementary References
Supplementary Data

Supplementary Data 1. Properties of strain No. 22.

Major features of strain No. 22: Rod shape, gram-positive, non-spore forming, non-motile, growth at 15°C and 37°C, no growth at 45°C, no growth under anaerobic conditions, growth at 4% NaCl, catalase-positive, oxidase-negative and acid/gas production from glucose negative/negative.

i) Colony morphology on culture on LB media for 48 h:
   Diameter, 1.0 - 2.0 mm; color, yellow; shape, circular; raised state, lenticular; circumference, all edge; shape of the surface, smooth; transparency, opacity and viscosity, butter-like.

ii) Biochemical tests:
   Positive: β-Galactosidase, acetoin production (VP), lipase (Tween80) activity
   Negative: Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, use of citric acid, H₂S production, urease, tryptophan deaminase, indole production, gelatinase, nitrate reduction, casein hydrolysis, starch hydrolysis.

iii) Acid production from the carbohydrates:
   Positive: Fructose, mannose, esculin.
   Negative: Glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-D-xylose, galactose, glucose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, N-acetyl glucosamine, amygdalin, arbutin, salicin, cellobiose, mannotose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid.
Supplementary Data 2. The nucleotide and deduced amino acid sequences of \textit{sesA}.

The nucleotide sequence of \textit{sesA}.

\begin{verbatim}
1  ATGACCGCGACAAGCAATTCAACGGGCGGTACGCTCGCGCGCCGAGCTTGGATTTGTGCCCGCTGGA
72  GTATCGCGGTCTACGCGGAGGAGCGGTGTGCTCGAGGAGGACGGCCTACGATGCAGCGGATTTCGGATC
143  CCAATGCGGGCTTTACGTCGACGACGGCGACGGCGCCTACGAGTCGCGCTGATGCTGGATTTGCTGCT
214  TACGCGGTCTGGGGCTACCGAGAATCGCGCGCCGCTTCTGTCAACGCGACACCGATCTCTGACGCTGCG
285  CGGGCTAGTCGCTCGCTGCTGCTTGCAGCGGAGCGGAGCGGAGCGGAGCGGAGCGGAGCGGAGCGGAG
356  CCACGCGTGCTGATGGATGGTACCGGGTCTCGACGGGGACTGCTCGCTGCTGCTGCTGCTGCTGCTGCT
427  CGGAGGATCGGCGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
498  CATGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
569  TCCACGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
640  GTGAAGCAGGCGCTGACGCTTCTCTGAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGC
711  CCCGCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
782  ACAAGTACGGGCTTTTCTACCCGCGTCGCTCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
853  GGCTGGGGGGAATGACGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCG
924  AGCCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
995  TTTACCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
1066  CTCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
1137  CAGACGCGCTCTACCCGCGTCGCTGACGCTTCTCTGAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGC
1208  CATGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
1279  TCTCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
1350  CGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
\end{verbatim}

The amino acid sequence of SesA.

\begin{verbatim}
1  MTAEQAINEGAFSLAASFGFVPLEYRGYAEVLASKAYTIGTALNGAMSPIYDVTGPDALFELRSVCINS
72  FRGFQVQIRHAVLCNDKQGITLDGVARIEDTDYTRYWTAPALEYRINVSGDVLKGEDQSSNEFFQLAG
143  PRLSEVLEAAEHELHDIAGFRHRMSLATAGPIVRLRMLMAGGLAVEHAGAATETAYAIAEQAQPGFL
214  VKQLGNYALMLQHTAGFPNNLNLMVPLWYPEDPMAAFFDTRPQNFNYKRRFFYGSVGPDEARFVFYPI
285  GLLKMDVNFHDFIGKeeQLQERAEDHAWAVTLYWHEDVADVASKYGRDVEPYKIDDRFIDYHNLQ
356  PGFAYHADLADGERIGTSRINSVYVRRRMSLGFIDKHHAEGETLTLWGRPGTPQKEIRVTGRYP
427  YFDLEKNAIDVASIPRPALVDSAGA
\end{verbatim}
Supplementary Data 3.  Metal analysis of SesA.

