Metabolic Adaptations to Marine Environments: Molecular Diversity and Evolution of Ovothiol Biosynthesis in Bacteria

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Abstract

Ovothiols are sulfur-containing amino acids synthesized by marine invertebrates, protozoans, and bacteria. They act as pleiotropic molecules in signaling and protection against oxidative stress. The discovery of ovothiol biosynthetic enzymes, sulfoxide synthase OvoA and β-lyase OvoB, paves the way for a systematic investigation of ovothiol distribution and molecular diversification in nature. In this work, we conducted genomic and metagenomics data mining to investigate the distribution and diversification of ovothiol biosynthetic enzymes in Bacteria. We identified the bacteria endowed with this secondary metabolic pathway, described their taxonomy, habitat and biotic interactions in order to provide insight into their adaptation to specific environments. We report that OvoA and OvoB are mostly encountered in marine aerobic Proteobacteria, some of them establishing symbiotic or parasitic relationships with other organisms. We identified a horizontal gene transfer event of OvoB from Bacteroidetes living in symbiosis with Hydrozoa. Our search within the Ocean Gene Atlas revealed the occurrence of ovothiol biosynthetic genes in Proteobacteria living in a wide range of pelagic and highly oxygenated environments. Finally, we tracked the evolutionary history of ovothiol biosynthesis from marine bacteria to unicellular eukaryotes and metazoans. Our analysis provides new conceptual elements to unravel the evolutionary and ecological significance of ovothiol biosynthesis.

Key words: enzyme evolution, ovothiol, bacteria, marine environment, secondary metabolic pathways, natural products.

Significance

Ovothiols are sulfur-containing amino acids mostly present in marine organisms. The enzymes involved in ovothiol biosynthesis have been recently discovered. The origins and evolution of this secondary metabolic pathway, as well as the reasons behind its wide distribution in marine environments are largely unknown. Data mining of bacterial genomes and Ocean Gene Atlas revealed the occurrence of ovothiol biosynthetic genes mostly in aerobic Proteobacteria, including symbiotic and parasitic species. Analyses of bacterial molecular diversification prompted the identification of horizontal gene transfer event from bacterial symbionts to hydrozoan species. Our findings highlight the importance of symbiosis in contributing to the evolution of ovothiol biosynthesis from bacteria to lower eukaryotes. The ocean genes distribution reveals metabolic adaptations to photic and oxygenated pelagic environments.

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Introduction

Life began in the seas and the oceans reflect the longest evolutionary changes enabling the development of the most complex organisms from primordial cells. Oceans thus offer a unique opportunity to study the evolution of metabolic pathways. At metabolic level, the process of adaptation is related to the ability of enzymes to evolve beneficial functions and improve the organismal fitness, which is crucial to guarantee survival in an environment of changing conditions (Sambamoorthy et al. 2019). In the earliest times of biological evolution, the emergence of metabolic pathways allowed primitive microorganisms to become increasingly less dependent on exogenous sources of organic compounds (Pál et al. 2005). Subsequently, the metabolic diversification in various phylogenetic lineages has depended on a myriad of factors, including the response to environmental constrains and the adaptation to different ecological niches. This is also evident considering the secondary metabolism, which refers to specific pathways producing molecules that interfere with the biology of other organisms (e.g., environmental competitors, hosts, symbionts).

Sulfur-containing molecules are common components of cellular redox systems since ancient bacteria. Most organisms living in aerobic conditions contain up to millimolar concentrations of intracellular glutathione or other cysteine derivatives (Krauth-Siegel and Leroux 2012). The energy-dependent balance of the reduced versus oxidized thiols controls the cellular redox potential. Several species of animals, plants, fungi, and bacteria, including cyanobacteria, have developed the potential for the biosynthesis of alternative sulfur-containing amino acids, like thiohistidine derivatives, at equally high concentrations (Seebeck 2013; Castellano and Seebeck 2018). Although their biological and ecological roles remain largely unexplored, increasing evidence suggests that thiohistidine may play a key role in the protection against reactive oxygen species (Krauth-Siegel and Leroux 2012; Seebeck 2013; Castellano and Seebeck 2018; Palumbo et al. 2018). The most important representatives of this compound family are ergothioneine (*N*-α-trimethyl 2-thiohistidine), mainly produced in fungi and mycobacteria (Seebeck 2013), and ovothiol (*N*-p-methyl-5-thiohistidine), present in marine invertebrates, protozoans, and proteobacteria (fig. 1) (Ariyanayagam and Fairlamb 2001; Braunshausen and Seebeck 2011; O'Neill et al. 2015; Castellano et al. 2016; Palumbo et al. 2018). Despite structural similarity, the two compounds have different chemical properties: ergothioneine is significantly less acidic (pKa $>$ 10) and less reducing than ovothiol (pKa $=$ 1.4) (Seebeck 2013); ovothiol is much more reactive toward peroxides than ergothioneine (Holler and Hopkins 1988; Marjanovic et al. 1995). Ovothiol forms stable disulfides, whereas ergothioneine is stable in its thionic form under physiological conditions. These differences indicate that these two compounds may take part in different cellular processes, and therefore may be the result of adaptation to different ecological niches (Castellano and Seebeck 2018). Moreover, although the biological activities of ergothioneine have been widely investigated (Cumming et al. 2018; Halliwell et al. 2018), ovothiol has only recently attracted interest for its therapeutic potentials as an antiproliferative and anti-inflammatory natural product (Russo et al. 2014; Brancaccio et al. 2018, 2019; Castellano et al. 2018; Milito et al. 2019). Ovothiols can be hitherto considered as pleiotropic molecules in living organisms: they act as protective molecules against the oxidative stress produced during fertilization and larval development in sea urchins (Shapiro 1991; Castellano et al. 2016), or as a defense against the immune system of host cells activated during parasite-induced infections (Ariyanayagam and Fairlamb 2001; Krauth-Siegel and Leroux 2012); they may act as signal molecules in the pathways induced by light in microalgae (O'Neill et al. 2015; Milito et al. 2020) and as pheromones in marine worms and cone snails (Torres et al. 2021); they are secreted in the urine of cephalopods (Palumbo et al. 2018) probably as signals for animal communication.

At metabolic level, ergothioneine and ovothiol are synthesized by similar enzyme reactions. Ovothiol biosynthesis begins with the sulfoxide synthase (OvoA), which in the presence of oxygen and iron, catalyzes the formation of a carbon–sulfur bond between the sulfhydryl group of the cysteine and the C5 on the imidazole ring of histidine (Vogt et al. 2001; Braunshausen and Seebeck 2011). Subsequently, the pyridoxal phosphate (PLP)-dependent lyase (OvoB) removes the cysteinyl moiety to give 5-thiohistidine (fig. 1*A*). Then OvoA catalyzes the methylation of the imidazole ring of histidine to lead to the final product ovothiol A (1) figure 1*B* (Braunshausen and Seebeck 2011; Naowarojna et al. 2018). It is reported that, besides histidine, *Erwinia tasmaniensis* OvoA (*Et*OvoA) can accept also mono- and dimethyl-histidine to give ovothiol B (2) and ovothiol C (3) (the other two derivatives of ovothiol A; fig. 1*B*) (Song et al. 2013). In detail, ovothiol B is the major product when OvoA uses monomethylated histidine as substrate, whereas ovothiol C is the minor species, when OvoA uses dimethyl-histidine as substrate. This finding support the different occurrence of these three products in nature, ovothiol A being the most abundant (i.e., sea urchins, cephalopods, sea stars, microalgae, and proteobacteria) (Castellano and Seebeck 2018), whereas ovothiol B (so far isolated only in clams and diatoms) (Milito et al. 2020) and ovothiol C (in two species of sea urchins) are rarer than ovothiol A (Palumbo et al. 2018). On the other hand, ergothioneine biosynthesis starts by trimethylation of the α-amino group of histidine by a histidine methyltransferase (EgtD). The resulting *N*-α-trimethyl-histidine (TMH) also called hercynine, is fused to either γ-glutamyl-cysteine (in actinomycetes) or cysteine (in fungi) by the sulfoxide synthase EgtB (Seebeck 2013). The sulfoxide product is converted into ergothioneine by removal of the glutamyl and cysteinyl moieties by

