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Enzyme evolution beyond gene duplication

A model for incorporating horizontal gene transfer

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Abbreviations: HGT, horizontal gene transfer; EAC, escape from adaptive conflict; DDC, duplication, degeneration and complementation; ProFAR, *N'*-(5'-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide; PRA, *N'*-(5'-phosphoribosyl)anthranilate

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Understanding the evolution of enzyme function after gene duplication has been a major goal of molecular biologists, biochemists and evolutionary biologists alike, for almost half a century. In contrast, the impact that horizontal gene transfer (HGT) has had on the evolution of enzyme specialization and the assembly of metabolic networks has just started to being investigated. Traditionally, evolutionary studies of enzymes have been limited to either the function of enzymes *in vitro*, or to sequence variability at the population level, where in almost all cases the starting conceptual framework embraces gene duplication as the mechanism responsible for the appearance of genetic redundancy. Very recently, we merged comparative phylogenomics, detection of selection signals, enzyme kinetics, X-ray crystallography and computational molecular dynamics, to characterize the sub-functionalization process of an amino acid biosynthetic enzyme prompted by an episode of HGT in bacteria. Some of the evolutionary implications of these functional studies, including a proposed model of enzyme specialization independent of gene duplication, are developed in this commentary.

The core view of enzyme evolution is that gene duplication, followed by functionalization, is the primary mechanism by which novel enzymes have evolved.^{1–5} Since it is assumed that at the dawn of life genetic resources were scarce, it is commonly believed that ancestral enzymes lacked specificity, and that this allowed

them to engage in many surrogate activities. This view led to the seminal proposal that modern enzymes are the result of substrate specialization processes prompted by gene duplication, and that current organisms evolved regulation of metabolism in a pathway-specific manner, only possible in the absence of substrate ambiguity.⁶ Indeed, it is now broadly believed that functional divergence prior to duplication is a fundamental driving force of enzyme evolution.^{4,7}

The most accepted models of enzyme evolution are sub-functionalization involving either escape from adaptive conflict (EAC) or duplication, degeneration and complementation (DDC). In the EAC model, gene duplication has been proposed to allow organisms to escape from adaptive conflicts imposed by 'generalist' or promiscuous enzymes that result from divergence prior to duplication.^{1,8,9} During EAC the duplicated copies acquire adaptive mutations and become specialized toward one of the original functions. On the contrary, in the DDC model, the mutations that lead to sub-functionalization are neutral. One of the duplicated gene copies retains the original function, while the other evolves by acquiring mutations that render its product into a sub-specialized enzyme.^{1–5}

These hypotheses might explain primitive mechanisms of enzyme evolution, mostly in the absence of available genetic resources; however, it has been proposed that horizontal gene transfer (HGT), and not gene duplication, is the main mechanism by which prokaryotes expand their gene and protein families.¹⁰ What is the

