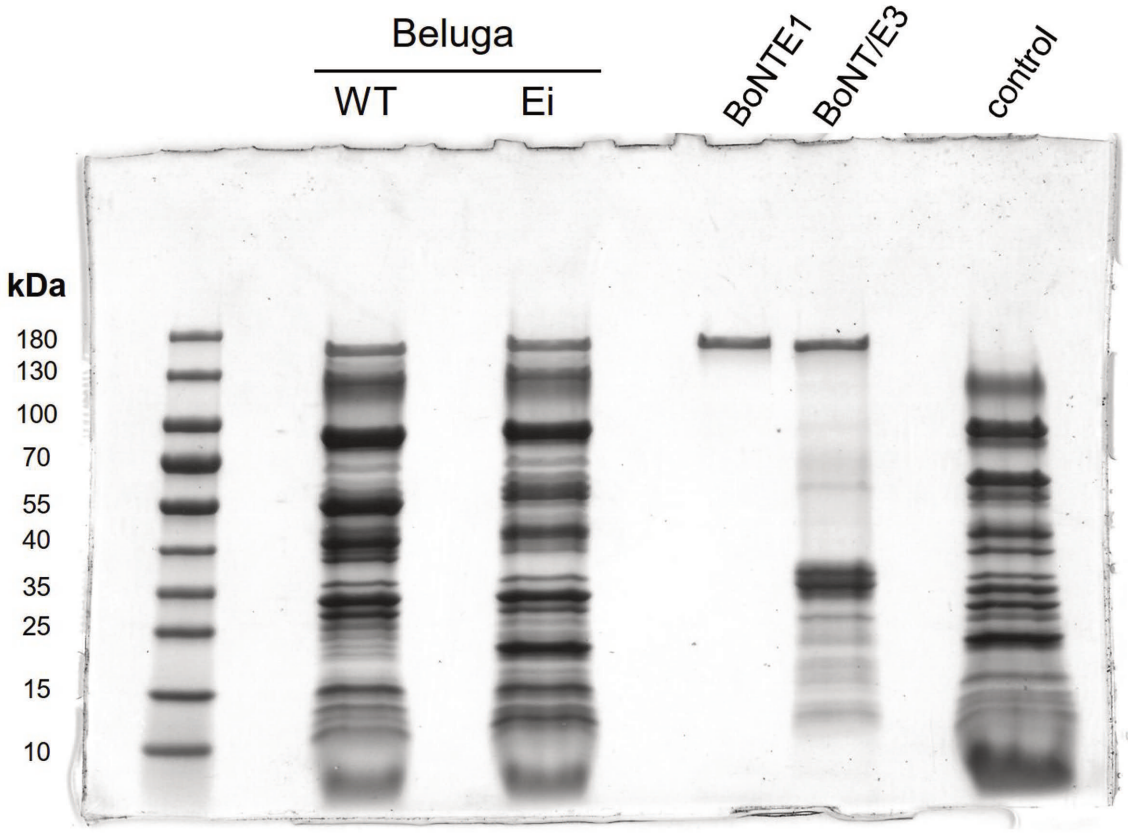

Figure S1. Coomassie-stained polyacrylamide gel of BoNT-containing samples. Prior to the gel electrophoresis, BoNT concentration in each sample was measured in BoNT/E-directed sandwich ELISA and volumes containing 1 µg of BoNT per lane were separated. The intensities of BoNT bands in samples from *Clostridium botulinum* Beluga WT and Beluga Ei were estimated to be similar based on densitometry image analysis (WT/Ei intensity ratio=1.08). Purified recombinant BoNTE1 and native BoNT/E3 in amounts 1 µg per lane were used as references. The control lane includes a sample from a Clostridial strain not producing BoNT.