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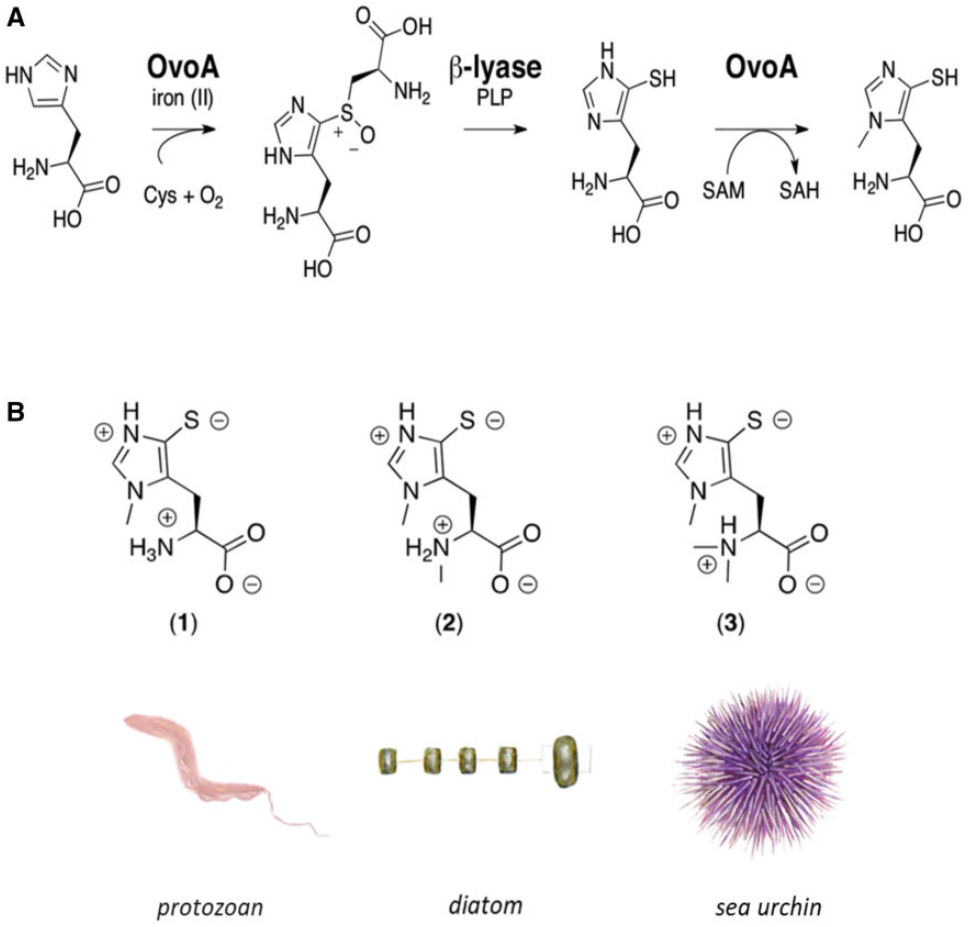

FIG. 1.—(*A*) Enzymatic reactions for ovothiol biosynthesis. Scheme of the sulfoxide synthase OvoA and *β*-lyase OvoB activities; (*B*) chemical structures of ovothiols in nature. Ovothiol A in the protozoan *Trypanosoma cruzi* (1); ovothiol B in the diatom *Skeletonema marinoi* (2); ovothiol C in the sea urchin *Strongylocentrotus purpuratus* (3).

a PLP-dependent reductive C–S lyase, named EgtE or Egt2 (Hu et al. 2014; Song et al. 2015; Irani et al. 2018). Most EgtB homologs are coencoded with or fused to EgtD (Liao and Seebeck 2017; Misson et al. 2018). Therefore, the main difference between the two sulfoxide synthases, OvoA and EgtB, is their regioselectivity: EgtB-like enzymes sulfurize the C2 of TMH to give ergothioneine, whereas OvoA-like enzymes sulfurize the C5 of histidine to give ovothiol. The two enzyme families share low sequence similarity (no more than 25%) and two structural domains: the N-terminal DinB-like domain and the formyl-glycine FGE-sulfatase domain. OvoA homologs, on the other hand, contain an additional C-terminal S-adenosyl methyl (SAM)-transferase domain that allows the final methylation of 5-thiohistidine on the imidazole ring (Braunshausen and Seebeck 2011; Naowarojna et al. 2018). Even though, these differences indicate a long evolutionary distance between EgtB- and OvoA-like enzymes, Liao et al. have recently highlighted a case of convergent evolution between OvoA and EgtB activities in a cyanobacterial OvoA able to catalyze both ovothiol and ergothioneine, depending on substrate availability of histidine or TMH (Liao and Seebeck 2017). In addition, *Et*OvoA was reported to display a broad substrate tolerance compared with EgtB (Song et al. 2013), for example, *Et*OvoA can also accept hercynine (TMH) to give ergothioneine to some extent. This feature accounts for a high promiscuity of OvoA compared with EgtB that may explain the adaptability of such an enzyme to different substrate availability and environmental niches.

Recently, OvoA orthologous have been also identified in unicellular eukaryotes like diatoms, which produce ovothiol B derivatives (figs. 1*B* and 2) (Milito et al. 2020). On the other hand, the origin of OvoA in metazoans has been traced back to an ancestral gene of the unicellular eukaryotes belonging to Choanozoa. The subsequent rapid molecular diversification of OvoA from basal phyla like Placozoa, Porifera, and Anthozoa, to protostomes and deuterostomes (Gerdol et al. 2019), has been then interrupted by several gene losses in specific phylum, like Nematoda, Arthropoda, and Vertebrata (Castellano et al. 2016; Gerdol et al. 2019). In addition, two events of horizontal gene transfer of OvoA from Bacteria and

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unicellular algae to basal metazoans (bdelloid rotifers and Hydrozoa) (Gerdol et al. 2019), might have contributed to OvoA evolution and diversification in the metazoan lineage.

In this study, we described OvoA distribution and enzyme diversification in bacteria, trying to link the evolutionary history of the ovothiol biosynthetic pathway in prokaryotes with its evolution in unicellular eukaryotes and metazoans. Moreover, we discussed molecular diversification in the light of aerobic and anaerobic conditions in which bacteria live, and we highlighted the importance of interspecific interactions like symbiosis in favoring metabolic exchanges between ovothiol-producing bacteria and the hosts in which this function was lost. Overall, the database of ovothiol biosynthetic enzyme sequences and of taxonomic and metabolic information produced in this work will provide a valuable source of concepts to shed light on the evolutionary and ecological role of this pathway and to identify the most performing enzyme in producing ovothiol for pharmacological purposes.

Results

Ovothiol Biosynthetic Genes Distribution in Bacteria

To obtain an exhaustive picture of the distribution of ovothiol biosynthetic pathway in Bacteria, we performed a sequence similarity search for OvoA- and OvoB-like enzymes in public reference genomes, using as reference sequences the characterized OvoA and OvoB proteins from *E. tasmaniensis* (*Et*OvoA and *Et*OvoB) (Braunshausen and Seebeck 2011; Naowarojna et al. 2018). We created a database with the corresponding accession numbers of OvoA and OvoB homologs supplied with information about taxonomy, habitat, metabolism, biotic interactions of bacteria featured with ovothiol biosynthetic genes (supplementary file 1, Supplementary Material online). Among these, we found (within the NCBI database) 230 full OvoA sequences belonging to different bacteria, 187 of which, besides OvoA, exhibited also OvoB-like sequences. Most of the bacteria characterized by the presence of ovothiol gene pathway belonged to the phylum Proteobacteria (96%; fig. 2*A*), followed by Bacteroidetes (3%), Cyanobacteria (1%), and Verrucomicrobia (1%). At class level, most belonged to Gammaproteobacteria (73%), followed by Betaproteobacteria (10%), Epsilonproteobacteria (6%), Deltaproteobacteria (4%), and Acidithiobacillia (2%); members of other classes included Bacteroidia, Flavobacteria, Oscillatoriophycideae, Verrucomicrobiae, and Alphaproteobacteria (1% each) (fig. 2*B*). At family level, most sequences (27%) belonged to Erwiniaceae; members of other families included Piscirickettsiaceae, Alteromonadaceae, Aeromonadaceae (6–8%). Interestingly, in some species like *Nitrosospira multiformis* (gene ID: 41370981), *Acidithiobacillus thiooxidans* (gene ID: 29720555), *Alteromonas macleodii* (gene ID: 29677280), *Alteromonas stellipolaris* (gene ID: 42913355), and *Pseudoalteromonas piscicida* (gene ID: 34018708) OvoA gene was found in cluster with an OvoB-like gene, annotated as C–S lyase or aminotransferase of class I/II, whereas in two cyanobacteria *Cylindrospermopsis raciborskii* (gene ID: 34474639) and *Microcystis aeruginosa* (gene ID: 5865063) OvoA was found in cluster with EgtD, confirming the possibility for these enzymes to accept also hercynine as substrate. Among the bacterial species analyzed, 68% were chemoorganotrophic, 24% were chemolithotrophic, 3% methylotrophic, 1% photosynthetic (fig. 2*C*); 54% exhibited an aerobic metabolism (including 11% of sulfur oxidizers and 1% of sulfur reducers), 38% exhibited a facultative anaerobic metabolism and 4% were classified as anaerobic (fig. 2*D*). Since annotations regarding anaerobicity may not be exhaustive, we performed a BlastP similarity search in the genomes annotated as belonging to anaerobic bacteria, for the presence of the cytochrome c oxidase, the enzyme responsible for the last step of aerobic respiration (Koepke et al. 2009). Although most of these genomes displayed significant gene similarity sequences for cytochrome c oxidase, only for the bacterial genomes of *Sulfurimonas hongkongensis* and *Proteiniphilum acetatigenes*, we found no significant similarity for the cytochrome c oxidase (see supplementary table, Supplementary Material online).

