Isolation of the (+)-Pinoresinol-Mineralizing *Pseudomonas* sp. Strain SG-MS2 and Elucidation of Its Catabolic Pathway

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ABSTRACT Pinoresinol is a dimer of two β-β’-linked coniferyl alcohol molecules. It is both a plant defense molecule synthesized through the shikimic acid pathway and a representative of several β-β-linked dimers produced during the microbial degradation of lignin in dead plant material. Until now, little has been known about the bacterial catabolism of such dimers. Here we report the isolation of the efficient (+)-pinoresinol-mineralizing *Pseudomonas* sp. strain SG-MS2 and its catabolic pathway. Degradation of pinoresinol in this strain is inducible and proceeds via a novel oxidative route, which is in contrast to the previously reported reductive transformation by other bacteria. Based on enzyme assays and bacterial growth, cell suspension, and resting cell studies, we provide conclusive evidence that pinoresinol degradation in strain SG-MS2 is initiated by benzylic hydroxylation, generating a hemiketal via a quinone methide intermediate, which is then hydrated at the benzylic carbon by water. The hemiketal, which stays in equilibrium with the corresponding keto alcohol, undergoes an aryl-alkyl cleavage to generate a lactone and 2-methoxyhydroquinone. While the fate of 2-methoxyhydroquinone is not investigated further, it is assumed to be assimilated by ring cleavage. The lactone is further metabolized via two routes, namely, lactone ring cleavage and benzylic hydroxylation via a quinone methide intermediate, as described above. The resulting hemiketal again exists in equilibrium with a keto alcohol. Our evidence suggests that both routes of lactone metabolism lead to vanillin and vanillic acid, which we show can then be mineralized by strain SG-MS2.

IMPORTANCE The oxidative catabolism of (+)-pinoresinol degradation elucidated here is fundamentally different from the reductive cometabolism reported for two previously characterized bacteria. Our findings open up new opportunities to use lignin for the bio-synthesis of vanillin, a key flavoring agent in foods, beverages, and pharmaceuticals, as well as various new lactones. Our work also has implications for the study of new pinoresinol metabolites in human health. The enterodiol and enterolactone produced through reductive transformation of pinoresinol by gut microbes have already been associated with decreased risks of cancer and cardiovascular diseases. The metabolites from oxidative metabolism we find here also deserve attention in this respect.

KEYWORDS *Pseudomonas*, pinoresinol, degradation, SG-MS2, lignin, lignan, pathway, metabolites, mineralization

Lignans are dimers formed through β-β’ linkages between monolignols (p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol), which in turn are formed from hydroxycinnamic acids (p-coumaric acid, ferulic acid, and sinapic acid, respectively) (1). Lignans are found in plants and many algae, where they are synthesized from inter-
mediates of the shikimic acid biosynthetic pathway and function as defense molecules against herbivores and microorganisms (2). One of the major lignans is (+)-pinoresinol (here referred to simply as pinoresinol), which contains two fused tetrahydrofuran rings and is synthesized by radical coupling of two coniferyl alcohol subunits (1, 3). Pinoresinol is also a component of lignin, the complex polymeric material that is crucial for the structural integrity of plants, which is also synthesized from monolignols but results from random polymerization in the cell wall (4). Cross-linkages among lignin subunits, such as \( \beta-\beta' \) linkages, not only are key to the rigidity of living plant structures but also render many of those structures relatively resistant to decay in dead plant material (5).

Aerobic brown rot and white rot fungi are thought to play major roles in degrading the lignin in dead plant material in open environments (6–8). Relatively little is known about the enzymology of delignification in brown rot fungi, but enzymes released by white rot fungi, such as lignin peroxidase, manganese peroxidase, versatile peroxidase, and various laccases, are known to catalyze at least the initial steps via free radical biochemistry (7–9). Products with progressively lower molecular weights are generated over time, including large pools of dimer and monomer subunits (10). There is also direct evidence that the white rot fungus, \textit{Phanerochaete chrysosporium}, and various strains of the soil fungus \textit{Fusarium} use such peroxidases to attack pinoresinol and another \( \beta-\beta' \)-linked lignin dimer, (±)-syringaresinol (a dimer of sinapyl alcohol), and various derivatives of the latter (11–13). In the case of syringaresinol and its derivatives, this attack initially involves oxidation at the benzylic position to generate \( \alpha \)-carbonyl compounds (Fig. 1), which are then cleaved at the alkyl-aryl bond (11, 13, 14). In the case of pinoresinol, however, the peroxidase-initiated attack involves carbon-carbon and carbon-oxygen polymerization reactions at the free positions \textit{ortho} to the phenolic hydroxyl groups, rather than \( \alpha \)-hydroxylation (13, 15). Notably, one \textit{Fusarium} strain, M-4-2, can catalyze the \( \alpha \)-hydroxylation of both pinoresinol and syringaresinol with an inducible intracellular enzyme that is assumed to be different from the extracellular peroxidases described above (12, 16); this fungal enzyme has not yet been characterized.