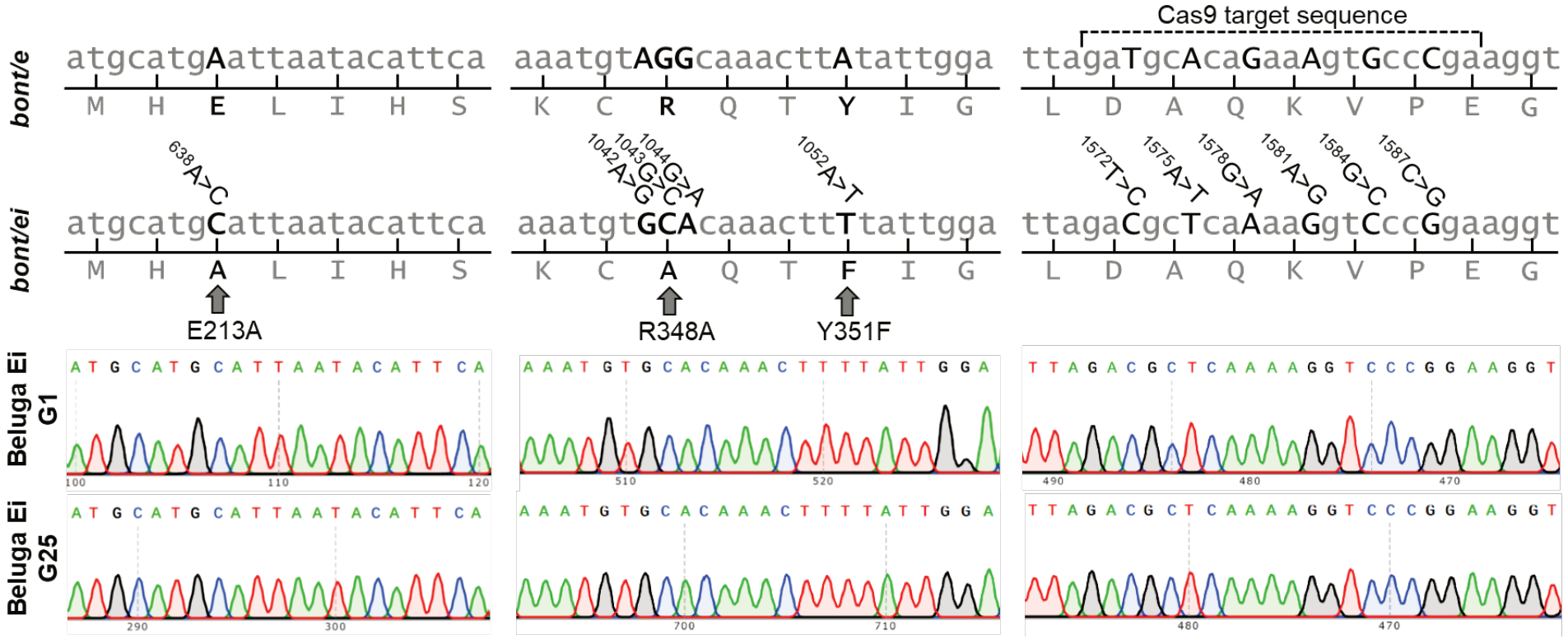

Figure S2. Alignment of wild-type *bont/e* and modified *bont/ei* gene sequences with Sanger sequencing chromatograms derived from 1st (Beluga Ei G1) and 25th (Beluga Ei G25) generations of *Clostridium botulinum* Beluga Ei strain. The altered base pairs and amino acids are capitalized and marked in black. The DNA sequence chromatogram of Beluga Ei G1 strain demonstrates the presence of all expected genome alterations. The DNA sequence chromatogram of Beluga Ei G25 strain still harbors all the pre-designed modifications confirming that the mutations are stably introduced into the genome for at least 25 subsequent generations.

