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Abstract	A large number of enzymes remain uncharacterized even in Escherichia coli (E.coli) K-12 whose genome has been known for over 15 years. Metabolomics can be useful to study such enzymes and particularly to identify their endogenous substrates. Here, we combined classical enzyme assay and metabolite profiling using Capillary electrophoresis-mass spectrometry (CE-MS) to characterize the activity of YhhY, a putative metal-inducible acetyltransferase. Screening using CoA assays revealed that the enzyme could transfer acetyl groups to the N-terminal of amino acids, preferably methionine, phenylalanine and histidine. The enzyme was found to be inhibited by thiol-reactive agents and kinetics date shows YhhY follows a ternary complex mechanism, and reaction rates differ among each amino acid. To confirm the in vitro results and rule out the possibility of activity resulting from a contaminating enzyme, we monitored metabolite changes by CE-MS in E.coli cells that overexpress YhhY. We observed elevated levels of acetylated methionine, phenylalanine and histidine in the overexpressing cells, confirming that the amino acid acetylating activity is connected with YhhY. Finally, in order to better understand the physiological activity of YhhY, we profiled metabolites in E.coli cells in which the yhhY gene has been disrupted. Following treatment with cobalt, we observed broad metabolite changes between wild type and knock out strain. The most significant changes were in nucleotide, pentose phosphate, and glutathione pathway intermediates. Overall, our results demonstrate that YhhY is an acetyltransferase displaying activity on several amino acids.
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Amino acid acetylation by the *E.coli* orphan enzyme YhhY.

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Summary

A large number of enzymes remain uncharacterized even in *Escherichia coli* (*E.coli*) K-12 whose genome has been known for over 15 years. Metabolomics can be useful to study such enzymes and particularly to identify their endogenous substrates. Here, we combined classical enzyme assay and metabolite profiling using Capillary electrophoresis-mass spectrometry (CE-MS) to characterize the activity of YhhY, a putative metal-inducible acetyltransferase. Screening using CoA assays revealed that the enzyme could transfer acetyl groups to the N-terminal of amino acids, preferably methionine, phenylalanine and histidine. The enzyme was found to be inhibited by thiol-reactive agents and kinetics date shows YhhY follows a ternary complex mechanism, and reaction rates differ among each amino acid.

To confirm the *in vitro* results and rule out the possibility of activity resulting from a contaminating enzyme, we monitored metabolite changes by CE-MS in *E.coli* cells that overexpress YhhY. We observed elevated levels of acetylated methionine, phenylalanine and histidine in the overexpressing cells, confirming that the amino acid acetylating activity is connected with YhhY. Finally, in order to better understand the physiological activity of YhhY, we profiled metabolites in *E.coli* cells in which the *yhhY* gene has been disrupted. Following treatment with cobalt, we observed broad metabolite changes between wild type and knock out strain. The most significant changes were in nucleotide, pentose phosphate, and glutathione pathway intermediates. Overall, our results demonstrate that YhhY is an acetyltransferase displaying activity on several amino acids.

Key Words

Escherichia coli, YhhY, Metabolomics, Capillary electrophoresis-mass spectrometry,

1. Introduction

Enzymes are important protein responsible for biological metabolism, and enzyme characterization has long been a principal field in biology (Yamada *et al.*, 2012). However, even in the model organism, *Escherichia coli* (*E.coli*), the function of only 30-40% of enzymes has not been experimentally verified (Chen and Vitkup, 2007). These orphan activities are today's bottleneck in molecular biology to understand living behavior. This is due to the difficulty of enzyme characterization. For example, some enzymes are expressed only limited condition like low pH or high temperature (Ma *et al.*, 2011).

Therefore, a new method using mass spectrometry, which allows faster assay of substances in the samples is extremely important for enzyme characterization. First report to the author's knowledge, Saghatelian *et al.*, characterized lipid synthesis activity using gene knockout mouse by mass spectrometry (Saghatelian *et al.*, 2005). Their method revealed enzyme activity by measuring living cell metabolome of gene knockout mouse. However if the targeted reaction has 1) reverse reaction, 2) Isozyme, 3) bypass pathway, gene deletion cannot always characterize the orphan enzyme. So, their method is not versatile approach. And then, Saito *et al.*, established Metabolic Enzyme and Reaction discovery by Metabolite profile Analysis and reactant IDentification (MERMAID) method as a solution (Saito *et al.*, 2006; 2009; 2010). And they revealed YihU of *E.coli* reduces succinate semialdehyde to gamma hydroxy butyrate depending on NADH by MERMAID method. They incubated some orphan enzyme in yeast extract as "Metabolite mixture" and measured decreased substrates and increased products by CE-MS. And Carvalho incubated RX1248c protein of Mycobacterium tuberculosis with Mycobacterium bovis extract and measure products with liquid chromatography-mass spectrometry. And they characterized as hydroxy-3-oxoadipix synthease (Carvalho *et al.*, 2010).

