Isolation and initial characterization of a novel type of Baeyer–Villiger monooxygenase activity from a marine microorganism

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Summary
A novel type of Baeyer–Villiger monooxygenase (BVMO) has been found in a marine strain of Stenotrophomonas maltophilia strain PML168 that was isolated from a temperate intertidal zone. The enzyme is able to use NADH as the source of reducing power necessary to accept the atom of diatomic oxygen not incorporated into the oxyfunctionalized substrate. Growth studies have establish that the enzyme is inducible, appears to serve a catabolic role, and is specifically induced by one or more unidentified components of seawater as well as various anthropogenic xenobiotic compounds. ABLAST search of the primary sequence of the enzyme, recovered from the genomic sequence of the isolate, has placed this atypical BVMO in the context of the several hundred known members of the flavoprotein monooxygenase superfamily. A particular feature of this BVMO lies in its truncated C-terminal domain, which results in a relatively small protein (357 amino acids; 38.4 kDa). In addition, metagenomic screening has been conducted on DNA recovered from an extensive range of marine environmental samples to gauge the relative abundance and distribution of similar enzymes within the global marine microbial community. Although low, abundance was detected in samples from many marine provinces, confirming the potential for biodiscovery in marine microorganisms.

Introduction
Enzymes play an important role as biocatalysts in the pharmaceutical and biotechnology industries (de Gonzalo et al., 2010; Wulgemuth, 2010). Compared with conventional chemical catalysis, enzyme-catalysed reactions are more efficient (e.g. exhibit greater atom efficiency, and higher regio- and/or enantioselectivity), are cheaper to undertake (e.g. operate at or near ambient temperature and atmospheric pressure), and are acknowledged to be more environmentally friendly (e.g. generate fewer by-products and less waste). The concomitant demand for enzymes with novel properties is being met by two strategically different approaches. Either known enzymes are subjected to protein engineering (e.g. Torres Pazmino et al., 2007; Reetz, 2009), or novel enzymes are sought by exploiting natural biodiversity, sourcing proteins from organisms that have evolved in un- or under-exploited environments (e.g. Wackett, 2004; Joint et al., 2010). Marine microorganisms are one such under-exploited source of biodiversity (Bull et al., 2000; Glöckner and Joint, 2010). As a part of an ongoing programme of biodiscovery, we have established a collection of pure cultures of environmentally representative marine bacteria (> 900) a large fraction (448) of which has been screened for a number of enzyme activities of potential use as commercial biocatalysts, resulting in the detection of putative Baeyer–Villiger monooxygenase (BVMO) activity in a relatively small number (16) of those isolates.

Baeyer–Villiger monooxygenases (EC 1.14.13.x) are flavoproteins that belong to a class of oxidoreductases that use diatomic oxygen as a co-substrate. In addition to oxyfunctionalizing various heteroatoms, they typically catalyse the insertion of one of the atoms of oxygen into carbon skeletons adjacent to carbonyl groups (Walsh and Chen, 1988). The reaction is a formal biological equivalent of the chemical Baeyer–Villiger reaction (Baeyer and...
Villiger, 1899), which established the use of peracid catalysts to oxidize ketones to esters. BVMOs have been extensively investigated as biocatalysts for organic synthesis as they exhibit consistently superior regio- and enantioselectivity compared with the currently available organometallic catalysts (Kamerbeek et al., 2003). BVMOs are dependent on both a flavin prosthetic group (FAD or FMN), to facilitate the single electron-based interactions with triplet ground-state diatomic oxygen, and a reduced nicotinamide nucleotide coenzyme [NAD(P)H] that serves as the source of the reducing power necessary to accommodate the non-integrated atom of oxygen (Massey, 1994). Most BVMOs can be categorized exclusively as either so-called type 1 or type 2 enzymes (Willett, 1997). Almost without exception, type 1 BVMOs, as typified by cyclohexanone monooxygenase from Acinetobacter calcoaceticus NCIMB 9871 (CHMO9871; EC 1.14.13.22; Donoghue et al., 1976) are obligate FAD plus NADPH-dependent members of the Class B flavoprotein superfamily (van Berkel et al., 2006). While most type 1 enzymes, including CHMO9871, are functionally active as monomers (α1), others are active either as α2 or α3 multimers. Conversely, type 2 BVMOs are obligate FMN plus NADH-dependent members of the Class C flavoprotein superfamily (van Berkel et al., 2006), and consist of a loosely bound trimeric assemblage (α2β) of two different polypeptide types.

