Supporting Information

Sirtuin-dependent reversible lysine acetylation of glutamine synthetases reveals an auto-feedback loop in nitrogen metabolism

Di You[1], Bin-Cheng Yin[1], Zhi-Hai Li[1], Ying Zhou[1], Wen-Bang Yu[1], Peng Zuo[1], Bang-Ce Ye[1,2]*

[1]Laboratory of Biosystems and Microanalysis, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China.
[2]School of Chemistry and Chemical Engineering, Shihezi University, Xinjiang, 832000, China

SI Appendix

SI Materials and Methods

Bacterial strains, growth conditions, and reagents

Saccharopolyspora erythraea wide-type strain NRRL2338 (from DSM 40517), Δ*glnR*, Δ*acuA* and Δ*srtN* strains were used in this study. *S. erythraea* strains were grown in TSB (Tryptone Soya Broth) medium or minimal medium (Evans) containing 25 mM TES (N-(Tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid sodium salt), 2 mM citric acid, 10 mM KCl, 0.25 mM $CaCl_2$, 1.25 mM $MgCl_2$, 2 mM Na_2SO_4, 1 mM Na_2MoO_4, 0.5% trace elements(0.02 mM $MnSO_4{\cdot}4H_2O$, 6 µM $ZnSO_4{\cdot}7H_2O$, 0.02 mM H_3BO_3, 1 µM KI, 2 µM $Na_2MoO_4{\cdot}2H_2O$, 0.05 mM $CuSO_4{\cdot}5H_2O$, 0.05 mM $CoCl_2{\cdot}6H_2O$), 2.5% (m/v) glucose, 2 mM NaH_2PO_4, and 2 mM /75 mM (nitrogen concentration) NH_4Cl (pH 7.2). Aerobic 100 ml batch cultures were grown in 1 L flasks at 30 °C on a rotary shaker at 250 rpm. Cultures were inoculated to an optical density (OD600) of 0.05 units with exponentially growing precultures.

All strains and plasmids used in this work are listed in Table S3. All media, growth conditions, and *S. erythraea* genetic techniques (construction of mutant strains) have been described previously (1,2). Acetyl lysine antibody (Cat# ICP0380) and acetylated BSA (Cat# ICP6090) were from ImmuneChem Pharmaceuticals Inc. (Burnaby, British Columbia, Canada). Trichostatin A (TSA) was purchased from Wako Chemicals (Japan). Protein A-conjugated-agarose beads were from Amersham Biosciences.

Overproduction and purification of proteins *in vitro*

All genes were amplified by PCR from the genomic DNA of *S. erythraea*. The primers used in this work are listed in Table S4. After restriction digest, the genes coding for GlnA1 (SACE_1623), GlnA2 (SACE_1613), GlnA3 (SACE_3095), GlnA4 (SACE_5355), GlnR (SACE_7101), *AcuA, SrtN, Sco*_GlnA1 (SCO2198), *Sco*_GlnR (SCO4159), *Msm*_GlnA1 (MSMEG_4290), and *Msm*_GlnR (MSMEG_5784) were cloned into pET-28a(+). The proteins were expressed using *E. coli* BL21(DE3) strain. A single colony was selected to start a 5 mL overnight culture, which was then used to inoculate 50 mL of lysogeny broth (LB) medium supplemented with 1‰ kanamycin. The cells were grown at 37 °C to about 0.7 OD_{600}, and then induced overnight with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 37 °C for 8 h.

Cells were harvested by centrifugation and re-suspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM $KHPO_4$). The cells were disrupted by sonication, and cell debris removed by centrifugation at 8000 g for 15 min. The resulting supernatants of GlnA1, GlnA2, GlnA3, GlnA4, GlnR, *Sco*_GlnA1, *Sco*_GlnR, *Msm*_GlnA1 and *Msm*_GlnR were loaded onto a 2 ml Ni-NTA agarose column (Merck) that was pre-equilibrated with the binding buffer. After discarding the flow through, the column was washed with 20 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0), and bound proteins were eluted using a linear gradient from 20 to 250 mM imidazole in 50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0. The fractions were analyzed by SDS-PAGE. The protein concentration was determined by the BCA method using bovine serum albumin as the standard.

In vitro glutamine synthetase (GS) assays

The biosynthetic assay, which measures the formation of γ-glutamyl hydroxamate from glutamate, hydroxylamine, and ATP was performed at pH 7.3 as described (3). The Mg^{2+}-dependent forword reaction was assayed in 0.3 ml final volume cotained 100 mM Tris (pH 7.4), 80 mM $MgSO_4{\cdot}7H_2O$, 20

mM Sodium glutamate, 20 mM L-Cysteine, 2mM EGTA, 80 mM Hydroxylamine, 40 mM ATP. The mixture was incubated at 37 °C for 30 min. After that, the color agent was added which cotained 0.2M TCA, 0.4 M $FeCl_3 \cdot 6H_2O$, 5% (v/v) concentrated hydrochloric acid. The absorbance was determined by microplate reader (Biotek, USA) in 540 nm. The enzyme activity was represented by the formation of γ-glutamyl hydroxamate per protein amount and time.

***In vitro* protein acetylation assays**

To determine whether GlnA1 and GlnA4 were substrates for *Sac*AcuA, 0.2 μM purified AcuA protein or BSA, and 5 μM purified unacetylated GlnA1 or GlnA4 proteins were added to a reaction mixture (200 μl total volume) contained 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5), 200 μM *tris*(2-carboxyethyl) phosphine (TCEP) hydrochloride, and 20 μM Ac-CoA. Reactions were incubated at 37 °C for 2 h. After reaction, the *Sen*_GlnA1 protein samples were divided into two portions: one portion was analyzed by SDS-PAGE and Western blot, and the other was used for measurement of the GS activity. The acetylated GlnA was isolated by SDS-PAGE and then analyzed by LC-MS/MS.

***In vitro* deacetylation assays**

Samples containing 5 μM unacetylated GlnA1 and GlnA4 were first incubated with 0.2 μM *Sac*AcuA, and 20 μM Ac-CoA at 37 °C for 2 h. After acetylation reaction, AcuA was removed using GST-Bind agarose and acetylated GlnA1 or GlnA4 was isolated from the reaction mixture by ultrafiltration. To examine whether acetylated GlnA was deacetylated, the purified acetylated GlnA protein was added to 50 mM HEPES (pH 8.5) buffer containing 1 mM $MgCl_2$, 1 mM NAD^+, and 0.5 μM SrtN. The mixture was incubated at 37 °C for 3 h. The samples were divided into two portions: one portion was resolved by SDS-PAGE and analyzed by Western blot, and the other was used for measurement of the glutamine synthetase activity.

***In vitro* acetylation with Ac-CoA/ acP**

2 μM of purified unacetylated GlnA1/GlnA4 was incubated at 37 °C for 6 h with 2 mM Ac-CoA in the presence of 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0) (4). The reaction was terminated by the addition of SDS loading buffer, and then separated by 10% SDS-PAGE. The acetylation level was analyzed by western blot using acetyl lysine antibody, HRP conjugates (anti-AcK; Immunechem). The acetylated GlnA1/GlnA4 was isolated by SDS-PAGE and then analyzed by LC-MS/MS.

2 μM of purified unacetylated GlnA1/GlnA4 and 20 mM acP were incubated at 37 °C in 150 mM Tris-HCl (pH 7.3 at room temperature), 10% glycerol, 10 mM $MgCl_2$, and 150 mM NaCl at the indicated amount of time. To stop the reaction, an equal volume of 3X SDS loading buffer was added and the reactions were heated at 95 ˚C for 10 minutes (5). And then the samples were separated by 10% SDS-PAGE. The acetylation level was analyzed by western blot using acetyl lysine antibody, HRP conjugates (anti-AcK; Immunechem). The acetylated GlnA1/GlnA4 was isolated by SDS-PAGE and then analyzed by LC-MS/MS.

Western blot analysis

The protein concentrations of the samples were determined using BCA Protein Assay Kit (TIANGEN) with BSA as the standard. Protein samples were isolated by SDS-PAGE and then transferred to a PVDF membrane for 30-60 min at 100 V. The membrane was blocked at 24 °C in 1 x TBST (20 mM Tris-HCl, pH

7.5, 150 mM NaCl, and 0.1% Tween-20) containing 5% non-fat dry milk (NFDM) for 2 h. Anti-acetyl-lysine(hereafter Anti-AcK) antibody which was conjugated with horseradish peroxidase and diluted 1:15000 in TBST/0.5% NFDM was used. After incubation at 4 °C for overnight, the blot was washed with TBST for 3 times. The ECL system (CTB, USA) was used for signal detection according to the manufacturer in conjunction with a luminescent image analyzer, Bio-Imaging Systems (DNR Bio-Imaging Systems, ISRAEL).

Site-Directed Mutagenesis of GlnA acetylated-site mutants

The acetylated-site mutants (K319Q, K319R, K179R, K179Q, K357R, K357Q, 2KR, and 2KQ) were introduced into the pET28a(+)::*senglnA1* plasmid using the Fast mutagenesis system (Transgen Biotech, China) with the primers listed in Table S5. The mutations were confirmed by DNA sequencing.

Immunoprecipitation and Immunoblotting

Cells of *S. erythraea* wild type strain was grown in TSB (Tryptone Soya Broth) medium for immunoprecipitation was harvested by centrifugation at 3000 g for 30 min, after ground and resuspended in PBS buffer in the presence of protease inhibitors (1 mM phenylmethanesulfonyl fluoride and Complete EDTA-free Protease Inhibitor Cocktail Tablets, Roche) and histone deacetylase inhibitors (100 μM Trichostatin A, 50 mM nicotinamide and 50 mM sodium butyrate). The cells were disrupted by sonication, and cell debris removed by centrifugation at 8000 g for 15 min. For immunoprecipitation, lysates (about 300 μg) were incubated with 2 μg of anti-GlnA1 antibody (Abmart, Shanghai) at 4 °C for 2 h, followed by the addition of 40 μl Protein A-Agarose (Santa Cruz Biotechnology, INC) overnight. After four washes with PBS washing buffer at 4 °C, bound proteins were eluted by boiling in SDS sample buffer, resolved by SDS-PAGE and then subjected to Western analysis. Primary antibodies used were: anti-GlnA1 antibody and acetyl lysine antibody, HRP conjugates (anti-AcK; Immunechem). Secondary antibodies were purchased from Abmart. Binding was visualized with the ECL Western Blotting method. Afterwards ECL detection, films were scanned by MF-ChemiBIS 3.2 (DNR Bio-Imaging Systems, ISRAEL) and quantified with Image J software.

Overexpression of GlnA and its mutants from *S. erythraea*

GS-overproducing strains were generated by protoplast transformation with pIB139-1623 or pIB139-5355 (Table S3). The plasmid, pIB139-1623 or pIB139-5355, was integrated onto *S. erythraea* genome by phiC31. The apra-resistance was examined by culture in plate added into apra 24 h after transformation. Selected mutants were verified by PCR and DNA sequencing.

With *S. erythraea* genomic DNA as a template, the genes of GlnA1^{WT} and its mutant genes (GlnA1^{K357Q}, GlnA1^{K179Q} and GlnA1^{2KQ}) was amplified by PCR using the primer pairs 5'GGAATTCCATATG CATCATCATCATCATCAT GTGTTCAAGAATCCAGACGAGG3' and 5'TGCAGGATATC CATCATCATCATCATCAT TCACACGTCGAAGTACAGCGC3'. The PCR products were respectively digested with EcoRV and NdeI, and inserted into the corresponding sites of integrative plasmid pIB (gifted from Anhui University), yielding pIB1623^{WT}, pIB1623^{K357Q}, pIB1623^{K179Q} and pIB1623^{2KQ}. By PEG-mediated protoplast transformation, pIB1623 was introduced into the *S. erythraea* wild type/Δ*glnR*/Δ*acuA*/Δ*srtN* strain. The overexpression strains were obtained by apramycin resistance screening and confirmed by PCR analysis with the primers apr-F and apr-R (Table S4). The GlnA4 overexpression strain was obtained the same way as GlnA1 .

The GlnA-overexpression strains grew on TSB medium were harvested by centrifugation at 3000 g for 30 min, after ground and resuspended in PBS buffer in the presence of protease inhibitors (1 mM phenylmethanesulfonyl fluoride and Complete EDTA-free Protease Inhibitor Cocktail Tablets, Roche) and histone deacetylase inhibitors (100 μM Trichostatin A, 50 mM nicotinamide and 50 mM sodium butyrate). The cells were disrupted by sonication, and cell debris removed by centrifugation at 8000 g for 15 min. Lysates for protein purification were purified with Ni-NTA agarose column (Merck) as above. The purified protein concentration was determined by the BCA method using bovine serum albumin as the standard. Samples with same amount were analyzed by SDS-PAGE and then subjected to Western analysis. Primary antibodies used were: anti-His antibody and acetyl lysine antibody, HRP conjugates (anti-AcK; Immunechem). Secondary antibodies were purchased from Abmart. Binding was visualized with the ECL Western Blotting method. Afterwards ECL detection, films were scanned by MF-ChemiBIS 3.2 (DNR Bio-Imaging Systems, ISRAEL) and quantified with Image J software. The purified GlnA1/GlnA4 from *S. erythraea* wild type sreains and Δ*acuA* strains was then analyzed by LC-MS/MS spectrometry.

RNA preparation and Real-time RT-PCR

S. erythraea NRRL 23338 and its mutants Δ*glnR* and Δ*acuA* strains were grown for 3 days at 30 °C in either the nitrogen-rich TSB medium (Tryptic Soy Broth) or nitrogen-limited N-Evans medium with NH_4Cl or Gln as unique nitrogen resource. *S. erythraea* strains with overexpression of GlnA and its mutants, including the control strain were grown for 24 h at 30 °C in nitrogen-limited N-Evans medium with 2 mM NH_4Cl as unique nitrogen resource. Cell pellets were collected after 20 min of centrifugation at 3000 rpm. Total RNA was prepared using RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA integrality was analyzed by 1% agarose gel electrophoresis and the RNA concentration was determined by microplate reader (BioTek, USA). Total RNA extracted from liquid cultures (1 μg) was reverse transcribed using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan) for RT-PCR and real-time RT-PCR, the DNase digestion was performed to remove genomic DNA before reverse transcription for 5 min at 42 °C. All this procedures above are following the manufacturer's instructions. PCR reactions were performed with primers listed in Table S6. The PCR products were quantified with 1% agarose gels and DNA was visualized by ethidium bromide staining.

For Real-time RT-PCR, SYBR premix Ex Taq™ GC Kit (Perfect Real Time, Takara) was used and about 100 ng cDNA was added in 20 μL volume of PCR reaction. The PCR was conducted using CFX96 Real-Time System(Bio-Rad,USA) and the PCR conditions were 95 °C for 5 min; then 40 cycles of 95 °C for 5 s, 60 °C~64 °C for 30 s; and an extension at 72 °C for 10 min.

Growth analysis

S. erythraea wild type strains with overexpression of GlnA1 and its mutants, including the control strain were grown in triplicate at 30 °C in nitrogen-limited N-Evans medium with 2 mM NH_4Cl as unique nitrogen resource. Growth was analyzed using a microplate reader (BioTek Instruments, Winooski, VT, USA). Cell density measurements at 600 nm were acquired every 8 h. Data were analysed using the GraphPad Prism 5 software package (GraphPad Software).

Mass spectrometry peptide fingerprinting

Protein digestion was performed according to the FASP procedure described by Wisniewski *et al.*(6). Briefly, the protein pellet (about 30 μg) was solubilized in 30 μl SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0) at 90 °C for 5 min. The detergent, DTT and other low-molecular-weight

components were removed using 200 µl UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) by multiple ultrafiltration (Microcon units, 30 kD). Then 100 µl 0.05 M iodoacetamide in UA buffer was added to block reduced cysteine residues and the samples were incubated for 20 min in darkness. The filter was washed with 100 µl UA buffer three times and then 100 µl 25 mM NH_4HCO_3 twice. Finally, the protein suspension was digested with 2 µg trypsin (Promega) in 40 µl 25 mM NH_4HCO_3 overnight at 37 °C, and the resulting peptides were collected as a filtrate.

Tryptic digests (approximately 30 µg of pre-digested protein) were solid phase extracted and analyzed by mLC–MS/MS using a Micromass (Waters) Q exactive spectrometer (Thermo Finnigan, San Jose, CA) to locate protein acetylation sites. Chromatography of peptides prior to mass spectral analysis was accomplished using HPLC. Columns were made using lengths of fused silica tubing (0.15 mm OD, 150 mm ID) with pulled tips (1 mm orifice) that were packed with Zorbax 300SB-C18 peptide traps (Agilent Technologies, Wilmington, DE). An Agilent HPLC delivered solvents A: 0.1% (v/v) formic acid in water, and B: 0.1% (v/v) formic acid in acetonitrile (84% v/v), 0.1% formic acid at either 1 ml/minute, to load sample, or 150–200 nl/minute, to elute peptides over a 50 minute 4% (v/v) B to 50% B gradient; another 4 minute 50% (v/v) B to 100% B gradient; and a 6 minute 100% (v/v) B. As peptides eluted from the HPLC-column/electrospray source, MS/MS spectra were collected.

MS/MS spectra were searched using MASCOT engine (Matrix Science, London, UK; version 2.2) against Uniprot Saccharopolyspora_NRRL23338 database (7165 sequences, download July. 1st, 2013). For protein identification, the following options were used. Peptide mass tolerance=20 ppm, MS/MS tolerance=0.1 Da, Enzyme=Trypsin, Missed cleavage=2, Fixed modification: Carbamidomethyl (C), Variable modification：Oxidation(M)，Acetylation (K, N-terminal). Decoy database pattern=Reverse. All reported data were based on 99% confidence for protein identification as determined by false discovery rate (FDR) $\leqslant$ 1%.

Electrophoretic mobility shift assay (EMSA)

The upstream region (-300 to +50) of genes predicted to contain a GlnR-binding site were amplified by PCR with gene-specific primers containing the universal primer (5’ AGCCAGTGGCGATAAG 3’) sequence (Table S6) and biotin-labeled by PCR with the 5’ biotin-modified universal primer. The PCR products were analyzed by agarose gel electrophoresis and purified by the PCR Purification Kit (Shanghai Generay Biotech Co., Ltd). The concentration of biotin-labeled DNA probes was determined with microplate reader (Biotek, USA). Electrophoretic mobility shift assays (EMSAs) were carried out according to the protocol accompanying Chemiluminescent EMSA Kit (Beyotime Biotechnology, China). The binding reaction contained 10 mM Tris·HCl pH 8.0, 25 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P40, 50 µg·ml^{-1} poly[d(I-C)], 10% glycerol. After binding, the samples are separated on a non-denaturating PAGE gel in ice-bathed 0.5 × Tris-borate-EDTA at 100 V and bands are detected by BeyoECL Plus.

Cross-Linking Experiments.

The cross-linking experiments were performed as described (7). The buffer for cross-linking reactions contained 20 mM Bicine (pH 8.3), 150 mM NaCl, 10 mM $MgCl_2$, 0.2% N-octyl glucoside, and 8% glycerol. After incubation at 25 °C for 30 min, reactions were initiated by adding DMS to a final concentration 15 mM. After incubation for 1h, the reactions were quenched by the addition of 0.1 volumes of 2 M Tris. Samples were fractionated by electrophoresis on 5% SDS-PAGE gels. Proteins were transferred to a

polyvinylidene difluoride membrane and probed with anti-GlnR antiserum. Protein bands were visualized with a secondary antibody conjugated to horseradish peroxidase and the chemiluminescent substrate luminol. All experiments were performed at least twice.

Circular Dichroism Spectroscopy and Dynamic Light Scattering.

The far-UV CD spectra were recorded at 20 °C using a Chirascan Plus instrument (Applied Photophysics). The spectra were collected from 190 to 260 nm using a rectangular quartz cell with a 1 mm path length. The proteins (0.2 mg/mL) were dissolved in a modified PBS buffer (pH 7.4) cotained 1.4 M KF, 100 mM K_2HPO_4, 18 mM KH_2PO_4. The spectra were analyzed for secondary structure content using CDNN CD spectra deconvolution software (Applied Photophysics). DLS measurements were performed with Zetasizer Nano ZS (Malvern, Herrenberg, Germany). Solvent-resistant micro cuvettes (ZN0040, Malvern, Herrenberg, Germany) have been used for experiments with a sample volume of 40 µL. The measurements were made at a fixed position with an automatic attenuator and at a controlled temperature. The samples were kept in PBS buffer. For each sample, three measurements were averaged.

Tryptophan Fluorecence and Surface Hydrophobicity.

The intrinsic tryptophan fluorescence spectra of the proteins (0.05 mg/mL) in 50 mM phosphate buffer (pH 7.5) at 25 °C were recarded using microplate reader (Biotek, USA) (8). The excitation wavelength was set to 295 nm, and the emission spectra were recarded between 315 and 400 nm. The surface hydrophobicity of the proteins (0.05 mg/mL) was measured using 10 µM 2-p-toluidinonaphthalene-6-sulfonate (TNS) (emission at 350-520 nm and excitation at 320 nm) (8). The data were collected at a 0.5 nm wavelength resolution.

Microscale Thermophoresis Measurements.

The thermophoresis measurement setup has been described previously (9). Here we used Monolith NT^{TM} Protein Labeling Kit (Nano Temper) to label protein and MST-NT.115 (Nano Temper) to measure the thermophoresis. GlnR was labeled by NT.647:NHS- and kept 100 nM unchanged. Label-free acetylatedatedated GlnA1 and native GlnA1 were assigned to 16 different concentrations which ranged from 0.26 nM to 8400 Nm. After a short time of binding reaction, the samples were loaded into the MST capillary and the thermophoresis was measured by Monolith.NT115. The buffer used in the experiments was PBS (pH 8.0) with 0.05% Tween-20.