There is relatively little evidence that bacteria play significant roles in the early steps of delignification in the environment (7). However, there is considerable evidence that cohabiting bacteria use the monomers generated by white rot fungi as a source of carbon, and there is growing evidence that they can similarly use at least some of the dimers (7, 17). The latter situation is best understood for other intermonomer cross-linkages, such as \( \beta-O-4 \), \( 5-5 \), and \( \beta-5 \) bonds; for example, \textit{Sphingobium} sp. strain SYK-6 and an alphaproteobacterium belonging to the family \textit{Erythrobacteraceae}, strain SG61-

![Figure 1](http://aem.asm.org/)

**FIG 1** Oxidative and reductive degradation of pinoresinol and syringaresinol, respectively, by microbes. It should be noted that reductive transformation of syringaresinol to 5,5-dimethoxylariciresinol by bacterial strain SYK-6 is not shown here.
1L, both express dehydrogenases that initiate mineralization of the β-aryl ether guaiacylglycerol-β-guaiacyl ether (GGE) (18, 19). Strain SYK-6, Enterococcus faecalis strain PDG-1, and Pseudomonas putida strain FK2 are the only three bacteria reported to degrade enantiomers of the β-β'-linked pinoresinol (20–22). While strain FK2 can use (±)-pinoresinol as a sole carbon source, the other two strains require additional carbon sources to transform (+)-pinoresinol (20–22). While little is known regarding the catabolic pathway in strain FK2, strains PDG-1 and SYK-6 reduce pinoresinol to secoisolariciresinol via lariciresinol (20–22). Furthermore, a reductase, PinZ, that can use NADH as a cofactor to reduce both (+)-pinoresinol to (+)-lariciresinol and syringaresinol to 5,5-dimethoxylariciresinol has also been observed in strain SYK-6 (Fig. 1) (23). Notably, this reductive pathway contrasts with the oxidative pathway in the fungi described above but is similar to the situation in plants, where pinoresinol is reduced to secoisolariciresinol via lariciresinol in two steps by a pinoresinol/lariciresinol reductase homologous to PinZ (24).

There is some evidence that intestinal fungi in some wood-eating insects can also degrade lignin (25). The initial steps in the process are oxidative, although the enzymes responsible have not been isolated (26). There is relatively little evidence for gastrointestinal degradation of lignin in mammals, although lignan transformation has been well documented (27). Pinoresinol is sequentially converted to lariciresinol, secoisolariciresinol, and matairesinol, before further transformation into enterodiol and enterolactone (28). Both of the latter products are absorbed in the large intestine of mammals, and there is growing evidence that they may then have various anticancer and beneficial cardiovascular effects (21, 28). The two initial reductions, generating secoisolariciresinol via lariciresinol (Fig. 1), are again thought to be catalyzed by PinZ/pinoresinol lariciresinol reductase homologues (29).

Here we describe a novel soil bacterium that efficiently mineralizes pinoresinol by a pathway initiated by an oxidative reaction similar to that in the Fusarium fungal species described above. Furthermore, we show that this oxidation is achieved intracellularly by an uncharacterized enzyme that is produced upon exposure to pinoresinol. Some of the next steps in the pathway are also elucidated and others are proposed, based on extensive characterization of metabolites.

RESULTS AND DISCUSSION

Enrichment, isolation, and characterization of SG-MS2. We set up three enrichment cultures, from garden soil, olive tree rhizosphere soil, and wheat straw/manure compost, by providing 55.8 μM pinoresinol as a sole carbon source in minimal salt medium (MSM). Only the wheat straw/manure compost enrichment was successful. The pinoresinol in this culture completely disappeared within 3.5 h after five rounds of enrichment. When diluted culture medium from the fifth enrichment round was plated on Luria-Bertani (LB) agar at 28°C, two morphologically distinct colonies appeared after 24 h. These colonies were picked, and both were found to mineralize pinoresinol as the sole source of carbon and energy. These colonies, designated SG-MS1 and SG-MS2, were identified as strains of the genera Burkholderia and Pseudomonas, respectively, based on 99% identities of their 16S rRNA genes (1,380 bp each) with known strains of the respective genera. Although the 16S rRNA genes of these two strains were also 99% identical to each other and the two strains produced the same catabolic intermediates in the culture supernatant, strain SG-MS2 showed much faster degradation of pinoresinol and therefore was selected for detailed analysis.

