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A Critical Examination of *Escherichia coli* Esterase Activity*[S]

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Alicja K. Antonczak, Zuzana Simova, and Eric M. Tippmann[1]

From the Cardiff University School of Chemistry, Cardiff CF10 3AT, United Kingdom

The ability of *Escherichia coli* to grow on a series of acetylated and glycosylated compounds has been investigated. It is surmised that *E. coli* maintains low levels of nonspecific esterase activity. This observation may have ramifications for previous reports that relied on nonspecific esterases from *E. coli* to genetically encode nonnatural amino acids. It had been reported that nonspecific esterases from *E. coli* deacetylate tri-acetyl *O*-linked glycosylated serine and threonine *in vivo*. The glycosylated amino acids were reported to have been genetically encoded into proteins in response to the amber stop codon. However, it is our contention that such amino acids are not utilized in this manner within *E. coli*. The current results report *in vitro* analysis of the original enzyme and an *in vivo* analysis of a glycosylated amino acid. It is concluded that the amber suppression method with nonnatural amino acids may require a caveat for use in certain instances.

The central question addressed in this paper is whether the glycosylated amino acids GlcNAc-Ser and GalNAc-Thr have been genetically encoded into proteins *in vivo* (1, 2). The reports for the incorporation of these two amino acids are unique from all other reports (3) that have incorporated unnatural amino acids using the recoded UAG codon and *Methanococcus jannaschii* orthogonal pairs in that these two amino acids required further processing by the host organism before incorporation (see Fig. 1). Here we posit that the primary barrier to their incorporation would appear to be the fact that the host organism used in the original reports, *Escherichia coli*, maintains very low levels of nonspecific esterase activity. In fact, the original reports used citations from mammalian biology to substantiate the nonspecific esterase mechanism (see below).

E. coli is likely the most thoroughly studied microorganism. This is especially true in regard to carbohydrate and amino acid uptake and utilization (4). Therefore, it should not be surprising that it has long been known that esterified carbon sources are not metabolized by *E. coli* in standard assays used to probe for microorganism lipase and esterase activity (5). Such results and our current analysis underscore the limitations of the reports that triacetyl *O*-linked glycosylated amino acids (GlcNAc-Ser and GalNAc-Thr) were deacetylated in *E. coli* by endogenous "nonspecific" esterases. The deacetylated amino acids were then believed to have been genetically encoded into full-length proteins *in vivo* (1, 2).

In these previous studies the glycosylated amino acids were provided to the growth media as their tetraacetate analogs, and it was construed from the mass spectra and lectin binding assays that the ester groups of the saccharide had all been hydrolyzed. The notion that *E. coli* rapidly hydrolyzes a simple ester is not easily reconciled with what is commonly observed when the ester functional group is introduced into cultures of *E. coli*. For example, we were prompted by reports that claimed to have harvested β-hydroxy esters from *E. coli* (6). There was nothing in such a report to indicate that the *E. coli* strain used had undergone a drastic genetic modification beyond the introduction of one enzyme derived from yeast. The enzyme from yeast was expressed in *E. coli* to asymmetrically reduce β-keto esters to the corresponding β-hydroxy esters. The reduction was accomplished in 87% yield and was performed in whole cells. It stands to reason that such a report having claimed to extract significant amounts of an esterified product would not be possible if *E. coli* maintained even moderate levels of nonspecific esterase activity. The fact that *E. coli* maintains low levels of endogenous esterases and lipases has been quite pivotal for a number of studies that have used this organism as the host to express esterase genes *in vivo* (see below).

Nonspecific esterase activity is common in eukaryotic organisms, for example, our ability to hydrolyze triacylglycerides to access an important energy source, but this stands in stark contrast to *E. coli* where it is possible to directly extract *O*-acetylated oligosaccharides (7) and other simple esters (6) in high yields. These reports are consistent with the observation that UDP-2,3-diacylglucosamine accumulates in *E. coli* when genes from lipid biosynthesis are deleted (8). *E. coli* is also the preferred host for evaluating esterase and lipase activity when screening genes from cultured and uncultured organisms (9, 10). Screening for lipase activity from various microorganisms is often performed on tributyrin agar plates (11). The results are typically the same as for triacetin, and it is repeatedly observed that *E. coli* does not naturally grow on triesters of glycerol (12, 13). These and many other similar esterase screens (14) would not have been feasible if *E. coli* produced even moderate levels of a lipase or nonspecific esterase.

