C. elegans flavin-containing monooxygenase-4 is essential for osmoregulation in hypotonic stress

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ABSTRACT

Studies in Caenorhabditis elegans have revealed osmoregulatory systems engaged when worms experience hypertonic conditions, but less is known about measures employed when faced with hypotonic stress. Inactivation of fmo-4, which encodes flavin-containing monooxygenase-4, results in dramatic hypoosmotic hypersensitivity; worms are unable to prevent overwhelming water influx and swell rapidly, finally rupturing due to high internal hydrostatic pressure. fmo-4 is expressed predominantly in hypodermis, duct and pore cells but is excluded from the excretory cell. Thus, FMO-4 plays a crucial osmoregulatory role by promoting clearance of excess water that enters during hypotonicity, perhaps by synthesizing an osmolyte that acts to establish an osmotic gradient from excretory cell to duct and pore cells. C. elegans FMO-4 contains a C-terminal extension conserved in all nematode FMO-4s. The coincidently numbered human FMO4 also contains an extended C-terminus with features similar to those of FMO-4. Although these shared sequence characteristics suggest potential orthology, human FMO4 was unable to rescue the fmo-4 osmoregulatory defect. Intriguingly, however, mammalian FMO4 is expressed predominantly in the kidney – an appropriate site if it too is, or once was, involved in osmoregulation.

KEY WORDS: Flavin-containing monooxygenase, FMO-4, C. elegans, Hypotonicity, Osmoregulation

INTRODUCTION

Alterations in cell and systemic osmolarity can have serious physiological consequences and the majority of animal species employ diverse osmoregulatory mechanisms to control water homeostasis (Hohmann et al., 2007; Hoffmann et al., 2009; Wood, 2011). At the cellular level changes in extracellular tonicity will drive water flux across the plasma membrane resulting in either cell swelling or shrinkage and consequent perturbations in intracellular osmolarity. To detect and readjust altered volume cells employ an array of sensory and effector systems that mediate either regulatory volume decrease (RVD) or regulatory volume increase in response to cell swelling and shrinkage, respectively (Lang, 2007). Nematodes, whether free-living or parasitic, require aqueous environments for survival resulting in exposure to often challenging osmotic conditions that may be subject to rapid change, for example following rainfall. A permeable cuticle and pseudocelomic cavity under hydrostatic pressure complicates these osmoregulatory challenges (Wharton and Perry, 2011). In its natural habitat of plant-derived organic material (Chen et al., 2006) nematodes are frequently exposed to differing osmotic microenvironments and has evolved a complex osmoregulatory armament that is slowly being experimentally revealed (Choe, 2013). Important sites for water homeostasis in C. elegans include intestine, hypodermis and, in particular, the excretory system and a number of significant osmoregulatory genes are expressed in one or more of these tissues (Choe, 2013).

To date, studies in C. elegans have focused primarily on identifying sensory, behavioural and recovery mechanisms employed during hypertonic stress. Worms exposed to conditions of hypertonicity may shrink dramatically with associated loss of mobility but can recover body volume and movement within a few hours (Lamitina et al., 2004). Recovery is associated with accumulation of glycerol (Lamitina et al., 2004) and regulation by WNK-1 (with-no-lysine) and GCK-3 (germinal centre kinase-3) serine-threonine protein kinases (Choe and Strange, 2007). In contrast, when exposed to water C. elegans swells gradually with reduced mobility, due to increased hydrostatic pressure following inward water flux, but recover when returned to normal osmotic conditions (Huang et al., 2005). The relative lack of research into mechanisms enabling C. elegans to endure and recover following sudden hypotonic exposure may be, in part, because hypoosmotic sensitivity phenotypes are observed relatively frequently often with genes that likely act indirectly rather than directly affecting a hypoosmoticity-related RVD pathway. For example, a number of genes associated with cuticle formation and stability, e.g. acs-20 (Kage-Nakadai et al., 2010), cuti-1 (Fritz and Behm, 2009) and tsp-15 (Moribe et al., 2004), exhibit hypoosmotic sensitivity presumably because cuticle integrity and permeability is compromised leading to water influx and increased turgor pressure. These indirect effects can complicate functional dissection of dedicated hypoosmoticity-related RVD mechanisms.

Flavin-containing monooxygenases (FMOs) comprise a large family of NADPH-, FAD- and O2-dependent enzymes active at heteroatom centres, particularly S and N, in structurally diverse compounds (Ziegler, 2002; Krueger and Williams, 2005). In mammals five distinct functional FMOs, numbered 1 to 5, exist each of which is encoded by a discrete gene (Phillips et al., 1995; Hernandez et al., 2004). Although classified as xenobiotic-metabolizing enzymes (XMEs) this may be a misnomer as FMOs with physiological or biocatalytic roles have now been described, e.g. in intracellular redox regulation in Saccharomyces cerevisiae (Suh et al., 1999), glucosinolate biosynthesis in plants (Schlaich, 2007) and alkaloid-based chemical defence in insects (Sehlmeyer...
et al., 2010). Very recently, *C. elegans fmo-2* has been demonstrated to promote health and life span via a HIF-1 pathway (Leiser et al., 2015). Mammalian FMOs also appear to have endogenous functions as recent reports have revealed that FMO1 and FMO5 play roles in the regulation of energy homeostasis and metabolic ageing, respectively (Veeravalli et al., 2014; Gonzalez-Malagon et al., 2015). Although human FMO3 null homozgyosity results in trimethylaminuria (Dolphin et al., 1997) there is no other apparent organic disease (Mitchell and Smith, 2001) suggesting it does not play any significant endogenous function. However, FMO3 has recently been associated, both directly and indirectly, with a range of conditions, including atherosclerosis, cholesterol balance and glucose and lipid metabolism indicating it may actually be a rather important modifier of human health and disease (Schugar and Brown, 2015). Thus, FMOs should no longer be defined exclusively in terms of xenobiotic detoxification but rather as enzymes with a wide functional repertoire including specific endogenous biological roles.