Growth with pinoresinol. When 558 μM pinoresinol was provided as the sole source of carbon and energy, a 1% (vol/vol) seed culture of strain SG-MS2 almost completely consumed pinoresinol within 6 h (Fig. 2A). The only other known pinoresinol-mineralizing bacterium, Pseudomonas putida FK2, was reported to consume 35 μM pinoresinol almost to completion in 48 h (30). The growth yield for strain SG-MS2 was 220.75 g of total protein/mole of pinoresinol across the range of pinoresinol concentrations tested (14 to 279 μmol) (see Fig. S1 in the supplemental material), which is equivalent to 11.04 g of protein (or 22.08 g [dry weight], assuming protein to
be 50% of the dry weight) per mole of carbon ($Y_{sub}$) (31). Assuming that bacterial cell dry weight is approximately 50% carbon (31), this represents an efficiency of pinoresinol assimilation of $\sim 91\%$. However, $Y_{sub}$ is known to vary widely among substrates and cannot be used to determine whether a substrate is completely mineralized; therefore, $Y_{av/e}$—(grams of dry cell yield per equivalent of available electrons utilized, either by transfer to oxygen or by incorporation in biomass (32))—was also calculated. A $Y_{av/e}$ value of $\sim 3.0$ indicates complete mineralization of any carbon source (32). We obtained a value of 2.6 for pinoresinol degradation, which indicates that pinoresinol may not be completely mineralized by strain SG-MS2.

Both microbial growth and pinoresinol degradation showed a discrete lag phase of $\sim 3 \text{ h}$, suggesting that the catabolic genes involved might require induction upon exposure to pinoresinol (Fig. 2A). Consistent with this, cell suspension studies with and without preexposure to pinoresinol showed a marked difference in the rate of pinoresinol degradation. Pinoresinol-induced cell suspension cultures degraded 125 $\mu$M pinoresinol within 40 min, while those without preexposure to pinoresinol showed a clear lag of 3 h (Fig. 2B). These results clearly indicate that genes involved in pinoresinol degradation in strain SG-MS2 are induced upon exposure to pinoresinol, as was also found to be the case for the Fusarium fungal strain M4-2 (12).

We note that previous studies of pinoresinol degradation by bacteria or fungi (16, 30) were carried out with racemic pinoresinol and no biphasic growth or degradation was reported, suggesting that there was no strong enantioselectivity in those cases. We have not tested our strain with either racemic pinoresinol or $(-)$-pinoresinol; therefore, we do not know whether the metabolic reactions are enantioselective.

**Evidence that SG-MS2 initially hydroxylates pinoresinol.** Liquid chromatography (LC)-time of flight (TOF) mass spectrometry (MS) analysis of filtered supernatants obtained at various times from growth and cell suspension cultures of SG-MS2 supplemented with pinoresinol showed that the progressive loss of pinoresinol was accompanied by the early but transient appearance of a major new peak (retention time [Rt], 8.76 min; termed metabolite 1) (Fig. S2), which was 16 Da larger than pinoresinol, and then the transient appearance of several other metabolites (Fig. S3B). The observed $m/z$ peaks for metabolite 1 perfectly matched the in silico isotopic profile of the deprotonated molecule ([M–H]$^-$) with the elemental composition ($C_{20}H_{22}O_7$) of hydroxylated pinoresinol (Fig. S3B). These data suggest that the first degradation step involves the incorporation of an oxygen atom.

Our next experiment compared the UV-visible spectra of neutral and alkalinized filtered supernatants of a pinoresinol-induced cell suspension of strain SG-MS2 incubated with 558 $\mu$M pinoresinol. We found increased absorbance at 358 nm in alkalinized samples after 2 h of incubation (Fig. 3). This suggests that the primary pinoresinol metabolite is transformed into a product containing an $\alpha$-carbonyl group conjugated
with a phenolic hydroxyl group (33). Such a metabolite has not been observed in any of the three previously reported pinoresinol-degrading bacterial strains (7, 23, 30, 34). Strain SYK-6, which cometabolizes pinoresinol via the well-characterized PinZ reductase enzyme, generates lariciresinol (23), which differs in molecular mass and retention time in LC-TOF MS analysis (data not shown). Instead, the hydroxylation of pinoresinol we see with SG-MS2 is consistent with the \(\beta\)-hydroxylation-initiated degradation of pinoresinol and \(\alpha\)-syringaresinol by the Fusarium fungal species (14).

**Chemical structures of early metabolites.** Pinoresinol-induced resting cells of strain SG-MS2 were incubated with 558 \(\mu\)M pinoresinol, and the resulting culture supernatant was extracted as described in Materials and Methods. Metabolite 1 from this extract was semipurified, together with a contaminating peak (later identified as metabolite 2; see below), using a preparative high-performance liquid chromatography (HPLC) column and was analyzed by gas chromatography (GC)-MS and nuclear magnetic resonance (NMR) spectroscopy. For GC-MS, the metabolites were derivatized with methoxyamine HCl (MOX) and \(N\)-methyl-\(N\)-(trimethylsilyl)trifluoroacetamide (MSTFA) for methoximation and silylation, respectively. Methoximation indicates the presence of a keto group and silylation a phenolic or carboxy hydroxyl group (35).