In the present article we use a combination of our current findings and a thorough review of the relevant literature to conclude that *E. coli* may not maintain sufficient levels of nonspecific esterase activity to permit the *in vivo* incorporation of the glycosylated amino acids by the mechanism reported (Fig. 1). Our conclusion is further supported by isothermal calorimetry measurements of Zhang *et al.* (1) original enzyme showing it

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[1] To whom correspondence should be addressed: Main Bldg., Park Place, Cardiff CF10 3AT, UK. Fax: 44-029-20873040; E-mail: tippmann@cardiff.ac.uk.

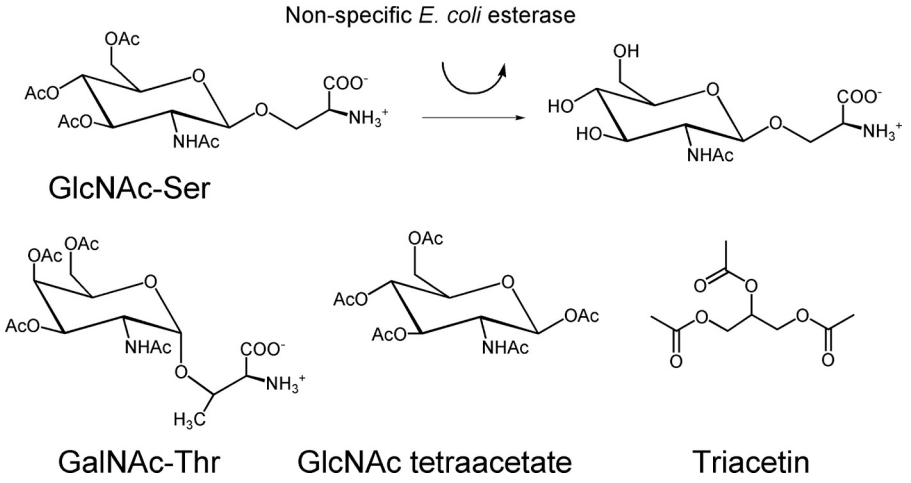

FIGURE 1. **Proposed product of an esterase with GlcNAc-Ser and other esterase substrates discussed in this study.**

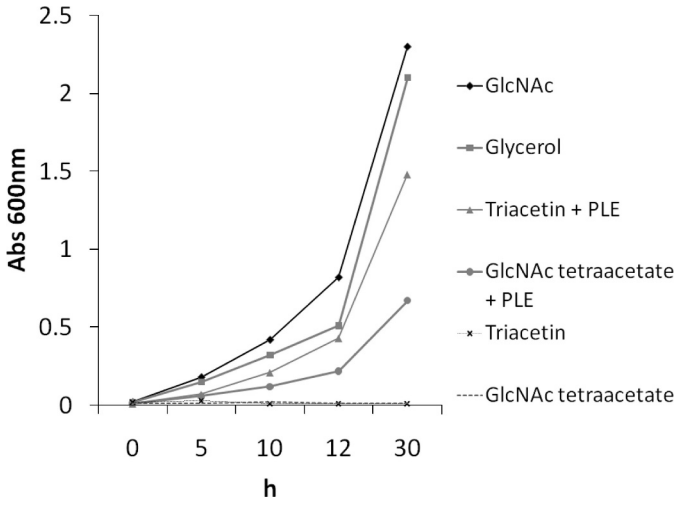

FIGURE 2. **Growth curves of *E. coli* on various carbon sources (1% v/v or w/v) in the presence and absence of porcine liver esterase (1760 units/liter).** *Abs*, absorbance.

retains considerable wild-type activity. We also show that the amino acid GlcNAc-Ser appears to be metabolized in *E. coli*.

