Biochemical and Biophysical Research Communications 450 (2014) 679–684

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Promiscuity of *Exiguobacterium* sp. AT1b *o*-succinylbenzoate synthase illustrates evolutionary transitions in the OSBS family

Ashley M. Brizendine [a,1], Denis Odokonyero [a,1], Andrew W. McMillan [a], Mingzhao Zhu [b], Ken Hull [b], Daniel Romo [b,c], Margaret E. Glasner [a,*]

[a] Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College Station, TX 77843-2128, United States
[b] Natural Products LINCHPIN Laboratory, Texas A&M University, 3255 TAMU, College Station, TX 77840, United States
[c] Department of Chemistry, Texas A&M University, 3255 TAMU, College Station, TX 77840, United States

ARTICLE INFO

Article history:
Received 2 June 2014
Available online 14 June 2014

Keywords:
Catalytic promiscuity
Protein evolution
Enzymes

ABSTRACT

Catalytic promiscuity, which is the ability to catalyze more than one reaction in the same active site, is thought to facilitate the evolution of new protein functions. Although many enzymes are catalytically promiscuous, there is little direct evidence to show how promiscuous activities evolved into biological functions. We are seeking evidence for this model by studying the *o*-succinylbenzoate synthase (OSBS) family. Most enzymes within this family only catalyze OSBS, which is a step in menaquinone synthesis. However, several characterized enzymes in one branch of the family (called the NSAR/OSBS subfamily) efficiently catalyze both OSBS and *N*-succinylamino acid racemization (NSAR). Based on genome context, NSAR appears to be the only biological function of some characterized NSAR/OSBS enzymes, while both activities are biologically relevant in others. The promiscuity model predicts that these enzymes evolved from an ancestral OSBS which promiscuously catalyzed NSAR as a side reaction that was not biologically relevant. If so, the model predicts that some extant OSBS enzymes would have low levels of promiscuous NSAR activity. This manuscript describes such an enzyme from *Exiguobacterium* sp. AT1b (ExiOSBS). We show that ExiOSBS efficiently catalyzes OSBS ($k_{cat}/K_M = 2.6 \times 10^6\ M^{-1}\ s^{-1}$), but its efficiency for the NSAR reaction is only 41 $M^{-1}\ s^{-1}$. Moreover, genome context indicates that OSBS is the only biologically relevant activity. ExiOSBS diverged from the NSAR/OSBS subfamily before NSAR emerged as a biologically relevant activity. These results provide evidence that NSAR activity originated as a promiscuous activity in an ancestor of the NSAR/OSBS subfamily.

1. Introduction

The manner in which enzymes evolve new functions is not well understood. This poses a challenge when using protein sequence and structure to predict function or when engineering proteins to catalyze novel reactions. Gene duplication followed by divergence is often proposed as a mechanism for generating new protein functions [1]. A fundamental problem with this model is that evolutionary intermediates are nonfunctional, so deleterious mutations would be much more frequent than the rare mutations that confer new activities [2].

An alternative model is that protein evolution occurs through promiscuous intermediates (Fig. 1) [2–6]. Promiscuous enzymes catalyze more than one reaction using the same active site. In this model, the ancestral enzyme has a single biological function but fortuitously catalyzes a promiscuous side reaction that does not serve a biological function. Typically, promiscuous activities are catalyzed at much lower efficiency than biologically relevant activities [7–10]. If the environment of the organism changes so that the promiscuous side reaction confers a selective advantage, mutations that increase the efficiency of the promiscuous activity would be selected, yielding a bifunctional enzyme that has two biologically relevant activities. If gene duplication occurs, mutations that enhance one activity while interfering with the other can accumulate in each copy. Subfunctionalization in this manner would yield paralogs that have different biological functions.

Abbreviations: OSBS, *o*-succinylbenzoate synthase; NSAR, *N*-succinylamino acid racemase; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; PMSF, phenylmethylsulfonyl fluoride.

* Corresponding author.
E-mail addresses: ashleybrizendine@neo.tamu.edu (A.M. Brizendine), denis.odokos@gmail.com (D. Odokonyero), amcmillan@tamu.edu (A.W. McMillan), mingzhao.zhu@mail.chem.tamu.edu (M. Zhu), ken.hull@chem.tamu.edu (K. Hull), romo@chem.tamu.edu (D. Romo), margy.glasner@tamu.edu (M.E. Glasner).

[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.bbrc.2014.06.034
0006-291X/