Reference

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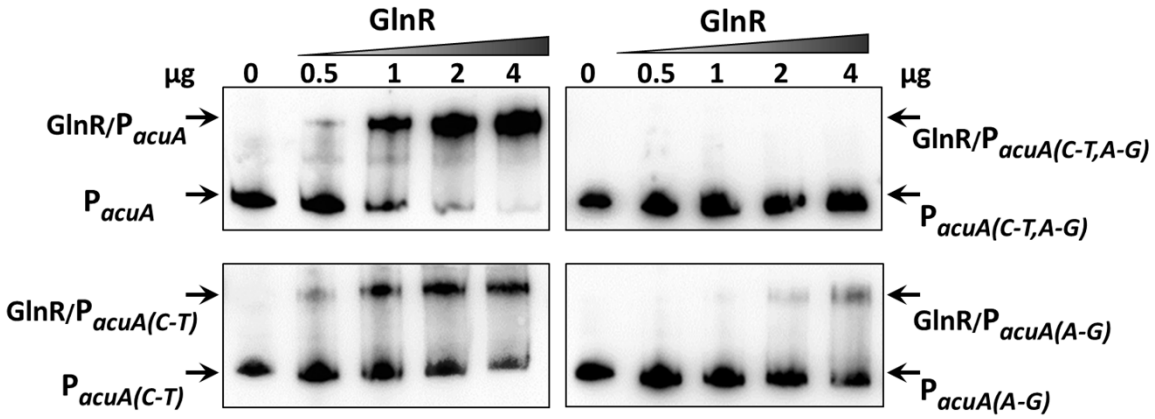


Figure S1. EMSAs of purified His-GlnR to the *acuA* promoter region with GlnR box or its variants. Equal amount of DNA probes (Free DNA, 300 bp) containing the GlnR-box or variants were incubated with GlnR and a 200-fold excess of nonspecific competitor DNA (sperm DNA).

P_{acuA}	5’- TTCA**C**GCGGCGGAA**A**C - 3’
$P_{acuA(C-T)}$	5’- TTCA**T**GCGGCGGAAAC - 3’
$P_{acuA(A-G)}$	5’- TTCACGCGGCGGAA**G**C - 3’
$P_{acuA(C-T,A-G)}$	5’- TTCA**T**GCGGCGGAA**G**C - 3’

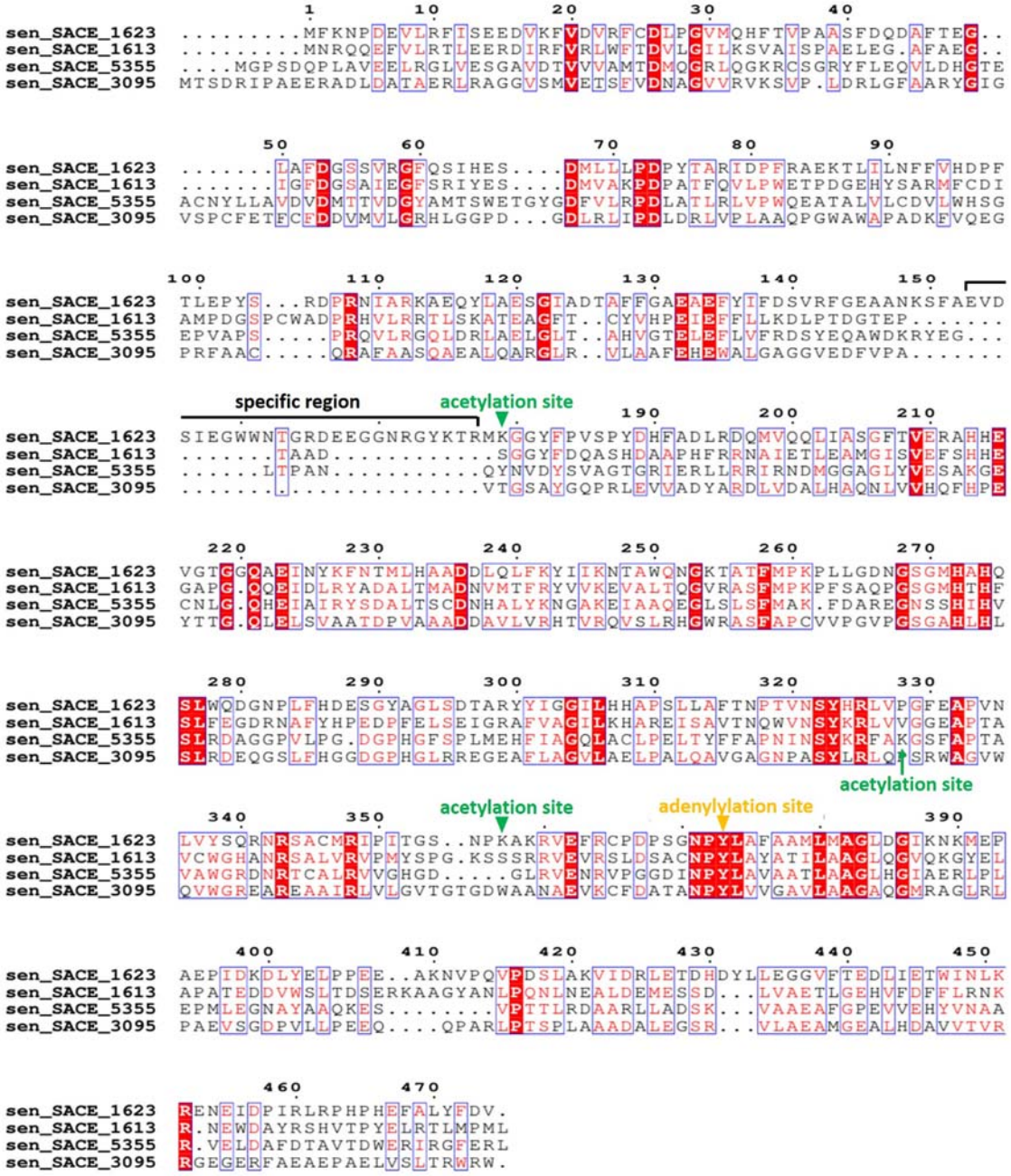


Figure S2. Multiple protein sequence alignment of the *S. erythraea* GlnA enzymes. A specific 25-amino-acid insertion (EVDSIEGWWNTGRDEEGGNRGYKTR) distinguishing GSI-β and GSII is in bold type; Acetylated site lysine is indicated in red and the active site tyrosine is indicated in yellow.

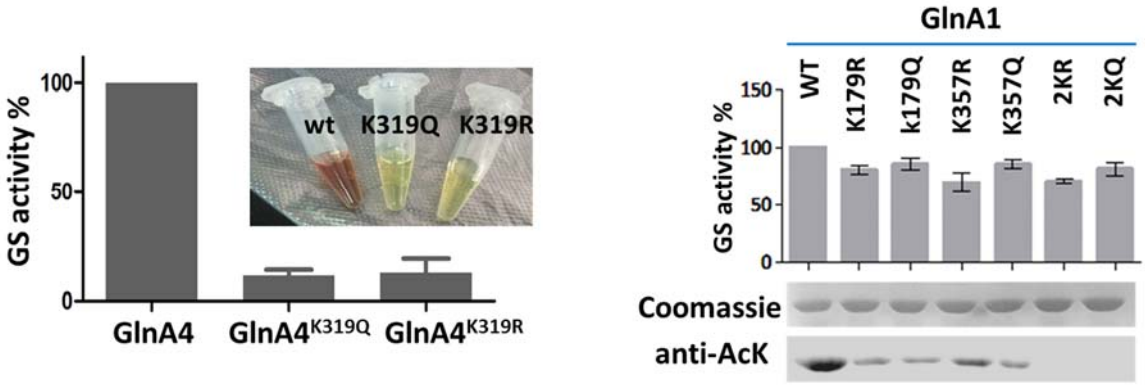


Figure S3. The effect of site-directed mutagenesis on GlnA1 and GlnA4 activity.

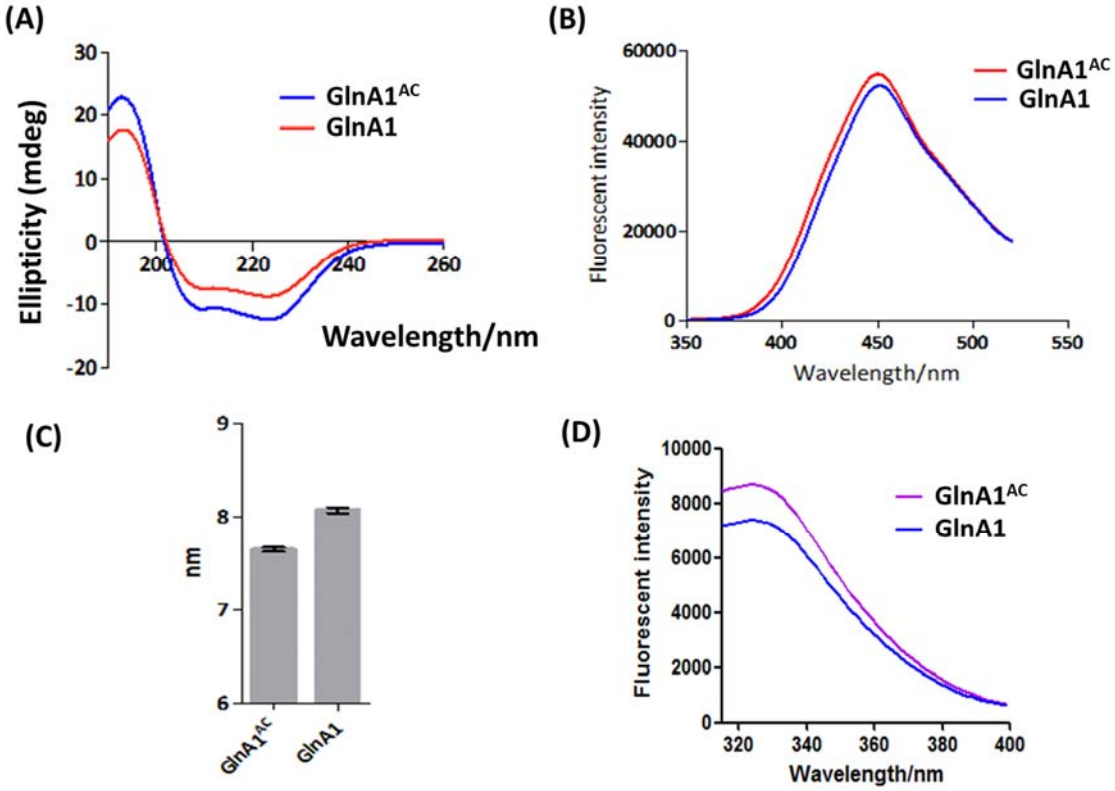


Figure S4. Effect of acetylation on secondary structure of GlnA1. (A) Circular dichroism spectra for native GlnA1 and GlnA1^{AC}. **(B)** Surface hydrophobicity of acetylated and native proteins. Surface hydrophobicity of acetylated GlnA-1 and native GlnA1 probed with TNS at 30 °C. **(C)** The hydrodynamic radius of acetylated GlnA1, native GlnA1. The radius is measured by dynamic light scattering. The proteins were kept in the PBS buffer. Each data point is the average of three independent determinations. (D) Tryptophan fluorescence of for the acetylated GlnA1 and native GlnA1

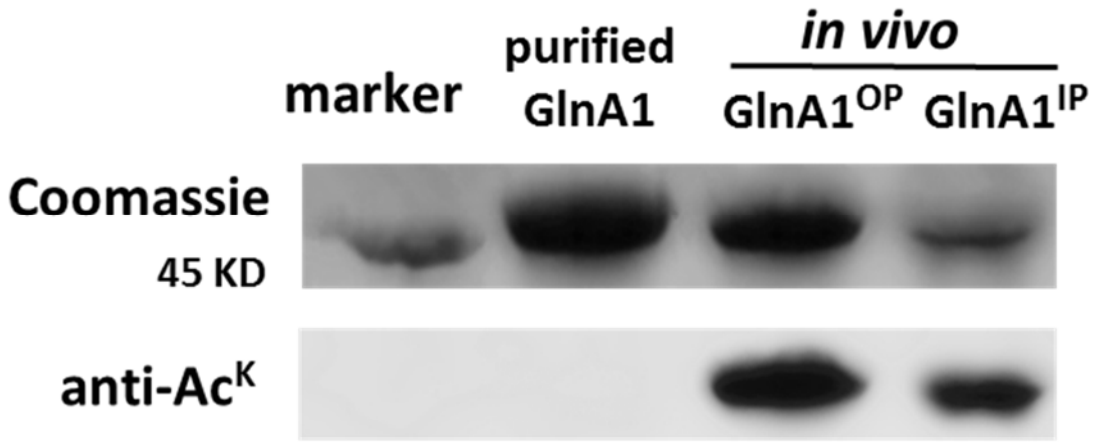


Figure S5. GlnA1 acetylation in *S. erythraea* was evaluated by IP (lane 4) and overexpression (OP, lane 3) and detected by western blotting using an anti-acetylated lysine (AcK) antibody. Also shown are purified recombinant GlnA1 (lane 2) and the protein marker (45 kDa, lane 1).

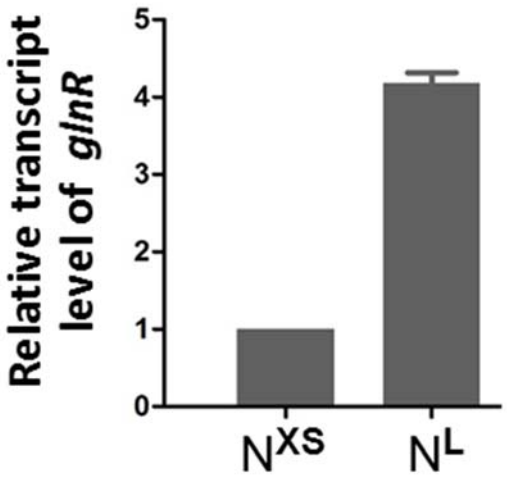


Figure S6. qRT-PCR was used to examine the transcriptional profiles of the *glnR* gene when *S. erythraea* was grown in the medium with excess/limited (N^{XS}/N^{L}) nitrogen. RNA was collected at the exponential phage. The relative expression of the *glnR* gene from the N^{XS} condition was set to 1.0. Error bars show the standard deviations from three independent experiments.

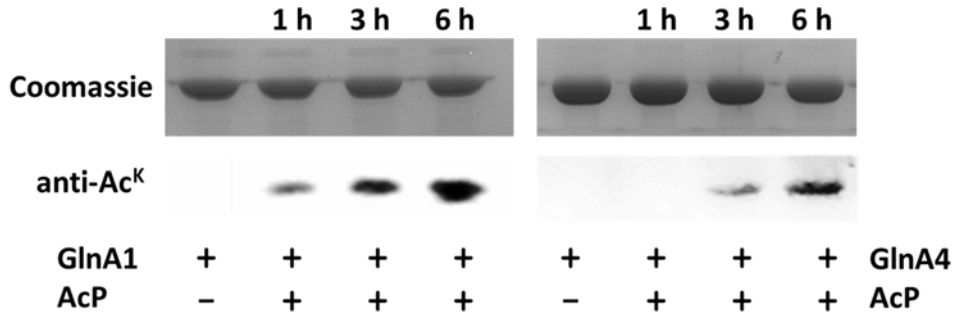


Figure S7. *In vitro* acetylation of GlnA1 and GlnA4 with acetyl-phosphate. Coomassie stain of SDS-polyacrylamide gel and antiacetyllysine Western immunoblot analysis of GlnA1 or GlnA4 (2 μM) incubated with 20 mM AcP in reaction buffer (150 mM Tris-HCl, pH 7.3, 10% glycerol, 10 mM $MgCl_2$, 150 mM NaCl) for various lengths of time (1, 3, and 6 hours) at 37 °C.

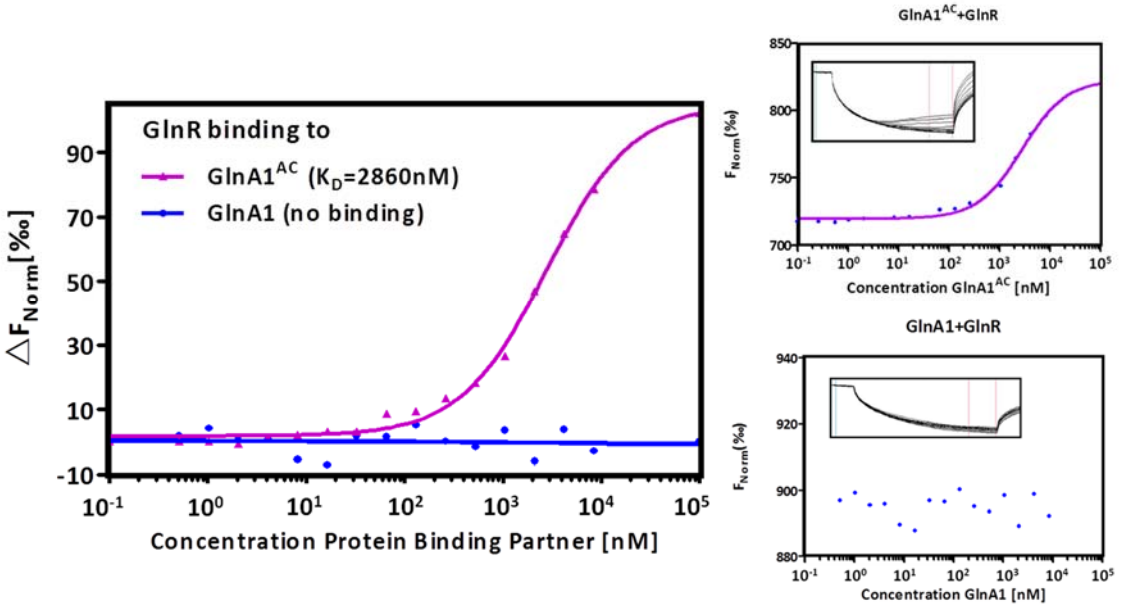
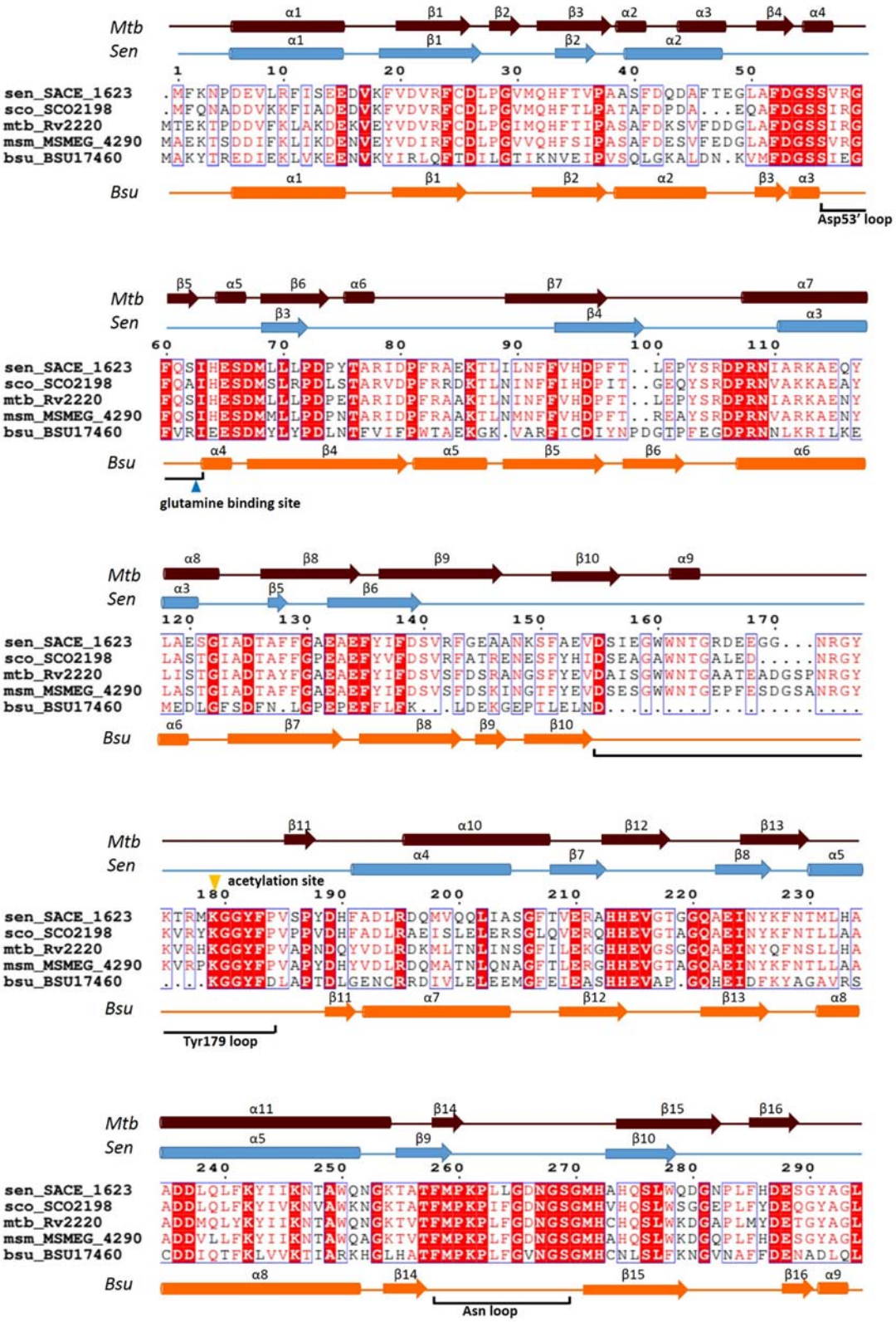


Figure S8. Equilibrium binding analysis of GlnR with acetylated GlnA1 and native GlnA1 by microscale thermophoresis assay. GlnR was labeled by NT.647:NHS- (Monolith NTTM Protein Labeling Kit) and kept 100 nM unchanged. Label-free acetyl-GlnA1 and native GlnA1 were assigned to 16 different concentrations which ranged from 0.26 nM to 8.4 μM.