However one big dilemma is these methods take a lot of time to measure substrates and products. Even if untargeted approach can characterize without knowledge about target's enzyme, it takes a lot of time and throughput is low. Here, I introduce a new method using both traditional enzyme assay and mass spectrometry for orphan enzyme characterization (Figure 1, Figure 2). YhhY is selected as a model for establishing method. YhhY is predicted as acetyltransferase, but there are no experimental evidence and target amino acids are unclear. The goal of this study is to characterize YhhY.

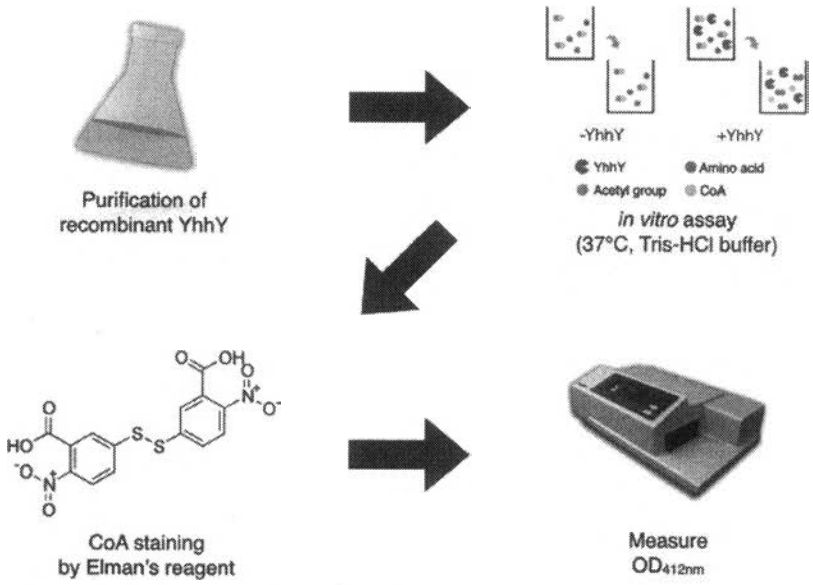

Figure 1 Scheme of *in vitro* assay

Four steps of *in vitro* assay. 1: Purification of recombinant protein from overexpressing cells. 2: *In vitro* enzyme assay using Acetyl CoA as acetyl group donor. 3: CoA staining using Ellman's reagent. 4: Measuring OD_{412}nm to quantify CoA.

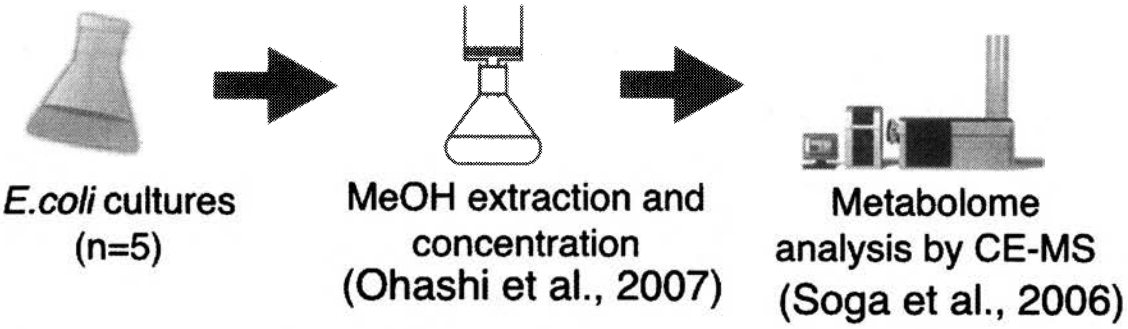

Figure 2 Scheme of metabolome analysis

Three steps of metabolome analysis. 1: *E.coli* culture using LB for YhhY overexpressing cells, M9 for *yhhY* deletion mutant.

2. Materials and Methods

2.1. Strains and conditions

JW3504 strain (Δ*yhhY*) and BW25113 strains were used as control strain (Baba *et al.*, 2006). Also I selected *yhhy* gene overexpression to obtain purified enzyme and metabolome analysis strain from ASKA clone (Kitagawa *et al.*, 2005). Control strain is prepared introducing plasmid pCA24N to AG1 strain. Culture for metabolome analysis is performed with 500ml flask. Glycerol stocks of each strains were streaked on 1.5% LB agar plate (10g tryptone, 5g east extract, 10g NaCl in 1L Milli-Q). Colonies were inoculated to 2ml fresh medium. After reaching stationary phase an aliqupt of medium inoculated to 50ml fresh medium, controlling the amount so that OD of the total volume equals 0.05 (110min-1, 37°C).

