Characterization of virulence potencies associated with the expression of Shiga toxin subtypes using cell-based assays & mass spectrometry

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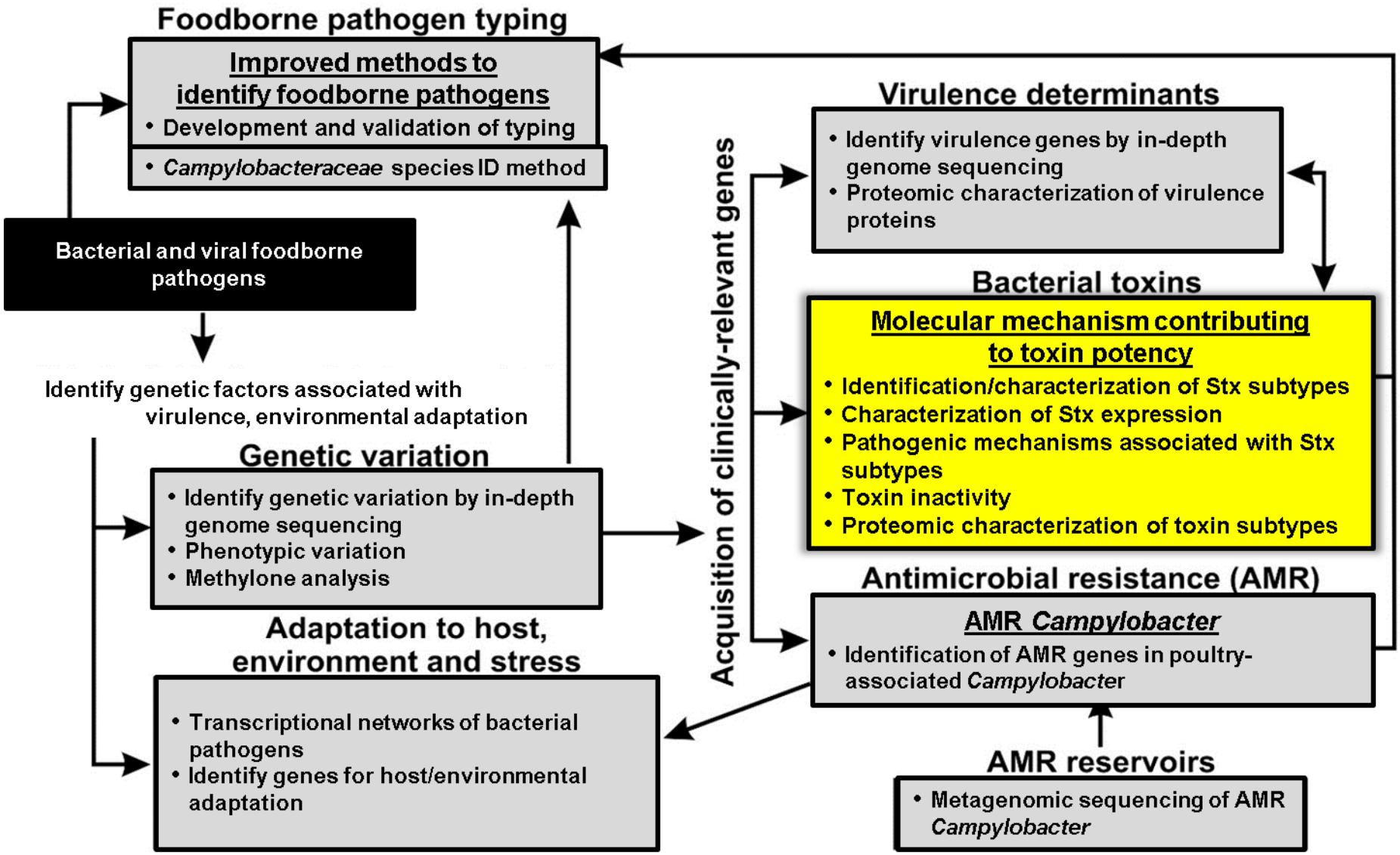
CRIS Project 051: Molecular identification and characterization of bacterial and viral pathogens associated with foods (Produce Safety & Microbiology Unit-WRRC, Albany, CA)



Sampling regions for STEC

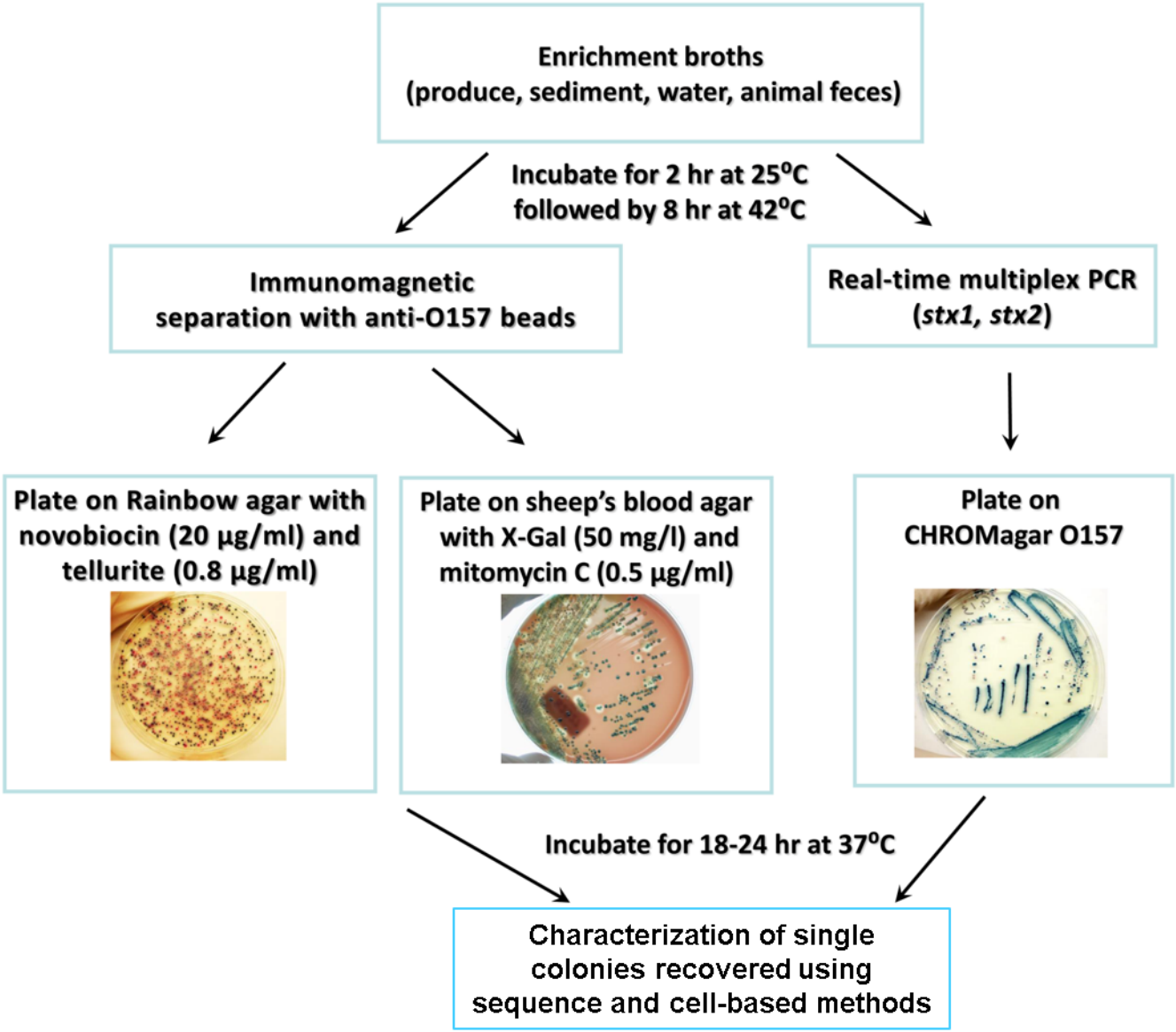
- Salinas Valley area considered the "salad bowl of the United States" major produce production region

- Sampling done in 3 counties in California: Monterrey, San Luis Obispo, and San Benito

- Sample locations in regions for leafy vegetable production
  - Fields (23)
  - Ranches (12)
  - Other locations (47): adjacent waterways and habitats

(Cooley et al., 2013 *PLoS ONE* 8(6): e65716)