Sequential methoximation and silylation of the semipurified metabolites yielded three major peaks and two minor peaks in GC-MS (Fig. 4A). The fragmentation pattern of the largest major peak (Rt, 16.86 min) was consistent with the derivative of downstream, \(\gamma\)-lactone, metabolite 2 (Fig. 4B). The two other major GC peaks (Rt, 23.08 and 23.77 min) showed similar fragmentation patterns, with both having a molecular ion at \(m/z\) 619 (Fig. 4D); this finding is consistent with them being the two diastereomers of the methoximated and silylated derivatives of metabolite 1B. The two minor metabolite peaks (Rt, 24.27 and 26.49 min; molecular ion at \(m/z\) 590) also had similar fragmentation patterns, which is consistent with them being two diastereomeric forms of a trisilylated

**FIG 3** Changes in the UV-visible absorption of culture filtrates containing pinoresinol from resting cells of strain SG-MS2 at 0 h (A) and 2 h (B).
Identification of metabolites 1A, 1B, and 2. The GC-MS chromatogram (A) and fragmentation patterns of the observed peaks (B to D) of HPLC-purified metabolites 1 and 2 after methoximation and silylation are shown. Panels C and D show fragmentation patterns of diastereomers of derivatized metabolites 1A and 1B as mirrored spectra, for comparison. Panels B to D also show the structures of metabolites and fragmentation schemes. TIC, total ion current; TMS, trimethylsilyl group.
hemiketal metabolite that we denote metabolite 1A (Fig. 4C). The equivalent α-hydroxylation of syringaresinol and subsequent ring-chain tautomerization between its hemital and keto alcohol were shown previously in *Fusarium solani* M4-2 (12).

NMR analysis of nonderivatized enriched fractions verified the identity of the hemiketal and keto alcohol metabolites 1A and 1B, respectively, and of the γ-lactone metabolite 2. Since only a small amount of material was available and the major components of the mixture consisted of at least five very similar chemical species, the NMR chemical shift assignments for the individual species were complex. Details of the NMR workflow and analysis are provided in Fig. S4 in the supplemental material.

All of the evidence presented above indicates that degradation is initiated by oxidation of pinoresinol at a benzylic position to generate a hemiketal intermediate (metabolite 1A), which is in equilibrium with the corresponding keto alcohol (metabolite 1B). A second oxidation at the aromatic ring of metabolite 1A would result in aryl-alkyl cleavage and generate the γ-lactone (metabolite 2). Such an oxidation scheme, known as *ipso*-hydroxylation, is a well-known microbial strategy to eliminate sulfonamide antibiotics and the environmental contaminant 4-nonylphenol (36, 37). Note that this scheme predicts that the other product of the aryl-alkyl cleavage of metabolite 1A would be 2-methoxyhydroquinone (metabolite 3) (also see Fig. 7 below). In fact, we observed a peak at *m/z* 284.1 (Rt, 8.34 min), corresponding to the disilylated derivative of this species in the GC-MS spectrum of a doubly derivatized culture supernatant extract; this peak yielded a MS fragmentation pattern perfectly matching that of the commercially available and derivatized authentic standard (Fig. S5E). As noted, initial oxidation at an α-carbon to generate an α-carbonyl compound was shown previously for both pinoresinol and (-)-syringaresinol in various fungi (11–13, 16).

**Enzymology and proposed reaction mechanism for the initial α-hydroxylation step.** Incubation of a pinoresinol-induced cell extract of strain SG-MS2 with 90 μM pinoresinol in the absence of any added metal or redox cofactors showed a steady but very slow transformation of pinoresinol to metabolites 1A and 1B (Fig. S6). This indicates either that a factor required for pinoresinol oxidation had largely been removed during preparation of the cell extract or that the enzyme in question is oxygen sensitive. In order to determine the class of enzyme(s) responsible for catalyzing the α-oxidation step and to identify the possibly missing factor, we repeated the experiment but separately added various metal ions (500 μM FeSO₄, MnCl₂, NiCl₂, MgCl₂, CoCl₂, CuCl₂, CaCl₂, and ZnCl₂), mediators of phenol-oxidizing enzymes (100 μM syringaldazine and 1,4-hydroquinone), and an artificial electron acceptor (2 μM oxidized phenazine methosulfate [PMS]). The only reagent found to significantly enhance the decrease in pinoresinol concentrations and the production of metabolites 1A and 1B was oxidized PMS, which increased the reaction rate from 0.26 nmol min mg⁻¹ to 12.82 nmol min mg⁻¹. This ∼50-fold increase in activity suggests that the pinoresinol-hydroxylating enzyme system is not oxygen sensitive but is linked *in vivo* to an electron-transporting system, which is replaced by PMS in the assay described above.