2.2. Purification of recombinant protein

Purification of recombinant protein was performed Overnight Express Autoinduction system (Merk). Protein purification was followed manufacture's instructions. Briefly, 2μl of saturated medium was inoculated to fresh 200μl LB medium after log phase and several solutions were appended according to the instruction manual. After 12 hours, medium was centrifuged (10,000rpm, 5min, 24 min.) and the supernatant discard. The cells were broken by lysis buffer (50mM HEPES, 300mM NaCl, 1mM Tween 20, 1 tablet /10ml complete Mini EDTA). After cells were lyzed, 30μl Magnehis™ Ni-Particles (Promega) ware appended. 10 minutes later beads were washed with washing buffer 1 (50mM HEPES, 300mM NaCl, 10mM Twee20, 80mM Imidazole) and 2 (50mM HEPES, 300mM NaCl, 10mM Twee20 80mM Imidazole 80mM DTT) twice each. The beads were stored at -20°C in storage buffer (50mM HEPES, 300mM NaCl, 10mM Tween20, 50% Glycerol) until usage. The confirmation of purified enzyme was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. In vitro assay

The beads with the enzymes were washed by washing buffer (50mM Tricine 20mM, KCl 5mM, $MgCl_2$, 0.2M MnCl, 0.1mM Ca_{2+}, 0.1mM $FeSO_4$, 0.1mM $ZnSO_4$) three times. The 40μl reaction solution (4μl washed enzyme beads, 10mM 20 amino acids, 10mM amino acids, 10mM Acetyl CoA, 50mM Tricine, 20mM KCl, 5mM MgCl2, 0.2M MnCl, 0.1mM Ca2+, 0.1mM FeSO4, 0.1mM ZnSO4, 1mM MES, 1mM 3AP) was incubated in the PCR tube at 37°C Overnight.

2.4. Transformation

Transformation was performed by chemical competent method. *E.coli* was inoculated to SOB medium (tryptone 2g, yeast extract 0.5g, NaCl 50mg, 0.4M KCl 625μl), and cultured at 30°C. After overnight culture, 50μl culture was inoculated to 5ml SOB medium at 30°C. After OD600 reached 0.440, cells were harvested (3000rpm, 15min, 4°C) and medium was reduced. 1.75 ml of TB buffer was added to the tube. After centrifuging (3000rpm, 15min, 4°C), the tube was added 30 μl DMSO. Plasmid was added 100μl competent cell and induce heat-shock (42°C, 45 seconds). 0.9ml SOC medium was added, and cultured 1 hour at 37°C without shaging.

2.5. Metabolome sampling, metabolite extraction and analysis

Sample preparation was performed using the minor modified method described by Ohashi *et al.*,(2008). Culture broth containing more than 20 OD (Absorbance (OD) × volume (ml)) was passed through a 0.45 μm pore size filter (Millipore). Cells were washed with 10 ml of Milli-Q water pre-incubated at 37°C (twice). Then, metabolism was stopped by immersing the membrane in 4 ml of methanol (4°C) containing 2 μM MES, 2 μM Trimesate, 2μM MS and 2μM 3AP as internal standard. Chloroform (4ml) and Milli-Q water (1.6ml) were added to the solution and fully mixed. The solution was centrifuged at 2,300g for 5 min at 4°C, and the separated methanol layer was filtered by centrifugation through a Millipore 5-kDa cutoff filter to remove high molecular weight compounds. Last of all, methanol was volatile by evaporator (Labconco). And These samples were stored at -80°C until metabolome analysis. The filtrate was dissolved in 50 μl Milli-Q water just before CE-TOFMS analysis.

2.6. Metabolome analysis

Metabolome analysis was performed using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). Metabolite values were normalized to unit OD_{600}. Cationic metabolites were separated using fused-silica capillary. Sheath liquid containing 0.1μM Hexakis (2,2-difluorothoxy) phosphazene for reference electrolyte in MeOH/water (50% v/v) was delivered at 10 μl/min. Sample solution was injected at 50mbar for 3 sec (less than 3nl). Anionic metabolites were separated using cationic polymer coated SMILE (+) capillary (Nacalai Tesque). Ammonium acetate (5 mM) in metanol/water (50% v/v) containing 0.1μM Hexakis (2,2-difluorothoxy) phosphazene for reference electrolyte. Sample solution was injected at 50 mbar for 30 sec (less than 30nl). In TOFMS, an automatic recalibration function was performed by using reference masses of reference standards. Exact mass data were acquired at the rate of 1.5 cycles/s over a 50 to 1,000 m/z range. The CE-TOFMS conditions have been described (Soga *et al.*, 2003; 2006). The raw data were processed house software for identification and quantification (Sugimoto *et al.*, 2010).

3. Results and Discussion

3.1. In vitro assay reveals YhhY acetylates 3 amino acids.

Next, I purified YhhY from *E.coli* cells overexpressing YhhY. Objective molecular size band was appeared by SDS-PAGE (Figure 3). And no contaminated protein appeared.