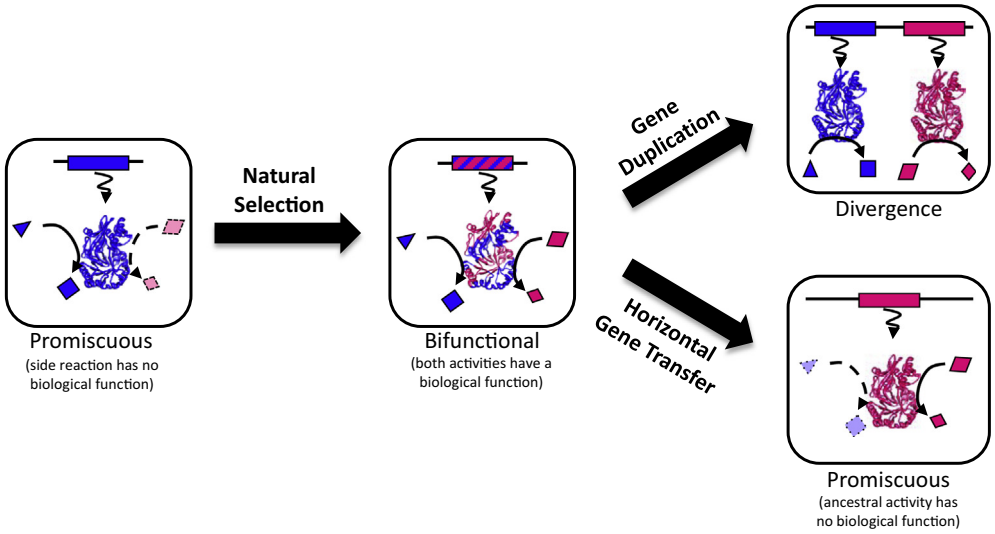

Fig. 1. Evolution of new protein functions through promiscuous intermediates. Promiscuous, non-biological reactions (faded pink) of an enzyme (blue) can be enhanced by natural selection if the promiscuous activity confers a selective advantage. This would result in a bifunctional enzyme (pink and blue striped). If gene duplication occurs, each activity could be optimized separately (subfunctionalization), yielding paralogs that have the original function (blue) or the new function (pink). Alternatively, horizontal transfer of the bifunctional gene into a species that does not require the original activity would yield a protein that has the new function (pink), which could promiscuously catalyze the original function (faded blue), as observed in the NSAR/OSBS subfamily.

Much of the evidence supporting this model is anecdotal. Many, if not most proteins catalyze promiscuous side reactions [9,10]. In addition, protein engineering typically works best if the template protein promiscuously catalyzes the target reaction, and the products of protein engineering usually catalyze both the target reaction and their ancestral reaction [11–14]. What has been lacking is a clear demonstration of an evolutionary trajectory of a natural protein from a promiscuous ancestor to an enzyme that has a novel biological function.

To discover direct evidence for this model, we are studying the *o*-succinylbenzoate synthase (OSBS) family. Most enzymes within this family only catalyze OSB synthesis, which is required for menaquinone (Vitamin K) synthesis [15,16]. However, many enzymes in one branch of the family (the NSAR/OSBS subfamily) catalyze both OSB synthesis and *N*-succinylamino acid racemization (NSAR; Fig. 2) [17–19]. In *Geobacillus kaustophilus*, NSAR activity is used to perform the racemization step in a pathway that irreversibly converts D-amino acids to L-amino acids [18]. Although OSBS and NSAR are very different chemical reactions (dehydration and racemization, respectively), they proceed through a common enolate anion intermediate. Similarities in their reaction mechanisms only partially explain how NSAR activity evolved from an OSBS ancestor, because the catalytic residues required for both reactions are also conserved in non-promiscuous OSB synthases and other related enzymes [15,20].

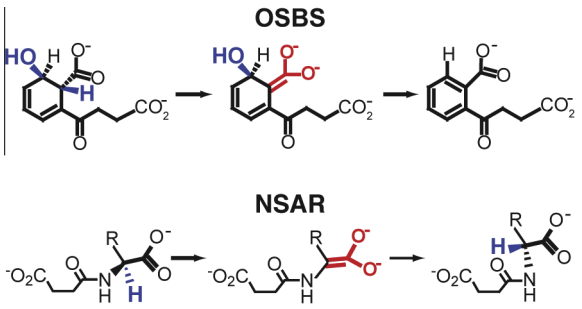

Fig. 2. Reactions catalyzed by promiscuous NSAR/OSBS enzymes. Structural similarities of the intermediates are red; blue atoms are lost or rearranged in the reactions. R = hydrophobic amino acid side chain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Five experimentally characterized proteins in the NSAR/OSBS subfamily catalyze both OSBS and NSAR reactions efficiently ($k_{cat}/K_M \sim 10^3$–10^5 M^{-1} s^{-1}), as expected if both activities are biologically relevant [18,19,21]. A sixth enzyme, *Bacillus subtilis* OSBS, lacks NSAR activity with *N*-acetylmethionine [17]. However, this is a poor substrate for the other enzymes that have NSAR activity; the catalytic efficiency using *N*-succinylphenylglycine is up to 1000-fold higher, so it is possible that *B. subtilis* OSBS could exhibit some NSAR activity with this substrate.

Biological functions of proteins in the NSAR/OSBS subfamily can be predicted from genome context. *B. subtilis* OSBS and many other subfamily members from the phylum Firmicutes are encoded in menaquinone synthesis operons, suggesting that OSBS is their only biological function [15,17]. In contrast, the NSAR/OSBS from *G. kaustophilus* is bifunctional, because both activities are required [18]. It catalyzes both reactions efficiently, and it has an operon encoding menaquinone synthesis genes. However, the NSAR/OSBS is encoded in a 3-gene operon encoding the D-amino acid conversion pathway. Many other members of the NSAR/OSBS subfamily are found in species that do not require OSBS activity to make menaquinone, suggesting that NSAR activity is their only biological function [15]. In addition, most of these species obtained the NSAR/OSBS gene by lateral gene transfer (Fig. 1) [15]. Thus, the NSAR/OSBS family provides examples that support two of the stages in the promiscuity model in Fig. 1: the bifunctional intermediate and a later stage in which only the newly evolved function is biologically relevant. In the NSAR/OSBS subfamily, ancestral OSBS activity is retained at this latter stage as a promiscuous activity.