We previously identified and described the fmo family of *C. elegans*, compared them with orthologous genes present in *C. briggsae* and reported that preliminary examination of *C. elegans fmo* fmo-1, 2, 3 or 5 are apparently phenotypically normal, the fmo-4+/− strain RB562 [fmo-4(ok294)] displayed hypoosmotic sensitivity (Petalcorn et al., 2005). We have reexamined this in detail and confirm fmo-4(ok294) exhibits a hypoosmotic hypersensitivity phenotype as following transfer to water worms swell rapidly resulting, in many case, in violent rupture. We confirm that fmo-4 is hypodernally expressed (Petalcorn et al., 2005) but report that, in addition, fmo-4 is also highly expressed in duct and pore cells. Thus, FMO-4 appears to function as an osmoregulatory brake to attenuate excessive body fluid accumulation during sudden hypotonic exposure. Although the precise underlying FMO-4-mediated role has not been identified we discuss possible related enzymatic activities and osmoregulatory mechanisms.

In comparison to other nematode FMOs, FMO-4 has a C-terminal extension that contains predicted membrane spanning domains and terminates in a conserved consensus sequence. Interestingly, mammalian FMO4 (numbering is purely coincidental) is also extended at the C-terminus with predicted secondary structure features. The trans-membrane helix (TMH) consensus was derived (Fig. 2). The conservation of this C-terminal extension prompted us to analyze it for potential secondary structure features. The trans-membrane helix (TMH) tool TMHMM ( Krogh et al., 2001), judged to be the most accurate at predicting TMHs (Moller et al., 2001), predicted with high probability (0.95 to 1.0) that, in all cases, the non-conserved hydrophobic region folds into two discrete TMHs, with a loop of 4 to 8 residues, while the final 10 to 12 residues, containing the consensus, lie outside the membrane (Fig. 3A,C).

Designated FMO2 when first cloned (Dolphin et al., 1992), human FMO4 (Lawton et al., 1994) encodes a longer FMO due also to the presence of a C-terminal extension (Fig. S3A). In the original report this was proposed to have arisen from a mutational event in an ancestral gene at, or near to, the original stop codon leading to transcriptional run-through to the next in-frame stop triplet. Orthologous FMO4 genes encoding FMO4s with C-terminal extensions exist in the genomes of many other mammalian species (Burnett et al., 1994 and this study) enabling an alignment of mammalian FMO4s to be compiled using a single species from the Prototheria, Metatheria and each Eutherian order (Fig. S3B). Examination of the aligned C-termini revealed that, apart from the Prototheria and Metatheria FMO4 examples in which the hypoosmotic sensitivity was due solely to loss of functional fmo-4 with no apparent redundancy with other fmo genes. However, rescue was not achieved, even partially, in strains in which transient production of FMO-4 was induced by heat-shock (Fig. 1D).

Although we statistically analysed the rescue experiment data (Fig. 1B-D), via ordered logistic regression, the essentially complete rescue observed by transgenic copies of constitutively expressed fmo-4 meant that in all cases the differences between groups were too extreme for odds ratios to be meaningful.

**RESULTS**

**Loss of fmo-4 results in severe hypoosmotic sensitivity**

DNA sequencing and PCR analysis confirmed RB562 [fmo-4 (ok294)] contains an inactivating deletion of ~1.5 kb in both copies of fmo-4 (Fig. S1). When placed in distilled water adult fmo-4(ok294) hermaphrodites rapidly become immobile, stiff and rod-like before, in many cases, eviscerating violently, usually at the vulva (Fig. 1A). An equivalent phenotype was observed in another fmo-4 deletion mutant tm765 (data not shown). The phenotype was not observed with any of four strains lacking functional fmo-1, -2, -3 or -5 (data not shown). The severity and rapid progression of the hypoosmotic sensitivity strongly resembles that of unc-29(e1072) (Lewis et al., 1980) and we used CB1072 homozygous for this allele as a positive phenotypic control (data not shown and Fig. 1B). fmo-4(ok294) hypoosmotic sensitivity was phenocopied effectively via fmo-4 RNAi (Fig. 1A) but not by RNAi targeting fmo-1, -2, -3 or -5 (data not shown). Furthermore, the phenotype was rescued completely in strains (Fig. 1B) carrying either fosmid WRM0636aA04 that contains fmo-4 located centrally (Fig. S2A), a PCR amplicon (PCR155) encompassing fmo-4 plus 5′ and 3′ intergenic sequences (Fig. S2A), or iMW002 in which fmo-4 was tagged, in-frame, with a gfp reporter at the C-terminus (Fig. S2C). These data confirmed that the hypoosmotic sensitivity was due solely to loss of functional fmo-4 with no apparent redundancy with other fmo genes. However, rescue was not achieved, even partially, in strains in which transient production of FMO-4 was induced by heat-shock (Fig. 1D).