Isolation method for STEC

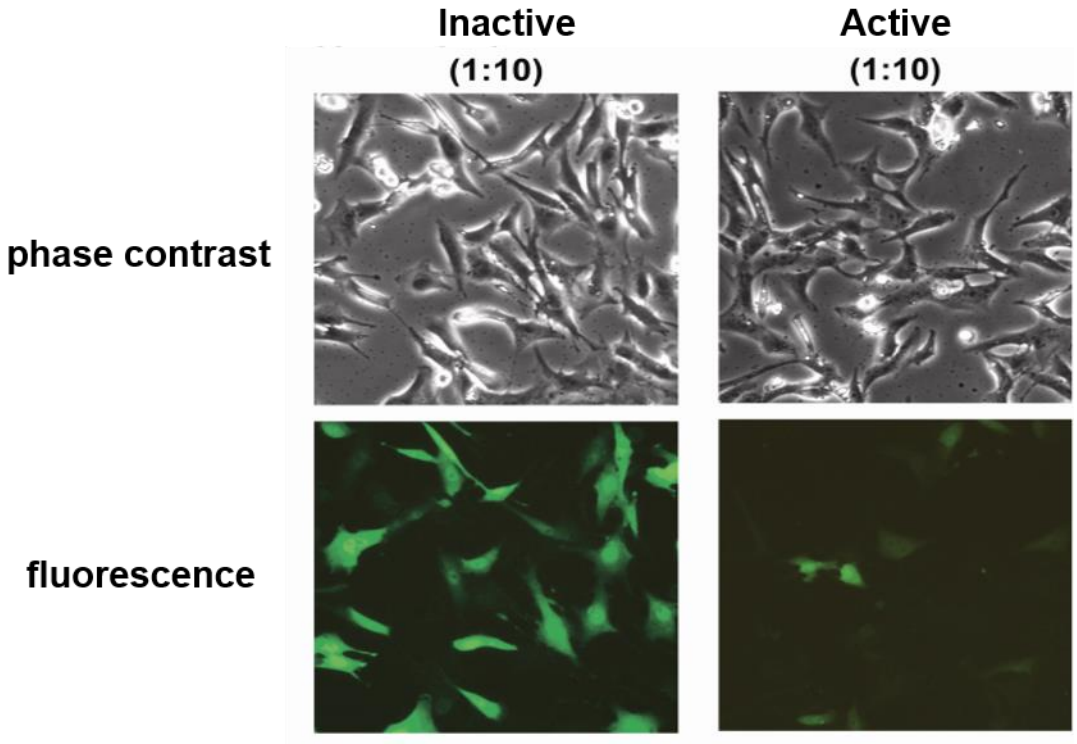


(Cooley et al., 2013 *PLoS ONE* 8(6): e65716)

Development of a Vero cell-based fluorescence assay

- **Vero-d2EGFP cell line that stably expresses a destabilized variant ($t_{1/2}$ = 2 hrs) of the enhanced green fluorescent protein (EGFP)**

- **Assay Format:** Vero-d2EGFP $\xrightarrow{\text{+/- Stx}}$ 96-well plate $\xrightarrow{\text{16 hrs}}$ measure fluorescence



- **Addition of Stx eliminates fluorescence from Vero-d2EGFP cells**

(Quiñones, B., et al., 2009 *Appl. Environ. Microbiol.* 75:1410-1416)

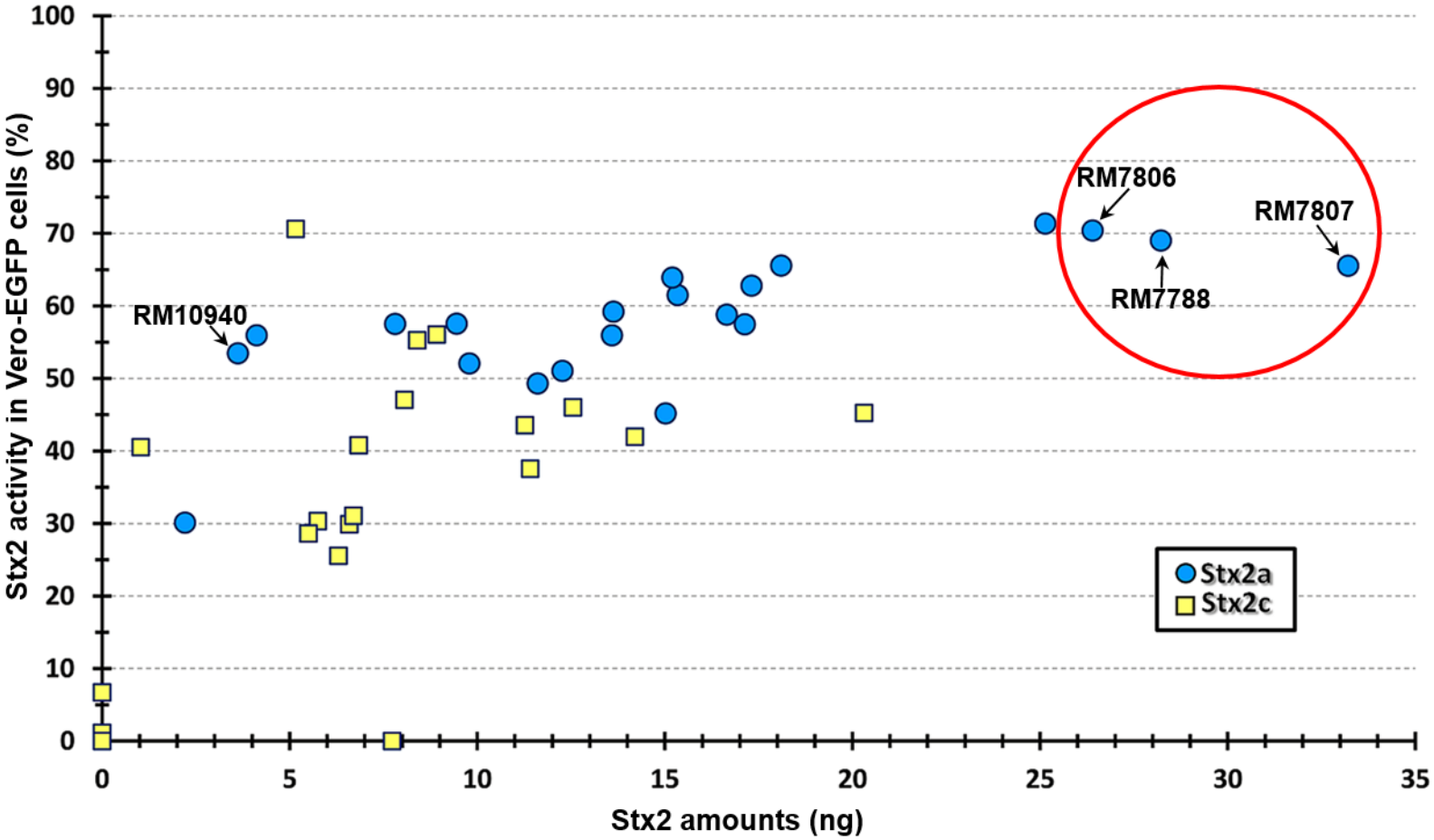
Stx2 subtypes

Stx Type	Amino acid homology to Stx2 (%) A subunit	B subunit	Characteristics
Stx1a	55	57	Shiga toxin produced by STEC and almost identical to Stx produced by *Shigella dysenteriae* serotype 1
Stx2a	100	100	Prototype of non-Stx1 toxins; associated with severe HUS disease in humans
Stx2b	99	97	Associated with *eae*-negative STEC and mild disease in humans
Stx2c	100	97	Associated with diarrhea and HUS in humans; common in ovine STEC
Stx2d	99	97	Strains with this toxin are highly virulent; Stx activity increased by elastase
Stx2e	93	84	A variant responsible for edema disease in pigs; rare in human disease and associated with mild diarrhea or asymptomatic infections in humans
Stx2f	71	82	A subtype frequently isolated from pigeon droppings; rare in human disease
Stx2g	96	94	A subtype common in bovine STEC

(Gyles, C. L., 2007 *J. Anim. Sci*. 85:E45-62)

- The Stx2 group is composed of a diverse and heterogeneous group of subtypes

Relationship between Shiga toxin activity and amounts in STEC recovered from produce production regions in California



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PROKARYOTES

Draft Genome Sequences of *Escherichia coli* O113:H21 Strains Recovered from a Major Produce-Production Region in California

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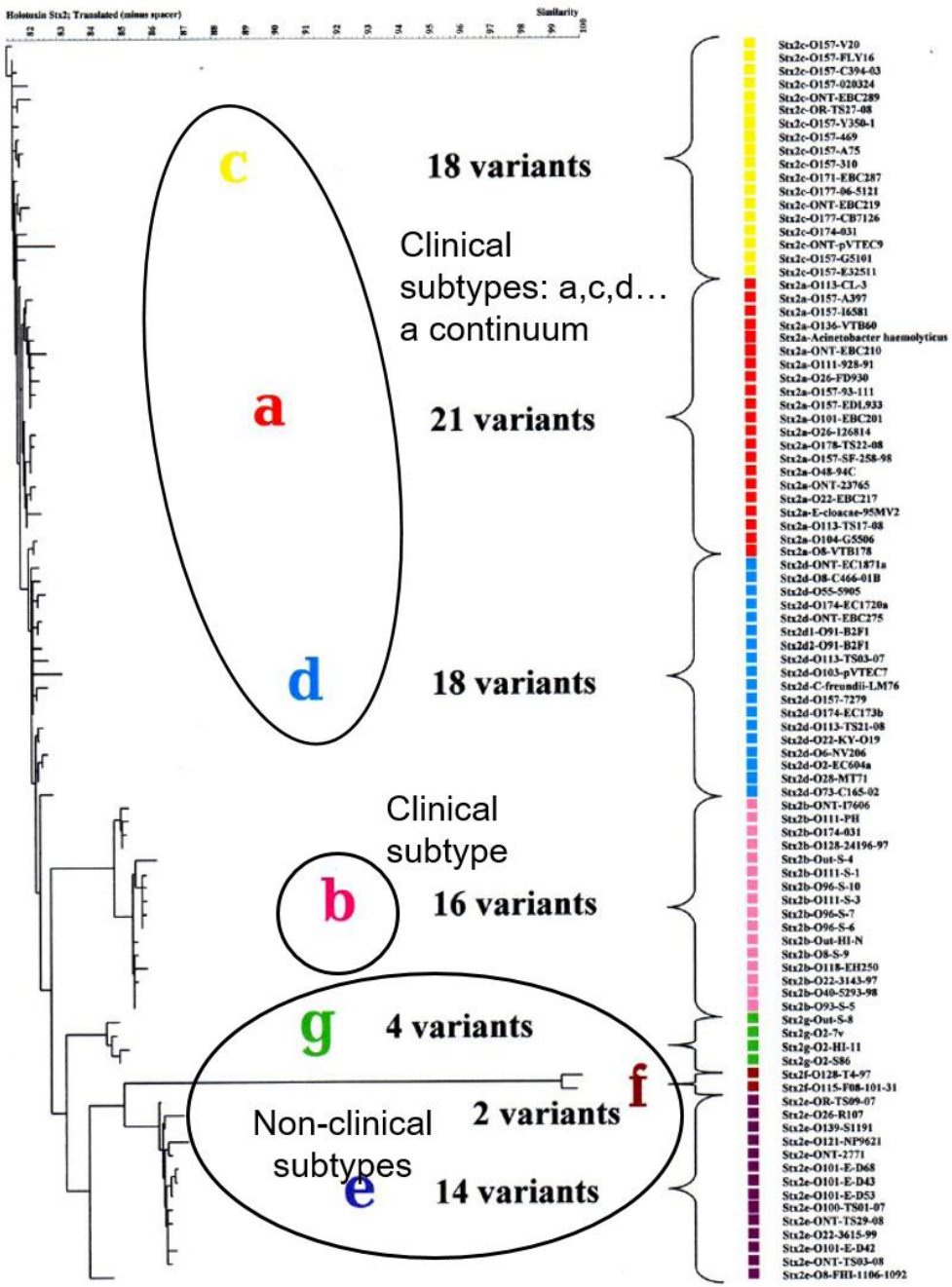
TABLE 1 Genome characteristics of *Escherichia coli* O113:H21 from produce-production regions in California