A few well-characterized α,β flavocytochrome dehydrogenases that belong to the 4-phenol-oxidizing subgroup of the recently described vanillyl alcohol oxidase (VAO)/*p*-cresol methylhydroxylase (PCMH) flavoprotein family are also known to be rate enhanced by the presence of oxidized PMS as an electron acceptor. Two well-studied members of this subgroup are the *p*-cresol methyl hydroxylase (EC 1.17.99.1) from *P. putida* NCIMB 9869 and *P. putida* NCIMB 9866 and an alkylphenol hydroxylase from *P. putida* JD1 (38). The flavin subunit of both these hydroxylases abstracts two hydrogen atoms from the respective phenolic substrates, forming a quinone methide intermediate, and then hydrates the latter at the benzylic carbon by abstracting an OH group derived from water (39). While the protons are lost to the bulk solvent, the two electrons are passed to the heme on the cytochrome subunit. The electrons are then transferred to azurin (a small copper-containing protein that is localized with the flavocytochrome in the periplasm), which transports them to the membrane for reduction of molecular oxygen. *In vitro*, PMS acts as a replacement for azurin by
accepting electrons from the cytochrome and subsequently being reoxidized with molecular oxygen.

To test the hypothesis that pinoresinol is hydroxylated via the mechanism described above, we incubated pinoresinol with a cell suspension of strain SG-MS2 in the presence of 50% H$_2$O$_{18}$O. The mass spectrum of the hydroxylated pinoresinol (metabolites 1A and 1B) that appeared in the culture supernatant showed an additional deprotonated molecule of m/z 375 ([M/H]$^{11002}$H$^{11001}$2]/H$^{11002}$) with equal intensity, confirming the incorporation of $^{18}$O from water and confirming our hypothesis (Fig. 5). The incorporation of an oxygen atom from water into pinoresinol also indicates that pinoresinol degradation in this strain is initiated by a dehydrogenase rather than an oxidase or oxygenase. The potential mechanism of pinoresinol hydroxylation in strain SG-MS2 is shown in Fig. 6. Although the aim of this $^{18}$O experiment was to observe mass changes for metabolites 1A and 1B, it also generated two additional metabolite peaks (Rt, 8.60 and 8.74 min) (Fig. 5C and D), which are discussed below.

**Downstream intermediates and a proposed catabolic pathway.** As shown in Fig. 7, several downstream catabolic intermediates were observed along with metabolites 1A, 1B, and 2 by LC-TOF MS or GC-MS analysis. Of particular interest were metabolites 5A (GC-MS Rt, 18.02 and 17.76 min) and 5B (GC-MS Rt, 17.24 and 17.50 min), which we propose are the hemiketal and keto alcohol products, respectively, of hydroxylation at the carbon of the $\gamma$-lactone (metabolite 2) by a mechanism similar to that proposed above for the $\alpha_2\beta_2$ flavocytochrome hydroxylases (Fig. 6). This hypothesis was strongly supported by the cell suspension experiment with 50% H$_2$O$_{18}$O described above, in which peaks of m/z 265 ([M−H]−), 267 ([M−H+2]−), and 269 ([M−H+4]−), with intensity ratios of 1:2:1, for these two metabolites confirmed the incorporation of two heavy oxygen labels in each (Fig. 6). Further support for our proposition that metabolites 5A and 5B are hemiketals and keto alcohols, respectively, is the fact that sequential methoximation and silylation of the culture supernatant extract yielded two forms of each on GC-MS analysis (Fig. 8). Two diastereomeric silylated keto alcohols are expected for metabolite 5A and the syn- and anti-isomers of the methoxyamine group are expected for metabolite 5B, analogous to the situation for metabolites 1A and 1B, respectively.

Based on the agreement between observed m/z values (Fig. S2) and predicted in silico elemental compositions in the LC-TOF MS analyses, we tentatively assigned two other peaks as metabolites 4 (Rt, 7.15 min; C$_{13}$H$_{16}$O$_{5}$) and 6 (Rt, 6.80 min; C$_{13}$H$_{16}$O$_{7}$) in the pathway summarized in Fig. 7B. These metabolites are hydrolysis products of the $\gamma$-lactone metabolites 2 and 5B, respectively.
Interestingly, we also observed 4-hydroxy-3-methoxybenzoic acid (vanillic acid, metabolite 8) and 4-hydroxy-3-methoxybenzaldehyde (vanillin, metabolite 7) peaks in the GC-MS analyses, and we confirmed their identities using authentic standards (Fig. S5). Vanillic acid has been proposed as a catabolic intermediate of (-)-pinoresinol in bacterial strain FK-2 (22). Consistent with those findings, we found that 2 mM vanillin and vanillic acid, as sole carbon and energy sources, were completely consumed by 1% (vol/vol) seed cultures of strain SG-MS2 within 4 and 6 h, respectively (Fig. S7). Furthermore, the degradation of vanillin in strain SG-MS2 was confirmed to occur via its conversion to vanillic acid (Fig. S7A), which is a well-established catabolic pathway in bacteria (7, 19). No other intermediates were observed in the culture supernatants of vanillin- or vanillic acid-degrading cultures of strain SG-MS2.

