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The tRNA synthetase paralog PoxA modifies elongation factor-P with (*R*)-β-lysine

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Abstract

The lysyl-tRNA synthetase paralog PoxA modifies elongation factor P (EF-P) with α-lysine at low efficiency. Cell-free extracts contained non-α-lysine substrates of PoxA that modified EF-P by a change in mass consistent with β–lysine, a substrate also predicted by genomic analyses. EF-P was efficiently, functionally, modified with (*R*)-β-lysine but not (*S*)-β-lysine or genetically encoded α-amino acids, indicating that PoxA has evolved an activity orthogonal to that of the canonical aminoacyl-tRNA synthetases.

The aminoacyl-tRNA synthetases family (aaRSs) encompasses 20 canonical enzymes that match amino acids with their corresponding tRNAs during translation[1]. The aaRSs are divided between classes I and II, each of which is characterized by a core catalytic domain responsible for ATP-dependent acyl-adenylate synthesis[2]. These core domains recognize and activate specific amino acids, which are transferred to the 3' end of tRNA. AaRSs are highly modular and contain domains appended to the catalytic core that assist in RNA binding and recognition, and in proofreading of incorrect aminoacylation products[3]. In addition to the canonical enzymes required for translation, the aaRS superfamily contains paralogs that recapitulate either the core or the appended domains of aaRSs[4]. AaRS paralogs generally retain some degree of specificity for canonical protein synthesis substrates, amino acid or tRNA, whether or not they function inside or outside translation[5]. One notable

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AUTHOR CONTRIBUTIONS

H.R., S.B.Z., T.B., B.W. and M.G. performed experiments and analyzed the resulting data; H.R., C.F., W.W.N. and M.I. designed experiments and wrote the manuscript.

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exception is PoxA, a paralog of lysyl-tRNA synthetase (LysRS), which does not recognize tRNA but instead post-translationally modifies elongation factor P (EF-P), a protein that mimics tRNA in both shape and size[6–8]. EF-P modification leads to specific alterations in the proteome and is required for virulence in *Salmonella*, but exactly how PoxA modifies EF-P to elicit these changes is unknown[7].

Structural studies suggest PoxA recognizes EF-P in a similar manner to that by which LysRS binds $tRNA^{Lys}$ [7,8]. Aminoacylation assays show the K_M of PoxA for EF-P (3.2 μM) is similar to values determined for bacterial class II-type LysRS with $tRNA^{Lys}$ (1–2 μM[9,10]). PoxA cannot aminoacylate $tRNA^{Lys}$, indicating that while it is structurally similar to LysRS its activity is different from that of a canonical aaRS[11]. The amino acid binding pockets of PoxA and LysRS also appear highly similar and super positioning of the α-carbons within the active sites shows an RMSD of 1.1 Å (Fig. 1a). Using the LysRS co-crystal structure, lysyl-adenylate can be docked into the active site of PoxA, consistent with previous findings that lysine is a substrate for EF-P modification *in vitro*[7]. While lysine was a substrate for PoxA, the reaction was extremely inefficient compared to amino acid activation by LysRS (Supplementary Methods, Table 1). Characterization of amino acid activation by PoxA under a variety of conditions did not lead to significant improvements in the efficiency of the reaction (Supplementary Results, Supplementary Fig. 1), suggesting that lysine may not be the cognate substrate. This was supported by comparing the PoxA and LysRS active sites, which indicated divergence of two conserved residues predicted to impact substrate specificity, Ser76 and Ala294 (Fig. 1a; *Escherichia coli* PoxA numbering). The effect of reverting these PoxA residues to their conserved LysRS identities was investigated by comparing the apparent amino acid binding affinities of the S76A and A294G variants to wild-type. The S76A mutation abolished all activity, while A294G showed a 25-fold decrease in the K_M for lysine compared to wild-type (Table 1). These data indicate PoxA is not optimized to use lysine as its preferred substrate, but has instead evolved amino acid specificity divergent from that of LysRS.