EXPERIMENTAL PROCEDURES

All cultures were saturated in minimal media with either 0.5–1% glycerol or *N*-acetylglucosamine and then pelleted and washed with minimal media lacking a carbon source before finally adding the acetylated carbon sources and pig liver esterase (PLE)[2] as prescribed. Cultures containing PLE were buffered to pH 7.4–7.8 with 20 mM Tris buffer to maintain activity of PLE. Figs. 2 and 3 were produced with Top-10 cells (Invitrogen). These cells are a derivative of DH10B. Cultures were grown under multiple conditions including the presence and absence of antibiotics to minimize the occasion of another microorganism being cultured. To impart antibiotic resistance, cells were transformed with a pUC19 plasmid to control propagation with ampicillin (50 μg/ml). Testing for esterase activity in the presence of *p*-iodophenylalanine (*p*-iodoPhe) and the methyl ester was performed on 2-YT (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl) agar plates. The amino acid and amino ester were added to media when the agar was ~50 °C and then quickly poured into plates. Fluorescence was readily visible after 20 h, and the plates were removed from 37 °C and placed at 4 °C for 48, after which time the fluorescence increased slightly for the final image in Fig. 4.

2-Acetamido-2-deoxy-β-D-glucopyranose 1,3,4,6-tetraacetate 99% (catalog no. 859990, Sigma-Aldrich). Triacetin was from Sigma-Aldrich. Porcine liver esterase was used from a suspension in 3.2 M ammonium sulfate solution, ~130 units/mg of protein (~10 mg/ml) (Sigma-Aldrich).

Cloning and Plasmids—Custom gene synthesis was performed by Epoch Biolabs. Reporter plasmid pAA was constructed from a synthetic T5 promoter and multicloning site (T5-MCS) fragment that was custom-designed to have two copies of the *M. jannaschii* $tRNA_{CUA}$ under control of the lpp promoter and rrnC terminator. The green fluorescent protein (GFP) amber mutant corresponded to residue 34 and was isolated from a screen of a transposon trinucleotide insertion protocol (TriNEx) (15). The synthetic T5-MCS fragment was cloned into a chloramphenicol-resistant derivative of pACYC184 and pETDuet. The GFP gene with a TAG mutation was cloned into the NdeI and XmaI sites of pAA to give pAAGFP-TAG34. The gene corresponding to the mutant *M. jannaschii* TyrRS for *p*-iodoPhe (16) was synthesized (Epoch) and cloned into the NdeI/PstI site of pTUL, a pBR322-derived plasmid with kanamycin resistance, under control of a Gln aminoacyl tRNA synthetase promoter and terminator. Oligonucleotides were purchased from IDT DNA. The aminoacyl tRNA synthetase (aaRS) for *p*-acetylPhe (and similarly for (S)1–90, as labeled in the original report from Zhang *et al.* (1)) was synthesized (with codons optimized for expression in *E. coli*) with the reported mutations and cloned into pET101TOPO (Invitrogen) according to the manufacturer's protocol. The aaRS was overexpressed in BL21(DE3) Gold (Stratagene), and the cells were disrupted by freeze-fracturing at −80 °C and sonication. The protein was purified using nickel-nitrilotriacetic acid resin (Qiagen) and then by anion exchange before being dialyzed (25 mM Tris, pH 7.7, 250 mM NaCl, 0.5 mM EDTA) for isothermal calorimetry (ITC) studies.