Purified YhhY was incubated with 20 amino acids and Acetyl CoA. And CoA produced by acetylation reaction was quantified using Ellman's reagent. Then, it was revealed that the enzyme could transfer acetyl

groups to the N-terminal of amino acids, preferably methionine, phenylalanine and histidine and reaction rates differ among each amino acid (Figure 5).

Figure 8 indicates relative amount of acetyl amino acids relative to internal standards. This results shows that YhhY can acetylate methionine, histidine and phenylalanine.

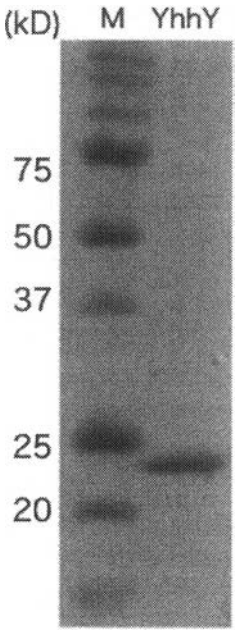

Figure 3 SDS-PAGE gel of recombinant protein
Left is maker, right is purified YhhY protein. 15% SDS-PAGE gel, 30mA, 30min.

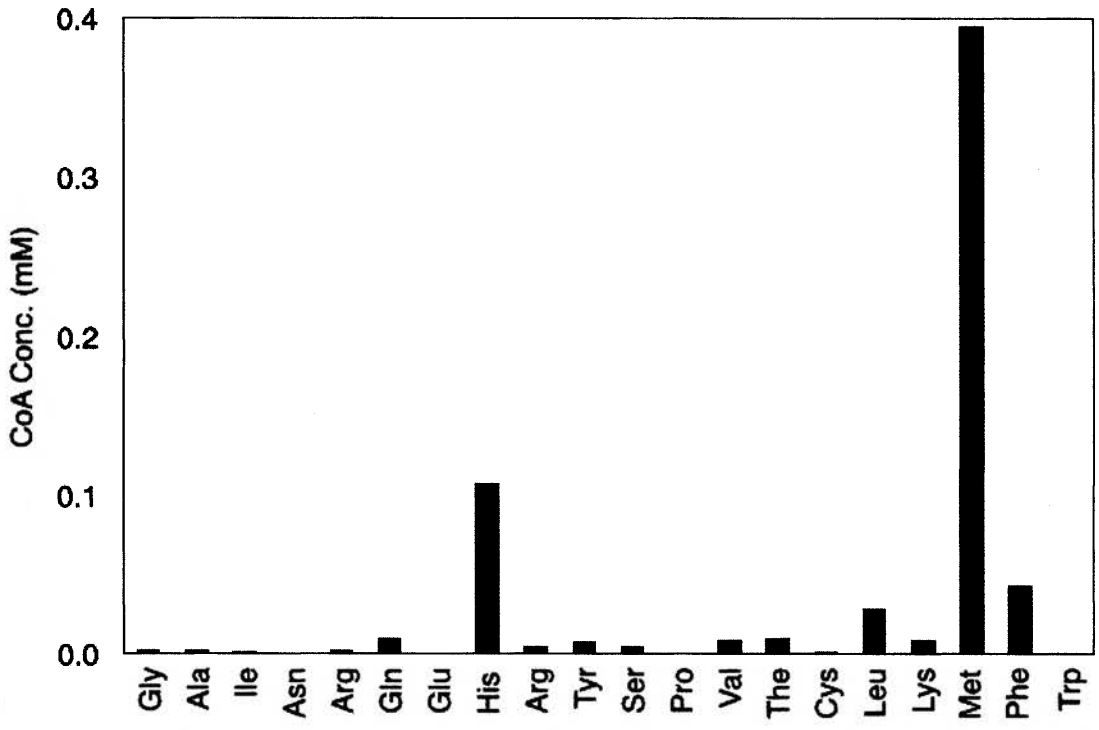

Figure 5 Amino acids acetylation by YhhY

Purified YhhY, 20 amino acids and Acetyl CoA were incubated at 37°C over night. CoA was stained by Elman's reagent. Value of blank (no amino acid) was subtracted from each value.

3.2. Kinetics of YhhY

To characterize YhhY in more details, its kinetic properties were examined. The data suggested that enzyme kinetics of YhhY follows ternary complex mechanism (Figure 6, Figure 7, Table 1, Figure 8). For example, YhhY may bind to methionine to form enzyme-substrate complex. The formation of this complex facilitates the binding of second substrate (Acetyl CoA) to form a ternary complex. Kinetic parameters were calculated as bellows;