Previous studies show different LysRSs preferentially recognize particular non-cognate amino acids[12], and the specificity of PoxA was similarly tested (Fig. 1b). PoxA did not activate either other non-cognate genetically encoded amino acids or various commercially available analogs more efficiently than lysine. This raised the possibility that another amino acid was the preferred substrate of PoxA *in vivo*. *E. coli* cell-free extracts showed significantly higher stimulation of ATP / PPi exchange activity than expected based solely upon their lysine content, suggesting the presence of a second, more efficient, substrate (Supplementary Fig. 2a). Cell-free extracts were fractionated and the resulting samples tested for ATP / PPi exchange activity using LysRS and PoxA (Supplementary Fig. 2b). Optimal activities for LysRS and PoxA did not co-fractionate during purification, supporting the idea that *E. coli* cell-free extracts contained a substrate for EF-P modification distinct from lysine (Fig. 1c). The identity of the amino acid attached by PoxA *in vivo* was investigated by mass spectroscopy of both affinity purified EF-P, and of EF-P modified *in vitro* using enriched cell-free extracts. In both cases EF-P was site-specifically modified at Lys34 by a mass consistent with the attachment of lysine (Supplementary Fig. 3). These data showed that while the genetically encoded amino acid α-lysine was not the optimal substrate

for PoxA, EF-P is nevertheless modified by a compound with the mass of lysine, consistent with previous reports[8,13].

Genomic and phylogenetic studies predict that β-lysine might be the natural substrate for PoxA[6,7], consistent with the mass observed for modified EF-P and with the detection *in vivo* of a substrate distinct from α-lysine. (*R*)-β-lysine was 100-fold more efficient as a substrate than either (*S*)-β-lysine or α-lysine (Fig. 2a), suggesting that (*R*)-β-lysine is the cognate substrate for EF-P modification (Table 1). In contrast, (*R*)-β-lysine was a poor substrate compared to α-lysine for LysRS (Supplementary Fig. 4a) indicating that the amino acid substrate specificity of PoxA is different to that of the ancestral aaRS from which it evolved. The ability of PoxA to post-translationally modify EF-P with β-lysine was examined *in vitro* and found to be considerably more efficient than modification with α-lysine (Fig. 2b). β-lysine was also a potent inhibitor of α-lysine modification of EF-P, consistent with this reaction being orthogonal to tRNA aminoacylation by LysRS (Supplementary Fig. 4b).

Previous studies established that EF-P, PoxA and the 2,3-β-lysine aminomutase encoded by *yjeK*[8,14] act together *in vivo* to establish virulence in *Salmonella*, and are important for maintenance of stress resistance phenotypes including resistance to gentamicin and growth on AB2 media[7,15]. The latter phenotype was used to assess how modification with β-lysine affects the functional activity of EF-P *in vivo*. Complementation of a *poxA yjeK* double knockout strain showed that neither wild-type PoxA nor the A298G variant could restore normal growth on AB2 media, suggesting that lysylation alone (as opposed to β-lysylation) is not sufficient to create functional EF-P (Supplementary Fig. 5). To more directly investigate the role of β-lysylation of EF-P *in vivo*, we attempted to restore the growth of the *yjeK* mutant on gentamicin by supplementation with different forms of lysine. β-lysine, but not α-lysine, was able to restore growth of the *yjeK* mutant in the presence of gentamicin (Fig. 2c). These data show that functional EF-P is dependent on post-translational modification by PoxA specifically with β-lysine, and that synthesis of β-lysine by YjeK occurs prior to addition rather than by a post-addition rearrangement of α-lysine.

Perturbation of the post-translational modification of EF-P by PoxA gives rise to changes in translation that attenuate *Salmonella* virulence[7,15,16]. The chemical nature of this novel modification is a critical determinant of EF-P activity; while both α- and β-lysine are substrates for PoxA, only the latter amino acid generates the biologically active form of EF-P. What remains unclear is how attachment of β-lysine changes the activity of EF-P. One possibility is that β-lysine acts as a recognition element for ribosome binding, although this may not be a universal role as unmodified isoforms of EF-P can also bind to bacterial ribosomes[17] and a number of bacteria encode EF-P but not PoxA or YjeK[6]. Comparisons to the eukaryotic homolog eIF5a[18] suggest that addition of β-lysine may contribute to the ability of EF-P to promote translation initiation and /or elongation[13,19]. The modification of eIF5a with hypusine, by an unrelated pathway, is required for its stimulatory effect on translation[20], and the availability of a defined *in vitro* modification system will now allow similar effects to be tested for with EF-P. The availability of a defined modification system *in vivo* will in addition facilitate investigation of the proposal that EF-P stimulates translation of specific messages[7,16], a role also proposed for eIF5a but for which there is no known mechanism[18].