RESULTS

E. coli cells were cultured in the presence of *N*-acetyl glucosamine, glycerol, and their acetylated analogs, 2-acetamido-2-deoxy-β-D-glucopyranose 1,3,4,6-tetraacetate (GlcNAc tetraacetate) and 2,3-diacetyloxypropyl acetate (triacetin). The growth curves show that *E. coli* is unable to grow on the acetylated carbon sources (Fig. 2). Growth was observed only in the

[2] The abbreviations used are: PLE, pig liver esterase; aaRS, aminoacyl tRNA synthetase; ITC, isothermal calorimetry; *p*-iodoPhe, *p*-iodophenylalanine; *p*-acetylPhe, *p*-acetylphenylalanine; GFP, green fluorescent protein.

cultures supplemented with a significant amount of the non-specific porcine liver esterase (Sigma). Commercially available PLE was used in this study because the enzyme's broad substrate tolerance and mechanism of action are well understood (17, 18). Using GlcNAc-Ser as the sole carbon source in the presence and absence of PLE suggests that *E. coli* is able to further metabolize both GlcNAc-Ser and *O*-deacetylated GlcNAc-Ser (Fig. 3).

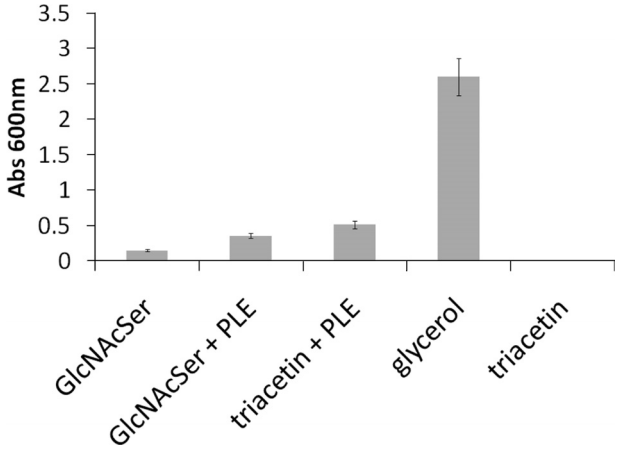

FIGURE 3. **Growth of *E. coli* on tri-acetyl-β-GlcNAc-serine, glycerol, and triacetin as the sole carbon sources in minimal media in the presence and absence of porcine liver esterase (4000 units/liter).** Cultures were measured at 15 h, and all carbon sources provided as 0.5% v/v or w/v.

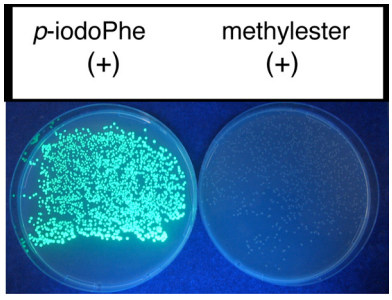

FIGURE 4. **Examination of *E. coli* esterase activity.** The experiment requires hydrolysis of a methyl ester of *p*-iodophenylalanine to complement the phenotype of an amber mutant of GFP. Identical cultures were supplemented with 1 mM free and protected amino acid as indicated and incubated 24 h.

A third experiment was designed to attempt to correlate endogenous *E. coli* esterase activity with an amber suppression contingent phenotype (Fig. 4). Here, a TAG mutation was incorporated into the GFP gene corresponding to residue 34. For the orthogonal $tRNA_{CUA}$/aaRS pair, we employed a known tyrosyl pair from *M. jannaschii* evolved to incorporate *p*-iodoPhe (16). The pair is reported to incorporate *p*-iodoPhe with high fidelity into proteins in response to the UAG codon. In this experiment efficient suppression of the amber stop codon, UAG, results in fluorescence which will only be observed as a result of the production of full-length GFP. The result in Fig. 4 showed that GFP fluorescence was only observed in colonies grown in the presence of *p*-iodoPhe. An absence of fluorescence indicates that the UAG codon is not suppressed. Here the UAG codon simply reverts to its canonical role as a stop codon, and ultimately this produces products of terminated translation. This result strongly suggests that the methyl ester of an amino acid is not appreciably hydrolyzed by whole *E. coli* cells to allow the free amino acid to be aminoacylated to $tRNA_{CUA}$.