To date, an example of the first stage – an OSBS with promiscuous NSAR activity – has not been identified. Finding such a protein would provide essential support for the promiscuity model, and it would also provide a point of comparison for determining what amino acid substitutions were required for evolving NSAR activity. Here, we report the biochemical characterization of such an enzyme, the OSBS from *Exiguobacterium* sp. AT1b (ExiOSBS). Our results show that this enzyme, which is on one of the earliest diverging branches of the NSAR/OSBS subfamily phylogeny, is an efficient OSBS but has very little NSAR activity. This supports the

idea that NSAR activity originated as a promiscuous activity in an ancestral OSBS that predates the divergence of ExiOSBS from the rest of the NSAR/OSBS subfamily.

2. Materials and methods

2.1. Cloning the Exiguobacterium sp. AT1b OSBS gene

The gene encoding ExiOSBS (gi 229918090) was obtained by gene synthesis with codon optimization for expression in *Escherichia coli* (GenScript, USA Inc) and cloned into a pUC57 vector. It was subsequently subcloned into a modified pET-21a expression vector, variant pMCSG7, with an N-terminal His_6-tag via ligation independent cloning [22].

2.2. Protein expression and purification

The expression plasmid encoding ExiOSBS was transformed into a *menC*$^-$ *E. coli* BW25113 strain (*menC*::*kan*, DE3) to ensure that the purified protein would not be contaminated with the native *E. coli* OSBS [23]. A single colony was used to inoculate 1.5 l of LB containing carbenicillin and kanamycin and incubated overnight at 37 °C.

After pelleting the culture, the cells were resuspended in a buffer containing 10 mM Tris pH 8.0, 5 mM $MgCl_2$, 500 mM NaCl, 5 mM imidazole, 0.02 mg/ml DNAse (Worthington), and 2 μM phenylmethylsulfonyl fluoride (PMSF; Thermo Scientific). The cells were lysed by sonication, and the cell debris was removed by centrifugation and filtration using a 0.22 μm Steriflip filter (Millipore). ExiOSBS was purified from the supernatant on a HisTrap FF column (GE) by elution with a step gradient of 10% elution buffer composed of 5 mM $MgCl_2$, 500 mM imidazole, 500 mM NaCl, and 10 mM Tris pH 8.0 for 7 column volumes, followed by a linear gradient of 10–100% elution buffer over 20 column volumes. Fractions containing ExiOSBS were pooled, concentrated and exchanged into storage buffer containing 10 mM Tris pH 8.0 and 5 mM $MgCl_2$ (Sartorius Stedim Biotech). Glycerol was added to a final concentration of 25%, and the purified protein was stored at −20 °C. The yield was 0.3 mg protein/g of wet cell pellet.

2.3. Kinetic assays

The substrates 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) and *N*-succinyl-L-phenylglycine were prepared as described in Refs. [23,19]. OSBS activity was measured at an enzyme concentration of 0.01 μM in 50 mM Tris pH 8.0 and 0.1 mM $MnCl_2$ at 25 °C. Enzyme activity was determined by following the disappearance of the substrate at 310 nm ($\Delta\varepsilon = -2400\ M^{-1}\ cm^{-1}$) in a SpectraMax Plus^{384} UV–VIS spectrophotometer (Molecular Devices). NSAR activity was measured at an enzyme concentration of 10 μM in 200 mM Tris pH 8.0 and 0.1 mM $MnCl_2$ at 25 °C. Activity was determined by following the change in optical rotation at 405 nm (specific rotation of 6.54 deg $M^{-1}\ cm^{-1}$) using a P2000 Polarimeter (Jasco). Both assays were performed in triplicate. Initial rates were determined by fitting the linear portion of the curve in Excel (Microsoft), and the resulting data was fit to the Michaelis–Menten equation using Kaleidagraph (Synergy Software).

2.4. Homology model construction

MODELLER was used to build a homology model of ExiOSBS using a sequence alignment of the *Amycolatopsis* sp. T-1-60 NSAR/OSBS (AmyNSAR/OSBS; PDB: 1SJB) and *D. radiodurans* R1 NSAR/OSBS (PDB: 1XPY) as the template [24–26]. These structures were used because they were solved with OSB or *N*-acetylglutamine bound, respectively. Using the resulting models, a flexible active site loop around position 20 (the 20s loop) was remodeled by generating 100 models using the DOPE loop modeling protocol [27,28]. The highest scoring model with residues 18, 20, and 22 (the loop residues that make active site contacts in the template structures) oriented towards the active site was used for docking.

2.5. Ligand docking

Docking was carried out with the Autodock Vina tool in UCSF Chimera using the Opal web service with default options [29–31]. Structures of OSB and *N*-succinyl-D-phenylglycine were taken from the crystal structures of *Amycolatopsis* NSAR/OSBS (1SJB and 1SJD, respectively). The structure of SHCHC was downloaded from the PubChem Compound Database (http://www.ncbi.nlm.nih.gov/pccompound). *N*-succinyl-D-phenylglycine was converted to *N*-succinyl-L-phenylglycine using Avogadro [32]. Docking was performed using a box with the dimensions 17 Å × 17 Å × 25 Å centered in the active site. The lowest energy models with the ligand in a similar orientation to that observed in 1SJB were analyzed.