Ki_{Met} = -1 / (X of primary plot 's intersection) = 9.41

Vmax = 1 / Y intercept of Or_{Met}= 0.021

Km_{Met} = (1- Y of primary plot 's intersection × Vmax) / Ki_{Met} = 0.0047

$Km_{Acetyl\ CoA}$ = slope of Or_{Met} × Vmax = 0.29

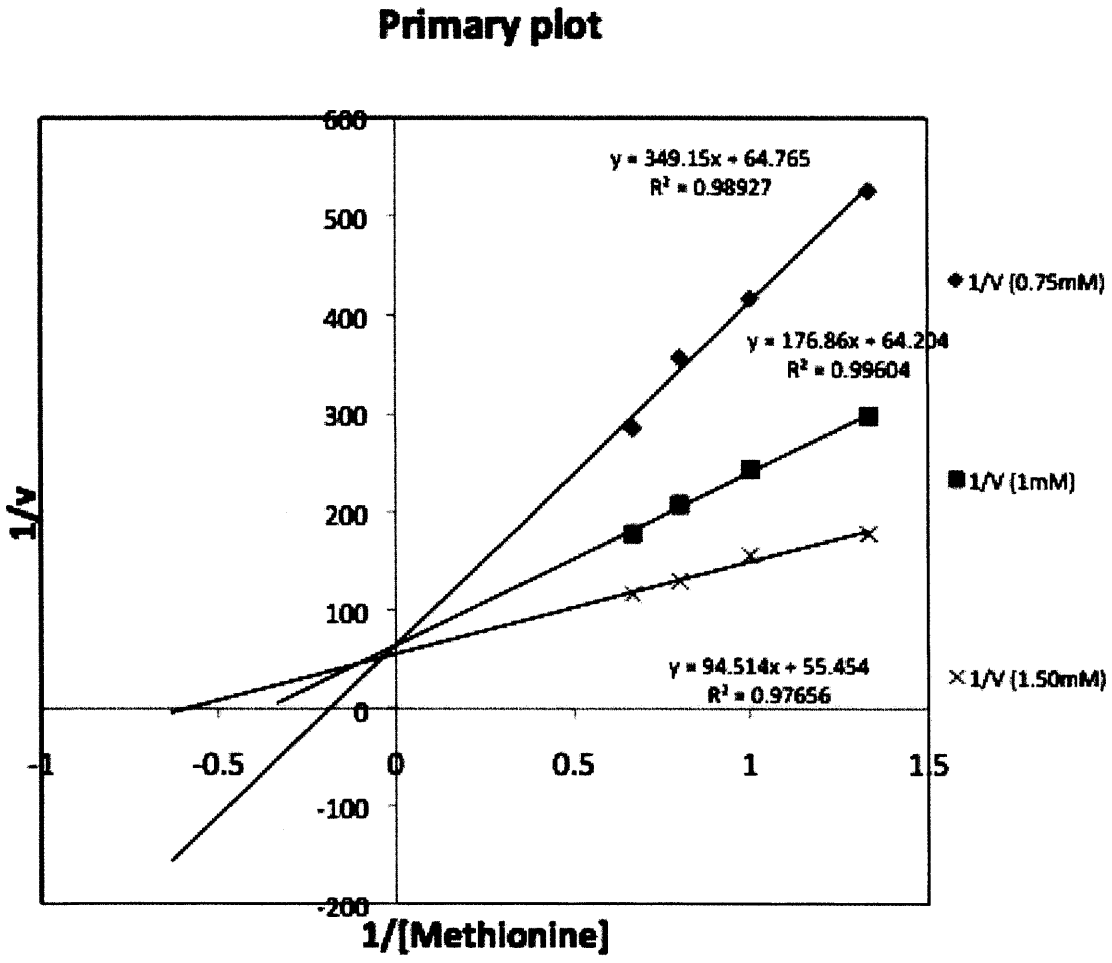

Figure 6 Lineweaver-Burk plot for YhhY

YhhY was incubated with Acetyl CoA (1mM) and Methionine (0.75, 1.00. 1.50 mM). And Lineweaver-Burk plot was draw. Color indicates Methionine concentration, Blue; 0.75mM, Red; 1.0mM, Purple; 1.50mM.