Regarding the environment, 43% of these bacteria were mainly found in aquatic environments, including 33% living in marine environments (2% of which in deep-sea hydrothermal vents) and 10% living in freshwater environments (including 6% isolated from lakes), 36% living exclusively in terrestrial environments, and 14% in multiple environments (fig. 2*E*). Finally, we highlighted that, although most were free living, a consistent number of prokaryotes established intimate interactions with other organisms. In detail, 34% were parasites/pathogens of plants, animal and/or humans and 12% were symbionts of marine invertebrates, plants, or insects, including species with commensal behavior (fig. 2*F* and *G*).

In addition, we retrieved a total of 96 OvoA-like proteins, characterized by the canonical three domain structure from the Ocean Gene Atlas database (see supplementary file 1, Supplementary Material online), and reflecting approximately the same taxonomic distribution.

Characterization of Bacterial OvoA Orthologous Proteins

To investigate the structural organization of the bacterial OvoA proteins, we compared the 230 bacterial sequences selected for the presence of the three canonical domains (DinB, FGE-sulfatase, and MT domain) with the previously characterized *Et*OvoA protein (fig. 3*A*). Interestingly, bacterial OvoA orthologous while exhibiting highly conserved DinB and FGE-sulfatase domain, differ in the C-terminal region for different annotated methyl-transferase SAM domains (MT11, MT12, MT25, and MT31); the most frequent being MT12, followed by MT31, MT25, and MT11. Regarding the residues

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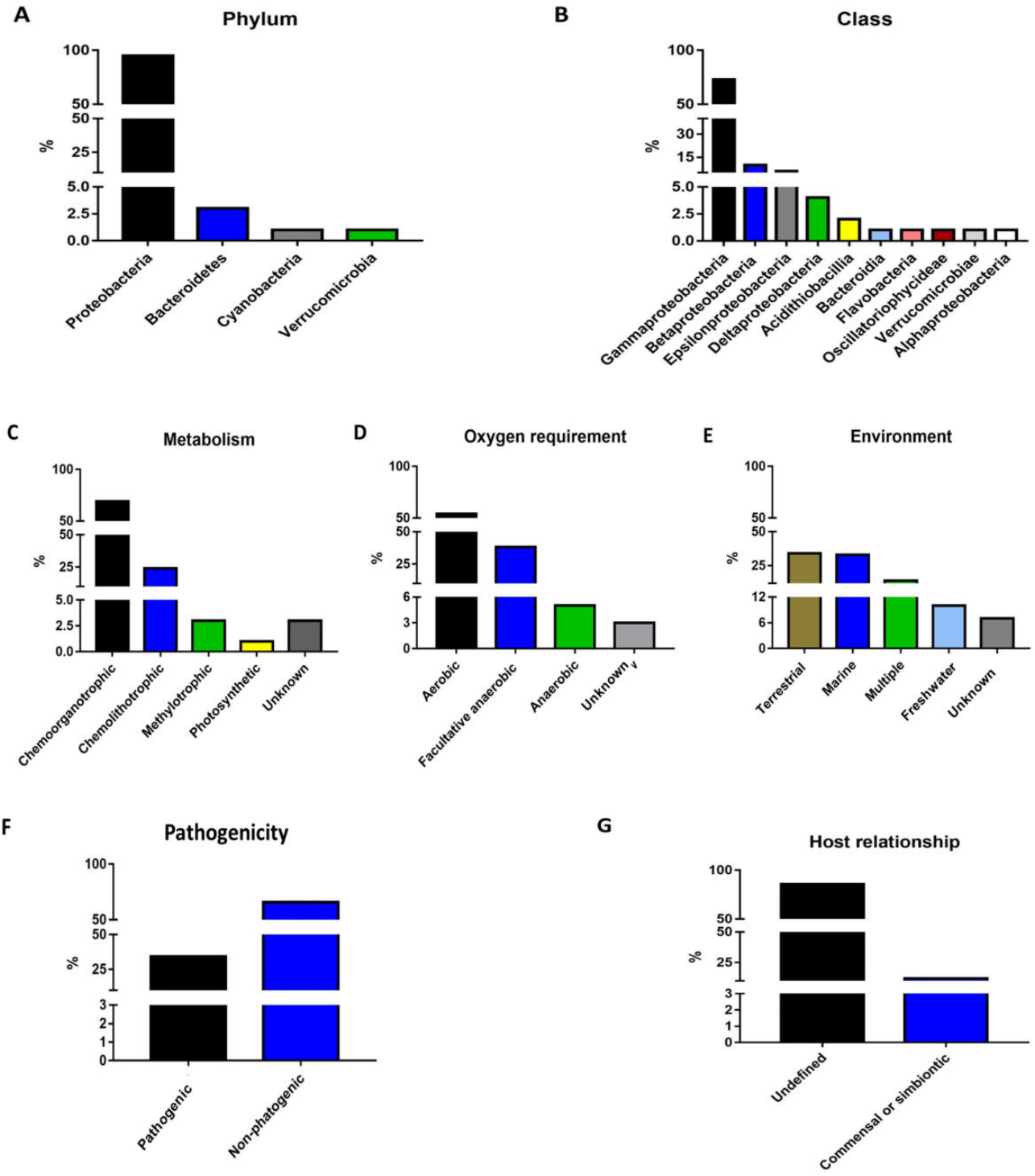

FIG. 2.—Taxonomy, habitat, metabolism, oxygen requirements, intimate relationship of bacteria with ovothiol-biosynthetic pathway. (*A*) *Phylum* classification. (*B*) *Class* categorization. (*C*) Metabolism diversification. (*D*) Oxygen requirements. (*E*) Habitat. (*F* and *G*) Biotic relationship.

necessary for enzymatic activities, all bacterial OvoA proteins preserved: the highly conserved iron binding motif HX3HXE in the DinB domain (Braunshausen and Seebeck 2011); all the residues proposed to be involved in the substrates (cysteine and histidine) recognition/binding in the FGE-sulfatase domain (Castellano et al. 2016) and more importantly Tyr417, which plays a key role in sulfoxide synthase activity (Chen et al. 2018); the residues G524, C525, G528, R529, D545, G599, D600, A601 considered to be involved in the formation of the SAM binding site. The conservation of such