3. Results

3.1. Biochemical activities of ExiOSBS

In this report, we present the experimental characterization of the OSBS from *Exiguobacterium* sp. AT1b. ExiOSBS was chosen for this study because it is on one of the earliest diverging branches in the NSAR/OSBS subfamily phylogeny (Fig. 3A) [23]. ExiOSBS is also encoded in the menaquinone synthesis operon in this species, suggesting that OSBS is its only biological function (Fig. 3B) [33].

The codon-optimized OSBS gene from *Exiguobacterium* sp. AT1b was cloned into an expression vector containing an N-terminal His_6-tag. The protein was purified to >95% homogeneity by metal-affinity chromatography. Assaying ExiOSBS for OSBS activity determined that the k_{cat}/K_M^{OSBS} was $2.6 \times 10^6\ M^{-1}\ s^{-1}$. This is comparable to the efficiencies of other characterized OSBS enzymes (Table 1).

In contrast, NSAR activity was difficult to detect. The enzyme concentration had to be increased to 10 μM (1000-fold higher than in the OSBS assay) in order to measure activity using *N*-succinyl-L-phenylglycine. The k_{cat}/K_M^{NSAR} was only 41 $M^{-1}\ s^{-1}$. No activity was observed with *N*-succinyl-D-phenylglycine or *N*-succinyl-L-valine. This efficiency is two to four orders of magnitude lower than k_{cat}/k_M^{NSAR} of several other NSAR/OSBS subfamily members. Unlike other characterized NSAR/OSBS subfamily members, operon context suggests that OSBS is ExiOSBS's only biological function. Thus, NSAR activity is a promiscuous, non-biological activity of ExiOSBS. ExiOSBS is the first example of an OSBS with promiscuous NSAR activity in the NSAR/OSBS subfamily, representing the first evolutionary stage in the promiscuity model (Fig. 1).

3.2. Modeling substrate and product binding in ExiOSBS

To compare ligand binding in ExiOSBS with *Amycolatopsis* NSAR/OSBS (AmyNSAR/OSBS) bound to OSB or *N*-succinyl-D-phenylglycine, we constructed a homology model of the protein and used computational ligand docking to determine how the substrates and products could fit in the active site. Both OSB and SHCHC could be docked into the model in almost the same orientation as the OSB in the crystal structure of AmyNSAR/OSBS, preserving most of the same active site contacts (PDB: 1SJB) (Fig. 4A–C). Two exceptions are I18 and T132 (homologous to F19 and S135 in AmyNSAR/OSBS). In AmyNSAR/OSBS, F19 is