**FMO-4 and human FMO4 both possess a C-terminal extension**

*C. elegans fmo-4* is ~50 amino acids longer than FMO1, 2, 3 and 5 and these form a distinct C-terminal extension (Fig. S3A). As the presence of this extension differentiates FMO-4 from other nematode FMOs the last 100 residues were used to search protein and genomic DNA databases for orthologous genes. Although fmo-4 orthologs were apparently absent from Nematoda clades I and II (Blaxter et al., 1998) and also from Annelida and Platycladins, complete or partial FMO-4 amino-acid sequences were retrieved for Nematoda clades III, IV and V and included a number of human disease-causing parasitic species (Fig. 2). Visual examination of a C-terminal section from an alignment of these FMO-4 primary sequences revealed a common stretch of hydrophobic residues followed by highly conserved extreme C-termini from within which a WFDLQYDM (ST)(FL) consensus was derived (Fig. 2). The conservation of this C-terminal extension prompted us to analyze it for potential secondary structure features. The trans-membrane helix (TMH) tool TMHMM ( Krogh et al., 2001), judged to be the most accurate at predicting TMHs (Moller et al., 2001), predicted with high probability (0.95 to 1.0) that, in all cases, the non-conserved hydrophobic region folds into two discrete TMHs, with a loop of 4 to 8 residues, while the final 10 to 12 residues, containing the consensus, lie outside the membrane (Fig. 3A,C).

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Although the global sequence identity between *C. elegans* FMO-4 and human FMO4 is only 33% (data not shown) it is tempting to propose that the existence in both of a C-terminal extension containing membrane spanning domains and highly similar conserved motifs towards the end is evidence that (1) rather than resulting from a random mutational event the extension evolved in an ancestral gene to serve a clear functional role and (2) nematode *fmo-4* and mammalian FMO4 evolved from an ancient common ancestor. The two genes would thus be, albeit now highly diverged, potential orthologs. If this is the case then use of the same numerical suffix, decided upon independently for nematode *fmo* (Petalcorin et al., 2005) and mammalian *FMO* (Lawton et al., 1994) nomenclatures, is apposite. However, such coincidental numbering should not be taken to infer orthology between other nematode *fmo* and mammalian *FMO* members.

**Fig. 1.** *fmo-4(ok294)* hypoosmotic sensitivity and rescue assay. Following transfer to distilled water adult RB562 [*fmo-4(ok294)*] (A1,A2) or *fmo-4* RNAi-treated N2 (A3,A4) worms rapidly stop thrashing, becoming first immobile then rod-like (arrowheads, A1,A3) and finally, in the majority of individuals, undergoing explosive evisceration (arrowheads, A2,A4). The ability of different strains to rescue the hypoosmotic sensitivity phenotype exhibited by RB562 was assayed by transferring individual worms to 0.8 ml of distilled water and scoring, at 5, 20 and 40 min time points, the phenotype of the worm as either mobile, immobile, rod-like or exploded (72 worms [three replicate *n*=24 assays] per time-point). The percentages of each phenotype at each time-point are shown graphically (B-D). Strains tested were RB562-derived and harbored extra-chromosomal *fmo-4* or human FMO4 transgenes that expressed either constitutively [CD1078 (Pfmo-4::fmo-4::GFP); B], CD1085 (Pfmo-4::fmo-4); B), CD1086 (WRM0636aA04; B) and CD1094 (Pfmo-4::CeoptHuFMO4; C), or were induced following heat-shock [CD1080 (Phsp-16.2::fmo-4), CD1081 (Phsp-16.41::fmo-4) and CD1094 (Phsp-16.2::HuFMO4); D]. Positive (CB1072; B) and negative (CD1079 [pRF4 (rol-6 su1006)]; B) control strains were included in some experiments.
**FMO4s exist in other species but not all retain C-terminal extensions**

Although FMO4 or FMO4-like genes are present in other vertebrate species, not all of these appear to encode proteins that include C-terminal extensions. For example, although BLASTP searches, with the complete human FMO4 sequence as string, identifies putative FMO4 orthologs in Amphibia, Reptilia and birds, none appear to encode a C-terminally extended FMO4 (data not shown). However, a number of FMO4 genes in the bony fishes do appear to encode proteins extended at their C-termini. Whilst the sequences of the many C-termini varies salmon FMO4 contains the sequence DLLQD that matches closely the [DE]KLQ[DN] consensus in mammalian FMO4 C-termini (Fig. S4).

