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Comparative Genomic and Genetic Evidence on a Role for the OarX Protein in Thiamin Salvage

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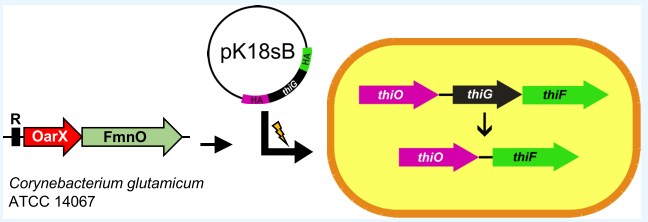
ABSTRACT: Salvage pathways for thiamin and its thiazole and pyrimidine moieties are poorly characterized compared to synthesis pathways. A candidate salvage gene is *oarX*, which encodes a short-chain dehydrogenase/reductase. In diverse bacteria, *oarX* clusters on the chromosome with genes of thiamin synthesis, salvage, or transport and is preceded by a thiamin pyrophosphate riboswitch. Thiamin and its moieties can undergo oxidations that convert a side-chain hydroxymethyl group to a carboxyl group, or the thiazole ring to a thiazolone, causing a loss of biological activity. To test if OarX participates in salvage of the carboxyl or thiazolone products, we used a genetic approach in *Corynebacterium glutamicum* ATCC 14067, which is auxotrophic for thiamin's pyrimidine moiety. This strain could not utilize the pyrimidine carboxyl derivative. This excluded a role in salvaging this product and narrowed the function search to metabolism of the carboxyl or thiazolone derivatives of thiamin or its thiazole moiety. However, a Δ*thiG* (thiazole auxotroph) strain was not rescued by any of these derivatives. Nor did deleting *oarX* affect rescue by the physiological pyrimidine and thiazole precursors of thiamin. These findings reinforce the genomic evidence that OarX has a function in thiamin metabolism and rule out five logical possibilities for what this function is.



1. INTRODUCTION

Thiamin (vitamin B1), in its active pyrophosphate form, is the cofactor for various enzymes that make or break carbon−carbon bonds. Thiamin is essential in all organisms, although not all can synthesize it, which makes thiamin biosynthesis and metabolism pathways attractive drug targets.[1−3] Bacterial biosynthesis pathways for thiamin and its constituent 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and 5-(2-hydroxyethyl)-4-methylthiazole (HET) moieties are well-known; the widely distributed canonical pathways have no missing steps or enzymes (Figure 1).[4−6] Salvage pathways are less well-known, in part because there are at least 14 potential degradation products of thiamin itself and its moieties.[5,7−10] As some of these products are toxic,[11−13] they may need detoxification; even less is known about detoxification routes than about salvage.[8,9,13]

Because all of the genes of thiamin biosynthesis have been identified in various organisms, genes of unknown function that comparative genomics evidence associates with thiamin are likely to participate in salvage or detoxification, not biosynthesis. One such gene, *oarX*, encoding a short-chain dehydrogenase/reductase broadly similar to the 3-oxoacyl-(acyl-carrier protein) reductase FabG, has long been known to cluster on the chromosome with thiamin synthesis and salvage genes and to be downstream of a thiamin pyrophosphate (TPP) riboswitch.[4,14,15] Binding of TPP to this riboswitch represses transcription and translation of the downstream coding sequence.[14] Also, the SEED comparative genomics database[16] has long computationally flagged a predicted FMN-dependent monooxygenase gene (henceforth: *fmnO*) as being in an operonic arrangement with *oarX*.

Rodionov et al. suggested 20 years ago that the "data seem to be sufficiently strong to warrant experimental analysis of the functional role of the *oarX* gene product in thiamin metabolism".[4] As many more genomes have been sequenced since then and an association with *fmnO* has emerged, we undertook a bioinformatic reanalysis of the function of *oarX* and a genetic analysis using *Corynebacterium glutamicum* as a test organism. *C. glutamicum* was chosen because it has an *oarX-fmnO* operon preceded by a TPP riboswitch (Figure 2) and is genetically tractable. We tested for potential salvage or detoxification activities based on the likelihood that some of these remain to be discovered (see above), on the fact that—like known salvage genes—*oarX* occurs in some organisms but not others,[4] and on the evolutionary premium on salvaging thiamin because it is so energetically expensive to synthesize de novo.[5,10,17,18] Further, we focused on the main five "orphan" degradation products that are known to be excreted in