Qualitative and quantitative analyses of the following 51 metals in the purified enzyme solution (1.48 mg/mL) were performed with an inductively coupled radiofrequency plasma spectrophotometer: Ba, B, Mg, Al, Si, P, S, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Sr, Zr, Mo, Pd, Ag, Cd, Sn, Sb, Ta, W, Pt, Au, Hg, Pb, Li, Na, K, Sc, Ga, As, Rh, In, Re, Ir, Ti, Bi, Ge, Y, Nb, Ru, Te, Hf, and Os.
Supplementary Methods

Chemicals

Sesamin, sesamol, curcumin and piperine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (-)-Asarinin, diasesamin, (+)-episesamin, samin, sesaminol and (+)-sesamolin were purchased from Nagara Science Co., Ltd. (Gifu, Japan). Isovanillin was purchased from Tokyo Chemical Industries (Tokyo, Japan). Protocatechualdehyde, vanillin and piperonal were purchased from Nacalai Tesque Co., Inc. (Kyoto, Japan). Pinoresinol and tetrahydrofolic acid (THF) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). 5-CH$_3$-THF and 5,10-CH$_2$-THF were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). All chemicals used were of analytical grade.

HPLC and LC/MS/MS analyses

A sample was applied to a Cosmosil πnap column (4.6 x 150 mm; Nacalai Tesque Co., Inc. Kyoto, Japan). The analyses were carried out using a Prominence system with a photodiode array detector (SPD-M20A) and an LCMS-8030 (Shimadzu, Kyoto, Japan). The HPLC conditions were as follows: flow rate, 1 ml min$^{-1}$; solvent A, 0.1%(v/v) HCOOH; and solvent B, acetonitrile. After column equilibration with 10% acetonitrile, a linear gradient system of acetonitrile, 10% to 100%, was applied over 15 min, followed by 100% acetonitrile for 5 min.

Structure determination

The structures of products of the SesA reaction were determined using LC/MS/MS data (LCMS-8030, Shimadzu), high-resolution mass spectral (HRMS) data (Synapt G2; Waters Inc., MA, USA), and nuclear magnetic resonance (NMR) spectral data (AVANCE-600; Bruker, Rheinstetten, Germany).

Purification of the sesamin-metabolizing enzyme from Sinomonas sp. No. 22

Sinomonas sp. No. 22 was cultured in PYS medium (1% Bacto Yeast Extract [DIFCO], 1.6% Bacto Peptone [DIFCO] and 0.5% NaCl) at 28°C for 48 h. Column chromatography was carried out using an AKTA purifier (GE Healthcare).
At first we carried out partial purification through the following steps.

Step 1: Cells were harvested and resuspended in 20 mM Tris-HCl buffer (pH 8.0) to disrupt them by sonication at 150 W for 30 min with an INSONATOR 201M (Kubota, Tokyo, Japan). The lysate was centrifuged at 27,000 × g at 4°C for 20 min.

Step 2: The resulting supernatant was applied to a Hiprep DEAE column (GE Healthcare) and eluted with increasing concentrations of NaCl (0 M to 1 M).

Step 3: The active fractions were concentrated and fractionated by gel-filtration (HiLoad superdex200 26/60, GE Healthcare). The active fractions were analyzed by SDS-PAGE.

After the partial purification of the sesamin-metabolizing enzyme, we determined its partial N-terminal amino acid sequence and identified the gene coding for the enzyme. Next, we carried out complete purification through the following steps.

Step 1: The preparation of cell-free extracts was the same as above.

Step 2: The cell-free extracts were fractionated with ammonium sulfate (35-55% saturation), followed by dialysis against 20 mM Tris-HCl buffer (pH 8.0).

Step 3: After dialysis, a sample was brought to 1 M ammonium sulfate, and then applied onto a Hiprep Butyl FF (GE Healthcare) column equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 M ammonium sulfate. The enzyme was eluted by decreasing the concentration of ammonium sulfate (1 M to 0 M).

Step 4: The active fractions were dialyzed and placed on a Mimetic Orange® 1 A6XL column (PROMETIC BIOSCIENCES, Ltd. Cambridge, U.K.) equilibrated with 20 mM Tris-HCl buffer. The enzyme was eluted with 20 mM Tris-HCl (pH 8.0) buffer containing 2 M NaCl.

Draft genome sequence of Sinomonas sp. No. 22

Total DNA from Sinomonas sp. No. 22 was prepared as follows: the strain was cultured at 28°C for 48 h in 100 ml of 2 × YT media. Cells were harvested by centrifugation, washed with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 100 mM NaCl, and then suspended in 10 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA and 15% (w/v) sucrose. The suspension was incubated with 7 mg/ml of lysozyme at 37°C for 3 h, and then 2 ml of 0.5 M EDTA (pH 8.0), 2 ml of 10% SDS and 2.7 mg of proteinase K were added to the solution, followed by incubation at 55°C for 16 h. DNA was purified by extracting the lysate with phenol/chloroform/isoamylalcohol (25/24/1; v/v/v), followed by precipitation with isopropanol, treatment with RNase, and then reprecipitation with ethanol. Draft genome sequencing of strain
No. 22 was performed using an Illumina Hiseq platform (Hokkaido System Science Co., Ltd., Sapporo, Japan). We obtained 27.9 million reads of a 100 bp paired-end read. A total of 69 contigs comprising 149 ~ 618,663 bp were assembled. The draft genome sequence was annotated with MiGAP (http://www.migap.org).