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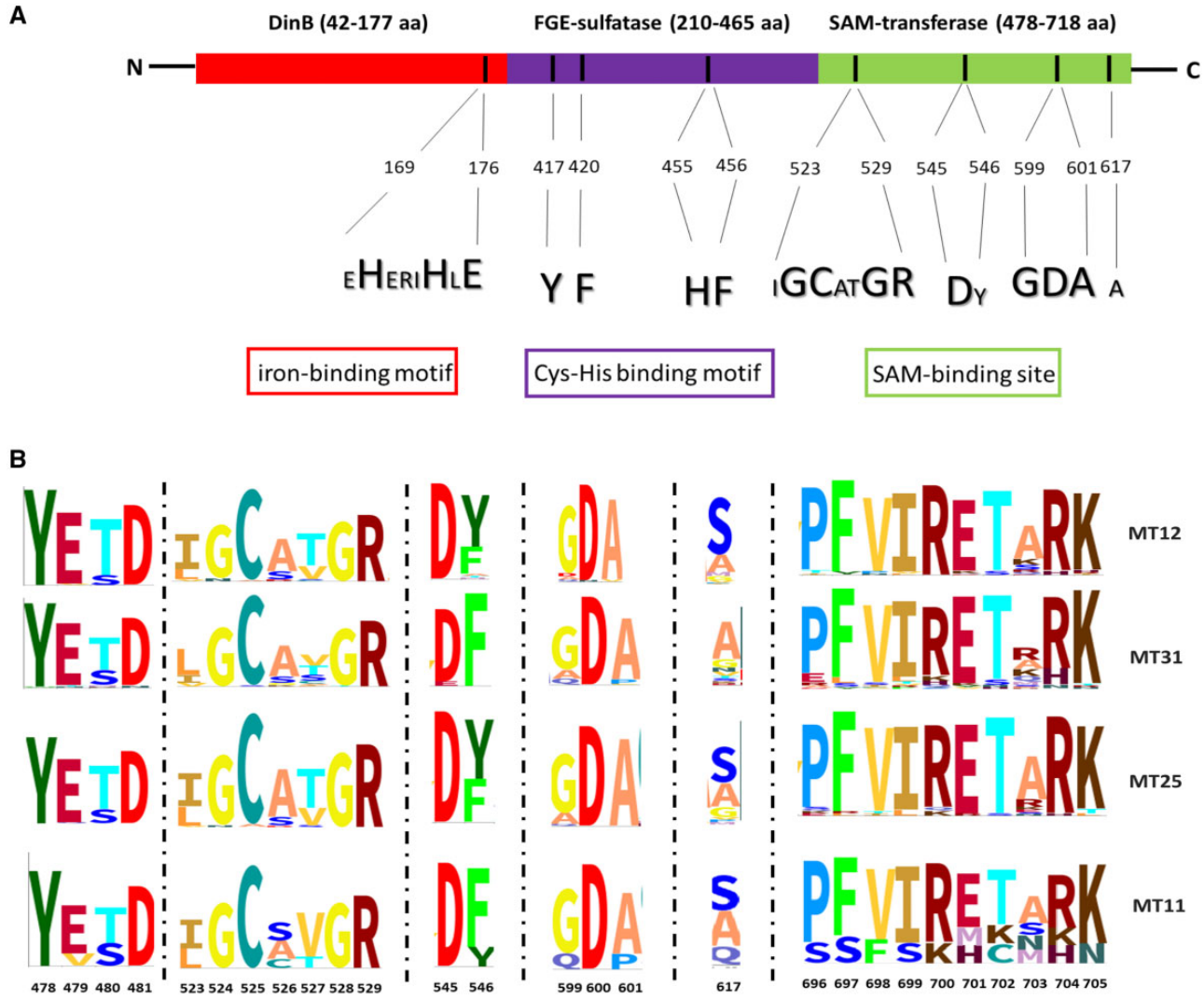

Fig. 3.—Characterization of OvoA proteins in Bacteria. (*A*) Schematic structural domain organization of OvoA proteins in Bacteria. DinB, FGE-sulfatase, SAM-transferase domains are highlighted in red, violet, and green, respectively. The residues involved in the iron-binding site, the putative residues involved in the *Cys–His* binding, the Tyr involved in the catalytic activity, the residues involved in the SAM binding sites are indicated by arrows. The indicated residues correspond to *Et*OvoA numeration. (*B*) Comparison of HMM profiles of the different annotated bacterial MT domains: MT12, MT31, MT25, and MT11 obtained from Skylign program.

key residues in bacterial OvoA sequences is represented in a scheme in figure 3*A*. On the other hand, the bacterial OvoA-like sequences did not conserve the residues known to be involved in the binding of EgtB with the substrates TMH and glutamyl-cysteine, thus excluding the possibility for these proteins to accept these substrates as favorites to produce ergothioneine.

To reveal whether the four different MT domain annotations had a specific sequence profile that could explain the diversification of their structure and function in bacterial OvoA orthologs, we compared the HMM profiles of all sequences restricted to the C-terminal region that included the SAM domain. The sequences of the MT domains showed a higher degree of similarity in the positions and motifs highlighted in figure 3*B*, while exhibiting a high variability in the remaining part. The overall similarity between the sequences classified by PFAM as having different SAM-methyltransferase domains are 37% for MT11, 61% for MT12, 59% for MT25, and 53% for MT31, respectively. In particular, MT12 and MT25 are mostly found in *Erwiniaceae*, the most represented family of bacteria exhibiting OvoA orthologs.

Identification and Characterization of OvoB Orthologs in Bacteria

To characterize the structural organization of bacterial OvoB orthologous proteins, we compared the 187 OvoB-like sequences found among bacteria with the previously

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characterized *Et*OvoB (PDB ID: 5Z0Q) (Naowarojna et al. 2018). These sequences shared the same structural organization of *Et*OvoB: the N-terminal (11–93 aa) domain, the catalytic domain (94–256 aa), and the C-terminal (257–389 aa) domain (supplementary fig. S1*A*, Supplementary Material online). Among the bacterial OvoB proteins, 91 shared from 50% to 93% aa identity with *Et*OvoB, whereas the others 96 shared from 30% to 49% aa identity. In all OvoB-like bacterial sequences sharing the higher identity with *Et*OvoB, all the residues in the active site responsible for the lyase activity (K240 in *Et*OvoB) and the interaction with the PLP cofactor: Y125, N176, D204, and H207, V100, and V101 (in the catalytic domain), and Y66 (in the N-terminal domain) (Naowarojna et al. 2018) were highly conserved (see supplementary fig. S1*A*, Supplementary Material online). In the sequences showing lower degree of similarity with *Et*OvoB, the residues N176, D204, and K240 were conserved; Y66 was present in all sequences except in *Halomonas sp. TD01* (WP_009724024.1). In addition, the substrate-binding pocket was conserved in bacterial OvoB sequences, in particular for the residues (S352, D356, and R364), previously identified by docking analysis as responsible for the selectivity of OvoB for its sulfoxide intermediate (Naowarojna et al. 2018). In addition, we retrieved a total of 141 OvoB-like proteins from the Ocean Gene Atlas database, sharing from 30% to 52% aa identity with *Et*OvoB, and reflecting the same conservation of key amino acidic residues (see supplementary file 1, Supplementary Material online).

Comparison of Bacterial OvoB Proteins with OvoB-Like Domain in Hydrozoa

In bacteria, the C–S sulfoxide synthase (OvoA) and the PLP-dependent lyase (OvoB) activities are performed by two different enzymes, encoded by two different genes. In metazoans, we did not found any OvoB-like sequence, except for Hydrozoa, which displays a unique polypeptide with both OvoA- and OvoB-like domains (Gerdol et al. 2019) (supplementary fig. S1*B*, Supplementary Material online). To verify whether the N-terminal OvoB-like domain fused with OvoA in Hydrozoa and encoded by a unique exon (Gerdol et al. 2019) could derive from an HGT event from bacteria, we carried out a similarity search comparison of the 292 aa of OvoB domain in *Hydra vulgaris* against bacterial genomes. We found two interesting cases of PLP-dependent enzymes from Bacteroidetes, which live in close association with Hydrozoa (Fraune and Bosch 2007; Di Camillo et al. 2012). In particular, the metagenome-assembled genomes of bacteria belonging to Bacteroidetes, isolated from Great Salt Lake, Utah, displayed both an OvoA-like sequence (KAA3624609.1) and a putative C–S lyase protein (NBC82978.1), this latter exhibiting 35.9% identity with *Et*OvoB. Another protein sequence annotated as aminotransferase class V-fold PLP-dependent enzyme (NBV57240.1), was found in the uncharacterized bacterium of the Bacteroidetes phylum, identified in freshwater metagenome. Comparative analysis highlighted that OvoA-like proteins from these Bacteroidetes exhibit only the first two domains; DinB and FGE-sulfatase domain; whereas both putative PLP-dependent proteins modeled trough HHpred gave as best templates C–S lyase or aminotransferase of class I/II (fig. 4*A*). In detail, the predicted models displayed superimposition with the 3D structure of *Et*OvoB (PDB ID: 5Z0Q) and high conservation of the residues involved in OvoB activity, especially for the putative C–S lyase from Bacterioidetes of Great Salt Lake (fig. 4*B*), which therefore was named *Bac*OvoB_1. In supplementary figure S2*A*, Supplementary Material online, we showed the corresponding multiple sequence alignment between *Et*OvoB and the two putative OvoB-like proteins from Bacteroidetes to underline the conservation of the amino acid residues involved in the binding of PLP, the substrate-coordination, and the catalytic activity. In detail, in the putative *Bac*OvoB_1 (in cyan) from Bacteroidetes of Great Salt Lake, we observed the conservation of histidine (K236) essential for the C–S lyase activity, and of the aspartic acid (D201) and valine (V97), both involved in the interaction with the PLP cofactor. In addition, the putative residues involved in substrate coordination in *Et*OvoB (S352 and R364) were conserved, whereas D356 was replaced by a lysine (see supplementary fig. S2*A*, Supplementary Material online). In the other putative aminotransferase class V-fold PLP-dependent enzyme from Bacterioidetes of freshwater genome, *Bac*OvoB_5 (in red), besides the conservation of the catalytic K255 and D229, and the conservative substitution of V100 with A126, the other residues were not conserved. In addition, the phylogenetic relationship between the three proteins, indicates how *Et*OvoB and the putative *Bac*OvoB_1 are more closely related compared with *Bac*OvoB_5 (supplementary fig. S2*B*, Supplementary Material online).