Whereas the significant potential of both type 1 and type 2 BVMOs as effective regio- and enantioselective biocatalysts has been demonstrated successfully in proof of principle chemoenzymatic syntheses (Willett, 1997), commercial interest in these enzymes has focused exclusively on the development of a relatively small number of representative type 1 BVMOs mainly because of the significant operational stability problems encountered when attempting to use the structurally unstable type 2 enzymes (van Berkel et al., 2006; de Gonzalo et al., 2010). However, despite their relative structural stability, the significantly higher (×10) cost of NADPH relative to NADH remains a major disincentive to the successful development of type 1 BVMOs as industrial biocatalysts.

Therefore, there would be considerable commercial interest in the identification of a novel type of BVMO that combines the favourable (relative) operational stability conferred by the subunit structure of the type 1 BVMOs with the dedicated NADH dependence of the type 2 enzymes. While some success has been achieved in switching the coenzyme specificity of the Class A flavoprotein 4-hydroxybenzoate hydroxylase (EC 1.14.13.3) to NADH by site-directed mutagenesis (Eppink et al., 1999), attempts to achieve this hybrid status by subjecting type 1 BVMOs to concerted programmes of ‘directed evolution’ (Reetz, 2009), or site-directed mutagenesis (Dudek et al., 2010) have so far achieved only limited success. Consequently, our primary strategy, as reported here, has been to conduct a concerted programme of conventional chromogenic screening, focussed on identifying and characterizing the potential for such novelty in microorganisms sourced from the under-explored marine environment, because of their recognized potential for biodiscovery (Joint et al., 2010).

Results
Detection of putative BVMO-positive marine isolates

A total of 448 marine bacteria were examined for the presence of putative BVMO and lactone hydrolase (LH) activities using solid-phase screening (Fig. S1). The extent of coloration of colonies after 14 days growth on 3AI agar indicated that 16 isolates were BVMO-positive; 12 of these 16 isolates contained an additional complementary LH activity. From this initial screen, Isolate 168 (BVMO+; LH+) was chosen as the most promising candidate for additional characterization. The isolate was confirmed as a strain of Stenotrophomonas maltophilia by performing a comparative BLAST analysis of the complete 16S rRNA gene retrieved from the whole genome sequence (which will be reported elsewhere) with the NCBI sequence database. This resulted exclusively in hits to 16S rRNA sequences of Stenotrophomonas spp., with a sequence similarity between 98% and 99% over 98–100% of sequence length.

Two important conclusions emerged from comparisons of the chromogenic responses of colonies of S. maltophilia strain PML 168 grown on 3AI agar variants formulated with an equivalent volume of doubly distilled water. 

### Table 1. Production of indigo by colonies of S. maltophilia strain PML 168 after growth (14 days at 15°C) on various formulations of marine agar containing 3-acetyl indole (3AI).

| Agar Type | Indole Production before addition of 0.1 M KOH | Indole Production after addition of 0.1 M KOH |
|-----------|---------------------------------------------|---------------------------------------------|
| 3AI-marine agar | + + | + + |
| 3AI-marine agar + cyclohexanone | + + | + + |
| 3AI-marine agar + acetophenone | + + + | + + + |
| 3AI-marine agar – filtered seawater | – | – |
| Marine agar – 3AI | – | – |

Table 1. Production of indigo by colonies of S. maltophilia strain PML 168 after growth (14 days at 15°C) on various formulations of marine agar containing 3-acetyl indole (3AI).