Mtb
Sen
Bsu
sen_SACE_1623
sco_SCO2198
mtb_Rv2220
msm_MSMEG_4290
bsu_BSU17460
Asp53' loop
glutamine binding site
acetylation site
Tyr179 loop
Asn loop

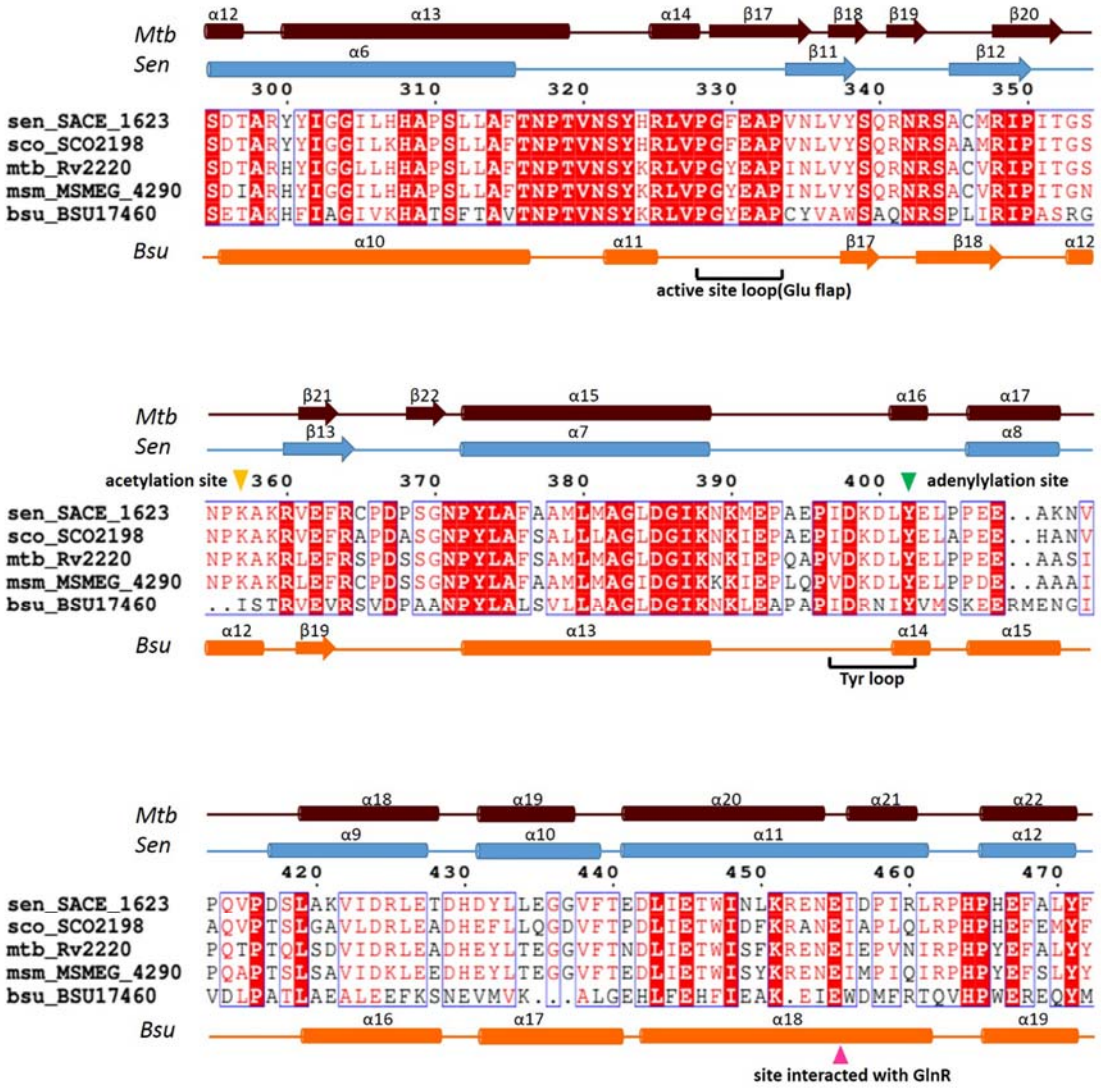


Figure S9. Alignment of the *S. erythraea* GlnA1 sequence with SCO2198 from *Streptomyces coelicolor*, MSMEG_4290 from *Mycobacterium smegmatis*, RV2220 from *Mycobacterium tuberculosis,* and BSU17460 (GSI-α) from *B. subtilis*.

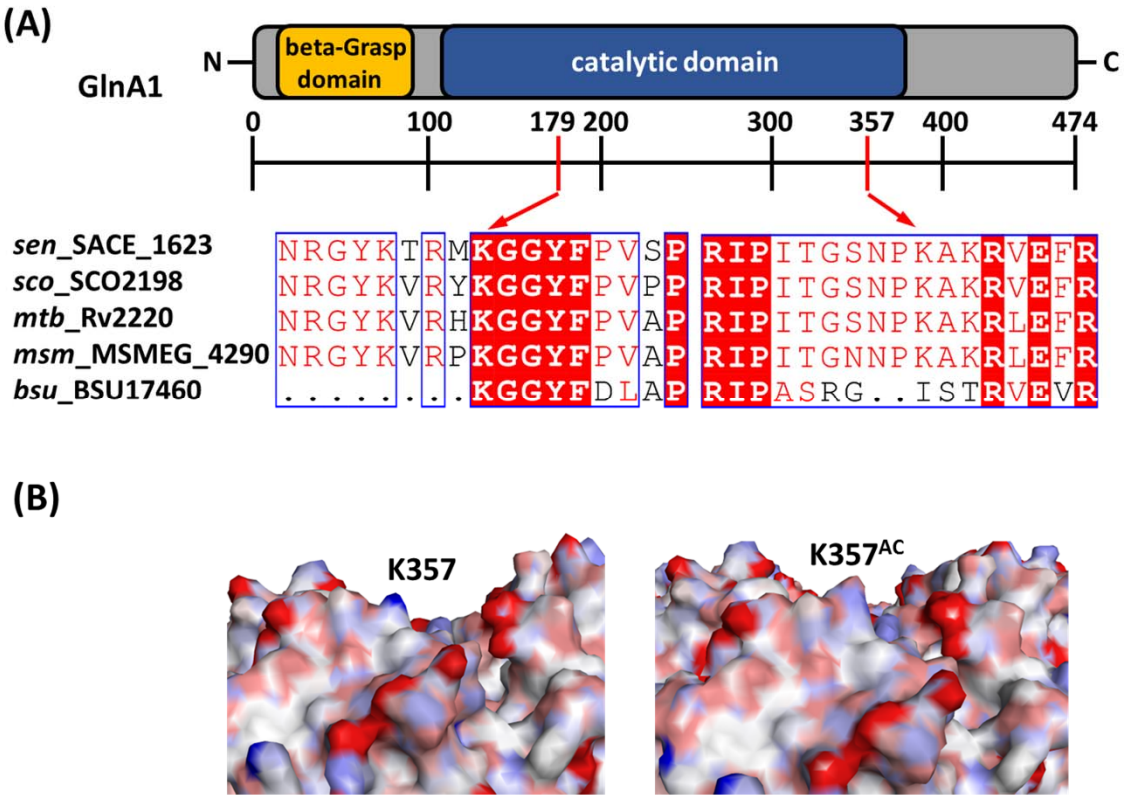


Figure S10. Structural analysis of GlnA1. (A) Two acetylation sites (K357 and K179) are conserved in GlnA1 GS across actinomycetes. **(B)** Molecular surface of GlnA1 mapped with electrostatic potentials for native and acetyl K357 of GlnA1. Positive and negative electrostatic potentials on isosurfaces and molecular surfaces are colored blue and red, respectively.

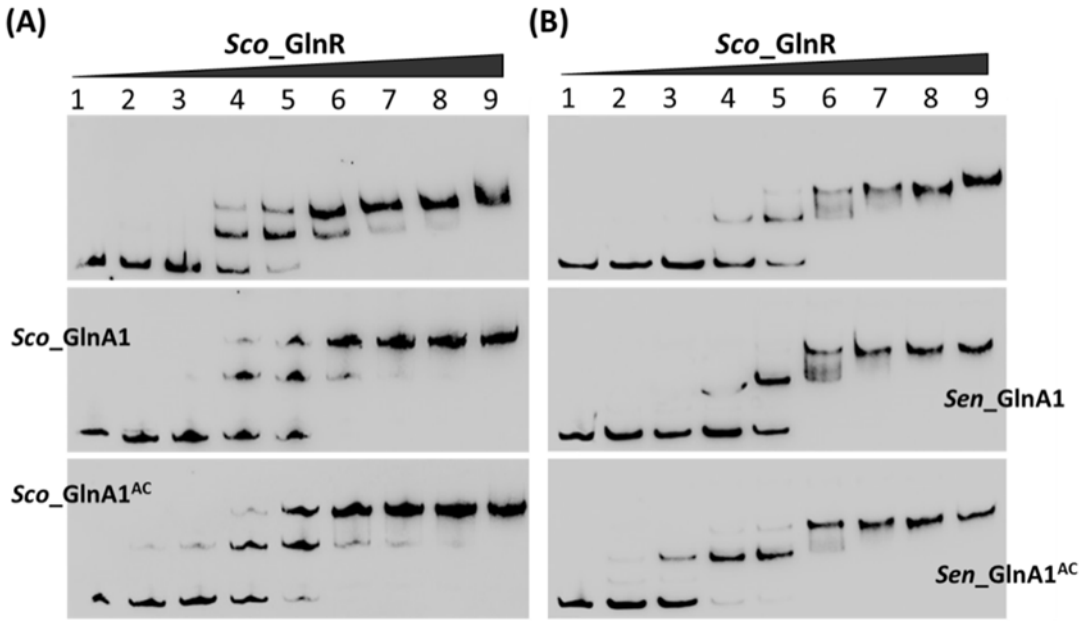


Figure S11. Chaperone activity of acetylated GSI-β is conserved in actinomycetes. (A) Binding of *Sco*_GlnR to the *S. coelicolor glnA1* promoter, as determined by EMSA. GlnR and a 300-bp fragment of the *glnA1* promoter were incubated in the absence of *Sco*_GlnA1 (upper panel), with 5 µM native *Sco*_GlnA1 subunit (middle panel), or with 5 µM acetylated *Sco*_GlnA1 subunit (lower panel). **(B)** Binding of *Sco*_GlnR to the *S. coelicolor glnA1* promoter. GlnR and a 300-bp fragment of the *glnA1* promoter were incubated in the absence of *Sen*_GlnA1 (upper panel), with 5 µM native *Sen*_GlnA1 subunit (middle panel), or 5 µM acetylated *Sen*_GlnA1 subunit (lower panel). Lane 1 contained no GlnR; lanes 2–9 contained increasing concentrations of GlnR in 2-fold increments ranging from 100 nM to 12.8 µM.

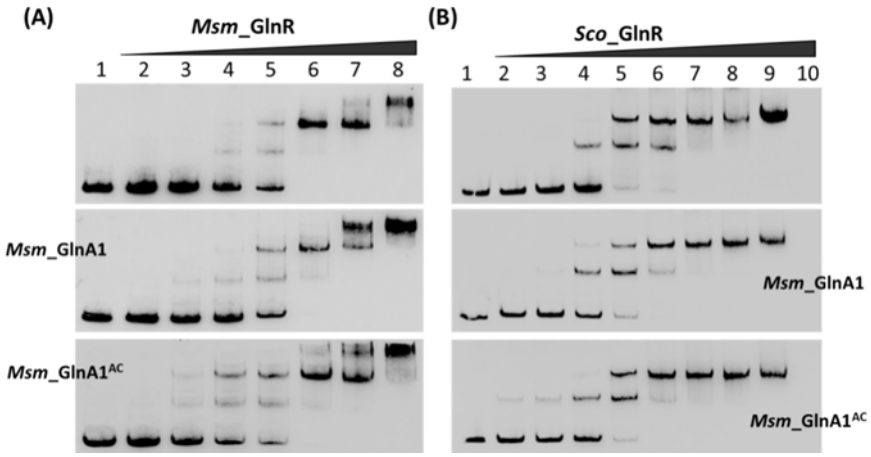


Figure S12. Chaperone activity of acetylated GSI-β is conserved in actinomycetes. (A) EMSA for the binding of *Msm*_GlnR to *glnA1* promoter DNA of *M. smegmatis*. GlnR and a 300-bp *glnA1* promoter DNA fragment were incubated in the absence of *Msm*_GlnA1 (up), the presence of 5 μM native *Msm*_GlnA1 subunit (middle) and the presence of 5 μM acetylated *Msm*_GlnA1 subunit (down). **(B)** EMSA for the binding of *Sco*_GlnR to *glnA1* promoter DNA of *S. coelicolor*. GlnR and a 300-bp *glnA1* promoter DNA fragment were incubated in the absence of *Msm*_GlnA1 (up), the presence of 5 μM native *Msm*_GlnA1 subunit (middle) and the presence of 5 μM acetylated *Msm*_GlnA1 subunit (down). Lane 1 contained no GlnR. Lanes 2-10 cotained increasing concentrations of GlnR in 2-fold increments that ranged from100 nM to 25.6 μM.

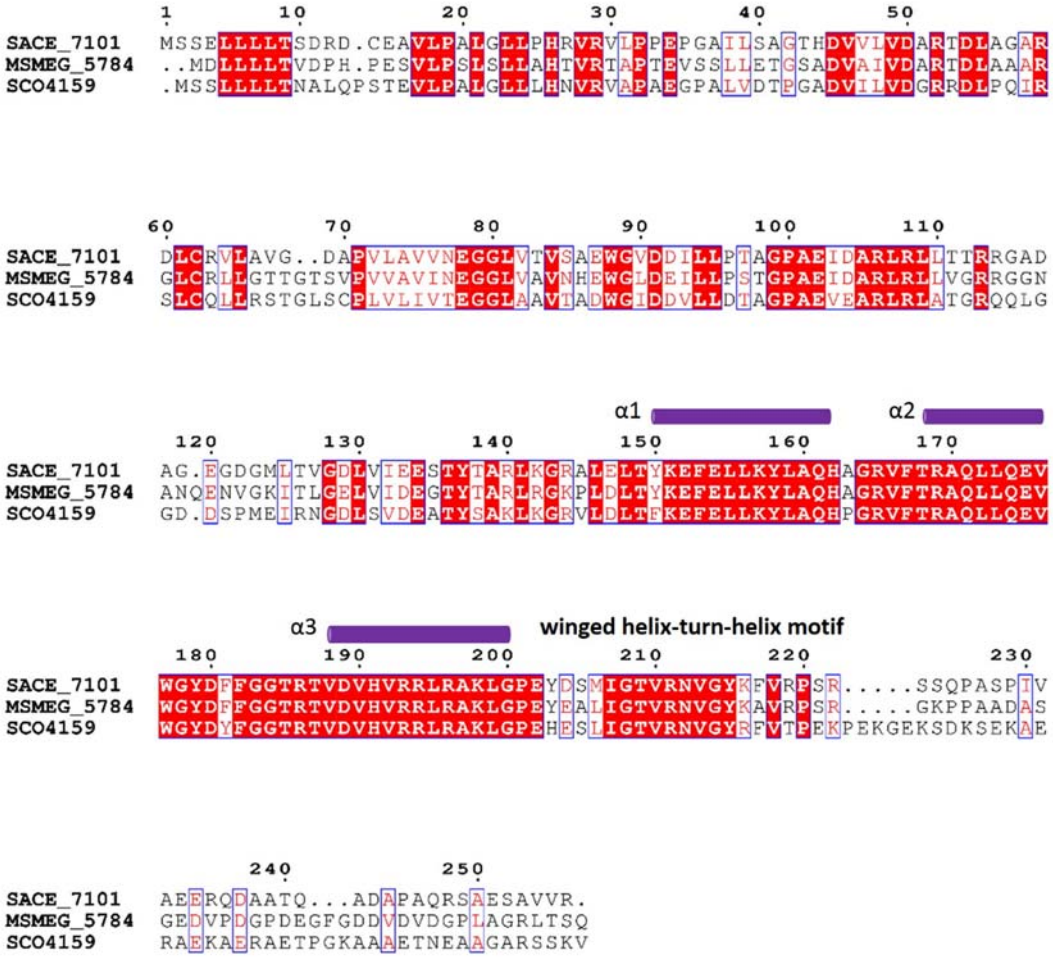


Figure S13. Alignment of the *S. erythraea* GlnR sequence (SACE_7101) with SCO4159 from *S. coelicolor*, MSMEG_5784 from *M. smegmatis*. α1 = helix 1, α2 = helix 2 and α3 = the DNA recognition helix.

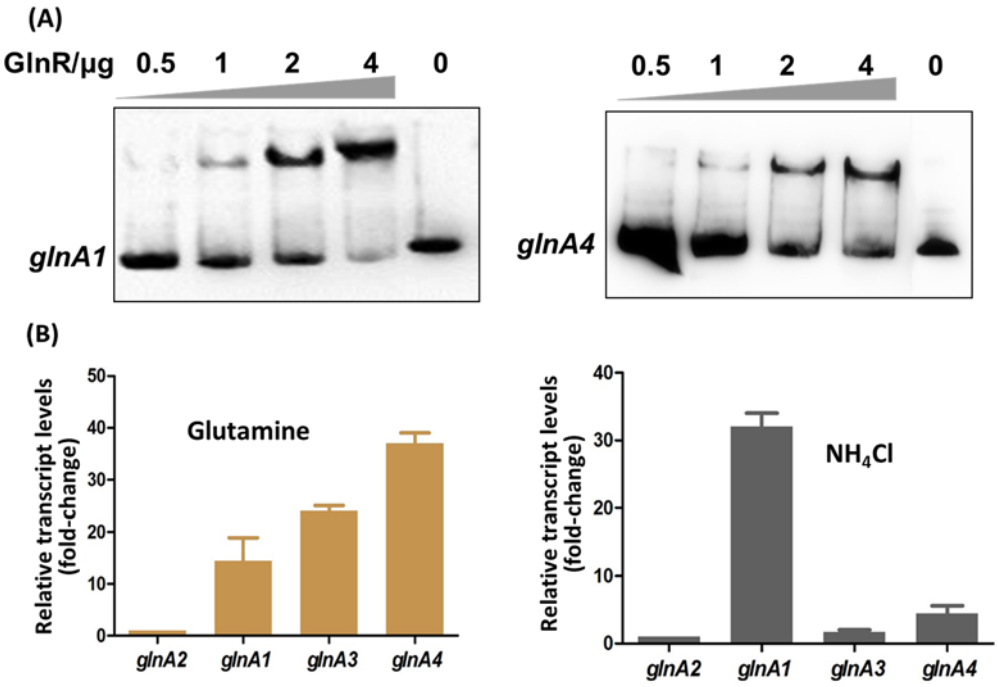


Figure S14. **Responses of the four GS genes to different nitrogen sources. (A)** EMSAs of purified His-GlnR to the promoter regions of *glnA1* and *glnA4* genes. The DNA probes were incubated with GlnR and a 200-fold excess of nonspecific competitor DNA (sperm DNA). **(B)** qRT-PCR was used to examine the transcriptional profiles of GS genes when *S. erythraea* was grown in glutamine or NH_4Cl. RNA was collected at the exponential phase after inoculating cells in minimal Evans medium with glutamine or NH_4Cl as the sole nitrgen source. The relative expression of the *glnA2* gene was set to 1.0. Error bars show standard deviations from three independent experiments.