Phylogenetic Analysis of OvoA and OvoB in Bacteria

The OvoA bacterial sequences retrieved from both NCBI database and the Ocean Gene Atlas were used to reconstruct OvoA phylogeny in bacteria. Since OvoA and EgtB share common structural features and belong to the same family of sulfoxide synthase, six EgtB sequences from bacteria and one from cyanobacteria were used as outgroups. The results showed that OvoA homologs clustered in 9 different clades, whereas EgtB homologs clustered separately from the others (fig. 5). Then, we compared the taxonomy, the distribution of metabolic traits, oxygen requirements, and the diversification of the MT domain among the different clades (supplementary fig. S3, Supplementary Material online). In particular, we found that most sequences of Gammaproteobacteria (90%), cluster in a single large group (clade IX) including most of bacteria belonging to the *Erwiniaceae* family most showing a facultative anaerobic chemoorganotrophic

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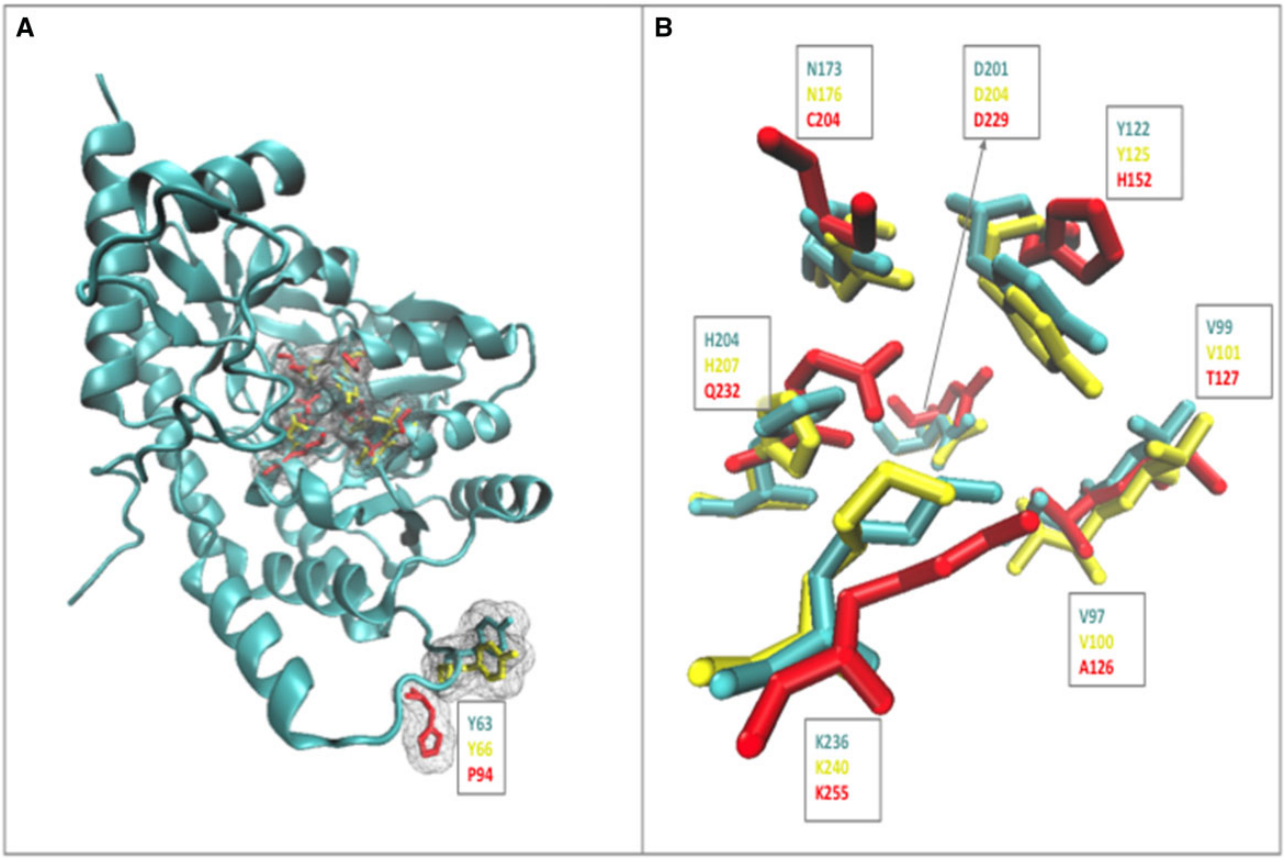

Fig. 4.—Protein structures superposition of the putative C–S lyase *Bac*OvoB_1 and *Bac*OvoB_5 with *Et*OvoB. (*A*) Three-dimensional model of the putative C–S lyase *Bac*OvoB_1 (in cyan); amino acids involved in the catalytic activity are showed in a licorice view (in cyan for *Bac*OvoB_1), in red for *Bac*OvoB_5, in yellow for *Et*OvoB). (*B*) Close-up view of amino acids in the active site and the corresponding position numbers (in cyan for *Bac*OvoB_1, in red for *Bac*OvoB_5, in yellow for *Et*OvoB).

metabolism. Marine prokaryotes were dominant among most clades, and especially in clade I, II, and IV (supplementary fig. S3*B*, Supplementary Material online). Chemoorganotrophic bacteria were dominant in five clades and clearly separated from chemolitotrophic ones, mainly found in clades II and III (supplementary fig. S3*C*, Supplementary Material online). MT 31 was found to be the main MT in 7 clades (supplementary fig. S3*D*, Supplementary Material online) and completely absent in clade III constituted only by aerobic chemiolitothrophic Acidithiobacilla.

We also performed phylogenetic analysis for bacterial OvoB-like sequences including also environmental sequences, and using Egt2 sequences as outgroup. Indeed, bacterial OvoB homologs form a polyphyletic cluster which however can be separated from Egt2 enzymes (supplementary fig. S4, Supplementary Material online).

In addition, we found that Ovo-like environmental sequences from the Ocean Gene Atlas webserver could be retrieved from a high number of sampling points at different depth layers across the water column (surface layer, subsurface with the maximum concentration of chlorophyll, mesopelagic) in different biomes (coastal, polar, trades, westerlies) with high abundance especially in surface and subsurface waters and polar regions (fig. 6).

Discussion

Ovothiols are small sulfur-containing natural products, which could have played a pivotal role in the evolution of redox homeostasis, providing an adaptive strategy for the response of marine organisms to environmental stressors (Castellano and Seebeck 2018). Little is known about the biological function/s and the ecological role of these natural products and different hypothesis have been proposed. Thanks to their ability to scavenge peroxides and make redox exchange with glutathione, they have the potential to protect eggs and early embryos against the oxidative burst occurring at fertilization in sea urchins (Shapiro 1991), to protect against the oxidative stress produced by the immune response of the host in *Trypanosoma* species (Ariyanayagam and Fairlamb 2001), and against environmental pollutants in the starlet sea anemone and in the common mussel *Mytilus galloprovincialis*