**Cloning and heterologous expression of sesA**

*SesA* was amplified using the listed primers (Table S2). The underlined letters represent the *Nde*I and *Bam*HI restriction sites, respectively. The PCR product was cloned into the linearized pET24a(+) vector by using In-fusion (Clontech Laboratories Inc.). The resulting plasmid was designated as pET24a(+)-sesA. *E. coli* BL21 CodonPlus (DE3) RIL cells harboring plasmid pET24a(+)-sesA were cultivated in 1 L of liquid 2×YT medium containing 50 µg/ml kanamycin, and grown at 37°C to an OD<sub>600</sub> of 1.5. The temperature was lowered to 18°C and isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM. The cells were cultured for a further 20 h and then harvested. Forty milliliters of 20 mM Tris-HCl buffer (pH 8.0) was added to the pellet (20 g). The cells were disrupted with a sonifier. The lysate was centrifuged at 27,000 × g at 4°C for 20 min. Purification was performed by the same method as that described under “Purification of the sesamin-metabolizing enzyme from Sinomonas sp. No. 22”.

**Determination of the molecular mass of SesA**

Gel-filtration chromatography was performed for determination of the molecular mass of SesA. A sample was applied to a Superdex x200 FPLC column (GE Healthcare). The analysis were carried out using an AKTA purifier (GE Healthcare). The conditions for the analysis were as follows: flow rate, 1 ml min<sup>-1</sup>; and buffer, 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl. The standard proteins, i.e., glutamate dehydrogenase (290 kDa), lactate dehydrogenase (140 kDa), enolase (67 kDa), myokinase (32 kDa), and cytochrome c (12.4 kDa) (MW-Marker [HPLC], Oriental Yeast Co., Ltd., Ohtsu, Japan), were applied before and after sample injection. The molecular mass of the enzyme was calculated from the mobilities of the standard proteins.
Time course of cell growth and enzymatic activity

*Sinomonas* sp. No. 22 was cultured in 10 ml of 2×YT liquid medium for 48 h at 28°C. The cells were harvested by centrifugation at 4,000 rpm and 4°C for 10 min, and then washed twice with 10 mM potassium phosphate buffer (pH 7.0). The cells were resuspended in 10 ml of potassium phosphate buffer (pH 7.0), and then inoculated 1% (v/v) into 100 ml of the following four types of medium; i) sesamin sole-carbon liquid medium, ii) glucose sole-carbon liquid medium, iii) 2×YT liquid medium supplemented with 0.1% (w/v) sesamin, and iv) 2×YT liquid medium. The cells were cultured at 28°C. During the cell growth, 1 ml samples were withdrawn every 4 h for measurement of OD$_{600}$, analysis of supernatants by HPLC and preparation of cell-free extracts. The cell-free extracts were subjected to measurement of the protein concentration and sesamin degradation activity.

Western blot analysis

*Sinomonas* sp. No. 22 was cultured in 10 mL of 2×YT liquid medium with or without 20 mg sesamin. After centrifugation at 4,000 × g for 10 min (CR-21N; Hitachi, Ibaraki, Japan), the pellets were resuspended in 1.25 mL of 20 mM Tris-HCl (pH 8.0), cell-free extracts being prepared. An equal volume of 2× electrophoresis sample buffer was then added and the samples were boiled for 5 min. Samples were subjected to 12% SDS-PAGE and then transferred to PVDF membranes (Sequi-Blot™ PVDF Membrane Roll; Nippon BioRad Laboratories Inc., Tokyo, Japan) using a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Trans-Blot SD; BioRad Laboratories). Pre-stained protein markers (Low range) (Nacalai Tesque, Inc., Kyoto, Japan) were used as molecular mass standards. SesA was detected with anti-SesA specific antibodies. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. ECL (ECL Western Blotting Detection Reagents; GE Healthcare) was used for protein detection. The protein bands were detected on film (Amersham Hyperfilm™ ECL; GE Healthcare). The purified SesA cross-reacted with the anti-SesA antibodies.