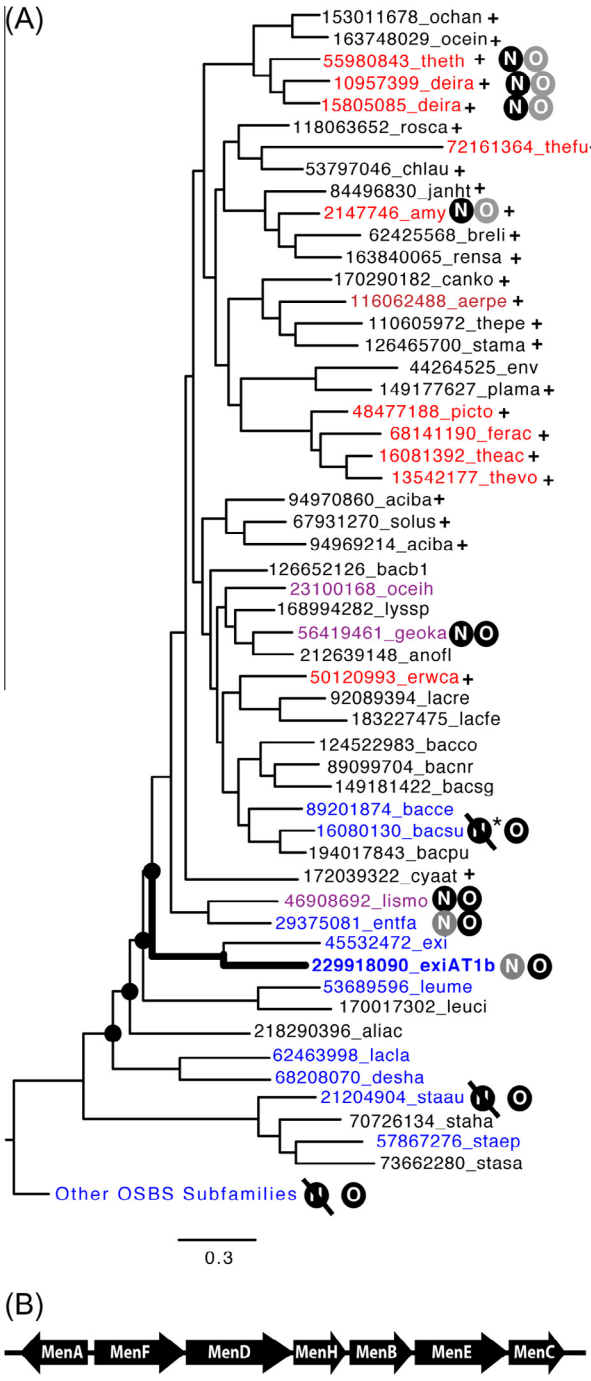

Fig. 3. Phylogenetic relationships and genome context of ExiOSBS. (A) Phylogenetic tree of the NSAR/OSBS subfamily (adapted from Ref. [23]). The location of ExiOSBS is shown with a thick branch. Biochemical activities are indicated with a circled N for NSAR and O for OSBS. Black circles indicate that the activity is predicted to be biologically relevant, and gray circles indicate that the activity is predicted to be a promiscuous, non-biological reaction, based on genome context. A line through the N indicates that NSAR activity was not detected using *N*-succinyl-L-phenylglycine, except for *B. subtilis* OSBS, which is marked with an asterisk and was assayed with *N*-acetylmethionine [17]. Plus signs indicate horizontal gene transfer to species outside the phylum Firmicutes [15]. Colors indicate predicted biological function based on genome context (red = NSAR, purple = bifunctional for NSAR and OSBS, blue = OSBS, black = not examined), as determined in Ref. [15]. Results reported in this work suggest that NSAR activity evolved in one of the ancestral nodes marked with black circles. Proteins are labeled with their gi number and an abbreviation consisting of the first three letters of the genus and first two letters of the species, if available. "env" = metagenomic sequence. (B) Genome context of the ExiOSBS gene (*menC*). All of the genes shown encode proteins in the menaquinone synthesis pathway.

required for both OSBS and NSAR activities [34], but I18 in ExiOSBS would not be able to contact the substrate. Some of this active site volume in ExiOSBS is instead filled by Y284, which is equivalent to I293 in AmyNSAR/OSBS. S135 in AmyNSAR/OSBS forms a hydrogen bond with the carbonyl of OSB. In the model of ExiOSBS, T132 does not make this interaction, but it should be possible because rotating the Cα-Cβ bond would not introduce a clash.

L- and D-succinylphenylglycine docked in an orientation with slight differences from the *N*-succinyl-D-phenylglycine in the AmyNSAR/OSBS 1SJD crystal structure, although they still seem positioned in a way that could be feasible for catalysis. (Fig. 4D–F). The phenyl ring of *N*-succinyl-D-phenylglycine is rotated ~90 degrees relative to its position in AmyNSAR/OSBS, while the ring of *N*-succinyl-L-phenylglycine is rotated smaller amount. *N*-succinyl-L-phenylglycine is also shifted farther away from R290, resulting in a weaker interaction with the carboxyl of the succinyl group. These shifts appear to be required to avoid clashing with Y284 or F50. In AmyNSAR/OSBS the residue homologous to F50 is a more flexible methionine, which could adopt different conformations to better accommodate succinylphenylglycine, as seen in the 1SJD crystal structure. It should be noted that this crystal structure might not reflect the catalytically competent form of the protein, because it is missing the catalytic magnesium ion.

These models do not demonstrate why ExiOSBS catalyzes NSAR poorly. However, using an efficient NSAR as a template may have resulted in a distorted model. Unfortunately, using an OSBS that lacks NSAR activity is problematic, because lower sequence identity and insertions and deletions led to poorer quality homology models [35]. The changes in active site packing discussed above are unlikely to account for the lack of NSAR activity, because *Listeria innocua* and *Enterococcus faecalis* NSAR/OSBSs also have aromatic side chains at positions homologous to Y284 and F50, even though they are able to catalyze the NSAR reaction at much higher rates than we observe in ExiOSBS [19].

4. Discussion

Characterization of ExiOSBS fills an important gap in understanding the evolution of a new protein function in the OSBS family. We have now identified examples representing each stage of evolution in the promiscuity model. It should be noted that these extant proteins do not directly represent intermediates in a stepwise evolutionary pathway; that is, ExiOSBS is not the ancestor of NSAR/OSBS subfamily proteins that diverged later. Instead, properties of these extant proteins can be used to infer the functional characteristics of their ancestors. The results presented here, along with our previous work, places the origin of promiscuous NSAR activity near the base of the NSAR/OSBS subfamily tree (Fig. 3A). Most proteins near the base of the tree are encoded in menaquinone biosynthesis operons, and they are from species that are in the phylum Firmicutes. In the absence of natural selection, NSAR activity could have been sporadically gained or lost among these proteins, as might be the case for *B. subtilis* OSBS (16080130_bacsu). Later branching Firmicutes, like *G. kaustophilus*, have bifunctional NSAR/OSBS enzymes, indicating that NSAR was recruited to be a biological function after it first appeared as a promiscuous activity. Recruitment into biological pathways could have occurred more than once, since *L. innocua* NSAR/OSBS (represented by the closely related 46908692_lismo in Fig. 3A) efficiently catalyzes both activities. However, *L. innocua* does not have an operon for the D-amino acid conversion pathway like *G. kaustophilus*, and the biological relevance of its NSAR activity is unknown.