**Human FMO4 fails to rescue fmo-4(ck294)**

Notwithstanding the relatively low global identity between C. elegans FMO-4 and human FMO4, we tested the latter for the potential to rescue fmo-4(ck294). To this end we constructed a fosmid-based transgene in which the complete fmo-4 CDS was replaced with a synthetic C. elegans-optimised human FMO4 mini-gene. The transgene failed to rescue the hypoosmotic sensitivity (Fig. 1C). In addition, rescue via heat-shock induced transient expression of human FMO4 was also unsuccessful (Fig. 1D).

**fmo-4 gene expression analysis**

We investigated fmo-4 expression indirectly in transgenic strains carrying transcriptional and translational reporter constructs (Figs 4, 5). In CD1002, transgenic for pMPFG1 (Pfmo-4::GFP) that contains ~4 kb of fmo-4 5′ flanking sequence (Fig. S2B), GFP was expressed diffusely throughout the syncytial hypodermis, including head and tail hyp cells, but was absent from seam cells. Prominent GFP expression was also observed in cells flanking the vulval slit and, most noticeably, in the duct and pore cells just outside the vulva (Fig. 5). In CD1002, transgenic for pMPFG1 (Pfmo-4::GFP) that contains ~4 kb of fmo-4 5′ flanking sequence (Fig. S2B), GFP was expressed diffusely throughout the syncytial hypodermis, including head and tail hyp cells, but was absent from seam cells. Prominent GFP expression was also observed in cells flanking the vulval slit and, most noticeably, in the duct and pore cells just outside the vulva (Fig. 5).
anterior to the posterior bulb of the pharynx (Fig. 4A1-A11). Essentially the same GFP expression was observed in both CD1018, transgenic for the fosmid-based reporter fMW002 (Pfmo-4::fmo-4::GFP) (Fig. 4B1-B10), and BC14787 (rCesF53F4.5::GFP) (McKay et al., 2003) (Fig. 4C1-C3) in which the PCR-generated Pfmo-4::GFP transgene drives gfp expression with ∼2.5 kb of upstream sequence (Fig. S2B). GFP expression in the duct and pore cell bodies was clearly visible under higher magnification in all three strains and was also observed in CD1002 and BC14787 outlining the distal section of the excretory duct up to the location of the excretory pore (Fig. 4C2,C3). GFP expression was not observed in the body or canals of the adjacent excretory cell. By targeting GFP to nuclei with a Pfmo-4::NLS::GFP::LacZ fusion intense, punctate GFP expression was observed in CD1090 not only in the hypodermal nuclei but also those of the duct and pore cells (Fig. 4D3,D4). The same pattern was observed in Pfmo-4::NLS::GFP transgenenic worms except nuclear expression was less punctate (data not shown). These data indicate that, in addition to the hypodermis, fmo-4 is expressed prominently in the duct and pore cells of the excretory system.

To confirm duct and pore cell expression we generated strains in which an fmo-4 reporter was coinjected with aqp-8 (Khan et al., 2013) or lin-48 (Wang and Chamberlin, 2002) co-markers (Fig. 5) to label the excretory cell and duct and pore cells, respectively. GFP expression in CD1091, which co-expressed Pfmo-4::GFP and Paqp-8::aqp-8::mCherry, was restricted to hypodermal, duct and pore cells but was absent from the excretory cell which, instead, clearly expressed Paqp-8-driven mCherry (Fig. 5A1-A4). In CD1092 (Fig. 5B1-B4) and CD1093 (Fig. 5C1-C4), in which Pfmo-4::GFP was replaced with Pfmo-4::NLS::GFP and Pfmo-4::NLS::gfp::LacZ, respectively, GFP was clearly expressed in the duct and pore cell nuclei located directly anterior to the mCherry-stained excretory cell body. Finally, strain CD1096, that co-expressed Pfmo-4::mCherry and Plin-48::GFP, was visualized in both green and red channels which, when merged, generated yellow fluorescence confirming co-localization of fmo-4 and lin-48 in duct and pore cells (Fig. 5D1-D4).

Cuticle permeability assay
We examined the integrity of the cuticle/hypodermal barrier in RB562, CB1072 and the epidermis-defective bus-8 mutants CB6193 and CB6147 (Partridge et al., 2008) with the DNA-binding fluorescent dye Hoechst 33258 (Fig. 6). Stained nuclei were visible in a small percentage of N2 worms (3%, n=165) whereas, under the same experimental conditions of incubation and microscopy there was, as expected, extensive and highly statistically significant nuclear staining in CB6193 (84%, n=193, P<0.001) and CB6147 (89%, n=197, P<0.001) (Fig. 6D-E). Although significant compared to N2 far fewer nuclei were unambiguously stained in RB562 (15%, n=184, P<0.05) and CB1072 (22%, n=193, P<0.01) in comparison to the extensive and distinct staining in the bus-8 mutants (Fig. 6B,C).