Post-translational modification of EF-P is based on molecular mimicry of a pathway hijacked from the translation machinery. To achieve the substrate specificity necessary to modify EF-P with β-lysine, PoxA evolved two specificities orthogonal to those of the protein from which it is derived, the class II-type LysRS. Elimination of the amino-terminal anticodon-binding domain of LysRS would have substantially reduced affinity for $tRNA^{10}$, and allowed PoxA to evolve the protein-protein interactions that permit it to specifically recognize the tRNA-mimic $EF\text{-}P^{8}$ but not $tRNA^{Lys}$. The evolution of amino acid specificity in PoxA appears to have been primarily driven by selection for β-lysine as a substrate, and may have been facilitated by the lack of selection to maintain a high turnover rate. The biological role of LysRS, providing Lys-$tRNA^{Lys}$ for protein synthesis, requires a high rate of product synthesis whereas PoxA would be required to have a far lower turnover rate. As a result, only very few active site replacements compared to LysRS are sufficient to provide PoxA with useful β-lysine specificity, since any accompanying loss in turnover number is still compatible with a biologically relevant rate of EF-P modification.

Many aaRS paralogs have been sequenced and annotated, but to date functions have only been assigned to a small fraction[4,21,22]. The relative ease with which PoxA apparently acquired new substrate specificities suggests that alternative mechanisms of post-translational modification, and amino acid-dependent transformations, may be associated with other aaRS paralogs. The substrate specificity of PoxA also opens up the possibility that aaRS paralogs could provide the basis for new tools for protein design and engineering. The orthogonality of PoxA to the translation machinery mirrors that of systems developed for co-translational insertion of unnatural amino acids[23], suggesting that similar approaches could be developed in the future to evolve new post-translational protein modification activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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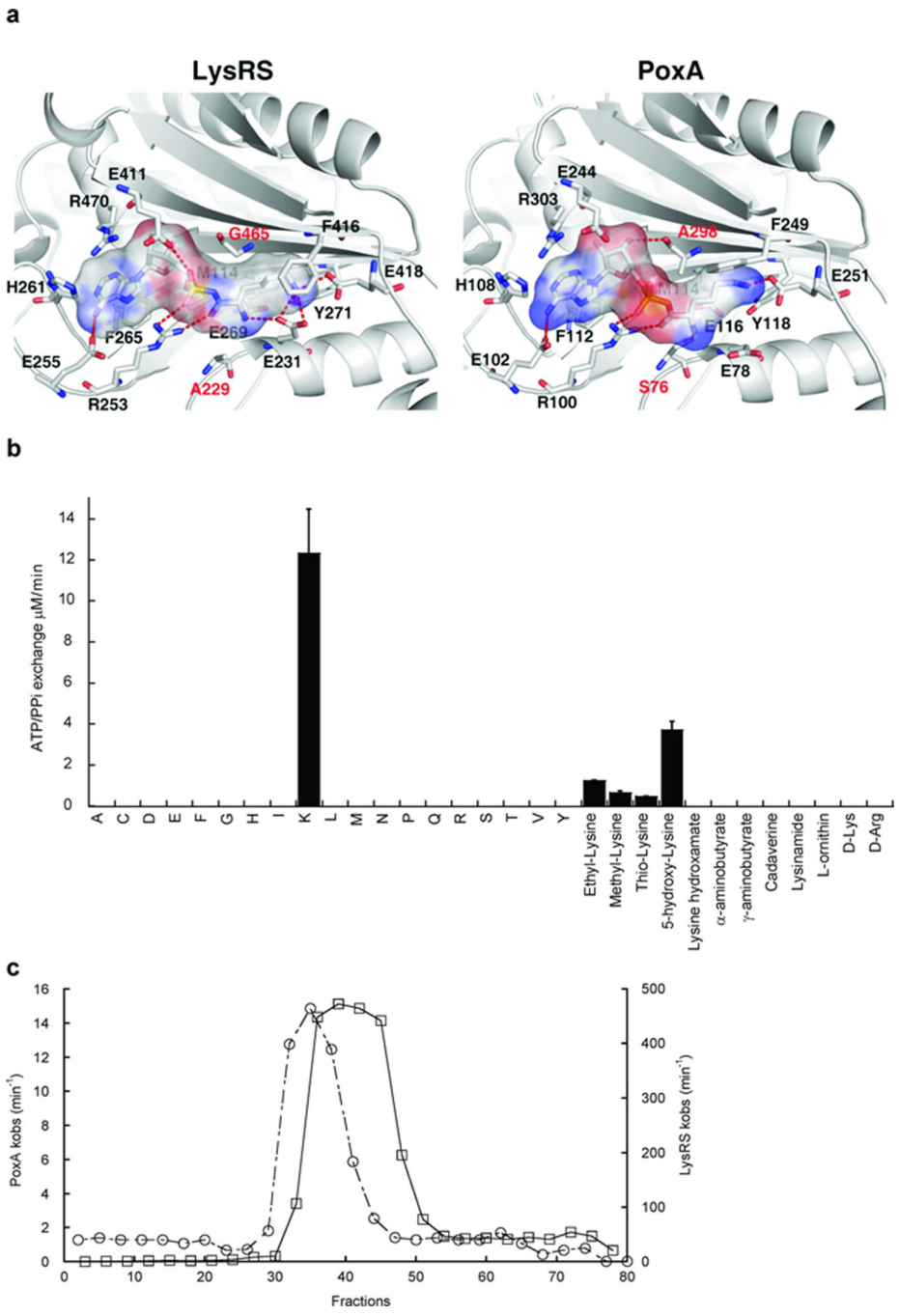