Together, the data presented here and in previous work supports the hypothesis that NSAR activity evolved through promiscuous intermediates. Knowing the biochemical activities of ExiOSBS

Table 1
Enzymatic activity of ExiOSBS and related enzymes from the NSAR/OSBS subfamily.

Species	OSBS			NSAR[a]		
	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
Staphylococcus aureus[b]	306 ± 23	287 ± 60	1.1×10^6	<0.00002	–	–
Exiguobacterium sp. AT1b	51 ± 5	20 ± 7	2.6×10^6	0.07 ± 0.006	1700 ± 500	41
Listeria innocua[b]	170 ± 10	59 ± 20	2.9×10^6	1.6 ± 0.2	640 ± 20	2.6×10^3
Amycolatopsis sp. T-1–60[c]	46 ± 5	550 ± 120	8.4×10^4	42 ± 2	1000 ± 100	4.2×10^4

[a] *N*-succinyl-L-phenylglycine was the substrate.
[b] OSBS and NSAR activities were measured in reference [19].
[c] OSBS and NSAR activities are reported in McMillan et al. [34].

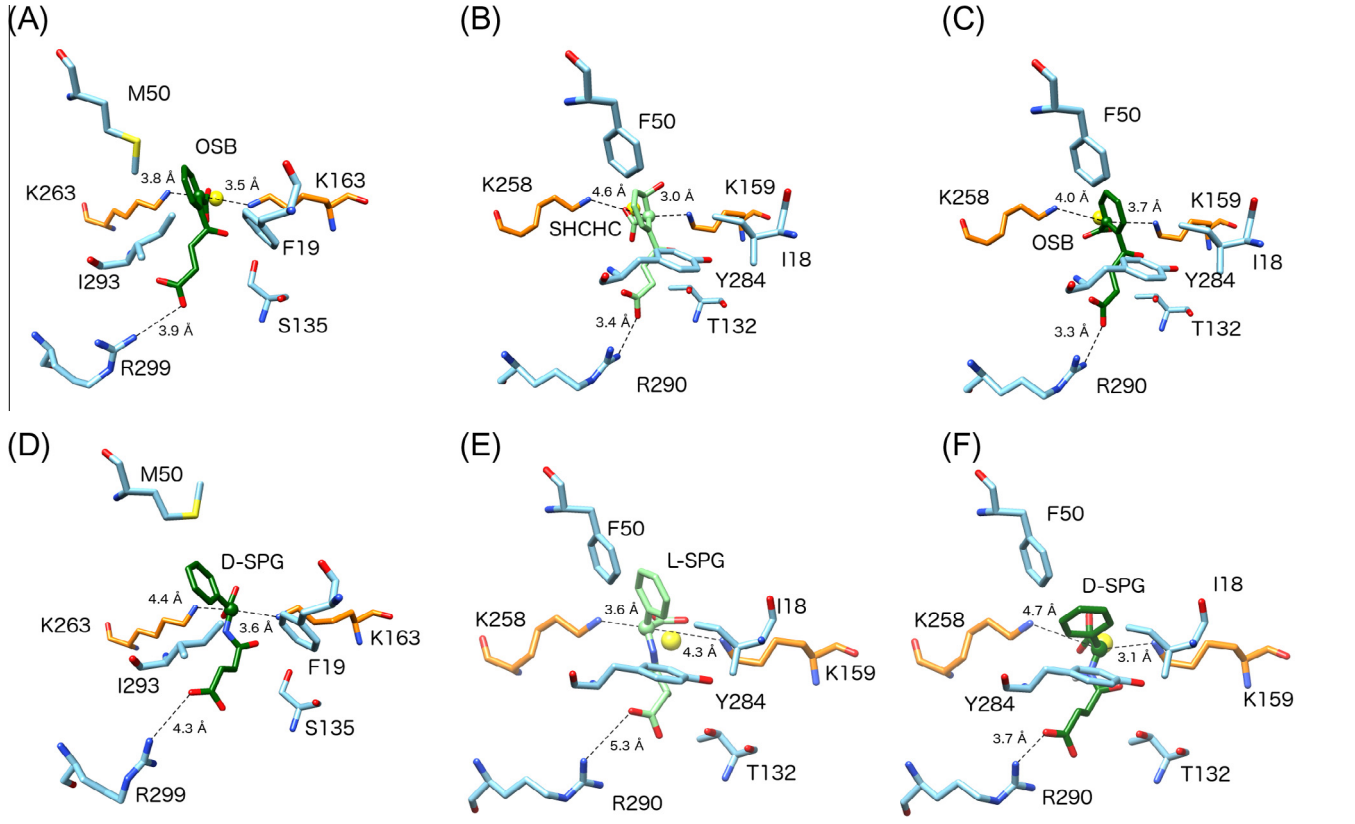

Fig. 4. Model of substrates and products docked into the ExiOSBS active site. Catalytic lysines are orange, and other active site residues are blue. Substrates of the assayed reactions are light green and the products are dark green. The magnesium that stabilizes the transition state is shown as a yellow sphere. The atom from which the proton is abstracted in both reactions is shown as a ball. Numbers indicate distances between atoms linked by dotted lines. (A) AmyNSAR/OSBS crystal structure with OSB (PDB: 1SJB) [25]. (B) ExiOSBS model with SHCHC. (C) ExiOSBS model with OSB. (D) AmyNSAR/OSBS with *N*-succinyl-D-phenylglycine (D-SPG; PDB: 1SJD) [25]. (E) ExiOSBS model with *N*-succinyl-L-phenylglycine (L-SPG). (F) ExiOSBS model with *N*-succinyl-D-phenylglycine.

is essential for future work to determine what sequence changes were required to improve the efficiency of NSAR activity in other subfamily members. Understanding how a new function evolved in the NSAR/OSBS subfamily will provide fundamental insights that will enhance protein engineering efforts and improve our ability to predict functional divergence between evolutionarily related proteins.