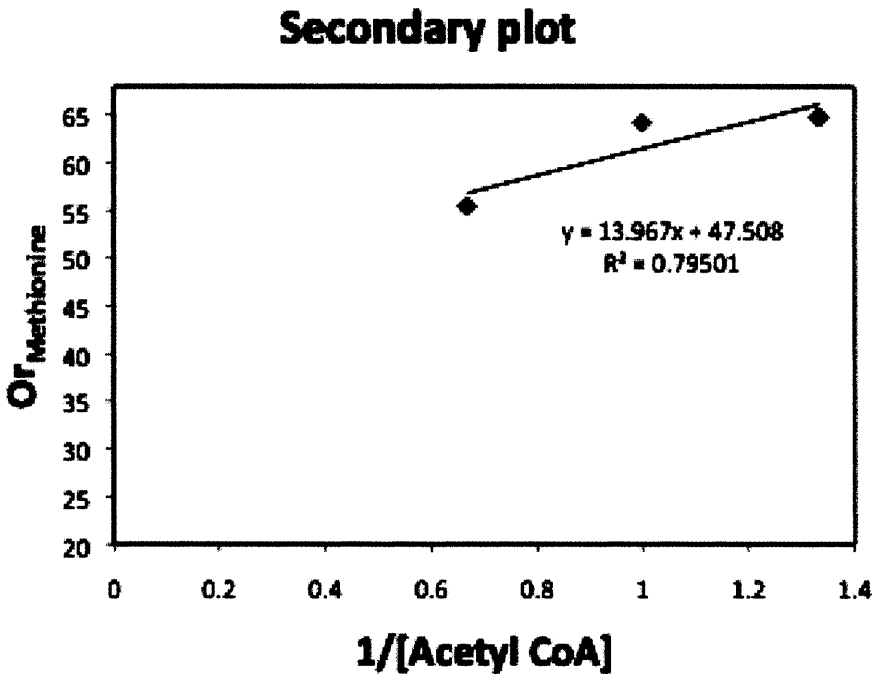

Figure 7 Ordinate plot of Figure 6 for $Km_{Acetyl\ CoA}$

To obtain $Km_{Acetyl\ CoA}$ secondary plot was draw. X-axis is 1/[Acetyl CoA], Y-axis is $Or_{methionine}$ (Y intercept of Figure 6).

Table 1 Kinetic parameters

Km_{met}	$Km_{Acetyl\ CoA}$	Vmax
0.0047	0.29	0.021

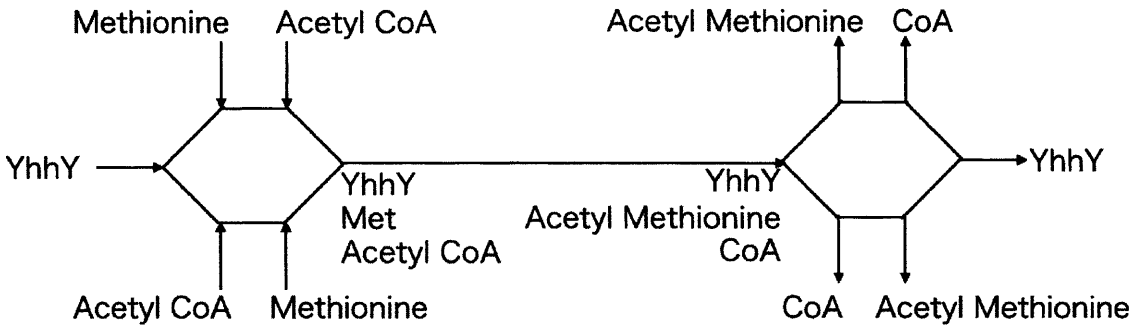

Figure 8 Ternary complex mechanism of YhhY

Free enzyme YhhY binds to first Methionine (or phenylalanine, histidine), the enzyme-substrate complex improves the binding of second substrate.

3.3. Inhibition by thiol reactive reagent

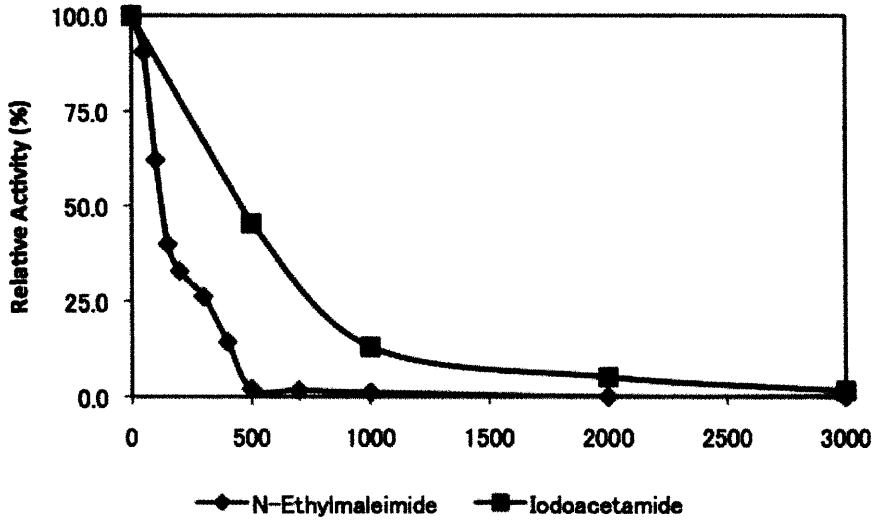

Figure 9 Inhibition by thiol reactive reagent
YhhY, Acetyl CoA and Methionine were incubated with N-Ethylmaleimide or Iodoacetamide (0 ~ 3000 μM).

Next to characterize enzyme activity, two inhibitors of known acetyltransferase activity were tested. Both N-Ethylmaleimide and Iodoacetamide are known to react with thiol group and modify cystein residues. Also, they inhibit acetyltransferase activity (Perez-Gil *et al.*, 1990). Then, YhhY activity was inhibited by both N-Ethylmaleimide and Iodoacetamide (Figure 9). Methionine was used as substrate for inhibition test. This results suggest N-Ethylmaleimide and Iodoacetamide inhibit YhhY activity and that YhhY has an active cysteine catalytic residue.