Table S1. Secondary Structual Elements in native and acetylated GlnA1

Protein	α-helix (%)	Antiparallel (%)	Parallel (%)	β-turn (%)	Random coil (%)	Total sum (%)
GlnA1	32.6	7.9	9.0	16.7	35.1	101.4
GlnA1^{AC}	41.5	3.7	7.3	15.2	29.0	96.8

Table S2. The acetylated sites of GlnA1 and GlnA4 under the different conditions

GS enzymes	*in vivo*		*in vitro*	
	WT strain	**Δ*acuA* strain**	**enzymatic (AcuA)**	**non-enzymatic (AcP)**
GlnA1	K227, K357, K390, K421	K149, K227, K242, K390	K179, K357	K246, K390, K421
GlnA4	K207, K319, K394, K412	K162, K207, K235, K412	K319	K162, K207, K235, K394, K412

Table S3. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
S. erythraea NRRL23338	used as parental strain, wild type	DSM 40517
E. coli DH5α	F^-Ø80d lacZΔM (lacZYA -argF)U169 deoR	Invitrogen
E. coli BL21(λDE3)-7101	The strain for expression of GlnR	(1)
E. coli BL21(λDE3)	F'ompTr^-_Bm-$_B$ (λDE3)	Invitrogen
S. erythraea OglnA1	The strain for over-expression of *glnA1,* NRRL23338 integrated with pIB139-1623	this study
S. erythraea OglnA4	The strain for over-expression of *glnA4,* NRRL23338 integrated with pIB139-5355	this study
S. erythraea Δ*glnR*	NRRL23338 *glnR*::*tsr* (*glnR* null mutant)	(1,2)
S. erythraea Δ*acuA*	NRRL23338 *acuA*::*tsr* (*acuA* null mutant)	this study
Δ*glnR/*pIB-*glnR(CglnR)*	*glnR complementary strain,* Δ*glnR* carrying pIB-*glnR*	(2)
plasmid		
pET28a(+)	vector with T7-RNA polymerase-based promoter for expression in *E. coli* BL21(λDE3), hexahistidine tag with thrombin cleavage	Thermo Scientific
p7101	pET28a(+)with *glnR* (SACE-7101) inserted in NcoI-HindIII	this study
P5148	pET28a(+)with *acuA* (SACE-5148) inserted in NcoI-HindIII	this study
pUC18-tsr	pUC18 with *tsr* gene for thiostrepton resistance inserted into *BamH1-Sma1*	(2)
pIB139	pSET152 with integrase of phiC31 and PermE, the strong promoter of *Streptomyces*	(2)
pIB139-1623	pIB139 with *glnA1* gene interted into Nde1-EcoRV	This study
pIB139-5355	pIB139 with *glnA4* gene interted into Nde1-EcoRV	This study

Table S4. Primers for overproduction of proteins

gene	Primer sequence (5'-3')
sace_3798	TAAGAATTCTTGTTCGGCGCGGCCC
	TATAAGCTTTCAGGTCCATGTGCTGGGGTC
sace_5148	TAAGAATTCATGAGGATCCAGCAGGTGCAGT
	TATGTCGACTCAGGCGCTGCGTTGTGC
sace_7101	TAAGAATTCATGAGCTCTGAGCTTCTCCTGCTC
	TATAAGCTTTCAGCGGACGACCGCGG
sace_1613	TAAGAATTCATGAACCGCCAGCAGGAGTTC
	TATAAGCTTCTACAGCATCGGCATGAGGGTG
sace_1623	TAAGAATTCGTGTTCAAGAATCCAGACGAGG
	TATAAGCTTTCACACGTCGAAGTACAGCG
sace_3095	TAAGAATTCATGACCTCCGACCGCATCC
	TATAAGCTTCTACCAGCGCCAGCGGGTC
sace_5355	TAAGAATTCGTGGGGCCAAGCGATCAAC
	TATAAGCTTTCAGAGCCGCTCGAAACCA
SCO2198	TAAGAATTCATGTTCCAGAACGCCGACGA
	TAAAAGCTT TCACACGTCGAAGTACATCTCGAAC
SCO4159	TAAGAATTCATGAGTTCTCTGCTGCTCCTGACCA
	TAAAAGCTTCGTTGTGCAGCAGCAGGCC
MSMEG_4290	TAAGAATTCGTGGCAGAAAAGACGTCCGA
	TAAAAGCTTTTACACGTCGTAGTAGAGCGAGAAC
MSMEG_5784	TAAGGATCCTTGGATCTACTGCTACTGACCGTCG
	TAAAAGCTTTCACTGACTGGTCAACCGCCC
apr-F	GCTCATCGGTCAGCTTCTCA
apr-R	TCGCATTCTTCGCATCCC

Table S5. The primers used in Site-Directed Mutagenesis of GlnA

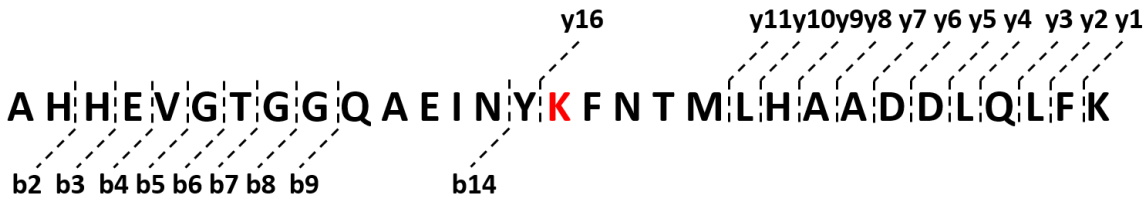
Name		DNA sequence (5'→3')
K179R	F	GACCCGGATGAGGGGCGGCTACTTCCCGG
K179R	R	CCTCATCCGGGTCTTGTAGCCCCGGTTGCC
K179Q	F	TACAAGACCCGGATGCAGGGCGGCTACT
K179Q	R	TTGGCCCCGATGTTCTGGGCCTACGTC
K357R	F	ACCGGCTCGAACCCGCGTGCCAAGCGCG
K357R	R	TAGGGCTAGTGGCCGAGCTTGGGCGCA
K357Q	F	ACCGGCTCGAACCCGCAGGCCAAGCGCG
K357Q	R	TAGGGCTAGTGGCCGAGCTTGGGCGTC
K319Q	F	TACAAGCGTTTCGCCCAGGGCAGCTT
K319Q	R	GGGCGAAACGCTTGTAGGAGTTGATG
K319R	F	ACAAGCGTTTCGCCAGGGGCAGCTTT
K319R	R	CTGGCGAAACGCTTGTAGGAGTTGATG

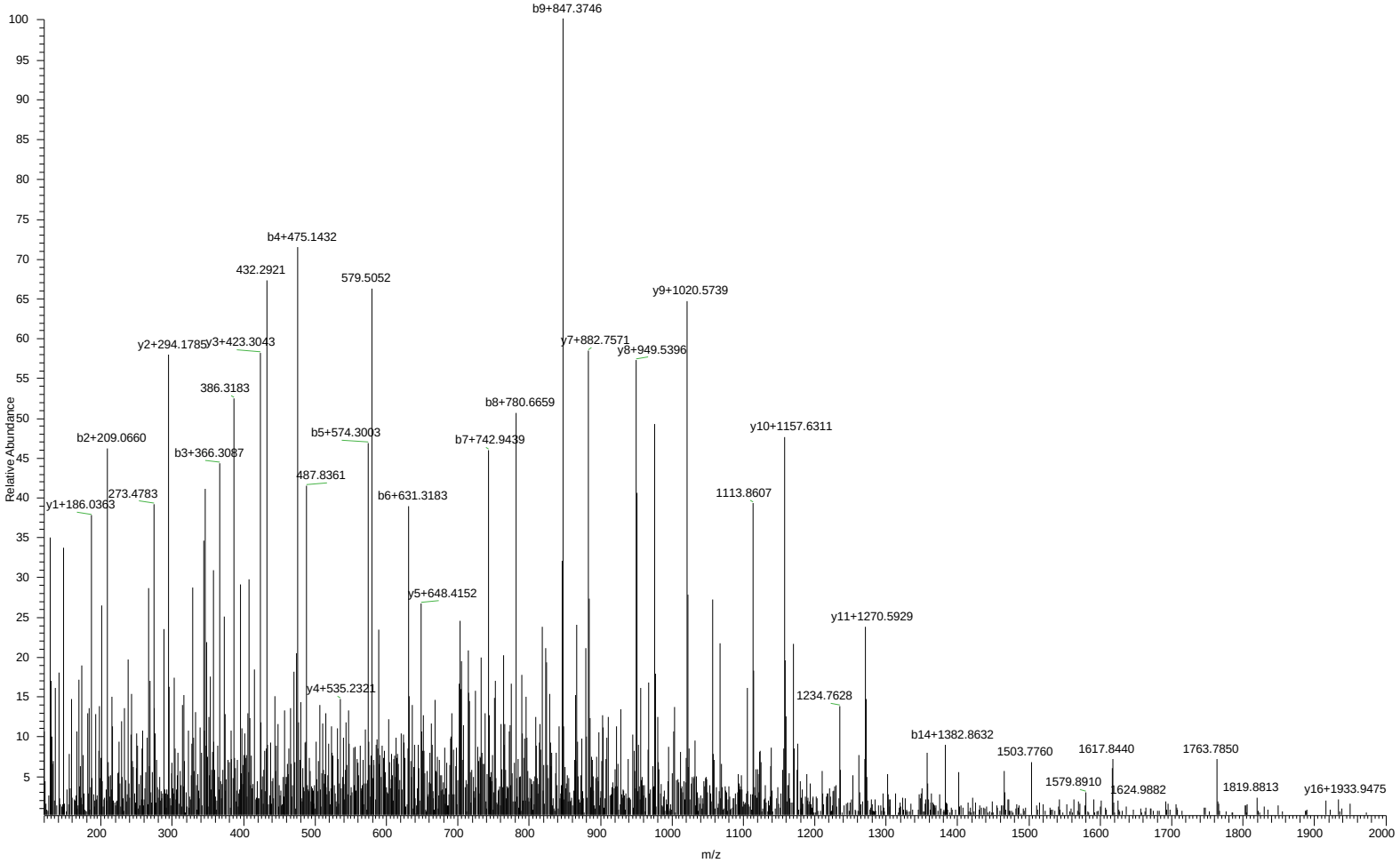
Table S6. Primers for RT-PCR and EMSA

gene	RT-PCR Sequences (5'-3')
sace_5148	CATCGCTGCGTTCTTC
	TACATCCGCTTGACCT
sace_1613	CCACTTCAGCCTCTTC
	CGCTTGTAGGAGTTGA
sace_1623	GATCAACTACAAGTTCAAC
	GGTGTTCTTGATGATGTA
sace_3095	TGGTGAGGGTCAAGTC
	GTCGTCGAAGCAGAAC
sace_5355	TGATGGAGCACTTCAT
	TTGTAGGAGTTGATGTTC
sace_8101	GTTGCGATGCCGTGAGGT
	CGGGTGTTACCGACTTTCA
gene	**EMSA Sequences (5'-3')**
sace_5148	AGTGGCGATAAGGGTGTCACGATCACTGGC
	AGTGGCGATAAGCACTGCACCTGCTGGATC
sace_1623	AGCCAGTGGCGATAAGGAGCCCTACAGCCGGGAC
	AGCCAGTGGCGATAAGGGGCCGGTGGCACCAG
sace_5355	AGCCAGTGGCGATAAGCAGGAGGCGACCGCGC
	AGCCAGTGGCGATAAGCGCCCCAGCTCGCGG
MSMEG_4290	AGCCAGTGGCGATAAG CCGGGAGCCGTTTG
	AGCCAGTGGCGATAAG TGAGTGTTCTCCTTTACTGGTA
SCO2198	AGCCAGTGGCGATAAG CCGAACACGGTACTGCGC
	AGCCAGTGGCGATAAG CCAGCTCCTCCTACTCCCG

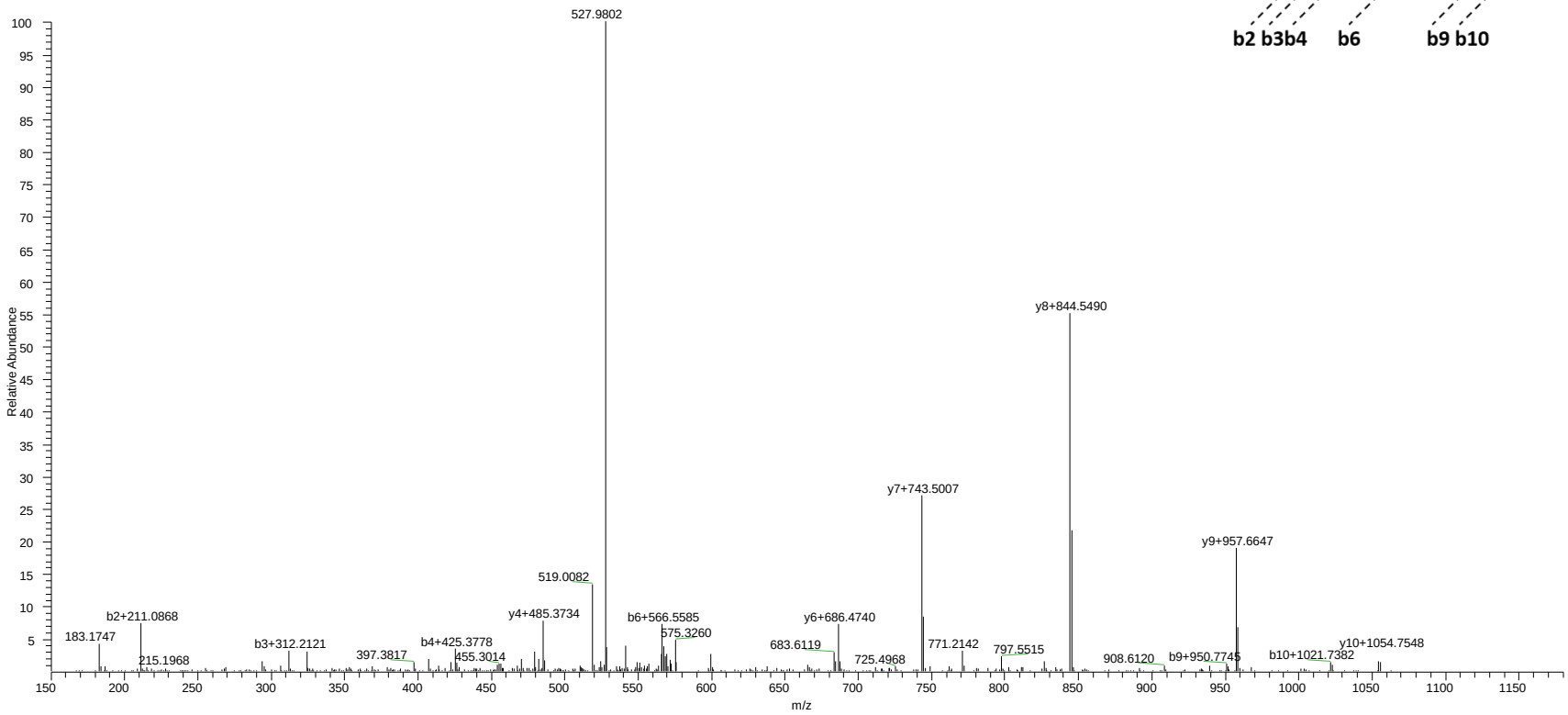
All MS/MS spectra for the identification of acetylated sites by LC/MS/MS analysis.

K227_GlnA1
***in vivo* WT strain**





K357_GlnA1
in vivo WT strain



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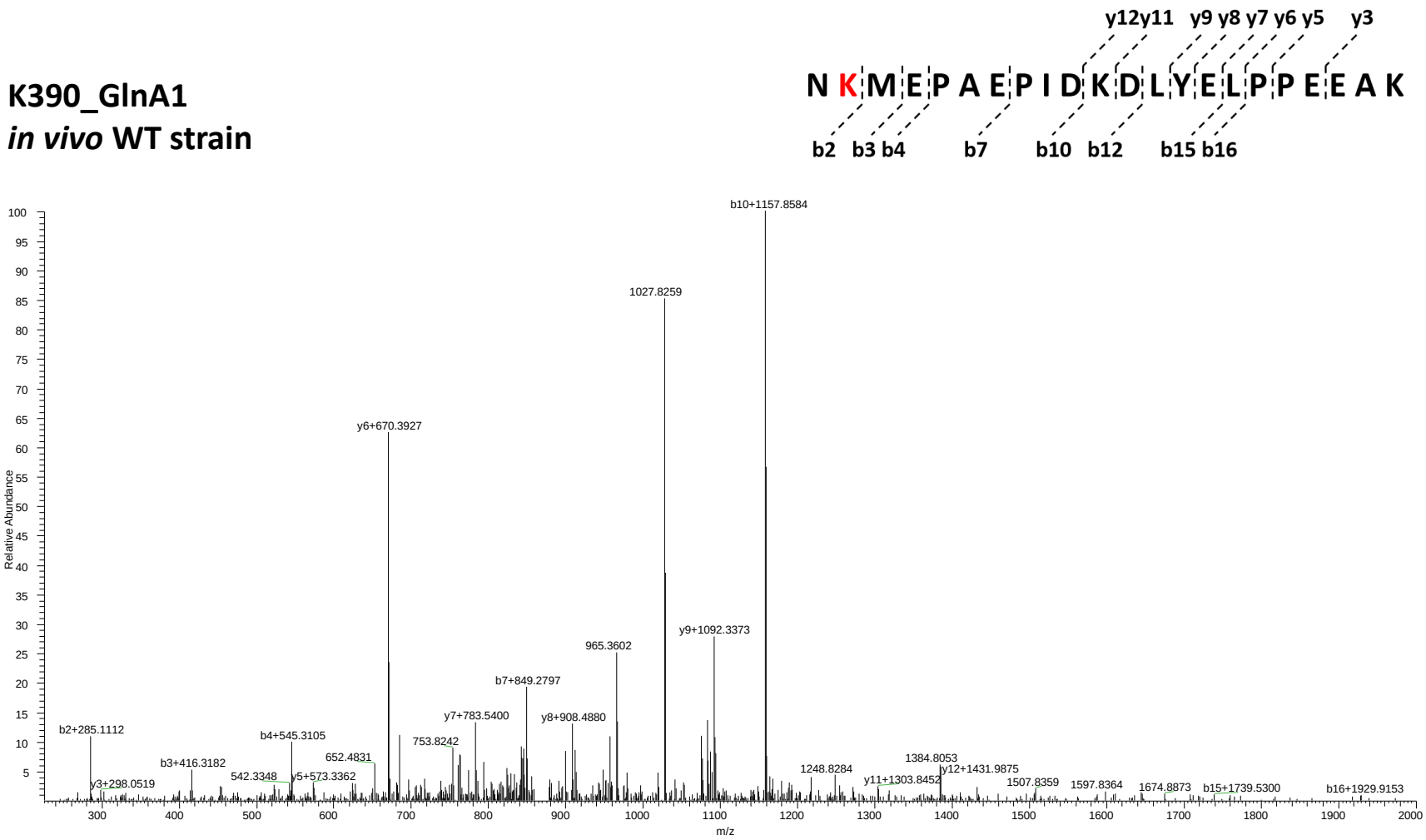

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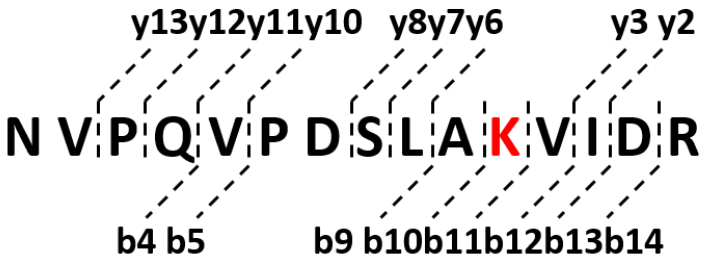
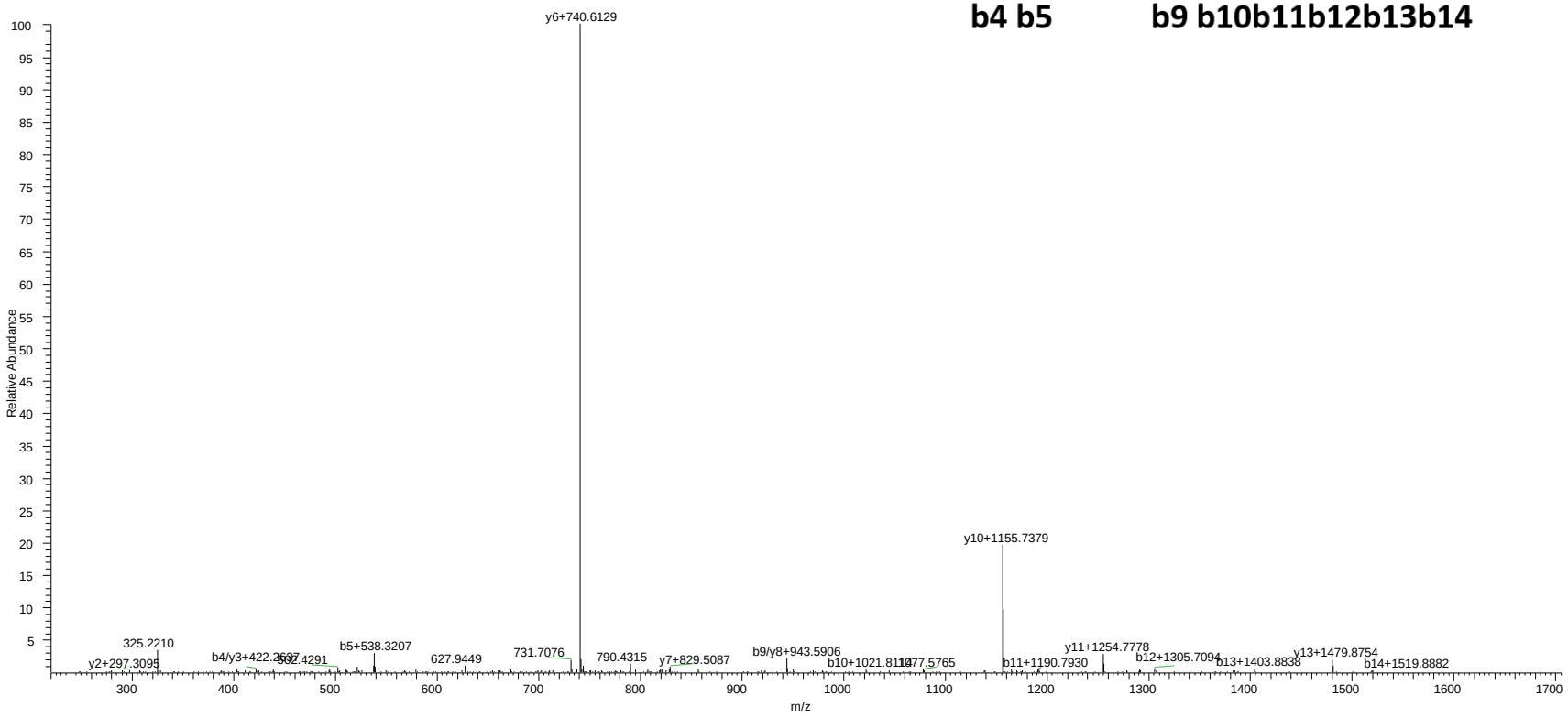
***in vivo* WT strain**