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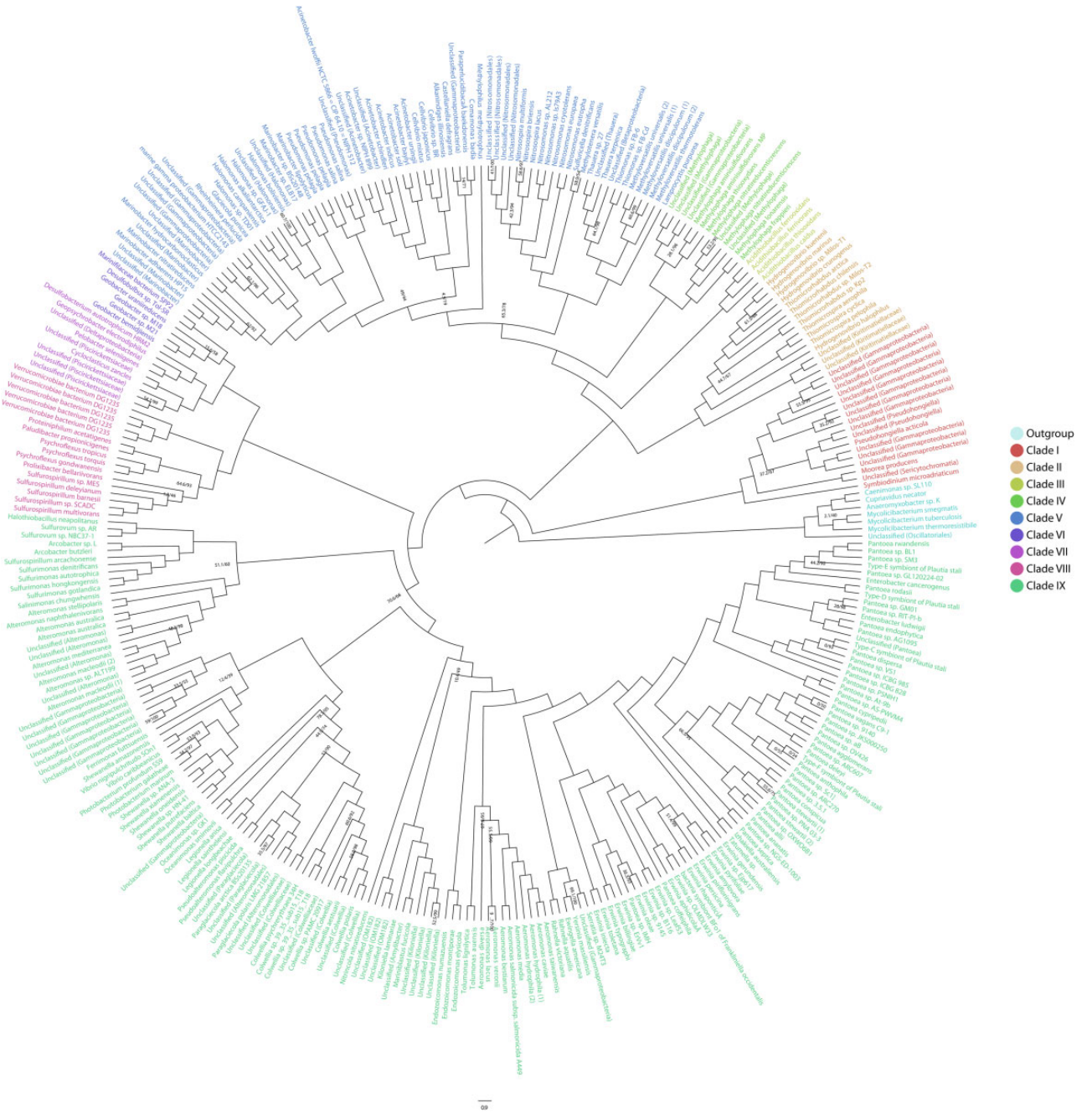

Fig. 5.—Phylogenetic tree of OvoA sequences in bacteria. The phylogenetic tree resulting from multiple sequence alignment of OvoA-like sequences from both the NCBI database and the Ocean Gene Atlas webserver was visualized using FigTree. Species names are colored according to cluster membership. Labels on branching nodes report both ultrafast bootstrap (UFboot) and the SH-aLRT test results. Bootstrap values above the threshold of 70 were omitted.

(Tarrant et al. 2018; Diaz de Cerio et al. 2020). However, although ovothiols are present in a broad range of organisms, their biosynthetic pathway has been hitherto characterized in detail only in a few microorganisms like the proteobacterium *E. tasmaniensis* and the trypanosome *Crithidia fasciculata* (Vogt et al. 2001; Braunshausen and Seebeck 2011), in the sea urchin *Paracentrotus lividus* (Castellano et al. 2016) and in the diatom *Skeletonema marinoi* (Milito et al. 2020). Recently, we revealed that the taxonomic spread of OvoA genes in Metazoa is characterized by the occurrence of lineage-specific gene losses and two independent HGT events in Bdelloidea and Hydrozoa (Gerdol et al. 2019).

Here, we describe ovothiol gene cluster distribution and enzyme diversification in Bacteria, trying to understand for

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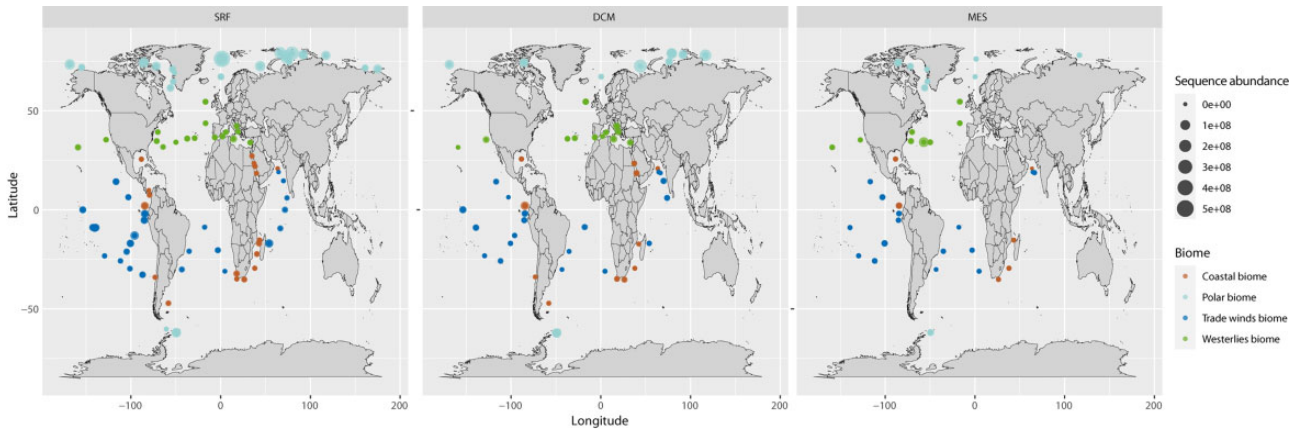

Fig. 6.—Distribution of OvoA-like sequences in marine environments. Verified *ovoA*-like sequences from Ocean Gene Atlas were mapped alongside marine biomes at different depth (SRF, surface layer; DCM, deep chlorophyll maximum; MES, mesopelagic). Circle size is referred to the abundance of sequences for each gene, whereas colors indicate the source biome.

the first time the ecological distribution of such a pathway in marine environment and to trace the evolutionary link of the ovothiol biosynthetic pathway from bacteria to unicellular eukaryotes and metazoans.

We found that ovothiol biosynthetic pathway was mostly present in Proteobacteria, which due to their abundance in marine environments and their wide range of metabolic traits, represent an ideal example of genome diversification and environmental adaptation (Zhou et al. 2020). Since life began in the ocean, the richest distribution of ovothiol gene cluster in marine niches could account for an ancient origin of this pathway. Indeed, 2% of bacteria preserving ovothiol pathway were found in deep-sea hydrothermal vents (which are hypothesized to represent "windows" into the early Earth ecosystem). In addition, the genomes of two bacterial species *Sulfurimonas hongkongensis* and *Proteiniphilum acetatigenes*, considered to be strictly anaerobic (Chen and Dong 2005; Cai et al. 2014), display an OvoA-like sequence, while completely lacking the most important enzymes involved in aerobic respiration. This is surprising because enzymes belonging to the sulfoxide synthase family are known to be typically oxygen-dependent (Mashabela and Seebeck 2013). Indeed, these OvoA-like sequences may represent attempts in ovothiol biosynthesis evolution. On the other hand, the first appearance of oxygen in the primordial atmosphere could have represented a selective pressure for the evolution of oxygen-dependent sulfoxidation. Interestingly, an alternative anaerobic pathway for the biosynthesis of the ergothioneine has been previously described in the green-sulfur bacterium, *Chlorobium limicola* (Leisinger et al. 2019), in which a rhodanese domain containing protein participate to the formation of a C – S bond, using polysulfide as the direct sulfur source (Cheng et al. 2020). Among the two strictly anaerobic bacteria displaying OvoA-like sequences, *S. hongkongensis* is involved in marine sedimentary sulfur oxidation and denitrification: it grew chemolithoautotrophically using thiosulfate, sulfide, or hydrogen as electron donor and nitrate as the electron acceptor under anoxic conditions (Chen and Dong 2005).