FMO-4 exhibits S-oxygenase activity
Preliminary functional characterization of C. elegans FMO-1 and FMO-4 plus human FMO3 and FMO4, produced via heterologous expression in insect cells, was undertaken. As specific antibodies against FMO-1 and FMO-4 were unavailable we chose to bypass examination of microsomal protein by immunoblotting and move directly to investigate the ability of membranes to catalyze S-oxidation of the archetypal mammalian FMO substrate methimazole (Dixit and Roche, 1984) as described (Dolphin

Fig. 3. Predicted transmembrane segments within C. elegans FMO-4 and human FM04. C. elegans FMO-4 (A) and human FM04 (B) are predicted, with high probability, to contain, respectively, two and one transmembrane segments within their C-termini. Additionally, the extreme C-termini of both C. elegans FMO-4 (C) and human FM04 (D) are predicted to lie outside the membrane lamella and contain, respectively, highly similar DLQYD and DKLQD motifs (boxed). Prediction of trans-membrane segments (A,B) and membrane models (C,D) were generated with TMHMM and HMMTOP, respectively, the latter using the last 100 amino acid residues of the respective protein as input.
Membranes from cells infected with vCD020 (C. elegans FMO-1) or vCD024 (C. elegans FMO-4) catalyzed S-oxidation with essentially maximal specific activities of, respectively, 0.02 and 0.005 nmol of methimazole oxide formed/min/mg microsomal protein (Fig. 7A). Although, the non-ionic detergent NP-40 had little effect on the activity of either FMO addition of the zwitterionic CHAPS increased FMO-1 and FMO-4 activities to 0.06 and 0.05 nmol/min/mg microsomal protein, respectively (Fig. 7A). We also expressed human FMO3 and FMO4 – the former as a positive control as we have expressed and characterized FMO3 previously (Dolphin et al., 1997) and the latter because of the putative relationship with FMO-4. Initial maximal S-oxidation rates with membranes from cultures infected with vFMO3 (human FMO3) or vCD030 (human FMO4) were, respectively, 0.15 and 0.22 nmol/min/mg (Fig. 7B). NP40 had little effect on the base activity of FMO4-catalyzed methimazole oxidation whereas the FMO3 activity was increased to 0.22 nmol/min/mg (Fig. 7B).

Fig. 4. fmo-4 expression patterns. Representative gene reporter expression patterns for young adult (A1-A8,B1,B5-B10,C1-C3,D3-D4) or L2/L3 (A9-A11,D1,D2) hermaphrodites (A1-A11,B1,B5-B10,C1-C3,D1-D4) and a single adult male (B2-B4) of strains CD1002 (Pfmo-4::GFP; A1-A11), CD1018 (Pfmo-4::fmo-4::GFP; B1-B10), BC14787 (Pfmo-4::fmo-4::GFP; C1-C3) and CD1090 (Pfmo-4::NLS::GFP::LacZ; D1-D4) visualized as a single-channel GFP fluorescence image (A2-A5,A7,A8,A10,A11,B3,B4,B6-B10,D1,D3), the corresponding DIC image (A6,A9,B2,B5,B8) or overlaid (A1,B1,C3,D2,D4). Arrowheads indicate either the vulva (A6-A8,B5-B7) or intense GFP expression localized to duct and pore cell nuclei (D3,D4). Deconvolution (nearest-neighbor) deblurring was applied on some image files (A3,A5,A8,A11,B4,B7,B10) to reveal additional detail. Images were captured at 100× (A1,B1) 200× (A9-A11,D1,D2), 400× (A2-A8,B2-B10,C1-C3,D3,D4) magnification. Scale bars are 100 µm (A1,B1), 50 µm (A9-A11,D1,D2) and 20 µm (A2-A8,B2-B10,C1-C3,D3,D4).
Again, addition of CHAPS had a clear stimulatory effect and both human FMO activities were increased to \(\sim 0.4\ \text{nmol/min/mg}\) microsomal protein (Fig. 7B). The base methimazole oxidation activity with FMO3-containing microsomes is less than we reported previously (2.0 nmol/min/mg microsomal protein) (Dolphin et al., 1997) and this likely reflects less than optimal expression conditions and/or microsomal membrane isolation resulting in lower specific yields of FMO. This may also explain the low activities observed with both recombinant worm FMOs. That said, the preliminary experiments reported here confirm both worm FMOs are indeed able to catalyze methimazole S-oxidation.

DISCUSSION

Loss of \(fmo-4\) had no apparent effect upon the ability of \(C.\) elegans to osmoregulate under laboratory conditions as there was no evidence of compromised water homeostasis, such as fluid accumulation or swelling, during passage on NGM plates or when transferred to M9 buffer (data not shown). However, following transfer to distilled water loss of \(fmo-4\) results in dramatic hypoosmotic hypersensitivity – worms are unable to prevent overwhelming water influx, swell and, in many cases, rupture violently due to the high internal turgor pressure. The phenotype was observed in two independent \(fmo-4\) deletion strains, was phenocopied by RNAi and rescued completely with extrachromosomal \(fmo-4\). These data confirm that the inability to osmoregulate under sudden hypotonic stress, as would likely be experienced by \(C.\) elegans in its natural habitat following rainfall, is due solely to loss of functional \(fmo-4\). Rescue was not achieved when transgenic \(fmo-4\) was induced following heat shock perhaps because this did not result in sufficient enzyme being synthesized to provide adequate protection from subsequent osmotic challenge. The \(fmo-4\) expression pattern (Figs 4,5), investigated here in some detail, supports such a role as reporter expression was detected in the hypodermis and duct and pore cells all of which are involved in water balance control (Choe, 2013). Expression was especially prominent in duct and pore cell nuclei (B1-B4,C1-C4) while \(lin-48\)-driven GFP expression in neuronal support cells is indicated with an arrow (D1-D4).