K149_GlnA1
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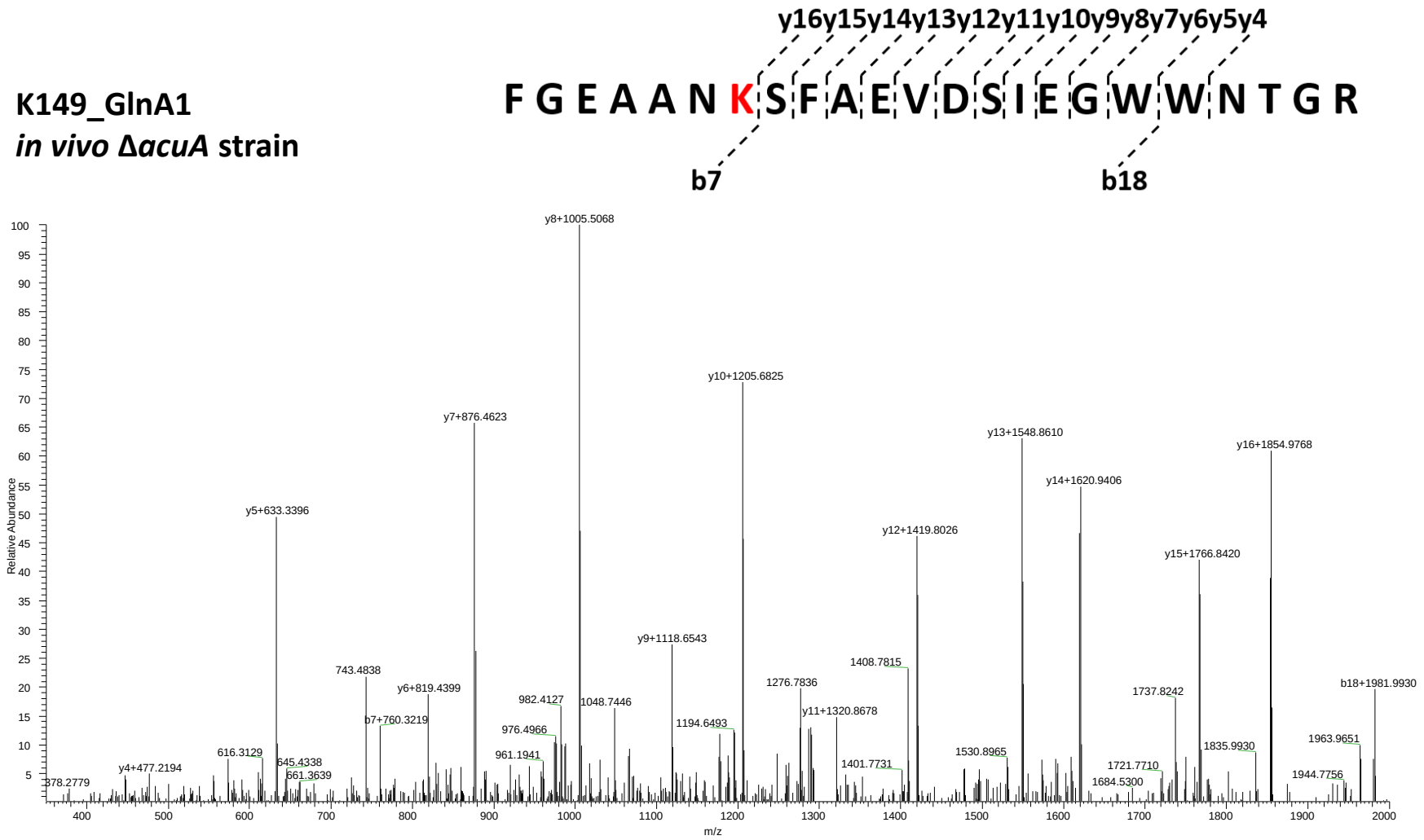
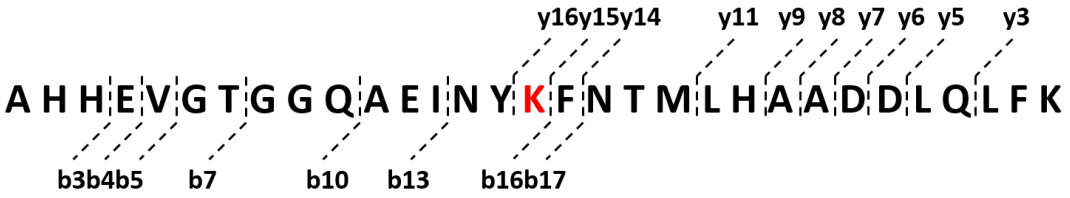
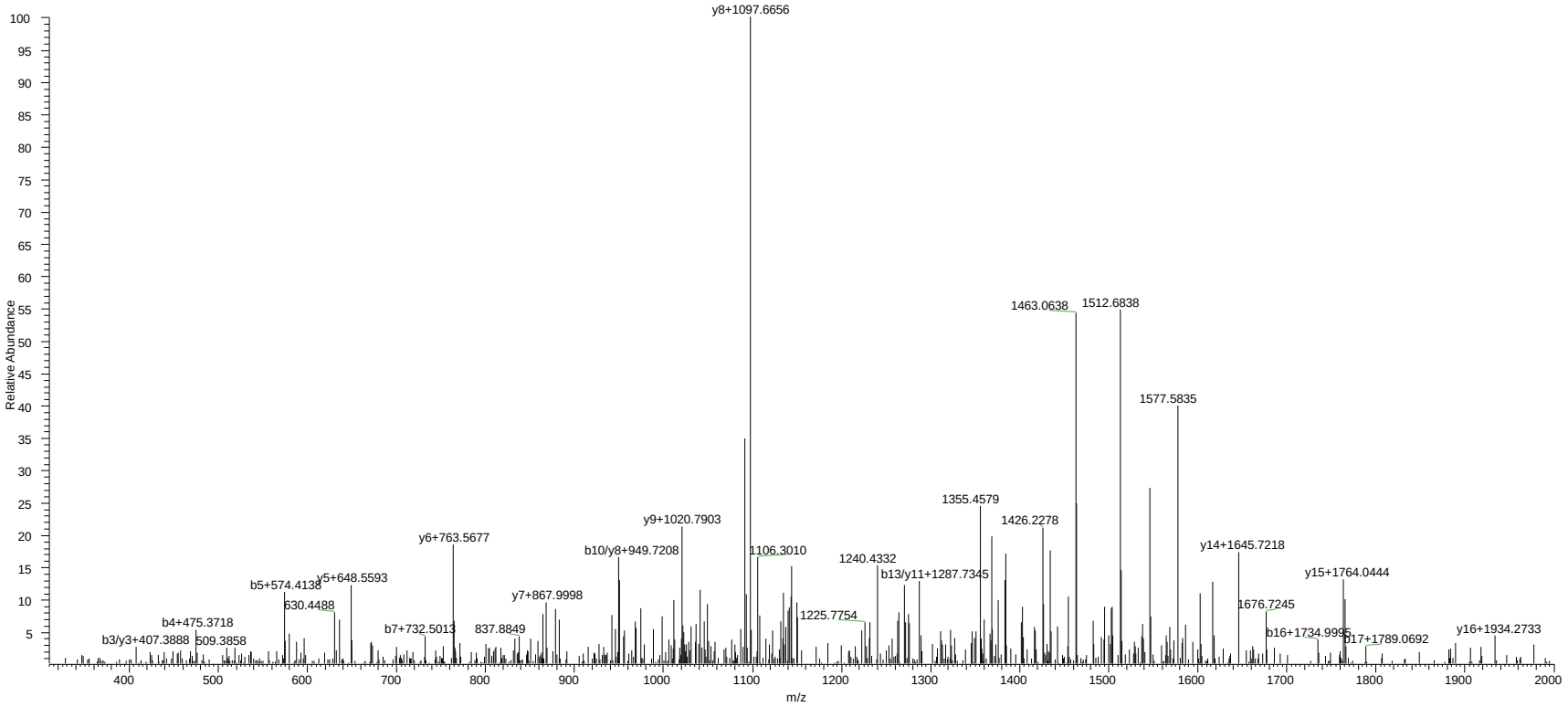
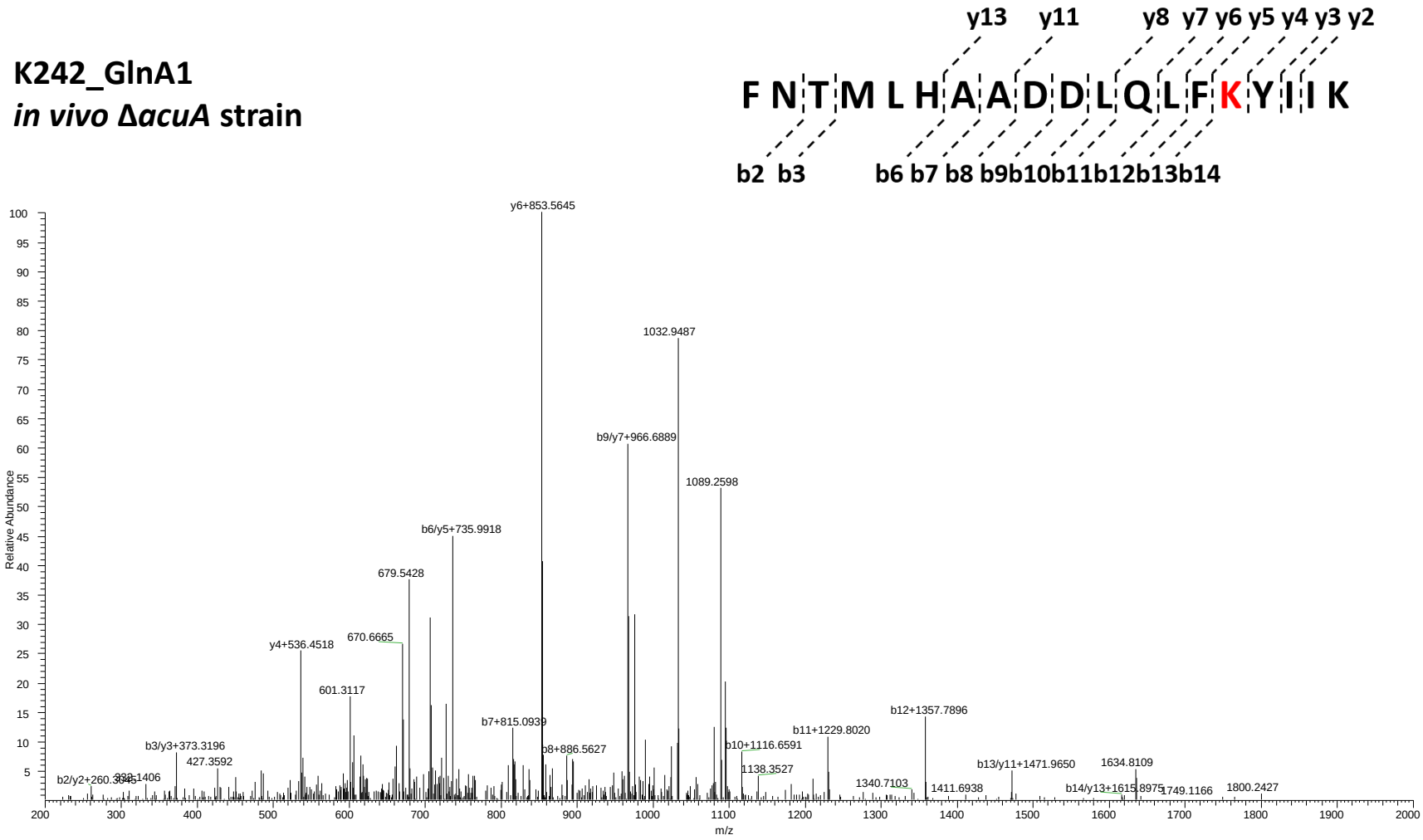


K227_GlnA1
***in vivo* Δ*acuA* strain**

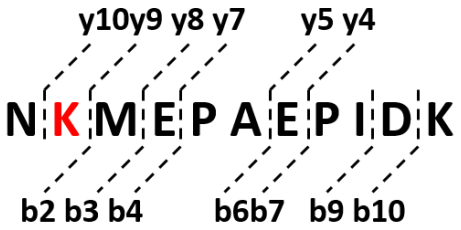


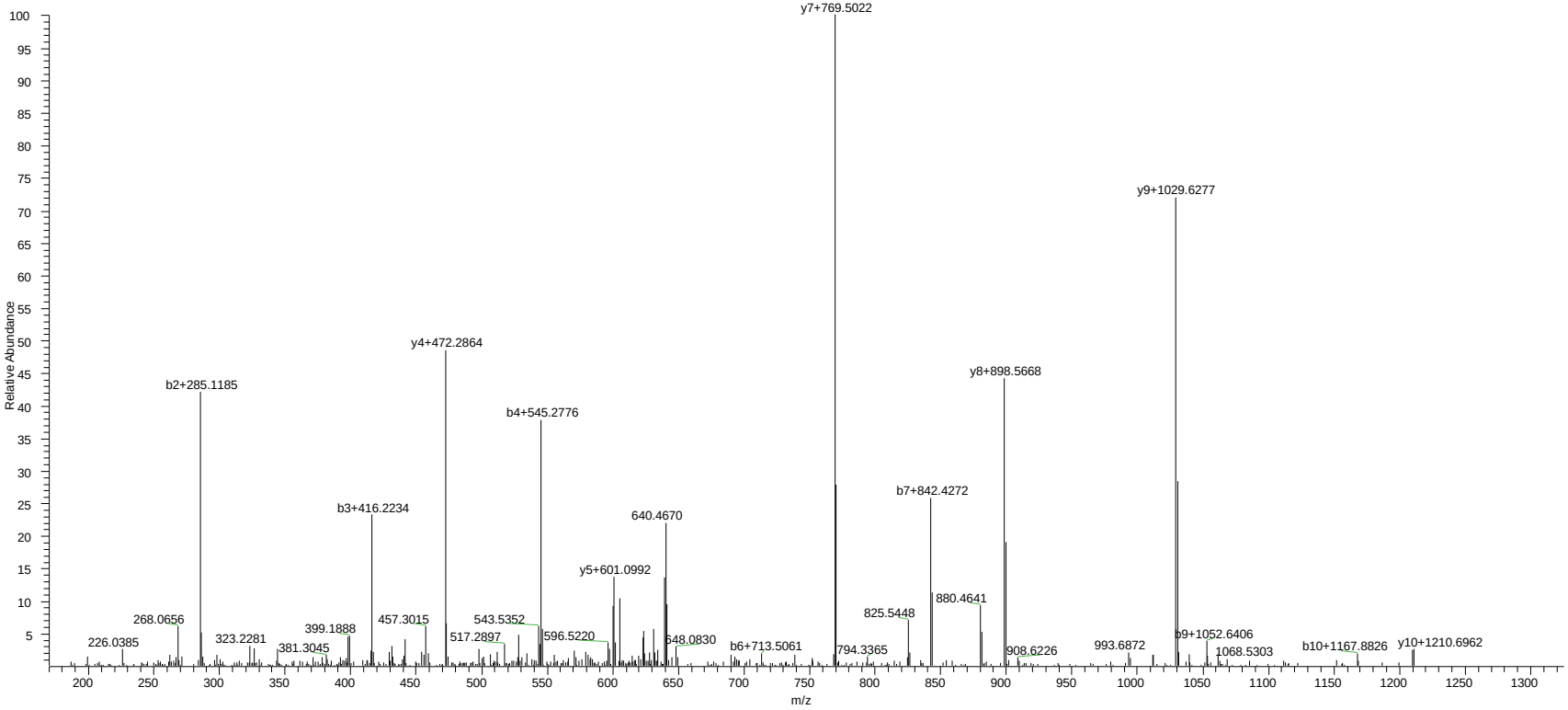


K242_GlnA1
***in vivo* Δ*acuA* strain**



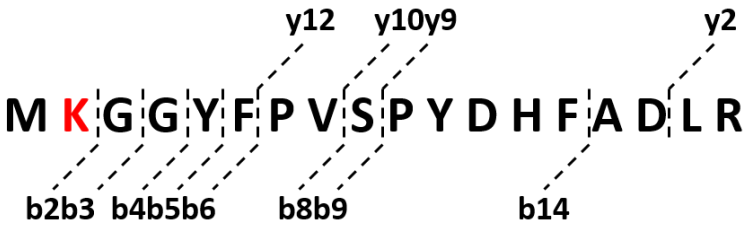
K390_GlnA1
***in vivo* Δ*acuA* strain**

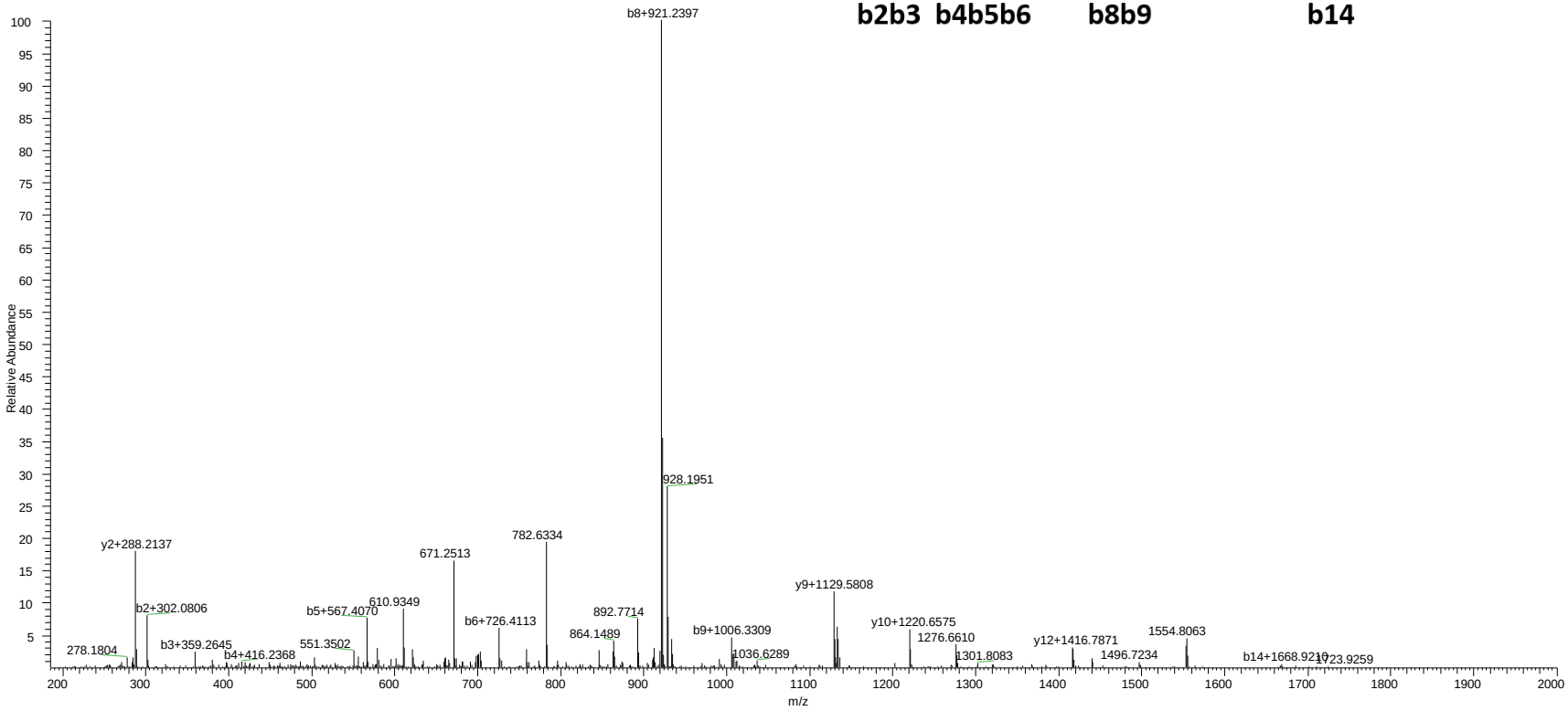




K179_GlnA1

***in vitro* AcuA-acetylation**





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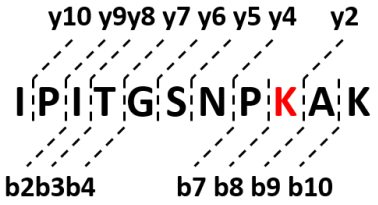
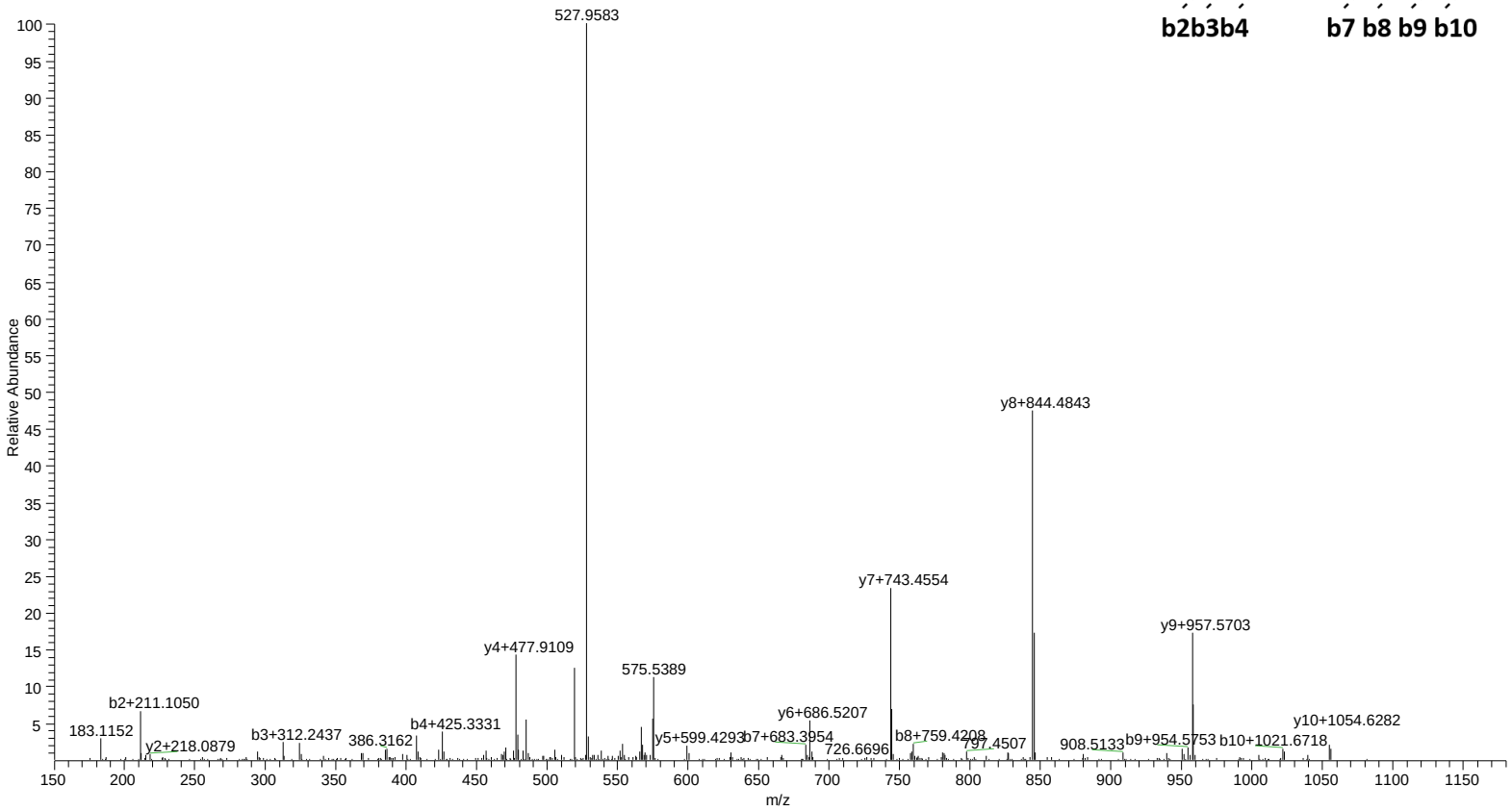
in vitro AcuA-acetylation