The final experiment used ITC to directly assess the affinity of the aaRS (labeled (S)1–90 in the original report; Zhang *et al.* (1)) for GlcNAc-Ser (Fig. 5). The results with the (S)1–90 aaRS were compared with a second aaRS specific for *p*-acetylphenylalanine (*p*-acetylPhe) (19). The enzymes were evaluated for binding their cognate unnatural amino acids and also tyrosine. For *p*-acetylPhe with cognate aaRS, the K_d values were 330–522 μM. For GlcNAc-Ser and the cognate (S)1–90 aaRS, our experimental setup was unable to measure K_d. However a K_d value for the (S)1–90 aaRS with tyrosine was easily measured (662–2659 μM). Thus, the affinity for the (S)1–90 aaRS and tyrosine is slightly reduced when compared with the result of *p*-acetylphenylalanine with its cognate aaRS.

DISCUSSION

Genomic sequencing efforts have revealed that *E. coli* has at least 13 genes encoding acetyltransferase or esterase-like activ-

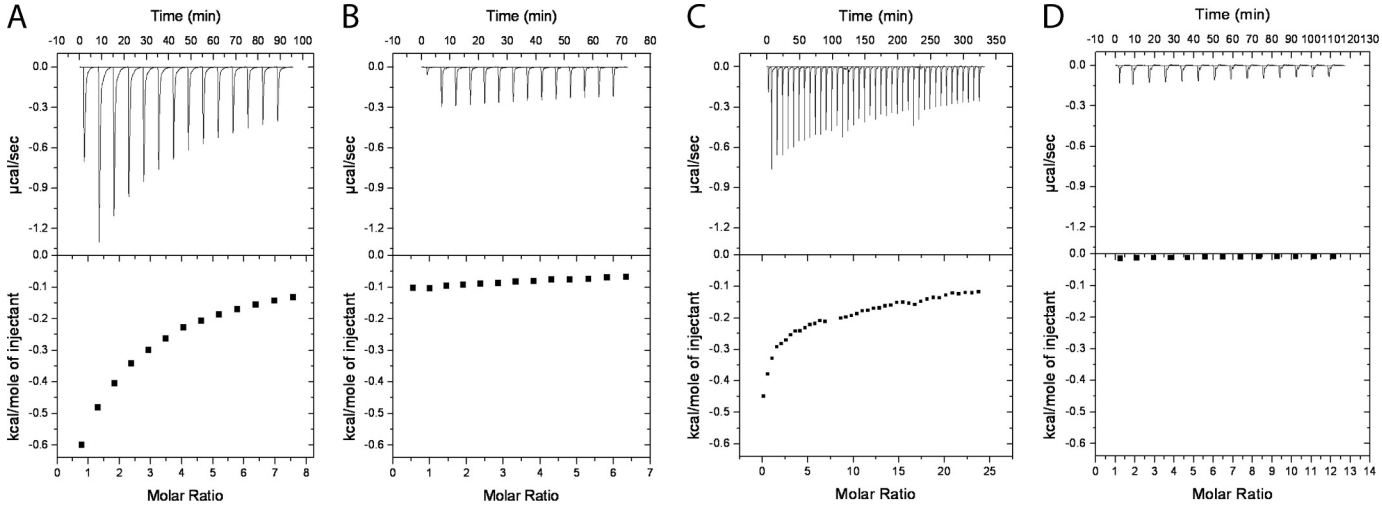

FIGURE 5. **Isothermal calorimetry measurements.** *A*, results for *p*-acetylphenylalanine with a mutant aminoacyl tRNA synthetase ($aaRS^{keto}$) evolved to be specific for the amino acid (36). *B*, results for tyrosine with aminoacyl tRNA synthetase ($aaRS^{keto}$) evolved for *p*-acetylphenylalanine. *C*, results for tyrosine with the (S)1–90 aaRS reported by Zhang *et al.* (1). *D*, results for GlcNAc-Ser with the (S)1–90 aaRS reported by Zhang *et al.* (1).