| Formulation of 3AI-marine agar: 5.0 g bactopeptone, 1.0 g yeast extract, 0.1 g ferric orthophosphate, 0.32 g (2.0 mM) 3-acetyl indole, 15.0 g agar, 1.0 l filtered seawater; autoclaved 15 min at 15 p.s.i. Supplements where appropriate: 0.99 g (1.0 mM) cyclohexanone or 1.2 g (1.0 mM) acetophenone. Agar without filtered seawater was formulated with an equivalent volume of doubly distilled water. |
Time-course studies of the titres of BVMO and LH during growth of S. maltophilia strain PML 168 in liquid culture

*Stenotrophomonas maltophilia* previously grown on un-supplemented marine agar grew well after a short lag phase when transferred into an equivalent marine broth liquid medium (Fig. S2). The timing and extent of changes in the specific activity of 3-acetylsindole monooxygenase (BVMO) and indoxyl acetate hydrolase (LH) in cell-free extracts of cells harvested at different times throughout the growth curve confirmed that both enzymes were inducible with their activities maximally expressed in early to mid log phase of growth (20–24 h post inoculation), suggesting an association with one or more functions of primary metabolism. Cell-free extracts prepared from cells harvested at the peak of BVMO activity (20–24 h after inoculation) biooxidized acetoephone to an equivalent acetate ester with a high molar yield, whereas cyclohexanone was a relatively poor substrate. In this respect, acetoephone served both as an effective substrate and an inducer (Table 1) for the BVMO from *S. maltophilia* strain PML 168, while cyclohexanone served solely as a poor substrate. Interestingly, the heterocyclic aldehyde 2-pyridine carboxaldehyde, a known substrate of the Class B flavin monooxygenase FMO1 (Chen et al., 1995), was also a competent substrate for the biooxidative activity of *S. maltophilia* strain PML 168, thereby forming the equivalent formate ester. However, it was not possible to assess the ability of this compound to serve as an inducer of BVMO activity because it was toxic to growth of the bacterium at all levels tested (>0.02 mM).

Genome annotation

No open reading frames (ORFs) were detected in the genome of *S. maltophilia* strain PML 168 that contained the original signal sequence motif for a type 1 BVMO proposed by Fraaije and colleagues (2002), or either of the two novel motifs proposed by Szolkowy and colleagues (2009). However, a single ORF was detected (Fig. 1a) that matched both the deleted version of the original type 1 BVMO-identifying sequence developed by Szolkowy and colleagues (2009), and the Class B FMO-identifying signal sequence motif proposed by Fraaije and colleagues (2002), though notably the identified ORF translated into an atypically short (357-amino-acid residues) BVMO. The ORF also contained two GxGXXG motifs, indicative of two classical Rossman [αβ]β-α folds that serve to bind the AMP moieties of FAD and NAD(P)H (Vallon, 2000).

A comparison of the ORF of the Class B flavoprotein gene from *S. maltophilia* strain PML 168 with that of the typical type 1 BVMO CHMO<sub>9871</sub> (ORF = 543 amino acid residues, Fig. 1b), and the BVMO-catalysing flavin monooxygenase FMO1 (ORF = 532 amino acid residues, Fig. 1c) confirmed that the lower MW (38.4 kDa) enzyme present in *S. maltophilia* strain PML 168 shared very similar sequence topology from the N-terminus to the second GxGXXG signal sequence motif. However, thereafter it was characterized by a truncated C-terminal sequence that was notably deficient in positively charged amino acid residues such as histidine (H), lysine (K) and arginine (R).