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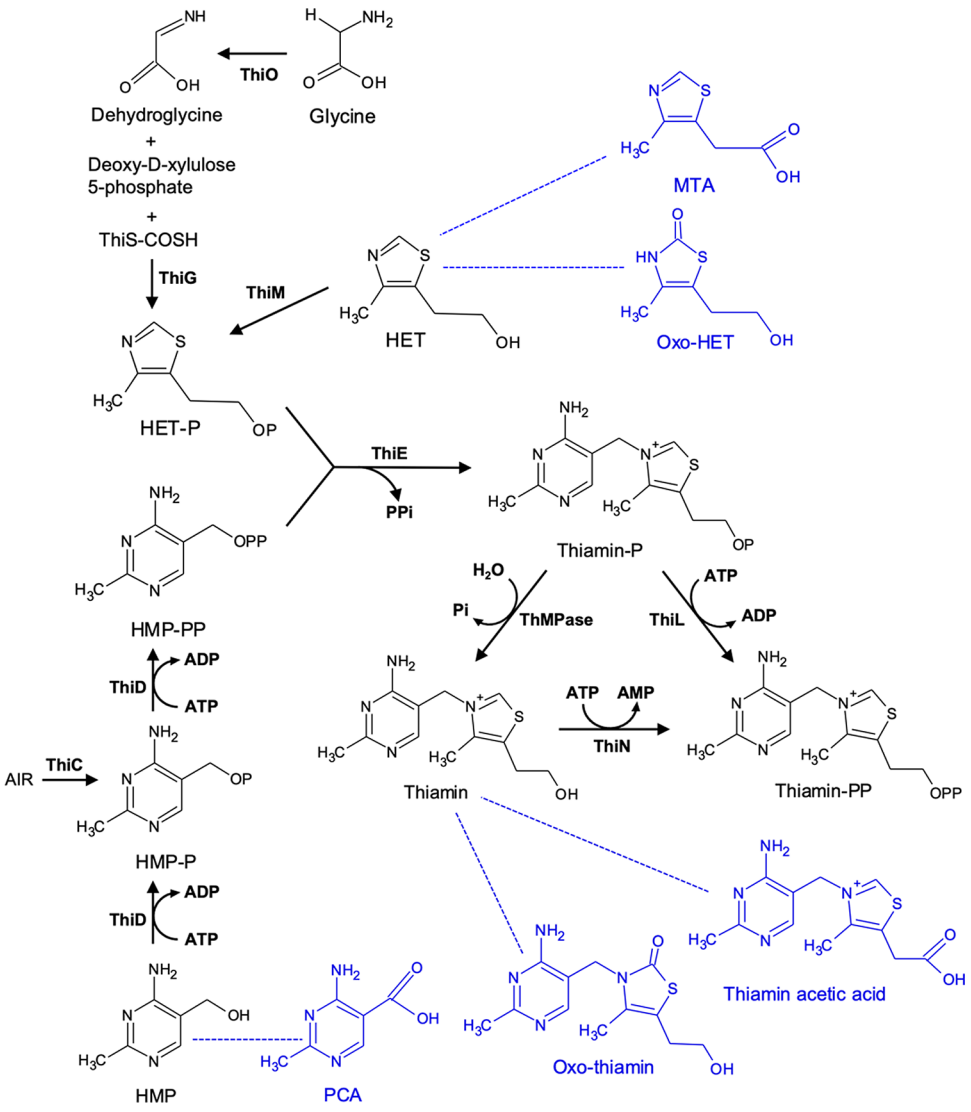