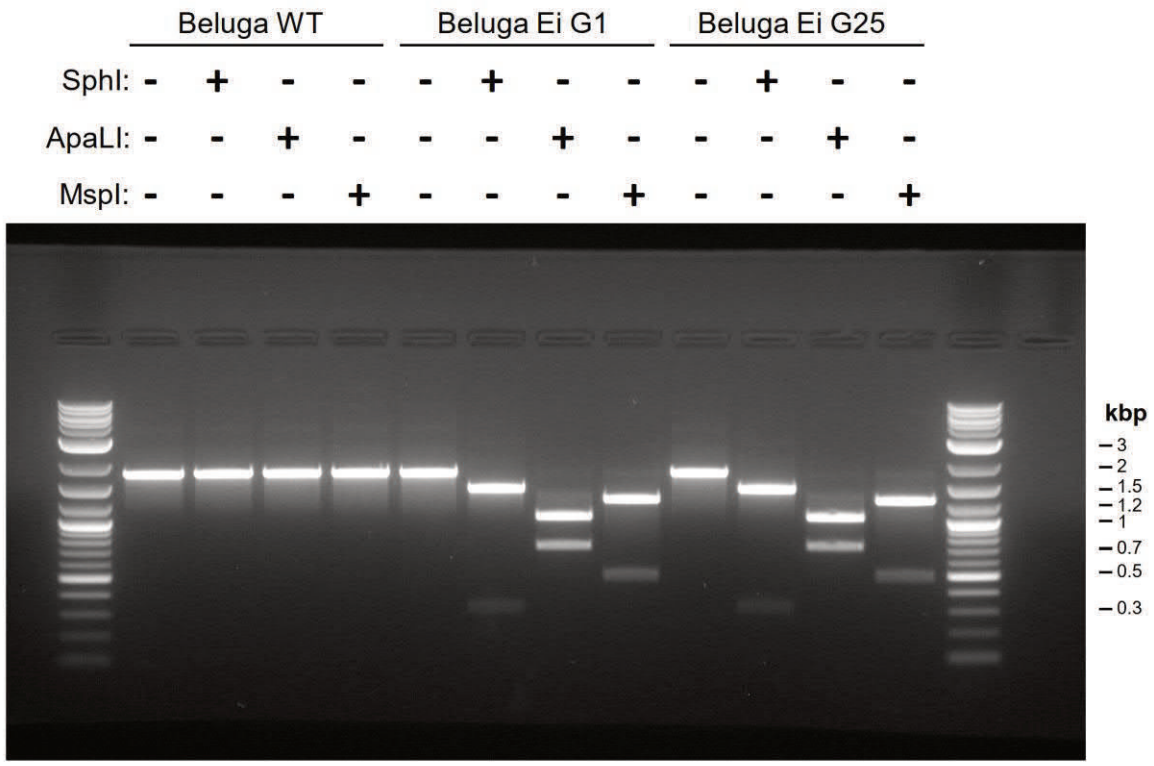

Figure S3. Full-length picture of the agarose gel electrophoresis visualizing the PCR amplicons after the restriction digestion. The image presents the restriction digestion-based verification of the presence of designed nucleotide modifications within *bont* locus of *Clostridium botulinum* Beluga wild-type (Beluga WT), Beluga Ei 1st generation (Beluga Ei G1) and Beluga Ei 25th generation (Beluga Ei G25).