3D structure

Because of the intriguing possibility that this flavoprotein of *S. maltophilia* strain PML 168 represents a novel type of BVMO activity, the FFAS server (Joachimiak, 2009; Jaroszewski et al., 2011) was used to generate a predictive 3D structure of the protein based on the known structure of phenylacetone monooxygenase (PAMO) from *Thermobifida busca* (Malito et al., 2004). The result (Fig. S3) predicted that the BVMO-catalysing flavoprotein present in *S. maltophilia* strain PML 168 had a relatively open 3D structure compared with Class B flavoproteins for which a crystal structure has been determined, namely the type 1 BVMOs PAMO (Malito et al., 2004) and CHMO<sub>8631</sub> from a *Rhodococcus* sp. strain Hl-31 (Mirza et al., 2009), plus the Class B FMO from *Methyliphaga* sp. SK1 (Alfieri et al., 2008). The significance of this relaxed structure in terms of the range and nature of carbonyl-containing compounds that can serve as competent substrates and, where relevant, any elements of regio- and enantioselectivity imposed on the oxyfunctionalized product(s) generated from racemic or prochiral substrates, can only be established by appropriate chemical characterization of the outcomes of biotransformations undertaken with samples of the flavoprotein from *S. maltophilia* strain PML 168.

Jensen and colleagues (2011) have established that the relevant gene from *S. maltophilia* strain PML 168 can be cloned and expressed in *E. coli*, and demonstrated conclusively that the resultant flavoprotein product serves as a BVMO. Biochemical and kinetic characterization of the purified expressed enzyme has confirmed that, as is idiosyncratic of a number of other purified BVMOs (Kampebeck et al., 2003), it can catalyse both the electrophilic biotransformation of prochiral sulfides to equivalent...
produced abundantly in a heterologous host, but also because the novel NADH-dependent BVMO can be from and Zhou, 2003). The successful cloning of the BVMO preference for NADH (Jensen et al., 2006), or individual basic amino acid residues important in distinguishing between NADPH and NADH dependency (Kamerbeek et al., 2004; Malito et al., 2004; Torres Pazmino, 2008; Mirza et al., 2009).

sulfoxides, and the nuleophilic biotransformation of various carbonyl-containing substrates, including racemic fused cyclobutanones, to equivalent lactones. However, perhaps most significantly, the purified enzyme is active with either NADH or NADPH, as the exogenous reduced nucleotide coenzyme in supporting the oxygenation process advantages by facilitating coenzyme recycling active with NADH offers significant environmental and especially cheaper commodity biochemical (van der Donk and Zhou, 2003).

The availability of a purified BVMO that is preferentially active with NADH offers significant environmental and processing advantages by facilitating coenzyme recycling via a coupled enzyme system deploying the NAD-dependent formate dehydrogenase from Candida boidinii that yields CO₂ and H₂O as co-products (van der Donk and Zhou, 2003). The successful cloning of the BVMO from S. maltophila strain PML 168 is a valuable prerequisite to any commercial exploitation of the enzyme not only because the novel NADH-dependent BVMO can be produced abundantly in a heterologous host, but also because it serves to segregate the flavoprotein from the subsequent lactone hydrolase-catalysed ring-opening step in the natural host.

Phylogeny and environmental distribution of similar BVMO genes – the results from genome mining and metagenomic screening

A BLASTp search within the NCBI database revealed a large number of similar sequences of which the 67 most similar sequences were selected for a subsequent phylogenetic analysis (Fig. 2). This analysis showed that a gene from S. maltophila strain K279a, annotated as a flavoprotein from a strain of Stenotrophomonas (S. maltophila R551-3) formed a separate cluster (Fig. 2). The putative monooxygenase (Crossman et al., 2008), was closely related to that of isolate PML 168 and that these ORFs together with the only other available Class B flavoproteins (Fraaije et al., 2001; van Berkel et al., 2006), or individual basic amino acid residues important in distinguishing between NADPH and NADH dependency (Kamerbeek et al., 2004; Malito et al., 2004; Torres Pazmino, 2008; Mirza et al., 2009).