Figure 1. Canonical bacterial thiamin synthesis and salvage pathways. Enzymes: ThiD, hydroxymethylpyrimidine/phosphomethylpyrimidine kinase; ThiE, thiamin phosphate synthase; ThiG, thiazole synthase; ThiL, thiamin phosphate kinase; ThiN, thiamin pyrophosphokinase; ThiO, glycine oxidase; ThMPase, thiamin monophosphatase. Dehydroglycine can also be derived from tyrosine via ThiH. Compounds: AIR, 5-aminoimidazole ribonucleotide; HET, 5-(2-hydroxy-ethyl)-4-methylthiazole; HMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine; HMP-P, HMP monophosphate; HMP-PP, HMP pyrophosphate; MTA, 4-methyl-5-thiazoleacetic acid; oxo-HET, oxo derivative of HET; oxo-thiamin, oxo derivative of thiamin; PCA, 2-methyl-4-amino-5-pyrimidinecarboxylic acid; ThiS-COSH, ThiS thiocarboxylate; HET-P, HET-phosphate. Damage products of thiamin and its thiazole or pyrimidine precursors are shown in blue and tied to the corresponding physiological compound with dashed blue lines.

mammalian urine[19,20] and formed by microbes[7,21] but whose metabolic fates are unknown (Figure 1). In three of these, thiamin acetic acid, 4-methyl-5-thiazoleacetic acid (MTA), and 2-methyl-4-amino-5-pyrimidinecarboxylic acid (PCA), a side-chain primary alcohol group, has been oxidized to a carboxyl group. In the other two, oxo-thiamin (the oxo derivative of thiamin) or oxo-HET (the oxo derivative of the thiazole precursor HET), the thiazole ring has been oxidized to a thiazolone. Each of these compounds could theoretically be recycled to thiamin or its HET or HMP precursor; after activation, a carboxyl group can be reduced via an aldehyde to an alcohol,[22] and ring oxo groups can likewise be reduced.[23] Salvage pathways for these compounds are accordingly predicted to include dedicated reductases; OarX is a plausible candidate for such a role.

2. MATERIALS AND METHODS

2.1. Bioinformatics Tools and Databases. The analyzed microbial genomes were downloaded from GenBank[24] and from the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (https://www.bv-brc.org/), formerly known as PATRIC.[25] The gene neighborhood and distribution analysis, functional gene assignments, and metabolic subsystem analysis for thiamin metabolism genes were performed using the SEED database and web tools.[16] The thiamin biosynthesis subsystem in SEED was adapted from the previously developed subsystem for 2228 microbial genomes representing the human gut microbiome[26] and was further enriched by additional genomes encoding homologues of the *oarX* and *fmnO* genes from the BV-BRC genomic database. Orthologs were identified as bidirectional best hits using protein BLAST. The analyzed functional roles of known and predicted thiamin

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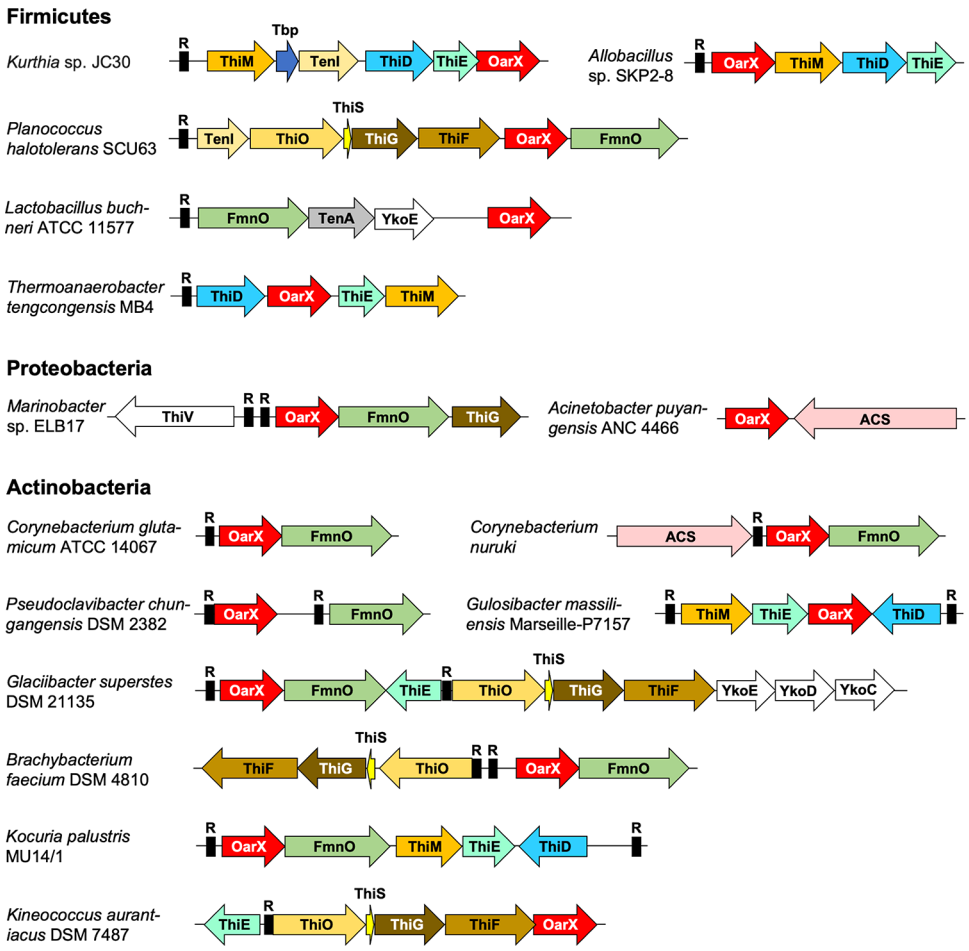

