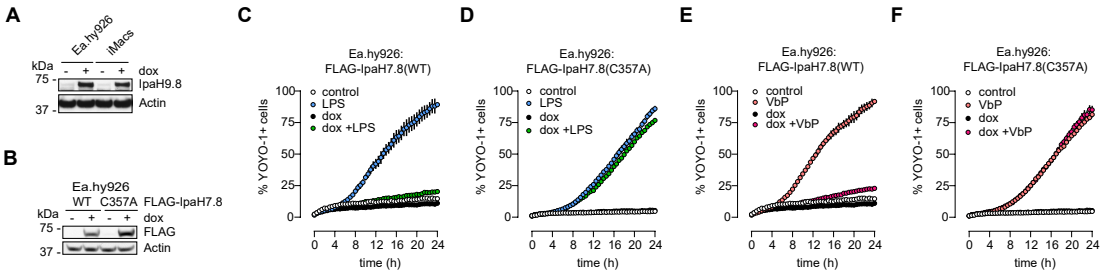

Figure S1. Doxycycline-inducible expression of IpaH7.8 in Ea.hy926 cells or iMacs and cell death kinetics. Related to Figure 1. **(A** and **B)** Immunoblots of human Ea.hy926 cells and murine iMacs induced with doxycycline for 24 h to express wild-type (WT) IpaH7.8 (A) or both WT IpaH7.8 and IpaH7.8 mutant CA (C357A) (B). In (A), anti-IpaH9.8 polyclonal antibody was used to confirm expression of untagged IpaH7.8 because this antibody cross-reacts with all IpaHs. Results representative of 3 independent experiments. (**C-F**) Percentage YOYO-1 positive Ea.hy926 cells after LPS transfection (C and D) or treatment with 25 μM Valboro-pro (E and F, VbP). Live cell images taken every 30 min up to 24 h. Where indicated, doxycycline (dox) was used to induce expression of wild-type (WT) IpaH7.8 or mutant IpaH7.8 (C357A). Each data point represents the mean ± SD of 4 biological replicates. Data representative of 3 independent experiments.