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Fig. 2. Phylogenetic analysis of the BVMO amino acid sequence of *Stenotrophomonas* isolate PML 168 and 67 similar sequences identified by BLASTp search. Evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved those 66 amino acid sequences which showed greatest similarity to the BVMO sequence of isolate 168 as judged by a BLASTp search within the NCBI database. All positions containing gaps and missing data were eliminated. There were a total of 265 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2007). The cluster containing the *Stenotrophomonas* sequences is indicated by a bar.
set comprising 79 303 Sanger reads from a GOS survey abundant location (0.09% – 69 reads) was found in a data <0.14% and 0.13% respectively, they compromised only the top three most relative abundant constituted 0.3%, effort were marine surface water samples sourced off average abundance was 0.04%. The locations with the (Rusch which was sampled as a part of the Global Ocean Survey Islands in the Galapogos Islands archipelago (GOS033), the Punta Cormorant hypersaline lagoon on Floreana The location with the greatest specific abundance was the Punta Cormorant hypersaline lagoon on Floreana Island in the Galapogos Islands archipelago (GOS033), which was sampled as a part of the Global Ocean Survey (Rusch et al., 2007). In this particular ecosystem the average abundance was 0.04%. The locations with the greatest relative abundance compared with sequence effort were marine surface water samples sourced off French Polynesia, again part of the GOS. However, while the top three most relative abundant constituted 0.3%, 0.14% and 0.13% respectively, they compromised only single or doubleton reads identified within data sets with < 800 reads (GS045, 43, 40). The fourth most relative abundant location (0.09% – 69 reads) was found in a data set comprising 79 303 Sanger reads from a GOS survey sample (GS009) sourced from the coast of Block Island, NY State, USA. There are no relevant environmental parameters associated with these metagenomic data that could serve as indicators for ascertaining which particular factors might favour the relative proliferation of the K279a flavoprotein ORF within specific communities. Using a notional cut-off value of 10 or more sequence identified fragments, the location with the lowest relative abundance identified was a pyrosequenced metagenomic data set from the surface waters of a long-term PML observatory, L4, located in the Western English Channel a site within 20 km of where strain PML 168 was isolated (Gilbert et al., 2010); this comprised 18 sequences out of 826 289 reads (relative abundance = 0.002%). A similarly low relative abundance (0.003%) was identified in the surface waters of the North Pacific gyre (MGRAST ID – 4443699.3) and this comprised 10 sequences out of 283 485 reads. While this analysis provides evidence of the widespread distribution of representative members of a relatively low MW (< 40 kDa) subset of the Class B flavoproteins, it also suggests that they occur with relatively low frequency in each of the sampled marine populations, comprising a low abundance component of the functional gene inventory of each examined location.

Discussion

The reported isolation and characterization of a Class B flavoprotein from S. maltophilia strain PML 168 is the first unequivocal demonstration of this type of enzyme in a marine microorganism. It is known that this type of flavoprotein, in both prokaryotic and eukaryotic terrestrial microorganisms, can have either a catabolic or anabolic role in metabolism. Catabolic Class B flavoprotein enzymes such as CHMO3871 serve as the initiating biooxidative enzyme in short metabolic pathways that promote the access of various low MW alicyclic ketones (Donoghue et al., 1976), or ketone-substituted aromatics (Cripps et al., 1978), into pathways of central intermediary metabolism such as the Embden–Meyerhof–Parnas (EMP) pathway or the tricarboxylic acid (TCA) cycle (Wackett, 1997). Conversely, anabolic Class B flavoproteins, such as the steroid monoxygenase from Cylindrocarpon radicicola ATCC11011 (Königsberger et al., 1990), catalyse various oxidative biotransformations of key steroid intermediates that serve to diversify the range of bioactive products formed (Trudgill, 1978), or act as ‘tailoring’ enzymes for various classes of secondary metabolites (e.g. Yabe et al., 2003; Banskota et al., 2006).