In conclusion, we have described a new pseudomonad, strain SG-MS2, which rapidly mineralizes the (-)–linked lignan pinoresinol via an inducible pathway, initiated by \(\alpha\)-hydroxylation, that has not been observed previously in bacteria, plants, or eukaryotes but whose initial steps resemble a partially characterized pathway from certain fungi (11–14, 16). Whereas most of those fungi use extracellular peroxidases or laccases to catalyze the initial \(\alpha\)-hydroxylation, strain SG-MS2 uses an intracellular enzyme with properties reminiscent of those of well-characterized \(\alpha_2\beta_2\) flavocytochrome hydroxylases from other *Pseudomonas* species. The downstream steps of the pathway we have elucidated involve alkyl-aryl cleavage to generate a \(\gamma\)-lactone, which is either \(\alpha\)-hydroxylated or hydrolyzed. Subsequent metabolites are converted into vanillin and vanillic acid, which is assimilated into central metabolism. Strain SG-MS2 was isolated from composting plant material, and we propose that its catabolic pathway could be
an important contributor to the recycling of carbon in at least some of the β-β'-linked lignans in decaying plant material. Components of this pathway may also have value in biologically based production of vanillin, vanillic acid, and various novel lactones from lignin feedstocks. Furthermore, given that lactones and diols produced by reductive pinoresinol catabolism by human gut microbes have been found to have various...

**FIG 7** Intermediates observed in the culture supernatant during pinoresinol degradation by strain SG-MS2 and proposed catabolic pathway. (A) Appearance of multiple metabolites in the HPLC chromatogram at 280 nm. (B) Proposed pinoresinol catabolic pathway based on GC-MS and LC-TOF MS analyses. (C) GC-MS total ion chromatogram for the culture supernatant. Different pathway intermediates are labeled in the GC-MS total ion chromatogram and the HPLC chromatogram. Metabolites 1A and 1B are not resolved in the HPLC chromatogram and appear as a single peak. Metabolites 5A and 5B have the same molecular weight in LC-TOF MS, and we cannot determine with confidence which of the two peaks each represents. The LC-TOF MS and GC-MS data for pinoresinol and its metabolites depicted here are provided in Fig. S3 and Fig. S5, respectively, in the supplemental material.
anticancer and cardiovascular effects (21, 28, 40), it will now be important to investigate
the possible health effects of the new lactones produced by this oxidative pathway.

MATERIALS AND METHODS

Chemicals. (+)-Pinorosinol (>98% purity) was purchased from Arbonova Sales (Turku, Finland). (+)-
Laricresinol was synthesized by bioconversion of (+)-pinorosinol using strain SYK-6 (obtained from the
National Institute of Technology and Evaluation Biological Resource Centre culture collection; Kisarazu, Chiba,
Japan), as described in the literature (23). Vanillin, vanillic acid, ferulic acid, 2-methoxyhydroquinone, phen-
zine methosulphate, 1,10-phenanthroline, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammom-
nium salt (ABTS), MOX, MSTFA, syringaldazine, veratyl alcohol, veratraldehyde, p-cresol (all at least 96%
purity), and hydrogen peroxide (30%) were purchased from Sigma-Aldrich (Castle Hill, Australia) and Chem
Supply (Port Adelaide, Australia). H218O at >97% atom purity was purchased from Miles Laboratory (Rohenov,
Israel). All other reagents were of the highest purity and analytical grade available from local vendors.

Culture media. MSM was prepared as described previously (41). Different concentrations of pinoros-
inol, vanillic acid, or vanillin, in powder form, were added individually to the MSM as the sole source of
carbon and energy and were dissolved overnight at 40°C, with constant stirring. The media were
sterilized by filtration (0.22 μm) and stored at room temperature.

Bacterial enrichment. Two soil samples and one wheat straw/manure compost sample were
obtained for enrichment culturing. The soil samples, i.e., garden soil and olive plant rhizosphere soil, were
collected in Canberra, and the wheat straw/manure compost was obtained from TM Organics (Stirling,
South Australia). One gram of each sample was transferred to 100 ml MSM containing 55.8 μM
pinorosinol (pH 7.0). No pinorosinol degradation was noticed in the enrichment cultures from the two soil
samples, but the pinorosinol was almost completely degraded overnight by the wheat straw/manure
compost sample; 1% (vol/vol) of the growth medium was then transferred to a fresh batch of the same
pinorosinol-containing MSM. After five rounds of such enrichment, serial dilutions of the growth medium
were plated on LB agar plates, which were incubated at 28°C until bacterial colonies were observed.
Individual colonies were tested for their ability to grow with 55.8 μM pinorosinol in MSM.