ity (20), but even a cursory study of the literature does not support the idea that *E. coli* maintains sufficient endogenous levels of any particular nonspecific esterase to hydrolyze the glycosylated amino acids in the manner that was reported. For example, in Zhang *et al.* (1) and Xu *et al.* (2), the complete hydrolysis of all *O*-linked esters on GlcNAc-Ser and GalNAc-Thr would have had to occur after just 8 h, after which time the protein was harvested as detailed in their experimental reports. The duration of the experiments is just one variable to be evaluated. The other variable unique to Zhang *et al.* (1) is the amber suppression method itself. It should, therefore, be mentioned that the method is analogous to most gene expressions (3). The difference between a standard expression and these expressions is that the host organism must supply the orthogonal pair ($tRNA_{CUA}$/aaRS) to incorporate just one nonnatural amino acid as well as the nonnatural amino acid itself. The final requirement is to mutate the codon corresponding to the target residue of incorporation to TAG. Because the amber suppression methodology works in concert with the existing translational machinery of the host, other affects from the method on the host organism should remain negligible. The method is also performed with the more common strains of *E. coli* (DH10B and BL21(DE3)), which have well characterized genotypes. We can, therefore, conclude that the amber suppression method does not provide an obvious rationale for the overexpression of an endogenous nonspecific esterase.

It is not disputed that *E. coli* produces enzymes that if overexpressed or purified to homogeneity could hydrolyze an ester (21); it is disputed that such a process proceeds on a relevant time scale to support the results of Zhang *et al.* (1). For example, no growth on the acetylated carbon sources, such as triacetin, was apparent after 7 days, which is consistent with the literature. Again, the experimental details reported by Zhang *et al.* (1) would have required the complete hydrolysis of the triacetylated saccharide in as little as 8 h.

In Fig. 3 it is even suggested that the glycosylated amino acids would be further processed by endogenous enzymes. Serine can be used as the sole source of carbon by *E. coli*, and free serine would result if GlcNAc-Ser were hydrolyzed at the α position (22). In the presence of porcine liver esterase the cultures had increased doubling times. This can be explained from the fact that *N*-acetylglucosamine is a preferred source of carbon and would also result if *O*-deacetylated GlcNAc-Ser were hydrolyzed at the α position (23). If the amino acid is metabolized by *E. coli*, as our results would seem to indicate, then the consequence is that the overall concentration of the free amino acid would be reduced. A lower concentration of amino acid would then equate into lower yields of recombinant protein. However, as much as 4 mg/liter recombinant protein was reported to be produced with the glycosylated amino acids. Such a yield would place the orthogonal pairs used to incorporate the glycosylated amino acids among the most efficient known (3, 24). Given what has been observed with other saccharide analogues, it seems reasonable to question how *O*-deacetylated GlcNAc-Ser might be present in *E. coli* and evade enzymes that have phosphorylated or otherwise modified structurally similar compounds (25–27). Thus, the result in Fig. 3 is hardly surprising given that *N*-acetylglucosamine is an integral component of the cell wall and a preferred carbon source for this organism (23).

For Fig. 4, we produced a variation of a standard assay to assess the activity of an orthogonal tRNA/aaRS pair (28). In such an experiment, an amber mutation is often incorporated into a gene essential for survival, and only the combination of a functional pair and the cognate amino acid will produce the necessary enzyme. However, our experiment only made the minor requirement of suppression of an amber stop codon in the gene for GFP. After 20 h there was no visible fluorescence in the presence of a methyl ester of *p*-iodoPhe. The result with free *p*-iodoPhe is a typical result using an efficient orthogonal pair (29). Here, the $tRNA_{CUA}$ is charged with the nonnatural amino acid by the mutant *M. jannaschii* tyrosyl aaRS, specific for that amino acid, and in-frame UAG codons are suppressed to produce full-length gene products (3). The fidelity for the *p*-iodoPhe aaRS is exquisite (16), the enzyme does not charge $tRNA_{CUA}$ with an endogenous amino acid such as tyrosine, and therefore, no suppression of the amber codon is observed in the absence of the amino acid. Again, in the absence of the noncanonical amino acid, the UAG codon is read as a stop codon, and translation terminates per the usual mechanism.