Figure 2. Examples of chromosomal clustering of genes encoding OarX, FmnO, and acyl-CoA synthetase (ACS) with a thiamin pyrophosphate riboswitch (R) and thiamin synthesis, salvage, and transport genes. ThiD, hydroxymethylpyrimidine kinase/phosphomethylpyrimidine kinase; ThiE, thiamin phosphate synthase; ThiF, ThiS adenylyltransferase; ThiG, thiazole synthase; ThiM, HET kinase; ThiO, glycine oxidase; ThiS, sulfur carrier protein; ThiV, predicted thiamin precursor transporter; YkoCDE, ECF thiamin transporter components; TenA, thiamin pyrimidine moiety salvage enzyme; TenI, thiazole tautomerase; and Tbp, thiamin-binding protein.

metabolism enzymes and uptake transporters are summarized in Supplementary Table 1, which also includes amino acid sequences of the identified OarX and FmnO proteins. The taxonomic distribution of TPP riboswitches was taken from the RegPrecise database (https://regprecise.lbl.gov/)[27] for the RF00059 family as described in the comparative genomics study of riboswitch regulons in bacterial genomes.[28] Additional TPP riboswitch sequences in target genomes were predicted using Riboswitch Scanner.[29] Phylogenetic trees were constructed with MEGA X[30] and the Robust Phylogenetic Analysis tool (https://www.phylogeny.fr/).[31]

2.2. *C. glutamicum* Gene Knockouts. *Corynebacterium glutamicum* ATCC 14067 (DSM 20411) was obtained from DSMZ (Braunschweig, Germany). Knockouts were obtained via gene displacement using pk18sB[32] obtained from Addgene. Primers were designed based on the ATCC 14067 genome sequence (GenBank Accession NZ_CP022614.1).[33] Molecular manipulations followed standard protocols[34] or kit manufacturers' instructions. Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA) was used to amplify DNA sequences in a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Primer sequences are listed in Supplementary Table 2. PCR products were gel-purified using GeneJET Gel Extraction Kits (Thermo Fisher Scientific, Waltham, MA) and assembled using NEBuilder HiFi DNA Assembly or KLD Enzyme Mix (New England Biolabs, Ipswich, MA). Assembly products were inserted into *Escherichia coli* Top10 via electroporation using an *E. coli* Pulser apparatus (Bio-Rad Laboratories, Hercules, CA). Candidate clones (kanamycin-resistant) were then screened by PCR to select those with successful ligation. Recombinant plasmids were purified using GeneJET Plasmid Miniprep Kits (Thermo Fisher Scientific, Waltham, MA) and sequence-verified. pK18sB harboring *C. glutamicum* ATCC 14067 *thiG* or *oarX-fmnO* deletion cassettes containing *thiG* or *oarX-fmnO* 800-bp flanking sequences (homology arms) were transformed into *C. glutamicum* as described.[35] Briefly, *C. glutamicum* was grown overnight on BHI medium and transferred to modified Epo medium, starting OD_{600} = 0.3. When the culture reached OD_{600} = 1.0, it was cooled on ice for 15 min, then cells were harvested by centrifugation (4 °C, 4000 × *g*, 10 min), washed four times with ice-cold 10% glycerol (w/v), and resuspended in 0.4% of the initial volume of 10% glycerol. For electroporation, 80 μL of cell suspension were mixed with ∼2000 ng of plasmid DNA and given a single pulse at 12.5 kV/cm for 3−5 ms. Cells were resuspended in 915 μL of BHIS medium, incubated at 46 °C for 6 min, recovered for 2 h at 30 °C with shaking at 250 rpm, and plated on LBHIS medium containing