Strain	Serotype	Source	stx_{2a} integration locus	Genome size (bp)	No. of contigs	G+C content (%)	GenBank accession no.
RM7788	O113:H21	Water	*potC, serT*	5,029,620	179	50.6	NWVS00000000
RM7806	O113:H21	Pig	*potC, serT*	5,155,541	18	50.6	NWVR00000000
RM7807	O113:H21	Pig	*potC, serT*	5,032,176	202	50.6	NWVQ00000000
RM9244	O113:H21	Cattle	*potC, yehV*	5,375,282	279	50.5	NWVP00000000
RM9245	O113:H21	Cattle	*potC, yehV*	5,295,742	311	50.5	NWVO00000000
RM9246	O113:H21	Cattle	*potC, yehV*	5,293,811	294	50.5	NWVN00000000
RM10940	O113:H21	Cattle	*yehV*	5,036,463	131	50.6	NWVM00000000



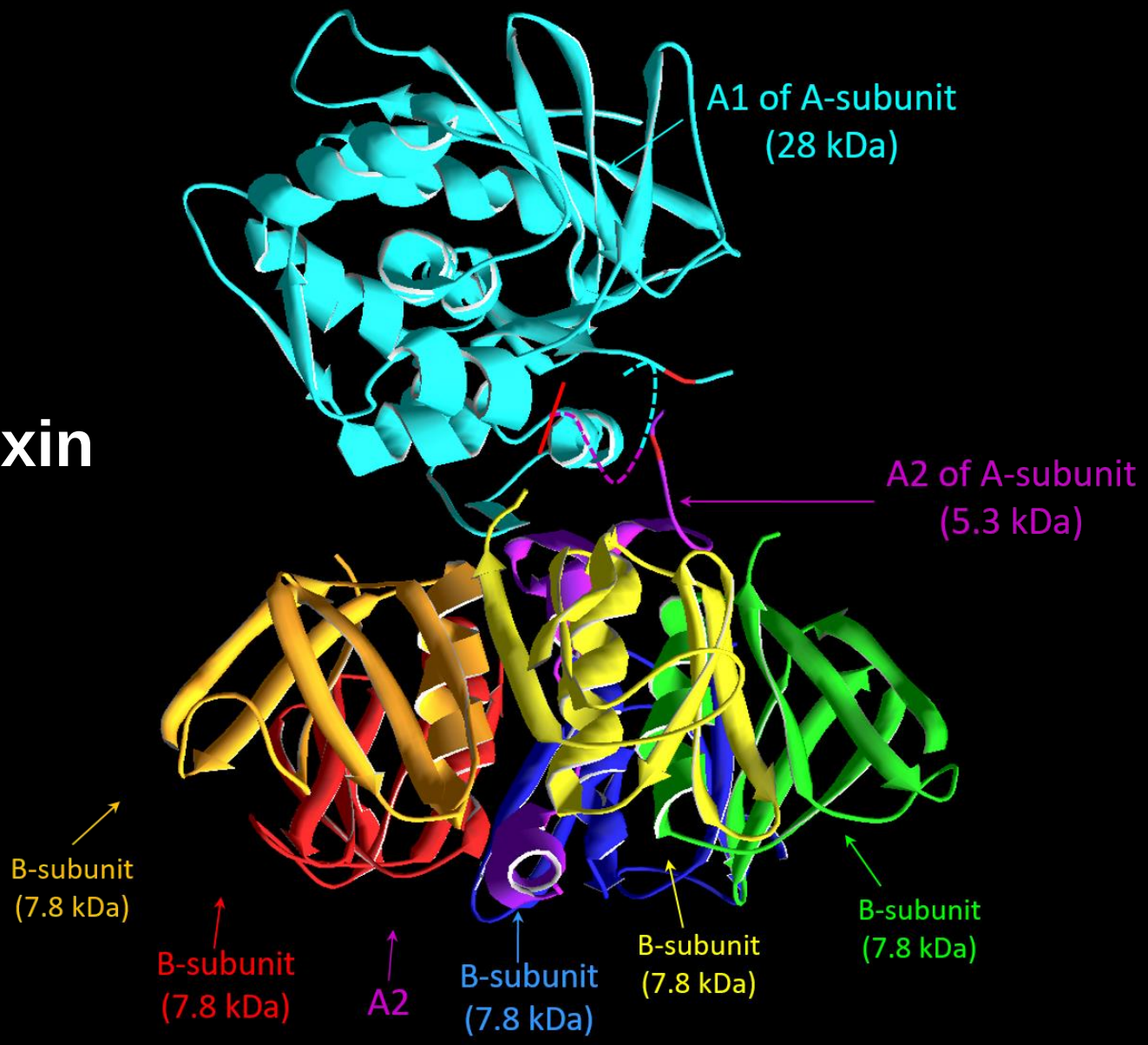
Stx2 subtypes

Modified from Scheutz, et al.
J. Clin. Microbio. **2012**

Stx2 subtypes

- Amino acid (AA) substitutions result in differences in subtype toxicity
- Most substitutions occur in the B-subunit & A2 sequence of the A-subunit
- Differences in toxicity due to AB_5/cell surface attachment, retro-translocation & processing
- Rapid methods are needed to detect & identify Stx types and subtypes

AB_5
Stx holotoxin



Protein Data Bank

Mass spectrometry of Stx from STECs

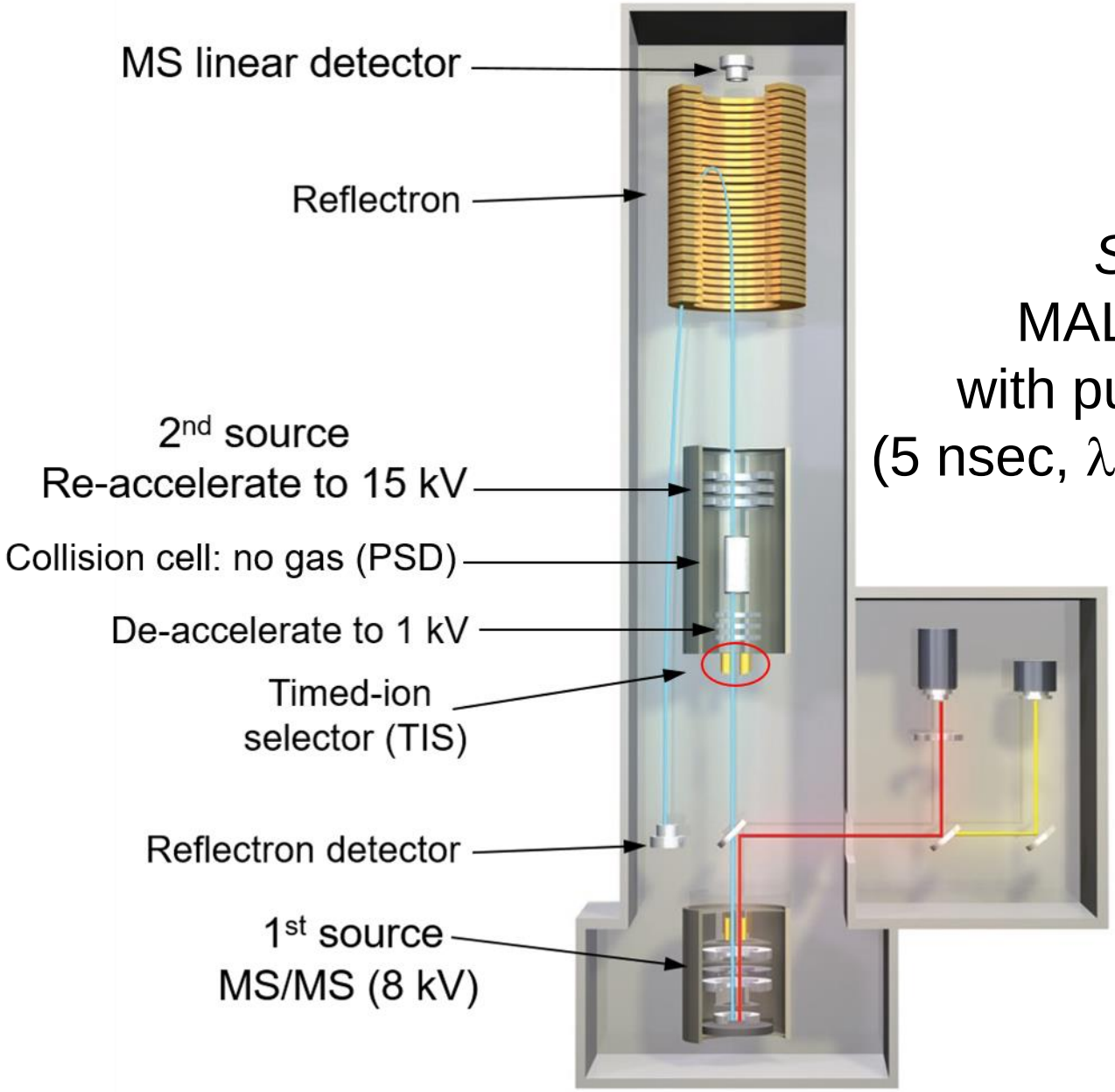
- 4 hr culture from glycerol stocks in LBB followed by overnight culture on LBA with ciprofloxaxin or mitomycin-C (MMC)
- Next morning: 1 μL loop of cells in 300 μL of water
- Brief vortexing and 2 min centrifugation
- B-subunit (reduced): 10 min DTT
- A2: 1 hr furin digestion, 10 min DTT reduction
- 0.5 μL supernatant spotted onto SS target. Let dry. Overlay sinapinic acid (33% CH_3CN, 67% H_2O, 0.2% TFA)
- MALDI-TOF-TOF-MS/MS & post-source decay (PSD)
- 10k laser shots. Data acquisition: less than 1 minute
- USDA software for top-down proteomic ID