For Fig. 5, another previously reported aaRS was used in isothermal calorimetry measurements as part of our control experiments. The noncanonical amino acid *p*-acetylPhe was reported to be incorporated into proteins with high fidelity, and we have reproduced similar results many times (19). This model synthetase/amino acid pair was compared with the synthetase/amino acid pair reported by Zhang *et al.* (1) because the yield of recombinant protein with *p*-acetylPhe (3.6 mg/liter) was highly similar for recombinant protein produced with the glycosylated amino acids (1–4 mg/liter). The K_d for *p*-acetylPhe and its cognate aaRS was measured as 330–522 μM. This is comparable with other methods and the K_m values reported by those for noncanonical amino acids and *M. jannaschii* Tyr aaRS variants (24). The average K_d for the Zhang *et al.* (1) (S)1–90 aaRS and tyrosine was found to be nearer to 1660 μM. This is only a factor of three less than the *p*-acetylPhe/aaRS pair, but still adequate for a fully functional synthetase (30). The discrepancy in the K_d values with the (S)1–90 aaRS may be because of some inherent instability of the enzyme, as we routinely yielded less S(1)-90 aaRS from similar sized cultures; thus, enzyme instability may have contributed to the disparate ITC measurements with this enzyme. Under identical conditions used for *p*-acetylPhe, we were unable to detect any appreciable interaction between the Zhang *et al.* (1) (S)1–90 and GlcNAc-Ser with our instrument. We have been able to reliably measure K_d values in the range of 2500 μM, so we conservatively estimate that the value of K_d for (S)1–90 and GlcNAc-Ser has to be greater than 2500 μM. More importantly, the ITC measurements would seem to indicate that the (S)1–90 enzyme seems to retain considerable affinity for tyrosine, which stands in contrast to the original report which claimed exquisite fidelity of this enzyme for GlcNAc-Ser (1).

Synthetases with drastically decreased K_m values for noncanonical amino acids have been reported to produce full-length proteins under auxotrophic conditions, and this could provide an explanation for the incorporation of GlcNAc-Ser or Gal-

NAc-Thr. Yet even in these studies the amino acid analogues were highly similar to the canonical amino acid and did not require pre-translational processing, and the concentrations of the analogues were four times greater than those reported for the glycosylated amino acids (30). Furthermore, an obvious paradigm has now emerged regarding how the amber suppression method (24) continually succeeds (3) in surmounting nature's original solution for achieving exquisite fidelity in translation. The PDB data base now has at least nine *M. jannaschii* Tyr aaRS structures bound with various nonnatural amino acids, and together they provide the method with an obvious structural rationale for success (31–35). For example, the minimum requirement needed to reduce wild-type enzyme affinity for tyrosine involves mutating Tyr-32 and Asp-158 to amino acids less capable of hydrogen bonding to the tyrosine hydroxyl group. The mutant aaRS, labeled (S)1–90, was reported with the following mutations: E107P, D159C, I159Y, L162R (1). As there was no Tyr-32 mutation, it can be argued here that any reduction in hydrogen bonding to free tyrosine caused by the D158C mutation may have been partially restored with the proximal hydrogen bonding partners introduced with the I159Y and L162R mutations. The (S)1–90 aaRS also shares three mutations common to aaRSs evolved for *p*-benzoyl-L-phenylalanine, but not the critical Tyr-32 mutation required to reduce affinity for tyrosine (36). At a minimum, the glycosylated amino acids would seem to require at least one novel mutation to account for differences in side chain stereochemistry or the entropic penalty that would be required to move the hydrophilic saccharide rings of *O*-deacetylated GlcNAc-Ser or GalNAc-Thr deep into the hydrophobic pocket of the Tyr aaRS; on a basic level it could be argued that the conversion of a Tyr aaRS to an enzyme that preferentially binds a GlcNAc derivative is tantamount to converting a Tyr aaRS to a lectin binding or sugar transport protein (37). The mechanism used by lectin binding proteins typically involves shallow binding pockets and other sequestering methods (38, 39), which contrast sharply with the mechanism and function of a tyrosyl aminoacyl tRNA synthetase.