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10 μg/mL kanamycin. The plates were incubated at 30 °C until colonies appeared (up to 10 days). Following sucrose selection (four passages on BHI + 10% sucrose), single colonies were analyzed by colony PCR to identify those with successful recombination.

2.3. Chemicals. Thiamin-related compounds were obtained from the following sources: thiamin, Sigma-Aldrich (St. Louis, MO); HMP, Ark Pharm Inc. (Arlington Heights, IL); HET, Sigma-Aldrich; PCA, AstaTech Inc. (Bristol, PA), MTA, AstaTech Inc.; oxo-thiamin (Toronto Research Chemicals, North York, ON, Canada); oxo-HET, Toronto Research Chemicals; thiamin acetic acid (hydrobromide bromide form), Enamine Ltd. (Kyiv, Ukraine). Tests confirmed that bromide ions did not affect growth at the maximum concentration (2 μM) present in the medium in experiments with thiamin acetic acid. Aqueous stock solutions of thiamin-related compounds (10−100 μM) were filter-sterilized and added to media to give the concentrations indicated in the text.

2.4. Growth Media and Culture Conditions. *E. coli* cultures were grown at 37 °C with shaking at 250 rpm in LB medium (composition in g/L: Tryptone, 10; yeast extract, 5; NaCl, 10). *C. glutamicum* precultures were grown at 30 °C with shaking at 250 rpm in BHI medium (BD, Franklin Lakes, NJ). Competent cells were prepared using modified Epo medium (composition in g/L: Tryptone, 10; yeast extract, 5; NaCl, 10; glycine, 25; Tween 80, 0.1%).[36] Cells were recovered after transformation in BHIS medium (BHI, 18.5 g/L, sorbitol, 91 g/L). Recombinants were selected on LBHIS agar (composition in g/L: Tryptone, 5; NaCl, 5; yeast extract, 2.5; BHI, 18.5; sorbitol, 91; agar, 18; pH 7.2). Growth experiments were conducted on CGXII medium, modified from the literature,[36] with the following composition in g/L: $(NH_4)_2SO_4$, 20; urea, 5; KH_2PO_4, 1; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.25; MOPS, 42; glucose, 40; $CaCl_2$, 0.013; biotin, 0.0002, $FeSO_4$, 0.001; and trace elements solution.[37]

3. RESULTS AND DISCUSSION

3.1. Sequence-Based Analyses of OarX and FmnO Proteins. Sequence-based analyses reinforced the original inference[4] that OarX proteins differ in function from FabG since they occur in genomes that encode a canonical FabG, share only ∼30% sequence identity and ∼50% similarity with FabG proteins, and belong to a different phylogenetic clade (Supplementary Figure 1). Nothing beyond membership of a short-chain dehydrogenase/reductase subgroup that includes FabG and other carbonyl reductases could be gleaned from OarX amino acid sequences. Similarly for FmnO, sequence and phylogenetic analysis of FmnO proteins and FMN-dependent monooxygenases with known activities indicated only that FmnOs share at most ∼45% sequence identity with other FMN-dependent monooxygenase family members and that they form a separate clade (Supplementary Figure 2).