Evidence strongly suggests this Class B flavoprotein in S. maltophilia strain PML 168 has a catabolic role. Specifically, induction of the enzyme in early-to-mid-log phase of growth, plus the coincident induction of a complementary lactone hydrolase activity, indicates catabolic activity.
Currently, the evolved role(s) of the enzyme in the marine environment remains uncharacterized. To date, all competent substrates (e.g. 3-acetyl indole) and the one identified inducer (acetophenone) for the flavoprotein enzyme are anthropogenic xenobiotic organic compounds. However, this study has shown that one or more components of filtered seawater can serve as an effective inducer for the flavoprotein, although no attempt has been made to identify specific competent molecule(s). The chemical composition of dissolved organic matter in seawater is currently too poorly characterized (Kujawinski, 2011) to allow speculation on which substrate(s) might be relevant for this enzyme in the natural environment.

In terms of structure, like all other Class B flavoprotein monooxygenases, which includes type 1 BVMOs (van Berkel et al., 2006), the enzyme is a single polypeptide moiety coded for by a specific single copy ORF in the genome of *S. maltophila* strain PML 168. It contains two 2|5\] Rossmann-fold motifs indicative of two separate binding domains, one located towards the N-terminus of the protein that is involved in interacting with a flavin cofactor, and a second located towards the C-terminus involved in interactions with a reduced nicotinamide coenzyme (Vallon, 2000). The presence of both binding domains is a definitive feature that distinguishes Class B flavoproteins from other flavin-dependent proteins, and serves to promote cooperation between the two ligand types, which results ultimately in the types of biooxidations that characterize this class of enzymes.

The BVMO of *S. maltophila* strain PML 168 was shown to be able to undertake typical BVMO-catalysed oxyfunctionalizations, such as the biooxidation of cyclohexanone to ε-caprolactone (Donoghue et al., 1976), acetophenone to phenylacetate (Cripps et al., 1978), and 3-acetylindole to indoxyl acetate (Kamerbeek, 2004). However, the BVMO from strain PML 168 also has a number of features that are not typical for a Class B type 1 BVMO flavoprotein. We suggest that it is a type 3 BVMO, and represents the first characterized example of a new sub-class of the Class B flavoproteins.

Novel features that clearly define the Class B type 3 BVMO flavoprotein were present in both the genome and proteome of *S. maltophila* strain PML 168. First, a relatively small number of constituent amino acid residues (357) were present compared with type 1 BVMOs (~ 510 residues). Second, the signal sequence motif FAGIQLH-SAHY was indicative of a hydrid structure between MO22, a type 1 BVMO from *Rhodococcus jostii* RHA1 (Szkolowy et al., 2009) and the Class B FMO from *Methyllophaga* sp. SK1 (Alfieri et al., 2008). In all Class B flavoproteins that have been studied in sufficient detail, each variant of this short sequence of amino acids is reported to be located at the surface of the enzyme. In simplistic terms, it can be considered as a linker region between the FAD-binding N-terminal domain and the NAD(P)H-binding C-terminal domain (Malito et al., 2004). However, its role may be more subtle, by acting as an atomic switch that serves to coordinate multiple unconnected parts of flavoprotein enzymes to coordinate the binding and subsequent electron transfer-based interactive biochemistry of the flavin and nicotinamide cofactors (Mirza et al., 2009); this, in turn, may assist in controlling the pattern(s) of oxygen atom incorporation into competent substrates able to be accommodated in the substrate binding pocket. It is possible that the particular variant of this mechanistically influential signal sequence present in the primary structure of the type 3 BVMO from *S. maltophila* strain PML 168 may be a defining factor in enabling the enzyme to perform oxyfunctionalizations characteristic both of some Class B type 1 BVMOs and some Class B FMOs. Thirdly, there was the ability to function with both NADH and NAPDH as the requisite reduced nicotinamide nucleotide coenzyme. Although relatively rare, such duality of reduced nicotinamide nucleotide dependence has been previously reported in some other Classes of flavoproteins; for example, the Class A flavoprotein MtmIVO from *Streptomyces argillaceus* (Gibson et al., 2005), and the Class C flavoprotein alkane sulfonate monooxygenase from *Escherichia coli* (Eichhorn et al., 1999). However, all previously known representatives of the Class B flavoproteins, with the exception of MekA from *Pseudomonas veronii* MEK700 (Volker et al., 2008), have been characterized as exclusively NADPH dependent. While the relatively small size and the distinctive signal sequence motif are extant features shared by the type 3 BVMO from *S. maltophila* strain PML 168 with the slightly larger (365 amino acid residues) protein coded for by an ORF (annotated as a putative monooxygenase) detected in the genome of *S. maltophila* K279a (Crossman et al., 2008), any putative BVMO-type enzyme activity of the K279a protein, and the consequent coenzyme dependency thereof, remains to be established.