Using the mutations reported by Zhang *et al.* (1), it is difficult, but possible, to hypothesize how a Tyr aaRS might be capable of binding *O*-deacetylated GlcNAc-Ser, but it becomes increasingly difficult to suggest how such mutations would lead to a binding event for GlcNAc-Ser to the complete exclusion of tyrosine. Both the ITC data and Tyr aaRS structural analysis would seem to support the interpretation that the mutations introduced into the enzyme reported by Zhang *et al.* (1) would not radically alter nature's original solution to charging $tRNA^{Tyr}$ with tyrosine.

We next sought to find literature precedents to substantiate the notion that an *E. coli* esterase might deacetylate the glycosylated amino acids. The strongest evidence contrary to our hypothesis was found in a report characterizing an esterase from *E. coli* (21). However, even this study noted that *E. coli* could grow on triacetin as the sole carbon source only after the *E. coli* esterase gene was cloned into an expression plasmid and subsequently overexpressed to high intracellular levels using a strong isopropyl 1-thio-β-D-galactopyranoside-inducible promoter.

The literature relevant to our work shows that *E. coli* is a common host for examining foreign esterase genes. The references used to support our hypothesis were selected because they reported using *E. coli* to study foreign esterase activity *in vivo* (40). In such experiments culturing is commonly performed on triacetin as the sole source of carbon to explore and validate gene products for esterase activity (41, 42). Even with the simplest of esters, one study found that *E. coli* B and strains KO11 and K-12, "…lacked the ability to hydrolyze ethyl acetate and failed to utilize ethyl acetate as a sole carbon source for growth" (43). These studies support the premise that *E. coli* maintains low levels of endogenous esterases, and they stand to further contrast the reports from Zhang *et al.* (1) and Xu *et al.* (2), which seem to imply that comparable molecules are processed similarly by taxonomically distinct organisms. For example, in Zhang *et al.* (1) the authors' citations for nonspecific esterase activity in *E. coli* lead to reports wherein all the pertinent biological experiments were performed in mammalian cell lines (44, 45). There are reports of nonspecific esterases hydrolyzing GlcNAc peracetylated compounds similar to the ones used by Zhang *et al.* (1), but these experiments also employed mammalian cell lines (46, 47).

The previous works also reported generating a 4-GlcNAc-Ser myoglobin variant that tested positive in lectin binding assays. We have not reproduced this series of experiments, but we have found one report (48) that detailed the production of a 4-Tyr myoglobin variant having the same mass (18,433 Da), within experiment error, as the Zhang *et al.* (1) 4-GlcNAc-Ser myoglobin variant (18,430.1 Da).

If we attempt to rationalize the apparent contradiction in activity for the esterified glycosyl amino acids and what has typically been observed with esterified compounds in *E. coli*, the simplest explanation might be that the genotype of the various strains of *E. coli* was radically different. However, the genotypes of the strains used by Zhang *et al.* (1), the references we cite, and in our experiments (DH10B and BL21(DE3)) do not suggest that any esterase gene such as *aes* (21) has been mutated or deleted. Because *E. coli* is known to have genes with sequence homology to lipases (49), it may be that further experiments and discussion are warranted. However, of the greater than 30 nonnatural amino acids incorporated *in vivo* using *E. coli* as the host organism with a *M. jannaschii* tyrosyl aaRS mutants, only GlcNAc-Ser and GalNAc-Thr lack an aromatic side chain (3). As mentioned previously, the structural basis for the highly successful amber suppression method is now well understood, and similar crystallography efforts involving the glycosylated amino acids would be equally informative.

In conclusion, the genetic incorporation of either GlcNAc-Ser or GalNAc-Thr in *E. coli* may require a reevaluation of the pathways involved in processing the amino acids. If these amino acids have privileged access to a nonspecific esterase previously unknown in *E. coli*, then this may indeed stimulate further investigation. Ultimately though, the current paradigms for *E. coli* esterase activity and *M. jannaschii* Tyr aaRS specificity would both seem to conflict with the currently proposed mechanism needed to genetically encode the amino acids reported by Zhang *et al.* (1) or Xu *et al.* (2).

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