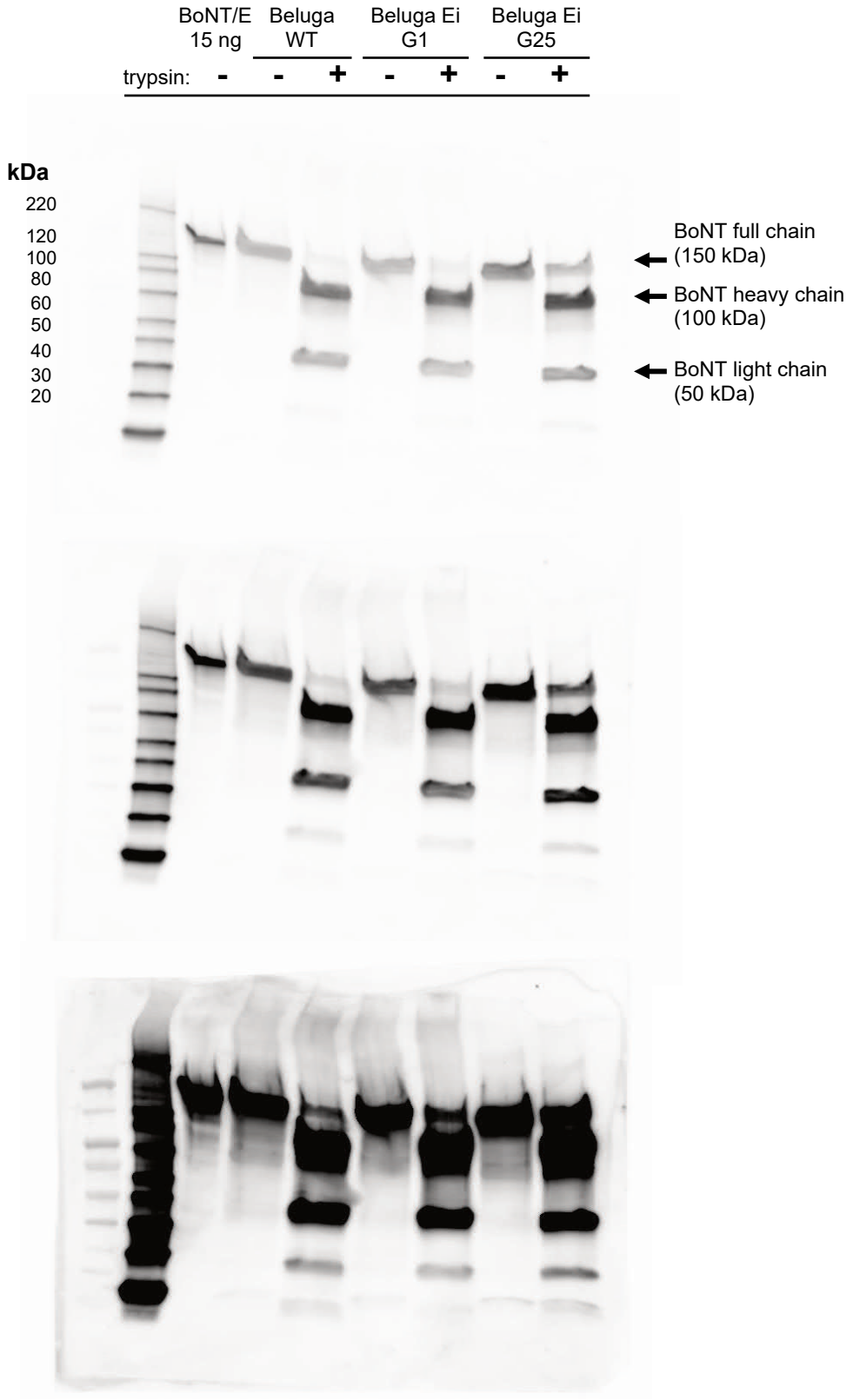

Figure S4. Full-length images of BoNT/E-directed Western blots presenting the validation of the correct trypsin-cleavage of BoNT/E and BoNT/Ei produced by 1st and 25th generations of the *Clostridium botulinum* Beluga Ei strain (Beluga Ei G1 and Beluga Ei G25). Upper membrane exposition time = 40 sec; middle membrane exposition time = 2 min; lower membrane exposition time = 11 min.