Top-down vs. bottom-up proteomic analysis

- "Top-down" means that a protein is <u>not</u> digested prior to mass spectrometry analysis

- "Bottom-up" means that the protein is digested (trypsin) into its constituent peptides prior to mass spectrometry analysis

- Pros & cons of "top-down"
  - 1st step is an accurate measurement of the mass of the mature, functional protein (including any PTMs). Bottom-up does not do this step
  - Fragment ions are from gas phase dissociation of the entire protein precursor ion
  - Although top-down spectra can be complex, it provides a more complete picture of the presence and location of PTMs.
- Pros & cons "bottom-up"
  - Peptides ions are easier to fragment, sequence & quantify
  - However, 50% protein sequence coverage is considered excellent!?
  - In consequence, PTMs can be missed because incomplete sequence coverage and/or facile dissociative loss of PTM



Sciex 4800
MALDI-TOF-TOF
with pulsed YAG laser
(5 nsec, λ = 355 nm, 200 Hz)

MS/MS: post-source decay (PSD)

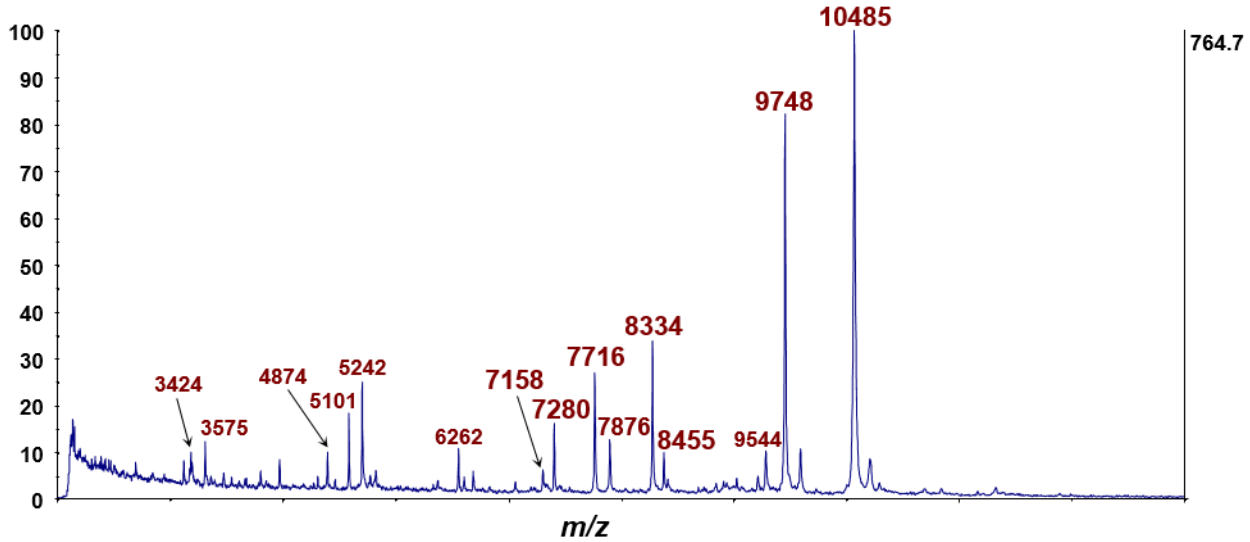
- PSD of MALDI protein ions (+1 or +2) fragment on the C-terminal side of aspartic acid (D) & glutamic acid (E) residues:
  NH_2- - - - - - D- - - - - - - - - - D- - - - E- - D- - - - - - CO_2H

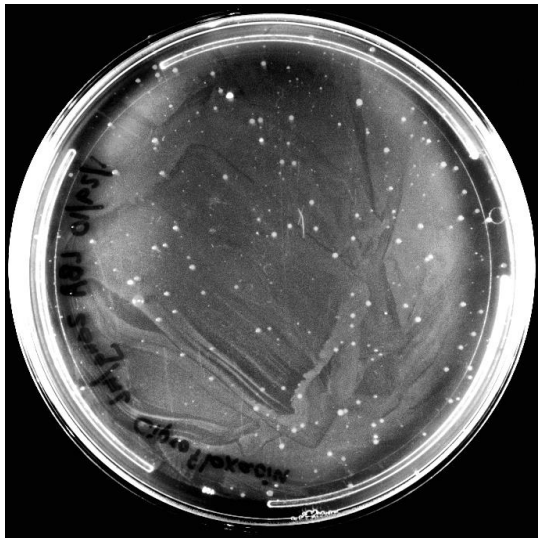
- <u>Fragmentation</u>: Proton transfer from the side-chain of D- and E-residues to the polypeptide backbone weakening the amide bond

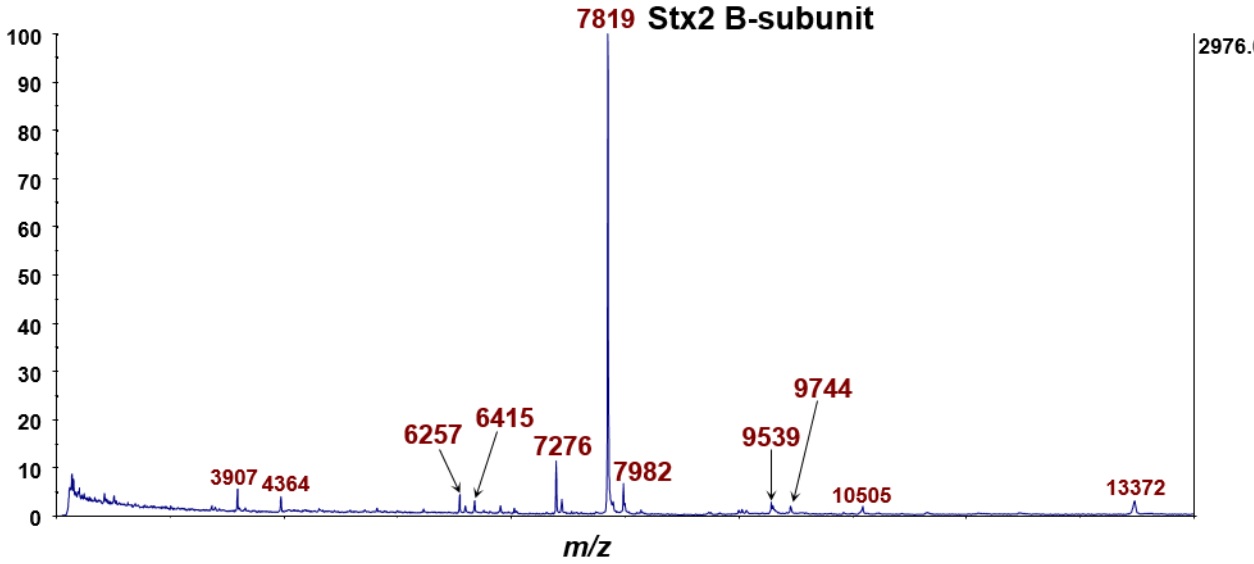
- <u>Mechanism</u>: “Aspartic acid effect”
  <u>Reference</u>: Yu W, Vath JE, Huberty MC, Martin SA. Analytical Chem. 1993, 65:3015-23.