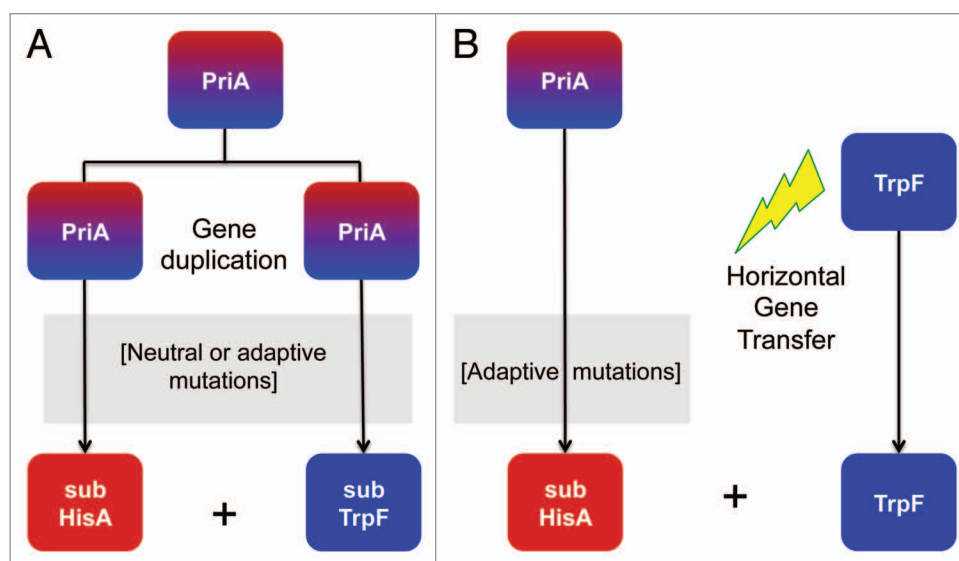


Figure 1. Models of enzyme sub-functionalization. When loss of a *trpF* gene occurs evolution of a proficient dual-substrate *PriA* enzyme takes place after positive selection. From that stage onwards, two possible scenarios can be envisaged. **(A)** The most accepted model of enzyme evolution implies gene duplication followed by narrowing of substrate specificity (*subHisA* and *subTrpF*), which is expected to occur after relaxation of the purifying selection. **(B)** Alternatively, differential gain of a *trpF* gene after HGT (marked with a lightning bolt), which does not imply the genetic and functional redundancy that is generated after gene duplication, could also take place. Thus, the sub-functionalization of *PriA* is constrained since purifying selection for the original function, i.e., *HisA*, is still in place. Moreover, the need for integrating the HGT-acquired whole-pathway *trp* operon into the receiving metabolic network, by means of altering substrate specificity, implies positive selection.

effect of HGT upon the topology of metabolic networks and enzyme features such as substrate specificity within the receiving organism is a question that remains unanswered. Indeed, although many reports show the pervasive occurrence of HGT in the prokaryotic kingdom, likely to be promoted by the social behavior of bacteria within the communities in which they evolve, insights into the mechanisms allowing the receiving organisms to adapt to the consequences of HGT are scarce.

Multi-specific enzymes from prokaryotic central metabolism, as they are well-integrated within the metabolic network, may advance our current understanding of enzyme evolution beyond what we have learnt using enzymes involved in peripheral pathways.¹¹ Ideally, these models should be phylogenetically traceable at a whole genome level. In our previous study we adopted and further developed, within a phylogenomics framework, an enzyme model from amino acid biosynthesis.¹² This model has been postulated and adopted as well by others as a suitable system for testing evolutionary hypotheses.^{11,13,14} At the center of this model stands a dual-substrate ($\beta\alpha$)₈-barrel phosphoribosyl isomerase (*priA* gene), which is a

close homolog (~50% ID) of the histidine biosynthetic *hisA* gene.¹²

The product of the *priA* gene has been shown to participate in both the biosynthesis of L-histidine [*HisA*, *N*'-[(5'-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) isomerase] and L-tryptophan [*TrpF*, *N*'-(5'-phosphoribosyl) anthranilate (PRA) isomerase] in the model actinobacteria *Streptomyces coelicolor* and *Mycobacterium tuberculosis*, Gram positive bacteria with high (G + C) content.^{12,15} The discovery of *PriA* stemmed from the realization that after whole genome sequencing of these organisms the *trpF* gene could not be found.

Given that the existence of a generalist enzyme like *PriA* could impose a metabolic disadvantage, we initially hypothesized the occurrence of gene duplication events that could lead to pathway-specific *PriA* enzymes with narrow substrate specificities for ProFAR and PRA substrates (Fig. 1A). Thus, we searched for *priA* duplicates, with an emphasis on actinobacterial genome sequences, including closely related organisms as out-groups. Despite it was found that the majority of the organisms analyzed lack a *trpF* gene,

these analyses failed to identify any sign of *priA* paralogy. In contrast, based in co-occurrence of *trpF* and *priA* genes, scenarios involving HGT or differential gene loss, could be identified (Fig. 1B).