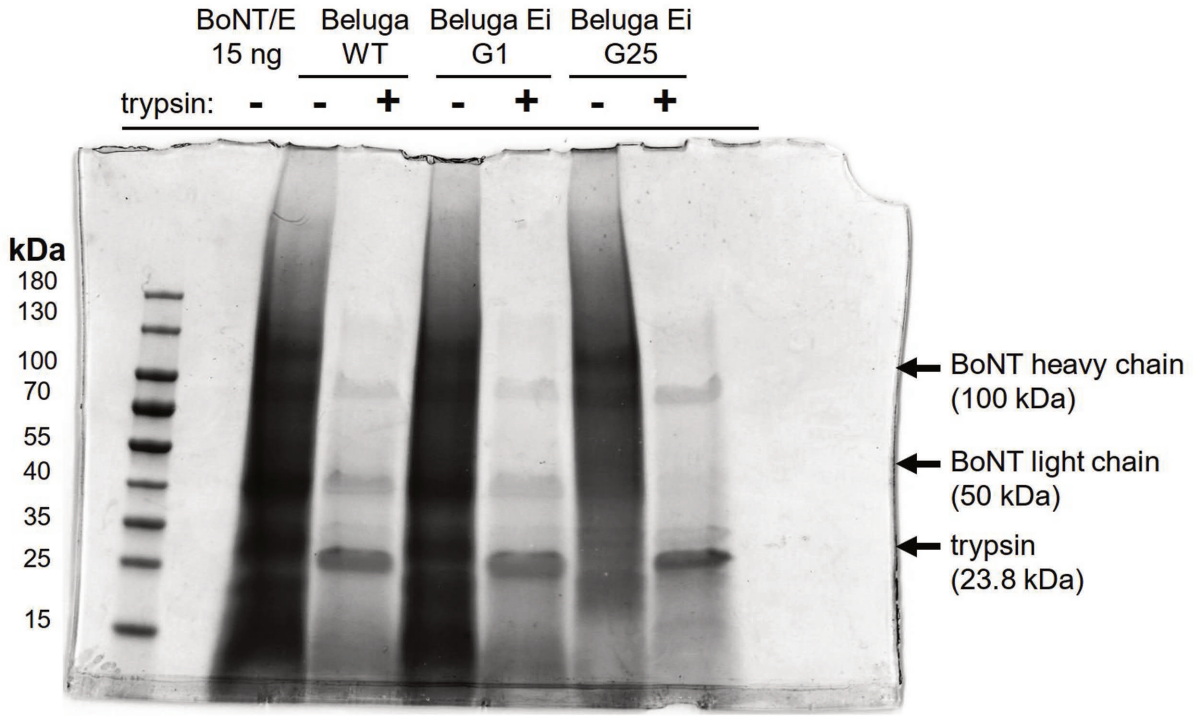

Figure S5. Full-length picture of Coomassie-stained polyacrylamide gel presenting the validation of the correct trypsin-cleavage of BoNT/E and BoNT/Ei produced by 1st and 25th generations of the *Clostridium botulinum* Beluga Ei strain (Beluga Ei G1 and Beluga Ei G25).

Table S1. List of variants detected in *C. botulinum* Beluga Ei G1 used in the present study compared to the genome sequence of Beluga WT; and the list of variants in *C. botulinum* Beluga Ei G25 compared to the genome sequence of *C. botulinum* Ei G1. Legend: AA, amino acid; CR, coding region; MNV, multiple nucleotide variation; n.a., not applicable; SNV, single nucleotide variation.

***Clostridium botulinum* Beluga Ei G1 (generation 1)**

Coordinate	Type	Reference	Allele	Frequency (%)	CR change	AA change	Comments
705125	SNV	A	G	100	^{1055}A>G	Y352C	Conserved protein
929871	SNV	C	A	99	^{298}G>T	G100*	Transcriptional regulator, Fur family
1097224	SNV	G	T	100	n.a	n.a.	Non-coding region
1217507	SNV	G	T	100	^{1582}G>T	E528*	Type II restriction-modification system
1385720	SNV	G	T	100	^{19}G>T	A7S	LSU ribosomal protein L20p
1568113	SNV	C	A	100	^{549}G>T	n.a.	Hypothetical protein
1687587	SNV	G	T	100	^{136}G>T	A46S	Hypothetical protein
2000599	SNV	C	A	99	^{847}C>A	P283T	tRNA dimethylallyltransferase
2536554	SNV	G	T	100	n.a	n.a.	Non-coding region
2628988	SNV	C	A	100	^{373}G>T	A125S	Oligoribonuclease A
2763800	SNV	C	A	100	^{210}G>T	K70N	GTP-binding protein TypA/BipA
2789844	SNV	A	C	99	^{638}A>C	E213A	Neurotoxin type E (toxoid modification)

Coordinate	Type	Reference	Allele	Frequency (%)	CR change	AA change	Comments
2790248	MNV	AGG	GCA	99	$^{1042\text{-}1044}$AGG del $^{1042\text{-}1044}$GCA ins	R348A	Neurotoxin type E (toxoid modification)
2790258	SNV	A	T	100	^{1052}A>T	Y351F	Neurotoxin type E (toxoid modification)
2790778	SNV	T	C	100	^{1572}T>C	n.a.	Neurotoxin type E (toxoid modification)
2790781	SNV	A	T	100	^{1575}A>T	n.a.	Neurotoxin type E (toxoid modification)
2790784	SNV	G	A	89	^{1578}G>A	n.a.	Neurotoxin type E (toxoid modification)
2790787	SNV	A	G	89	^{1581}A>G	n.a.	Neurotoxin type E (toxoid modification)
2790790	SNV	G	C	96	^{1584}G>C	n.a.	Neurotoxin type E (toxoid modification)
2790793	SNV	C	G	95	^{1587}C>G	n.a.	Neurotoxin type E (toxoid modification)
2912731	SNV	C	A	100	^{3}G>T	n.a.	Hypothetical protein
3015605	SNV	C	A	100	^{298}G>T	G100*	Nucleoside diphosphate kinase
3423844	SNV	C	A	100	^{884}G>T ^{1118}G>T	R295I R373I	Aminoacyl-histidine dipeptidase
3461123	SNV	C	A	100	^{2042}G>T	S681I	RecD-like DNA helicase YrrC
3759932	SNV	G	T	100	^{57}C>A	n.a	DNA integrity scanning protein DisA