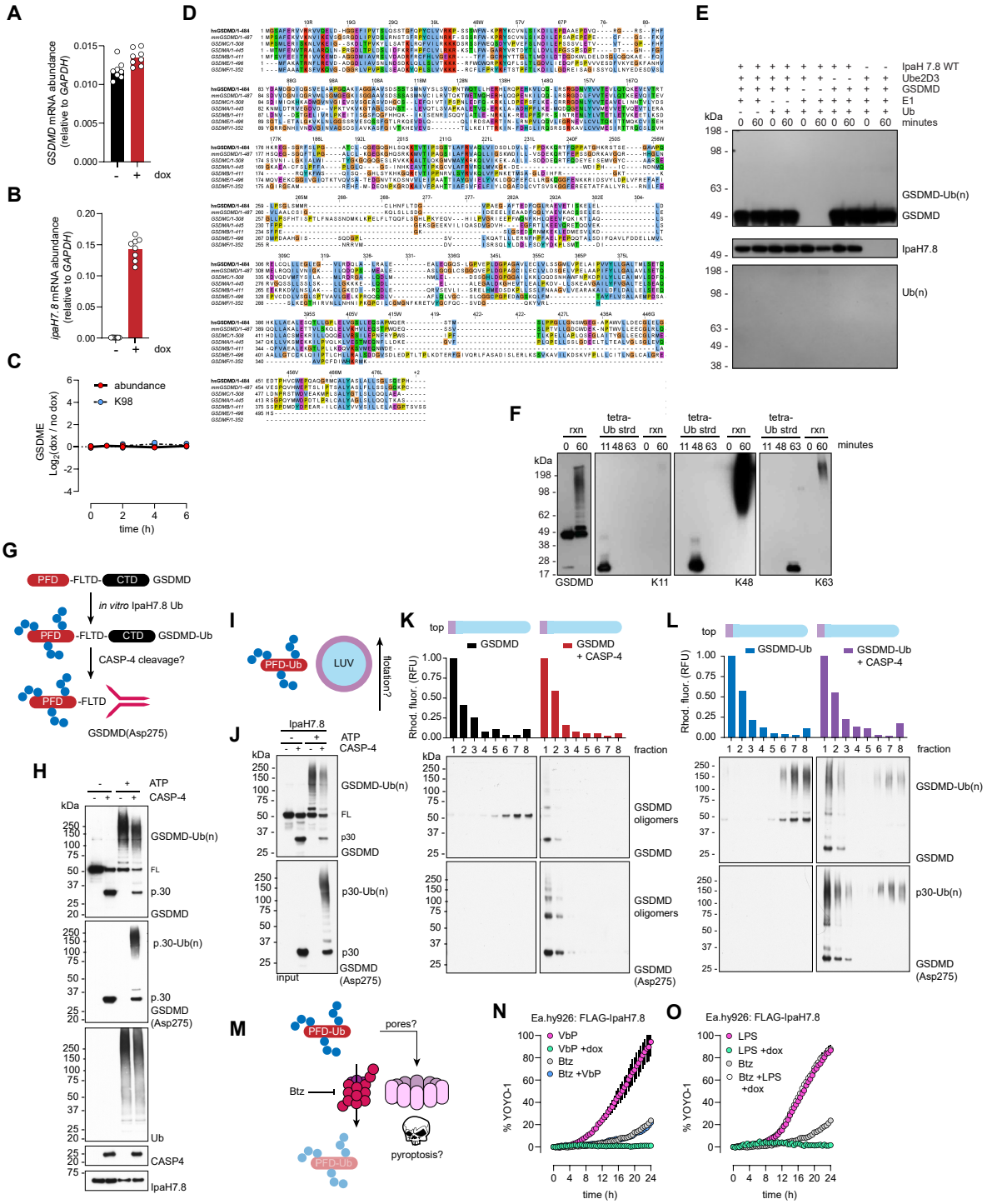

Figure S2. IpaH7.8 does not reduce expression of *GSDMD* mRNA, does not target GSDME, and requires the proteasome to prevent pyroptosis. Related to Figure 2. (**A** and **B**) Graphs indicate the relative expression of *GSDMD* (A) and *ipaH7.8* (B) mRNAs in Ea.hy926 cells with and without

doxycycline-induced (dox) expression of IpaH7.8 for 24 h. Bars represent the mean of 4 biological and 2 technical replicates, each plotted as a single data point. **(C)** Abundance (red circles) and ubiquitination (blue circles, indicated lysines) of GSDME in Ea.hy926 cells treated with doxycycline to induce expression of IpaH7.8. Results are derived from the lysates used in figures 2A and 2B. Each circle represents the Log_2 ratio of dox treated / no dox (2 biological replicates each). **(D)** Multiple-sequence alignment of gasdermin family proteins. **(E** and **F)** Immunoblots of *in vitro* ubiquitination reactions. In (F), reactions are probed with linkage-specific polyubiquitin antibodies alongside tetra-ubiquitin chain standards. Results representative of 3 independent experiments. **(G)** Strategy for testing whether IpaH7.8-mediated ubiquitination of GSDMD blocks GSDMD cleavage by caspase-4. **(H)** Immunoblots depicting *in vitro* processing of GSDMD by caspase-4 before and after IpaH7.8-mediated ubiquitination. An antibody detecting total GSDMD (upper blot) reveals full-length (FL) GSDMD and the cleaved p30 PFD. An antibody detecting cleaved GSDMD(Asp275) only detects the p30 fragment (lower blot). **(I)** Strategy for testing whether ubiquitination of the GSDMD PFD alters its interactions with membranes by liposome flotation assay. LUV, large unilamellar vesicles. **(J)** Immunoblots of input samples incubated with fluorescently-labeled LUVs. **(K** and **L)** Graphs indicate the fluorescence signal from each gradient fraction. Tubes depict the gradient fraction orientation. Immunoblots of each gradient fraction are shown below. **(M)** Strategy for testing whether ubiqutination of the GSDMD PFD is sufficient to prevent pyroptosis. Btz, Bortezomib. **(N** and **O)** Percentage of YOYO-1 positive Ea.hy926 cells transfected with LPS (N) or treated with 25 μM Valboro-Pro (O, VbP), or in combination with 1 μM Btz over 24 h. Live cell images were taken every 30 min up to 24 h. Where indicated, doxycycline (dox) was used to induce the expression of IpaH7.8. Data points represent the mean ± SD of 4 biological replicates. Results representative of 3 independent experiments.