K246_GlnA1
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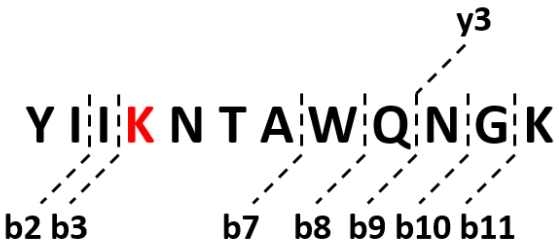
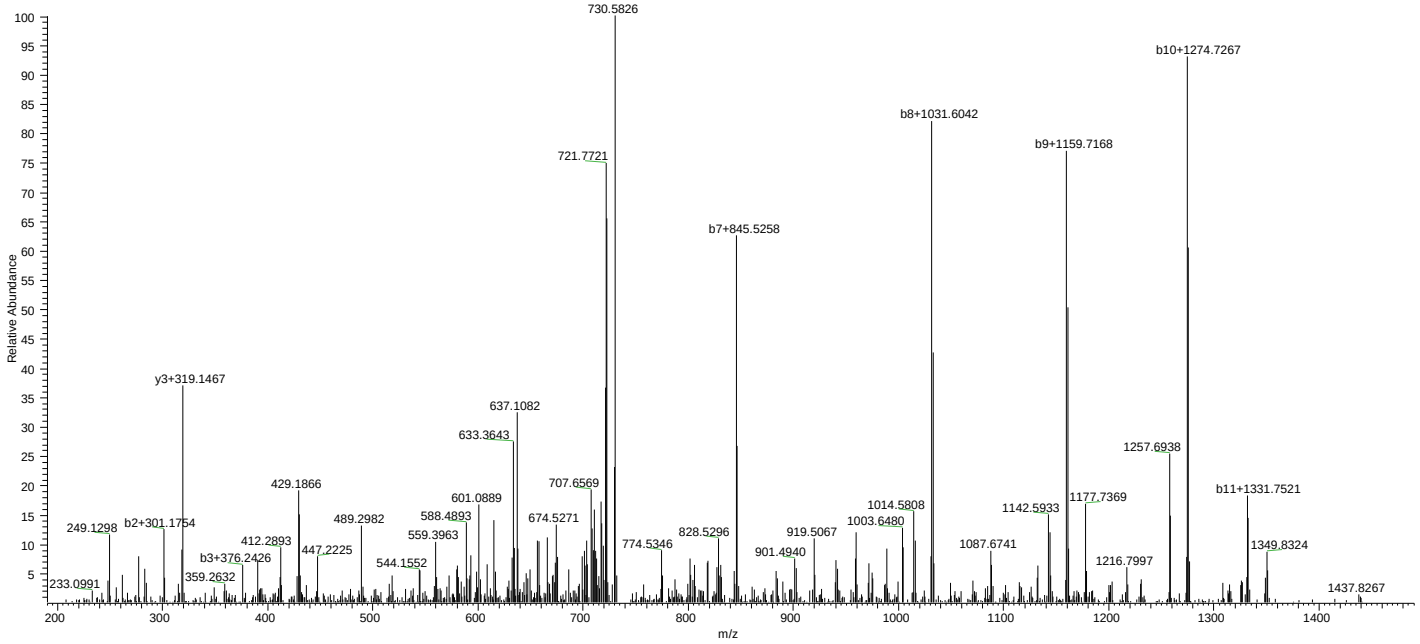
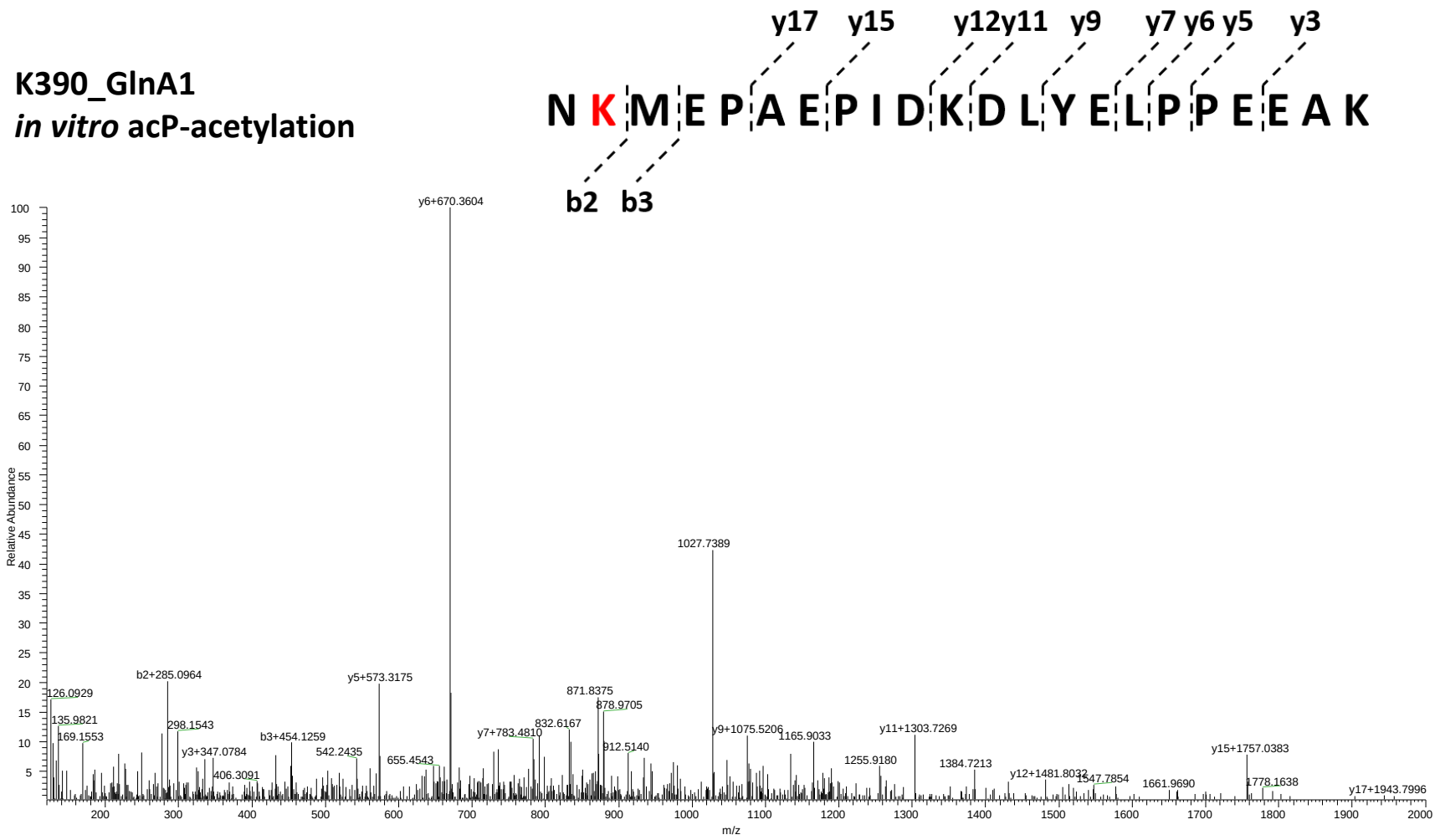




K390_GlnA1
***in vitro* acP-acetylation**



K421_GlnA1
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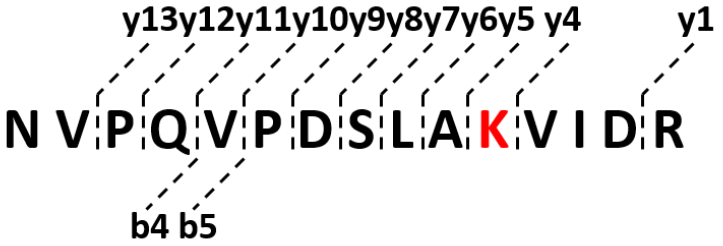
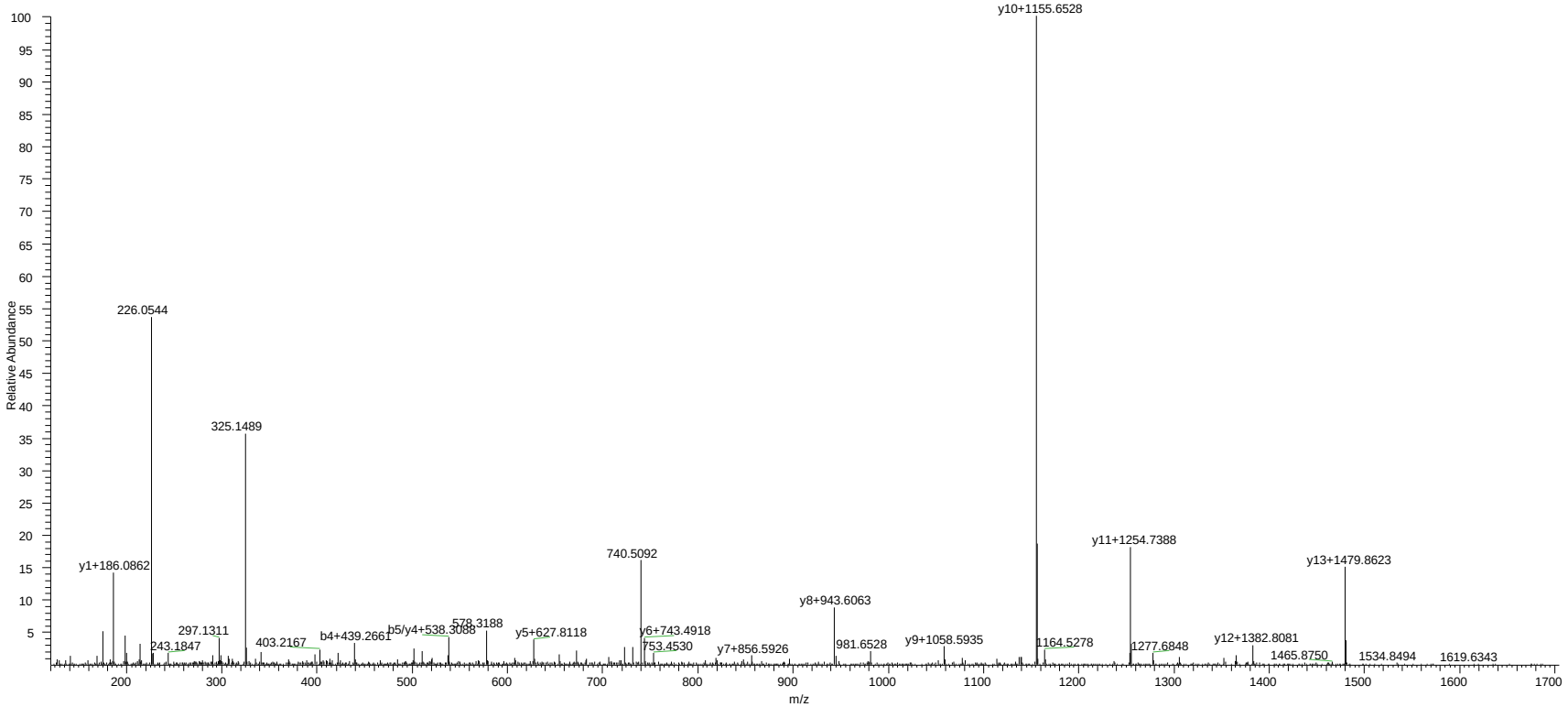
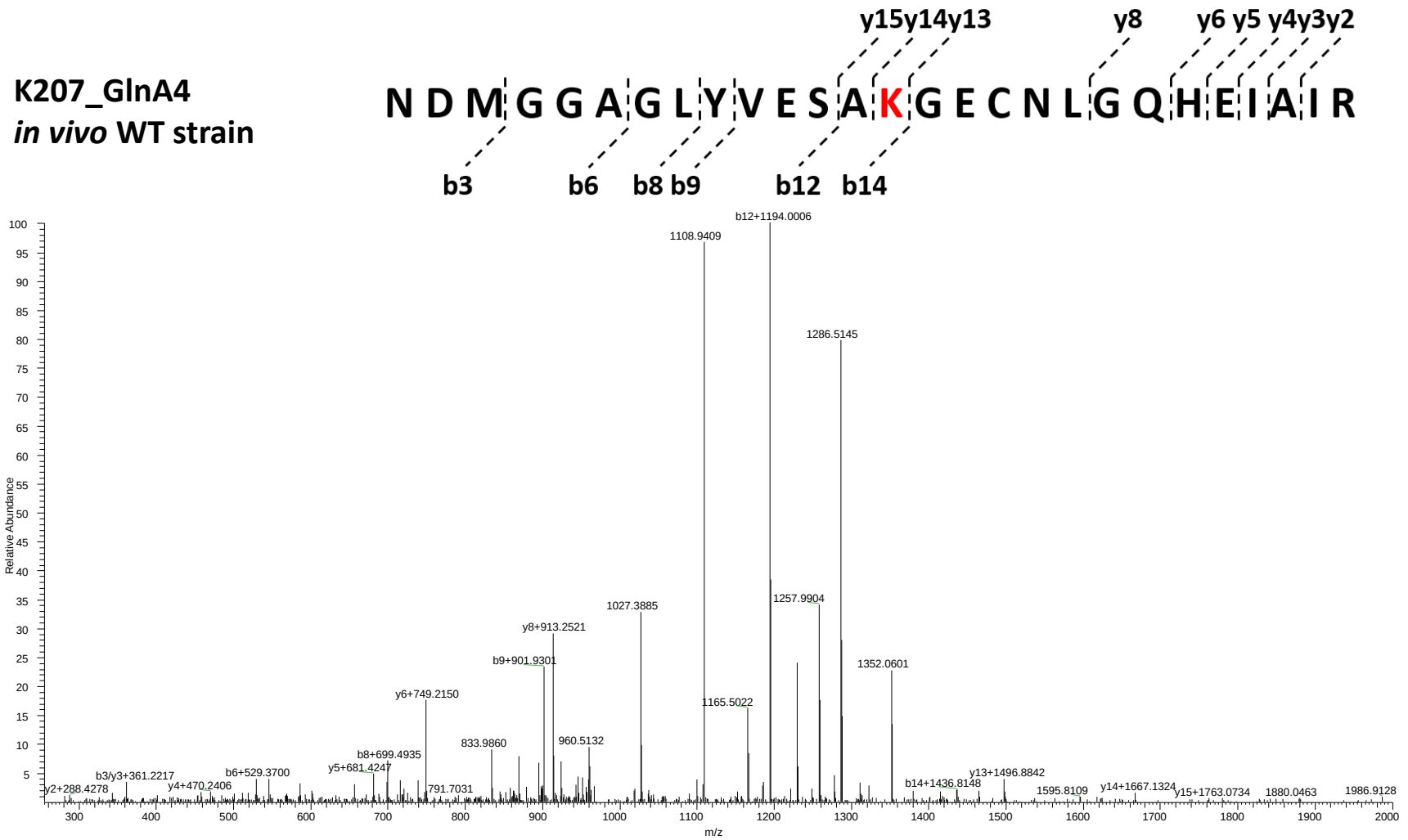
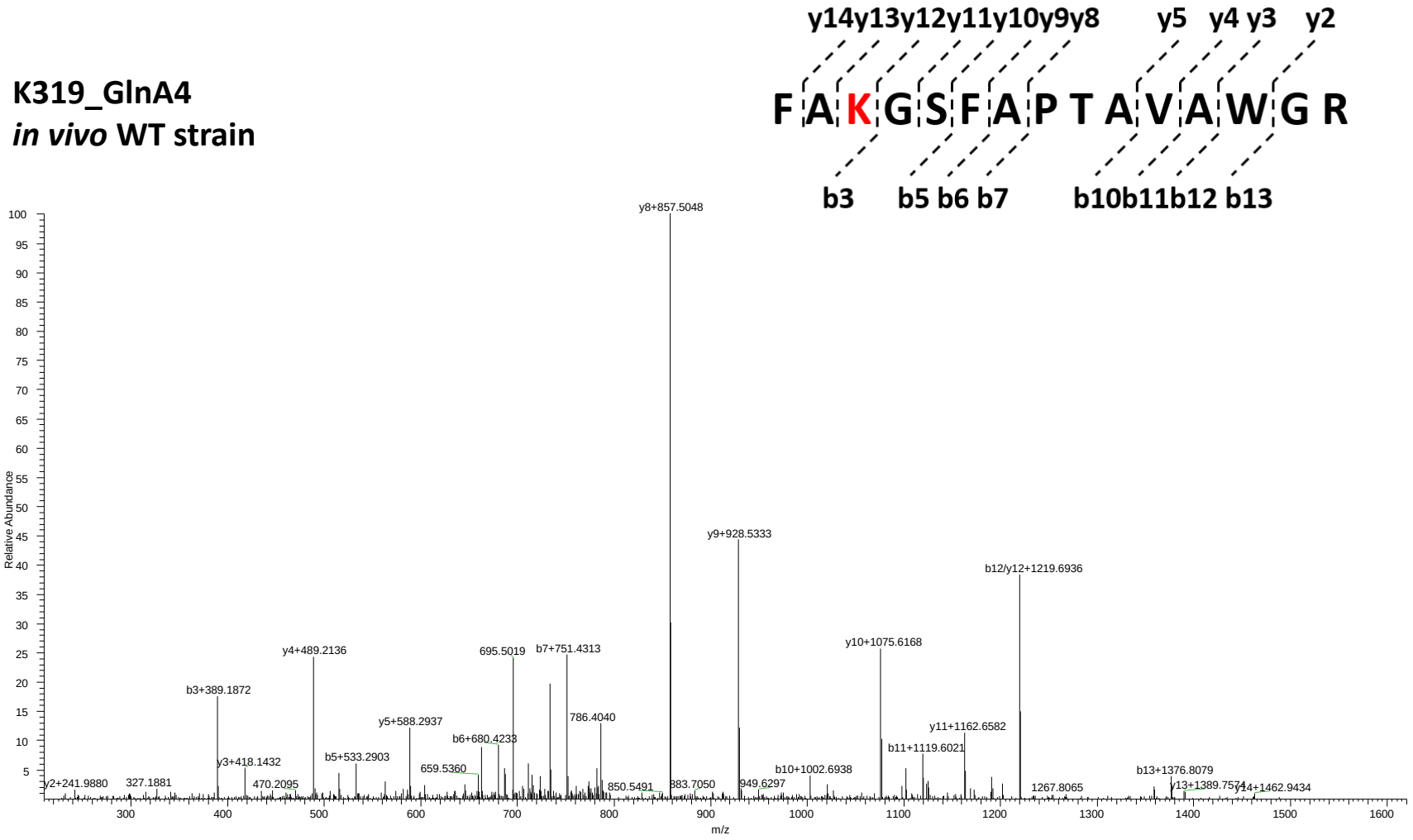