Measurement of folates

A sample was applied to a Kinetex 1.7 µm 100Å column (2.1 x 50 mm; Phenomenex, USA). The analysis was carried out using a Prominence system with a photodiode array detector (SPD-M20A) and LCMS-8030. The HPLC conditions were as follows: Flow rate, 0.4 ml min$^{-1}$; solvent A, water; and solvent B,
acetoni. After column equilibration with 5 % B, a linear gradient system of B from 5% to 25% was applied over 5 min, followed by 25% to 100% over 2 min, and 100% of B for 1 min. The extracted ions following MRM transitions were monitored at \( m/z \) 458.10→311.10 for 5,10-\( \text{CH}_2 \)-THF, and \( m/z \) 460.10→313.10 for 5-\( \text{CH}_3 \)-THF.

**Temperature dependency and stability**

One hundred microliters of the reaction mixture consisted of 1 µl of 0.0731 mg/ml SesA, 5 µl of 1 M Tris-HCl (pH 8.0), 3 µl of 10 mM sesamin (in DMSO), 10 µl of 10 mM THF and 2 µl of Tween 80. The experiments were carried out in triplicate at 10, 20, 25, 30, 35, 40, 45, 50, 60 and 70°C for 10 min. A CHILL HEAT CHT-101 (IWAKI Asahi Techno Glass, Japan) was used for incubation at 10 to 50°C, and a Dry Thermo Unit DTU-1B (TAITEC, Japan) was used for incubation at 60 and 70°C. For thermal stability estimations, SesA was pre-incubated for 15 min at temperatures between 10 and 70°C. The remaining activity was determined by incubating the enzyme at 28°C for 10 min. The amounts of the reaction products were determined by HPLC-PDA.

**pH dependency and stability**

One hundred microliters of the reaction mixture consisted of 1 µl of 0.0731 mg/ml SesA, 25 µl of 0.2 M Britton-Robinson buffer (pH 2.0-12.0 (0.5 pH units)), 3 µl of 10 mM sesamin (in DMSO), 10 µl of 10 mM THF and 2 µl of Tween 80. The experiments were carried out for 10 min at 28°C. For pH stability estimations, SesA was pre-incubated on ice for 30 min at each pH and then the reaction was carried out at 28°C in Tris-HCl buffer (pH 8.0) for 10 min. The reaction was stopped by adding 50 µl of acetonitrile. The amount of the reaction product was measured by HPLC-PDA.

**Substrate specificity**

One hundred microliters of the reaction mixture consisted of 1 ml of 0.73 mg/ml SesA, 5 ml of 1 M Tris-HCl (pH 8.0), 3 ml of 10 mM each substrate (in DMSO), 10 ml of 10 mM THF and 2 ml Tween 80. Large-scale production of products in a total volume of 50 ml was carried out under the same conditions as those for the enzyme assay described above. The reaction mixtures were incubated at 28°C overnight and
then extracted with 50 ml ethyl acetate three times. The extracts were evaporated and the residues were dissolved in 2 ml of methanol. The products were purified by HPLC on a Cosmols πnap column, 20 x 250 mm (Nacalai Tesque, Inc., Kyoto, Japan). The chemical structures of the reaction products were elucidated by analyzing \(^1\)H NMR, \(^{13}\)C NMR, HMBC, and HMQC spectra, and LC/MS/MS and high resolution MS (HRMS) data. The stereochemistry was determined by previous report (1).

**Circular dichroism analysis**

CD measurements were carried out with a Jasco spectropolarimeter, model J-720W (Japan Spectroscopic Co., Ltd., Tokyo, Japan), equipped with a thermal incubation system at 20°C with a 1 mm path length cell. CD spectra were obtained at a protein concentration of 0.1 mg/ml in the far-UV region (200 - 240 nm).

**Site-directed mutagenesis**

Site-directed mutagenesis was performed using a KOD-plus mutagenesis kit (Toyobo Co., Ltd., Osaka, Japan), following the instructions of the manufacturer. The primers used for iPCR are given in Table S2. A clone with the sequence for the desired D95A, E195A and Y221A mutation was chosen and transformed into *E. coli* BL21-CodonPlus(DE3)-RIL. The recombinant cells were used for the overproduction and purification of the mutant enzymes. Each of the mutant enzymes was purified to homogeneity. Each purification step was the same as that for *E. coli* BL21-CodonPlus(DE3)-RIL carrying pET24a(+)–sesA, as described above.
Supplementary Tables

Supplementary Table 1. Bacterial strains and plasmids used in this study.