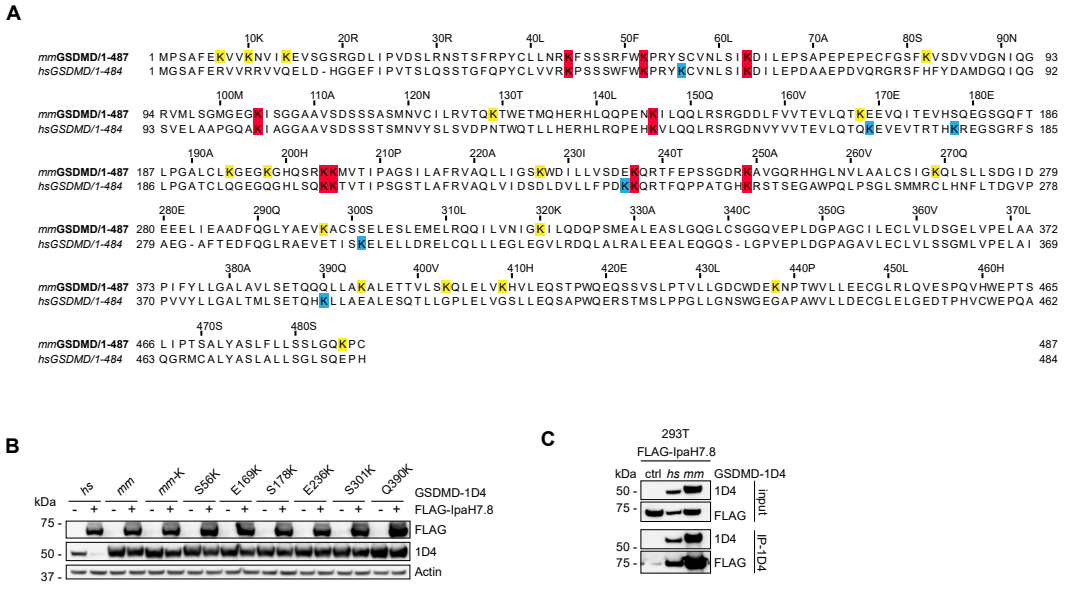

Figure S3. IpaH7.8 ubiquitinates human GSDMD. Related to Figure 3. **(A)** Alignment of mouse (*mm*) and human (*hs*) GSDMD protein sequences with lysines colored by conservation (red, conserved; blue, human only; yellow, mouse only). **(B)** Immunoblots of 293T cells transfected with the constructs indicated. *mm*, mouse. *hs*, human, *mm*-K, introduces the 6 non-conserved lysine residues from human GSDMD into mouse GSDMD. **(C)** Immunoblots showing co-immunoprecipitation of IpaH7.8(C357A) and GSDMD from co-transfected 293T cells. Results representative of 3 independent experiments.