***Clostridium botulinum* Beluga Ei G25 (generation 25)**

Coordinate	Type	Reference	Allele	Frequency (%)	CR change	AA change	Comments
222801	SNV	C	A	99	^{785}C>A	S262Y	Glucose-1-phosphate adenylyltransferase
442373	SNV	C	A	99	^{560}C>A	A187D	Choline binding protein A
964192	SNV	G	T	99	^{2077}G>T	G693C	5-methyltetrahydrofolate-homocysteine methyltransferase
1207656	SNV	C	A	99	^{1208}C>A	A403E	Endonuclease
2396875	SNV	G	T	99	^{729}C>A	F243L	PTS system, beta-glucoside-specific IIB component
2482821	SNV	C	A	99	^{1000}G>T	E334*	Two-component response regulator
3227216	SNV	G	T	99	^{22}C>A	P8T	Hypothetical protein
3279633	SNV	C	A	100	^{36}G>T	K12N	Endonuclease IV
3322036	SNV	G	T	99	^{211}G>T	D71Y	Transcriptional regulator, DeoR family
3963865	SNV	G	T	99	^{3245}C>A	A1082E	ATP-dependent nuclease, subunit A

Table S2. List of strains and plasmids used.

Strains/plasmids	Description	Source
Clostridium botulinum		
Beluga WT	Wild-type strain isolated from fermented Beluga whale flippers	IFR[a]
Beluga Ei G1	1st generation of the mutant containing three amino acid substitutions E213A/R348A/Y351F in *bont/e* gene	This study
Beluga Ei G25	25th generation of the mutant containing three amino acid substitutions E213A/R348A/Y351F in *bont/e* gene	This study
Escherichia coli		
CA434	Conjugation donor	Purdy et al.
CA434 + pMTL431511-*bont/ei*	Conjugation donor	This study
NEB 5-alpha	Cloning strain	New England BioLabs
Plasmids		
pMTL431511	Empty CRISPR/Cas9 vector	SBRC[b].
pMTL431511-*bont/ei*	CRISPR/Cas9 vector for constructing Beluga Ei mutant	This study

[a]Culture Collection of the Institute of Food Research, Norwich, UK

[b] Synthetic Biology Research Centre, Nottingham UK. CRISPR/Cas9 vectors may be sourced at http://www.plasmidvectors.com

Table S3. List of primers. The underlined sequences indicate the restriction enzyme cleavage sequence.

Primer name	Sequence (5´→3´)	Information
F_MC1_*bont/ei*-AsiSI	TATATAGCGATCGCGGGATGAGGTAA TATTTTCTGTATTAGATG	Construction of the first segment of the modification cassette for pMTL431511-*bont/ei.* Primer used for SOE-PCR amplification of final MC.
R_MC1_*bont/ei*	CATGTAATGAATGTATTAATGCATGCA TTAATGTAAG	Construction of the first segment of the modification cassette for pMTL431511-*bont/ei*. Primer introduces E213A substitution into *bont/e* sequence (^{638}A>C).
F_MC2_ *bont/ei*	CTTACATTAATGCATGCATTAATACATT CATTACATG	Construction of the second segment of the modification cassette for pMTL431511-*bont/ei*. Primer introduces E213A substitution into *bont/e* sequence (^{638}A>C).
R_MC2_ *bont/ei*	CAATAAAAGTTTGTGCACATTTAACTT GAAATTTAGTTGCTAAATCAAATTCC	Construction of the second segment of the modification cassette for pMTL431511-*bont/ei*. Primer introduces R348A and Y351F substitutions into *bont/e* sequence (^{1042}A>G, ^{1043}G>C, ^{1044}G>A, ^{1052}A>T).
F_MC3_ *bont/ei*	CAAGTTAAATGTGCACAAACTTTTATT GGACAGTATAAATACTTCAAACTTTC	Construction of the third segment of the modification cassette for pMTL431511-*bont/ei*. Primer introduces R348A and Y351F substitutions into *bont/e* sequence (^{1042}A>G, ^{1043}G>C, ^{1044}G>A, ^{1052}A>T).
R_MC3_*bont/ei*	CGGGACCTTTTGAGCGTCTAAATAGA AAAATACATTAAGTTCATTAACATC	Construction of the third segment of the modification cassette for pMTL431511-*bont/ei*. Primer introduces six silent mutations into the CRISPR/Cas9 targeting *bont/e* sequence (^{1572}T>C, ^{1575}A>T, ^{1578}G>A, ^{1581}A>G, ^{1584}G>C, ^{1587}C>G).