The presence of ovothiol biosynthetic genes in sulfur-oxidizing and sulfur-reducing chemosynthetic bacteria might suggest that these molecules could have played a role in the evolution as electrons and sulfur donors in chemical reactions. On the other hand, life in extreme environments, such as hydrothermal vents (which are rich in heavy metals and hydrogen sulphide) may have favored the bacteria producing ovothiol as a defense against these metals, as already reported in *P. lividus* (Eukaryota) (Castellano et al. 2016).

Bacterial OvoA proteins exhibit a canonical three-domain organization, with a higher conservation for the DinB and FGE-sulfatase domains especially for the residues involved in the iron binding and the sulfoxide synthase activity, whereas the MT domain is more diversified. This finding supports the hypothesis of a selective pressure to keep the residues involved in the essential activity of the enzyme, that is apparently the production of 5-thiohistine compound. The final methylation on the imidazole ring of histidine may not be always necessary, as also suggested by the finding that some OvoA-like proteins from Bacteroidetes lack the MT domain. In addition, both ovothiol and the unmethylated form of 5-thiohistidine were found in nature in complex with other marine secondary metabolites, like adenochromines in cephalopods, discorhabdins in sponges, imbricatine in sea stars (Castellano and Seebeck 2018), and conazolium in mollusks (Torres et al. 2021), even though the biological implications of such building blocks are still obscure. Moreover, 5-thiohistidine chemical and biological properties are similar to ovothiol, as also demonstrated by the effectiveness of the unmethylated 5-thiohistidine form in inhibiting γ-glutamyl-transpeptidase activity in human cancer cells at the same extent of ovothiol (Brancaccio et al. 2019; Milito et al. 2019).

In bacteria, we observed a strong association between the presence of both OvoA- and OvoB-like proteins encoded by

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two different genes, whereas our previous analysis on ovothiol biosynthesis in metazoans revealed no OvoB-like proteins in animals except for the case of hydrozoans, in which an N-terminal OvoB-like domain is fused with OvoA (Gerdol et al. 2019). This finding supports a bacterial origin of the OvoB-like domain in *Hydra* likely from symbionts. Indeed, our analysis showed that 14% of bacteria displaying ovothiol biosynthetic genes are symbionts, and that the hydrozoan OvoB-like domain shares a significant similarity and high conservation of key catalytic residues with OvoB-like proteins of uncultured Bacteroidetes, a phylum to which symbiotic bacteria in Hydra belong (Fraune and Bosch 2007; Di Camillo et al. 2012). The OvoB-like domain fused with OvoA in *Hydra* represents the unique case reported so far of a single polypeptide chain that combines all the three enzymatic activities (sulfatase, lyase, and methyltransferase) responsible for ovothiol biosynthesis (Gerdol et al. 2019). Moreover, the *ovoB–ovoA* fused gene in *Hydra* displays a significantly lower number of exons (5 instead of 17) compared with the other metazoans including other cnidarians like *Nematostella* (Chapman et al. 2010); this may be due to the high rate of intron loss in the *Hydra* lineage, compared with other anthozoans (Chapman et al. 2010). Interestingly, the additional N-terminal OvoB-like domain in *Hydra* is entirely encoded by the first exon (Gerdol et al. 2019), further confirming our hypothesis that its acquisition may be the result of the horizontal transfer of an intron-less gene of bacterial origin, subsequently fused with OvoA. Phylogenetic analysis of OvoA sequences in Metazoa revealed that the closest relative to the OvoA-like hydrozoan gene was a sequence from the chromerid *Vitrella brassicaformis* (Gerdol et al. 2019), a species of photosynthetic protozoan associated with the corals of the Great Barrier Reef (Oborník et al. 2012). This finding indicated a possible HGT, at least for the C-terminal OvoA domains, from an ancestral chromerid or apicomplexan symbiont, which might have lived in close association with an ancestor of the hydrozoan lineage (Janouškovec et al. 2015). However, no significant homology was observed between the hydrozoan OvoB-like domain and sequences encoded by the genome of *V. brassicaformis* and other alveolates (Gerdol et al. 2019). Therefore, subsequent events of genome reorganization could have driven the fusion of a pre-existing *ovoA*-like sequence in the ancestral hydrozoan genome with a second gene encoding a *β*-lyase OvoB-like, likely acquired by HGT from symbiotic Bacteroidetes. Since also in bdelloid rotifers we found a secondary reacquisition of OvoA by HGT, likely from Gammaproteobacteria (Gerdol et al. 2019), symbiotic relationships between bacteria and marine invertebrates could have had a crucial role for the evolution of this pathway.

The presence of ovothiol biosynthetic genes in parasites and pathogens of host organisms which do not display the same metabolic pathway is another feature that might have important evolutionary and ecological implications. Almost 36% of bacteria displaying *ovoA* and *ovoB* genes are parasites and/or pathogens of plants or animals, which lack the same biosynthetic gene cluster. This finding suggests that these pathogens could use ovothiol to protect themselves from the immune response of those hosts which do not produce this antioxidant, on the other hand organisms (hosts) producing ovothiols can be protected by the infection of these pathogenic microbes. This hypothesis is also consistent with the lack of OvoA in obligated parasites which lost primary metabolic pathways provided by the host (e.g., Platyhelminthes) (Jackson 2015; Zarowiecki and Berriman 2015) and Myxozoa (Chang et al. 2015). In addition, the protective role of ovothiol in the host/pathogens interactions strongly support our recent findings on the antioxidant and anti-inflammatory activities of ovothiols in mammalian models (Brancaccio et al. 2018, 2019; Castellano et al. 2018; Milito et al. 2019). Since Vertebrata have lost ovothiol biosynthetic genes during their evolution, the discovery of these biological activities open promising scenarios for the potential exploitation of these molecules as new marine drugs. On the other hand, ovothiol biosynthesis in pathogens may emerge as a target for novel anti-infective therapeutics (Krauth-Siegel and Leroux 2012).

Overall, the reasons of the broad taxonomic spread of ovothiol biosynthetic genes especially in marine environment, their loss in some phyla and secondary reacquisition by HGT in others are likely the product of multiple independent factors, which may have acted with different relative pressures in different organisms adapted to different environmental conditions, such as different oxygen concentrations, iron and sulfur availability, as well as light intensity. The clustering of OvoA bacterial sequences reflected to some extent differences in metabolic traits and oxygen requirements. Indeed, when considering environmental-derived OvoA-like sequences, we found that they were spread across a wide range of marine environments, with higher abundance in surface and subsurface waters, especially in polar biomes. Since surface and subsurface waters are characterized by the maximum concentration of chlorophyll and high photosynthetic rate, this distribution suggests that the ovothiol gene pathway might represent a widespread and important evolutionary marker of adaptation of prokaryotes to light-exposed and highly oxygenated extreme environments.

Tracking the evolutionary history of ovothiol biosynthesis from bacteria to unicellular and higher eukaryotes, and piecing together the results obtained from the analysis of bacterial OvoA sequences in this work with those carried out for metazoans and microalgae (Gerdol et al. 2019; Milito et al. 2020), we can summarize: 1) Archaea do not display any OvoA enzyme (Gerdol et al. 2019), while exhibiting EgtB (ergothioneine pathway); 2) some fungi and mycobacteria contain EgtB in cluster with EgtD (Seebeck 2013); 3) some Cyanobacteria are known to display both OvoA- and EgtB-like activities (Liao and Seebeck 2017); and 4) in a few bacteria, we found OvoB in cluster with OvoA, whereas in some cyanobacteria, OvoA was in cluster with EgtD. These findings

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let us to hypothesize that an ancestral event of gene duplication of an ancient sulfoxide synthase enzyme with a broader substrate promiscuity might have occurred in primitive bacteria followed by the subsequent divergence in more substrate-specific enzymes like EgtB and OvoA. During metabolic evolution, the ergothioneine pathway spread in Archaea, fungi, and mycobacteria, whereas the ovothiol pathway evolved in other uni- and multicellular eukaryotes. For example, OvoA evolved in unicellular eukaryotes like diatoms through the endosymbiotic events involving cyanobacteria (Milito et al. 2020); and in Protozoa and Choanozoa, whose ancestor gave rise to metazoan lineages (Gerdol et al. 2020), through the ancient endosymbiosis of heterotrophic Proteobacteria.