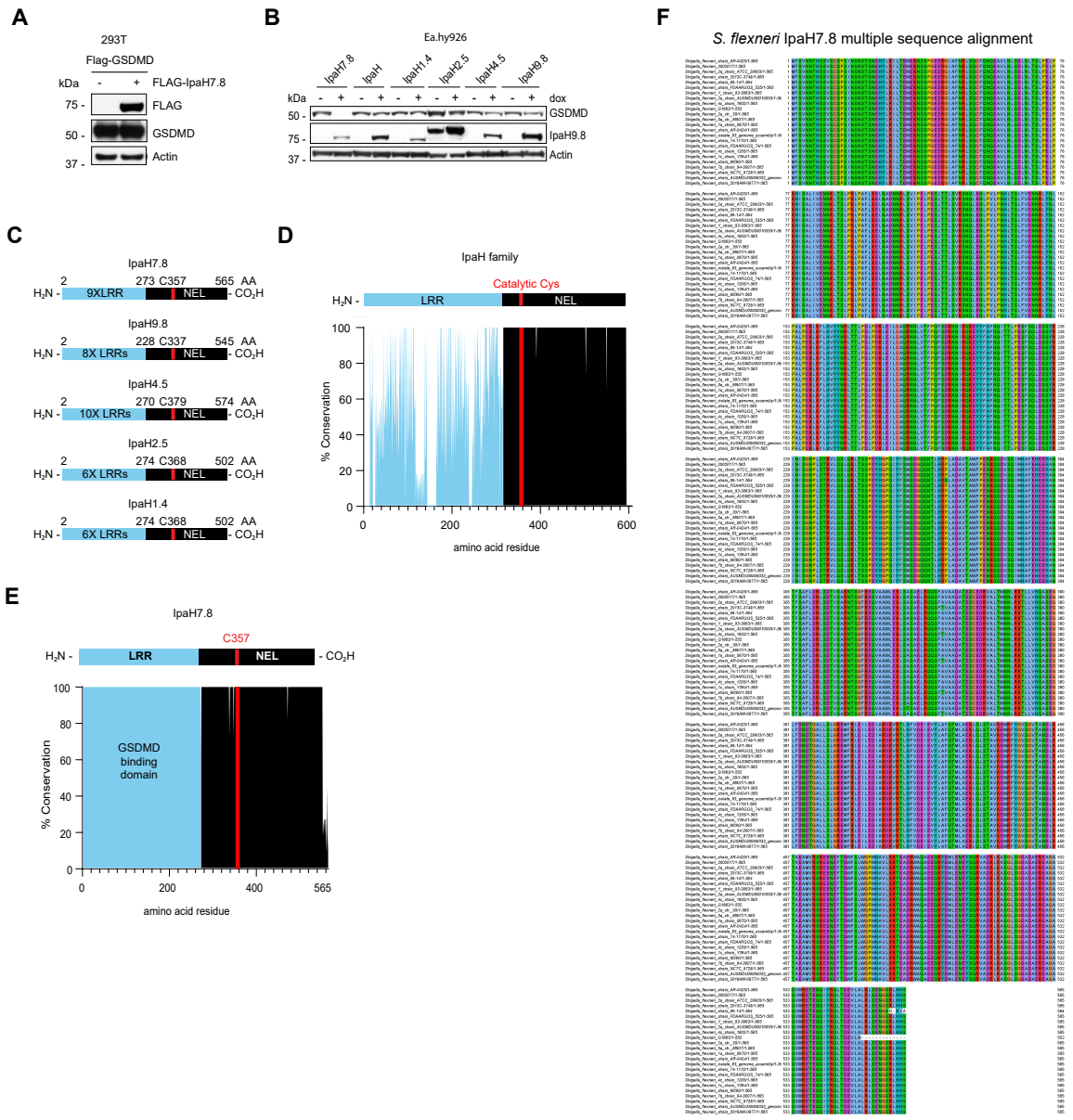

Figure S4. IpaH7.8 is a prevalent and evolutionarily conserved virulence factor that specifically targets GSDMD. Related to Figure 4. **(A)** Immunoblot of 293T cells co-transfected with IpaH7.8 and FLAG-N GSDMD. **(B)** Immunoblots of Ea.hy926 cells expressing doxycycline-inducible IpaH family members. Polyclonal IpaH9.8 Ab cross-reacts with all indicated IpaH family members.