3.2. Comparative Genomics Analysis of *oarX* and *fmnO* Genes. We analyzed the distribution and genomic neighborhood of thiamin metabolism genes in bacterial genomes possessing orthologs of the *oarX* and/or *fmnO* genes using the SEED and BV-BRC databases.[16,25] Additionally, we identified TPP riboswitches in regulatory regions of thiamin metabolism genes using the RegPrecise resource.[27] We found *oarX* genes in 87 genomes representing the phyla Firmicutes (46 genomes), Actinobacteria (22 genomes), and Proteobacteria (19 genomes); we found *fmnO* genes only in a subset of 22 bacteria from these phyla, always clustered on the chromosome with *oarX* (Supplementary Table 1).

Genomics-based reconstruction of thiamin metabolic pathways showed that *oarX* genes are frequently preceded by a TPP riboswitch and clustered with thiamin synthesis, salvage, or transport genes (Figure 2 and Supplementary Table 1). In particular, *oarX* is clustered with one or more de novo thiazole synthesis genes (*thiOSGF* and *tenI*) in four actinobacterial genomes, in the Firmicutes *Planococcus halotolerans* and *Kurthia* sp. JC30, and in the Proteobacterium *Marinobacter* sp. ELB17, with the thiamin/HMP transporter gene *thiV*[38] in four proteobacterial genomes, and with *ykoCDE* thiamin transporter genes[39] in two genomes. In addition, *oarX* genes cluster with the thiazole salvage gene *thiM* in five genomes, with the pyrimidine salvage gene *tenA* in one genome, and with thiamin synthesis genes *thiD* and *thiE* in eight genomes. Lastly, *oarX* genes occasionally cluster with genes encoding acyl-CoA synthetase, e.g., in *Acinetobacter puyangensis* ANC 4466 and *Corynebacterium nuruki* (Figure 2).

By analyzing the genomic distribution of thiamin synthesis and transport genes, we assigned requirements for thiamin or for its HET or HMP precursors (see "Auxotrophy" column in Supplementary Table 1) and capacities to take up these molecules. Of the 87 OarX-encoding genomes, all encode ThiE and so can synthesize thiamin from its HET and HMP moieties. Of the 87 genomes, 45 are predicted prototrophs that require no supplied precursors, while the other 42 are predicted auxotrophs that require HET and/or HMP. Most of the genomes (77 out of 87) are predicted to encode transporters of thiamin, HET, or HMP that may also transport derivatives of these compounds.[10,40]

This comparative genomic evidence points to four deductions about the functions of OarX and FmnO. First, the clustering and/or co-occurrence of *oarX* with thiamin, thiazole, and pyrimidine transporter genes and with the core thiamin synthesis gene *thiE* is consistent with a role for OarX in salvaging a thiamin-related breakdown product that can be taken up and reused for thiamin synthesis. Second, that *oarX* clusters with genes for synthesis and salvage of the thiazole moiety (*thiOSGF*, *tenI*, and *thiM*) and for transport of thiamin (*thiV*, *ykoCDE*) suggests that OarX acts on a thiazole or thiamin derivative. Third, the clustering of *oarX* with acyl-CoA synthetase genes, although weaker than other associations, is suggestive because CoA thioester formation is a common activation step in carboxyl reduction pathways.[22] This clustering thus suggests that OarX might catalyze reduction of the CoA thioester derivative of thiamin acetic acid, MTA, or PCA. Other members of the short-chain dehydrogenase-reductase family carry out such a reduction on a fatty acyl-CoA substrate.[41,42] Fourth, that *oarX* occurs in many genomes that lack *fmnO* and—among the genomes analyzed—that *fmnO* is always in an operonic arrangement with *oarX* implies that the function of OarX does not depend on FmnO, although that of FmnO could depend on OarX.