Deconvolution (nearest-neighbor) deblurring of both fluorescence channels was applied to each original file to reveal additional detail (A2,A4,B2,B4,C2,C4,D2,D4). Images were captured at 400× (A1,A2,C1-C4,D1-D4) or 600× (A3,A4,B1-B4) magnification. Scale bars are 20 µm (A1,A2,C1-C4,D1-D4) or 10 µm (A3,A4,B1-B4).
Although we did not investigate it here loss of FMO-4, whether via deletion (Shen et al., 2005) or RNAi (Rual et al., 2004), has also been associated with reduced viability, e.g. the former report described a ∼60% reduction in brood size that was further reduced under hypoxia. Whether the embryonic lethality under normoxia is associated with the compromised osmoregulatory competence we report here is not clear. Interestingly, null mutations in *rdy-2*, a gene encoding a novel tetraspan protein, are associated with osmoregulatory defects and lethality as larvae die with a fluid-filled and rod-like phenotype (Liegeois et al., 2007). Intriguingly, although there is no evidence that *fmo-4* and *rdy-2* are located together in an operon, they are adjacent and on the same strand on chromosome V.

FMOs are classified conventionally as XMEs that detoxify and, occasionally, bioactivate diverse xenobiotics (Ziegler, 2002; Krueger and Williams, 2005). However, individual plant and

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**Fig. 6. Comparative cuticle permeability in *fmo-4*, *unc-29* and *bus-8* strains.** (A) Representative DAPI (A1,A2,B1,B2, C1,C2, D1,D2, E1,E2) and corresponding DIC (A2,B2,C2, D2,E2) tail images of N2 (A1,A2), RB562 (*fmo-4*; B1,B2), CB1072 (*unc-29*; C1,C2), CB6147 (*bus-8*; D1,D2), CB6193 (*bus-8*; E1,E2) after incubation with Hoechst 33258. 400× magnification; scale bar=20 µm. (B) Percentage (*y*-axis) of adult animals containing ≥10 distinctly stained nuclei for strains N2 (A), RB562 (B), CB1072 (C), CB6193 (D), CB6147 (E). Error bars represent s.e.m. from four independent replicates (∼50 animals per replicate). *P<0.05, **P<0.01, ***P<0.001.
animal, including human, FMOs have now been identified or implicated in a range of physiological or metabolic activities in many cases mediated via the oxygenation of a specific endogenous substrate. Although FMO-4 may still function in xenobiotic detoxification the clear involvement of FMO-4 in water balance suggests a major role is one of endogenous osmoregulation. Although it is not clear at this stage how this role is enzymatically accomplished we speculate below on possible mechanisms.

There was evidence for at least partial loss of epidermal barrier function in \textit{fmo-4} (\textit{ok294}) as dye uptake studies revealed a small but significant increase in nuclear staining compared to N2. The degree of staining was, however, far less than that observed in two \textit{bus-8} mutants that exhibit clear disorganized body wall structures reflecting loss of a glycosyltransferase activity required for epidermis morphogenesis (Partridge et al., 2008). We did not observe any gross epidermal or cuticle defects in adult RB562 (data not shown). Interestingly, the single FMO of yeast promotes protein disulphide bond formation in the ER by generating permissive oxidative conditions via biological thiol oxidation (Suh et al., 1999). Thus, it is conceivable that, were FMO-4 to have the same activity, hypodermally-expressed FMO-4 may contribute to cuticular collagen disulphide bond formation by a similar mechanism.

FMOs classically catalyze heteroatom oxidation via the C(4a) hydroperoxide flavin derivative. There are, however, reports of FMOs catalyzing Baeyer–Villiger oxidations which, by inserting oxygen into the C-C bond adjacent to a carbonyl, convert ketones to esters and cyclic ketones to lactones (Chen et al., 1995; Lai et al., 2011; Meng et al., 2015). Thus, unless FMO-4 has a yet-to-be recorded enzymatic activity the osmoregulatory role is likely mediated either via heteroatom oxygenation or perhaps even Baeyer–Villiger oxidation. There is, however, one other perhaps less likely mechanism – generation of H$_2$O$_2$. Considering this option first, heterologously expressed mammalian FMO can generate H$_2$O$_2$ not only during enzymatic uncoupling but also with substrate present and this NADPH oxidase activity can result in not insubstantial H$_2$O$_2$ production (Siddens et al., 2014). H$_2$O$_2$ and other reactive oxygen species increase rapidly in a number of mammalian cell lines following hypotonic exposure (Hoffmann et al., 2009), including NIH3T3 fibroblasts (Lambert, 2003) and hepatocytes (Varela et al., 2004), and activate or potentiate swell-inducible osmolyte and ion efflux channels. Thus, it is feasible FMO-4 might reduce hypotonicity-induced water influx via FMO-4-generated H$_2$O$_2$-mediated transporter activation.