Figure 1. Amino acid recognition by PoxA

(**a**) The 20 amino acids forming the lysyl-adenylate binding pocket of PoxA and LysRS are identical, apart from the two positions indicated in red. For clarity, only 14 residues are represented. Crystal structures are LysRS from *Bacillus stearothermophilus*[24] and PoxA from *Salmonella enterica* Typhimurium[7]. The Van der Waals surface of the lysyl-adenylate is represented. *S. enterica* Ala298 corresponds to *E. coli* Ala294. (**b**) PoxA-catalyzed ATP/PPi exchange in the presence of non-cognate amino acids (single letter code) and lysine analogs. The concentration of each amino acid was 20 mM. Errors correspond to the

standard deviation from three independent experiments. (**c**) Metabolite fractionation by LC on silica gel. Fractions were separately tested for stimulation of ATP/PPi exchange catalyzed by PoxA (○) or LysRS (□).

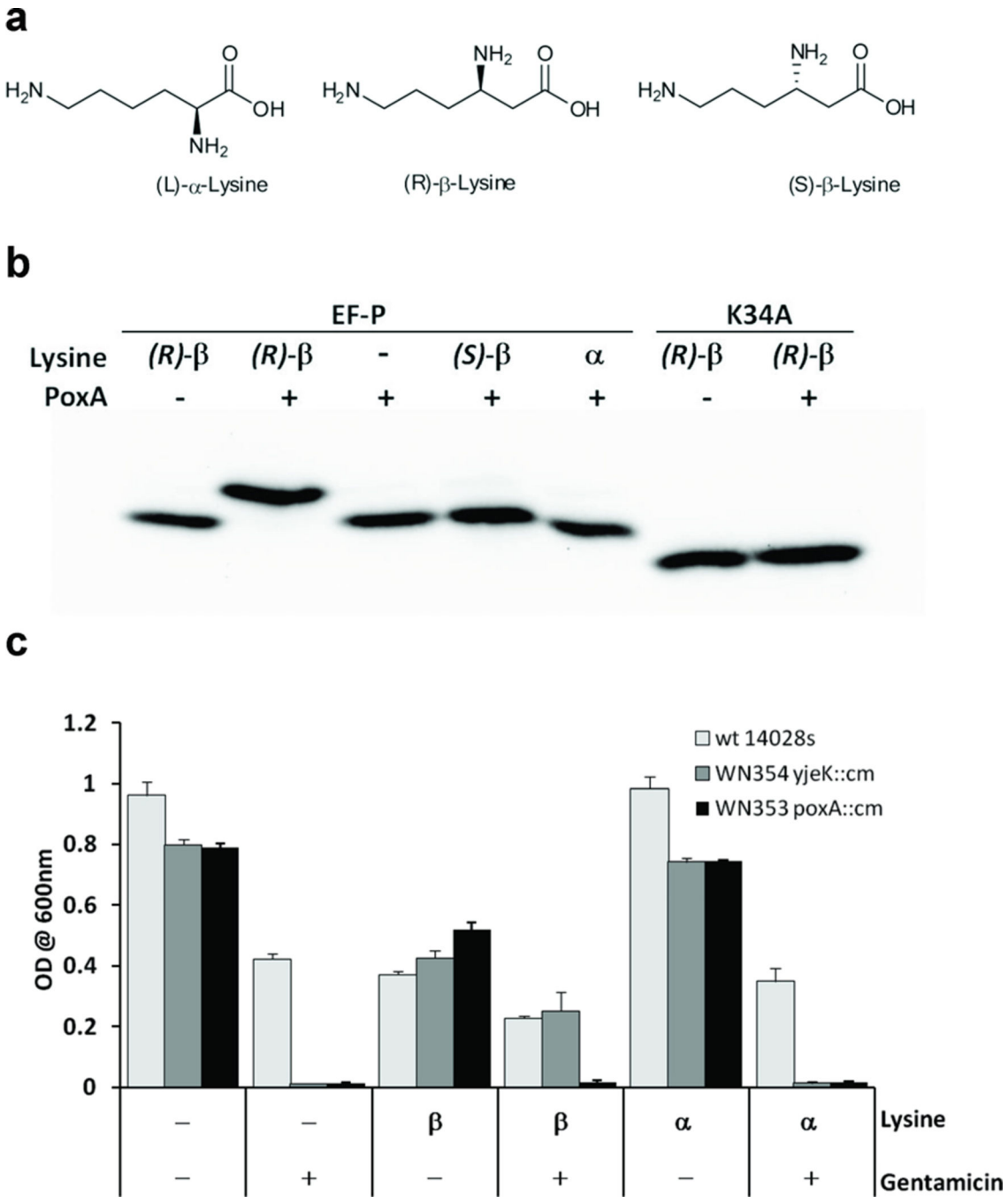

Figure 2. Modification of EF-P with β-lysine

(a) Lysine structures (**b**) PoxA catalyzed aminoacylation of EF-P with (*R*)-β-lysine. Aminoacylation of EF-P or the inactive K34A variant was assayed in the presence or absence of 20μM (*R*)-β-lysine, (*S*)-β-lysine, or α-lysine. Modified