K207_GlnA4
***in vivo* WT strain**



K319_GlnA4
***in vivo* WT strain**



K394_GlnA4
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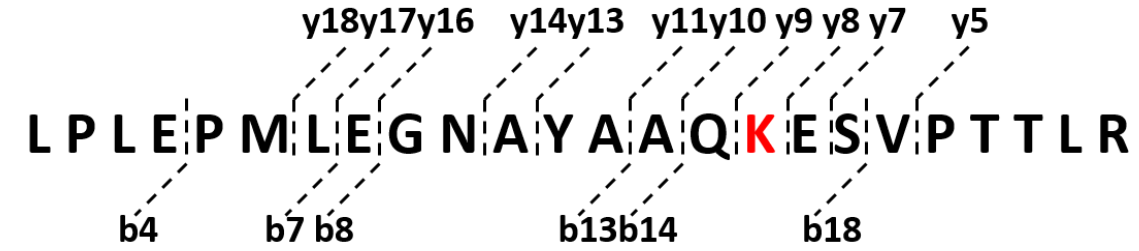
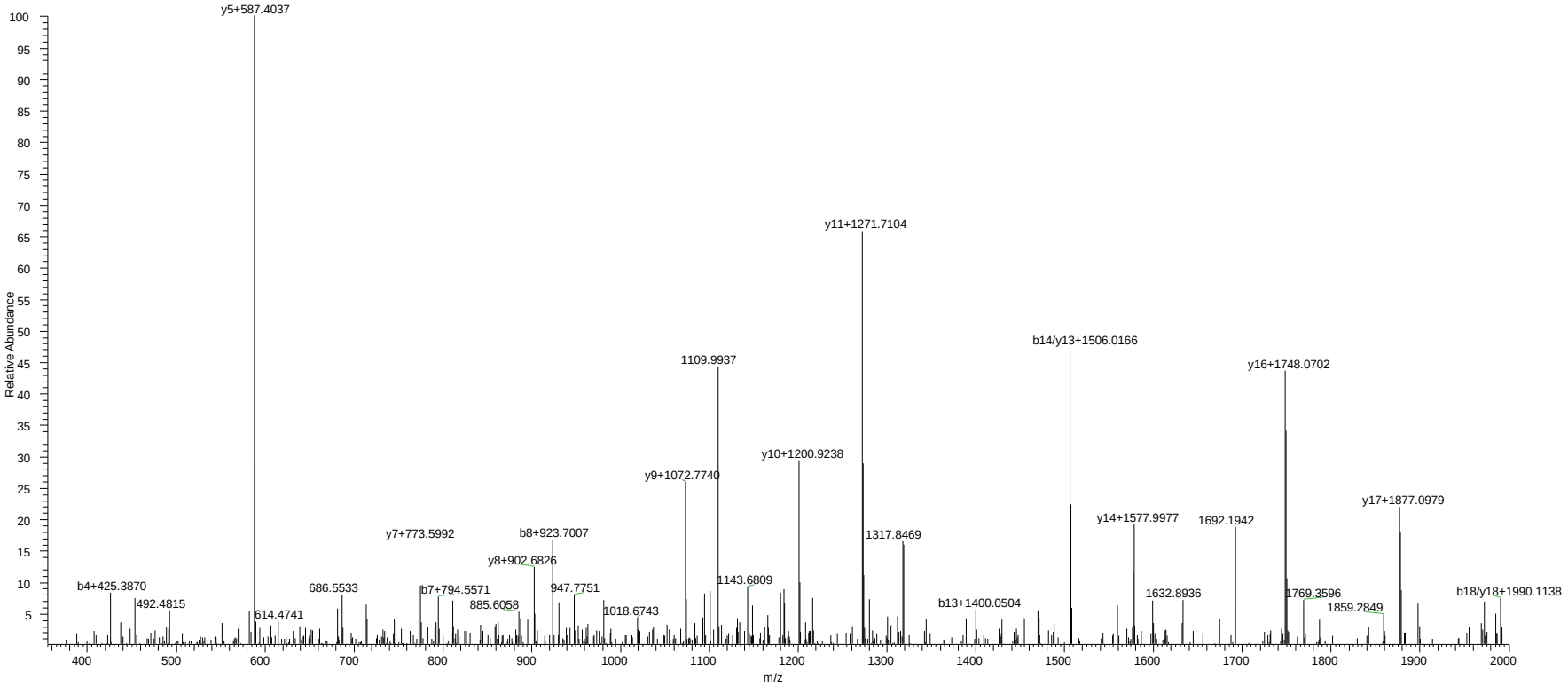
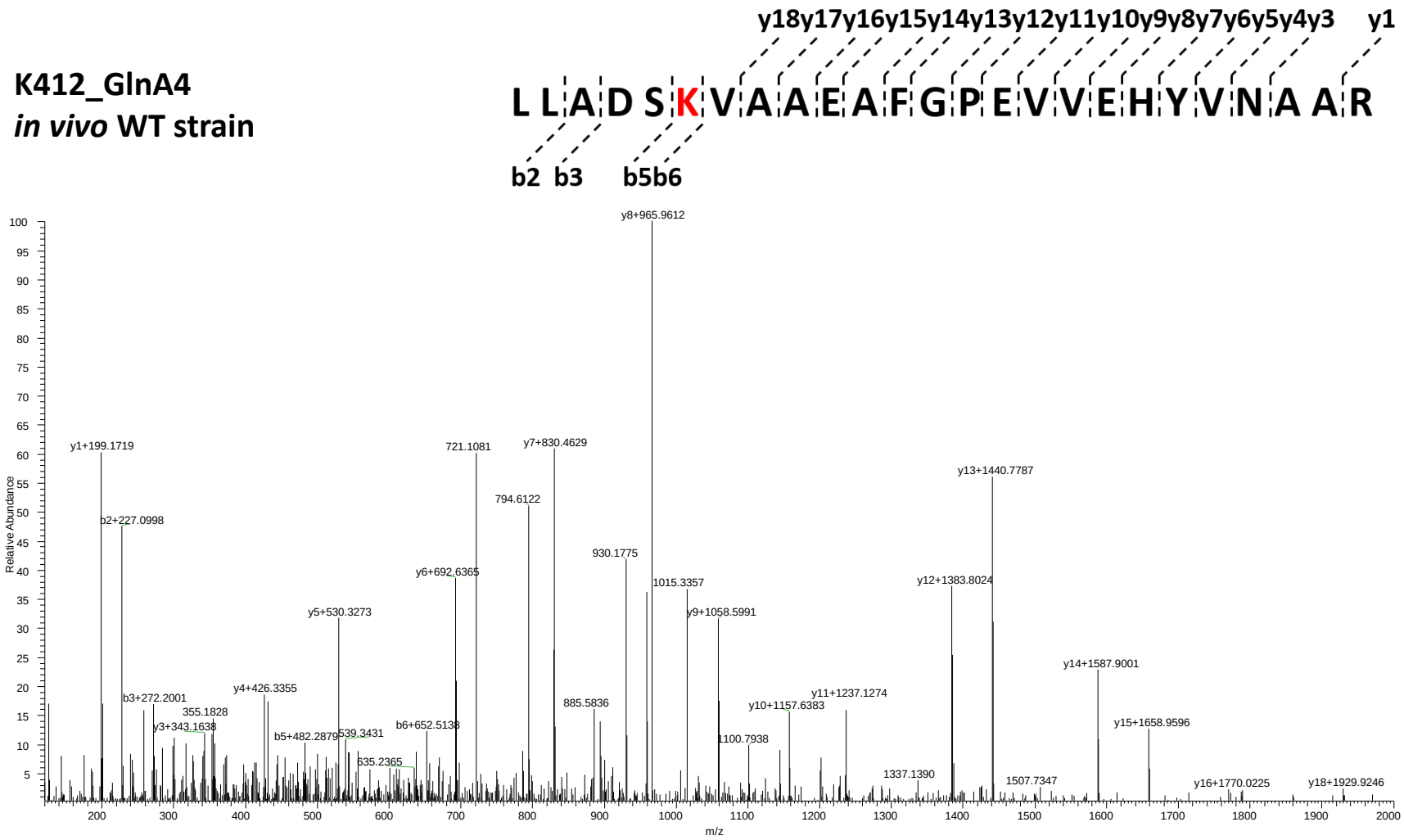
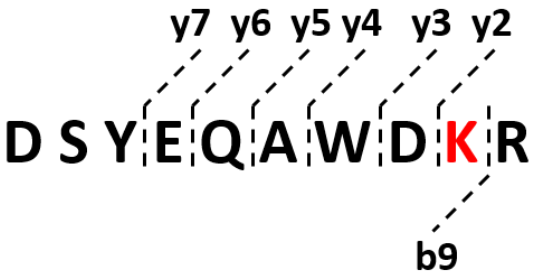