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<th>Strains and plasmids</th>
<th>Relevant characteristics</th>
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<td><strong>Strains</strong></td>
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<td><em>E. coli</em> DH10B</td>
<td>Cloning host; F−, mcrA, Δ(mrr-hsdRMS-mcrBC), F80d lacZ, DM15, DlacX74, deoR, recA1, araD139, Δ(ara leu)7697, galU, galK, λ−, rpsL, endA1, nupG</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 CodonPlus® (DE3) RIL</td>
<td>Strain for recombinant protein expression; F−ompT hsdS(rB− mB−) dcm+ Tetgal λ(DE3) endA Hte [argU ileY leuW Cam']</td>
</tr>
<tr>
<td><em>Sinomonas</em> sp. No.22</td>
<td>Wild-type sesamin-catabolizing strain</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pET24a(+)</td>
<td>T7 RNA polymerase-dependent recombinant protein expression vector, KanR.</td>
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<td>pET24a(+)−sesA</td>
<td>The sesA fragment (1359 bp) was inserted into the <em>Ndel</em> and <em>BamHI</em> sites of pET24a(+)</td>
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Supplementary Table 2. The primers used in this study.

**Primers used for cloning of sesA**

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<th>Primers for cloning of sesA</th>
<th>Sequence</th>
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<tr>
<td>sesA Fw (<em>NdeI</em>)</td>
<td>AGAAGGAGATATACATATGACCGCAGAAGGCAATCAACGAGG</td>
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<tr>
<td>sesA Rv (<em>BamHI</em>)</td>
<td>ACCGGAGCTCGAATTCCGATCCCTACGCGCCGACAGC</td>
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**Primers used for site-directed mutagenesis**

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<th>Primers for site-directed mutagenesis</th>
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<td>D95A_F</td>
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<tr>
<td>D95A_R</td>
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</tr>
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<td>E189A_R</td>
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<td>Y221A_F</td>
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</tbody>
</table>

The restriction site is underlined.
### Supplementary Table 3.  Partial purification of SesA.

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<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
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<tbody>
<tr>
<td>Cell-free extract</td>
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<td>0.47</td>
<td>0.00089</td>
<td>100</td>
<td>1</td>
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<tr>
<td>Hiprep DEAE FF</td>
<td>75</td>
<td>0.12</td>
<td>0.0016</td>
<td>25</td>
<td>1.8</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>14</td>
<td>0.016</td>
<td>0.0011</td>
<td>3.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

### Supplementary Table 4.  Activity of each fraction obtained on gel-filtration.

The SDS-PAGE of each fraction is shown in Supplementary Fig. 4.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10</td>
<td>5.4</td>
<td>0.0034</td>
<td>0.00063</td>
</tr>
<tr>
<td>A11</td>
<td>13</td>
<td>0.0130</td>
<td>0.00100</td>
</tr>
<tr>
<td>A12</td>
<td>14</td>
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</tr>
<tr>
<td>B1</td>
<td>8.2</td>
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<td>0.00099</td>
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<tr>
<td>B2</td>
<td>1.7</td>
<td>0.0021</td>
<td>0.00125</td>
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Supplementary Figures

Supplementary Figure 1. NMR and HRMS data for compounds A and B.

<table>
<thead>
<tr>
<th>HRMS</th>
<th>Compound A</th>
<th>Compound B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical formula</td>
<td>C_{19}H_{18}O_{6}</td>
<td>C_{18}H_{18}O_{6}</td>
</tr>
<tr>
<td>Calculated mass</td>
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</tr>
<tr>
<td>Observed mass</td>
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<td>329.1022</td>
</tr>
</tbody>
</table>