Indeed, and in accordance with previous reports,^{16,17} we found *trpF* homologs in several species from the genus *Corynebacterium*, which is closely related to the genus *Mycobacterium* (Fig. 2). These organisms, such as *C. glutamicum*, have acquired the *trpF* gene by HGT as part of a whole-pathway *trp* operon from a member of the *Gammaproteobacteria*.^{16,17} After this HGT event, genomic and enzyme evolution events occurred, including (1) partial homologous displacement of the original corynebacterial *trp* genes and (2) complete loss of PRA isomerase activity of their cognate *PriA* homologs, which we explained after identification of mutations within the active sites of these enzymes and loss of protein conformational diversity.¹⁸ Thus, the *PriA* homologs from this sub-clade of the genus *Corynebacterium* were renamed as *subHisA*, in order to reflect the sub-functionalization process—involving HGT and not gene duplication—that renders these enzymes with narrow substrate specificity with metabolic implications.

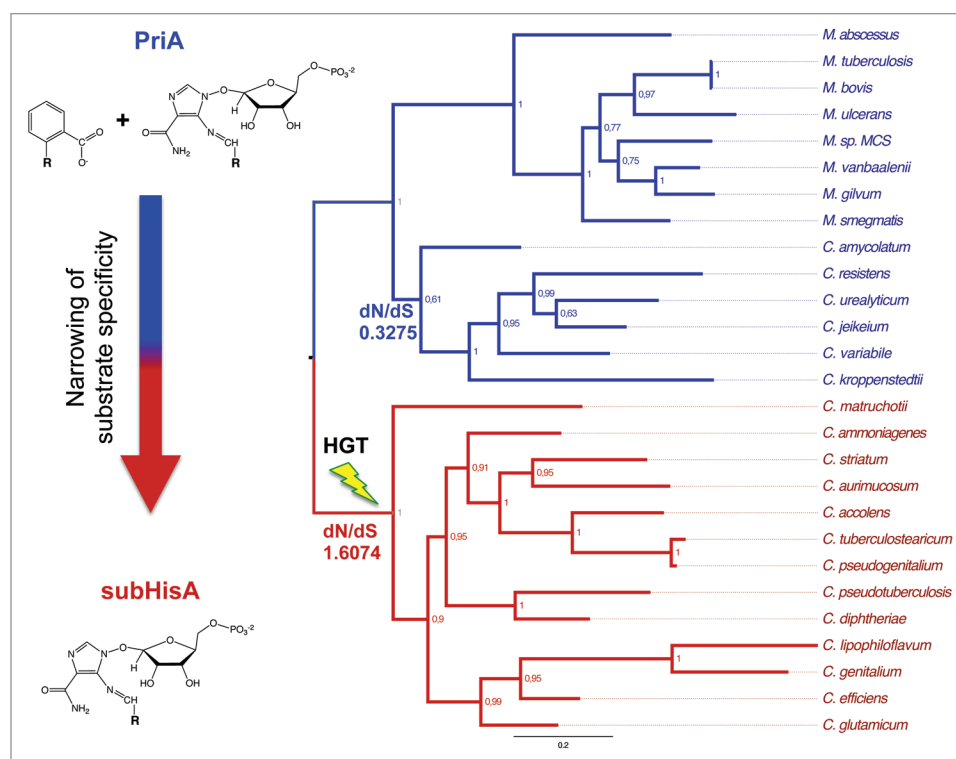


Figure 2. Positive selection during evolution of subHisA from PriA after HGT. PriA/subHisA phylogeny was constructed with MrBayes (posterior probability at each node is indicated). PriA and subHisA are shown with blue and red branches, respectively. The tree is rooted using the genus *Mycobacterium*. The proposed HGT event of *trpF* together with other *trp* genes, which coincides with speciation of one of the *Corynebacterium* lineages, is marked with a lightning bolt. The dN/dS values for ancestral PriA (blue) and subHisA (red) nodes are specified. The common phosphoribosyl moiety of ProFAR and PRA, whose chemical structures are shown, is denoted with an R.