(C) Domain architecture of IpaH family proteins highlighting the relative numbers of leucine-rich repeats (LRR). **(D** and **E)** Amino acid (aa) conservation of IpaH family (D) and IpaH7.8 variants (F) as scored by a numerical index reflecting the conservation of physico-chemical properties in the alignment. Values at each aa position are normalized to the highest numerical value of conservation from the alignment, and expressed as percentages. **(F)** Multiple-sequence alignment of IpaH7.8 variants from virulent *Shigella flexneri* strains (E). Results representative of 3 independent experiments.

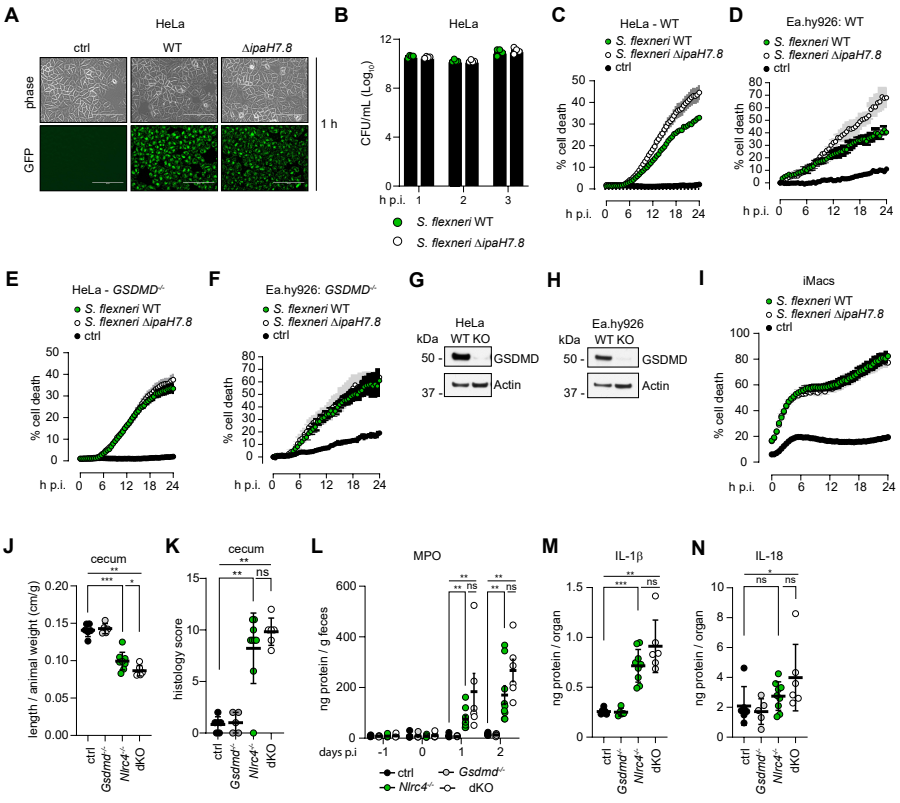

Figure S5. *S. flexneri* deficient in IpaH7.8 induces more GSDMD-dependent cell death than wild-type *S. flexneri.* Related to Figure 5. **(A)** Micrographs of HeLa cells at 1 hour post infection (h.p.i) with GFP-expressing *S. flexneri* 5a strain M90T (wild-type, WT, or ΔipaH7.8). Micrographs obtained using an inverted digital microscope at 10X magnification (scale bar, 200 µm), and are representative of 5 total fields from 3 independent experiments. **(B)** CFU recovered from gentamicin-treated HeLa cells at 1, 2, and 3 h.p.i with *S. flexneri* 5a strain M90T WT or ΔipaH7.8. CFUs assayed at 1 h correspond to the same cells imaged in (A). **(C-F**, and **I)** Percentage of infected HeLa cells (C and E), Ea.hy926 cells (D and F), or iMacs (I) killed by GFP-expressing *S. flexneri* 5a strain M90T (WT or ΔipaH7.8) based on their uptake of propidium iodide (GFP/PI++ cells over total GFP+ cells). Circles represent the mean ± SD calculated from 6 infected wells. Data representative of 3 independent experiments. **(G** and **H)** Immunoblots of HeLa (G) or Ea.hy926 (H) after CRISPR-cas9 knockout of *GSDMD*. **(J-N)** Characterization of control (black