3.4. Confirmation of YhhY activity in vivo by metabolome analysis

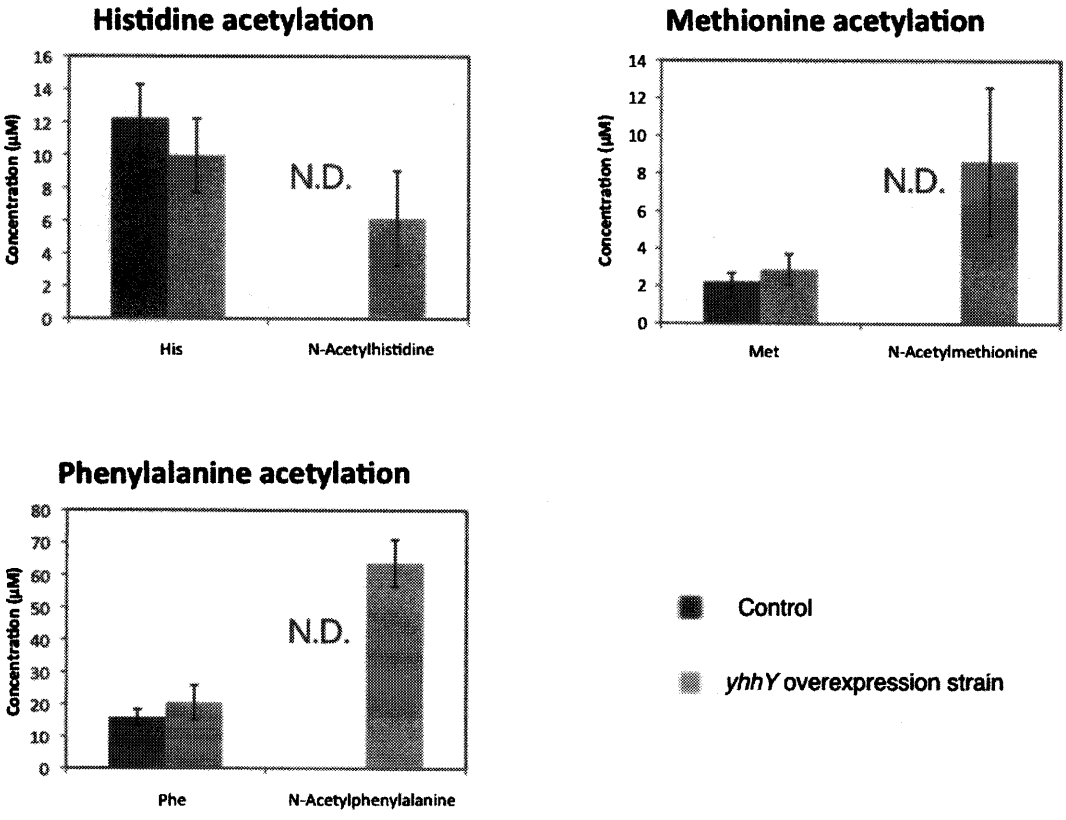

Figure 10 Accumulation cells of 3 compounds acetylated by YhhY *in vivo*

Accumulation of substrates and products catalyzed by YhhY. A: Histidine acetylation, B: Methionine acetylation, C: Phenylalanine acetylation. Black bar means control strain, grey bar means YhhY overexpression strain. Error bar indicates standard deviation (n=3). N.D. indicates not detected.

The three major acetylated products observed *in vitro* by YhhY were also detected in *yhhy* overexpression cell (Figure 8). These compounds were not detected in control strain, so the acetylation was likely to be caused by YhhY. These results support the hypothesis that YhhY acetylates several amino acids *in vivo*.