Acknowledgments

This work was funded by NSF CAREER Award 1253975 to M.E.G. Seed funding to the Natural Products LINCHPIN Laboratory to foster this collaboration was from the Office of the Vice President for Research, the College of Science, and the Department of Chemistry of Texas A&M University.

References

[1] S. Ohno, Evolution by Gene Duplication, Springer-Verlag, New York, 1970.
[2] A.L. Hughes, The evolution of functionally novel proteins after gene duplication, Proc. Biol. Sci. 256 (1994) 119–124.
[3] R.A. Jensen, Enzyme recruitment in evolution of new function, Annu. Rev. Microbiol. 30 (1976) 409–425.
[4] M. Ycas, On earlier states of the biochemical system, J. Theor. Biol. 44 (1974) 145–160.
[5] P.J. O'Brien, D. Herschlag, Catalytic promiscuity and the evolution of new enzymatic activities, Chem. Biol. 6 (1999) R91–R105.
[6] O. Khersonsky, D.S. Tawfik, Enzyme promiscuity: a mechanistic and evolutionary perspective, Annu. Rev. Biochem. 79 (2010) 471–505.
[7] P.J. O'Brien, D. Herschlag, Sulfatase activity of *E. coli* alkaline phosphatase demonstrates a functional link to arylsulfatases, an evolutionarily related enzyme family, J. Am. Chem. Soc. 120 (1998) 12369–12370.
[8] S.C. Wang, W.H. Johnson Jr., C.P. Whitman, The 4-oxalocrotonate tautomerase- and YwhB-catalyzed hydration of 3E-haloacrylates: implications for the evolution of new enzymatic activities, J. Am. Chem. Soc. 125 (2003) 14282–14283.