As mentioned, the subHisA sequences are limited to the genus *Corynebacterium*. Interestingly, however, this genus also includes species with PriA enzymes, sharing up to a ~70% sequence identity (Fig. 2). Inspection of this enzyme sub-family within the phylogenetic tree suggests sequence divergence, which could be related to the evolution of enzyme substrate specificity. Indeed, sequence analyses revealed that the shape parameters of PriA ($\alpha = 1.6631$) and subHisA ($\alpha = 0.8311$) independent sub-trees are different and show higher rate heterogeneity for subHisA. Moreover, and in disagreement with the DDC model of enzyme sub-functionalization,¹⁻³ preliminary dN/dS () analysis showed signs of positive selection within the subHisA branch, which correlates with acquisition of the whole-pathway *trp* operon by HGT (Fig. 2; see ref. 19).

Narrowing of enzyme substrate specificity in subHisA shows that, even within a highly constrained active site, loss of one of the ancestral activities can occur

without compromising the catalytic efficiency of the remaining enzyme function. Thus, given that most random mutations are deleterious to protein stability, which implies a trade-off during evolution²⁰ and that in the DDC sub-functionalization model beneficial mutations need not be invoked,² detection of positive selection in the branch where subHisA has evolved, may question the universality of this model to include enzyme substrate sub-functionalization in purely biochemical grounds. Indeed, the DDC model calls upon evolution of gene duplicates in eukaryotes, mainly through tissue-specific gene regulation,² and thus this may be the origin of this discrepancy.

Moreover, the occurrence of positive selection, originally implied by the EAC model¹ but which still lacks 'smoking gun' evidence involving a generalist ancestral enzyme,^{8,21} can be explained if the mutations identified in the active site of subHisA were positively selected to accommodate the feedback regulated HGT-acquired whole-pathway *trp* operon

within the metabolism of the cognate *Corynebacterium* species. Interestingly, in *C. glutamicum*, where we confirmed the existence of a subHisA enzyme, it has been shown that feedback gene regulation of L-tryptophan^{16,17,22,23} and L-histidine²⁴ biosynthesis has recently evolved. The implication of this observation is that multi-specific enzymes connecting central metabolic pathways will impose a conflict to the cell if pathway-specific regulatory regimens are needed.

Therefore, our model of enzyme evolution independent of gene duplication proposes that during dynamic genome processes involving HGT strong positive selection is needed to drive both (1) narrowing of enzyme substrate specificity from a generalist enzyme; and (2) efficient assembly of HGT-acquired biosynthetic pathways within the receiving metabolic network. In the presence of a PRA isomerase encoded by *trpF*, these mutations may have provided subHisA with an adaptive mechanism to avoid productive binding of PRA within a promiscuous-prone enzyme

active site. More generally, our model emphasizes the need for an integrated view on the evolution of enzyme substrate specificity, which shall accommodate prokaryotic genetics and physiology, as well as HGT.

In conclusion, we anticipate that the discovery of a novel ($\beta\alpha$)₈-isomerase subfamily, i.e., subHisA, will open the door to explore the mutational effects, within a naturally occurring evolutionary trajectory, upon the fitness landscape both in terms of

the biochemical and biophysical features of enzymes, something that has remained elusive.²⁵ Our proposed evolutionary model of enzyme sub-functionalization after HGT or lineage-specific gene loss emphasizes the need to further investigate the neglected role of prokaryotes in evolutionary theory. It remains to be seen whether our model can be formalized within bacterial population genetics theory, once more examples similar to the evolution of subHisA from PriA become available.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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