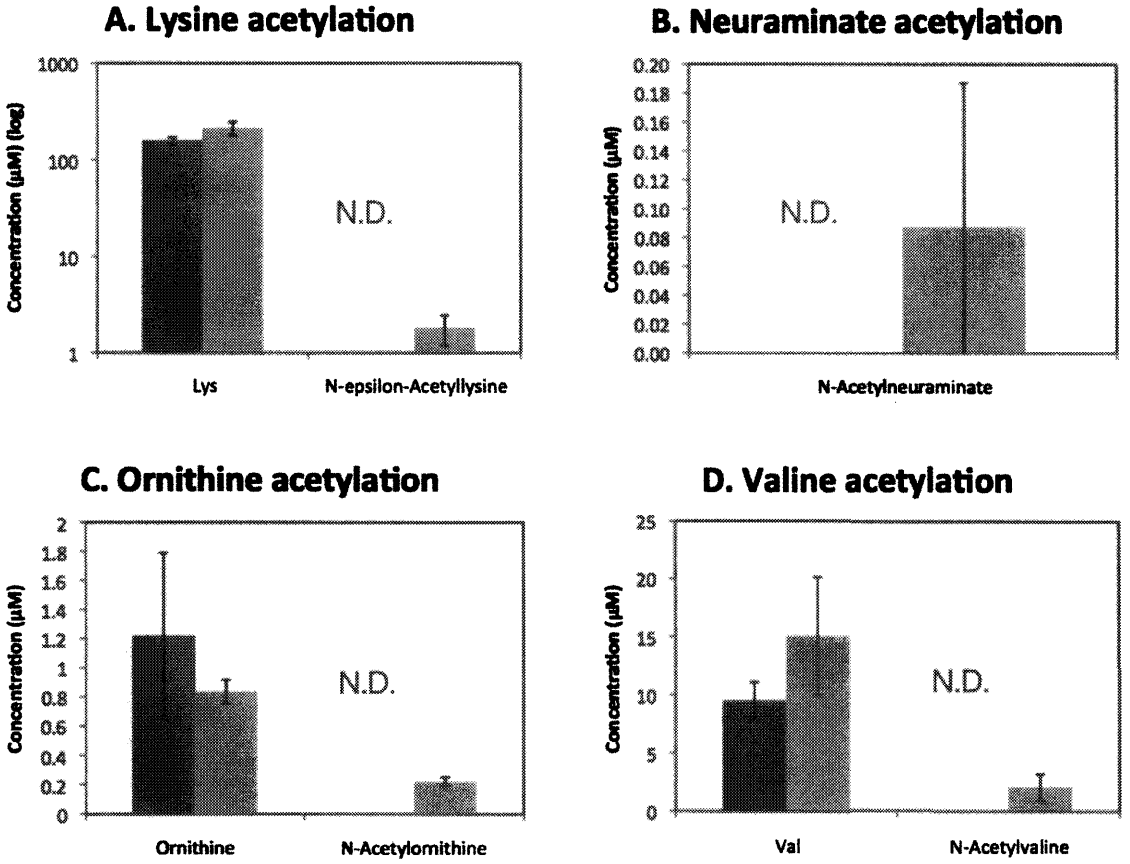

Figure 11 Accumulation of possible products acetylated by YhhY *in vivo*

Accumulation in vivo of possible product acetylated by YhhY. y-axis indicates concentration (μM). Black bar indicates control strain, grey bar indicates YhhY overexpression strain. A: lysine acetylation, B: Neuraminate acetylation, C: Ornithine acetylation, D: Valine acetylation. Error bar indicates standard deviation (n=3). Neuraminate was not able to be detected because we had no standard. N.D. means not detected.

Acetyllycine, Acetylneuraminate, Acetylornithine and Acetylvaline were detected not in the control strain but in the overexoression strain (Figure 9). These compounds also seemed to be acetylated by YhhY. However lysine and valine acetylation were not detected *in vitro* assay. This result may be due differences in conditions and concentrations between the in vitro and *in vivo* assays.

3.5. Metabolome analysis of yhhY deletion mutant

Last of all, in order to better understand the physiological activity of YhhY, we profied metabolites in *E. coli* cells in which the *yhhY* gene has been disrupted. Then, Previous report suggested *yhhY* gene expression was induced by metal stress (Nichols *et al.*, 2011). Following treatment with cobalt, the data shows broad metabolite changes between wild-type and knock-out strain (Figure 12). The most significant changes were in nucleotide, pentose phosphate, and glutathione pathway intermediates. This fact suggests strong connection between *yhhY* gene and cobalt resistance. Thus, metabolome analysis of wild type and *yhhY* gene deletion mutant revealed metabolic differences following cobalt treatment.

4. Conclusion

The author tried to characterize *E.coli* orphan enzyme YhhY using both generic enzyme assays and CE-MS based metabolomics approach. First, purified YhhY was incubated with 20 amino acids and Acetyl CoA. And CoA produced by acetylation event was stained and quantified by Ellman's reagent. The results revealed that YhhY could acetylate preferentially three amino acids. Methionine, histidine and phenylalanine were the major acetylated products. Next, metabolome analysis of *yhhY* gene overexpressed strain revealed only the strain overexpressing YhhY contained significant levels of Acetylmethionine, Acetylhistidine and Acetylphenylalanine. This results shows YhhY acetylates free amino acids *in vivo*. Then, metabolome analysis of *yhhY* deletion mutant revealed neither wild type nor Δ*yhhY* mutant accumulated these acetyl amino acids. And difference between wild type and mutant was not big. However, after cobalt treatment, drastic change in metabolome was occurred. These result is consistent with precious report that *yhhY* is metal-induced enzyme (Vassinova *et al.*, 2000; Nichols *et al.*, 2011). Thus, I characterized *E.coli* orphan enzyme YhhY using traditional enzyme assay and modern technology, CE-MS based metabolomics. These results presented here contribute to expand our knowledge on *E. coli* metabolic function and its response to metal stress.

5. Acknowledgements

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