circles, n = 6), *Gsdmd*$^{-/-}$ (grey circles, n = 5), *Nlrc4*$^{-/-}$ (green circles, n = 9) and *Nlrc4*$^{-/-}$*Gsdmd*$^{-/-}$ (dKO, white circles, n = 6) female mice at 48 h.p.i. with 10^7 CFU (colony forming units) of *S. flexneri* 2a strain 2457T. **(J)** Normalized cecum lengths (cecum length (cm) / mouse weight (g) before infection). (**K**) Blinded quantification of histology score (cumulative) for tissues (Figure 5I). (**L**) Fecal myeloperoxidase (MPO) levels measured by ELISA. (**M** and **N**) Tissue IL-1β and IL-18 levels measured by ELISA. (**J** -**N**) Each symbol represents one mouse, lines represent mean ± SD. Mann-Whitney test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns = not significant ($p > 0.05$).

Table S1. *Shigella* effector library. Related to Figure 1A.

The 30 *Shigella* effectors used for positive selection screens in Ea.hy926 cells.

number	effector	number	effector
1	ospE1	16	ospC2
2	ospE2	17	ospC3
3	ipgB2	18	icsB
4	ospG	19	ipgD
5	ipgB1	20	ipaH9.8
6	ospI	21	ospD3
7	ospD1	22	ipaH2.5
8	ospF	23	ospD3/senA
9	ipaJ	24	ipaH7.8
10	ospB	25	ospD2
11	ipaD	26	ipaH4.5
12	ipaC	27	ipaH1.4
13	virA	28	ipaB
14	ospC4	29	ipaH
15	ospC1	30	ipaA

Table S2. Genbank assembly accessions of the 24 *Shigella flexneri* complete genomes that contain the 200+ kb virulence plasmid. Related to Figures S4E and S4F.

1	GCA_903987015	*S. flexneri* strain AUSMDU00022017
2	GCA_010231485	*S. flexneri* strain 2013C-3749
3	GCA_001579965	*S. flexneri* 4c strain 1205
4	GCA_004799585	*S. flexneri* 5a str. M90T
5	GCA_003855135	*S. flexneri* strain FDAARGOS_535
6	GCA_002741635	*S. flexneri* 7b strain 94-3007
7	GCA_000006925	*S. flexneri* 2a str. 301
8	GCA_008727235	*S. flexneri* strain AR-0425
9	GCA_903987005	*S. flexneri* strain AUSMDU00021847
10	GCA_002240075	*S. flexneri* 4c strain 1602
11	GCA_002240135	*S. flexneri* 1a strain 0670
12	GCA_002950255	*S. flexneri* strain 74-1170
13	GCA_900659665	*S. flexneri* strain AUSMDU00008332
14	GCA_008727255	*S. flexneri* strain AR-0424
15	GCA_904066025	*S. flexneri* isolate 83
16	GCA_002442995	*S. flexneri* 1c strain Y394
17	GCA_000022245	*S. flexneri* 2002017
18	GCA_000783735	*S. flexneri* strain FDAARGOS_74
19	GCA_001021855	*S. flexneri* G1663
20	GCA_002949575	*S. flexneri* Y strain 93-3063
21	GCA_013402835	*S. flexneri* strain M2901
22	GCA_002946695	*S. flexneri* strain 89-141
23	GCA_003719775	*S. flexneri* strain 2016AM-0877
24	GCA_009664535	*S. flexneri* 2a strain AUSMDU00010535