E. coli O157:H7 strain EDL933
Stx2a subtype

LBA





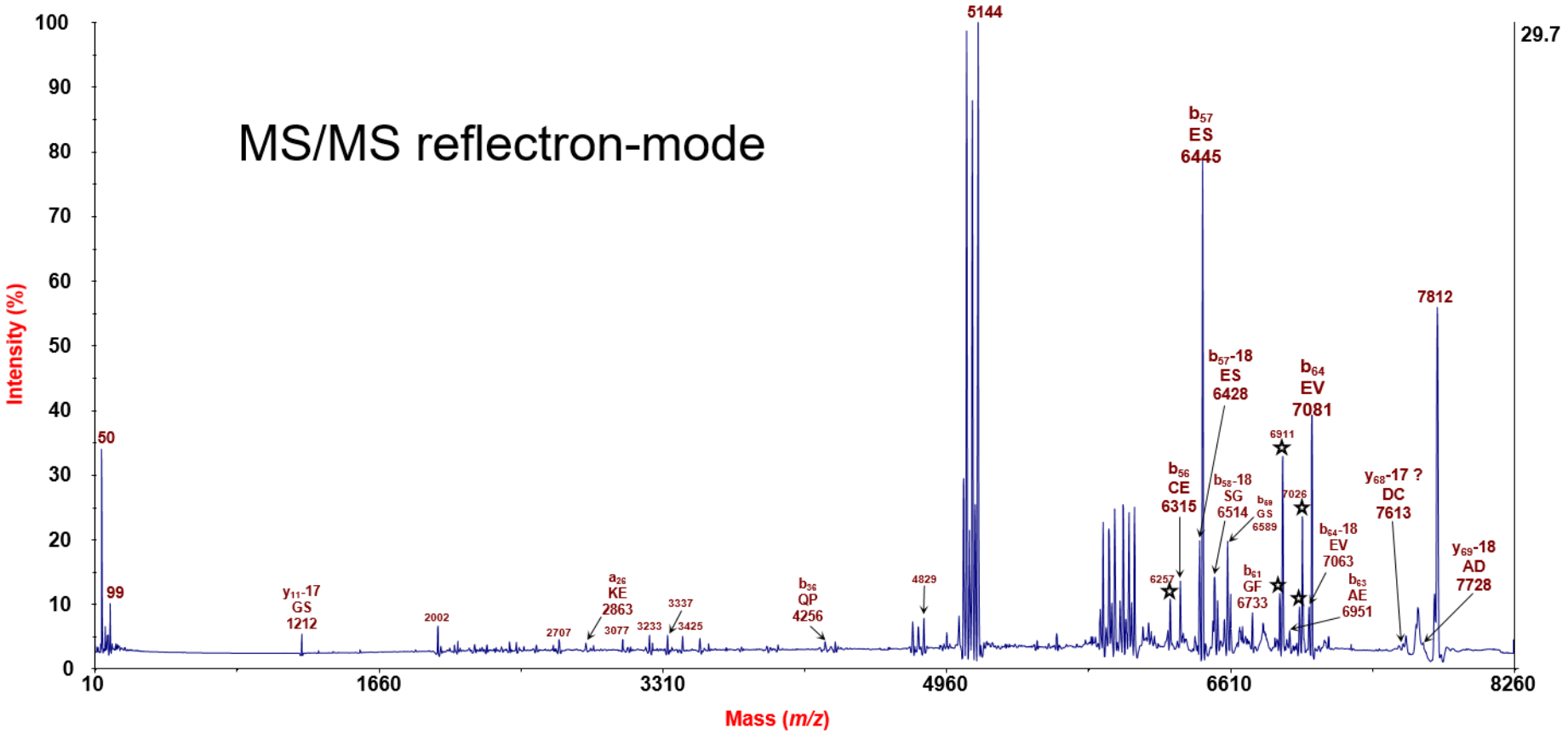


LBA with ciprofloxacin

MS/MS of *m/z* 7819 B-subunit (-*S*-*S*-) of EDL933

S.....*S*

ADCAKGKIEFSKYNE*D*D*TFTVKVD*GKE*YWTSRWNLQ*PLLQSAQLTGMTVTIKSSTCE*SGSGFAE*VQFNND



Dissociation: post-source decay (PSD)

- **<u>Lasso-looped 2° structure of B-subunit:</u> polypeptide cleavage at D & E residues between C residues of disulfide bond…**