[9] W.M. Patrick, E.M. Quandt, D.B. Swartzlander, I. Matsumura, Multicopy suppression underpins metabolic evolvability, Mol. Biol. Evol. 24 (2007) 2716–2722.
[10] J. Kim, J.P. Kershner, Y. Novikov, R.K. Shoemaker, S.D. Copley, Three serendipitous pathways in *E. coli* can bypass a block in pyridoxal-5'-phosphate synthesis, Mol. Syst. Biol. 6 (2010) 436.
[11] I. Matsumura, A.D. Ellington, *In vitro* evolution of beta-glucuronidase into a beta-galactosidase proceeds through non-specific intermediates, J. Mol. Biol. 305 (2001) 331–339.
[12] U.T. Bornscheuer, R.J. Kazlauskas, Catalytic promiscuity in biocatalysis: using old enzymes to form new bonds and follow new pathways, Angew. Chem. Int. Ed. Engl. 43 (2004) 6032–6040.
[13] M.E. Glasner, J.A. Gerlt, P.C. Babbitt, Mechanisms of protein evolution and their application to protein engineering, in: E.A. Toone (Ed.), Advances in Enzymology and Related Areas of Molecular Biology, Volume 75: Protein Evolution, Wiley & Sons, 2007, pp. 193–239.
[14] A. Aharoni, L. Gaidukov, O. Khersonsky, Q.G.S. Mc, C. Roodveldt, D.S. Tawfik, The 'evolvability' of promiscuous protein functions, Nat. Genet. 37 (2005) 73–76.
[15] M.E. Glasner, N. Fayazmanesh, R.A. Chiang, A. Sakai, M.P. Jacobson, J.A. Gerlt, P.C. Babbitt, Evolution of structure and function in the *o*-succinylbenzoate synthase/*N*-acylamino acid racemase family of the enolase superfamily, J. Mol. Biol. 360 (2006) 228–250.
[16] R. Meganathan, Biosynthesis of menaquinone (vitamin K_2) and ubiquinone (coenzyme Q): a perspective on enzymatic mechanisms, Vitam. Horm. 61 (2001) 173–218.
[17] D.R. Palmer, J.B. Garrett, V. Sharma, R. Meganathan, P.C. Babbitt, J.A. Gerlt, Unexpected divergence of enzyme function and sequence. "N-acylamino acid racemase" is *o*-succinylbenzoate synthase, Biochemistry 38 (1999) 4252–4258.
[18] A. Sakai, D.F. Xiang, C. Xu, L. Song, W.S. Yew, F.M. Raushel, J.A. Gerlt, Evolution of enzymatic activities in the enolase superfamily: *N*-succinylamino acid racemase and a new pathway for the irreversible conversion of D- to L-amino acids, Biochemistry 45 (2006) 4455–4462.
[19] D. Odokonyero, A. Sakai, Y. Patskovsky, V.N. Malashkevich, A.A. Fedorov, J.B. Bonanno, E.V. Fedorov, R. Toro, R. Agarwal, C. Wang, N.D.S. Ozerova, W.S. Yew, J.M. Sauder, S. Swaminathan, S.K. Burley, S.C. Almo, M.E. Glasner, Loss of quaternary structure is associated with rapid sequence divergence in the OSBS family, Proc. Natl. Acad. Sci. (2014) (Epub date May 28, 2014).
[20] J.A. Gerlt, P.C. Babbitt, M.P. Jacobson, S.C. Almo, Divergent evolution in enolase superfamily: strategies for assigning functions, J. Biol. Chem. 287 (2012) 29–34.
[21] E.A. Taylor Ringia, J.B. Garrett, J.B. Thoden, H.M. Holden, I. Rayment, J.A. Gerlt, Evolution of enzymatic activity in the enolase superfamily: functional studies of the promiscuous *o*-succinylbenzoate synthase from *Amycolatopsis*, Biochemistry 43 (2004) 224–229.
[22] W.H. Eschenfeldt, S. Lucy, C.S. Millard, A. Joachimiak, I.D. Mark, A family of LIC vectors for high-throughput cloning and purification of proteins, Methods Mol. Biol. 498 (2009) 105–115.
[23] W.W. Zhu, C. Wang, J. Jipp, L. Ferguson, S.N. Lucas, M.A. Hicks, M.E. Glasner, Residues required for activity in *Escherichia coli o*-succinylbenzoate synthase (OSBS) are not conserved in all OSBS enzymes, Biochemistry 51 (2012) 6171–6181.
[24] N. Eswar, B. Webb, M.A. Marti-Renom, M.S. Madhusudhan, D. Eramian, M.-Y. Shen, U. Pieper, A. Sali, Comparative protein structure modeling using MODELLER, Curr. Protoc. Protein Sci. (2001) (John Wiley & Sons, Inc.).
[25] J.B. Thoden, E.A. Taylor Ringia, J.B. Garrett, J.A. Gerlt, H.M. Holden, I. Rayment, Evolution of enzymatic activity in the enolase superfamily: structural studies of the promiscuous *o*-succinylbenzoate synthase from *Amycolatopsis*, Biochemistry 43 (2004) 5716–5727.
[26] W.C. Wang, W.C. Chiu, S.K. Hsu, C.L. Wu, C.Y. Chen, J.S. Liu, W.H. Hsu, Structural basis for catalytic racemization and substrate specificity of an *N*-acylamino acid racemase homologue from *Deinococcus radiodurans*, J. Mol. Biol. 342 (2004) 155–169.
[27] A. Fiser, R.K. Do, A. Sali, Modeling of loops in protein structures, Protein Sci. 9 (2000) 1753–1773.
[28] M.-Y. Shen, A. Sali, Statistical potential for assessment and prediction of protein structures, Protein Sci. 15 (2006) 2507–2524.
[29] O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, J. Comput. Chem. 31 (2010) 455–461.
[30] J.Y. Ren, N. Williams, L. Clementi, S. Krishnan, W.W. Li, Opal web services for biomedical applications, Nucleic Acids Res. 38 (2010) W724–W731.
[31] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF Chimera – a visualization system for exploratory research and analysis, J. Comput. Chem. 25 (2004) 1605–1612.
[32] M.D. Hanwell, D.E. Curtis, D.C. Lonie, T. Vandermeersch, E. Zurek, G.R. Hutchison, Avogadro: an advanced semantic chemical editor, visualization, and analysis platform, J. Cheminform. 4 (2012) 17.
[33] R. Overbeek, T. Begley, R.M. Butler, J.V. Choudhuri, H.Y. Chuang, M. Cohoon, V. de Crecy-Lagard, N. Diaz, T. Disz, R. Edwards, M. Fonstein, E.D. Frank, S. Gerdes, E.M. Glass, A. Goesmann, A. Hanson, D. Iwata-Reuyl, R. Jensen, N. Jamshidi, L. Krause, M. Kubal, N. Larsen, B. Linke, A.C. McHardy, F. Meyer, H. Neuweger, G. Olsen, R. Olson, A. Osterman, V. Portnoy, G.D. Pusch, D.A. Rodionov, C. Ruckert, J. Steiner, R. Stevens, I. Thiele, O. Vassieva, Y. Ye, O. Zagnitko, V. Vonstein, The subsystems approach to genome annotation and its use in the project to annotate, 1000 genomes, Nucleic Acids Res. 33 (2005) 5691–5702.
[34] A.W. McMillan, M.S. Lopez, M. Zhu, B.C. Morse, I.-C. Yeo, J. Amos, K. Hull, D. Romo, M.E. Glasner, Role of an active site loop in the promiscuous activities of *amycolatopsis* sp. T-1-60 NSAR/OSBS, submitted for publication.
[35] U. Pieper, B.M. Webb, D.T. Barkan, D. Schneidman-Duhovny, A. Schlessinger, H. Braberg, Z. Yang, E.C. Meng, E.F. Pettersen, C.C. Huang, R.S. Datta, P. Sampathkumar, M.S. Madhusudhan, K. Sjolander, T.E. Ferrin, S.K. Burley, A. Sali, ModBase, a database of annotated comparative protein structure models, and associated resources, Nucleic Acids Res. 39 (2011) D465–D474.