Bacterial identification. Pinorosinol-degrading bacteria were grown in LB medium at 28°C, and
genomic DNA was isolated from them using a genomic DNA extraction kit (Qiagen, Germany), according

FIG 8 GC-MS fragmentation patterns for metabolites 5A (A) and 5B (B), in mirrored format. TMS, trimethylsilyl group.
to the manufacturer's instructions. The 16S rRNA genes were PCR amplified using the 27F and 1492R universal primers (42). Amplifications were performed in 50-μl reaction mixtures containing 1× Phusion high-fidelity DNA polymerase (NEB, USA), 200 μM each deoxyribonucleoside triphosphate (dNTP), 0.4 μM each primer, 20 to 50 ng of DNA template, 2 units of Phusion high-fidelity DNA polymerase (NEB), and 33 μl of sterile deionized water. The temperature cycling program comprised one step of 98°C for 2 min, 30 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min, and a final extension of 72°C for 10 min. DNA sequencing was outsourced to Macrogen (Seoul, Republic of Korea).

**Growth, resting cell, and cell suspension studies.** A few colonies of the isolate of interest were inoculated in 10 ml of LB medium, and the sample was incubated overnight at 28°C, with shaking at 180 rpm. Cells were washed twice by centrifugation (4,000 × g for 10 min at 4°C), and the pellet was resuspended with 10 ml of carbon-free MSM. Finally, the pellet was resuspended in 10 ml of carbon-free MSM for use as a seed culture. For growth experiments, 1% (vol/vol) of the seed cultures was inoculated into MSM containing the compounds of interest as the sole carbon sources (558 μM pinoresinol and 2 mM vanillin and vanillic acid), and 0.5-ml aliquots were taken at different time points. The optical density at 600 nm (OD<sub>600</sub>) of the aliquots was measured, and the aliquots were then filtered and analyzed by UV spectroscopy, LC-TOF MS, or GC-MS. Where required, protein content (as a measure of bacterial growth) was measured using a Pierce modified Lowry protein assay kit (Thermo Scientific, USA), according to the manufacturer's instructions. A control without any carbon source was used in the growth experiments. This control was also used to adjust protein concentrations in growth yield calculations; a Y<sub>sw-e</sub> value was calculated from this control as described previously (32). UV-visible spectroscopy was carried out by scanning the filtered samples (at neutral pH) from 250 to 425 nm using a SpectraMax M3 spectrophotometer (Molecular Devices, USA). The filtered samples were then made alkaline (pH 9.5) with NaOH and rescanned.

For resting cell studies, 1% (vol/vol) of the seed culture was inoculated into 100 ml of LB medium and grown at 28°C in LB medium, with shaking at 180 rpm, to mid-exponential phase (OD<sub>600</sub> of 0.6). The cells were washed twice by centrifugation (4,500 × g for 10 min at 4°C) and resuspended (to an OD<sub>600</sub> of 2.0) in MSM containing 279 μM pinoresinol. At different time points, 0.5-ml aliquots were removed, filtered (0.2 μm; Merck Millipore, Darmstadt, Germany), and analyzed by LC-TOF MS (see below).

Cell suspension studies with pinoresinol were carried out like the resting cell studies except that the second suspension also contained 9.34 mM NH<sub>4</sub>Cl as a nitrogen source. Cell suspensions were incubated with 139 μM pinoresinol.

Pinoresinol-induced cells or resting cells were prepared as described above except that they were incubated with 55.8 μM pinoresinol for 3.5 h at 30°C, with shaking at 180 rpm, for the induction of the pinoresinol-degrading gene-enzyme system. The control (uninduced) culture was incubated with minimal medium containing no carbon. After induction, cells were washed with MSM as described above.

**LC-TOF MS.** Quantitative and/or qualitative data on pinoresinol and its catabolic intermediates were obtained using an Agilent-1100 series HPLC system with a diode array detector (Agilent Technologies, USA) operating at 270 to 280 nm. For metabolite identification, a TOF mass spectrometer (Agilent Technologies) was run in negative-ion mode with a scan range of m/z 100 to 400 and a fragmentation voltage of 100 V. Compound separation was achieved using a C<sub>18</sub> reverse-phase column (150 by 4.6 mm, 3 μm; Phenomenex, Lane Cove, Australia) heated to 30°C. The two mobile phases were water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B), with a gradient starting at 95% solvent A/5% solvent B and increasing to 90% solvent B/10% solvent A over 10 min. The flow rate was maintained at 0.8 ml min<sup>−1</sup> throughout the gradient.