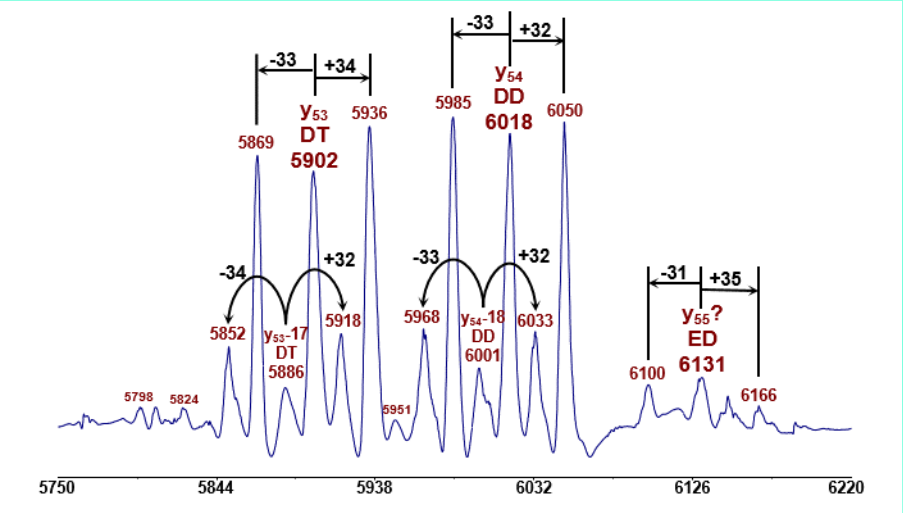
S.....*S*

ADCAKGKIEFSKYNE*D*D*TFTVKVD*GKE*YWTSRWNLQ*PLLQSAQLTGMTVTIKSSTCE*SGSGFAE*VQFNND

Symmetric –S–S–

Asymmetric –S–S– & –S–S–

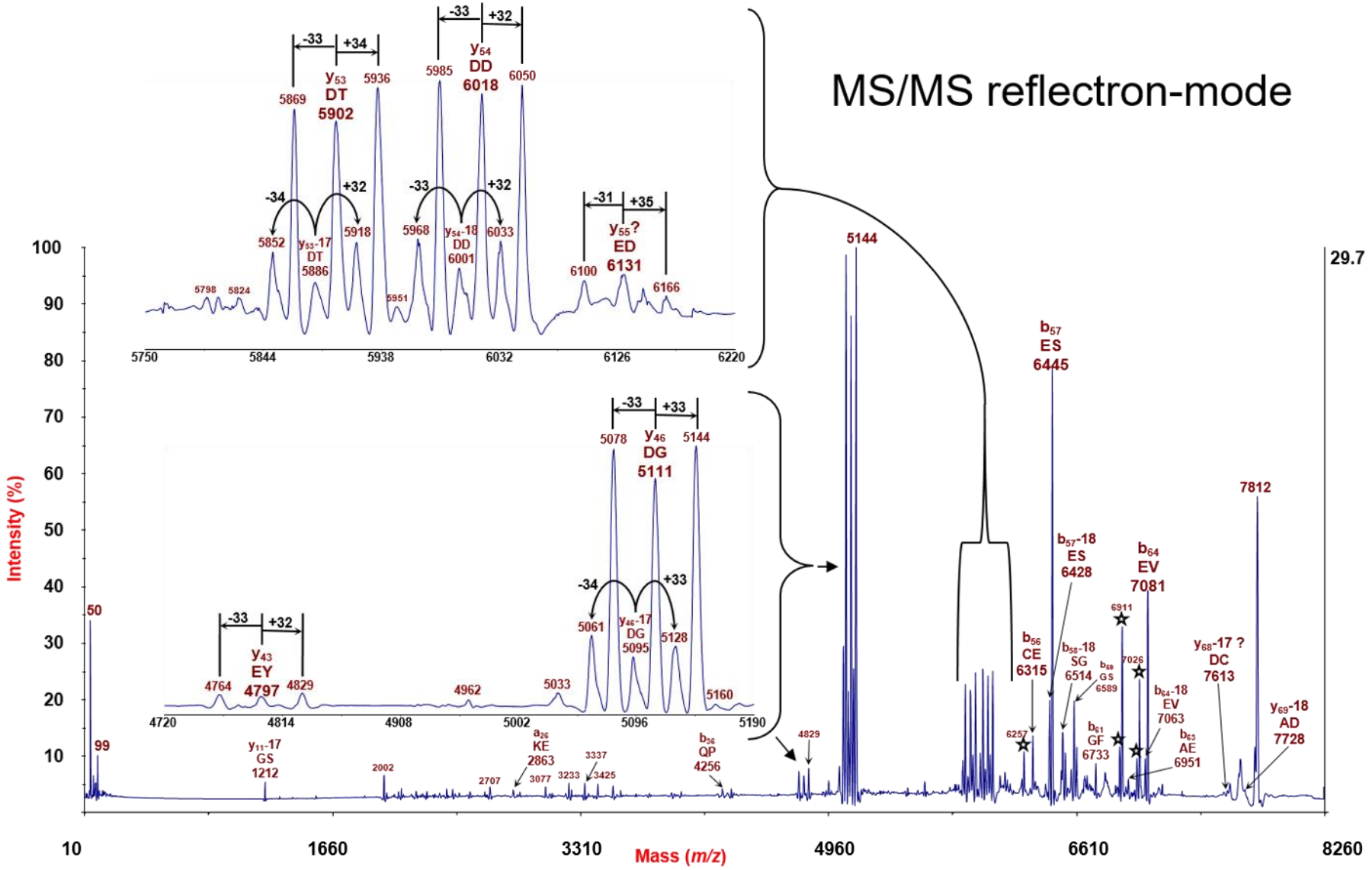
…fragment ion triplet…



MS/MS of *m/z* 7819 B-subunit (-*S*-*S*-) of EDL933

S……S

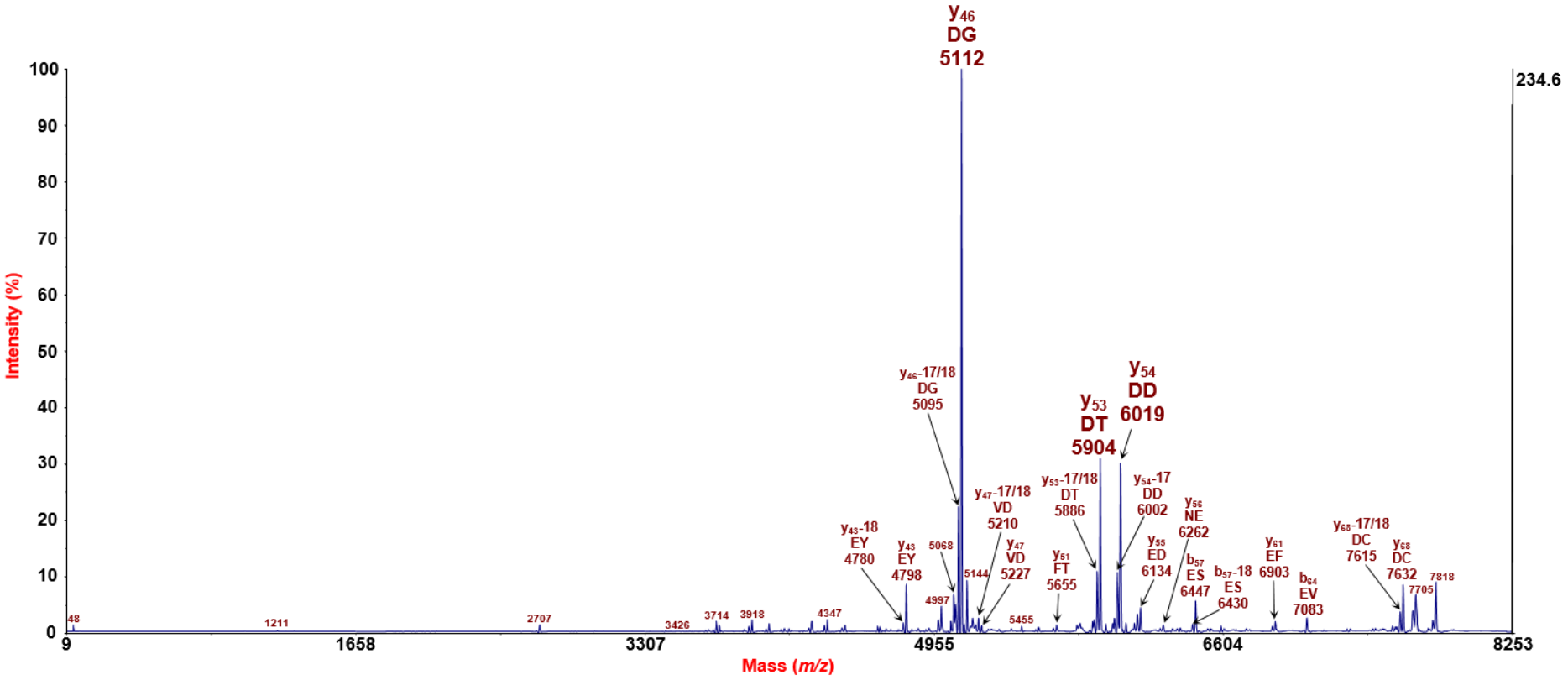
ADCAKGKIEFSKYNE*D*D*TFTVKVD*GKE*YWTSRWNLQ*PLLQSAQLTGMTVTIKSSTCE*SGSGFAE*VQFNND



MS/MS of B-subunit (-SH) of EDL933

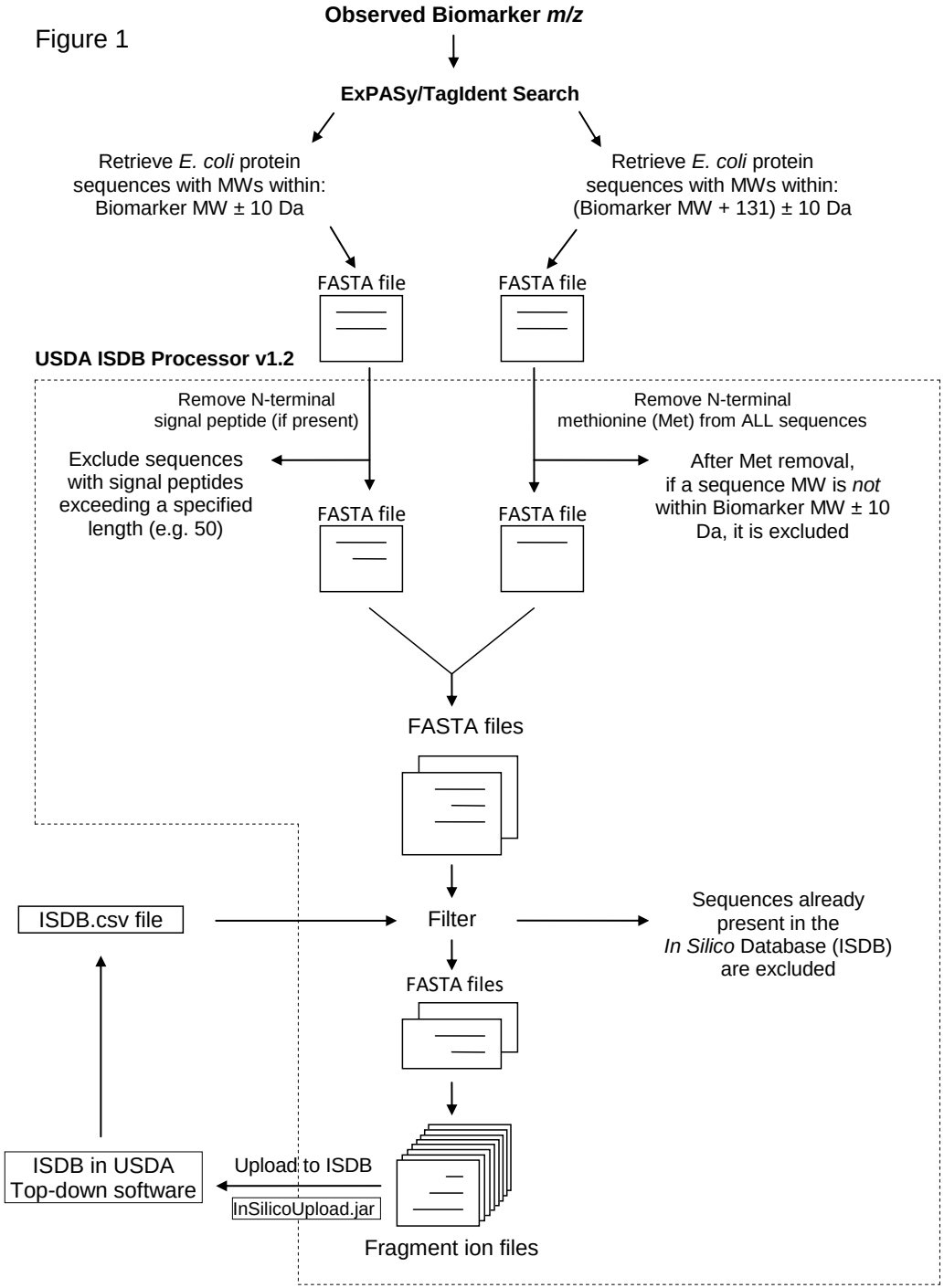
SH *SH*

AD*CAKGKIE*FSKYN*E*D*D*TF*TVKV*D*GKE*YWTSRWNLQ*PLLQSAQLTGMTVTIKSSTCE*SGSGFAE*VQFNND



Top-down proteomic analysis

Figure 1

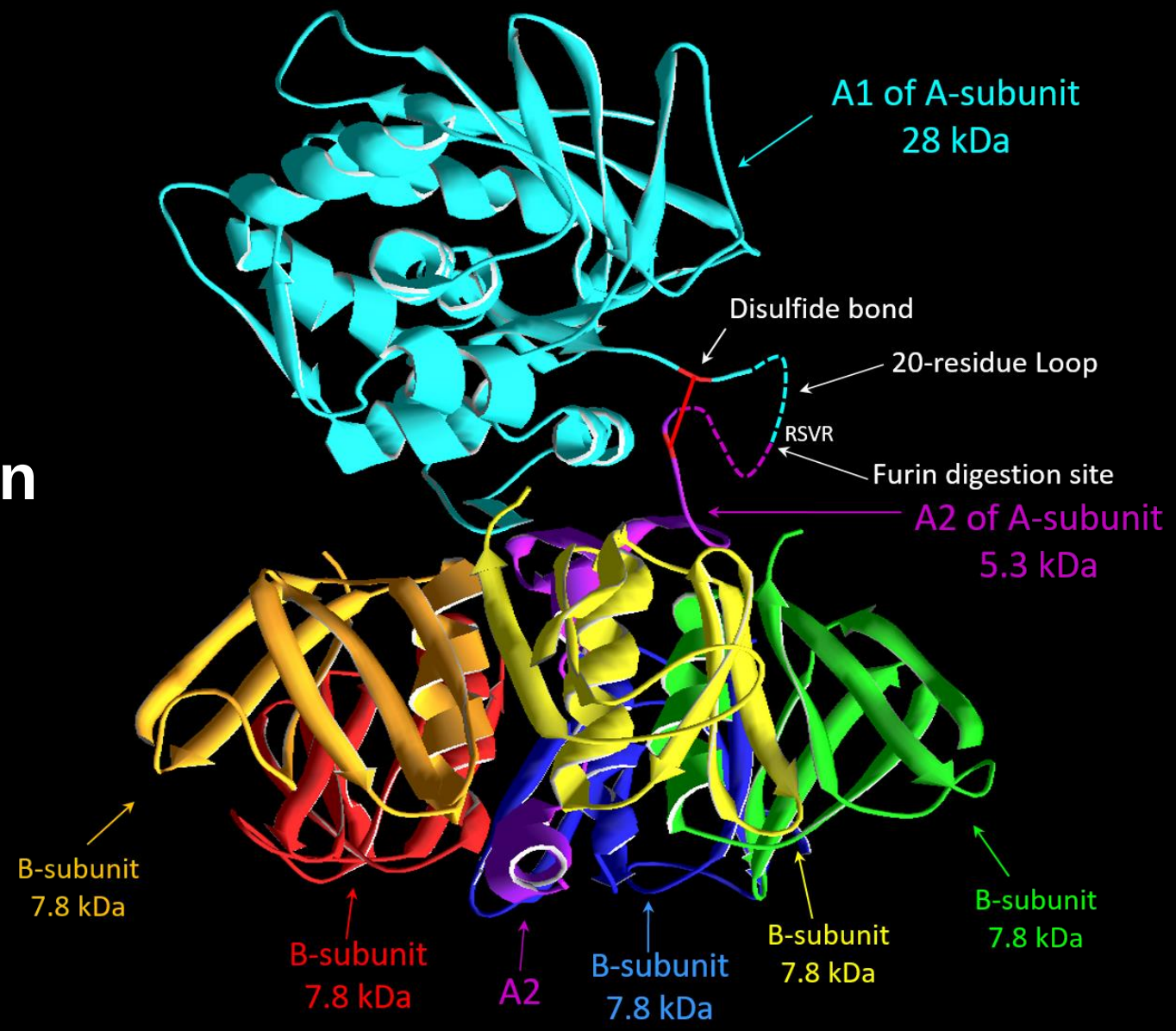