The relatively small flavoprotein (38.4 kDa) from strain PML 168 exhibits both similarities to and differences from previously characterized Class B type 1 BVMOs. The most apparent key differences are the low MW and the relatively open 3D structure predicted by using the FFAS server compared with the known crystal structures of PAMO (Malito et al., 2004) and CHMO_{0.81} (Mirza et al., 2009). Neither the relatively low MW nor the open 3D structure of the type 3 BVMO is due to a uniform attrition of amino acids from the equivalent structures of typical type 1 BVMOs, such as CHMO_{0.871}, and PAMO. Both enzyme types share a similar number of approximately 200 amino acids and matching sequence motifs [N-term . . . GXGXXG . . . FXGXXXXXXX(W/Y) . . . GXGXXG . . ] from the respective N-termini up to, and extending beyond, the second glycine-rich motif (which
signifies the reduced nicotinamide nucleotide binding Rossmann fold present in both Class B flavoprotein sub-classes); the notable exception is the substitution of histidine 194 in the type 3 BVMO for an equivalent arginine (R207) in CHMO9871, R203 in CHMOH31, R217 in PAMO, and R339 in 4-hydroxyacetophenone monoxygenase (HAMPO; EC 1.14.13.84; Kamerbeek et al., 2003).

The type 3 BVMO from S. maltophilia strain PML 168 differs most significantly from typical type 1 BVMOs in possessing a truncated C-terminal domain. Most notably, the outcome of a CLUSTAL W alignment predicts that the truncated C-terminal domain of the proposed type 3 BVMO contains no amino acid residue at the equivalent positions to lysine 326 and arginine 327 in CHMO9871, K328 and R329 in CHMOH31, K336 and R337 in PAMO, and K439 and R440 in HAMPO. The significance of these sequence differences in the substituted and absent arginine and lysine residues is that these particulate positively charged residues have been shown to be critical in accommodating the 2′-phosphate group of the adenosine ribose that distinguishes NADPH from NADH (Kamerbeek et al., 2004; Torres Pazmino, 2008), thereby serving to help establish NADPH-dependency as a definitive characteristic of almost all known Class B flavoproteins (van Berkel et al., 2006). MeKA, the only other type 1 BVMO able to accept reducing power from NADH, is also deficient in an equivalent key lysine residue (Volkert et al., 2008).

These critical differences between the strictly NADPH-dependent type 1 BVMOs and the type 3 BVMO from S. maltophilia strain PML 168 may help explain the demonstrated coenzyme duality of the latter, a cloned and purified sample of which has been shown to undertake Baeyer–Villiger biooxidation of a range of substrates with NADH serving as the preferred reduced nicotinamide nucleotide coenzyme (Jensen et al., 2011). While the ability of the type 3 BVMO from S. maltophilia strain PML 168 to use NADH resulting from the absence of the controlling influence of key arginine and lysine residues may be seen as a potential commercial advantage by lowering the projected operating costs of undertaking strategically important BVMO-dependent biotransformations, it should be noted that the key positively charged arginine (R217) in PAMO is influential in dictating catalytic activity by serving a key anchoring role for binding the ketone substrate in an orientation that favours nucleophilic attack by the oxygen-activating flavin-peroxide intermediate (Orru et al., 2011). Similarly, the key lysine (K336) in PAMO, by serving to anchor the NADP+ generated from NADPH in the biocatalytic cycle, has been shown to exert a significant positive influence in controlling the catalytic activity and stability of this flavoprotein, plus the extent of enantioselectivity it achieved in the oxygenfunctionalization of racemic ketone substrate (de Gonzalo et al., 2005; Dudek et al., 2010). In turn, this may help to account for both the decreased catalytic efficiency and increased Km with NADPH previously observed with the K439A site-directed mutant of HAMPO (Kamerbeek, 2004).