**Extraction and purification of the first metabolite.** Resting cells (induced with 55.8 μM pinoresinol) were incubated in nitrogen-free MSM containing 558 μM pinoresinol. After 3.5 h of incubation, when approximately 80% of the pinoresinol had disappeared (data not shown), the culture supernatant was separated by centrifugation (4,000 × g for 10 min at 4°C) and extracted twice with an equal volume of chloroform. The combined organic layers were then passed through anhydrous sodium sulfate to remove residual water, and the solvent was removed using a rotary concentrator/dryer (miVac; Genevac, Ipswich, UK). The dried residue was redissolved in 1 ml of acetonitrile/water (50:50), vortex-mixed and incubated at 37°C for 1.5 h. For silylation, 20 μl of MSTFA was added, and the sample was vortex-mixed and incubated at 37°C for 30 min, with intermittent mixing.

The doubly derivatized samples were analyzed in an Agilent 7890A GC system equipped with an Agilent 597 series mass selective detector, in electron ionization (EI) mode, and a CTC PAL autosampler (G6500 Combi PAL; CTC Analytics AG, Zwingen, Switzerland). Helium (2 ml min<sup>−1</sup>) was used as the carrier gas. The oven temperature was initially kept at 150°C for 3 min before being increased to 320°C at a rate of 10°C per min. The injector temperature was set at 300°C, and the split ratio was 10:1. The mass spectrometer was operated in scan mode (starting after 6 min; mass range, m/z 50 to 800).
Heavy oxygen labeling. To identify the origin of the hydroxyl group in the first steps of pinoresinol transformation, a resting cell experiment was performed as described above except that 50% (vol/vol) H$_2$O$_2$ was used. The culture supernatant containing heavy oxygen-labeled metabolites was analyzed with the Agilent HPLC system described above but with a XDB C$_{18}$ column (4.6 by 50 mm, 1.8 μm; Agilent Technologies) instead of the Phenomenex C$_{18}$ reverse-phase column (which was no longer available to us). The two mobile phases were water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B), with a gradient starting at 95% solvent A/5% solvent B and increasing to 90% solvent B/10% solvent A over 15 min. The flow rate was maintained at 0.3 ml min$^{-1}$ throughout the gradient.

Enzyme assays. Enzyme assays were performed to characterize the enzyme(s) performing the first step of pinoresinol degradation in strain SG-MS2. Pinoresinol-induced cells of strain SG-MS2 were incubated for 30 min with 8,000 units of lysozyme per ml of cell culture in 50 mM potassium phosphate buffer (pH 7.2), with gentle shaking. Following incubation, the cell suspension was lysed by three passages through a homogenizer (Microfluidics M-110P; Microfluidics, USA) operating at 18,000 lb/in$^2$. The lysate was centrifuged at 21,000 × g for 15 min, and the resulting supernatant was filtered through a 0.22-μm syringe filter (Merck Millipore). All steps in the preparation of this pinoresinol-induced cell-free extract (PI-CFE) were carried out at 4°C. The laccase, lignin peroxidase, manganese peroxidase, and general peroxidase activities of the PI-CFE were then determined using a SpectraMax M3 spectrophotometer, as follows. Laccase activity (phenol oxidation) was measured in 100 mM potassium phosphate buffer (pH 6.5) as the change in absorbance at 530 nm due to oxidation of 88 μM M$_4$Fe$_{10}$O$_{16}$H$_{2}$O$_2$ (45).

To test for metal dependence, the PI-CFE was dialyzed for 12 h (at 4°C) against 20 mM sodium phosphate buffer (pH 7.2) containing EDTA (10 mM) and 1,10-phenanthroline (10 mM), to remove existing metals. A second dialysis was then performed with the same buffer for 12 h (at 4°C) without EDTA or phenanthroline, to remove metal chelators. Following the dialysis, 500 μM FeSO$_4$, MnCl$_2$, NiCl$_2$, MgCl$_2$, CoCl$_2$, CuCl$_2$, CaCl$_2$, and ZnCl$_2$ were added (separately) to an assay mixture containing PI-CFE as the enzyme source and 125 μM pinoresinol as the substrate. Oxidized PMS (0.15 mM) was added to the enzyme assay as an electron acceptor. The disappearance of pinoresinol and the formation of metabolites 1A and 1B were analyzed by LC-TOF MS as described above. All enzyme assays were performed as technical triplicates.

Accession number(s). The 16S rRNA gene sequences from strains SG-MS1 and SG-MS2 have been deposited in GenBank under accession numbers MF150302 and MF150296, respectively.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02531-17.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

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We have no conflicts of interest to declare.

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