If the evolutionary success of ovothiol metabolic pathway were related to its ability to provide an efficient regulation of redox-sensitive signaling paths, then the loss of ovothiols may be linked to the independent evolution of alternative mechanisms for controlling redox homeostasis. These mechanisms might involve the biosynthesis of ergothioneine in mycobacteria and fungi (Seebeck 2013), as well as of the widespread glutathione, or cysteine derivatives in Vertebrata. In addition, besides reduction and simplification in genome evolution (Wolf and Koonin 2013; Lu et al. 2019), the appearance of specific membrane transporters to acquire ovothiol from diet might explain the progressive loss of the enzymes involved in the biosynthesis of these molecules in teleosts and other vertebrates. For example, the accumulation of the metabolite in the lens and gills of bony fish (Yanshole et al. 2019) may come from food available in the environment (microalgae, clams, mussels), as well as from metabolic exchanges with ovothiol-producing bacteria, living in close association with the host' tissues. Additional in silico analyses aimed at studying the co-evolution of ovothiol gene clusters and other thiols biosynthetic pathways together with their receptors/transporters may shed light on these aspects in the next future.

Conclusions

Data presented here suggest that the evolution of ovothiol biosynthesis might have involved symbiosis as a key process in contributing to metabolic and genetic materials exchange and in the emergence of functional innovations. Indeed, HGT events driven by different evolutionary pressures might have played a pivotal role in the diversification of lower eukaryotes, like hydrozoans, inhabiting very different marine habitats. The conservation of such secondary metabolic pathway especially in aquatic organisms suggests that ovothiol-like molecules can improve the organismal fitness in light exposed and oxygenated marine environments. An interesting question remains as to when and in which species OvoA-like sulfoxide synthases diverged from EgtB-like enzymes to make ovothiol instead of ergothioneine. Since this event could possibly be traced back almost two to three billion years ago, probably in the first photo-autotroph cyanobacteria, this will be a challenging question to answer in the future solely based on genome mining and comparative analysis. Further studies on the biochemistry and evolution of sulfoxide synthases, besides providing a better knowledge of marine life adaptation in general, may offer important insights into the technologies that rely on metabolic engineering and their exploitations.

Materials and Methods

Creation of OvoA- and OvoB-Like Sequence Databases

The fully sequenced and annotated bacterial genomes available in NCBI genome databases were screened for the presence of OvoA and OvoB based on homology criteria. In detail, *Et*OvoA (WP_012439783.1) and *Et*OvoB (PDB ID: 5Z0Q), previously characterized for enzymatic activities (Braunshausen and Seebeck 2011; Naowarojna et al. 2018), were used as a query for BlastP searches against the predicted protein collections of each species, using as e-values 1E-50 for an initial detection of possible matches. Positive hits were further inspected with HMMER v. 3.2.1 (Finn et al. 2011) to assess the presence of the three expected conserved domains in OvoA, that is, the DinB-like domain (PF12867), FGE-sulfatase domain (PF03781), and methyltransferase 11 domain (PF08241), pertaining to the SAM-dependent methyltransferase homologous superfamily (SSF53335). All bona fide complete OvoA sequences deposited in sequence databases displayed e-value much closer to 0. Multiple sequence alignments performed with Clustal Omega (Sievers et al. 2011) allowed us to assess the completeness of the inferred encoded protein sequences. Only sequences displaying all the three domains were kept for downstream analyses. A second search run was performed on the Ocean Gene Atlas webserver (Villar et al. 2018), using the OM-RGCv2+G data set with the E-value set at $1e^{-05}$, utilizing *Et*OvoA and *Et*OvoB as a query to retrieve other environmental protein sequences, which were then curated utilizing the criteria outlined for sequences retrieved from the NCBI database. OvoA-like confirmed homologs were plotted on a map according to the coordinates provided by the Ocean Gene Atlas database using the ggplot2 R package.

OvoA and OvoB Primary Structural Analysis

For primary structural analysis, both OvoA and OvoB protein sequence alignments were obtained by Clustal-Omega (Sievers et al. 2011). The conserved residues necessary for substrates binding and catalytic activities of the two enzymes were manually annotated comparing each OvoA- and OvoB-like bacterial sequence with *Et*OvoA and *Et*OvoB, respectively. After the final curation step, the C-terminal regions of all OvoA sequences were trimmed, and aligned to create HMM profiles for the methyl transferase (MT) domains (PF13847, PF08242, PF13649, and PF08241) using the

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HMMer suite v3.2 (Eddy 2011); profile logos were created with Skylign program (Wheeler et al. 2014).

Phylogenetic Analysis

The sequences gathered for OvoA and OvoB were first clustered to remove identical entries, and then aligned using MAFFT (Katoh et al. 2019) after adding characterized EgtB homologs as outgroup for OvoA-like sequences (Seebeck 2010; Goncharenko et al. 2015; Stampfli and Seebeck 2020) (*Mycolicibacterium smegmatis* [WP_011731158.1], *Mycolicibacterium thermoresistibile* [WP_050811957.1], *Mycobacterium tuberculosis* [WP_003899647.1], *Oscillatoriales cyanobacterium* [TAD81928.1], *Caenimonas sp. SL110* [WP_048439477.1], *Anaeromyxobacter sp. K* [WP_012525714.1], and *Cupriavidus necator* [WP_011616473.1]) and two characterized ergothioneine biosynthesis PLP-dependent enzymes as outgroup for OvoB-like sequences (EgtE from *M. smegmatis* [WP_011731155.1], Song et al. 2015 and Egt2 from *Neurospora crassa* [pdb|5UTS], Hu et al. 2014). The resulting alignment was used as an input for tree building using the IQ-TREE software (Minh et al. 2020) with the LG+ R10 model for OvoA- and OvoB-like proteins (as determined by the model finding algorithm implemented in IQ-TREE), using both ultrafast bootstrap (UFboot) and the SH-aLRT test to infer branching support values. Both trees were subsequently visualized using FigTree. Clades were manually defined on the maximum-likelihood OvoA tree based on ultrafast bootstrap values >70% (R Core Team 2019).

3D Models of Bacteroidetes OvoB-Like Sequences

Two selected proteins from *Bacteroidetes* species (NBV57240.1; NBC82978.1) were modeled using HHpred partition at MPI Bioinformatics Toolkit (Zimmermann et al. 2018) and selecting the best scoring hits. The atomic coordinates of the templates were obtained by scanning the PDB database (Berman et al. 2000). To predict 3D models, two independent multiple sequence alignments, performed by Clustal-Omega (Sievers and Higgins 2018), obtained in PIR format, were submitted to the homology modeling software Modeller 9v20 (Webb and Sali 2016). Modeller algorithm was set to generate 100 structural models for each protein structure. In order to evaluate the stereochemical quality of the generated structures and to select the best, the models were uploaded, in the standard PDB file format, to the PDBsum server (de Beer et al. 2014) to carry out a full set of Procheck structural analyses (Laskowski et al. 1993). The obtained models were displayed by using the molecular graphics software VMD (Humphrey et al. 1996). The comparisons between the modeled structures and the already characterized *Et*OvoB structure (Naowarojna et al. 2018) were carried out by VMD software and mTM-align (Dong et al. 2018).

Characterization of Habitat and Interspecific Interactions

The habitat and interspecific interactions of the selected bacterial species were inferred according to data available in the Pathosystems Resource Integration Center (PATRIC 3.6.2) free database and other public databases. PATRIC provides integrated data and analysis tools to support biomedical research on bacterial infectious diseases (Wattam et al. 2014). Information on strains not available within the PATRIC database was gathered through extensive literature searches. When strain information was not available for either isolation environment, oxygen requirement, or metabolism, we labeled the entries as "unknown." To further assess the anaerobicity of those bacteria annotated as anaerobic, we checked in their genomes for the presence of the cytochrome c oxidase, using *Paracoccus denitrificans* aa3 subunit 1 (3HB3_1) as template for pairwise similarity search (Koepke et al. 2009).

Statistical Analysis of OvoA-Like Sequence Cluster Diversifications

The frequency of prokaryotic taxonomic classes, methyltransferase domains, metabolic strategies, and isolation environment among the different clusters identified on the OvoA-like protein tree was assessed using the *ggstatsplot* R package using χ^2 goodness of fit test for statistical significance of differences (Kassambara 2017).

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Author Contributions

M.B. carried out data analysis and sequence alignments, participated in the design of the study, and drafted the methods and results of the manuscript. M.T. carried out in silico search, statistical analyses and drafted the methods and results of the manuscript. R.D. participated in the design of the study and critically revised the manuscript. I.C. conceived, designed, and coordinated the study, and drafted the manuscript. All authors revised the final version of the manuscript, gave final approval for publication, and agreed to be held accountable for the work performed therein.

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Data Availability

All data are incorporated into the article and its Supplementary Material online. Other data underlying this article will be shared on reasonable request to the corresponding author.

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