In conclusion, this is the first characterized BVMO isolated from a marine microorganism. Both its significantly smaller size compared with all other known type 1 BVMOs, and crucially its unique dependence on NADH as the preferred reduced nicotinamide nucleotide, define it as a novel enzyme type – the first characterized member of a new sub-class of Class B flavoprotein – a type 3 BVMO. Using both genome mining and metagenomic and metatranscriptomic screens, we also provide evidence that this type of flavoprotein is widely distributed in a range of marine provinces. This discovery of this novel biocatalyst substantiates the generic argument that the various marine environments are a fruitful, but greatly under-exploited source of biotechnological potential.

**Experimental procedures**

**Strain isolation**

The strain was isolated in January 2001. The surface of intertidal rocks at Church Reef, Wembury, Devon (50°19′W) were scraped and streaked onto R2A media (Difco); this medium was developed by Reasoner and colleagues (1979) as a ‘low nutrient’ medium to detect coliforms in potable water. Once isolated, the strain was grown routinely in marine broth (5.0 g bactopeptone, 1.0 g yeast extract, 0.1 g ferric orthophosphate, 1.0 l filtered seawater).

**Chromogenic screening**

Enzyme detection was based on the BVMO-dependent biooxidation of 2 mM 3-acetylindole to indoxylacetate (Kiernan, 2007; Fig. S1).

**Enzyme assays**

Cell-free extracts of S. maltophilia strain PML 168 were prepared after growth for various periods of time on unsupplemented marine broth, and any BVMO and LH activities therein subsequently assayed with various authentic substrates as previously described (Donoghue et al., 1976; Adger et al., 1997).

**DNA extraction and genome sequencing**

For a complete description of the DNA extraction and sequencing protocols, refer to the Supporting Information. The genome of isolate 168 was sequenced on a Roche 454 FLX Titanium sequencer by the Gene Pool, which is part of the Institute of Evolutionary Biology at the University of Edinburgh, Scotland. Details on the genome sequencing, annotation and assembly are will be reported elsewhere. Both the nucleotide sequence for the full-length 16S rRNA gene and
that of the BVMO gene were retrieved from this genome sequence. Open reading frames, corresponding to putative BVMO genes in the sequenced genome of isolate PML 168, were identified by performing a pattern-hit search via the PENDANT database (http://pendant.gsf.de).

**Comparative analysis of metagenomic sequences**

For a complete description of the metagenomic screening protocols and procedures, refer to the Supporting Information.

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marine isolates. The assay was developed from examples in a review of related methodologies (Kiernan, 2007). Formulation of 3-acetyl indole agar is detailed in Table 1.

**Fig. S2.** Changes in specific activity of 3-acetyl indole monooxygenase (●) and indoxyl acetate hydrolase (▲) during growth of *S. maltophilia* strain PML 168 (○) in marine broth liquid medium. Specific activities of both enzymes are expressed as U mg⁻¹. Growth of *S. maltophilia* strain PML 168 is recorded as A₅₅₀ read against a distilled water blank.

**Fig. S3.** 3D structure of the Class B flavoprotein from *S. maltophilia* strain PML 168 predicted by the FFAS server (Joachimiak, 2009). The predicted structure was generated based on 21% sequence identity to the known structure (1W4X: PubMed 15328411) of phenylacetone monooxygenase from Thermobifida busca (Malito *et al.*, 2004).

**Table S1.** The percentage relative abundance and actual abundance of BVMO homologues in the 99 marine metagenomic data sets in which homology was identified.

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