Fagerquist CK, Garbus BR, Williams KE, Bates AH, Boyle S, Harden LA. Web-based software for rapid top-down proteomic identification of protein biomarkers, with implications for bacterial identification. *Appl Environ Microbiol*. 2009; 75: 4341-53.

Top-down proteomic ID of B-subunit

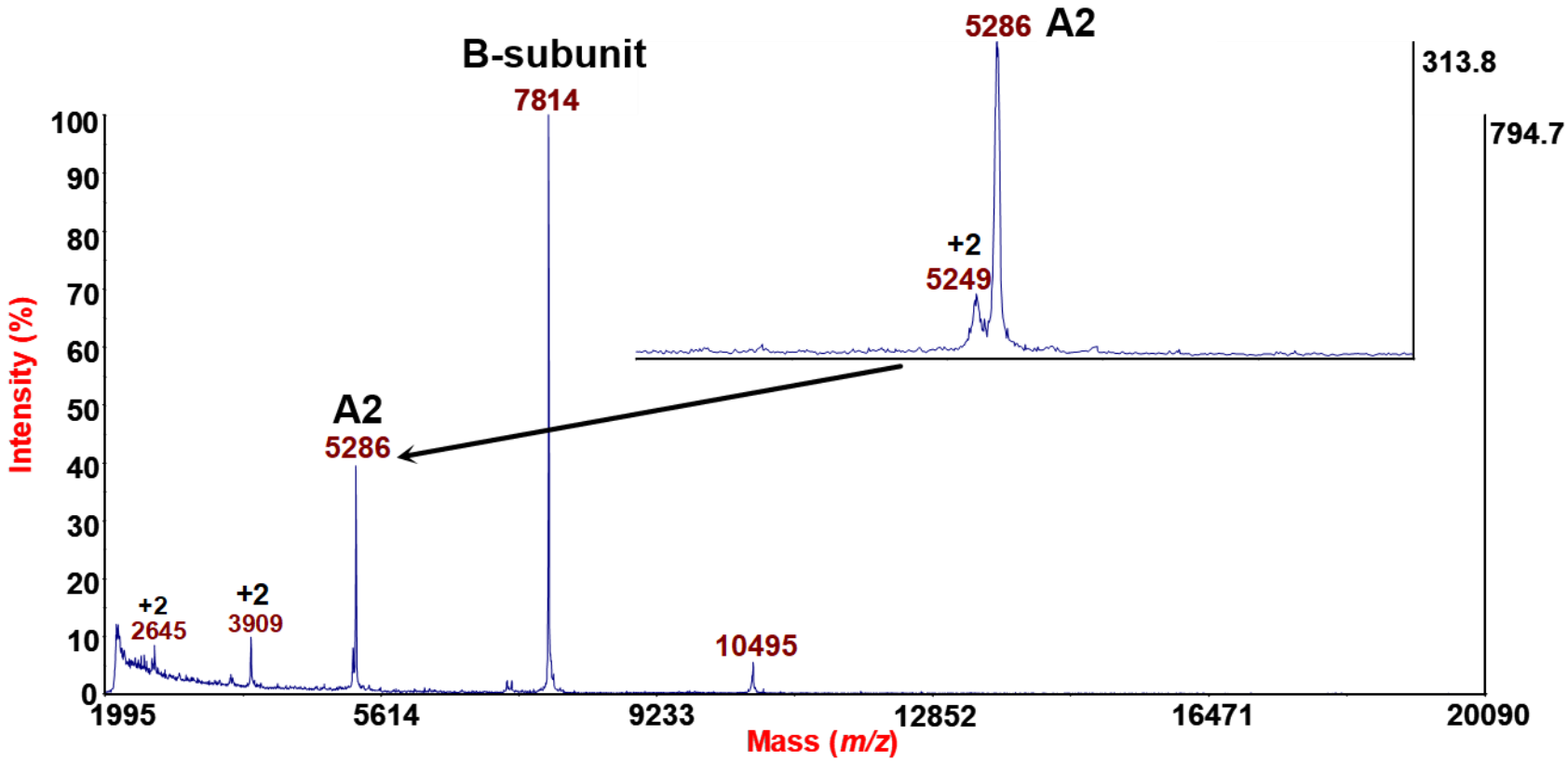
- Top-down ID: Shiga-like toxin II B-subunit BP-933W (reduced) *E. coli* O157:H7 (EDL933)
- % of matched peaks (USDA score): 47.62%
- P-value score: 3.4E-14

AB_5
Stx holotoxin



Protein Data Bank

Furin & DTT of EDL933

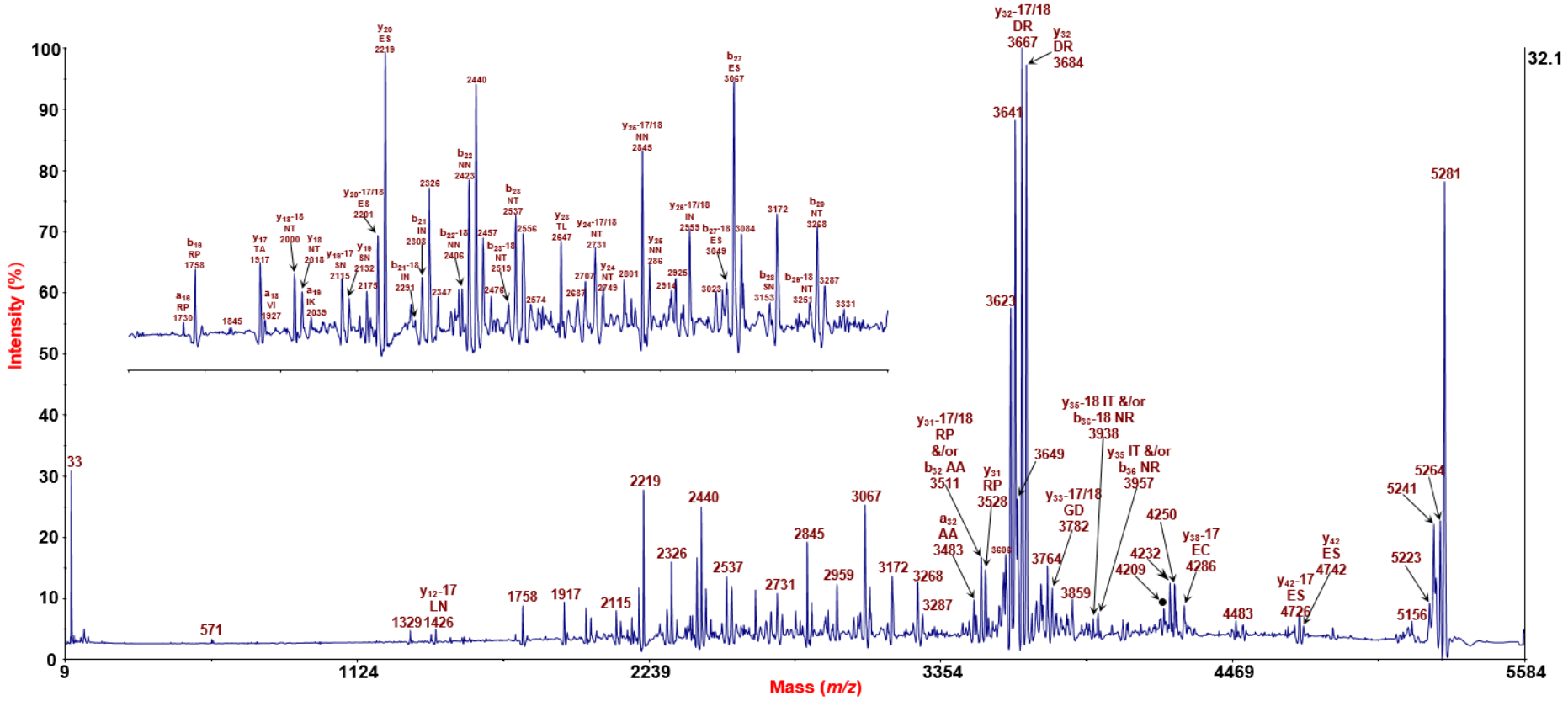


MS/MS of A2 of EDL933

y_{42} y_{38} y_{35} y_{33} y_{32} y_{31} y_{26} y_{25} y_{24} y_{23} y_{20} y_{19} y_{18} y_{17} y_{12}