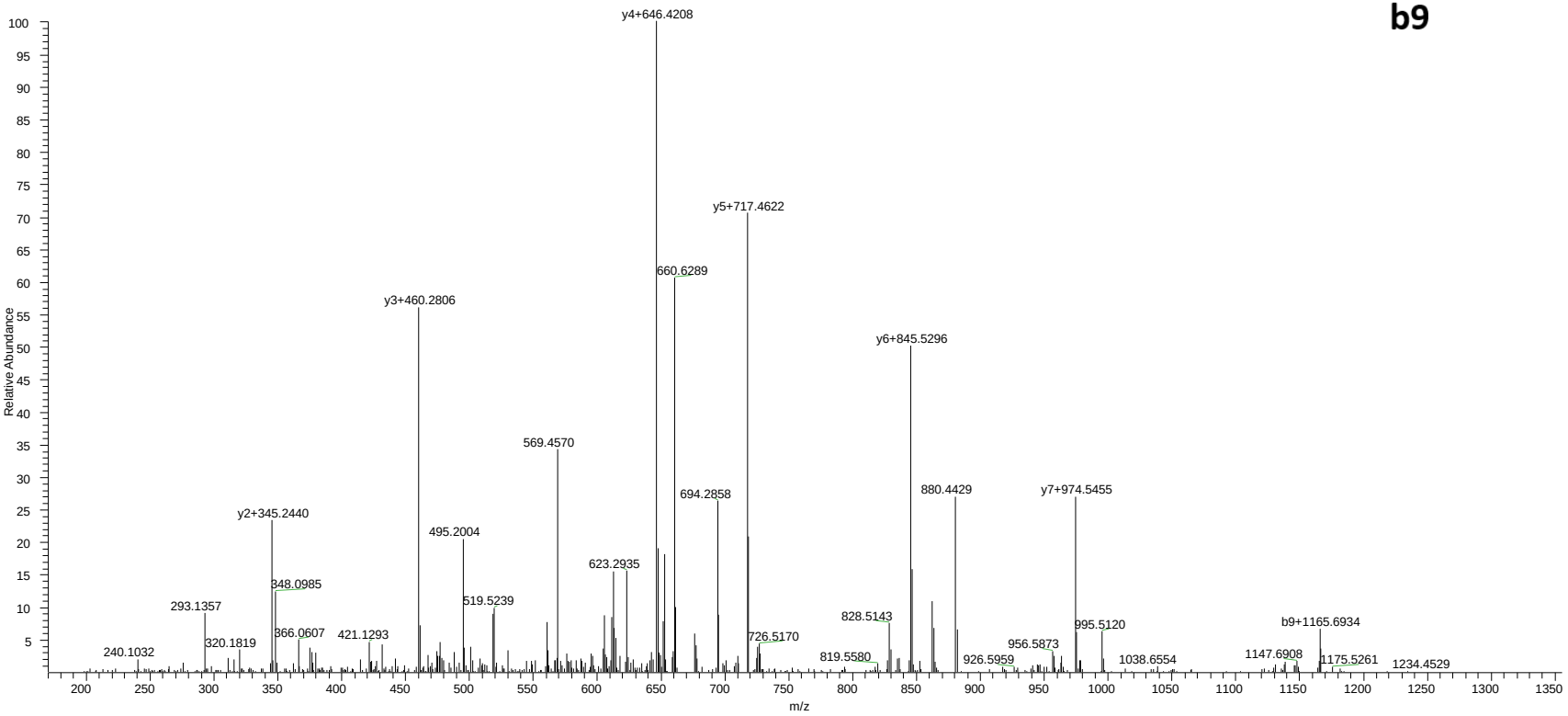


K412_GlnA4
***in vivo* WT strain**

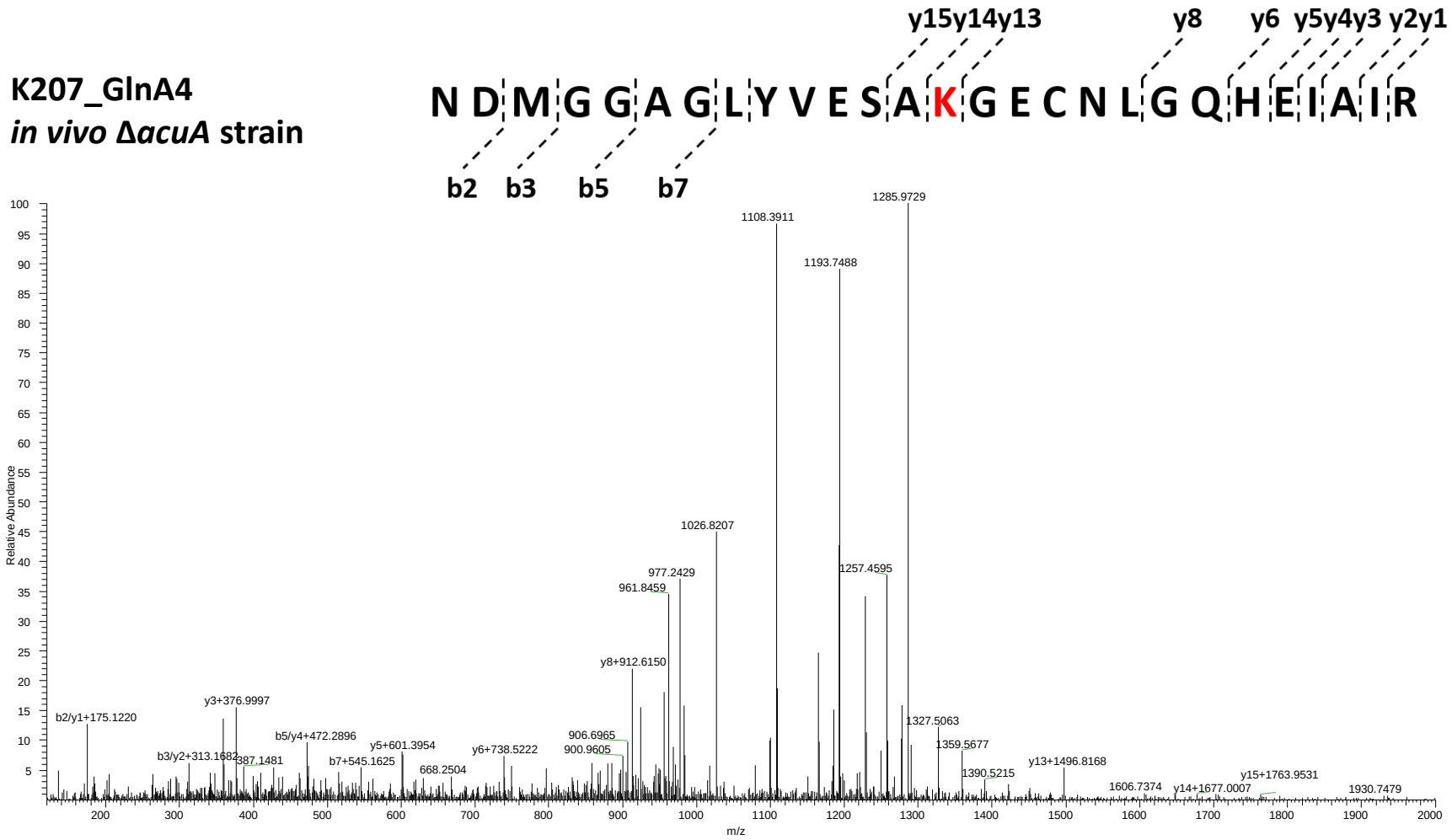


K162_GlnA4
***in vivo* Δ*acuA* strain**



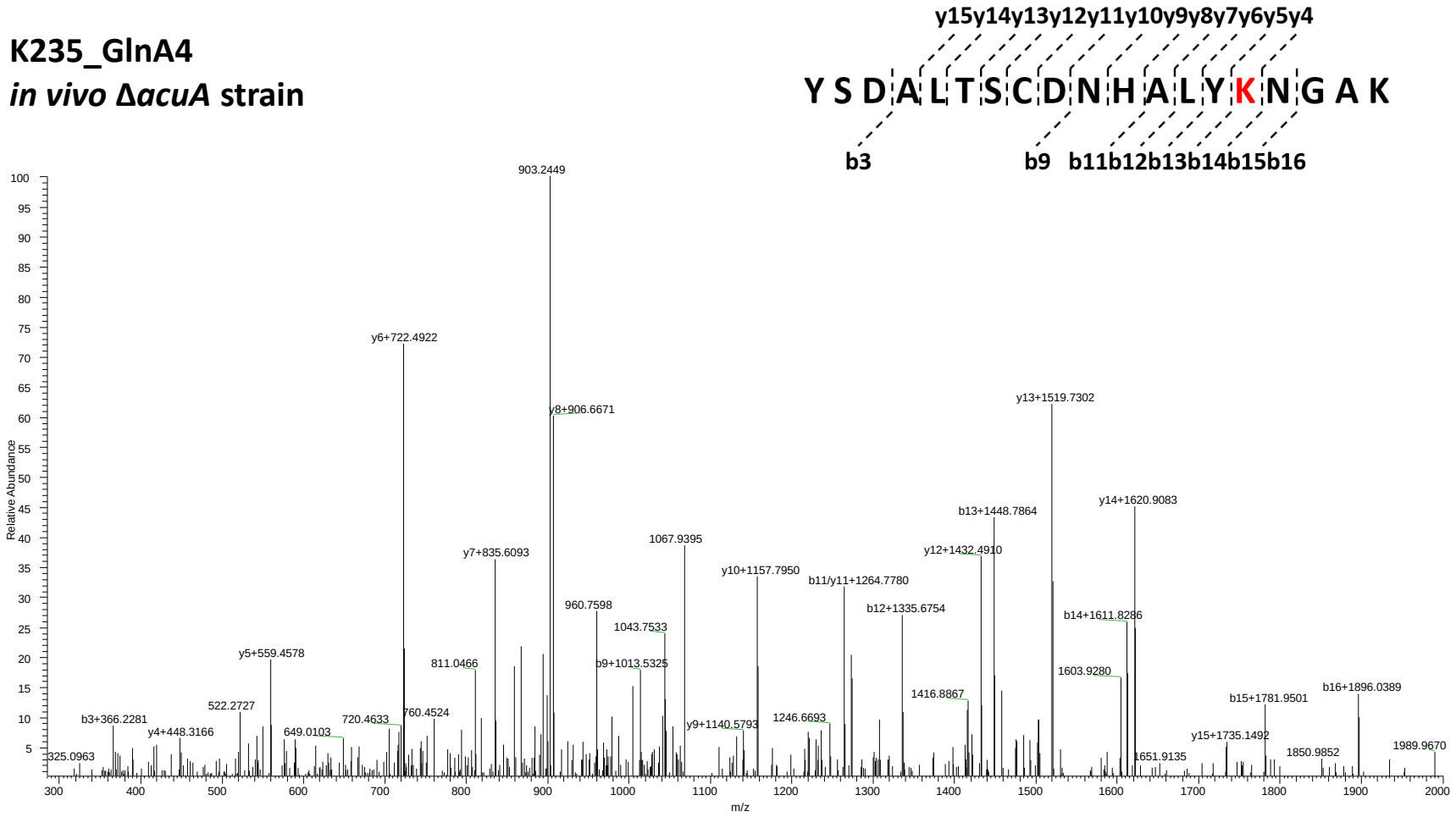


K207_GlnA4
***in vivo* Δ*acuA* strain**

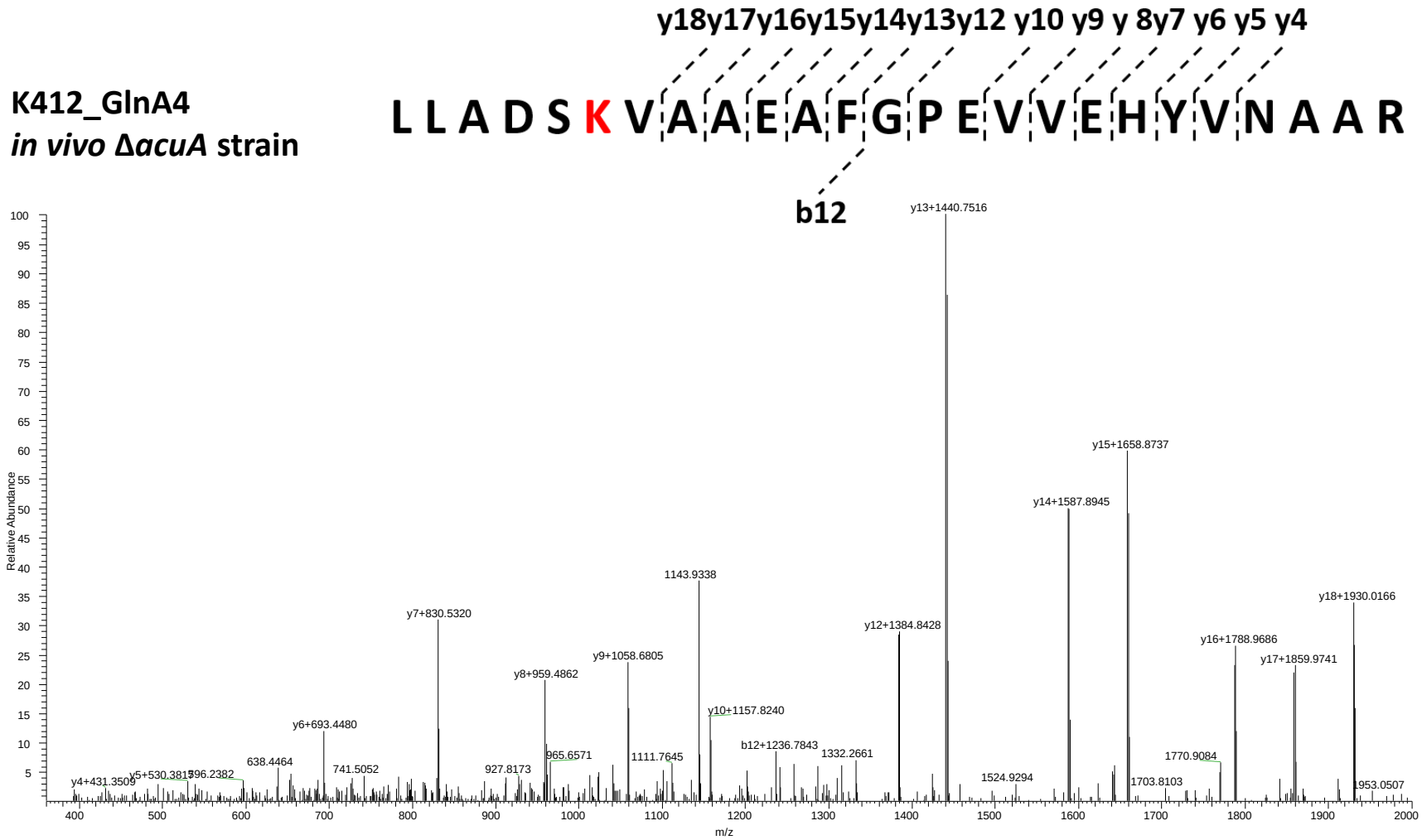


K235_GlnA4

***in vivo* Δ*acuA* strain**

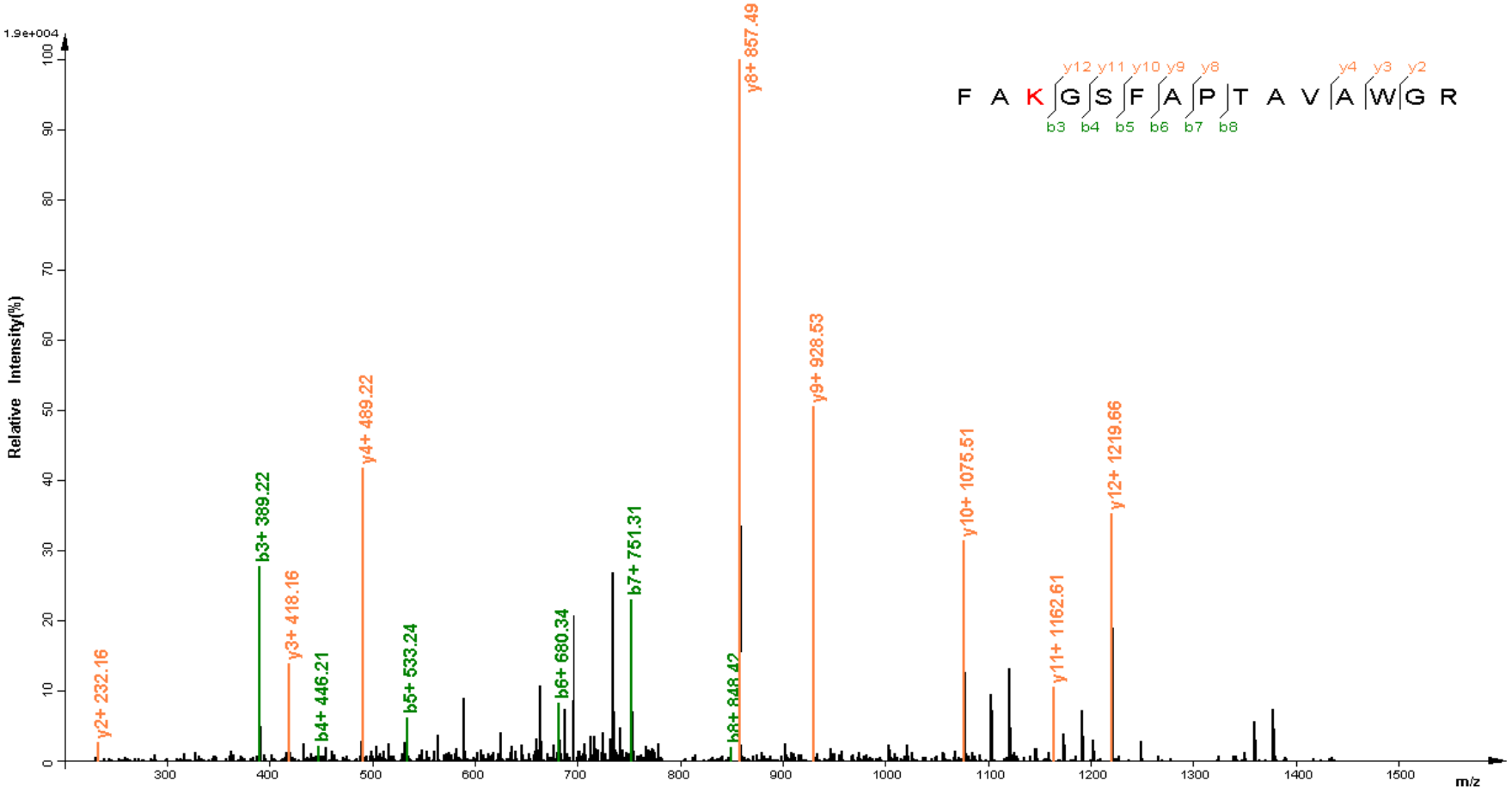


K412_GlnA4
***in vivo* Δ*acuA* strain**



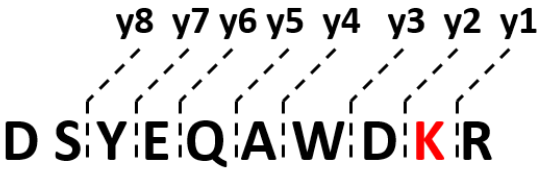
K319_GlnA4

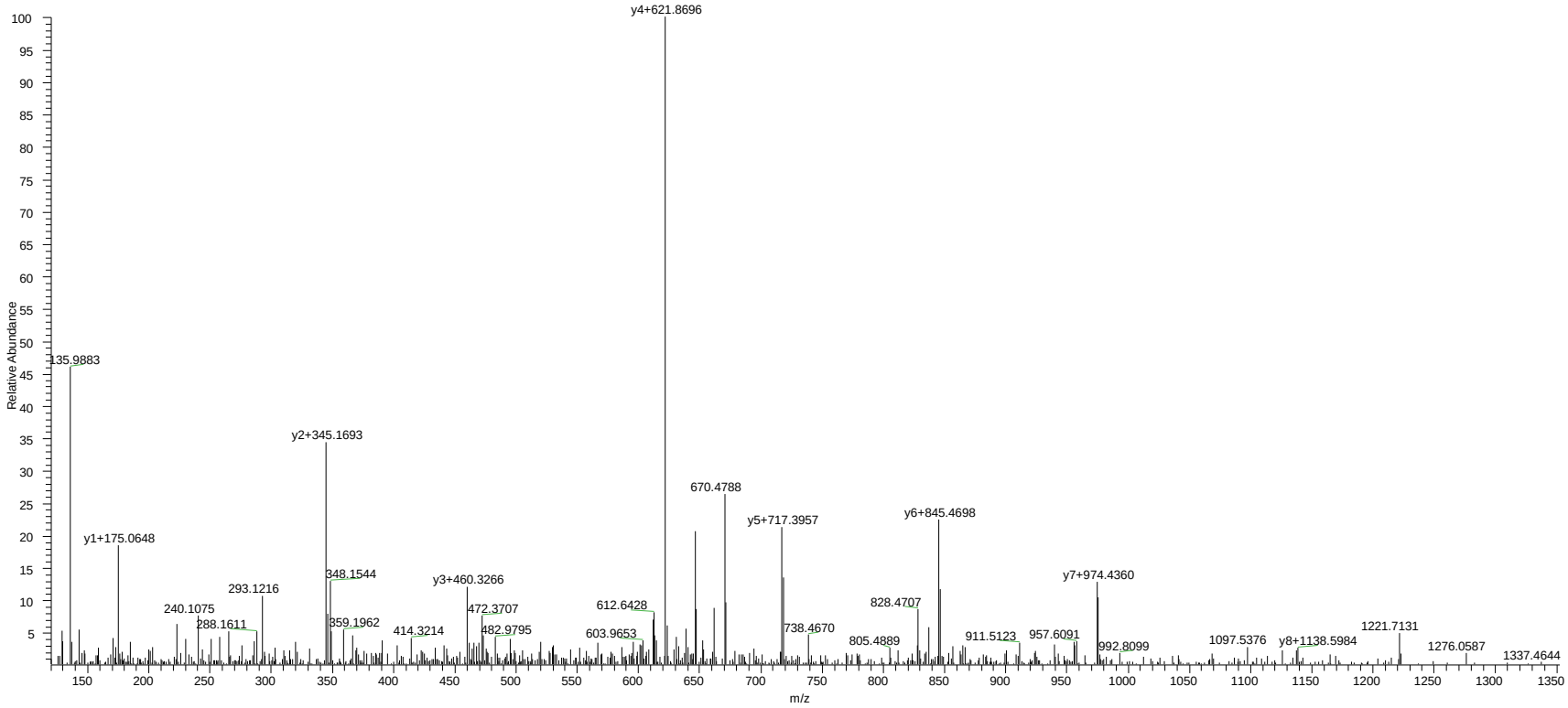
***in vitro* AcuA-acetylation**



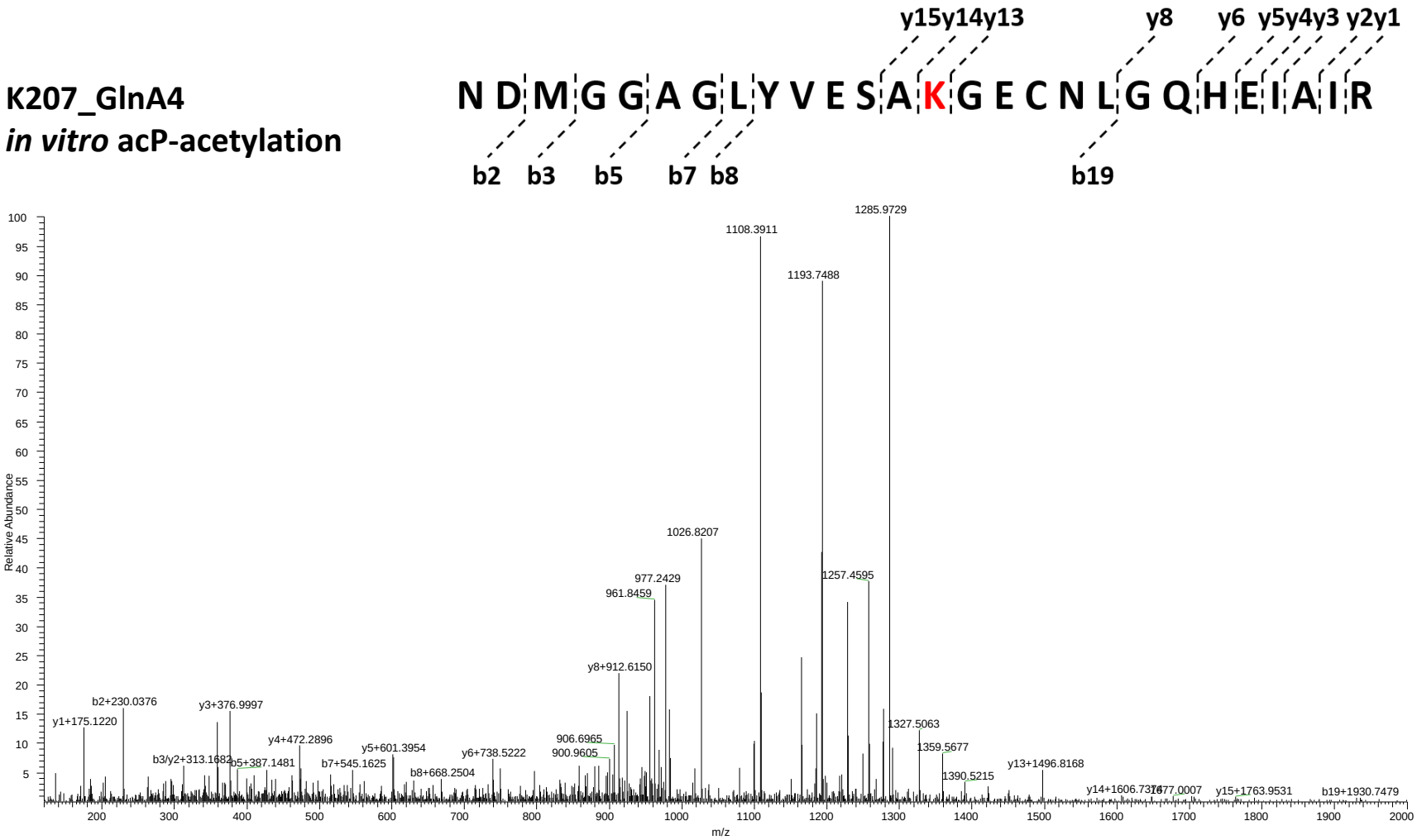
K162_GlnA4

***in vitro* acP-acetylation**

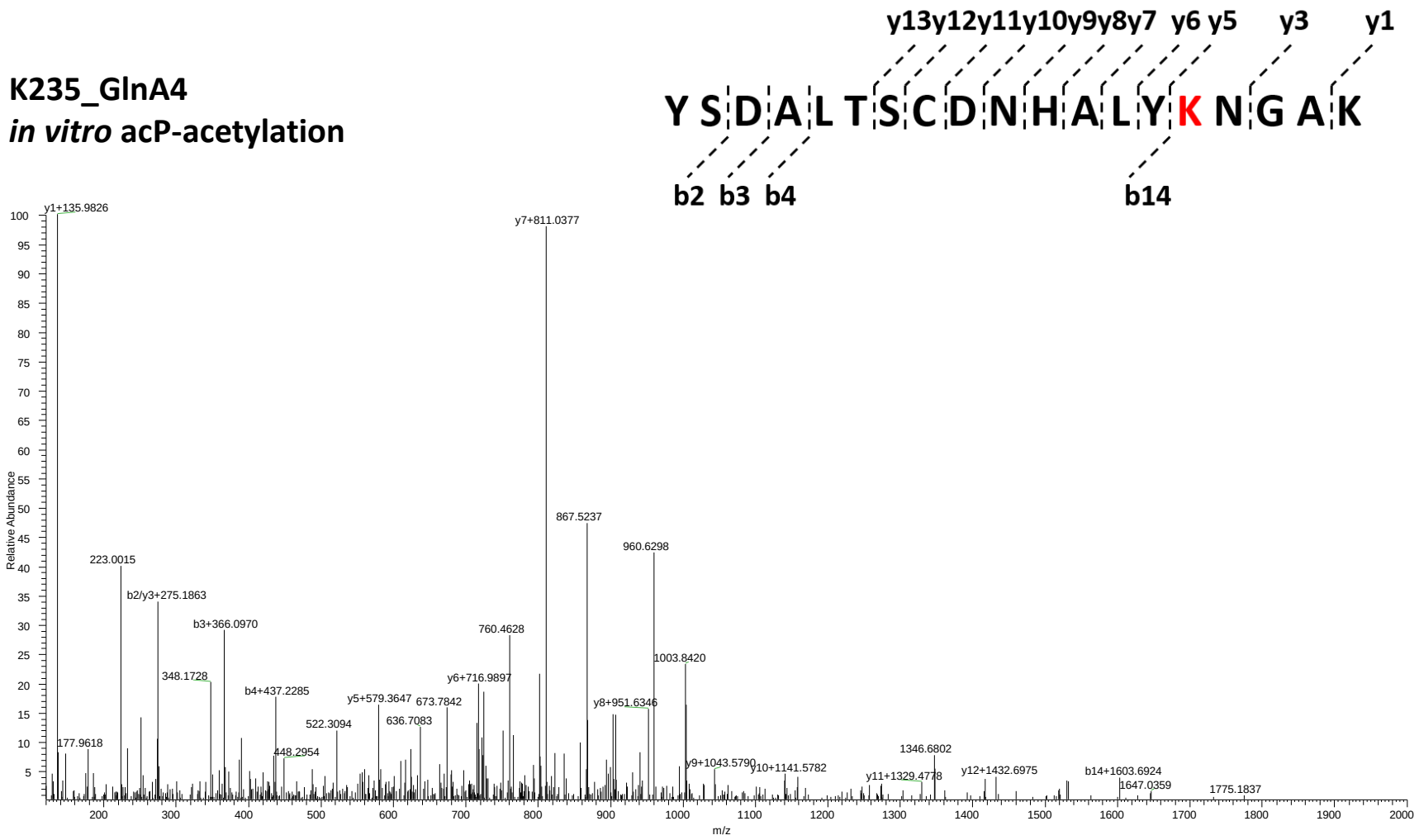




K207_GlnA4
***in vitro* acP-acetylation**

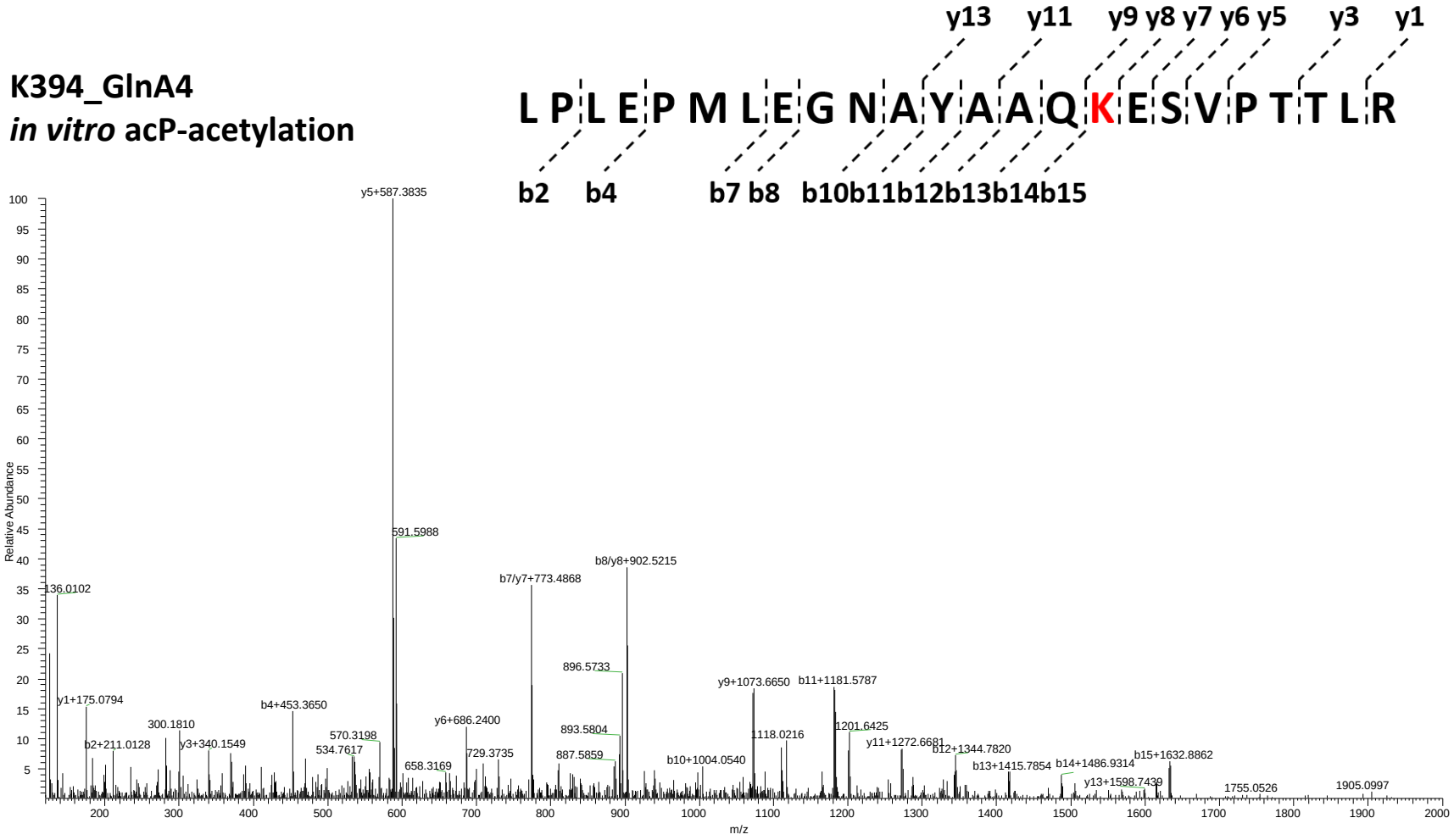


K235_GlnA4
***in vitro* acP-acetylation**



K394_GlnA4

***in vitro* acP-acetylation**



K412_GlnA4
***in vitro* acP-acetylation**