| NMR spectrum (\textsuperscript{1}H=600 MHz, in methanol-\textit{d}_4) |
|--------------------------|-----------------|-----------------|
| position                 | Compound A      | Compound B      |
|                          | $\delta_H$ (J$_H$= Hz) | $\delta_H$ (J$_H$= Hz) |
| 1                        |                 |                 |
| 2                        | 6.87 (d, J$_H$=1.7, 1H) | 6.79 (d, J$_H$=2.0, 1H) |
| 3                        |                 |                 |
| 4                        |                 |                 |
| 5                        | 6.77 (d, J$_H$=7.9, 1H) | 6.72 (d, J$_H$=8.0, 1H) |
| 6                        | 6.83 (dd, J$_H$=7.9, 1.7, 1H) | 6.68 (dd, J$_H$=8.1, 2.1, 1H) |
| 7                        | 4.68 (d, J$_H$=5.0, 1H) | 4.62 (d, J$_H$=4.4, 1H) |
| 8                        | 3.08 (m, 1H)    | 3.08 (m, 1H)    |
| 9ax                      | 3.81 (m, 1H)    | 3.78 (dd, J$_H$=9.1, 3.6, 1H) |
| 9eq                      | 4.20 (m, 1H)    | 4.20 (dd, J$_H$=9.1, 6.9, 1H) |
| 10                       | 5.92 (s, 2H)    |                 |
| 1'                       |                 |                 |
| 2'                       | 6.79 (d, J$_H$=2.1, 1H) | 6.79 (d, J$_H$=2.0, 1H) |
| 3'                       |                 |                 |
| 4'                       |                 |                 |
| 5'                       | 6.72 (d, J$_H$=8.1, 1H) | 6.72 (d, J$_H$=8.0, 1H) |
| 6'                       | 6.67 (dd, J$_H$=8.2, 1.9, 1H) | 6.68 (dd, J$_H$=8.1, 2.1, 1H) |
| 7'                       | 4.63 (d, J$_H$=4.9, 1H) | 4.62 (d, J$_H$=4.4, 1H) |
| 8'                       | 3.08 (m, 1H)    | 3.08 (m, 1H)    |
| 9ax'                     | 3.81 (m, 1H)    | 3.78 (dd, J$_H$=9.1, 3.6, 1H) |
| 9eq'                     | 4.20 (m, 1H)    | 4.20 (dd, J$_H$=9.1, 6.9, 1H) |
| -OH                      | 8.54 (br s)     | 8.54 (br s)     |
Supplementary Figure 2. $^1$H NMR spectrum of compound A.

Supplementary Figure 3. $^1$H NMR spectrum of compound B.
Supplementary Figure 4.  SDS-PAGE for partial purification of the sesamin-metabolizing enzyme by gel-filtration.

M, molecular weight marker; A10-B2, the active fractions obtained by gel-filtration chromatography. The relative molecular mass of the enzyme was calculated from the mobilities of the marker proteins, phosphorylase $b$ (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa).

Supplementary Figure 5.  SDS-PAGE for complete purification of SesA.

Lane 1, molecular weight marker; Lane 2, cell-free extract of strain No. 22; Lane 3, the fraction of ammonium sulfate (35-55% saturation); Lane 4, the active fraction obtained by Hiprep Butyl FF column chromatography; Lane 5, the active fraction obtained by Mimetic Orange® 1 A6XL column. The relative molecular mass of the enzyme was calculated from the mobilities of the marker proteins, phosphorylase $b$ (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa).
Supplementary Figure 6. Estimation of the molecular mass of SesA from *Sinomonas* sp. by gel-filtration.

The standard proteins are shown in (■) and purified SesA is shown in (○).
Supplementary Figure 7.  Growth curve, and SDS-PAGE and Western blot analyses.

Time course of cell growth, sesamin degradation and SesA activity during culture using media containing sesamin (solid line) or glucose (dashed line) as a sole-carbon source (A); 2×YT media supplemented with (solid line) or without sesamin (dashed line) (B); SDS-PAGE (C); Western blot analysis (D). Symbols: (●) amount of sesamin; (△) amount of sesamin di-catechol; (○) protein concentration in cell-free extracts; (■) OD_{600}; (□) specific activity of cell-free extracts. Experiments were carried out twice independently. M, molecular weight markers; C1, cell-free extracts of strain No. 22 grown in 2×YT medium; C2, cell-free extracts of strain No. 22 grown in 2×YT medium with 0.1% (w/v) sesamin; S, purified SesA. The relative molecular mass of the enzyme was calculated from the mobilities of the marker proteins for SDS-PAGE, phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). And Pre-stained Protein markers (Low range) (Nacalai Tesque, Inc., Kyoto, Japan) were used as molecular mass standards for Western blot analysis.
Supplementary Figure 8. Effects of temperature and pH.

A, temperature dependency; B, pH dependency; C, temperature stability; D, pH stability. Error bars denote the standard error for three independent experiments.
Supplementary Figure 9. Absorption spectrum of the purified SesA.
The concentration of the enzyme was 0.41 mg/ml in 20 mM Tris-HCl (pH 8.0).

Supplementary Figure 10. CD spectrum of SesA.
The concentration of SesA was 0.1 mg/ml.
Supplementary Figure 11. Stoichiometric study of the SesA reaction and Michaelis-Menten analysis of SesA.

The amounts of sesamin and reaction products are indicated in panel (A): sesamin (□), sesamin monocatechol (▲), and sesamin dicatechol (◇). The amounts of 5,10-CH₂-THF are indicated in panel (B): the assay sample (●) and the negative control (incubated without SesA) (○). The Michaelis-Menten kinetics of the purified SesA with sesamin as a substrate are indicated (C): Values represent formation of sesamin mono-catechol. Experiments were carried out three times.
Supplementary Figure 12.  LC/MS analysis of a reaction product of (+)-episesamin.