EF-P containing an additional $–NH_3^+$ group was separated from the unmodified EF-P by 1D isoelectric focusing and detected by western blotting. (**c**) Complementation of *Salmonella* Δ*yjeK* strain with (*R*)-β-lysine. Growth in liquid LB with (+) or without (−) 8 μg/ml gentamicin for 24 hours at 37 °C. Growth was monitored in the presence of either 0.8 mM (*R*)-β-lysine, 0.8 mM α-lysine,

or no lysine (−). Optical densities of replicate cultures are plotted. Growth rates were consistently slower on β-lysine than on α-lysine, suggesting a modest inhibitory effect at the concentrations used, the reason for which is presently unclear.

Table 1

Steady-state kinetics of amino acid activation by *E. coli* LysRS and PoxA.

	K_M (μM)	k_{cat} (min^{-1})	k_{cat} / K_M ($min^{-1}μM^{-1}$)
	L-α-lysine		
LysRS[1]	43	3000	70
PoxA	8600 ± 1200	15 ± 1.3	17×10^{-4}
PoxA A294G	344 ± 44	3.8 ± 0.6	111×10^{-4}
	(*R*)-β-lysine		
PoxA	213 ± 31	36 ± 3.3	1690×10^{-4}
PoxA A294G	414 ± 63	17 ± 0.5	410×10^{-4}
	(*S*)-β-lysine		
PoxA	6950 ± 1894	2.8 ± 0	4×10^{-4}

[1]Values previously determined for the *lysS*-encoded protein[25]. Errors correspond to the standard deviation from three independent experiments.