Primer name	Sequence (5´→3´)	Information
F_MC4_ *bont/ei*	GACGCTCAAAAGGTCCCGGAAGGTGAAAATAATGTCAATCTCAC	Construction of the fourth segment of the modification cassette for pMTL431511-*bont/ei*. Primer introduces six silent mutations into the CRISPR/Cas9 targeting *bont/e* sequence (^{1572}T>C, ^{1575}A>T, ^{1578}G>A, ^{1581}A>G, ^{1584}G>C, ^{1587}C>G).
R_MC4_ *bont/ei*-AscI	TATATAGGCGCGCCCATATCCTGAAGTATCTACGTATTTATC	Construction of the fourth segment of the modification cassette for pMTL431511-*bont/ei.* Primer used for SOE-PCR amplification of final MC.
F_*bont/e*_sgRNA-SalI	TTTTCGTCGACGATGCACAGAAAGTGCCCGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC	Construction of a fragment containing *bont/e*-specific sgRNA template.
R_sgRNA-AsiSI	CGCGCGCGGCGATCGCATAAAAATAAGAAGCCTGCAAATGCAGGCTTCTTATTTTTATAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTG	Construction of a fragment containing sgRNA template.
F_ *bont/ei*_RE	GAAGAACTGTCAAAAGCTAATCCA	Amplification of modified region within *bont/e* and *bont/ei* for further restriction enzyme digestion, sequencing of pMTL431511-*bont/ei*
R_*bont/ei*_RE	GTGTATTAATTTTAGTCATCCAATTCG	Amplification of modified region within *bont/e* and *bont/ei* for further restriction enzyme digestion, sequencing of pMTL431511-*bont/ei*
F_sgRNA_seq	CTAGATTTATATTTAGTCCCTTGCC	Sequencing of pMTL431511 plasmids series.

Primer name	**Sequence (5´→3´)**	**Information**
83XXX-LR	ACGGCTTGATGTGTTGGTAG	Sequencing of pMTL431511 plasmids series.
F_ *bont/ei*_scr	CAACTAGTAGATAATAAAAATAATGCACAG	Screening and sequencing of Beluga Ei mutant.
R_*bont/ei*_scr	GATTTTTATTAGTTGGATATTTATATACATCTCC	Screening and sequencing of Beluga Ei mutant.

Table S4. Read mapping summary report and accession numbers of sequenced *Clostridium botulinum* samples. Data were deposited under the project number PRJNA751216 in Sequence Read Archive (SRA). Beluga WT reads were mapped against the reference genome available in NCBI database. Beluga Ei G1 reads were mapped against assembled consensus sequence of Beluga WT, and Beluga Ei G25 reads mapped against Beluga Ei G1 assembled consensus sequence.

Feature	**Beluga WT**	**Beluga Ei G1**	**Beluga Ei G25**
BioSample Accession Number	SAMN20513657	SAMN20511721	SAMN20511722
SRA Accession number	SRR15317859	SRR15312153	SRR15312152
Mapping Summary			
Total reads	11,150,642	27,176,720	23,838,926
Mapped reads	10,820,072	27,124,936	23,727,788
Unmapped reads	330,570	51,784	111,138
% mapped reads	97.04%	99.81%	99.53%
% unmapped reads	2.96%	0.19%	0.47%

Table S5. Toxin concentration of the supernatant samples used in mouse bioassays measured in BoNT/E-directed sandwich ELISA. Each sample was diluted in at least 3 technical replicates.

C. botulinum **strain**	**BoNT concentration (ng/ml)**	**Negative error (ng/ml)**	**Positive error (ng/ml)**
Beluga WT	5606	200	307
Beluga Ei G1	4532	553	600
Beluga Ei G25	4384	547	735