Most likely, however, FMO-4 acts via classic heteroatom monoxygenation in an endogenous substrate and the resulting product acts to attenuate water accumulation under hypoosmotic conditions. Such a product might be generated in response to water influx or, alternatively, generated constitutively and sequestered and thus be available for immediate use under hypotonic stress. The product would likely be electroneutral and tolerated at high concentration to avoid disturbance of intracellular ionic and protein homeostasis – characteristics typical of osmolytes (Yancey et al., 1982). The product remains to be identified but glycerol, the osmolyte accumulated in \textit{C. elegans} under hyperosmotic stress, can be excluded as it is synthesized by glycerol-3-phosphate dehydrogenase (Lamitina et al., 2004). Although trimethylamine N-oxide (TMAO), an osmolyte utilized by many marine species (Yancey et al., 1982) and generated via FMO (Lang et al., 1998), is a possible candidate it is not considered likely as, if this were the case, \textit{C. elegans} would be commonly associated with a fish odour as TMAO is readily reduced to the malodorous, volatile base.

Fig. 7. Functional characterization of heterologously expressed \textit{C. elegans} and human FMOs. Methimazole oxidation-dependent nitro-5-thiobenzoate oxidation, monitored indirectly as the time-dependent difference in absorbance at 412 nm, was catalyzed via microsomal membrane protein isolated from S9 cells infected with baculoviruses vCD020 (950 µg, A), vCD024 (930 µg, A), vCD030 (330 µg, B) or vFMO3 (315 µg, B) encoding, respectively, \textit{C. elegans} FMO-1, \textit{C. elegans} FMO-4, human FMO4 and human FMO3. Reactions contained microsomal protein resuspended in Tricine buffer (pH 8.4) alone or with additional NP40 or CHAPS detergent (1% v/v).
Setting identification to one side how might this product act in hypotonicity? One possibility is that, stored in the hypodermis as an osmolyte, it would, during hypotonicity-induced water influx, be available for efflux via transporter systems thereby reducing the osmotic gradient and water ingress. Another, potentially compatible model can be built from the observation that FMO-4 is expressed prominently in duct and pore cells but is absent from the excretory cell. Assuming the putative FMO-4 product is concentrated in these two cells this could establish a strong osmotic gradient from excretory cell to duct and pore cells encouraging water movement down the gradient through the excretory system exiting at the excretory pore. The presence of aquaporin channels in the excretory cell (Huang et al., 2007) would be available to transfer water, drawn from the hypodermis and/or pseudocoelom, into the excretory cell canals before passing into the excretory duct at the secretory-excretory junction. If this osmotic gradient is compromised in fmo-4(ok294) worms water flow rate may be insufficient to prevent swelling and rupture following acute hypotonic exposure. There are, of course, limitations with this model – how, for example, would excess water be encouraged to remain in, and move down, the duct lumen? One possibility is that the cuticle surrounding the duct is less water permeable than cuticle elsewhere. Also, Nelson and Riddle (1984) reported that, although muscle-like filaments were not observed in duct or pore cells, the excretory duct pulsates in dauer larvae resulting in fluid exiting at the pore. Pulsation has also been observed in excretory ducts of larval stages of other nematodes (Atkinson and Onwuliri, 1981). Thus, it is possible that similar, transient pulsations might occur in the adult excretory duct under conditions of excess water influx. Another potential complication is the reported presence of gap junctions between excretory, duct and pore cells (Nelson et al., 1983) as these would presumably permit diffusion of any FMO-4 other potential activity modulators nor were we able to examine detergents. Unfortunately, lack of expressed protein and time precluded further investigations into additional detergent effects or other potential activity modulators nor were we able to examine N-oxidation. We also expressed human FMO3 and FMO4 – the former as a positive control and the latter because of the putative relationship with FMO-4. FMO4 was expressed successfully as a full-length protein containing the C-terminal extension sequence. Previous attempts to express FMO4 as a functional enzyme in yeast or bacteria were only successful when this region was deleted (Itagaki et al., 1996). Future work will explore the expression and functional characterizations of FMO-4 and FMO4 as C-terminally truncated proteins.

Although the specific catalytic activity underlying the osmoregulatory role of FMO-4 has yet to be determined it would be theoretically possible to screen a series of candidate substrates with heterologously expressed enzyme. An alternative and non-biased approach would be to employ a metabonomics-LC-MS-based system to interrogate C. elegans extracts following incubation with recombinant FMO-4 (de Carvalho et al., 2010; Prosser et al., 2014). This approach has been used successfully to identify endogenous ligands for a number of enzymes (Saghatelian et al., 2004; Saito et al., 2006). By combining the approach with \(^{18}\text{O}_2\) labeling the method has also been applied to deorphanize human cytochrome P450 enzymes (Tang et al., 2009) – a strategy that could also be applied to identify endogenous FMO-4 ligand(s).