AVNEE*SQPE*CQI*TG*D*R*PV*I*KI*N*N*T*LWE*S*N*T*AA*AFL*N*RKSQFLYTTGK

a/b_{16} $a_{18}a_{19}$ b_{21} b_{22} b_{23} b_{27} b_{28} b_{29} a/b_{32} b_{36}

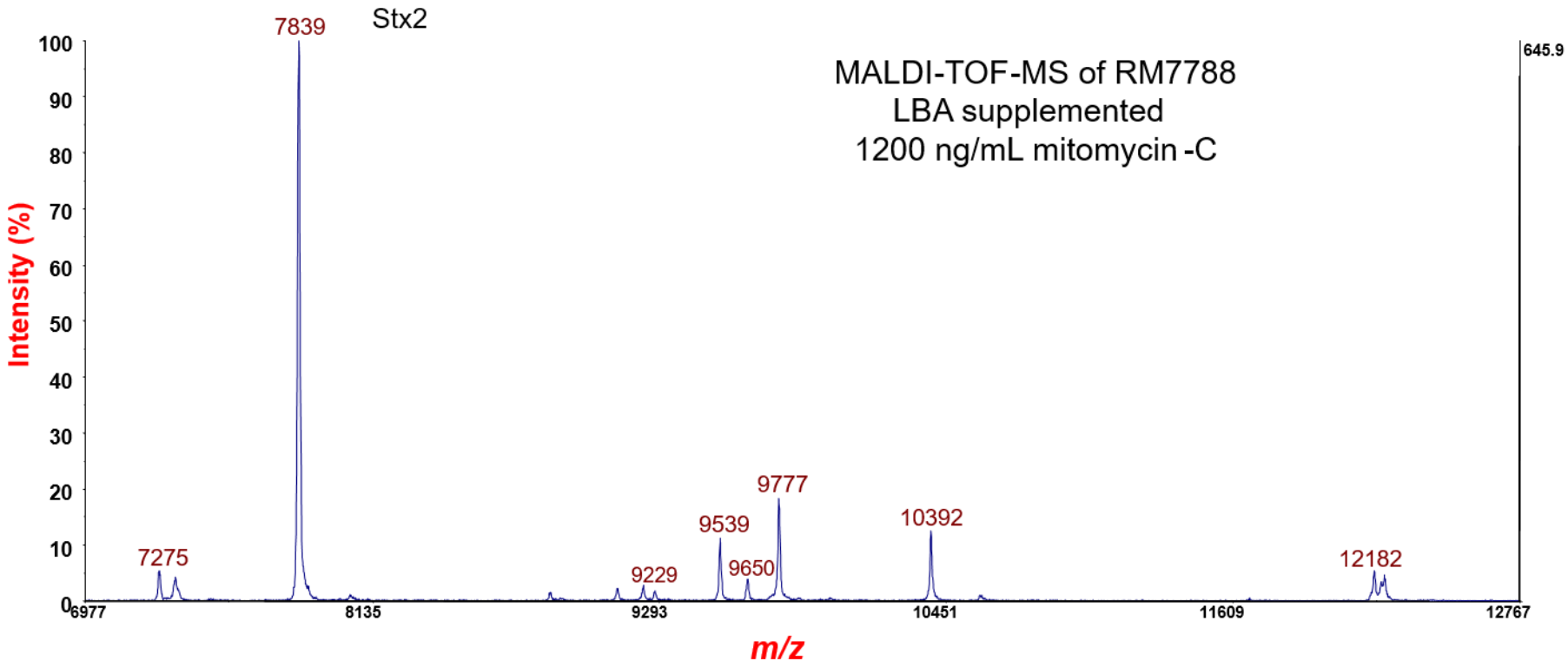


Top-down proteomic ID of A2 of A-subunit

- Top-down ID: Shiga-like toxin II A2 of A-subunit BP-933W *E. coli* O157:H7 (EDL933)
- % of matched peaks (USDA score): 42.37%
- P-value score: 2.2E-13

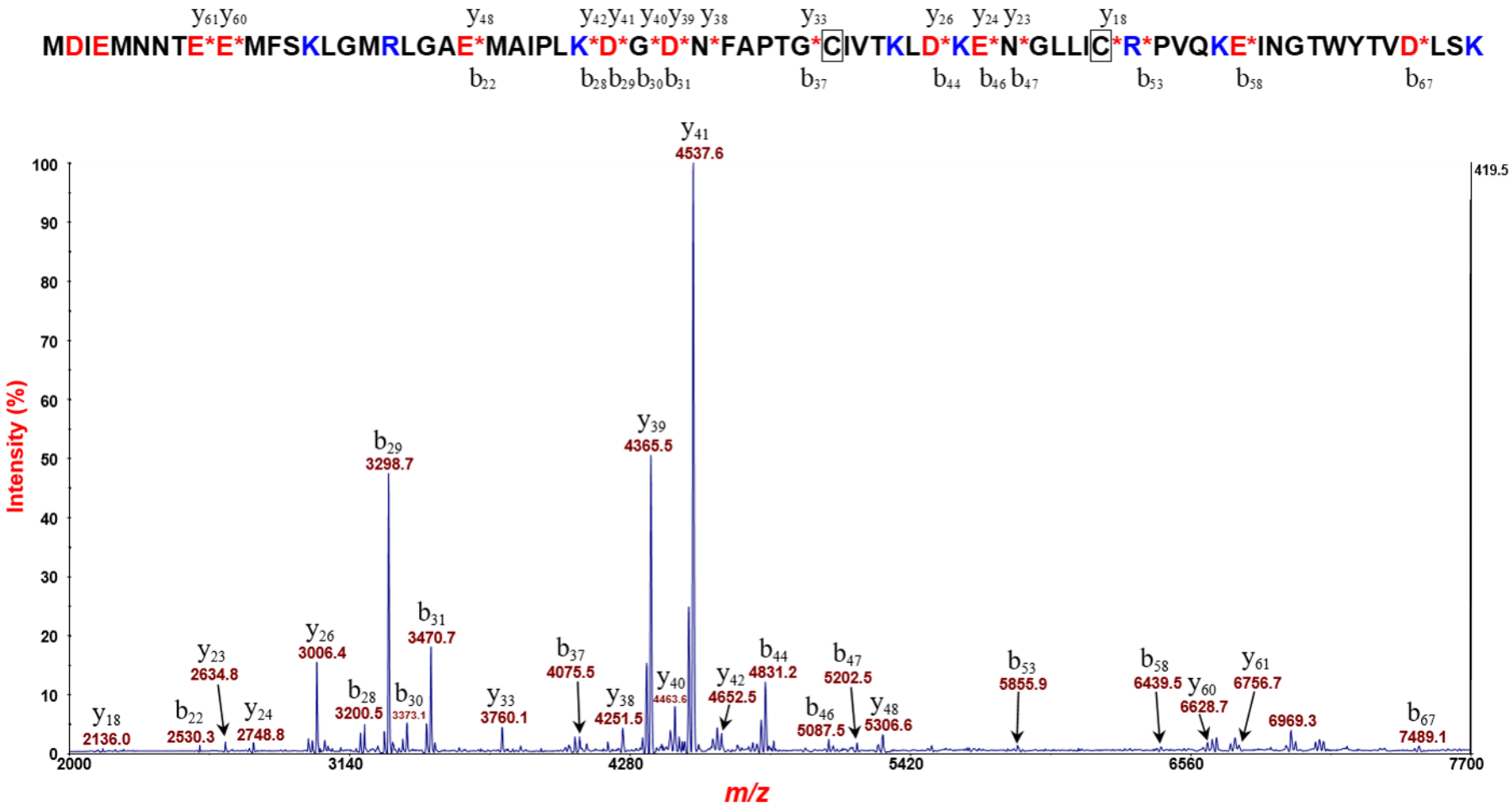
Top-down ID of antibiotic-induced protein biomarker in six STEC strains

MS of STEC RM7788



Hypothetical protein CDS (plasmid-encoded factor)

MS/MS of *m/z* 7839 protein biomarker



Top-down proteomic ID of plasmid-encoded factor

- Top-down ID: Plasmid-encoded factor: hypothetical protein CDS in six STEC strains
- % of matched peaks (USDA score): 44.8%
- P-value score: 8.5E-24

Summary & Conclusions

- 49 environmental STEC strains analyzed by MALDI-TOF-TOF-MS/MS & top-down proteomics
- 18 strains were ID as Stx2a
- 10 strains were ID as Stx2c
- 4 strains were ID as either Stx2a or 2c
- 2 strains were ID as either Stx2c or 2d
- 3 strains expressed both Stx2a & plasmid-encoded factor
- 3 strains expressed only plasmid-encoded factor
- 8 strains that weakly expressed Stx2 had a transposon insertion in the A-subunit
- 3 negative control strains were negative
- 1 positive control strain (EDL933) was positive
- Results were consistent with PCR and WGS

Summary & Conclusions 2

- Our top-down proteomic technique is looking for collaborators who wish to identify protein biomarkers and toxins from pathogenic strains especially if the strains have already been genomically sequenced

Summary & Conclusions 3

- Vero cell-based fluorescence assay was developed to measure Stx activity at concentrations of pg/mL
- Stx2 subtypes showed differential cytotoxicities in mammalian cells
- Environmental STEC isolates expressing Stx2a were more effective at inhibiting protein synthesis than Stx2c
- Environmental STEC O113:H21 co-expressing plasmid-encoded factor also had the highest levels of Stx2a amounts & activity in mammalian host cells

Support & Acknowledgments

ARS, Food safety (NP108): Jim Lindsay (NPL)

FSIS: John J. Johnston (Scientific Liaison)