This is a chromatogram and mass spectrum of the reaction mixture after incubation of (+)-episesamin with SesA. (A) A UV-vis chromatogram of the control reaction without SesA. (B) A UV-vis chromatogram of the assay with SesA. (+)-Episesamin monocatechol 3 is indicated. (C) A mass chromatogram at m/z 341 in the negative ion mode.
Supplementary Figure 13. NMR and HRMS data for 3.

**NMR spectrum** (\(^1\)H=600 MHz and \(^13\)C=150 MHz, in methanol-\(d_4\))

<table>
<thead>
<tr>
<th>position</th>
<th>(\delta_H) ((J_H=) Hz)</th>
<th>(\delta_C)</th>
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<tr>
<td>1</td>
<td>8.90 (br s)</td>
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<tr>
<td>2</td>
<td>6.74 (s, 1H)</td>
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</tr>
<tr>
<td>3</td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td></td>
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<td>6.68 (d, (J_H=7.98), 1H)</td>
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<td>7</td>
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<td>3\text{' }</td>
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<td>147.5</td>
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<tr>
<td>4\text{' }</td>
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<td>81.7</td>
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<tr>
<td>8\text{' }</td>
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<tr>
<td>10\text{' }</td>
<td>6.01 (s, 2H)</td>
<td>101.3</td>
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Supplementary Figure 14. ¹H NMR spectrum of 3.

Supplementary Figure 15. ¹³C NMR spectrum of 3.
Supplementary Figure 16.  HMBC spectrum of 3.
Supplementary Figure 17. LC/MS analysis of a reaction product of (-)-asarinin.

This is a chromatogram and mass spectrum of the reaction mixture after incubation of (-)-asarinin with SesA. (A) A UV-vis chromatogram of the control reaction without SesA. (B) A UV-vis chromatogram of the assay with SesA. (-)-Asarinin mono-catechol 4 and (-)-asarinin dicatechol 5 are indicated. (C) A mass chromatogram at m/z 341 in the negative ion mode. (D) A mass chromatogram at m/z 329 in the negative ion mode.
**Supplementary Figure 18.** NMR and HRMS data for 4.

**NMR spectrum** \(^{1}H=600\text{ MHz and }^{13}C=150\text{ MHz, in methanol-}d_4\)

<table>
<thead>
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<th>(\delta_C)</th>
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<tr>
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<td>4'</td>
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<td>145.3</td>
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<tr>
<td>5'</td>
<td>6.69 (d, (J_H=8.04), 1H)</td>
<td>115.8</td>
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<tr>
<td>6'</td>
<td>6.59 (d, (J_H=5.94), 1H)</td>
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<td>7'</td>
<td>4.69 (d, (J_H=6.84), 1H)</td>
<td>81.8</td>
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<td></td>
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</tbody>
</table>
Supplementary Figure 19. $^1$H NMR spectrum of 4.

Supplementary Figure 20. $^{13}$C NMR spectrum of 4.
Supplementary Figure 21. HMBC spectrum of 4.

Supplementary Figure 22. HMQC spectrum of 4.
Supplementary Figure 23. LC/MS analysis of a reaction product of sesaminol.
This is a chromatogram and mass spectrum of the reaction mixture after incubation of sesaminol with SesA.  
(A) A UV-vis chromatogram of the control reaction without SesA.  
(B) A UV-vis chromatogram of the assay with SesA.  
Sesaminol mono-catechol 6 and sesaminol de-catechol 7 are indicated.  
(C) A mass chromatogram at m/z 357 in the negative ion mode.  
(D) A mass chromatogram at m/z 345 in the negative ion mode.

<table>
<thead>
<tr>
<th>Observed mass</th>
<th>Calculated mass</th>
<th>Chemical formula</th>
<th>ΔmDa</th>
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<tbody>
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<td>357.0997</td>
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<td>C_{19}H_{17}O_{7}</td>
<td>2.3</td>
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</table>

Supplementary Figure 24. MS/MS analysis of 6.
Supplementary Figure 25. MS/MS spectrum of 6.

Supplementary Figure 26. LC/MS analysis of a reaction product of (+)-sesamolin.

This is a chromatogram and mass spectrum of the reaction mixture after incubation of (+)-sesamolin with SesA. (A) A UV-vis chromatogram of the control reaction without SesA. (B) A UV-vis chromatogram of the assay with SesA. (+)-Sesamolin mono-catechols 8, 9 and (+)-sesamolin di-catechol 10 are indicated. (C) A mass chromatogram at m/z 357 in the negative ion mode. (D) A mass chromatogram at m/z 345 in the negative ion mode.
Supplementary Figure 27. MS/MS analyses of 8 and 9.