These deductions prompted genetic tests in *C. glutamicum* for roles for OarX and FmnO in salvage of PCA, MTA, oxo-HET, thiamin acetic acid, or oxo-thiamin. For these tests, the *oarX-fmnO* operon was deleted as a unit for the sake of efficiency. We reasoned that if knocking out both genes does not give a phenotype, this excludes a role for either gene and that if it does give a phenotype, this would warrant making single knockouts to dissect which gene is responsible. We tested all compounds at the physiological concentrations

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appropriate for thiamin and its precursors (≤1 μM);[10,38] concentrations higher than this are most unlikely to occur in natural environments.[43]

3.3. Evidence That OarX and FmnO Do Not Salvage PCA. We first tested whether OarX and FmnO are needed to salvage the pyrimidine carboxylic acid PCA, which seemed relatively unlikely given the association of *oarX* and *fmnO* with thiazole synthesis and salvage genes (see above). We used *C. glutamicum* ATCC 14067, which has a full set of thiamin synthesis genes except *thiC*[33] and consequently requires HMP (or thiamin) for growth.[44] We simply compared the ability of various PCA concentrations to support the growth of ATCC 14067 with that of HMP or thiamin (Figure 3A). No concentration of PCA allowed growth, meaning that neither OarX nor FmnO enabled its salvage. Nor are OarX and FmnO likely to detoxify PCA, as there is no indication that PCA is toxic (Supplementary Figure 3).[45,46]

3.4. Evidence That OarX and FmnO Do Not Salvage MTA, Oxo-HET, Oxo-thiamin, or Thiamin Acetic Acid. To test for roles of OarX and FmnO in salvage of thiamin or thiazole oxidation products, we deleted *thiG* in *C. glutamicum* ATCC 14067 to create a thiazole auxotroph (i.e., to force dependence on an external source of thiazole) and attempted to rescue the deletant by supplying 100 nM or 1 μM MTA, oxo-HET, oxo-thiamin, or thiamin acetic acid using HET and thiamin as benchmarks. HET or thiamin supported growth as expected, but the other compounds did not (Figure 3B–E); the slight growth at the highest concentration of oxo-HET could be due to trace contamination with HET. Tests with wild-type*C. glutamicum*ATCC 14067 showed that, at 100 nM or 1 μM, none of the thiamin degradation products inhibited growth except for a minor transient effect of oxo-HET (Supplementary Figure 3). These results exclude roles for OarX and FnmO in salvaging MTA, oxo-HET, oxo-thiamin, or thiamin acetic acid.

The above data left open the possibility that OarX and FmnO have an unrecognized accessory (i.e., nonessential) role in salvaging thiamin itself or its physiological precursors HMP or HET, or in detoxifying an unidentified product of thiamin breakdown. We therefore created a triple Δ*thiG* Δ*oarX*–*fmnO* *C. glutamicum* ATCC 14067 knockout strain and compared its responses to the normally used concentrations of thiamin, HMP, and HET with those of the Δ*thiG* single knockout. There were no significant differences between the single and triple knockout strains (Supplementary Figure 4), which makes a cryptic role in salvage or detoxification unlikely.