The increasing number of reports describing FMO involvement in endogenous activities, including life span (Leiser et al., 2015), energy balance (Veeravalli et al., 2014), metabolic ageing (Gonzalez-Malagon et al., 2015) and even atherogenesis and cholesterol metabolism (Schugar and Brown, 2015), illustrate these XMEs also have, perhaps in many cases, specific biological roles. We have now added another entry to this list by describing an osmoregulatory role for C. elegans FMO-4. Sequence features shared between C. elegans FMO-4 and mammalian FMO4 suggest the corresponding genes may have evolved from a common, ancient ancestor. If this reflects a shared, or once shared, functional role then is it possible FMO4 may also have, or once had, an osmoregulatory function. Interestingly, human FMO4 expression, whether measured via mRNA (Yanai et al., 2005; Nishimura and Naito, 2006; Zhang and Cashman, 2006; Dezsö et al., 2008) or protein (Novick et al., 2009) abundance, reveals that while constitutively expressed in many tissues human FMO4 is often most abundantly expressed in the kidney, a site that would certainly appear to support such a proposal.

**MATERIALS AND METHODS**

**General C. elegans and molecular biological methods**

Worm maintenance was performed as described (Sulston and Hodgkin, 1988) under approved conditions. RNAi by feeding was performed...
essentially as described (Timmons and Fire, 1998). Construct fidelity was confirmed by sequencing. Strains carrying heat shock-inducible transgenes were heat shocked (32°C, 4 h) and recovered (20°C, 2 h). Counter-selection recombineering of *C. elegans* fosmid clones in *E. coli* MW005 (Westenberg et al., 2010) using a *rpsL*-*tet(A)* selection cassette (RT-cassette), amplified from pBAC-RT (Stavropoulos and Strathdee, 2001), was performed as described (Dolphin and Hope, 2006; Hirani et al., 2013). Cloning strategies and sequence analyses were performed with MacVector (MacVector Inc.). For creation of transgenic lines young adult hermaphrodites were coinjected, into the syncytial gonad, with a mixture of plasmid and/or fosmid DNA (10-50 µg/ml) and, where appropriate, single-worm RT-PCR. 

Chromosomal constructs was confirmed by expression of the transformation part of this study (Table S2) carrying either gene expression or rescue extra-worms containing point, was used with ODN 4368 (Table S1) to selectively amplify from addition, ODN 4565 (Table S1), by annealing across the 5′-3′ deletion in pMW002 in which *Pfn-4*: *fmo-4::gfp* is flanked by ~19 kb and 10 kb of 5′ and 3′ genomic sequence, respectively (Fig. S2C).

**Bioinformatics**

Nematode FMO-4 sequences were obtained either directly from WormBase (WS242) or via BLASTP or TBLASTN searches with the last 100 amino acids of *C. elegans* FMO-4 as string. Amino acid sequence alignments were generated with ClustalW and optimised ‘by eye’. Transmembrane predictions were performed with TMHMM ( Krogh et al., 2001) and membrane models generated with HMMTOP (Tusnady and Simon, 2001).

**Hyposmotic sensitivity assay**

For each strain 24 individual 5-7 day adult hermaphrodites were transferred to distilled water (1 ml in each of 24 wells of a 48-well plate) and each worm scored at 5 min, 20 min and 40 min as either mobile, immobile, rod-like or exploded. Each assay was performed in triplicate and proportions calculated for each phenotype at each time point. Ordered logistic regression (Long and Freese, 2004) was performed by investigating three outcomes: time to worm becoming immobile, rodlike or exploded; each with four possible values (≤5 min, 5-20 min, 20-40 min, ≥240 min) corresponding to time when first seen in the condition (5 min, 20 min, 40 min and Never) and results expressed as odds ratios. Formal statistical significance was set at *P*<0.05. Data was analysed in Stata (version 13.1).

**Construction of recombinant baculoviruses**

pFastBac1-based shuttle vectors pCD020, pCD024 and pCD075, containing FMO-1, FMO-4 and human FMO4 CDSs, respectively, were constructed as follows. pCD020 was built by inserting a FMO1 CDS PCR-amplified (ODNs 4458 and 4459; Table S1) into pCD024 CDS, amplified from clone 13W (Dolphin et al., 1992) using *KpnI* and *SacI*, with mCherry CDS, PCR-amplified from pNH077 (Hirani et al., 2013) (ODNs 4359 and 4360; Table S1), to generate IMW002 in which *Pfn-4*: *fmo-4::gfp* is flanked by ~19 kb and 10 kb of 5′ and 3′ genomic sequence, respectively (Fig. S2C).
Baculovirus-mediated expression and preparation of microsomal membrane vesicles

S9 cells were passaged using SF-900III (Thermo Fisher) media containing penicillin-G (100 units/ml) and streptomycin sulphate (0.1 mg/ml). Virus was amplified using a multiplicity of infection (MOI) of ≤0.1. For expression, S9 cells [200 ml, 106 cells/ml, 1 litre Spinner flask (Bellco)] were infected (MOI 10) and incubated (72 h, 100 rpm, 28°C) in SF-900III supplemented with FAD (10 μg/ml). Cells were pelleted, resuspended (0.154 M KCl, 50 mM Tris pH 7.4, 0.2 mM PMSF) and lysed by sonication. Debris was removed by centrifugation (10,000 g, 15 min, 4°C) and microsomal membrane vesicles were pelleted from the resulting supernatant (Sf expression, the experimental work and wrote the manuscript. pseudocoelomic fluid.

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