Supplementary Figure 28. MS/MS spectrum of 8 and 9.
Supplementary Figure 29. NMR and HRMS data for 5.

**NMR spectrum** (\(^1\)H=600 MHz and \(^{13}\)C=150 MHz, in methanol-d\(_4\) )

<table>
<thead>
<tr>
<th>Position</th>
<th>(\delta_H ) ((J_H) Hz)</th>
<th>(\delta_C)</th>
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</thead>
<tbody>
<tr>
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<td>6.73 (s, 1H)</td>
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<td>7</td>
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<tr>
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</tr>
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</table>
Supplementary Figure 30. $^1$H NMR spectrum of 5.

Supplementary Figure 31. $^{13}$C NMR spectrum of 5.
Supplementary Figure 32.  HMBC spectrum of 5.

**General**

DATE = 2014/09/02  
TIME = 08:33  
INSTRUM = spect  
PULPROG = inv4gplplrndqf

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SF = 600.13  
SW_p = 6613.757

**F1 [13C]**

SI = 1024  
SF = 150.903  
SW_p = 33557.047
Supplementary Figure 33.  HRMS analysis of 11.

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</table>

Supplementary Figure 34.  \(^1\)H NMR spectrum of 11.
Supplementary Figure 35.  HMQC spectrum of 11.
Supplementary Figure 36. SDS-PAGE of mutants of SesA and wild type SesA.

1, molecular weight marker; 2, purified SesA (D95A); 3, purified SesA (E189A); 4, purified SesA (Y221A); 5, purified SesA (wild type); The relative molecular mass of the enzyme was calculated from the mobilities of the marker proteins, phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).
Supplementary Figure 37.  Phylogenetic analysis of GcvT family proteins.

Vanillin demethylase is from *S. paucimobilis* (LigM) (UniProtKB: Q60FX1). Syringate demethylase is from *S. paucimobilis* (DesA) (UniProtKB: Q7WST1). Dimethylsulfoniopropionate demethylase is from *P. ubique* (DmdA) (UniProtKB: Q4FP21). GcvT proteins are from *E. coli* (Ec) (UniProtKB: P27248), *T. maritima* (Tm)(UniProtKB: Q9WY54), *B. subtilis* (Bs)(UniProtKB: P54378), and *P. aeruginosa* (Pa)(UniProtKB: Q9HTX5). The other proteins were searched for by using these amino acid sequences as query sequences by means of a blastp search. Clade A includes homologues of Ec and Tm. Clade B includes homologues of SoxA. Clade C includes homologues of LigM and DesA. Clade D includes homologues of SesA. Clade E includes homologues of DmdA.
Supplementary Figure 38. Amino acid sequence alignment of bacterial GcvT, SesA, LigM, DesA and DmdA.

Vanillin demethylase is from *S. paucimobilis* (LigM) (UniProtKB: Q60FX1). Syringate demethylase is from *S. paucimobilis* (DesA) (UniProtKB: Q7WST1). Dimethylsulfoniopropionate-dependent demethylase is from *P. ubique* (DmdA) (UniProtKB: Q4FP21). Dimethylglycine oxidase is from *A. globiformis* (DMGO) (UniProtKB: Q9AGP8). GcvT proteins are from *E. coli* (Ec) (UniProtKB: P27248), *T. maritima* (Tm) (UniProtKB: Q9WY54), *B. subtilis* (Bs) (UniProtKB: P54378), and *P. aeruginosa* (Pa) (UniProtKB: Q9HTX5). Alignment was performed using Clustal W (2). Identical residues are shown in white in red boxes and similar residues in red. The asterisks highlight residues that were substituted with alanine in this study. The black squares above the sequence indicate the residues that are predicted to give a proton to sesamin in SesA reaction.
**Supplementary Figure 39. SesA homologous genes and the flanking genes.**

SesA homologs are shown in blue. The numbers on the blue arrows indicate the protein sequence identity/similarity to SesA. The numbers on the orange arrows indicate the names of enzymes as follows: 1, 5,10-methylene-tetrahydrofolate cyclohydrolase; 2, tetrahydrofolate dehydrogenase; 3, formate-tetrahydrofolate ligase; 4, formyltetrahydrofolate deformylase; 5, bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/cyclohydrolase; 6, 5,10-methylenetetrahydrofolate reductase; 7, 5,10-methylene-tetrahydrofolate dehydrogenase.
Supplementary References