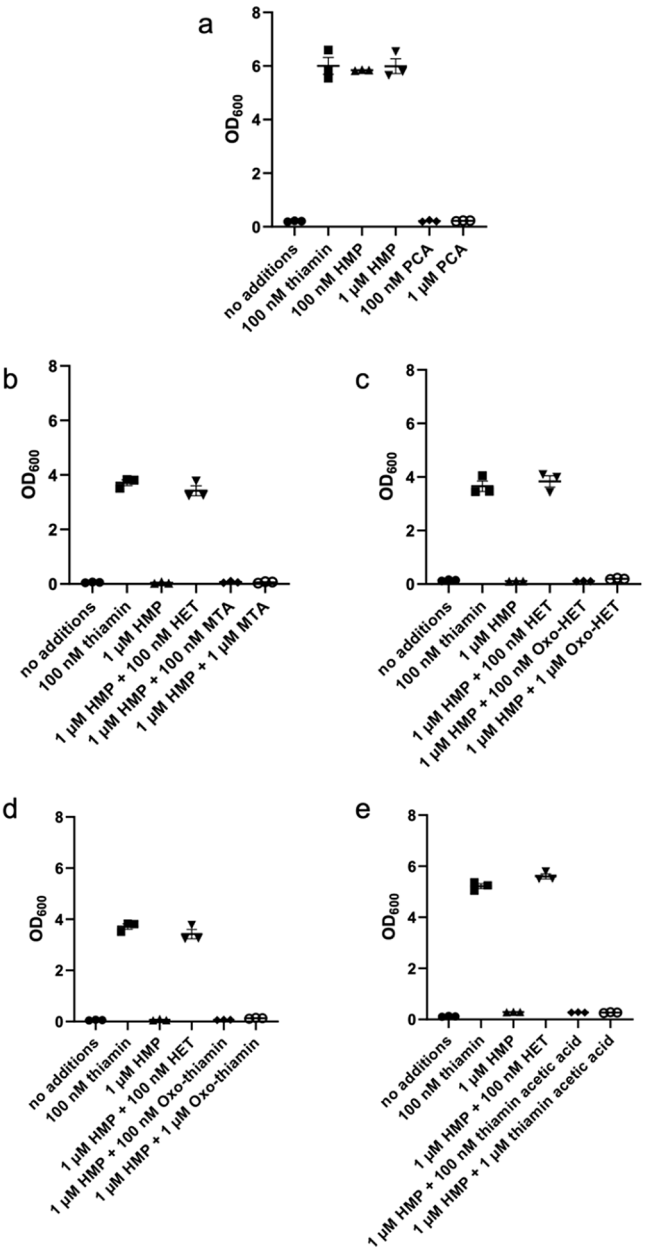

Figure 3. Growth responses of the *C. glutamicum* ATCC 14067 wild-type strain or its Δ*thiG* mutant to oxidative degradation products of thiamin. Growth was measured after 24 h. The corresponding physiological precursor (HMP or HET) and thiamin served as benchmarks. (a) Response of the wild-type strain to PCA. (b) Response of the Δ*thiG* strain to MTA. (c) Response of the Δ*thiG* strain to oxo-HET. (d) Response of the Δ*thiG* strain to oxo-thiamin. (e) Response of the Δ*thiG* strain to thiamin acetic acid. Data are from three independent cultures. Mean values are shown by horizontal bars (±standard error of the mean).

4. CONCLUSIONS

Our comparative genomic analysis, based on ∼600× more genomes than were available in 2002[47] when the OarX-thiamin association was first flagged,[4] greatly strengthens the case for this association and for its being functional rather than fortuitous. The fact that (i) OarX belongs to a subgroup of short-chain dehydrogenase/reductases whose members typically mediate carbonyl reduction reactions and (ii) carboxylate and thiazolone oxidation products of thiamin are prevalent favored the simple hypothesis that OarX mediates a reductive step in the salvage of one or more of these products. Our genetic data invalidate this hypothesis with respect to five known oxidation products. These negative results are important to document because the products that we tested are obvious ones that anyone probing the function of OarX would be likely to test (fruitlessly, in fact). The hypothesis of a salvage role for OarX, and possibly also for FmnO, nevertheless still stands because the thiazole and pyrimidine moieties of thiamin and thiamin itself can carry other modifications besides carboxyl or oxo groups,[7] i.e., the substrate for OarX could be a derivative of MTA, oxo-HET, thiamin acetic acid, oxo-thiamin, or PCA, not one of these compounds themselves. Future deep characterization of thiamin breakdown products by modern mass spectrometric methods (as opposed to the classical radiochemical methods used previously) could show

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whether such derivatives are formed in sufficient amounts to make them plausible candidates for salvage by OarX.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c03514.

Phylogenetic tree of representative OarX and FabG proteins; phylogenetic tree of representative FmnO proteins and FMN-dependent monooxygenase family proteins with known enzymatic activities; growth responses of *C. glutamicum* ATCC 14067 to HMP only (no additions) or HMP + thiamin or thiazole oxidation products; and growth responses of the *C. glutamicum* ATCC 14067 Δ*thiG* and Δ*thiG* Δ*oarX-fmnO* mutants to thiamin or its physiological precursors (HMP + HET) (PDF)

Thiamin metabolism subsystem in OarX-encoding bacterial genomes (XLSX)

Primer sequences used in this work (XLSX)

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Author Contributions

E.R.O.-F.: conceptualization, methodology, investigation, visualization, and writing—original draft. D.A.R.: formal analysis, data curation, and writing—review and editing. A.D.H.: conceptualization, writing—original draft, funding acquisition, and project administration.

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Notes

The authors declare no competing financial interest.

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